

COGNITIVE IMPAIRMENT IN TREATED HIV-DISEASE

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

Jonathan Underwood

*Department of Medicine | Division of Infectious Diseases
Imperial College London*

Declaration

The work presented here is my own unless referenced or stated otherwise.

Jonathan Underwood

October 2016

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Acknowledgements

Firstly, I would like to thank all the volunteers who participated in this research, without your altruism, time and commitment none of this work would have been possible. Secondly, I would like to thank the European Union for their funding through their Seventh Framework Programme initiative. Thirdly, I am indebted to all the CHARTER (Appendix 1), COBRA (Appendix 2) and POPPY (Appendix 3) study teams, as well as the Jefferiss Wing and Clinical Trials Centre staff at St Mary's Hospital, London. In particular, Matthew Stott, Linda McDonald and Lavender Tembo for their help collecting data for the POPPY and COBRA studies.

In addition, I would like to thank Dr James Cole of the Computational, Cognitive and Clinical Neuroimaging Laboratory, Imperial College London for all his neuroimaging expertise, guidance, patience and teaching, and Davide De Francesco at the Research Department of Infection & Population Health, University College London for his statistical advice and expertise.

For their expert supervision, support and encouragement, I am extremely grateful to my supervisors Dr Robert Leech and Prof Alan Winston.

Lastly, to Lauren and Esme, I would be lost without your inspiration, humour, patience, perspective and love. Thank you.

Abstract

Background

Combination antiretroviral therapy has dramatically improved the outlook for people living with HIV-infection worldwide. As such, the focus of care in well-treated individuals has shifted to the management of long-term comorbidities, such as cognitive impairment. However, the pathogenesis of cognitive impairment in virologically suppressed individuals is unclear.

Aims

To determine the prevalence, characteristics and understand the pathogenesis of cognitive impairment in well-treated HIV-positive individuals and to assess biomarkers for their ability to predict cognitive impairment and longitudinal changes in cognition.

Methods

Cross-sectional analysis of two European cohorts (POPPY and COBRA) and longitudinal analysis of the CHARTER cohort using blood, cerebrospinal fluid, clinical, cognitive and neuroimaging data with advanced statistical techniques including machine-learning.

Results

Firstly, cognitive impairment was prevalent in ~20% of successfully treated patients compared to ~5% in demographically comparable controls. However, it was mild, not clearly associated with symptoms and remained stable over time. Additionally, the prevalence depended on the diagnostic method used, with simulation data demonstrating that the commonly used 'Frascati criteria' classifies impairment in ~25-50% of a *normative* control population. Secondly, cognitive impairment in well-treated patients was predominantly associated with white matter microstructural injury rather than grey or white matter atrophy and using multivariate machine learning techniques could be predicted with up to 80% accuracy. Thirdly, greater exposure to efavirenz and nevirapine were associated with clinical and neuroimaging signals of CNS neurotoxicity. However, these results should be interpreted with caution given their cross-sectional nature and limited sample size (n=60). Nevertheless, they justify further, prospective study given that millions are prescribed these drugs worldwide.

Conclusions

Cognitive impairment was more prevalent in well-treated HIV-positive patients compared to well matched controls, with white matter microstructural injury sustained before sustained suppression of HIV-viraemia the likely pathogenic driver. Reassuringly however, this impairment was generally mild, asymptomatic and remains stable over time.

Table of Contents

DECLARATION	2
ACKNOWLEDGEMENTS.....	4
ABSTRACT.....	5
ABBREVIATIONS.....	8
CHAPTER 1 – GENERAL INTRODUCTION	10
CHAPTER 2 – METHODS.....	57
CHAPTER 3 – THE PREVALENCE OF HIV-ASSOCIATED COGNITIVE IMPAIRMENT AND ITS ASSOCIATIONS WITH PATIENT REPORTED MEASURES OF MENTAL AND PHYSICAL HEALTH	91
CHAPTER 4 – MEDICALISING NORMALITY? USING SYNTHETIC DATA AND SIMULATION TO ASSESS THE PERFORMANCE OF DIFFERENT DIAGNOSTIC CRITERIA.	108
CHAPTER 5 – STRUCTURAL BRAIN ABNORMALITIES AND THEIR ASSOCIATION WITH COGNITIVE FUNCTION IN WELL-TREATED HIV-DISEASE.....	134
CHAPTER 6 – BLOOD, CEREBROSPINAL FLUID AND CENTRAL NERVOUS SYSTEM BIOMARKERS AND THEIR ASSOCIATION WITH COGNITIVE FUNCTION IN TREATED HIV-DISEASE.....	159
CHAPTER 7 – PLASMA AND CSF NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR EXPOSURE AND THEIR RELATIONSHIP WITH COGNITIVE FUNCTION.	187
CHAPTER 8 – MULTIVARIATE PREDICTION OF HIV-ASSOCIATED COGNITIVE IMPAIRMENT	211
CHAPTER 9 – CONCLUSIONS	237
LIST OF COMMUNICATIONS.....	250
REFERENCES	251
APPENDIX 1.....	268
APPENDIX 2.....	269
APPENDIX 3.....	270
APPENDIX 4.....	271

Abbreviations

¹ H-MRS	Proton magnetic resonance spectroscopy
AD	Axial diffusivity
AIDS	Acquired immunodeficiency syndrome
CHARTER	CNS HIV Anti-Retroviral Therapy Effects Research
Cho	Choline containing compounds
CI	Confidence interval
COBRA	Co-morBidity in Relation to AIDS
Cr	Creatine
CSF	Cerebrospinal fluid
DARTEL	Diffeomorphic Anatomical Registration using Exponentiated Lie algebra
DTI	Diffusion tensor imaging
DTI-TK	Diffusion Tensor Imaging ToolKit
EACS	European AIDS Clinical Society
EDTA	Ethylenediaminetetraacetic acid
FA	Fractional anisotropy
FMRIB	Functional Magnetic Resonance Imaging of the Brain
FNIRT	FSL's FMRIB's Linear Image Registration Tool
FSL	FMRIB Software Library
FWHM	Full width at half maximum
GDS	Global deficit score
Glx	Glutamate and glutamine
HAD	HIV-associated dementia
HAND	HIV-associated Neurocognitive Disorder
HIV	Human immunodeficiency virus-I
ICBM	International Consortium of Brain Mapping
MD	Mean diffusivity
MI	Myo-inositol
MNC	Multivariate normative comparison
MNI	Montreal Neurological Institute
MRI	Magnetic resonance imaging

NAA	N-acetyl aspartate
NFL	Neurofilament light
NMR	Nuclear magnetic resonance
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
PET	Positron emission tomography
POPPY	Pharmacokinetic and Clinical Observations in People Over Fifty
PRESS	Point-resolved spectroscopy sequence
PROMs	Patient reported outcome measures
RD	Radial diffusivity
RNA	Ribonucleic acid
sCD14	Soluble CD14
sCD1463	Soluble CD163
SPM	Statistical Parametric Mapping
TARQUIN	Totally automatic robust quantification in NMR algorithm
TBSS	Tract-based spatial statistics
TFCE	Threshold-free cluster enhancement
VBM	Voxel-based morphometry
WET	Water suppression enhanced through T1 effects

Chapter I

General Introduction

Table of contents

CHAPTER 1 GENERAL INTRODUCTION.....	10
1.1 HYPOTHESES	12
1.2 COGNITIVE IMPAIRMENT IN THE MODERN ANTIRETROVIRAL ERA.....	12
1.3 METHODS OF DIAGNOSIS & CONTROVERSIES.....	21
1.3.1 Initial classification	21
1.3.2 HIV-associated Neurocognitive Disorder	21
1.3.3 The Global Deficit Score	22
1.3.4 Multivariate Normative Comparison	23
1.3.5 Is cognitive impairment associated with other aspects of ill health?	24
1.3.6 Subtypes of HIV-associated cognitive impairment	24
1.4 PATHOGENESIS OF COGNITIVE IMPAIRMENT	25
1.4.1 The pre-treatment era	25
1.4.2 The antiretroviral era.....	26
1.4.3 Antiretroviral neurotoxicity?.....	29
1.5 NEUROIMAGING IN HIV-DISEASE	36
1.5.1 Structural imaging (sMRI).....	37
1.5.2 Diffusion weighted imaging.....	40
1.5.3 Magnetic resonance spectroscopy (MRS).....	42
1.5.4 Other neuroimaging modalities.....	45
1.5.5 Neuroimaging analysis approaches.....	46
1.6 SOLUBLE BIOMARKERS	49
1.6.1 Neuronal injury	50
1.6.2 Neuroinflammation	53
1.7 SUMMARY	54
1.7.1 Thesis aims.....	54
1.7.2 Major research question of this thesis.....	54

Figures

FIGURE I-A. MODEL OF HIV NEUROTROPISM.	13
FIGURE I-B. THE CHANGING AETIOLOGY OF COGNITIVE IMPAIRMENT IN HIV-POSITIVE INDIVIDUALS OVER TIME.	19
FIGURE I-C. HYPOTHETICAL GRAPH OF HIV VIRAL LOAD AND COGNITIVE FUNCTION OVER TIME.	35
FIGURE I-D. A SIMPLIFIED NEURON AND ANATOMICAL REGIONS PARTICULAR NEUROIMAGING MODALITIES MAY DETECT INJURY. .	37
FIGURE I-E. ILLUSTRATION OF THE MAJOR STEPS OF A GREY MATTER VOXEL-BASED MORPHOMETRY PIPELINE.	38
FIGURE I-F. ILLUSTRATION OF THE MAJOR STEPS OF A TRACT-BASED SPATIAL STATISTICS PIPELINE.	42
FIGURE I-G. EXAMPLE SPECTRA WITH LABELLED METABOLITES OF INTEREST.	43
FIGURE I-H. AN EXAMPLE OF NON-LINEAR CLASSIFICATION USING A MULTIVARIATE TECHNIQUE.	49
FIGURE I-I. A SIMPLIFIED NEURON AND THE ANATOMICAL REGIONS WHERE CERTAIN SOLUBLE BIOMARKERS MAY DETECT INJURY. .	52

Tables

TABLE I-A. MAJOR STUDIES REPORTING THE PREVALENCE OF HIV-ASSOCIATED COGNITIVE IMPAIRMENT IN THE MODERN COMBINATION ANTIRETROVIRAL ERA.	16
TABLE I-B. COMPARISON OF THE THREE COMMONLY USED METHODS OF DEFINING HIV-ASSOCIATED COGNITIVE IMPAIRMENT.	23
TABLE I-C. SUMMARY OF AVAILABLE EVIDENCE FOR ANTIRETROVIRAL CNS TOXICITY.	36

I.1 Hypotheses

Cognitive impairment remains reportedly prevalent despite the widespread use of antiretroviral therapy. However, the prevalence and pathogenesis in well-treated cohorts is uncertain. Three overarching hypotheses will be tested in this thesis, namely:

1. **Cognitive impairment remains more prevalent in HIV-positive individuals compared with demographically comparable controls, despite effective antiretroviral therapy.**
2. **Biomarkers will be associated with cognitive function and can be used to predict impairment and longitudinal changes in cognition.**
3. **Greater antiretroviral exposure will be associated with markers of central nervous system neurotoxicity.**

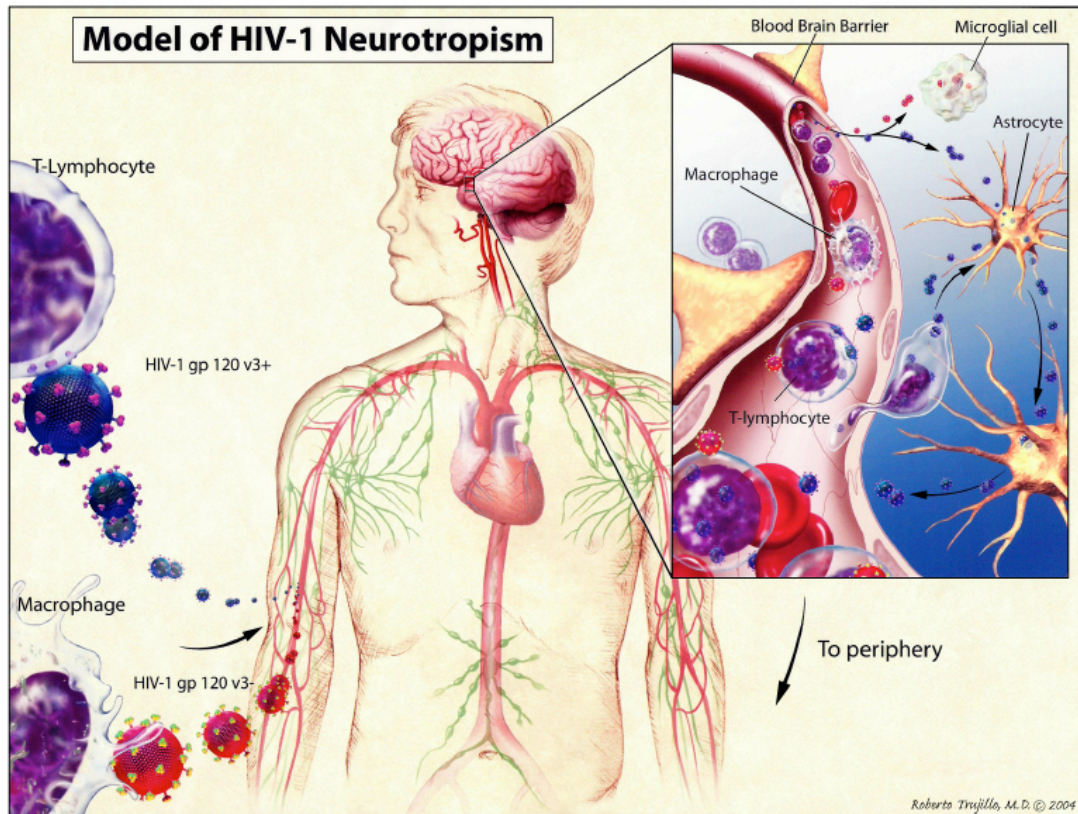
I.2 Cognitive impairment in the modern antiretroviral era

Human immunodeficiency virus type-1 (HIV) is a neurotropic virus with productive central nervous system (CNS) replication occurring days after infection¹ (see Figure I-A for an illustration). In the pre-antiretroviral era, HIV-associated dementia, a debilitating and often fatal disease, was estimated to affect up to 15% of those with other AIDS-defining conditions.² Because of the efficacy and tolerability of modern antiretroviral combinations, HIV-infection is now a chronic, manageable disease with life expectancy for those living with HIV now approaching normal.³ As such, the cohort of people living with HIV is ageing and the treatment paradigm has changed from the management of immunosuppression and opportunistic infections to the long-term consequences of HIV-infection and its treatment.

In tandem with improved longevity, there has been a dramatic decline in the incidence of CNS AIDS-defining conditions.^{4,7} This includes HIV-associated dementia as well as opportunistic

infections. Despite these improvements, cognitive impairment still reportedly affects up to 50% of HIV-positive individuals. This has been reported in both resource-rich and resource-poor settings⁸⁻¹⁸ and has been associated with poorer quality of life, poorer clinical outcomes and a higher mortality.^{19,20}

Figure I-A. Model of HIV neurotropism.



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Despite reportedly of high rates of cognitive impairment in HIV-positive cohorts in the modern antiretroviral era, controversy remains regarding its true prevalence given the wide range of prevalence rates reported, heterogeneity of cohorts studied and methodology used.^{21,22} Determining the true prevalence of cognitive impairment and understanding its aetiology is therefore crucial given the huge potential burden of morbidity if the upper end of the reported prevalence estimates is correct. Table I-A details the major studies reporting the prevalence of cognitive impairment in the modern antiretroviral era with prevalence rates reported ranging from 19-69%. This disparity is likely to be multifactorial, with the main factors being differences in study populations, antiretroviral use and cognitive function testing strategies employed. For example, the lowest prevalence of 19% reported by Garvey *et al*¹⁷ excluded subjects who had neurological symptoms, which is therefore likely to underestimate

the true prevalence of cognitive impairment given that those with more significant impairment may be more likely to report symptoms such as memory complaints. Nadir CD4+ cell count, a proxy marker for duration of untreated HIV-infection, which may be associated with brain injury, varied widely between the studies from 60-330 cells/ μ L.^{11,16} However, this does not seem to explain all the variance given that the greatest prevalence of cognitive impairment was reported from a cohort with median nadir CD4+ cell count of 171 cells/ μ L.¹²

Given the drop in the incidence of the most severe form of HIV-associated brain injury, namely dementia, with the advent of modern combination antiretroviral therapy,^{4,7} the prevalence rate reported from the large CHARTER cohort, where only 41% had HIV RNA <50 copies/ml,⁸ may overestimate the prevalence and severity of cognitive impairment relative to well-treated cohorts that are typical in the UK. Similarly, the exclusion of participants with potentially confounding comorbidities has not been uniform. The largest study of cognitive impairment in HIV-positive individuals is the CHARTER study⁸ (n=1,555) which deliberately included a population that was representative of patients receiving care at the participating institutions. The prevalence of cognitive impairment reduced from 83% in those with potentially confounding comorbidities to 40% if these participants were excluded. Even in this 'minimally confounded' population only 70% were receiving antiretroviral therapy and just 40% had undetectable plasma HIV RNA. Furthermore, the prevalence of 'traumatic brain injury with loss of consciousness and other neurological sequelae' was 3.4% and current major depression was 14%. Therefore, the generalisability of these findings to an individual with sustained suppression of viraemia (i.e. well-treated) without any significant comorbidities is limited.

Differences in cognitive batteries and methods of diagnosing cognitive impairment will be explored in more detail in the next section but it is interesting to note that Su *et al*¹³ reported the prevalence of cognitive impairment to vary between 5-48% depending on the method used to classify participants as impaired or not. Furthermore, there was only a significant difference in the rate of cognitive impairment, between patients and demographically matched HIV-negative controls, for only one of the three methods used (and only using a one-sided significance test). The lack of difference between HIV-positive and demographically similar HIV-negative controls was also reported by McDonnell *et al*¹⁵ who reported lower rates of impairment in the patient group of 21% vs 29% and Crum-Cianflone *et al*²³ who similarly reported rates of impairment of 19% vs. 30% for their HIV-positive and HIV-negative study

groups respectively. These data raise serious doubts about the amount of cognitive impairment reported in HIV-positive cohorts that is actually attributable to HIV-infection and how much is due to artefacts of classification and other potential aetiologies. These two studies also highlight the importance of a matched control group to put results in perspective. Many studies (see Table 1-A for details) did not recruit specific HIV-negative control groups, as such they relied on normative neuropsychological data for each test to compare the HIV-positive group too and only report the prevalence of cognitive impairment in a patient population.^{8,10-12,14,16,17} This is potentially problematic when studying HIV-positive cohorts, particularly in a high-resource setting such as Northern Europe where the burden of HIV-infection falls in distinct sub-groups of the general population (such as men who have sex with men – MSM)²⁴ with high rates of potentially confounding substance misuse^{13,15} that are likely to differ from the general population from which the normative control group for each particular neuropsychological test was derived.

One significant limitation of the data is its cross-sectional nature. Whilst it is possible to describe the prevalence of cognitive impairment in HIV-positive cohorts it is impossible to determine the proportion caused by HIV-infection. This limitation is unlikely to be overcome given the impracticality of prospectively following up a cohort of individuals at risk of HIV-infection and serially performing neuropsychological tests and subsequently comparing trajectories of cognitive function before and after HIV-acquisition. Even with this data, proving causation is problematic as people who develop HIV-infection from an at-risk population may differ in aspects that could potentially confound the measurement of cognitive function. For example, frequent use of methamphetamine may lead to impulsive behaviour such as high-risk sexual activity, which may lead to HIV-infection, as well as potentially resulting in brain injury and poorer cognitive function. Differences between study and control populations and unmeasured confounders must be borne in mind when interpreting the results of many studies including the results presented in this thesis.

Table I-A. Major studies reporting the prevalence of HIV-associated cognitive impairment in the modern combination antiretroviral era.

Study	Year published	Location	n*	Control group	Age	Nadir CD4 call count (cells/ μ L)*	Current nadir CD4 call count (cells/ μ L)*	HIV RNA <50 copies/ml (%)	Prevalence of cognitive impairment (%)***	Comments
CHARTER ⁸	2010	USA	1,555	No**	43	174	420	41	52	Included patients with potentially confounding comorbidities
ALLRT ¹⁰	2007	Multinational	1,160	No**	41	213	424	70	40	Combined analysis of subjects in 14 ACTG RCTs
PIVOT ¹⁴	2013	UK	557	No**	44	117	553	100	51	Baseline cognitive function results pre-randomisation to PI onotherapy.
MACS ¹¹	2015	USA	364	No**	47	330	589	70	33	Excluded confounding conditions
CIPHER ¹⁵	2014	Europe	248	Yes (not matched for age)	45	N/A	85% >350	80	21	All participants MSM (including controls). Higher prevalence of impairment in control group (29%)
Crum-Cianflone <i>et al</i> ²³	2013	USA (military)	200	Yes (matched)	36	319	546	55	19****	Did not report using the Frascati method of defining cognitive impairment. 91% documented seroconverters. No specific exclusion but low rates of confounding disorders. Higher rate of impairment in controls (30%)
Cysique <i>et al</i> ¹⁸	2011	Australia	116	Yes	49	87	326	51	42	Excluded subjects with confounding conditions. Subjects recruited 2001-2002.
AGE _h IV ¹³	2015	Netherlands	103	Yes (matched)	54	185	635	100	48	Excluded confounding conditions

Study	Year published	Location	n*	Control group	Age	Nadir CD4 cell count (cells/ μ L)*	Current nadir CD4 cell count (cells/ μ L)*	HIV RNA <50 copies/ml (%)	Prevalence of cognitive impairment (%)***	Comments
Garvey <i>et al</i> ¹⁷	2011	UK	101	No**	53	185	525	100	19	Excluded subjects with neurological symptoms and potentially confounding diseases
Simioni <i>et al</i> ¹²	2010	Switzerland	100	No**	46	171	597	100	69	Prevalence extrapolated for aviraemic population. Excluded recent confounding conditions
ART-NeCo ⁹	2015	Netherlands	95	Yes (matched)	48	214	N/A	100	41	Excluded confounding conditions.
2NN ¹⁶	2010	Thailand	64	No**	41	60	521	98	38	Results from the follow-up 2NN trial (90% on nevirapine)

* HIV-positive group only

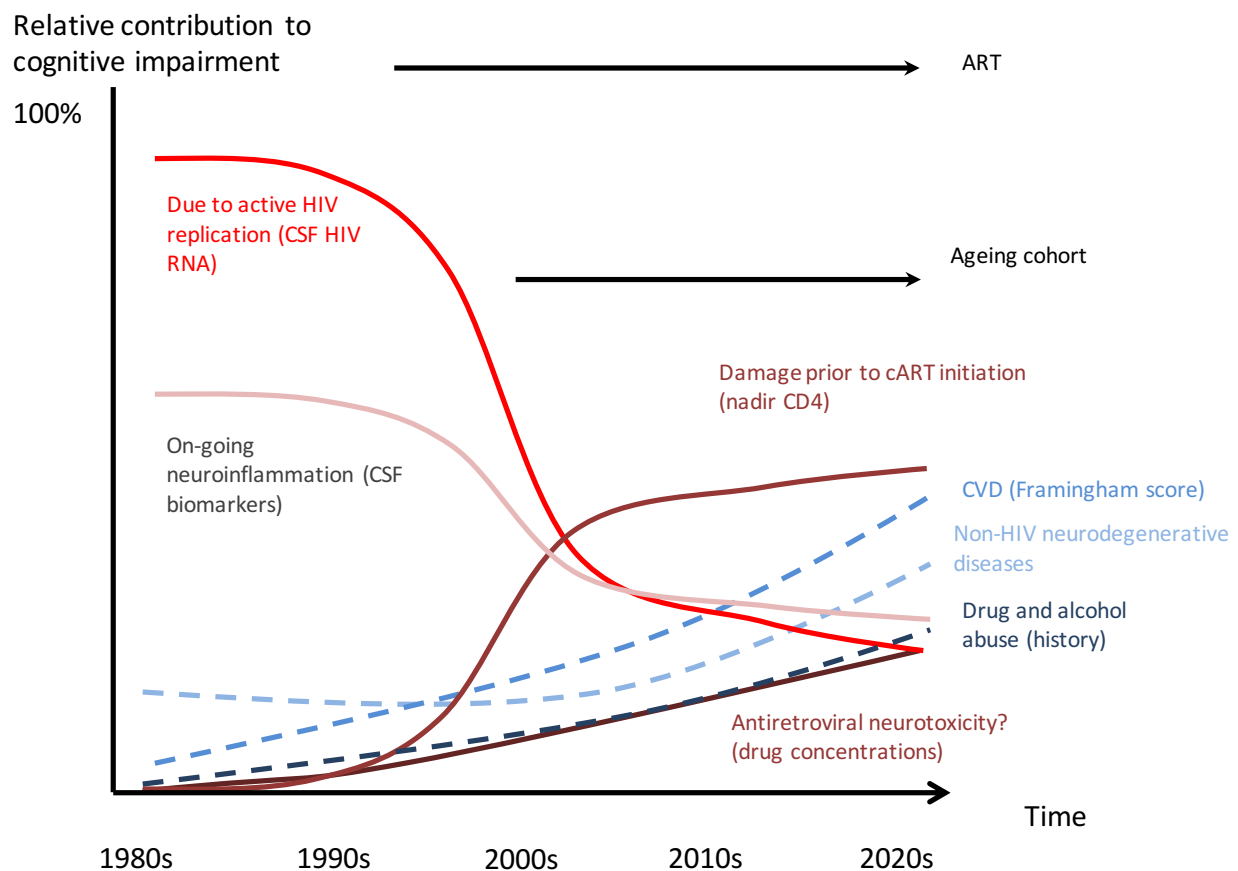
** population based norms for neuropsychological tests only

*** Frascati²⁵ defined cognitive impairment in the HIV-positive study group only

**** Global deficit score²⁶ defined cognitive impairment in the HIV-positive study group only

It is unclear at the moment whether HIV-infection leads to accelerated or premature ageing²⁷ but given that HIV-positive cohorts are ageing,²⁴ the number of HIV-positive individuals at risk of cognitive impairment as a result of HIV-infection or other neurodegenerative processes is increasing. The implicit assumption is that those who present with cognitive impairment who are also HIV-positive, have impairment because of HIV. This assumption was reasonable in the early years of the HIV pandemic as the majority of HIV-positive individuals were young and co-morbidity free. As such, an HIV-positive individual previously presenting with cognitive impairment had a high pre-test probability that this impairment was caused by uncontrolled HIV-replication in the central nervous system. The management of such a patient was clear – namely the initiation of antiretroviral therapy. This was borne out by the first randomised controlled trial of zidovudine monotherapy in the then called ‘AIDS dementia complex’, which improved cognitive performance.²⁸ This is no longer the case in 2016 given the previously mentioned demographic changes occurring worldwide and the recognition that HIV-positive individuals have an increased burden of co-morbidities even when compared to a matched HIV-negative control population, particularly as they get older.²⁹ These include cardiovascular, hepatic and renal disease, all of which may contribute to or may be associated with cognitive impairment.²⁹ Furthermore, the incidences of other forms of neurodegenerative disease in HIV-positive individuals, also presenting as cognitive impairment, are likely to increase. This now presents a diagnostic conundrum when faced with an HIV-positive patient presenting with cognitive impairment as there are many possible causes of cognitive impairment in an HIV-positive individual presenting with cognitive impairment, with HIV-infection being only one. This is illustrated in Figure I-B with the hypothetical aetiological burden of cognitive impairment in HIV-positive individuals changing over time. This could similarly be thought to occur at an individual level whereby an untreated, individual with no co-morbidities who presents with cognitive impairment in their forties has a greater a priori probability that the cause is directly related to central nervous system HIV-replication or its associated inflammation.

Figure I-B. The changing aetiology of cognitive impairment in HIV-positive individuals over time.



However, if the same individual, having initiated treatment and remained generally well into their seventies, aside from developing hypertension and hypercholesterolaemia in the interim, then presented with cognitive impairment the differential diagnosis is broader. In fact, as the majority of HIV-positive individuals receiving antiretrovirals have durable suppression of HIV-replication in both the plasma and CNS compartments^{30,31} the likelihood that cognitive impairment is directly caused by HIV-replication and associated neuroinflammation is now decreasing. This is further complicated as it is likely that the cause of many cases of cognitive impairment is likely to be multifactorial. Brain injury prior to the initiation of antiretrovirals may lower the threshold for symptomatic cognitive impairment later in life, by decreasing 'physiological reserve'. This potentially increases the impact of further insults such as drug and alcohol misuse,³² vascular disease³³ on the background of age-related changes and potentially antiretroviral neurotoxicity from chronic exposure.³⁴ In this thesis, I will examine, in detail, cognitive impairment in well-treated patients and determine the pathogenic mechanisms underpinning it.

The changing cohort

The widespread introduction of antiretroviral therapy has resulted in dramatic declines in the incidence of AIDS-defining conditions with associated reductions in mortality. Consequently, the demographics of HIV-positive cohorts have changed. In the UK, it is estimated that there are approximately 108,000 individuals infected with HIV, of those diagnosed 90% are receiving antiretroviral therapy with 90% achieving viral suppression (defined as HIV RNA <200 copies/mL). The main change is that the cohort is ageing, with the proportion of the UK cohort aged over 50 nearly doubling in the last decade (from 13% in 2004 to 27% in 2013).²⁴

Before the advent of antiretroviral therapy, the lifetime risk of developing HIV-associated dementia was estimated to be 15-20%.² Prognosis was poor with a median survival only six months. Since the widespread use of effective antiretroviral therapy, the incidence of HIV-associated dementia has decreased precipitously.⁴⁻⁷ However, more recently a large burden of mild-to-moderate cognitive dysfunction, affecting up to 50%, has been extensively reported worldwide.⁸⁻¹⁸

With UNAIDS ambitious 90-90-90 target, whereby 90% of those infected are diagnosed and 90% of those are treated with 90% achieving undetectable plasma HIV RNA, viral suppression will now be the norm. As such, co-morbidity data relevant to virally suppressed patients are crucial and will be the focus of this thesis. However, most data pertaining to HIV-associated cognitive impairment comes from populations with treatment that would be considered suboptimal by today's standards and therefore its relevance to today's virally suppressed populations is questionable. Furthermore, over time it has been appreciated that much data comes from studies with potentially inappropriate control populations. This may have led to overestimates of the prevalence of HIV-associated cognitive impairment as well as over attributing brain injury to HIV-infection. Additionally, the diagnosis of cognitive impairment itself is fraught with controversy with several different methods proposed in the literature. This has important implications for understanding pathogenesis. If the foundations on which we assess associations with other measures in order to deduce pathology are uncertain, extrapolations are likely to be inconsistent and our understanding limited. This thesis aims to address some of these shortcomings by describing the prevalence and elucidating the pathogenesis of cognitive impairment in contemporary, well-treated populations.

I.3 Methods of diagnosis & controversies

I.3.1 Initial classification

Neurological complications of HIV-infection were described early in the HIV pandemic. Originally the constellation of cognitive, mood and motor symptoms were grouped together as the AIDS dementia complex.³⁵ This name was later revised to HIV-associated dementia, with milder forms of impairment referred to as mild cognitive motor disorder. With the declining prevalence of HIV-associated dementia and the recognition of the existence of milder forms of cognitive impairment despite antiretroviral therapy, a consensus research definition of HIV-associated cognitive impairment was defined.²⁵ Other methods were subsequently developed, which will also be discussed. For a summary of the three main methods see Table I-B.

I.3.2 HIV-associated Neurocognitive Disorder

The HIV-associated neurocognitive disorder (HAND) classification,ⁱ known commonly as the 'Frascati criteria', did not include motor or mood disturbances whilst making it clear that alternative causes of cognitive impairment should be excluded. For mild forms, cognitive impairment is defined as scoring one standard deviation below the normative mean on tests from two or more cognitive domains. Patients with this degree of impairment are further subdivided into symptomatic and asymptomatic groups (so called 'mild neurocognitive disorder' [MND] and 'asymptomatic neurocognitive disorder' [ANI] respectively). HIV-associated dementia, the most severe form of impairment, is defined as scoring lower than two standard deviations below the normative mean on tests from at least two cognitive domains with "marked impairment of day-to-day functioning". The clear problem with this system of classification is the false positive rate. Approximately 16% of people would be expected to score one standard deviation below the normative mean. It is recommended that "the examination would include tests of the following ability domains (if possible, with at least two test measures per domain): verbal/language; attention/working memory;

ⁱ To avoid confusion with the term 'HAND', which is often used in the literature as a catch-all term for HIV-associated cognitive dysfunction not specifically referring to this particular classification method, I will throughout this thesis refer to this particular method of diagnosis of HIV-associated cognitive impairment as the Frascati criteria unless explicitly stated.

abstraction/executive; memory (learning; recall); speed of information processing; sensory-perceptual; motor skills” – i.e. at least fourteen separate tests. The recognition that individual tests of cognition are correlated was first reported by Spearman in 1904 and was hypothesised to be related to the general intelligence factor or ‘g’.³⁶ Simulation data, based on scoring less than one standard deviation below the mean in five tests with inter-test correlation coefficients varying from 0 to 0.8, has shown that the expected prevalence of ‘cognitive impairment’ in a normal population always exceeds 16% and is usually around 20%.²² Moreover, this would be expected to increase with the number of tests performed – particularly if the fourteen tests are performed with no attempt to account for multiple comparisons. This probably explains the high reported prevalence of cognitive impairment (Table 1-A) in HIV-positive cohorts.^{8,10,11,13,37} More importantly, using this definition liberally the prevalence of cognitive impairment in demographically comparable HIV-uninfected control groups is reported as 29-36%.^{13,15} This does not tally well with clinical experience, which may be related to the difference in severity between those labelled as ‘impaired’ in research studies and those with clinically relevant/apparent impairment. To explore these statistical concerns, in this thesis I will model the expected prevalence of cognitive impairment in a normal population for different diagnostic criteria and assess agreement between them *in silico* using synthetic data for a ‘normal’ population.

1.3.3 The Global Deficit Score

An alternative scoring system, known as the global deficit score (GDS), is obtained by converting demographically adjusted test data to deficit scores and then averaging them with a score ≥ 0.5 used to define as impairment.²⁶ The purpose of converting demographically adjusted test scores to deficit scores is to attach more weight to impaired performance with less significance placed on normal or superior scores, which could offset impairment if a simple averaging approach across cognitive domains was used. This method aims to be most comparable to clinician rating, the purported ‘gold standard’ and has shown to have good predictive and discriminatory power in HIV-positive individuals.²⁶ Furthermore, by averaging over domains it is less affected by the multiple comparison issue.

1.3.4 Multivariate Normative Comparison

Another method, known as the multivariate normative comparison (MNC), uses the control group as a reference to calculate a multivariate statistic (Hotelling's T^2) taking into account performance in all cognitive domains and covariance between different tests.^{38,39} As only one test is performed the multiple comparison issue is solved and the family-wise false positive rate is controlled. This has advantages over using a Bonferroni correction as this assumes that the different cognitive tests are uncorrelated and independent, which they are not.^{36,40} For the diagnosis of HIV-associated cognitive impairment, this method has been shown to potentially optimise the balance between sensitivity and specificity in the context of a lack of a true gold standard.¹³ It should be pointed out that in this study, the first to compare different methodologies directly, the most liberal definition of the Frascati criterion was used (i.e. applied to individual cognitive tests rather than limiting comparisons to cognitive domains), which is likely to increase the false positive rate significantly. The reported prevalence of cognitive impairment according to this definition was 48% vs. 17% using MNC. Furthermore, they did not use the GDS method and only used one-tailed tests with $\alpha=0.05$, which again may overstate the case for the superiority of MNC as a diagnostic method. In this thesis, I will compare different methods of diagnosing cognitive impairment and test whether they identify the same individuals.

Table I-B. Comparison of the three commonly used methods of defining HIV-associated cognitive impairment.

Criteria	Definition of cognitive impairment	Domains considered
Frascati	1 SD below normative mean in ≥ 2 tests/domains	≥ 2
GDS	Mean deficit score ≥ 0.5	All
MNC	Hotelling's T^2 statistic exceeds a critical value (defined using control population)	All

Abbreviations: GDS: global deficit score; MNC: multivariate normative comparison; SD standard deviation.

1.3.5 Is cognitive impairment associated with other aspects of ill health?

Severe forms of HIV-associated cognitive impairment have previously been associated with impairment of objective measures of everyday functioning. However, these data are from patient populations with lower CD4 cell counts than would be typical in the current era, and in whom treatment would now be considered suboptimal.^{41,42} The associations between more minor forms of cognitive impairment, which are more prevalent now, and other patient-reported outcomes are unclear. The implicit assumption is that those with cognitive impairment are more likely to have cognitive symptoms, like memory complaints or attention difficulties¹² as well as a higher frequency of physical complaints as part of a general syndrome of ill health. Additionally, whilst these previously reported associations of cognitive impairment and other patient-reported outcomes may be statistically significant, effect sizes are often small, calling into question the clinical relevance of these findings. In this thesis, I will perform robust analyses to determine the relationships between cognitive impairment and patient reported outcomes of mental and physical health in a cohort of patients reflective of those attending for care today.

1.3.6 Subtypes of HIV-associated cognitive impairment

Different sub-types of HIV-associated cognitive impairment have been proposed with some histopathological evidence of different phenotypes.^{43,44} It has also been suggested that the clinical phenotype has shifted from 'sub-cortical' deficits such as speed of information processing to 'cortical' or 'mixed' phenotypes where learning and executive function are more affected.⁴⁵ One explanation for this is that the pathogenesis of cognitive impairment may have changed from being directly related to central nervous system HIV-replication in untreated individuals/cohorts to other aetiologies, such as other neurodegenerative diseases or antiretroviral neurotoxicity, in treated individuals/cohorts (see Figure I-B). Another possible explanation is that some of the HIV-related changes may improve with antiretroviral treatment⁴⁶ but residual deficits or 'burnt out encephalitis'⁴³ may serve to lower the threshold to further insults or accentuate the normal ageing process. This has not been robustly

investigated in well-treated populations. As HIV-related mortality is much lower than previously, post-mortem data in well-treated populations are rare. Neuroimaging can potentially be used to study brain morphology and function *in vivo*. One study⁴⁷ applied the diagnostic criteria for mild cognitive impairment, a possible intermediary state between normal cognitive ageing and Alzheimer's disease, to a group of HIV-positive individuals with suppressed plasma viraemia. They found a significantly increased rate of mild cognitive impairment compared to HIV-uninfected controls particularly in those aged over 60. There was minimal correspondence with those diagnosed with ANI, which interestingly was not associated with age. This disparity may be explained by the application of these criteria to a different subset of all the cognitive tests performed (e.g. motor tests were only considered for the Frascati criteria) which does not permit a fair comparison. However, these findings do suggest different pathologies may be responsible for cognitive impairment, particularly in older patients on suppressive therapy. In this thesis, I will apply multivariate techniques to multi-modal neuroimaging data to investigate whether there are different subtypes of HIV-associated cognitive impairment in well-treated patients.

I.4 Pathogenesis of cognitive impairment

HIV is a neurotropic virus with productive CNS replication occurring days after infection¹ with the primary sites of replication in the brain being microglia, perivascular macrophages and to a lesser extent astrocytes.^{48,49} Conceptually, neuropathology can be divided into effects mediated by direct or indirect toxicity of HIV. HIV proteins produced during replication, such as gp120 and *tat*, exhibit toxicity by both synaptic/dendritic injury and impaired neurogenesis.⁵⁰ Indirect mechanisms of HIV toxicity would include microglial activation and neuroinflammation. This is further complicated in an era of ubiquitous treatment with antiretroviral neurotoxicity.

I.4.1 The pre-treatment era

The histopathological correlate of HIV-associated dementia, HIV encephalitis, was characterised by the presence of multinucleated giant cells. These were observed particularly in the central white matter and associated with myelin damage but with axons usually spared.^{51,52} Interestingly the largest loss of neurons, up to 30%, was observed in the calcarine

cortex but this occurred independently of the histopathological changes of HIV-encephalitis. This suggests that there may be differing pathologies occurring simultaneously in the white and grey matter, which may be related to the cell types HIV infects. As infection is not thought to occur in neurons, neuronal loss is therefore likely to be the consequence of indirect effects of central nervous system HIV replication such as production of neurotoxic proteins, astrocyte dysfunction or neuroinflammation.⁵⁰ On the other hand, the white matter injury described may be related to productive HIV-replication in oligodendrocytes, although this is an area fraught with controversies.⁴⁹

1.4.2 The antiretroviral era

Post-mortem studies in the antiretroviral era are understandably much less frequent given the precipitous drop in mortality coinciding with the advent of 'highly active' antiretroviral therapy. As such, the histopathological changes associated with HIV-associated cognitive impairment in the current era are unclear but generally tend to be less severe.⁵³ In the absence of a large post-mortem examination series in a comparable patient group, non-invasive biomarkers are needed as a substitute for histopathology to understand the pathogenesis in well-treated patients.

The underlying pathogenesis in well-treated individuals/cohorts remains elusive with several mechanisms proposed, including: i) previous irrecoverable nervous-system damage prior to the initiation of cART or the so called 'legacy' effects of initial infection; ii) neuronal damage from persistent low level central nervous system HIV replication despite effective control of HIV-replication in the plasma compartment; iii) ongoing central nervous system immune activation either in response to low level central nervous system HIV replication, systemic immune activation or microglial activation; iv) the presence of non-infectious co-morbidities resulting in additional neurological insults; v) accelerated or accentuated ageing and vi) antiretroviral neurotoxicity. In all likelihood, a combination of some/all of the above factors to varying degrees is likely to be responsible for cognitive impairment in well-treated individuals.

Much work assessing the role of many of the above pathogenic mechanisms has been undertaken previously. However, due to difficulties in recruiting virally suppressed individuals and conservative use of antiretroviral therapy in the past, previous studies included untreated

patients with variable suppression of HIV replication (see Table I-A for details).^{8,10,11,15,18} Additionally, in recent years it has become apparent that standard control populations, often used for reference purposes in neuropsychological research settings, have different and potentially confounding demographic characteristics compared to HIV-positive populations. Lastly, making comparisons between different studies is problematic because different definitions of cognitive impairment with varying methodologies (see section 1.3 for further details) have been used combined with a lack of standardisation to cognitive testing. Therefore, given the ageing cohort and frequent co-morbidities,²⁹ neuropsychological, neuroimaging and histopathological abnormalities reported previously may not extrapolate to the well-treated populations that are the norm today. These challenges may have contributed to the widely varying prevalence of HIV-associated cognitive impairment quoted in the literature (Table I-A) and to the confusion about its aetiology. In this thesis, I aim to mitigate some of these shortcomings by examining the pathogenesis of cognitive impairment, with numerous complementary techniques, in exclusively well-treated populations. As central nervous system antiretroviral neurotoxicity has barely been studied *in vivo* and because it is an easily modifiable risk factor given the number of currently licenced antiretrovirals, in this thesis, I will focus on exploring this as a possible pathogenesis mechanism.

Legacy effects

Given the prevalence of HIV-associated dementia in the pre-antiretroviral era,^{2,5} there is little doubt that untreated HIV-infection is associated with significant brain injury. In common with other neurodegenerative conditions, such as Alzheimer's dementia, atrophy and neuronal loss associated with prolonged untreated HIV-infection is unlikely to recover. However, milder HIV-associated cognitive deficits have been associated with synaptodendritic injury,⁵⁴ a mechanism of injury that is potentially reversible.⁵⁰ Therefore, the improvements in cognitive function associated with initiation of antiretroviral therapy^{46,55} are likely to be secondary to a reduction in further damage and associated inflammation with accompanying synaptodendritic repair. This may then lead to the more efficient functioning of the distributed brain networks that underpin cognition.⁵⁶ Irreversible damage, such as neuronal loss, may lower the threshold for clinically apparent cognitive impairment from subsequent insults such as ageing, vascular disease or drug or alcohol misuse. To thoroughly investigate legacy effects, follow-up of patients for decades after HIV-infection is necessary. This is beyond the scope of this thesis; however, cross-sectional data can provide suggestive evidence. I will test whether cognitive

impairment, as well as biomarker evidence of brain injury are associated with markers of prolonged untreated infection, such as the nadir CD4⁺ cell count, duration of known untreated infection and CD4⁺:CD8⁺ ratio.

Persistent brain injury and neuroinflammation

Modern antiretroviral therapy results in durable suppression of both plasma and cerebrospinal fluid HIV viraemia.^{30,31} However, concerns remain that HIV replication below the limit of detection is responsible for the high prevalence of cognitive impairment still reported. There is some evidence that persistent HIV-replication despite successful antiretroviral therapy, quantified by cell associated HIV DNA, is associated with brain injury.^{57,58} However, how these measures of the so called '*viral reservoir*' relate to central nervous system HIV replication and whether they exert their effects on the brain through indirect mechanisms, such as systemic immune activation, are unclear. Evidence of ongoing *in vivo* central nervous system injury despite antiretroviral therapy comes largely from longitudinal studies of cognitive function. Longitudinal data from the Multicenter AIDS Cohort Study (MACS) and Central nervous system HIV Anti-Retroviral Therapy Effects Research (CHARTER) cohorts suggests the majority (>60%) of HIV-positive individuals remain stable cognitively over time.^{11,59} Approximately 10% experienced deteriorating cognitive function and this was associated with ineffective antiretroviral therapy or treatment interruption. It should be noted that antiretroviral therapy was not universal in these cohorts and effective suppression of plasma HIV RNA was only evident in 70% and 41% respectively, which makes drawing conclusions for successfully treated cohorts which are common in the UK difficult. These data provide some evidence that persistent brain injury is associated with uncontrolled HIV replication. Although it remains unclear if clinically significant deterioration occurs in those on effective antiretroviral therapy, these data would suggest it is unusual. However, longitudinal tests of cognitive function are noisy measures and subject to practice effects,^{60,61} which limits their sensitivity for detecting ongoing subtle brain injury. More specific evidence of persistent injury and neuroinflammation come from neuroimaging studies or indirectly from cerebrospinal fluid examination. Two positron emitting tomography (PET) studies^{62,63} have suggested that despite effective antiretroviral therapy, HIV-positive patients have evidence of persistent microglial activation, which may be associated with cognitive impairment. However, due to the expense of PET imaging these findings have only been documented in small numbers of patients (n=7⁶³ and n=12⁶²) and have not been consistent, at least in non-demented HIV-positive

individuals,^{64,65} although these differences may reflect less sensitive analysis techniques in these older studies. Further evidence of persistent injury and neuroinflammation are discussed later in sections 1.5 (neuroimaging) and 1.6 (soluble biomarkers).

Non-infectious comorbidities and ageing

Given the demographic changes of HIV-positive cohorts discussed earlier, prevalence of age-associated comorbidities and their occurrence at younger ages than would be expected²⁹ there is concern from physicians and patients alike that long term infection is associated with ‘*accelerated ageing*’.⁶⁶ One of the most prevalent comorbidities reported in HIV-positive cohorts is vascular disease and its management features in most HIV treatment guidelines.^{29,67,68} Vascular disease is an important cause of cognitive impairment regardless of HIV-serostatus and its association with cognitive impairment has been reported in otherwise optimally treated HIV-positive patients.⁶⁹ Some of these effects may also be mediated by the metabolic consequences of particular antiretroviral agents which will be discussed in the following section. The evidence for accelerated cellular ageing and its pathogenic mechanisms that may explain the increased prevalence of an comorbidities in HIV-positive cohorts include epigenetic changes,⁷⁰ mitochondrial ageing⁷¹ (discussed in some detail in the following section) and telomere shortening.^{72,73} Furthermore, the underlying cause of these cellular abnormalities in treated individuals may be secondary to antiretrovirals themselves⁷⁴ and will be discussed in some detail in the next section.

1.4.3 Antiretroviral neurotoxicity?

Generally speaking initiating antiretroviral therapy in antiretroviral naïve patients is associated with improvements in cognitive function, at least in the short term, particularly in those with impairment.^{46,55} However, there has been little focus on the potential toxicity of antiretroviral drugs to the central nervous system over the longer term, despite this being a potentially simple risk factor for cognitive impairment to modify. Given the increased longevity of HIV-positive individuals, lifetime exposure to antiretrovirals is now measured in decades. Therefore, understanding the aetiological role, if any, that antiretroviral neurotoxicity plays in cognitive impairment in well-treated individuals is important.

Pharmacokinetic considerations in an ageing cohort

With regards to cognitive impairment, the appropriate exposure of antiretroviral drugs is important as too little may lead to uncontrolled HIV replication, whereas too much may increase the risk of antiretroviral neurotoxicity. The key elements of antiretroviral pharmacokinetics (absorption, distribution, metabolism and elimination) may be affected by ageing.⁷⁵ Many antiretroviral drugs require an acidic gastric environment for optimal absorption. Although this may not change substantially with age, the prescribing of drugs to lower gastric acidity, such as proton pump inhibitors, is more frequent as HIV-positive individuals age.⁷⁶ Additionally, age associated changes that may affect drug absorption, such as delayed gastric-emptying, decreased gastrointestinal motility and reduced intestinal enzymatic (e.g. CYP3A4) and transport protein (e.g. p-glycoprotein) activity all have potentially complex interactions with absorption of antiretroviral drugs. Body fat changes, which are thought to occur because of altered lipid metabolism, weight loss caused by untreated infection and mitochondrial toxicity secondary to specific antiretroviral drugs (e.g. zalcitabine) may affect antiretroviral distribution.⁷⁷ Delivery of antiretrovirals into the central nervous system compartment is largely dependent on transmembrane-transporters such as p-glycoprotein.⁷⁵ Their activity may wane physiologically with ageing, with one study reporting age as the most important determinant of the cerebrospinal fluid (CSF) antiretroviral exposure.⁷⁸

Hepatic CYP450 iso-enzyme activity may diminish with age, therefore drugs metabolised via this route (e.g. protease inhibitors and non-nucleoside reverse transcriptase inhibitors) may be more prone to increased exposure with age.⁷⁹ In contrast, drugs metabolised via other metabolic pathways that may vary less with age, e.g. raltegravir via glucuronidation,⁸⁰ may be less prone to such interactions. Elimination of antiretrovirals differ by class. Faecal elimination is important for protease inhibitors, whereas renal elimination is the major route for the nucleotide reverse transcriptase inhibitors (particularly tenofovir). Reduced elimination and increased plasma exposure in older individuals is likely given age-associated declines in glomerular filtration rates. This has been shown previously for tenofovir.⁸¹

Potential pathophysiology of antiretroviral neurotoxicity

Similar to the effects of HIV itself, conceptually antiretroviral central nervous system toxicity may be mediated by either direct or indirect mechanisms. Indirect mechanisms whereby

antiretroviral exert central nervous system toxicity potentially include interactions with vascular disease mechanisms and accelerated or accentuated brain ageing. As previously discussed, vascular disease is an important risk factor for cognitive impairment regardless of HIV-serostatus. However, it may be increased by the metabolic consequences of particular antiretroviral agents and combinations, although the evidence for such effects is limited.⁸² Another potential mechanism is interference with the normal cellular processes that are associated with ageing. 'Accelerated ageing' and 'frailty phenotypes' have been reported to occur prematurely in well-treated patients, with reports of relationships between antiretroviral exposure and markers of cellular ageing such as telomerase length providing a potential pathogenic mechanism independent of the effects of HIV-infection.^{66,72,73}

Direct toxicity to peripheral nerves is a well-documented side effect associated with the older nucleoside reverse-transcriptase inhibitors (NRTIs), particularly the dideoxy-nucleosides. There is emerging evidence, albeit in mice, that this effect is not limited to peripheral nerves where reductions in mitochondrial DNA in murine cerebral cortex neurons has been described after the chronic administration of NRTIs.⁸³ In common with their toxic effects on peripheral nerves, this toxicity is thought to be mediated by partial inhibition of mitochondrial gamma DNA polymerase.⁷⁷ Payne *et al*⁷¹ elaborated this mechanism of mitochondrial toxicity associated with NRTIs. Using skeletal muscle biopsies, individuals exposed to NRTIs had defects in mitochondrial oxidative function, with their severity proportional to cumulative lifetime NRTI exposure. The changes were comparable to those reported in healthy elderly adults, despite all participants being aged 50 or under. Mitochondrial toxicity was not thought to be secondary to a direct mutagenic effect of NRTIs but rather clonal expansion of pre-existing, age-related, mutations in mitochondrial DNA. However, it is unclear whether this observed toxicity in skeletal muscles is also present in the central nervous system. However, there is comparable evidence of toxicity in murine models⁸³ and evidence of depletion of mitochondrial DNA in the frontal cortex in HIV-positive individuals with the most marked changes observed in those with HIV-associated cognitive impairment.⁸⁴ Furthermore, similar abnormalities have been reported in patients with Alzheimer's disease, as well as its suspected precursor, mild cognitive impairment.⁸⁵ Additionally, mitochondrial dysfunction has been described as a crucial step in the pathogenesis of other neurodegenerative diseases such as Parkinson's disease.⁸⁶

Evidence for the direct toxicity of many antiretrovirals in current clinical use has been shown in laboratory models. Robertson et al⁸⁷ exposed rat neuronal cell cultures to fifteen antiretrovirals at varying concentrations. Mitochondrial dysfunction and neuronal injury, manifested by dendritic beading and loss of dendrites, were observed with most antiretrovirals at exposures comparable to those expected with standard clinical dosing. However, extensive cell death was rare and was only observed at the highest concentrations of certain drugs (notably efavirenz). Another experiment utilising rat neuronal cell cultures confirmed the neurotoxic effect of efavirenz and its 8-hydroxy metabolite, which was found to be more toxic than the parent compound.⁸⁸ The clinical relevance of these findings was then demonstrated by sampling plasma and cerebrospinal fluid of efavirenz treated patients. Concentrations of 8-hydroxy-efavirenz were three times the minimal toxic concentration determined in vitro. The putative mechanism of this toxicity was due to alterations in calcium homeostasis⁸⁸ associated with glutamate excitotoxicity,⁸⁹ which have been implicated in other neurodegenerative diseases as well as HIV-associated cognitive impairment.^{90,91} Further evidence for efavirenz induced mitochondrial toxicity is provided by experiments performed in both human and rat neuronal and glial cell lines.⁹² Interestingly, neuronal cells were more vulnerable than glial cells to mitochondrial dysfunction, due to differences in cellular bioenergetics and the ability of glial cells to activate glycolytic pathways in response to a mitochondrial insult. Preferential mitochondrial toxicity of neuronal cells has also been observed in mice chronically administered NRTIs.⁸³

Clinical evidence of antiretroviral central nervous system neurotoxicity

The majority of clinical research to date has focused on drug penetration and suppression of HIV-replication rather than toxicity in the central nervous system.⁹³ This was due to the perceived concern that cognitive impairment in treated individuals was the result of sub-therapeutic central nervous system antiretroviral exposure. In response to these concerns the 'clinical penetration effectiveness' (CPE) score was devised. This aimed to rank antiretrovirals in their ability to penetrate and inhibit HIV-replication in the central nervous system.⁹³ As expected, since it formed the basis of how the CPE score for each drug was initially determined, antiretrovirals with a higher CPE score have greater suppressive effects on cerebrospinal fluid HIV-replication. However, the data regarding cognitive outcomes are

mixed. One may expect that if antiretrovirals were potentially neurotoxic, drugs with greater central nervous system exposure (i.e. have a higher CPE score) would be associated with higher rates of cognitive impairment. Some have found that regimens with higher CPE scores were associated with poorer cognitive outcomes despite more effective suppression of CSF HIV-replication, whilst other studies have shown the opposite.^{46,94,95} Discrepancies between different combinations and their ability to suppress HIV-replication and improve cognitive function in naïve patients have also been noticed, highlighting the potential differences between efficacy and toxicity, as noted earlier.⁹⁶ One of the most suggestive studies of potential toxicity associated with high CPE scores comes from a large prospective study from six European countries and the United States of antiretroviral naïve patients (n≈56,000). The incidence of HIV-associated dementia was increased by more than 50% after initiating a combination with a high CPE score vs. a low CPE score.⁹⁷ There were no significant changes in the incidence of central nervous system opportunistic infections, suggesting this effect was not mediated by uncontrolled HIV-replication with associated immunosuppression. The major drawback is the cohort nature of the study, which makes proving causation impossible.

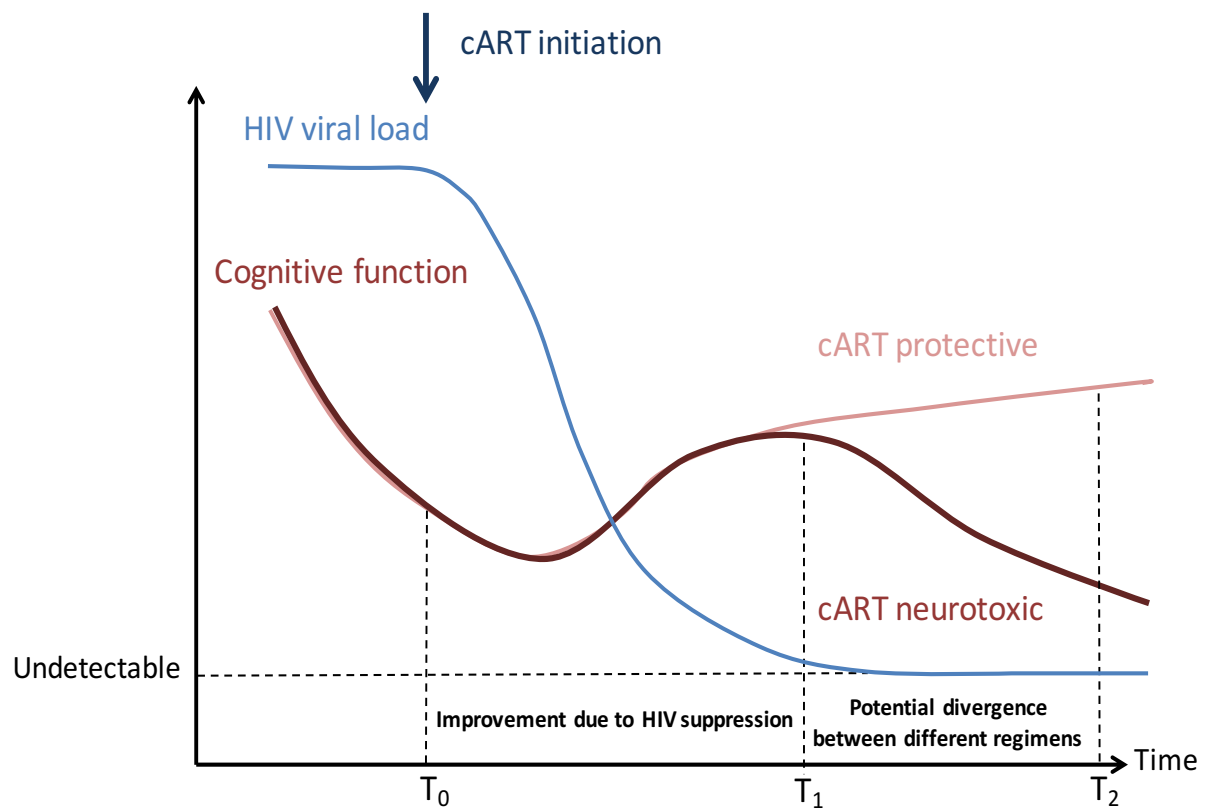
Further clinical evidence of toxicity comes from Robertson *et al*⁹⁸ who reported improvements in cognitive function after a planned interruption of therapy. Since effective antiretroviral therapy has been associated with improvements in cognitive function, these results were surprising and were contrary to the investigators' hypothesis. They may be explained by the patients they enrolled who had commenced antiretroviral therapy before significant immune dysfunction and probably before significant HIV-associated cognitive impairment had developed therefore accentuating any potential toxicity. Interestingly, while cognitive and psychiatric side effects of medications are well recognised in clinical practice, particularly with efavirenz, where early toxicity has been well described,⁹⁹⁻¹⁰³ long term toxicity and cognitive impairment has not been a major concern or robustly studied. However, recent evidence from cross-sectional studies^{104,105} has suggested a more subtle form of efavirenz neurotoxicity secondary to long-term.

Uncertainty exists regarding the aetiological role that antiretroviral central nervous system toxicity plays in cognitive impairment in treated HIV-disease. Although laboratory evidence of toxicity exists, data from clinical studies is conflicting. Some of the difficulty is likely to be explained by the dynamics of cognitive function in HIV-positive individuals commencing antiretroviral therapy. Prior to antiretroviral initiation, central nervous system HIV-replication

exerts toxic effects by direct and indirect mechanisms, which may subsequently lead to cognitive impairment. HIV-replication is suppressed after commencing antiretroviral therapy, ameliorating its toxic effects and cognitive function may subsequently improve. After prolonged exposure to antiretroviral drugs, secondary declines in cognitive function may then be observed if antiretrovirals exhibit appreciable toxicity. Therefore, the timing of assessment is crucial (illustrated in Figure I-C).

Most studies report benefits in cognition after initiating antiretroviral therapy, however, follow up is often limited to 48 weeks. Over this time frame dynamic changes to cognitive function are dominated by the positive effects of suppression of HIV-replication, which overwhelm any potentially modest neurotoxicity. Prolonged follow up is needed to truly determine the longitudinal effects of drug exposure given previously reported potential differences between antiretroviral combinations.¹⁰⁶ However, due to cost and logistical issues this sort of research is rarely undertaken. Therefore, in this thesis I will investigate whether this suggestive evidence of potential neurotoxicity (summarised in Table I-C) translates into clinically significant cognitive impairment using detailed measures of cognitive function and antiretroviral exposure. Furthermore, I will explore potential pathogenic mechanisms *in vivo* using a combination of advanced neuroimaging and soluble biomarkers.

Figure I-C. Hypothetical graph of HIV viral load and cognitive function over time.



Relative to T_0 (at antiretroviral initiation), measurement of cognitive function at T_1 will show an improvement due to suppression of HIV-replication outweighing any potential neurotoxicity. At T_2 , a difference between neurotoxic and neuro-protective may become apparent after a suitable delay.

Table I-C. Summary of available evidence for antiretroviral CNS toxicity.

Antiretroviral	In vitro evidence	Animal evidence	Imaging evidence	Clinical evidence
NRTIs				
Tenofovir ^{87,107}	+/-	+		
Abacavir ^{87,96,108,109}	++		+/-	
Emtricitabine ⁸⁷	+/-			
Lamivudine ^{84,87,108,109}	+/-	+	-	
Zidovudine ^{84,87,96,107-109}	+/-	+	+/-	
Stavudine ^{84,109}	+/-	+	+	
Didanosine ^{84,87,109}	+	+	+	
Zalcitabine ⁸⁷	+/-			
NNRTIs				
Efavirenz ^{87,88,96,104,106,110-112}	++	+	+/-	++
Nevirapine ^{87,113,114}	+	+		
Etravirine ⁸⁷	+			
PIs				
Darunavir ⁸⁷	-			
Atazanavir ^{87,96,106,107}	+	+	+/-	
Amprenavir ⁸⁷	+			
Ritonavir ^{87,107}	+/-	+		
Saquinavir ¹⁰⁷	+	+		
Indinavir ¹⁰⁸	+			
Others				
Maraviroc ⁸⁷	-			
Raltegravir ^{80,115}				+/-

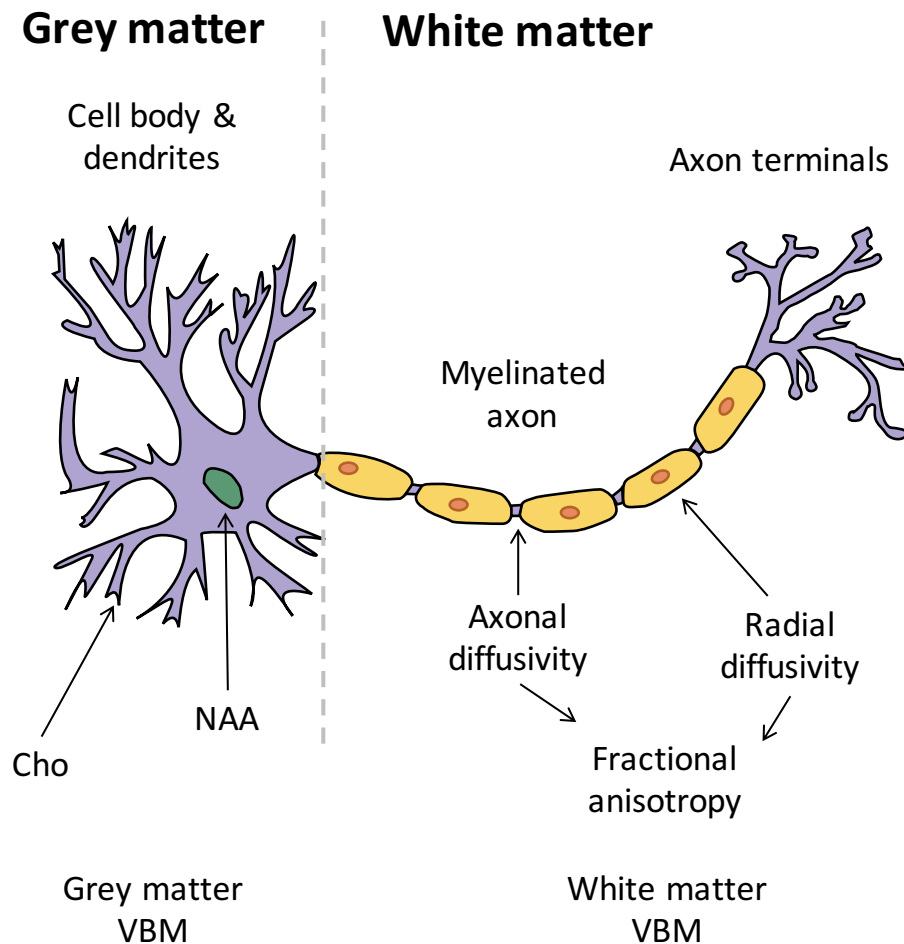
Key: - Significant toxicity unlikely at clinical doses / no evidence of toxicity; +/- Possible evidence of toxicity but probably not significant at clinical doses or conflicting reports; +Some evidence of toxicity at clinical doses; ++Toxicity likely at clinical doses/reasonable evidence of toxicity. Abbreviations: NRTI: nucleoside reverse transcriptase inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; PI: protease inhibitor.

I.5 Neuroimaging in HIV-disease

With the dramatic improvements in life expectancy of HIV-positive individuals, most post-mortem examination data pertain to the pre-treatment era. In the absence of similar data in the modern era, studying neuropathology in well-treated patients relies on non-invasive biomarkers such as neuroimaging. MRI has many advantages over other imaging modalities such as computed tomography (CT) or positron emission tomography (PET) due to its use of non-ionising radiation, image resolution, cost, availability and versatility. Different pulse sequences allow the acquisition of data with complementary information regarding brain structure (see Figure I-D for a simplified summary). For example, T₁-weighted images give excellent delineation of anatomy and allow assessment of grey and white matter volume.

Whereas, diffusion weighted images are sensitive to the free movement of water and provide detail regarding white matter microstructure.¹¹⁶

Figure I-D. A simplified neuron and anatomical regions particular neuroimaging modalities may detect injury.



Abbreviations: Cho: choline containing compounds; NAA: N-acetylaspartate; VBM: voxel-based morphometry.

1.5.1 Structural imaging (sMRI)

T_1 -weighted imaging allows high resolution delineation of brain tissue anatomy permitting segmentation of brain and non-brain tissue. This permits the accurate quantification of brain tissue volumes as well as localisation of atrophy in disease states. Originally, segmentation of grey and white matter from cerebrospinal fluid, bone and soft tissues had to be undertaken painstakingly by hand. This is exceptionally labour intense making large scale studies challenging. Newer automated techniques are comparable to this 'gold-standard', have high test re-test reliability and are well suited to multi-subject studies.^{45,117} Different analytical

techniques can be used with the simplest method being to quantify total volumes of grey and white matter. To study specific anatomical areas, regions of interest can be demarcated either using atlas based or specific segmentation techniques and subsequent quantification of tissue volumes. The drawback of using a region of interest approach is the reliance on *a priori* knowledge of areas likely to show abnormalities. Given the histopathological evidence that HIV-associated brain injury is likely to be diffuse in nature^{51,118} this approach may be problematic. An alternative is to perform whole brain analyses which allows the simultaneous study of the entire brain without relying on possibly biased/incorrect prior knowledge. Using a comprehensive spatial assessment is particularly important when assessing structure/function relationships given the uncertainties about how imaging changes relate to cognitive function in treated HIV-disease.

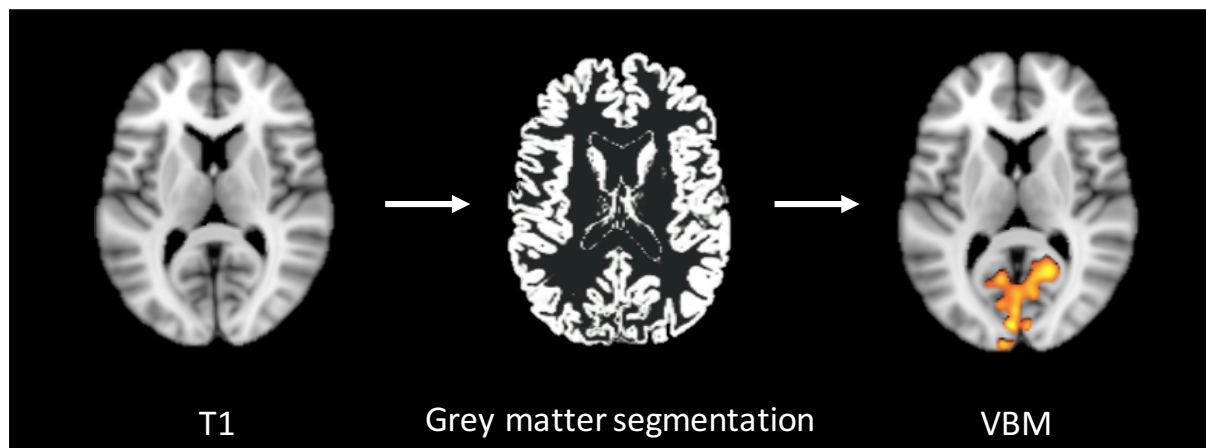
Voxel-based morphometry is a technique whereby the concentration of grey or white matter can be compared on a voxel by voxel basis (see Figure I-E for a simplified illustration of the main steps). This relies on the accurate segmentation of grey and white matter followed by registration of all images to a common space, the process of spatial normalisation, so that each particular voxel represents the same area of the brain in each subject. Following a smoothing step, a mass univariate statistical approach can be used to perform group comparisons or regression analysis accounting for multiple comparisons. In order to compare actual amounts of grey or white matter volume voxelwise, the degree of deformation during the registration step must be taken of account. This augmented voxel-based morphometry approach takes advantage of information required in the deformation process of image registration. This process is summarised by Ashburner and Friston in their seminal methodological paper.¹¹⁹

HIV-associated changes

Cerebral atrophy has been recognised as a feature of HIV-associated dementia since the 1990s.^{120,121} HIV-associated grey matter atrophy has been described in numerous locations previously including: frontal areas,^{122,123} the primary and association sensorimotor areas,^{124,125} anterior cingulate cortex,¹²⁶ subcortical structures,^{124,127} temporal lobes,^{122,126} occipital cortex¹²⁵ and cerebellum.¹²² White matter volumetric changes have been studied less frequently but HIV-associated white matter atrophy has also been reported, most consistently in the corpus callosum.^{124,127} However, in common with studies of cognitive function, many

subjects in these studies were not receiving antiretroviral therapy and, if they were, frequently did not have suppressed plasma HIV RNA. Furthermore, the relationship between these observed abnormalities and cognitive function remains unclear, particularly in those without HIV-associated dementia, and several studies have reported no association between neuroimaging changes and cognitive impairment.¹²⁷⁻¹³⁰

Figure I-E. Illustration of the major steps of a grey matter voxel-based morphometry pipeline.



Abbreviations: VBM: voxel-based morphometry.

There have been few longitudinal structural MRI studies of HIV-positive individuals. An early study by Stout *et al*¹³¹ in untreated individuals reported more rapid cerebral atrophy compared to HIV-uninfected controls. These changes were most marked in the grey matter and were worse in those with the most advanced HIV-disease. More recently, two studies have investigated longitudinal changes in brain tissue volumes, both reported accelerated atrophy in HIV-positive individuals compared to HIV-uninfected controls.^{132,133} However, in neither study were the HIV-positive patients successfully treated. In the one study¹³² to split up the HIV-positive group into those with and without viral suppression Cardenas *et al* showed the greatest rates in grey and white matter loss in HIV-positive individuals with detectable plasma HIV RNA.

Evidence for antiretroviral neurotoxicity

With regards to structural imaging evidence of potential antiretroviral neurotoxicity, one large (n=251) study¹²⁸ showed that longer duration of antiretroviral therapy was significantly associated with lower white matter and increased CSF volumes. This was despite adjusting for other covariates including duration of HIV-infection. These sorts of changes in HIV-

positive individuals have been associated with poorer performance on neuropsychological tests.^{122,134} A potential role for antiretroviral toxicity to white matter is supported by a longitudinal study of brain tissue volumes.¹³² In this study, the greatest rates of white matter atrophy were observed in those on treatment with suppressed plasma viral loads. However, caution should be noted with this finding given the small number of subjects in this group (n=14) and that this study was conducted in 1999-2002, when antiretrovirals use would differ significantly from now. Additionally, these findings have not been replicated with another study finding no relationship between duration of antiretroviral exposure and measures of brain volume.¹³⁵ This disparity may reflect the differences in duration of antiretroviral exposure in the two studies (mean duration 5.6 years vs. median duration 1.6 years respectively) as a shorter duration of exposure would be expected to result in more subtle and therefore possibly statistically insignificant/undetectable effects. Furthermore, antiretroviral therapy was not uniform or analysed by agent, which may mask differential toxicity.

1.5.2 Diffusion weighted imaging

Diffusion MRI techniques renders the measured signal to be sensitive to the diffusion of water. In an unrestricted medium, such as the middle of a glass of water, the movement of water molecules is stochastic or *isotropic*. However, in the brain anatomical boundaries may restrict the free movement of water molecules resulting in *anisotropic* diffusion. This is particularly the case for axons where the diffusion of water is analogous to the flow of water through a tube, i.e. perpendicular diffusion is more restricted than parallel diffusion.¹³⁶ In isotropic conditions a single scalar unit can be used to describe diffusion – the apparent diffusion coefficient. However, in anisotropic conditions this is no longer appropriate because at each measured unit, such as a voxel, diffusion has a degree of directionality as well as magnitude. Consequently, a more complicated mathematical description is needed – an ellipsoid that can be represented mathematically as a tensor.¹³⁷ Using this construct, diffusion tensor imaging allows the quantification of several biologically useful measures at each voxel: mean diffusivity (MD) or apparent diffusion coefficient; the degree of diffusion directionality - fractional anisotropy (FA); the diffusion rate along the principle diffusion axis of diffusion – axial diffusivity (AD) and the diffusion rate perpendicular to the principle axis of diffusion - radial diffusivity (RD). Increases in mean diffusivity have often been associated with pathology in a variety of clinical conditions.¹¹⁶ Fractional anisotropy, which varies from 0 (unrestricted, isotropic diffusion) to 1 (fully anisotropic diffusion), has been also shown to be sensitive to

many pathological brain states.^{116,138-140} Generally, these have been associated with reductions in fractional anisotropy, due to a loss of so called ‘white matter integrity’. However, some caution is necessary in the interpretation as if a voxel contains crossing fibres, loss of some fibres associated with disease may lead to a paradoxical increase in fractional anisotropy.^{141,142} Changes in axial and radial diffusivity measures can be used to delineate between axonal and demyelinating injury respectively,¹⁴³ which can be used to provide non-invasive evidence of pathogenic changes.

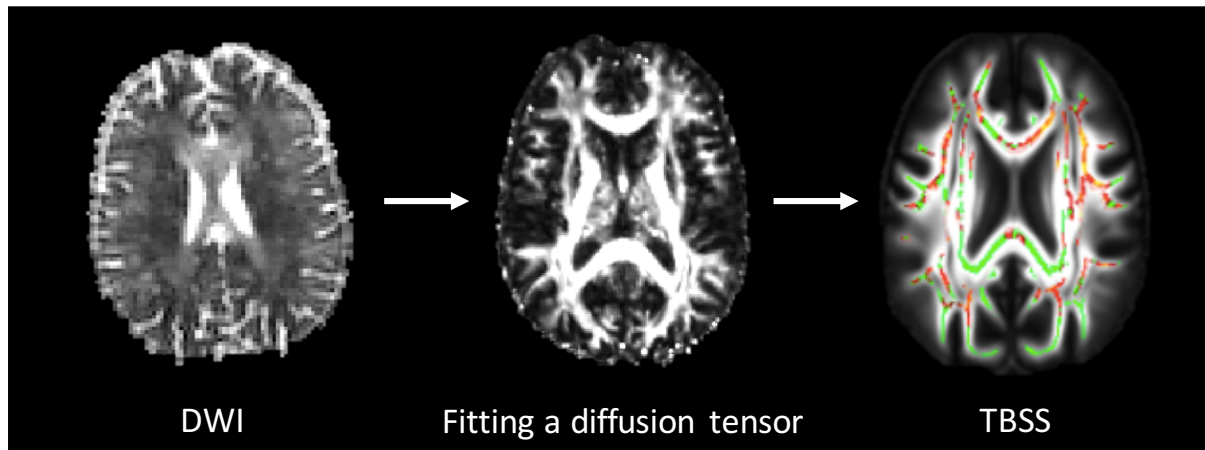
Diffusion tensor imaging may be particularly sensitive to partial volume effects due to misregistration errors, whereby white matter voxels may contain signal from cerebrospinal fluid and may artificially lower fractional anisotropy.¹⁴⁴ This may be particularly problematic in diseases where atrophy of white matter is a possible feature, especially in white matter tracts adjacent to cerebrospinal fluid such as the corpus callosum,¹⁴⁵ such as chronic HIV-infection.^{124,127} These effects can be mitigated only performing an analysis of the centre of white matter tracts common to all participants.¹⁴⁶ This is accomplished by a ‘skeletonisation’ step whereby only the centres of white matter tracts, common to all subjects, are considered for further voxelwise analysis – *tract based spatial statistics* (TBSS). This removes the need for smoothing, potentially ameliorates misregistration and partial volume effects and improves sensitivity by dimensionality reduction¹⁴⁶ (see Figure I-F for an illustration of the major analysis steps).

HIV-associated changes

Despite reports of HIV-associated white matter atrophy occurring mainly in the corpus callosum, widespread abnormalities on diffusion weighted imaging have been reported.^{129,130,147-}

¹⁵⁰ With the exception of Su et al¹³⁰ HIV-positive participants were not all receiving suppressive therapy, which makes drawing definitive conclusions in well-treated individuals problematic. An interesting study by Wright et al¹⁵¹, including subjects with primary HIV-infection, sheds some light of the potential natural history of HIV-associated brain injury.

Figure I-F. Illustration of the major steps of a tract-based spatial statistics pipeline.



Abbreviations: DWI: diffusion weighted imaging; TBSS: tract-based spatial statistics.

They showed that untreated individuals within one-year of primary HIV-infection had similar diffusion tensor imaging metrics to HIV-uninfected controls, in contrast to chronically HIV-infected individuals (also not on treatment).¹⁵¹ However, a relationship was observed between duration of HIV-infection and fractional anisotropy in the corpus callosum, with longer duration associated with lower fractional anisotropy. These results suggest diffusion tensor imaging is very sensitive to HIV-associated white matter microstructural injury and that this begins early in the course of infection and is progressive without antiretroviral treatment.

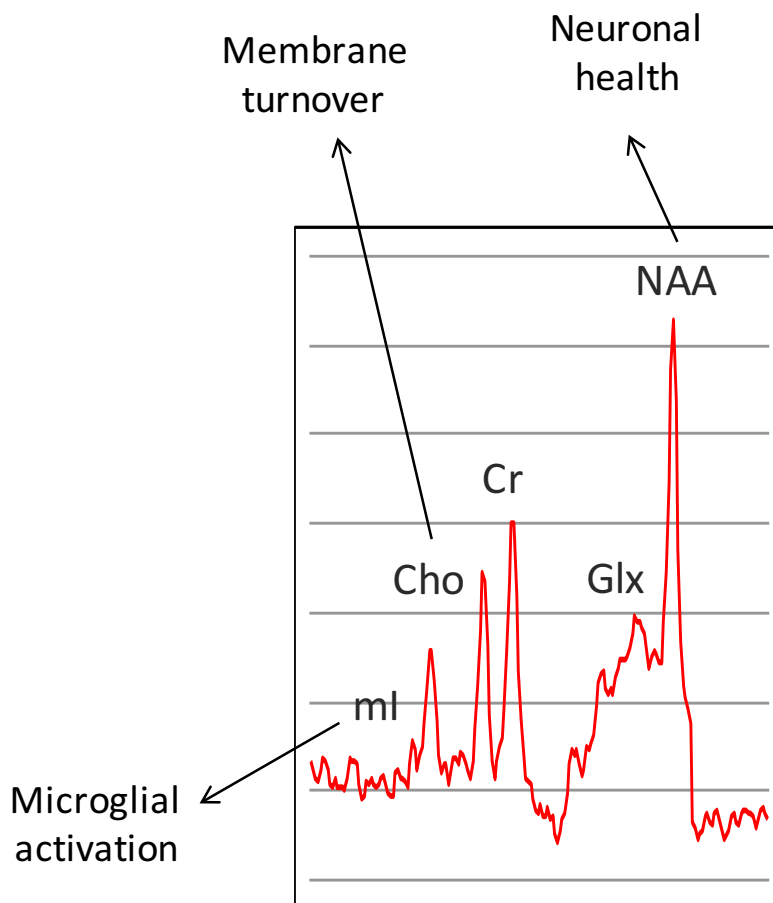
In common with volumetric data, the relationships between HIV-associated diffusion abnormalities and cognitive function are inconsistent, with some studies reporting lower fractional anisotropy in those with impairment but others showing no differences.^{129,130,147,149,150}. The lack of association between diffusion abnormalities in primary HIV-infection and cognitive function¹⁵¹ suggests that these sorts of changes may predate overt cognitive impairment. This may explain the inconsistent relationships with cognitive function whereby widespread changes in diffusion measures have not associated with cognitive impairment. To date there have been no studies specifically looking at the effects of antiretroviral exposure on white matter microstructure using diffusion weighted imaging.

1.5.3 Magnetic resonance spectroscopy (MRS)

Conventional MRI relies on the nuclear properties of protons in water molecules. With suppression of the water signal, proton magnetic resonance spectroscopy (¹H-MRS) allows

the non-invasive quantification of other protons in other metabolites by taking advantage of their differing resonance depending on their surrounding molecular environment.¹⁵² Using a single voxel technique with a short echo time the concentrations of the following metabolites can be accurately determined: choline containing compounds (Cho), which are markers of membrane turnover; creatine/phosphocreatine (Cr), a marker of often used as an internal control to express other metabolites as ratios; myo-inositol (ml), a putative neuroinflammatory marker of microglia; the excitatory neurotransmitter glutamate (Glu) and its metabolite glutamine (Gln) – quantified together as 'Glx' without specific pulse sequences; and N-acetylaspartate (NAA), a marker of neuronal health/viability (see Figure I-G for an example spectra).

Figure I-G. Example spectra with labelled metabolites of interest.



Abbreviations: Cho: choline containing compounds; Cr: creatine; Glx: glutamate/glutamine; ml: myo-inositol; NAA: N-acetylaspartate.

HIV-associated changes

In the 1990s, cerebral atrophy was noted in those with HIV-associated dementia but not in those with more subtle neuropsychological impairment.¹²⁰ This led to interest in using other neuroimaging modalities, such as spectroscopic techniques, which may be more sensitive to brain injury prior to the development of macroscopic atrophy. Many studies have documented a reduction in *N*-acetylaspartate and elevated concentrations of both myo-inositol and choline in HIV-positive individuals, including those on suppressive antiretroviral therapy, with the most marked reductions in those with HIV-associated dementia.^{33,153-157} Generally, inflammatory metabolite changes to frontal white matter seem to occur earliest, i.e. those with modest cognitive impairment, with neuronal loss occurring later and in cortical and sub-cortical grey matter.¹⁵⁴⁻¹⁵⁷ Longitudinal changes in cerebral metabolite concentrations have rarely been studied with one study reporting decreases in all metabolites despite successful antiretroviral therapy in HIV-positive individuals.¹⁵⁸ However, the lack of a control group makes the findings difficult to interpret, however the authors state that the observed declines are greater than would be expected in a healthy population. Most spectroscopy studies have been performed at 1.5T, which limits the ability to resolve metabolites certain metabolites such as glutamate and glutamine. Data acquisition at 3T improves the ability to resolve these metabolites.¹⁵⁹ Lower concentrations of Glx in frontal white matter have been shown in patients with HIV-associated dementia compared to HIV-positive but cognitively normal controls.¹⁵⁷ Concentrations were also negatively correlated with tests of cognitive function. Longitudinal decreases in Glx in the frontal white matter have also been associated with neurocognitive decline.¹⁵⁸

Evidence for antiretroviral neurotoxicity

Few studies have specifically studied the effects of antiretrovirals on cerebral metabolite concentrations. An early study by Schweinsburg *et al*¹⁰⁹ reported significant reductions in *N*-acetylaspartate concentrations, in patients receiving didanosine and/or stavudine compared with those taking zidovudine and lamivudine. This disparity was ascribed to differences in the relative inhibition of DNA-polymerase-gamma and consequent mitochondrial toxicity. Three-year follow-up of a cohort randomised to initiate three different initial antiretroviral regimens showed the reversal of initial improvements in cognitive function and myo-inositol concentrations, despite ongoing successful viral suppression.¹⁰⁶ These data emphasise the

potential dichotomy between antiretroviral efficacy and central nervous system toxicity and highlight the utility of prolonged follow-up to detect dynamic changes. The longitudinal study¹⁵⁸ that reported progressive cerebral injury, both in tests of cognitive function and by reductions in cerebral metabolites may be explained, at least to some degree, by antiretroviral neurotoxicity given the changes occurred despite suppressive antiretroviral therapy.

1.5.4 Other neuroimaging modalities

Other, less commonly used, neuroimaging modalities for the study HIV-disease are functional MRI (fMRI) and PET, neither of which will be studied in this thesis. fMRI takes advantage of the different magnetic properties of oxygenated and deoxygenated blood. Neurovascular coupling, where local blood flow changes in response to neuronal activity, gives rise to the blood-oxygenation-level dependent (BOLD) signal. This is measured with fMRI to provide spatial information about brain activity both in response to a task and at rest. PET is a nuclear medicine technique that uses a tracer that emits positrons and relies on the detection of collinear gamma rays emitted following the annihilation of positron electron pairs. Different tracer compounds provide allow the study of different processes in the brain. For example, fludeoxyglucose (FDG) is used to measure tissue metabolic activity, whereas translocator protein (TSPO) ligands can be used to measure *in vivo* microglial activation.¹⁶⁰

HIV-associated changes

Many fMRI studies have been performed on HIV-infected individuals, with the majority being small, of cross-sectional design comparing HIV-infected and un-infected individuals and task-based.¹⁶¹ Generally, patients and controls performed similarly in terms of behavioural measures (such as test accuracy) but had greater brain activation, particularly in attention networks.¹⁶¹⁻¹⁶⁴ Resting state fMRI studies, where functional connectivity is measured both in and between brain networks whilst the subject is at rest in the scanner (i.e. not performing a task), are less frequent in HIV-disease. Functional connectivity has been reported to be lower in HIV-infected individuals^{165,166} and in those with cognitive impairment.¹⁶⁷ However, none of these studies was performed in exclusively well-treated populations, and changes in brain activation may be driven by unsuppressed central nervous system HIV-replication.¹⁶⁸

As mentioned above, two recent positron emitting tomography (PET) studies^{62,63} have reported persistent microglial activation in HIV-positive individuals, despite effective antiretroviral therapy. These findings contrast with older PET studies,^{64,65} that may reflect differences in tracers used and analysis pipelines.

Effect of antiretrovirals

As fMRI and in particular PET have been performed less frequently in HIV-disease the effects of antiretroviral therapy are largely unknown. There have been no PET studies looking specifically at the effects of antiretroviral therapy. Differences in BOLD signal, between antiretroviral combinations of low and high clinical penetration effectiveness score have been reported.¹⁶⁸ This was hypothesised to be due to combinations with lower penetrance resulting in greater central nervous system HIV-replication and increased BOLD signal. However, only a quarter of patients in this study had suppressed plasma HIV RNA and CSF HIV RNA was not measured, which limits drawing any definite conclusions. Chang *et al*¹⁶² reported that antiretroviral therapy was associated with increased use of reserve brain networks, suggestive of reduced cognitive reserve, and poorer cognitive performance. In contrast, a recent resting state fMRI study reported increased functional connectivity in treated vs. untreated patients, which was more comparable to HIV-negative controls.¹⁶⁵ Taken together, these data suggest antiretroviral therapy may exert a protective effect on brain function but further study is needed to assess whether different combinations of drugs have more or less favourable effects.

1.5.5 Neuroimaging analysis approaches

Univariate and mass univariate approaches

The simplest approach to data analysis is the univariate approach, whereby only one outcome variable is analysed. This approach can be extended to include multiple explanatory variables for example in the case of multiple regression. If multiple outcome variables are measured in the course of a study they will all need to be analysed independently. If many measurements are taken multiple comparisons become more problematic. As the number of tests between two groups increases so does the chance of finding a significant difference by chance (a type I error). Various corrections can be employed with Bonferroni correction the most

conservative. This method divides the significance level (α) by the number of number of tests to determine the corrected significance level for each individual test. Although free of distributional assumptions, the drawback of this method is that it is overly conservative given that most outcome variables in clinical studies will not be fully independent of each other. The traditional approaches for analysing multi-dimensional data, such as neuroimaging, is to reduce the dimensionality of the data and create summary measures (e.g. anterior cingulate grey matter volume), which can then be analysed in a traditional sense with analysis of variance and its relations or alternatively to adopt a 'mass univariate approach'. The mass univariate approach treats the value of each voxel, for example grey matter volume in the context of volumetric data, as an outcome variable and fits a statistical model to each voxel. The traditional approach to model fitting is to use the framework of the general linear model and for inference to test the null hypothesis using *parametric* tests (such as a t-test). This relies on assumptions regarding the null distribution (e.g. the distribution of the residuals from a model are Gaussian), which may not hold for neuroimaging data. A better approach may be to use *non-parametric* methods, such as permutation techniques, where few assumptions about the distribution of data need to be made.¹⁶⁹ The downside is the computational expense, which prior the advent of fast and inexpensive computing made them practically impossible to use for large datasets.

Given the 100,000+ voxels in a typical neuroimaging study multiple comparisons are made and must be accounted for. Using the Bonferroni method is especially conservative in this setting given the inherent correlation between adjacent voxels particularly when spatial smoothing is used as part of the analysis pipeline. The spatial information that neuroimaging data contains can be an asset to distinguish signal from noise. A biological signal is unlikely to be seen at a single voxel as effects are likely to be spatially distributed to some extent, due to the nature of brain pathology and image acquisition. Therefore, finding clusters of significant voxels is more likely to represent a true signal than the same number of voxels with the same significance distributed spatially at random. Using cluster based thresholding is more sensitive than voxel-wise thresholding.¹⁷⁰ However, an arbitrary cluster-forming threshold is needed, which can affect the type of signal detected. An improvement in this method, known as threshold-free cluster enhancement (TFCE) has been proposed.¹⁷¹ This method aims to keep the advantages of cluster-based thresholding while minimising its problems and has been shown to have improved sensitivity compared with other cluster- or voxel-based techniques.¹⁷¹

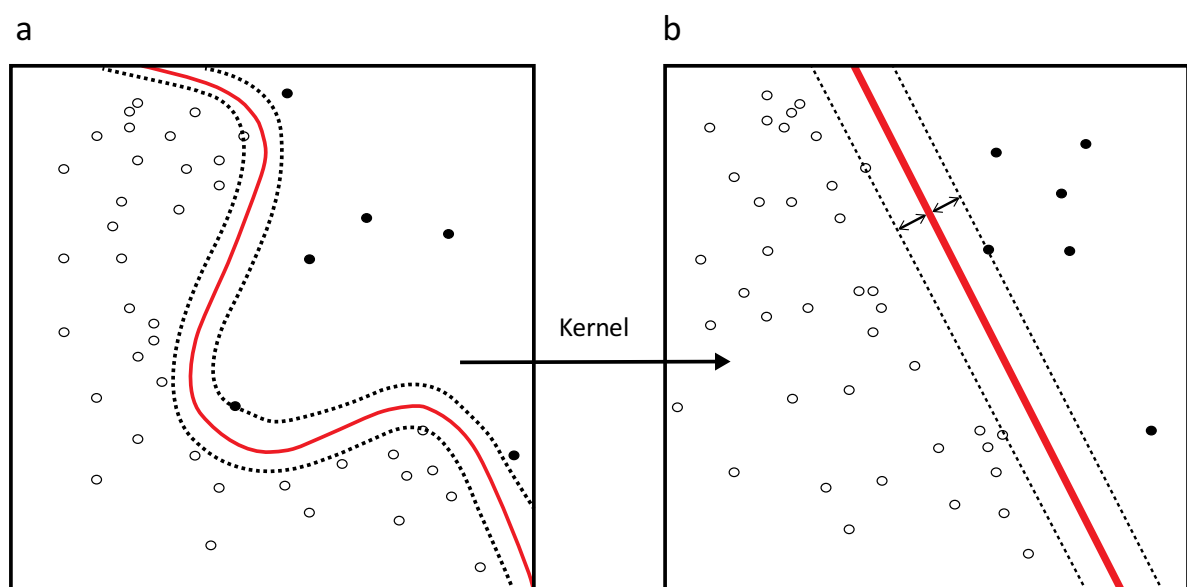
Multivariate techniques

While useful for inference and for building the foundations of modern neuroscience, these mass univariate approaches have limitations, particularly when it comes to applying findings to new data. The mass univariate approach defines a model at each voxel based on data for this voxel across a group of subjects. The model is then tested at each voxel, with contrasts depending on the hypothesis of the experiment, and inferences about brain structure are drawn. This results in comparisons that are valid at a group level. However, these findings have limited application at the individual level thereby limiting their clinical impact.¹⁷² A multivariate approach, i.e. having many outcome variables, aims to do the reverse, namely use many voxels to predict a variable of interest.¹⁷³ This approach may be more suited to neuroimaging data, given its inherent multidimensionality, and has potentially greater sensitivity to detect subtle, spatially distributed patterns of anatomy or activation and can be applied to data at an individual level.^{89,172} Furthermore, machine learning techniques and multivariate pattern analysis have the useful ability of being able to define non-linear functions to allow classification or regression from the data, which can then be applied to new data. This is illustrated graphically in Figure I-H. Conceptually, this is attractive as it would be very difficult to define such a function, for example to predict cognitive impairment from brain volume data, *a priori*.

Multivariate techniques, such as machine learning models, can broadly speaking be used for classification or regression problems. The difference between them is whether the outcome variable to be predicted is discrete (classification) or continuous (regression). A further distinction is the difference between supervised and unsupervised learning. Unsupervised learning is an exploratory technique for the discovery of hitherto unknown structure to the data (i.e. inferring a function from unlabelled data). Whereas, supervised learning uses labelled training data to find structure in the data which can then be applied to new data. In the realm of neuroimaging, supervised classification of structural neuroimaging data has proved useful. For example, support vector machines have yielded accurate classification of Alzheimer's disease¹⁷⁴ and white matter injury following traumatic brain injury¹⁷⁵ as well as in many other disease states.¹⁷² Additionally, the accurate prediction of age in healthy individuals from neuroimaging data alone is possible using an alternative techniques called Gaussian processes regression. This approach has been used to detect deviations from the normal brain ageing trajectory following traumatic brain injury, suggestive of accelerated ageing.¹⁷⁶ These

multivariate approaches have yet to be applied in HIV-disease but conceptually HIV-associated cognitive impairment may be accurately classified on neuroimaging data alone. In this thesis, I will explore both supervised and unsupervised multivariate techniques for the classification and prediction of HIV-associated cognitive impairment. Whilst diagnosis of impairment or prediction of cognitive function solely from an MRI scan is potentially useful clinically, the ability to be able to predict longitudinal changes has the most clinical utility. If accurate, these methods could potentially allow interventions to prevent cognitive impairment from developing.

Figure I-H. An example of non-linear classification using a multivariate technique.



In the original data space (panel a) the groups are only separable with a non-linear function (red) but in a high-dimensional space (panel b) they can be separated with a linear function.

I.6 Soluble biomarkers

Biological measures, or biomarkers, provide the ability to diagnose, monitor and predict disease. They are particularly useful when studying diseases of the central nervous system because of the impracticalities of obtaining tissue for histopathological examination. Soluble biomarkers provide complementary information to the previously discussed neuroimaging biomarkers. Cerebrospinal fluid (CSF) is the optimum fluid to study the central nervous system *in vivo* given that the relative concentration of markers of interest are order of magnitude higher compared to blood.¹⁷⁷ Furthermore, it can be collected safely with lumbar

puncture, a technique that is widely practiced by medical staff and accepted by patients. Broadly speaking, with regards to HIV-associated cognitive impairment, CSF biomarkers can be categorised into directly measuring HIV RNA and indirectly by measuring markers of neuronal injury and neuroinflammation.

CSF HIV RNA has been reported in humans as early as eight days after estimated HIV transmission.¹⁷⁸ In the pre-antiretroviral era CSF HIV RNA correlated with neurological dysfunction with the highest viral load found in those with HIV-associated dementia.¹⁷⁹ In the modern antiretroviral era, the relationship is not as clear due to the different kinetics of CSF HIV RNA and cognitive function in response to suppressive antiretroviral therapy. Plasma HIV RNA is usually an order of magnitude or more greater than in the CSF, with most patients having undetectable levels with standard assays in response to suppressive antiretroviral therapy.^{30,31,178,179} As discussed earlier, changes in cognitive function in response to initiating antiretroviral therapy are variable although generally some improvements are expected.⁵⁵ However, there is clearly a threshold to improvement if permanent brain injury has occurred, supported by the high prevalence of impairment in treated individuals.^{8,13,180} Therefore, as almost all patients can expect to have undetectable CSF HIV RNA in response to antiretroviral therapy the relationship between this biomarker and cognitive function may be lost. However, most studies have used assays of standard sensitivity (<40/50 copies/mL) and therefore it is uncertain if low level CSF viraemia is a useful biomarker of cognitive impairment in well-treated patients. Nevertheless, a recent study¹⁸¹ has suggested that discordant plasma and CSF HIV RNA is associated with up-regulation of many neuroinflammatory mediators, even in patients with discordance only measurable by ultrasensitive methods.

1.6.1 Neuronal injury

Neurofilament proteins

Neurofilaments are structural components of the neuronal cytoskeleton particularly in large calibre myelinated axons (see Figure I-I for an illustration).¹⁸² Cerebrospinal fluid neurofilament light (NFL) concentrations are elevated in numerous neurological disorders and provide a reliable reflection of disease progression including Alzheimer's disease, multiple sclerosis and following traumatic brain injury.¹⁸²⁻¹⁸⁴ In HIV-disease, CSF NFL concentrations have been shown to be elevated in primary HIV-infection¹⁸⁵ but this finding is inconsistent.¹⁸⁶

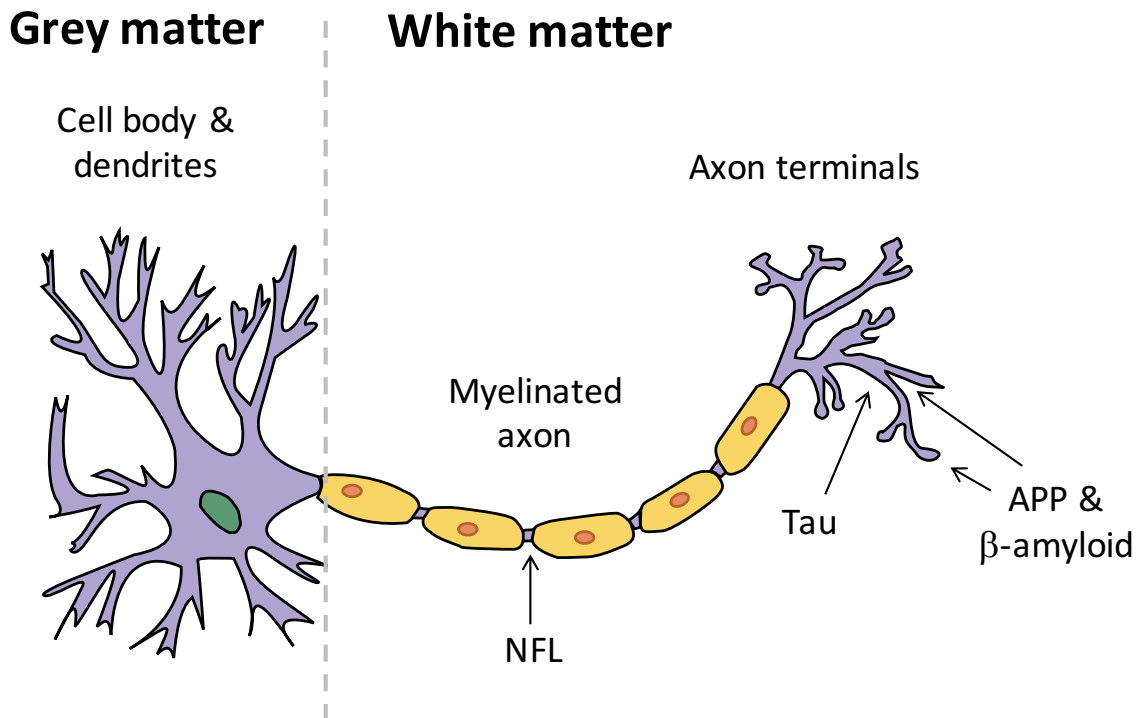
However, elevated CSF NFL concentrations have been consistently documented in chronically infected individuals, with the highest concentrations found in those with the most advanced disease (i.e. lowest CD4⁺ cell counts) and in those with HIV-associated dementia.¹⁸⁵⁻¹⁸⁷ The lowest concentrations have been reported in patients on suppressive antiretroviral therapy suggesting that active injury is correlated with viral replication.^{185,186} This makes NFL a potentially attractive biomarker for distinguishing between active and quiescent HIV-associated brain injury.

Amyloid and Tau proteins

Amyloid precursor protein is an ancient and highly conserved membrane protein expressed in many tissues, particularly in the synapses of neurons (see Figure I-I for an illustration).¹⁸⁸ It is cleaved by various secretases and, in the context of neurodegeneration, it is best known as the precursor protein of the β -amyloid 42 (A β 42) plaques found in Alzheimer's dementia. Together with plaques, the other major histopathological hallmark of Alzheimer's disease is the neurofibrillary tangle, which is comprised of accumulated intracellular hyperphosphorylated tau protein.¹⁸⁹ Tau is a protein involved in the stabilisation of microtubules found mainly in axons and dendrites.

In the context of HIV-associated cognitive impairment, reductions in cerebrospinal fluid A β 42 concentrations comparable to Alzheimer's disease have been reported in those with AIDS-dementia complex.¹⁹⁰ Generally speaking, reductions have been described in those with the most severe HIV-associated cognitive impairment although this finding is not consistent.^{185,191,192} Interestingly, reductions of cerebrospinal fluid A β 42 concentration with a concurrent reduction in soluble amyloid precursor protein were also observed in those with other central nervous system infections^{191,192} suggesting that this may be in response to central nervous system immune activation/inflammation rather than neurodegeneration. This contrasts with Alzheimer's disease where increased concentration of soluble amyloid precursor proteins were observed compared to controls.^{191,192} Further differences in amyloid metabolism between HIV- and Alzheimer's diseases have been shown by positron emitting tomography (PET) imaging, where, even in the context of cognitive impairment, no elevation in amyloid deposition was seen, in contrast to Alzheimer's disease.¹⁹³

Figure I-I. A simplified neuron and the anatomical regions where certain soluble biomarkers may detect injury.



Abbreviations: APP: amyloid precursor protein; NFL: neurofilament light.

Elevation of cerebrospinal fluid Tau concentration is described in many neurodegenerative diseases as well as normal ageing.¹⁸⁹ Elevations of total Tau (t-Tau) but not phosphorylated Tau (p-Tau) are generally described in HIV-disease, with the greatest abnormalities observed in those with HIV-associated dementia.^{185,190,191} In treated individuals, concentrations of A β 42, t-Tau and p-Tau are similar to HIV-uninfected controls. However, a small histopathological study has reported increased p-Tau in the brains of HIV-positive individuals compared to age matched controls including those on treatment.¹⁹⁴

There are some data suggestive of potentially neurotoxic effects associated with these proteins. Well-treated patients in the previous study had elevated p-Tau in an absence of other HIV-related neuropathology, whereas patients with HIV-encephalopathy did not. Another post-mortem examination study by the same group,¹⁹⁵ observed microglial activation in treated patients comparable to that seen in HIV-encephalitis. However, the usual accompanying lymphocytic infiltration was not observed and it was most marked in the hippocampus, an area not traditionally associated with HIV-associated brain injury. These distinct patterns may represent the histopathological hallmarks of antiretroviral neurotoxicity as they are not easily explained by HIV-infection per se. Antiretroviral therapy has also been

associated with potentially toxic effects on neuronal amyloid metabolism. In a neuronal cell culture experiment, the NRTIs zidovudine, lamivudine and abacavir were associated with increased neuronal production and reduced microglial clearance of β -amyloid.¹⁰⁸ Similar changes have been observed in mice and murine neuronal cell lines in association with the NNRTI efavirenz.¹⁹⁶

1.6.2 Neuroinflammation

Many inflammatory biomarkers have been studied in HIV-disease. The biomarkers most consistently associated with HIV-associated cognitive impairment are those pertaining to monocyte, macrophage and microglial activation.¹⁹⁷ Neopterin, a metabolite of guanosine triphosphate, is secreted by macrophages and microglial cells in response to infection, autoimmune and neurodegenerative diseases.¹⁹⁷ Cerebrospinal fluid concentrations have been shown to be elevated in HIV-disease generally,¹⁹⁸ in primary HIV-infection¹⁷⁸ and more specifically in those with cognitive impairment despite suppressive treatment.¹⁹⁹ Soluble CD14 (sCD14) and CD163 (sCD163) are both soluble receptors, for lipopolysaccharide and haemoglobin respectively, secreted by activated monocytes and macrophages.¹⁹⁷ Elevated plasma sCD14 and sCD163, suggestive of systemic immune activation, have both been associated with HIV-associated cognitive impairment.^{199,200} Elevated plasma concentrations in successfully treated individuals are less consistent but have been reported to be elevated compared to HIV-uninfected controls.^{130,199} CSF relationships are less clear but sCD14 correlates with Cho/Cr ratios measured by MRS indicating it may be a useful biomarker of central nervous system inflammation.^{197,199,201} More recently, perturbations of tryptophan metabolism have been recognised as crucial components of HIV-associated immune dysfunction.^{202,203} Expression of indoleamine 2,3-dioxygenase by antigen presenting cells metabolises tryptophan, an essential amino acid, via the kynurenine pathway. This plays an important part of the innate immune response leading to antibacterial and antiviral effects.^{202,203} Other tryptophan metabolites via the kynurenine pathway, such as quinolinic acid have been associated with the AIDS dementia complex in untreated patients.²⁰⁴ Furthermore, improvements in the kynurenine:tryptophan ratio following antiretroviral therapy may explain some of the improvements in mood associated with initiation of antiretroviral therapy.²⁰⁵ These findings provide some evidence for a neuroinflammatory aetiology of depression in HIV-disease and highlight the potential neurological implications of this metabolic pathway. However, its role as a biomarker of cognitive impairment in treated HIV-disease it is unclear.

I.7 Summary

In summary, many questions remain regarding cognitive impairment in treated HIV-disease. The reported prevalence of impairment may exceed what would be considered clinically significant due to methodological problems of diagnosis. The associations with other aspects of health in well-treated individuals remains unclear. As do the pathogenic mechanisms and whether antiretroviral central nervous system toxicity has an aetiological role. In this thesis, I will address these questions to test my hypotheses (section I.1) using synthetic, clinical, neuroimaging and soluble biomarker data with advanced exploratory techniques.

I.7.1 Thesis aims

The aims of this thesis are to:

1. Describe in detail the prevalence and the nature of cognitive impairment in contemporary, well-treated HIV-positive cohorts. Including:
 - a. assessing its association with other patient reported outcome measures, such as independence with activities of daily living.
 - b. Comparing different methodologies for the diagnosis of cognitive impairment.
2. Understand the pathogenesis of cognitive impairment in well-treated patients using neuroimaging and soluble biomarkers.
3. To develop predictive models that can identify cognitive impairment and potentially predict longitudinal changes in cognitive function.

I.7.2 Major research question of this thesis

1. What is the prevalence of HIV-associated cognitive impairment in well-treated, cohorts?
2. How does the prevalence of HIV-associated cognitive impairment vary depending on the methodology used to define it?
3. Is there an optimal way to define cognitive impairment?
4. What are the characteristics of HIV-associated cognitive impairment in treated HIV-disease?
5. Is HIV-associated cognitive impairment associated with other symptoms as a part of a syndrome of general ill health in treated HIV-disease?

6. What is the underlying pathophysiology of HIV-associated cognitive impairment treated HIV-disease?
7. Is there any evidence antiretrovirals are associated with central nervous system toxicity?
8. Is HIV-associated cognitive impairment a progressive neurodegenerative disease in virologically suppressed individuals?
9. Can HIV-associated cognitive impairment be predicted using just neuroimaging data?
10. Can longitudinal cognitive function be predicted using just neuroimaging data?

Chapter 2

Methods

Table of contents

CHAPTER 2 METHODS	56
2.1 CLINICAL STUDIES.....	58
2.1.1 <i>The POPPY study.....</i>	<i>58</i>
2.1.2 <i>The COBRA study.....</i>	<i>61</i>
2.1.3 <i>The CHARTER study</i>	<i>64</i>
2.2 CLASSIFICATION OF COGNITIVE IMPAIRMENT	66
2.2.1 <i>HIV-associated Neurocognitive Disorder ('Frascati' criteria).....</i>	<i>66</i>
2.2.2 <i>Global deficit score (GDS).....</i>	<i>66</i>
2.2.3 <i>Multivariate normative comparison (MNC)</i>	<i>67</i>
2.2.4 <i>Other methods</i>	<i>67</i>
2.3 NEUROIMAGING.....	68
2.3.1 <i>Neuroimaging acquisition</i>	<i>68</i>
2.3.2 <i>Image processing.....</i>	<i>70</i>
2.4 BLOOD AND CEREBROSPINAL FLUID SAMPLING.....	75
2.5 ANTIRETROVIRAL PHARMACOKINETICS	75
2.6 SOLUBLE BIOMARKERS	76
2.6.1 <i>Neuronal injury.....</i>	<i>76</i>
2.6.2 <i>Neuroinflammation</i>	<i>77</i>
2.6.3 <i>HIV RNA.....</i>	<i>77</i>
2.7 STATISTICS	77
2.7.1 <i>Simple group comparisons</i>	<i>77</i>
2.7.2 <i>Comparing different methods of cognitive impairment</i>	<i>78</i>
2.7.3 <i>Cognitive impairment and patient reported outcome measures</i>	<i>79</i>
2.7.4 <i>Group comparisons of volumetric and data</i>	<i>79</i>
2.7.5 <i>Combining imaging and cognitive data.....</i>	<i>81</i>
2.7.6 <i>Multivariate neuroimaging analysis.....</i>	<i>82</i>

Figures

FIGURE 2-A. FRONTAL WHITE MATTER VOXEL PLACEMENT	69
FIGURE 2-B. GREY MATTER SEGMENTATION.....	71
FIGURE 2-C. CALCULATING GREY MATTER VOLUME FOR REGIONS OF INTEREST.	71
FIGURE 2-D. AN EXAMPLE FRACTIONAL ANISOTROPY MAP COLOURED BY THE PRINCIPAL DIRECTION OF DIFFUSION.	72
FIGURE 2-E. EXAMPLE FRACTIONAL ANISOTROPY, AXIAL, MEAN AND RADIAL DIFFUSIVITY MAPS AND FORMULAE FOR CALCULATION.	73
FIGURE 2-F. SKELETONISATION.....	73
FIGURE 2-G. EXAMPLE SPECTRA OF VARYING QUALITY.	75
FIGURE 2-H. GRAPHICAL ILLUSTRATION OF NON-LINEAR CLASSIFICATION USING SUPPORT VECTORS.....	84

Tables

TABLE 2-A. COGNITIVE TESTS ADMINISTERED IN THE POPPY STUDY BY COGNITIVE DOMAIN WITH DATA TRANSFORMATIONS.	60
TABLE 2-B. COGNITIVE TESTS ADMINISTERED IN THE COBRA STUDY BY COGNITIVE DOMAIN.	63
TABLE 2-C. COGNITIVE TESTS ADMINISTERED IN THE CHARTER STUDY BY COGNITIVE DOMAIN.	65
TABLE 2-D. DATA SOURCES FOR NORMATIVE HEALTHY BRAIN AGE SAMPLE USED TO DEVELOP PREDICTIVE MODEL OF BRAIN AGEING.	88

2.1 Clinical studies

2.1.1 The POPPY study

The Pharmacokinetic and Clinical Observations in PeoPle Over fiftY (POPPY) study (ClinicalTrials.gov Identifier: NCT01737047) is a multi-centre, prospective cohort study primarily investigating the effects of ageing and comorbidities in HIV-positive individuals in the UK and Ireland.

Participants

Participants were prospectively enrolled into the POPPY study at seven HIV-outpatient sites across the UK and Ireland. I recruited patients at one site (St Mary's Hospital) with the help of a study nurse. Inclusion criteria were the documented presence or absence of HIV-infection, self-defined white or Black-African ethnicity, age >50 years at study entry (HIV-negatives only) and the ability to comprehend the study patient information leaflet. Additional inclusion criteria for the HIV-positive participants were probable route of HIV acquisition via sexual exposure (either by male to male exposure if white or by heterosexual exposure if white or Black-African). These particular inclusion criteria were chosen as these groups make up the vast majority of people living with HIV in the UK.²⁴ Subjects with a life expectancy less than 6 months or those unable or unwilling to comply with the requirements of the study (in the opinion of the investigator) were excluded given the original planned follow-up for the study was two years (with an interim study visit at one year). Considerable care was taken to recruit an appropriate HIV-uninfected control group from sexual health clinics and targeted community groups. They were also frequency matched by gender, ethnicity, sexual orientation and location (in or out London). For the purposes of analysis in this thesis only HIV-positive and HIV-negative participants aged >50 years were included for analysis. An additional HIV-positive cohort aged <50 years was also recruited but as there was no comparable HIV-negative control group aged <50 years they were not included for analysis in this thesis.

All participants provided written informed consent. The study was approved by the UK National Research Ethics Service (NRES; Fulham, London, UK -reference number 12/LO/1409).

Cognitive testing

POPPY participants underwent cognitive function testing using a computerised battery (CogState™, CogState Ltd, Melbourne, Australia) covering six cognitive domains (see Table 2-A for details of tests performed and how they map to each cognitive domain). This was performed by trained research staff at all sites. The battery consisted of eleven individual tasks in the form of card-games or shapes to minimise language and cultural differences and took approximately 45-60 minutes to complete. This has been shown to be a sensitive diagnostic tool for the assessment of HIV-associated cognitive impairment and allows standardised assessment to be completed in a reasonable amount of time across different sites in multicentre studies.^{206,207}

Due to the non-normality of the raw data, raw test scores were log-transformed or arcsine root-transformed where necessary (as recommended by the CogState guidelines for analysis – see Table 2-A for details) and converted into demographically-adjusted T-scores (with a mean of 50 and a standard deviation of 10) using the HIV-uninfected control group as the reference population. This method was chosen as the CogState norms do not have adequate normative data for older individuals. The amalgamation of data from different sites and this normalisation step was performed by Davide De Francesco at the Research Department of Infection and Population Health, University College London using SAS v9.4. These adjusted T-scores took into account age, level of education, gender and ethnicity as appropriate. A single domain T-score was calculated for each of the six cognitive domains by averaging individual T-scores within each domain. A global T-score was also obtained by calculating the mean of the six individual domain T-scores. For all T-scores, higher scores represent better cognitive function.

Table 2-A. Cognitive tests administered in the POPPY study by cognitive domain with data transformations.

Cognitive domain	Test administered	Scoring system
Attention/Working Memory	One back task	Arcsine root-proportion correct
	Two back task	Arcsine root-proportion correct
Executive Function	Groton Maze Learning test	Total errors
	Set shifting task	Total errors
Processing speed	Detection task	Log reaction time (ms)
Visual Attention	Identification task	Log reaction time (ms)
Verbal Learning/Memory	International Shopping list task	Total correct
	International Shopping list task – delayed recall	Total correct
Visual Learning/Memory	Continuous paired associate learning test	Total errors
	Groton Maze Learning test – delayed recall	Total errors
	One card learning task	Arcsine root-proportion correct

Patient reported outcome measures (PROMs)

All participants answered the previously recommended European AIDS Clinical Society (EACS) cognitive complaints screening questions²⁰⁸: ‘Do you experience frequent memory loss?’; ‘Do you feel that you are slower when reasoning, planning activities, or solving problems?’ and ‘Do you have difficulties paying attention?’. Participants also completed validated questionnaires detailing: physical & mental health with the Short Form Health Survey²⁰⁹ (SF-36); activities of daily living with the Lawton Instrumental Activities of Daily Living²¹⁰ (IADL); depression, using the Patient Depression Questionnaire²¹¹ (PHQ-9) and the Centres for Epidemiologic Studies Depression scale²¹² (CES-D). Additionally, falls and sexual desire were assessed by asking ‘over the past 28 days have you had any falls?’ and ‘how often have you worried about minimal sexual desire during the last 4 weeks’ respectively. Outcomes were then dichotomised as follows:

- *Cognitive screening*: responses of ‘never’ or ‘hardly ever’ were recoded as negative with ‘yes, definitely’ being recorded as positive;
- *Activities of daily living*: IADL scores of 8 were recorded as ‘no impairment’ with scores of <8 being recoded as ‘impairment’;
- *Depression*: PHQ-9 scores of <10 and CES-D scores of <16 were each recoded as ‘no signs of depression’, with PHQ-9 scores ≥ 10 /CES-D scores ≥ 16 being recorded as ‘signs of depression’;

- *Falls*: responses of 'I have not fallen' were recorded as 'no', with any responses indicating >1 fall being recoded as 'yes';
- *Sexual desire*: responses of 'never' or 'rarely' were recorded as 'no problems with sexual desire' with responses of 'now and then', 'often' or 'always' being recoded as 'problems with sexual desire'.

2.1.2 The COBRA study

The Co-morBidity in Relation to AIDS (COBRA) study is an EU FP7-funded (grant agreement number 305522), prospective, multi-centre, study of the pathogenesis of age-associated non-communicable comorbidities in HIV-positive individuals.

Participants

Participants were prospectively enrolled into the COBRA study from HIV-outpatient sites in London and Amsterdam, where I was responsible for recruitment of all study participants in London. To minimise confounding, a demographically comparable HIV-negative control group was recruited from sexual health clinics and specific community groups, with considerable care taken to ensure similar demographic, sexual and other risk taking characteristics to the HIV-positive group.

To determine the prevalence and pathogenesis of HIV-associated cognitive impairment in older individuals, participants over 45 years of age were recruited. Participants were eligible only if they did not have potentially confounding neurological conditions, substance or alcohol abuse or moderate/severe depression. Specific exclusion criteria were: current major depression (PHQ-9 score of >14 at study screening), the presence of any confounding neurological diseases, history of severe head injury (defined as loss of consciousness for >30 minutes), a history of cerebral infections (including AIDS defining illnesses), self-reported current intravenous drug use (in the past six months), daily use of recreational drugs (with the exception of cannabis) or excess alcohol use (>48 units per week), severe psychiatric disease, a contraindication for MRI or lumbar puncture examination. As the purpose of the study was to investigate the prevalence and pathogenesis of cognitive impairment in treated HIV-disease, all HIV-positive participants were required to be taking combination

antiretroviral therapy and to have had an undetectable plasma HIV RNA (defined as <50 copies/mL) recorded for at least 12 months prior to enrolment.

All participants gave written informed consent before screening. This study was approved by the institutional review board of the Academic Medical Center (AMC) (reference number 2009_327 / NL 30802.018.09) and a UK Research Ethics Committee (REC) (reference number 13/LO/0584 London – Stanmore).

Cognitive testing

COBRA subjects completed a comprehensive battery of neuropsychological tests assessing attention, executive function, language, memory, speed of information processing and motor function (see *Table 2-B* for further details), which lasted approximately 90-120 minutes. This was performed by trained research staff at both sites using a harmonised protocol to ensure comparable data. These domains and individual tests were chosen for their previous sensitivity to detect HIV-associated cognitive impairment^{8,10,13,213} and following previously published international guidelines.²⁵

Consistent testing and scoring of neuropsychological tests across study sites was achieved with a harmonised study protocol, standardised training and supervision of research staff. Raw cognitive function test scores were converted to standardised T-scores accounting for age and level of education with higher T-scores representing better cognitive function according to published norms for each test (with language/country specific norms where appropriate and available). As with the POPPY study, test scores were averaged within domains to calculate domain specific T-scores and across domains to calculate a global T-score.

Table 2-B. Cognitive tests administered in the COBRA study by cognitive domain.

Cognitive domain	Test administered	Scoring system
Attention	PASAT 3	Total correct summations
	WAIS-III Letter-number sequencing	Total correct sequences
Executive function	Trail Making Test-B	Total time to complete (s)
	Wisconsin Card Sorting Test	Number of total errors
	Wisconsin Card Sorting Test	Number of perseverative errors
	Wisconsin Card Sorting Test	Number of perseverative responses
Language	Category Fluency	Total number of animals in 1 minute
	Category Fluency	Total number of occupations in 1 minute
	Letter Fluency	Total number of words, 1 minute for each of 3 letters
Memory	Rey Auditory Verbal Learning	Total recalled words trials 1-5
	Rey Auditory Verbal Learning	Total words recalled
	WMS-IV Visual Reproduction	Immediate recall
	WMS-IV Visual Reproduction	Delayed recall
Motor function	Grooved pegboard	Dominant hand: Time to complete (s)
	Grooved pegboard	Non-dominant hand: Time to complete (s)
	Finger tapping	Dominant hand: Median number of taps
	Finger tapping	Non-dominant hand: Median number of taps
Processing speed	Trail Making Test-A	Total time to complete (s)
	WAIS-III Digit Symbol	Total correct symbols
	WAIS-III Symbol Search	Total correct symbols
	Stroop colour-word test	Number of items completed

Abbreviations: PASAT = Paced auditory serial-addition task; WAIS-III = Wechsler Adult Intelligence Scale - Third Edition; WMS-IV = Wechsler Memory Scale - Fourth Edition.

2.1.3 The CHARTER study

The CNS HIV Antiretroviral Therapy Effects Research (CHARTER) study was designed to examine cognitive function in a large cohort reflective of HIV-positive patients attending for care at university-affiliated HIV treatment centres in the USA.⁸

Participants

In total, 1,555 subjects were initially enrolled in the CHARTER study from 2003-2007 from six participating university centres. To ensure the cohort was representative of people living with HIV attending for care at the study sites, participants were recruited with minimal exclusions. Subsequently, subjects were enrolled into a neuroimaging sub-study (n=251) from five participating university sites in the USA: Johns Hopkins University (Baltimore); Mt. Sinai School of Medicine (New York); University of California at San Diego (San Diego); University of Texas Medical Branch (Galveston) and the University of Washington (Seattle).¹²⁸ Due to the broad inclusion and minimal exclusion criteria of the CHARTER study, all participants' historical and contemporary data was reviewed to determine the presence of potentially confounding comorbid conditions. These were classified as 'incidental', 'contributing' and 'confounding' according to the Antinori et al²⁵ guidelines. For further details please see Heaton et al⁸. To study the relationships between structural imaging measures and cognitive function in an independent cohort, I arranged for clinical, neuroimaging and neuropsychological data from the CHARTER study to be transferred for further analysis. To ensure comparability with contemporary cohorts and to assess relationships between brain structure and cognitive function without the confounding effect of untreated HIV-infection, only participants with plasma HIV RNA <50 copies/ml were included (n=139) for all analyses in this thesis.

Written informed consent was obtained from all study participants. CHARTER study procedures were approved by the Human Subjects Protection Committees of each participating institution.⁸

Cognitive testing

All participants completed a comprehensive neurocognitive test battery, covering seven cognitive domains, administered by trained research staff at each site using a harmonised

protocol (Table 2-A summarises the cognitive tests performed and scoring system) - see Heaton *et al*⁸ for further details of the tests of cognitive function administered in the CHARTER study and for the results of the entire cohort. As before, raw test scores were subsequently adjusted for age, education, sex and ethnicity, as appropriate, using normative data,⁸ and converted to demographically corrected T-scores.

Longitudinal changes in cognitive function

Longitudinal cognitive data was available for 111 subjects who had baseline neuroimaging data and suppressed plasma HIV viraemia at 6 monthly intervals for up to 2 years. Longitudinal changes in cognitive function were defined using the first and last available global T-scores. The global deficit score was not used as the global T-score allows more granularity of measurement. Clinically significant cognitive decline was defined as a global T-score >5 points lower at follow-up compared to baseline. This definition was chosen as a longitudinal change in global z-score of 0.5 (equivalent to T-score changes of 5) has been used as an endpoint in a therapeutic clinical trial of HIV-associated cognitive impairment²¹⁴ and has been deemed a 'clinically significant' change in cognitive function.

Table 2-C. Cognitive tests administered in the CHARTER study by cognitive domain.

Cognitive Domain	Test administered	Scoring system
Attention/ Working Memory	WAIS-III Letter-Number Sequencing	Total correct sequences
	PASAT (1 st channel only)	Total correct summations
Executive function	Wisconsin Card Sorting Test	Number of perseverative responses
	Trail Making Test, Part B	Total time to complete (s)
Learning	Story Memory Test	Total points divided by number of trials
	Figure Memory Test	Total points divided by number of trials
Memory	Story Memory Test	Percentage recalled
	Figure Memory Test	Percentage recalled
Motor function	Grooved Pegboard Test	Dominant hand: Time to complete (s)
		Non-dominant hand: Time to complete (s)
Processing speed	WAIS-III Digit Symbol	Total correct symbols
	WAIS-III Symbol Search	Total correct symbols
	Trail Making Test, Part A	Time to complete (s)
Verbal Fluency/ Language	Controlled Oral Word Association Test	Total number of words, 1 minute for each of 3 letters
	Category Fluency	Total number of animals in 1 minute

Abbreviations: PASAT = Paced auditory serial-addition task; WAIS-III = Wechsler Adult Intelligence Scale - Third Edition; WMS-IV = Wechsler Memory Scale - Fourth Edition.

2.2 Classification of cognitive impairment

For all three clinical studies in this thesis I have applied consistent definitions and methodology to ensure valid comparisons. The following sections detail how cognitive impairment was defined.

2.2.1 HIV-associated Neurocognitive Disorder (‘Frascati’ criteria)

The HIV-associated neurocognitive disorder (HAND) classification,ⁱ known commonly as and hereby referred to the ‘Frascati criteria’ defines cognitive impairment in those that score one standard deviation below the normative mean in two or more cognitive test/domains. These criteria were applied to mean domain T-scores to minimise spurious false positives due to multiple comparisons (see introduction for further discussion of this issue). Participants with Frascati defined impairment were further subdivided using Lawton Instrumental Activities of Daily Living (IADL),⁴² for descriptive purposes only to avoid circularity in assessing the relationships between objective measures of cognition and subjective symptomology as well as allowing comparison with other methods that do not subdivide those with impairment.

2.2.2 Global deficit score (GDS)

This global deficit score aims to be most comparable to clinician rating, the purported ‘gold standard’ and has shown to have good predictive and discriminatory power in HIV-positive individuals.²⁶ A deficit score was obtained for each cognitive domain by converting the domain T-scores as follows: 0: T-score $\geq$ 40, 1: 34<T-score<40, 2: 29<T-score $\leq$ 34, 3: 24<T-score $\leq$ 29, 4: 19<T-score $\leq$ 24, 5: T-score $\leq$ 19. The global deficit score was calculated as the mean of the domain deficit scores with cognitive impairment defined as a global deficit score $\geq$ 0.5. By averaging over cognitive domains, the multiple comparison issue is addressed.

ⁱ To avoid confusion with the term HAND, which is often used in the literature as a catch-all term for HIV-associated cognitive dysfunction not specifically referring to this particular classification method, I will throughout this thesis refer to this diagnostic method as the Frascati criteria unless explicitly explained.

2.2.3 Multivariate normative comparison (MNC)

The Hotelling's T^2 statistic was calculated using the HIV-uninfected controls as a reference. This takes into account performance in all cognitive domains as well as the covariance between them.⁴⁰ In the context of a lack of a gold standard, this method has been shown to optimally balance sensitivity and specificity for the diagnosis of HIV-associated cognitive impairment.¹³ As the Hotelling's T^2 reflects the distance from a multivariate mean it has no direction. As such, to determine whether its value represented better/worse cognitive performance relative to the multivariate mean (i.e. to give it direction as well as magnitude) the Hotelling's T^2 statistic for each participant was multiplied by -1 if the global T-score > 50, so that increasing values represented poorer cognitive performance. A critical value was defined based on the F -distribution (degrees of freedom: the number of cognitive tests, p ; and $n-p$) so that the family-wise false discovery rate was approximately 5% (i.e. type I error rate of 0.05), which, if exceeded, defined a subject as cognitively impaired. For further details please see Follman²¹⁵ and Huizenga *et al.*⁴⁰

2.2.4 Other methods

For the purposes of assessing relationships between cognitive impairment and patient reported outcome measures in the POPPY study other methods of defining impairment were tested. A more stringent, combined definition of cognitive impairment was assessed, whereby participants had to meet the Frascati, GDS *and* MNC criteria to be defined as impaired. Furthermore, another novel definition of cognitive impairment, using a global T-score threshold of <45, was also tested. This value was chosen as it is equivalent to a change in z-score (an alternative way of expressing standardised cognitive function with normative mean of 0 and standard deviation of 1) of 0.5. This has been used as a primary outcome in clinical trials assessing interventions for the management of HIV-associated cognitive impairment and is thought to represent a clinically significant difference in cognitive function.²¹⁴ These methods were not used in the other studies because of the smaller sample size and because detailed analyses using cognitive measures as continuous rather than categorical variables were preferable to study relationships in more depth.

2.3 Neuroimaging

2.3.1 Neuroimaging acquisition

Neuroimaging for the COBRA study was acquired at 3T using a harmonised protocol (within given hardware constraints) across the two study sites in London and Amsterdam. In Amsterdam, images were acquired initially with a Philips Intera (n=66) and then using a Philips Ingenia (n=59 both Philips Healthcare, Best, the Netherlands) due to a scanner upgrade – subsequently to be referred to as scanner 1 and 2 respectively. In London, images were acquired using a Siemens Verio scanner (n=88, Siemens Healthcare, Erlangen, Germany) - subsequently to be referred to as scanner 3.

Neuroimaging for the CHARTER study was performed on General Electric 1.5T scanners, with annual assessment of quality (see Jernigan *et al*^{185,191,192} for further details) at Johns Hopkins University (n=30); Mt. Sinai School of Medicine (n=25); University of California at San Diego (n=47); University of Texas Medical Branch (n=29) and the University of Washington (n=8).

Structural MRI (COBRA and CHARTER)

COBRA

High-resolution 3D T1-weighted structural images were acquired at both sites using the following parameters, in London: magnetisation-prepared rapid gradient-echo (MPRAGE), TR = 2300 ms, TE = 2.98 ms, TI = 900ms, flip angle = 9°, field-of-view = 256 mm, 160 slices of 1.0 mm thickness, in-plane resolution = 1.0 × 1.0 mm. In Amsterdam: sagittal Turbo Field Echo (T1-TFE), TR = 6.6ms, TE = 3.1ms, flip angle = 9°, field-of-view = 270 mm, 170 slices of 1.2 mm thickness, in-plane resolution = 1.1 × 1.1 mm.

CHARTER

3D T1-weighted images were acquired sagittally with spoiled gradient recall (SPGR), TR = 20ms, TE = 6ms, flip angle = 30°, field-of-view = 240mm, 124 slices of 1.3mm, in-plane resolution 0.94x0.94mm.

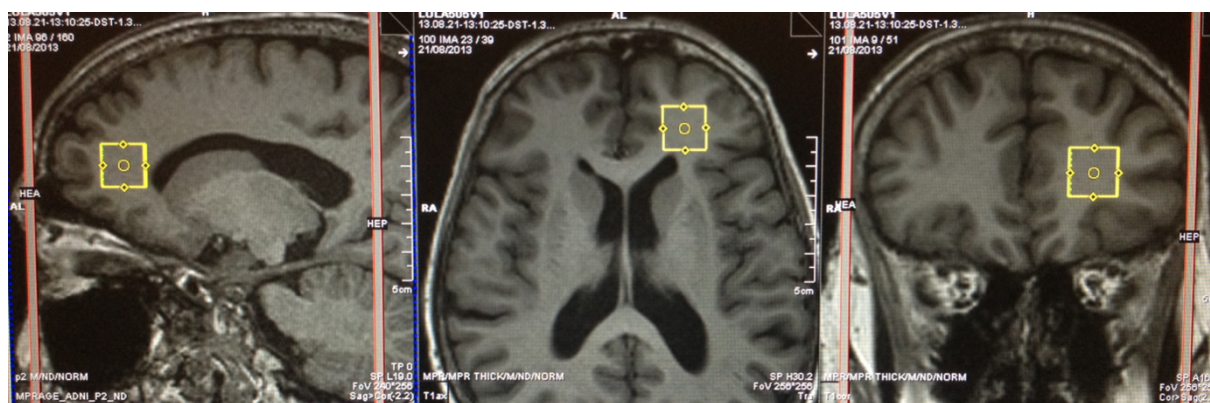
Diffusion weighted imaging (COBRA only)

Diffusion weighted images were acquired at both sites along 64 non-collinear directions with $b=1000\text{s/mm}^2$ and four averages with $b=0\text{s/mm}^2$. In London: TE/TR 103/9,500 ms, 64 contiguous slices, FoV 256 mm, voxel size 2mm^3 . In Amsterdam: TE/TR 92/7081-9665 ms, 64 contiguous slices, FoV 270 mm, voxel size 2mm^3 .

Magnetic resonance spectroscopy (COBRA only)

Single voxel spectroscopy was performed with $15\text{ mm} \times 15\text{ mm} \times 15\text{ mm}$ voxels placed in the left frontal white matter, left putamen and the anterior cingulate cortex (ACC, in London only) – frontal grey matter. These voxels were chosen to represent white matter, sub-cortical and cortical grey matter respectively. Voxel placement was performed by an experienced radiographer and was standardised within subjects and across sites while taking account of different brain sizes (see Figure 2-A for an example of frontal white matter voxel placement). Spectra were obtained at each voxel with and without water suppression using a water suppression enhanced through T1 effects (WET)²¹⁶ algorithm. Water suppressed spectra were obtained using a double spin-echo point-resolved spectroscopy sequence (PRESS) with the following parameters in London: TR 2,000 ms, TE 30ms, 96 averages; in Amsterdam TR 2,000ms TE 37ms, 64 averages.

Figure 2-A. Frontal white matter voxel placement.



Example of left frontal white matter voxel (yellow box) placement, from left in sagittal, axial and coronal views.

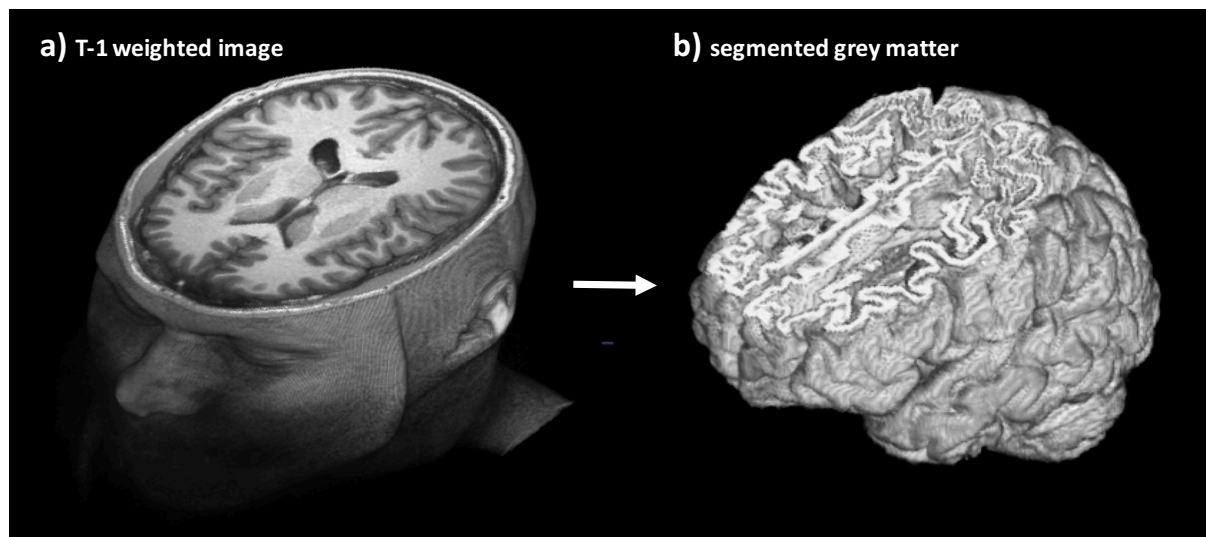
2.3.2 Image processing

Structural imaging

3D T1-weighted images were pre-processed using SPM12 (University College London, London, UK). Firstly, images were bias corrected to remove the smooth, spatially varying artefact that often modulates the intensity a T1-weighted image and can impede automated processing of the images. This was achieved with a 60mm full width at half maximum (FWHM) kernel. Next, bias-corrected images were segmented into grey matter, white matter and cerebrospinal fluid with removal of non-brain tissue using 'segment' (see *Figure 2-B* for an example of grey matter segmentation). Segmentation was achieved using tissue probability maps with two Gaussian intensity distributions for grey matter, two for white matter, two for CSF, three for bone, four for soft tissue and two for air to account for possible partial volume effects. A study specific registration template was generated using all the subjects segmented grey and white matter for all participants. Segmented grey and white matter were subsequently registered to the custom template and normalised to Montreal Neurological Institute (MNI) space using the diffeomorphic anatomical registration using exponentiated lie algebra (DARTEL) algorithm.²¹⁷ This method of image registration has been shown to be superior to many other commonly used methods such as FSL's FMRIB's Linear Image Registration Tool (FNIRT).²¹⁸ To account for the expansion and contraction resulting from spatial normalisation, images were modulated to retain the volumetric characteristics of the original data. A 6mm FWHM smoothing kernel was used prior to voxelwise inference testing, which was chosen given the sample size of the COBRA and CHARTER studies as per Shen and Sterr.²¹⁹ After each step, the images for each subject were visually inspected to ensure each process had been successful. Brain tissue volumes (total grey matter, white matter and cerebrospinal fluid) were then calculated for each participant using SPM12.

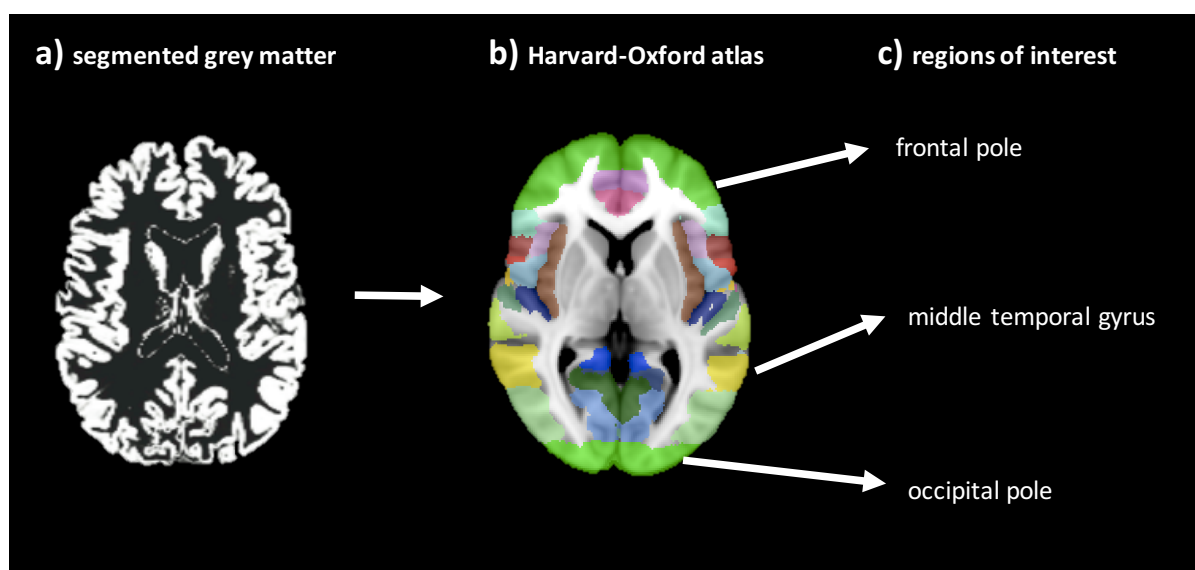
To obtain data from regions of interest and as a data reduction step prior to subsequent unsupervised machine learning with cluster analysis each individual's normalised grey matter was projected through masks created for each region of the Harvard-Oxford cortical and subcortical atlases (threshold 0.25) to calculate the total grey matter volume for each region. This process is illustrated in *Figure 2-C*.

Figure 2-B. Grey matter segmentation.



Automated segmentation allows the accurate delineation of grey matter (panel b) from the raw T1 image (panel a).

Figure 2-C. Calculating grey matter volume for regions of interest



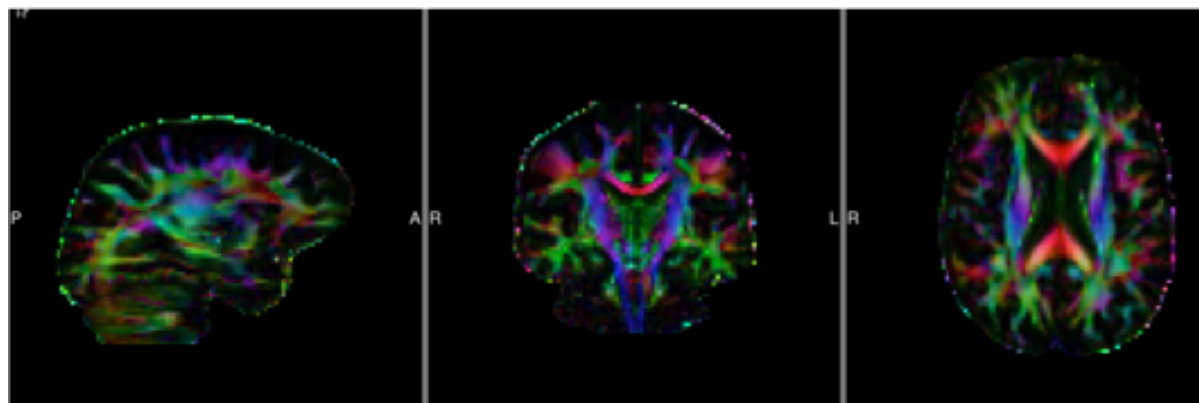
Grey matter volume for regions of interest were calculated by overlaying a specific mask for each region (panel b, defined using the Harvard-Oxford cortical and sub-cortical atlases) over segmented grey matter (panel a).

Diffusion tensor imaging

Diffusion data from the COBRA study were pre-processed using FSL v5.0.6 (FMRIB, University of Oxford). Firstly, images were corrected for eddy currents and head motion by rigid-body registration to each subject's initial B0 image using FMRIB's Diffusion Toolbox (FDT) 'eddy_correct' algorithm. Visual inspection of the scans before and after this process was

to ensure that head motion was not excessive prior to attempted correction and to ensure the correction process was successful. Non-brain tissue was deleted using the ‘*brain extraction tool*’ (BET).⁴⁵ A diffusion tensor model was then fitted at every voxel using FDT’s ‘*dtifit*’ algorithm (see Figure 2-D for an example).

Figure 2-D. An example fractional anisotropy map coloured by the principal direction of diffusion.



An individual subject's fractional anisotropy map coloured using the principal diffusion direction (blue: superior-inferior; green: anterior-posterior; and red: left-right).

Next, a study specific registration template was created using data from all subjects. These were then registered to a custom template and standard space, to allow further analysis at the group level, using Diffusion Tensor Imaging ToolKit (DTI-TK) v 2.3.1. This registration method uses the full information of the diffusion tensor (rather than just fractional anisotropy) and has been shown to increase registration accuracy relative to the standard FSL pipeline.^{145,220}

Spatially normalised fractional anisotropy, axial, mean and radial diffusivity maps for each participant were then created (see Figure 2-E for examples). These were then ‘skeletonised’ using FSL prior to performing ‘tract based spatial statistics’ (TBSS). This method has several advantages over previous approaches to the voxelwise analysis of diffusion data and aims to improve sensitivity and reliability of multi-subject diffusion studies.¹⁴⁶ This is accomplished by a ‘skeletonisation’ step whereby only the centres of white matter tracts that are common to all subjects are considered for further voxelwise analysis (illustrated in Figure 2-F). This removes the need for smoothing, potentially ameliorates misregistration errors and partial volume effects as well as improves sensitivity by dimensionality reduction.¹⁴⁶ Fractional anisotropy skeletons were thresholded using fractional anisotropy ≥ 0.2 to exclude areas of low mean fractional anisotropy with considerable inter-individual variability. Similar steps

were carried out to obtain the mean, axial and radial diffusivity images. As a quality control step, after each stage, images were inspected visually to ensure each process was completed successfully.

Figure 2-E. Example fractional anisotropy, axial, mean and radial diffusivity maps and formulae for calculation.

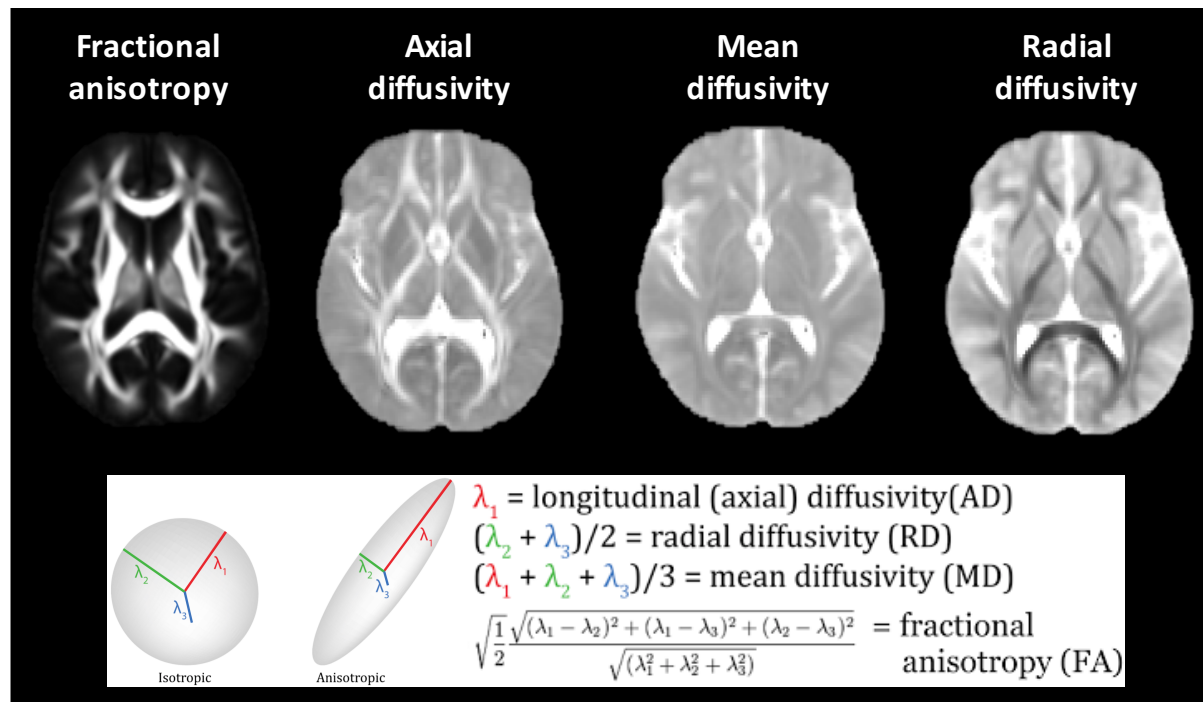
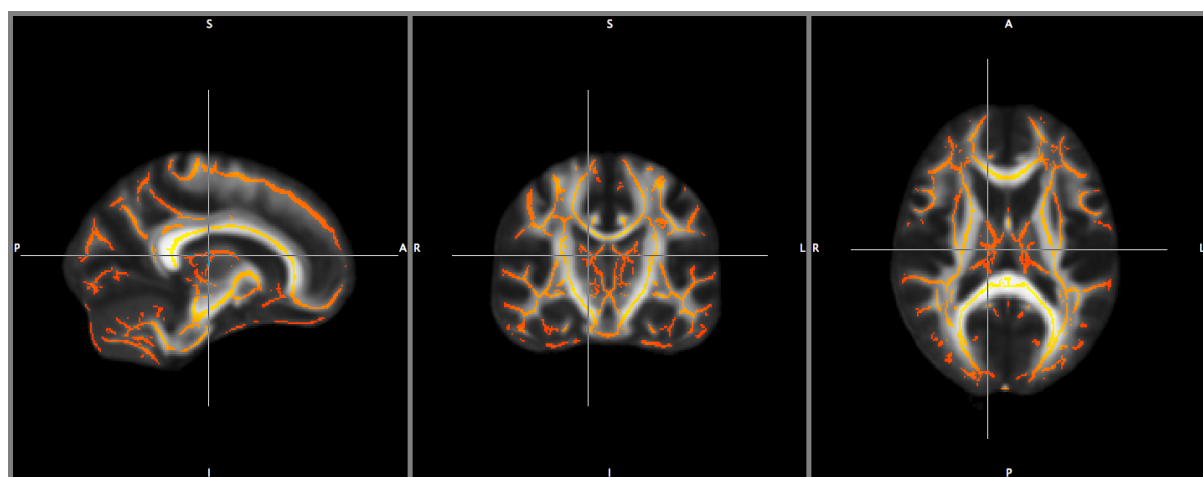


Figure 2-F. Skeletonisation.



The skeleton (red-yellow) represents the centre of each white matter tract common to each study participant. This is overlaid on the mean fractional anisotropy image (greyscale).

Again, to obtain region of interest data and as a data reduction step prior to unsupervised machine learning with cluster analysis, each participant's white matter was summarised by projecting each individual's normalised fractional anisotropy skeleton through masks created

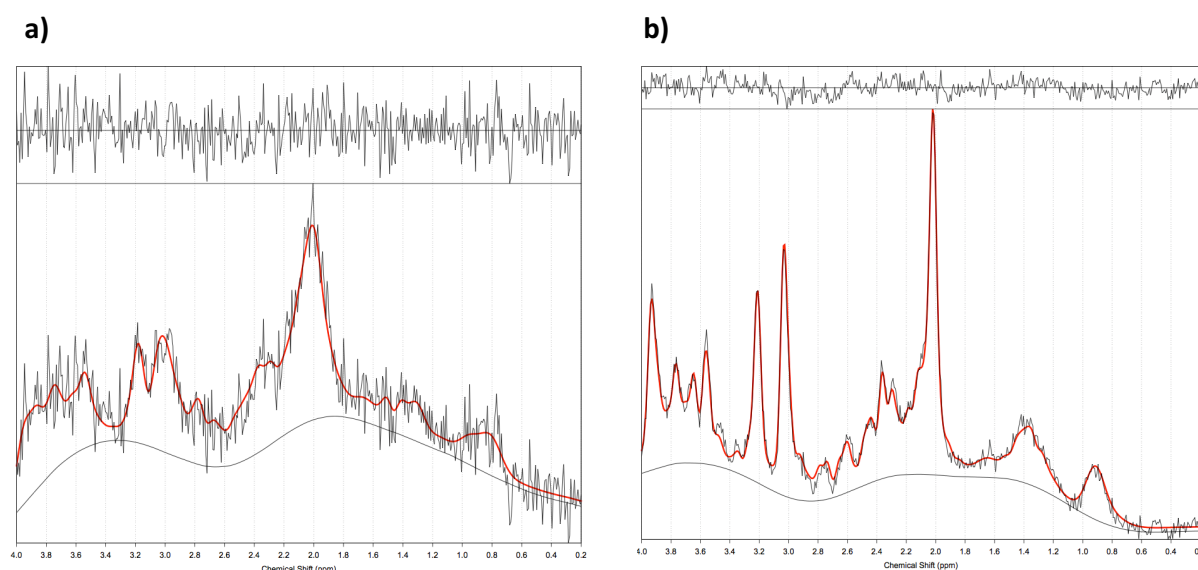
for each region of the International Consortium of Brain Mapping (ICBM) DTI-81 atlas to calculate the mean fractional anisotropy for each region. White matter structure was summarised using fractional anisotropy data in preference to volumetric data as the former is likely to be the more sensitive to HIV-associated white matter disease particularly in ‘normal’ appearing areas on conventional imaging.^{129,148,150,221}

Magnetic resonance spectroscopy

Absolute cerebral metabolite concentrations were quantified using the water unsuppressed data as a reference signal using the totally automatic robust quantification in NMR (nuclear magnetic resonance) algorithm – TARQUIN version 4.3.2. This algorithm analyses data in the time domain using simulated basis sets that include lipid and macromolecules.²²² This method has been shown to yield comparable results to LCModel™ in both healthy volunteers and with clinically acquired samples²²³ but has the advantages of being available across different computer platforms and it is free to use and modify under the GNU general public licence (GPL). Furthermore, in the context of HIV-disease it has been shown to potentially be more accurate than the java-based version of the magnetic resonance user interface package (jMRUI),²²⁴ another popular algorithm for spectroscopy analysis that has been used in HIV-disease.

The absolute concentration of the following metabolites of interest were quantified after eddy current correction, using water unsuppressed data as the reference signal:²²³ choline containing compounds (Cho), creatine (Cr); glutamate and glutamine (as Glx); myo-inositol (MI), N-acetyl-aspartate (NAA). In addition, metabolite ratios using creatine as a reference were also calculated, which may have advantages in multi-scanner studies and to remove partial volume effects.^{106,225} All spectra were visually inspected to assess model fit and were rejected if it was poor. Examples of poor and good quality spectra are illustrated in **Figure 2-G**. As additional quality control steps, spectra were excluded if the signal to noise ratio was <4 or the metabolite full width half maximum of >0.1 ppm as per Ernst *et al.*²²⁶

Figure 2-G. Example spectra of varying quality.



An example of poor quality spectra (panel a) – with low signal (black line) to noise and broad metabolite peaks. In contrast, panel b demonstrates good quality spectra with a good model fit (red line)

2.4 Blood and cerebrospinal fluid sampling

Blood samples for the COBRA study were collected using venepuncture in citrate, serum separator, Li and Na heparin and EDTA vacutainers. Serum was left to clot for 30-60 mins prior to centrifuging for 10 mins (2000g) at room temperature. Other samples were centrifuged for 10 minutes (1000g) at 20°C prior to storage at -80°C prior to shipment for further processing.

Cerebrospinal fluid from the COBRA was obtained following lumbar puncture, which was performed under local anaesthetic using aseptic technique. Cerebrospinal fluid was centrifuged for 10 minutes (400g) at 20°C and the supernatant was stored at -80°C prior to shipment for further processing.

2.5 Antiretroviral pharmacokinetics

Plasma and cerebrospinal fluid sampling for all COBRA participants was undertaken at the same visit. Self-reported time since their last dose of each antiretroviral was recorded. Blood samples were collected in Na heparin vacutainers and then centrifuged for 10 minutes (1000g)

at 20°C within 1 hour. The resulting plasma was stored at -80°C prior to shipment for further processing. Cerebrospinal fluid was obtained as previously described, centrifuged for 10 minutes (400g) at 20°C within an hour of collection and the supernatant was stored at -80°C prior to shipment for further processing.

Plasma and cerebrospinal fluid efavirenz and nevirapine concentrations were assayed using ultra high-performance liquid chromatography (HPLC) at the Department of Clinical Pharmacy, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands. For efavirenz, this method produced linear results over the range of 0.0517 to 15.51 mg/L. The lower limit of quantification was 0.05 mg/L. The intra-assay and inter-assay coefficients of variation (CoV) were 1.01–5.31% and 0.1–1.63%, respectively.²²⁷

2.6 Soluble biomarkers

2.6.1 Neuronal injury

Neuronal biomarker assays were performed on the supernatant of centrifuged and stored cerebrospinal fluid samples as described earlier at the Laboratory of Neurochemistry at the University of Gothenburg. Cerebrospinal fluid quantification of total Tau (t-Tau) and phosphorylated Tau (p-Tau) were performed using enzyme linked immunosorbent assay (ELISA) as previously described.²²⁸⁻²³⁰ The detection limits of the different assays were: A β 42: 125 ng/L, t-Tau: 75 ng/L, p-Tau: 15 ng/L. Values below the detection limit were estimated as the detection limit divided by two in calculations.

Cerebrospinal fluid neurofilament light (NFL) protein was measured using the sensitive sandwich ELISA method (NF-light ELISA kit) as described by the manufacturer (UmanDiagnostics AB, Umea, Sweden) as previously described.¹⁸⁷ The lower limit of quantification was 50 ng/L and intra- and inter-assay coefficients of variation were 4.1–13%. The upper normal reference limits of CSF NFL were <380 ng/L (18–30 years), <560 (30–39 years), <890 (40–59 years) and <1850 (>59 years); these limits were described by Krut et al¹⁸⁷ and were established from 108 cerebrospinal fluid samples from neurologically healthy control individuals aged 18–76 years.

2.6.2 Neuroinflammation

sCD163 and sCD14 concentrations were determined in stored plasma and cerebrospinal fluid samples (-80°C) by an enzyme-linked immunosorbent assay (Human CD14 DuoSet and Human CD163 DuoSet, R&D Systems, Minneapolis, Minnesota) according to the manufacturer's instructions as previously described.²³¹ These assays were performed by the Department of Experimental Immunology, Academic Medical Center of the University of Amsterdam, The Netherlands.

Neopterin, kynurenine and tryptophan were measured in stored cerebrospinal fluid samples (-80°C) at the Laboratory of Neurochemistry at the University of Gothenburg. Neopterin concentrations were determined by enzyme-linked immunosorbent assay (BRAHMS GmbH, Hennigsdorf, Germany) following the manufacturer's instructions as previously described.²³² Sensitivity of the test was 2 nmol/L neopterin. Kynurenine and tryptophan were quantified using reverse-phase high-performance liquid chromatography as previously described.²³³

2.6.3 HIV RNA

Cerebrospinal fluid HIV RNA concentration was measured in stored cerebrospinal fluid samples (-80°C) using the Abbott RealTime M2000 assay (Abbott, Chicago, USA) with a lower limit of detection of 40 copies/mL. This assay was performed at the Department of Experimental Immunology, Academic Medical Center of the University of Amsterdam, The Netherlands.

2.7 Statistics

2.7.1 Simple group comparisons

Throughout this thesis, differences in characteristics between groups (e.g. between HIV-positive and -uninfected groups) were assessed using Fisher's exact or χ^2 test (with continuity correction for 2x2 tables) for categorical data and independent sample t-tests or Wilcoxon rank-sum tests, as appropriate, depending on counts in the case of categorical data and distributions for continuous data.

2.7.2 Comparing different methods of cognitive impairment

Agreement between different diagnostic criteria of HIV-associated cognitive impairment were assessed using Cohen's κ statistic²³⁴ and interpreted by following the commonly used Landis and Koch guidelines²³⁵ where 0.4-0.6 represents moderate agreement and >0.8 is regarded as excellent. The asymptotic test was used to reject the null hypothesis that there was no agreement between diagnostic criteria (i.e. Cohen's $\kappa=0$) other than what would be expected by chance.²³⁶ All analyses were performed using R v3.2.1. Only p-values <0.05 (two-tailed) were considered statistically significant.

Synthetic data to test assumptions

Due to my concerns about the multiple comparisons and the over diagnosis of HIV-associated cognitive impairment using the Frascati criteria (see 1.3.2 in introduction), I created synthetic data to test the prevalence of cognitive impairment diagnosed with differing methods in a normal population.

Firstly, I created random data for 6 cognitive domains with a population mean of 50 and standard deviation of 10 (i.e. a T-score) for 10,000 individuals assuming a multivariate normative distribution. This allows correlation between different random variables and allows control of the correlation between different variables. Secondly, cognitive impairment was defined according the Frascati criteria, global deficit score, a combination of both of these and using a global T-score threshold <45 as previously described (in section 2.2). Thirdly, as cognitive tests are correlated to some degree and reflect underlying intelligence (see introduction for discussion), steps one and two were repeated over a range of correlations (0-1) between the different cognitive domains. Finally, agreement between cognitive impairment defined using the Frascati criteria and global deficit score across the range of inter-domain correlation coefficients was assessed as described above using Cohen's κ statistic. These simulations are described in more detail in chapter 4 and were performed in R v3.2.4 using the 'MASS' package v7.3-45. The code for these simulations is included in Appendix 4.

2.7.3 Cognitive impairment and patient reported outcome measures

Associations between and classification performance of different definitions of cognitive impairment and patient-reported outcome measures (as dichotomised questionnaire results) were assessed using the concordance (or 'c') statistic. This statistic is equivalent to the area under the receiver operating characteristic curve. It gives an indication of the ability of the different definitions of cognitive impairment to discriminate between those with and without symptoms (based on their questionnaire data). Concordance of 0.5 is equivalent to what would be expected by chance alone and is typically considered reasonable when the c-statistic >0.7 and strong >0.8 .²³⁷ In addition, sensitivity and specificity were also calculated. Optimal global T-score cut-offs were calculated for each patient-reported outcome measure by maximising the c-statistic. The amalgamation of data from different sites and calculation of c-statistics was performed by Davide De Francesco at the Research Department of Infection and Population Health, University College London using SAS v9.4 with all subsequent analysis performed by myself using R v3.2.1. Only p-values <0.05 (two-tailed) were considered statistically significant.

2.7.4 Group comparisons of volumetric and data

Summary measures

Group comparisons of brain volumes (total grey matter, white matter and cerebrospinal fluid) and diffusion measures (mean skeleton fractional anisotropy, axial, mean and radial diffusivity) were assessed using a multiple regression model adjusting for age, intracranial volume (grey matter volume + white matter volume + CSF volume) and scanner type. Multicentre studies are becoming more commonplace in neuroimaging to improve consistency of results and to increase statistical power. This potentially introduces bias due to differences in scanning parameters, however, reliability studies suggest that these effects are small for both volumetric and diffusion studies.^{238,239} Scanner was therefore represented as a three level factor in all analyses to account for the scanner upgrade in Amsterdam and different scanner in London – a method that has been used in other multi-site studies of HIV-positive individuals.^{122,128} To investigate differences between the scanners, ANOVA was performed

with Tukey's honest significant difference used to perform post-hoc tests. This method accounts for differences in sample size to give more representative 95% confidence intervals as well as controlling for the inherent multiple testing.

Least squares means, a potentially better estimate of the true population mean adjusted for covariates, and 95% confidence intervals were calculated from a linear model for each summary imaging measure and compared across groups. A similar method was used to assess the effects of current and nadir CD4⁺ cell counts, CD4⁺:CD8⁺ ratio, known duration of untreated infection and a prior history of AIDS on summary imaging measures in the HIV-positive group. These analyses were performed using R v3.1.3 and the 'lsmeans' package v2.18 with p-values <0.05 (two-tailed) considered as statistically significant.

Voxelwise inference testing

Voxelwise comparisons allow localised changes in brain structure to be delineated within the framework of a general linear model. Group differences in grey matter volume, white matter volume and diffusion measures were calculated using non-parametric permutation testing with 10,000 permutations with FSL's '*randomise*'¹⁶⁹ adjusting for age, intracranial volume and scanner type. Accounting for intracranial volume in volumetric studies is recommended and the use of SPM12 estimates of intracranial volume in this manner is thought to be superior to other approaches of accounting for intracranial volume, such as 'non-linear only modulation', used in other software packages such as FSL.²⁴⁰ While adjusting for intracranial volume is common in VBM studies, it is infrequent in diffusion studies. Evidence suggests that it is a potential 'nuisance' variable in diffusion analyses and that it may explain previously documented sex differences due to correlations between intracranial volume and fractional anisotropy.^{241,242} It was therefore included in models of volumetric and diffusion data. Correction for multiple comparisons was accomplished using threshold-free cluster enhancement (TFCE) within *randomise*. This method confers advantages over other cluster-based methods by removing the need for a user defined arbitrary threshold for the minimum cluster size.¹⁷¹ TFCE corrected p-values <0.05 were considered significant. These analyses were performed using FSL v5.0.6.

2.7.5 Combining imaging and cognitive data

Using summary measures

To assess the relationship between imaging and cognitive measures summary measures were extracted for each participant (total grey matter volume and mean skeleton fractional anisotropy). For each cognitive domain, a multiple linear regression model was fitted with age, intracranial volume, scanner, HIV-status and the summary imaging measure as independent variables and the relevant cognitive domain T-score as the dependent variable. To assess whether HIV modified the relationship between structure and function (i.e. to assess whether an interaction was present) a separate multiple regression model was fitted with an additional HIV-status and imaging measure interaction term. These analyses were performed using R v3.1.3 with p-values <0.05 (two-tailed) considered as statistically significant.

Voxelwise

To investigate the relationship between localised brain structure and measures of cognitive function, voxelwise regressions were calculated in the same manner as the earlier group comparison with the following variables in the design matrix: age, intracranial volume, HIV-serostatus, scanner and the cognitive T-score of interest. The brain regions that were significantly associated with cognitive function (thresholded with TFCE corrected $p < 0.05$) were then used as masks to extract summary measures (total grey matter volume and mean fractional anisotropy) for each participant. These were then used to determine the effect of HIV-infection by comparing the groups, as before, using least squares means.

To quantify the degree of spatial overlap between two different statistical images from the previously described voxelwise analyses (e.g. HIV-associated changes in fractional anisotropy and radial diffusivity) Dice's coefficient was calculated by dividing the overlapping volume between two images by the mean of the two volumes. This gives a value between 0 (no overlap) to 1 (perfect overlap).

2.7.6 Multivariate neuroimaging analysis

To take advantage of the inherent complexity and multidimensionality of neuroimaging data and to assess potentially more complicated relationships between brain structure and other outcomes, various multivariate machine learning techniques were used. Broadly speaking, these techniques can be divided into unsupervised or supervised techniques. Unsupervised techniques, such as cluster analysis, aim to find hidden structure in data without prior knowledge. Supervised techniques, on the other hand, require data labels and can be used for classification and regression problems and can assess model performance against a known outcome (e.g. presence or absence of cognitive impairment).

Unsupervised classification with k-means clustering

Volumetric and diffusion data provide complementary information on underlying brain structure. Whilst their independent effects on cognition are important, their combination potentially allows a better understanding of grey and white matter pathology at the individual level. As such, k-means cluster analysis was performed to integrate these two disparate imaging modalities. This method aims to define each individual as a member of a cluster iteratively so that the within-cluster sum of squares of the Euclidean distance from each individual's data point to its cluster centre is minimised. Furthermore, this method of cluster analysis allows one to test for the presence of sub-groups defined on neuroimaging characteristics in a data driven way. This is useful as subgroups of HIV-associated cognitive impairment have been proposed in the literature but this has not been rigorously tested before.^{37,44,243}

As a dimension reduction step prior to cluster analysis, each participant's brain was summarised by 48 measures of cortical volume, 8 measures of subcortical volume and 53 measures of white matter microstructure as previously described. Briefly, a specific mask was made for each region (defined using Harvard-Oxford atlases for cortical and sub-cortical regions and the ICBM DTI-81 atlas for white matter regions). Next, these were overlaid on top of each participant's spatially normalised grey matter or spatially normalised fractional anisotropy skeleton and the grey matter volume or mean fractional anisotropy was calculated. To ensure comparable distributions and to reduce bias due to different measuring scales, grey matter volume and mean skeleton fractional anisotropy values for each region were

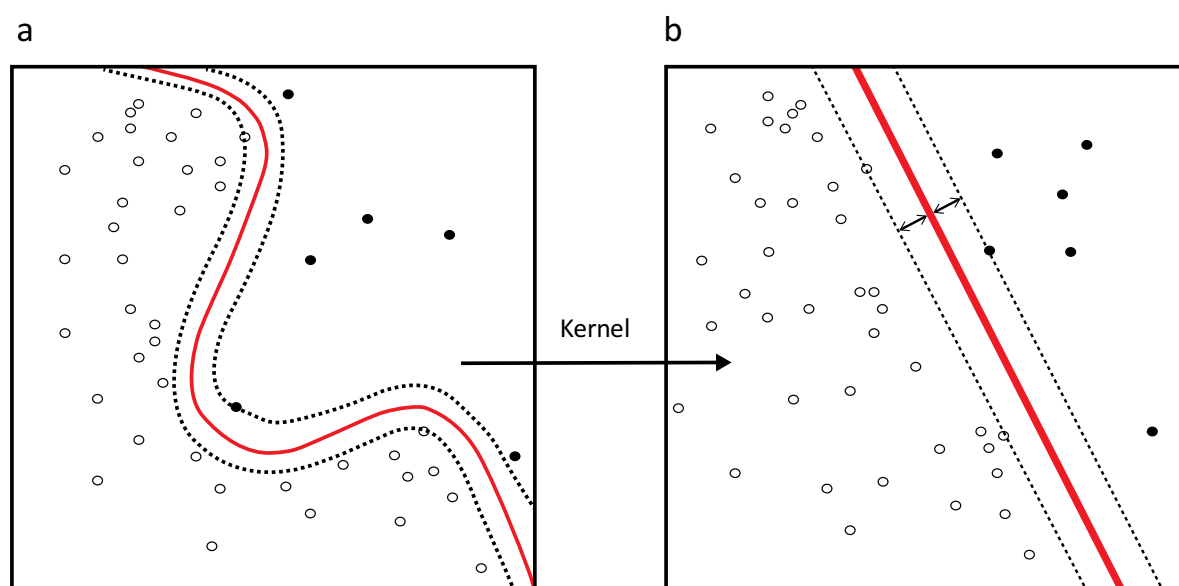
standardised by subtracting their mean and dividing by their standard deviation, before entering into the clustering model.

The optimal number of clusters in the data is usually unknown. Therefore, separate models with 2-10 clusters (i.e. k ranging from 2-10) were calculated and compared to assess the optimal partitioning of the data. These were compared using the Calinski-Harabasz index²⁴⁴ (analogous to a multivariate Fischer's F) and the average silhouette width.²⁴⁵ These are both measures of cluster structure, assessing both cluster similarity and between cluster difference, and allows comparison between models with different values of k , with the highest value representing the optimal partitioning of the data. The Duda-Hart test²⁴⁶ was used to confirm cluster structure to the data (i.e. $k > 1$ is better than $k = 1$). Cluster membership may be affected by initial values of cluster means, therefore the k -means algorithm, for each value of k , was initialised 100 times with randomly chosen points from the data so that the within-cluster sum of squares was minimised. Cluster stability to resampling was assessed using the method of Hennig²⁴⁷ by calculating the Jaccard coefficient using bootstrapping with 10,000 resamples. This provides a measure of how stable cluster membership is to resampling the data. It ranges from 0 (no stability) to 1 (perfect stability). Cluster analyses were performed with the 'fpc' package v2.1.9 in R v3.1.3.

Supervised classification and regression

The ability of structural neuroimaging data to predict cognitive impairment was assessed using two different methods: support vector machines and Gaussian processes classification. A support vector machine aims to classify data by finding a separating hyperplane between two group's data. It achieves this by representing the data in a high-dimensional space, and defining a separating hyperplane so that the margins between the hyperplane and nearest data points, the so-called *support vectors*, are maximised. This ensures the optimum partitioning of the data (illustrated graphically in Figure 2-H).

Figure 2-H. Graphical illustration of non-linear classification using support vectors.



Graphical illustration of a non-linear classifier (a) using the kernel method to transform data into a higher dimensional space (b) where it becomes linearly separable (red line). The arrows in (b) represent the support vectors from the margin (dotted line) that are maximised to find the optimum separating hyperplane.

An alternative technique is to use Gaussian processes, which are multi-variate statistical techniques for modelling and therefore predicting numerical data assuming the covariance between any set of points follows a multivariate Gaussian distribution. A Gaussian process can be partnered with any dataset and is described by its 'covariance function', which relates one observation to another, and can be calculated from a training dataset. Gaussian processes regression is a non-linear technique that predicts the value of a model (or function) at a given point by computing a weighted average of the output of the function in the nearby location of the point of interest from the training data. For the purposes of this thesis, this equates to predicting a value of interest for an individual, e.g. the global T-score, from a test subject's neuroimaging data, e.g. spatially normalised segmented grey matter, from a model derived from a larger training set of similar neuroimaging data. In this example, the predicted global T-score is weighted towards the global T-scores associated with the spatially normalised segmented grey matter from the training dataset that is most similar to test data. For classification purposes, the output of a Gaussian process can be transformed into the range 0-1 where it now represents the probability of the input data belonging to a particular class (or group). This is one potential advantage over support vector machine classification where the probabilistic interpretation is less clear. Using a similar example to before, a Gaussian process can be described that models the likelihood of cognitive impairment from a training

dataset of neuroimaging data. This can then be applied to new data to predict whether this test subject has cognitive impairment or not.

An alternative approach to multivariate regression is kernel ridge regression. This is equivalent to performing ridge regression (linear least squares but with L_2 regularisation) on data in kernel form (explained in more detail in the next section). L_2 regularisation is a process loss function is introduced to prevent over-fitting of data. This is especially important with multivariate neuroimaging data given that the number of data points vastly outnumbers the number of subjects. Therefore, kernel ridge regression learns a linear function in kernel space, which maps to a non-linear function in the original data space and can be used for non-linear prediction.

Predicting cognitive function from neuroimaging data

To assess the ability of structural neuroimaging data to predict cognitive impairment the Pattern Recognition for Neuroimaging Toolbox (PRoNTo)⁸⁹ software package v2.0 (Machine Learning and Neuroimaging Lab, University College London) was used to implement various machine learning techniques. These methods aim to find linear relationships/patterns in a high-dimensional space which maps to a non-linear relationship in the original data space (see Figure 2-H for a graphical explanation). Initially, volumetric and diffusion data were pre-processed as described previously so that spatially normalised data were entered into subsequent models. This step serves as a data reduction step and renders data between subjects comparable and reduces errors due to different head sizes/positioning. Next, a linear kernel in the form of a similarity matrix was calculated by computing the dot product of a pair of subjects' image data (converted to vector form) for each position in the matrix.⁸⁹ This step serves to greatly reduce computation time and is known as the '*kernel trick*' by allowing computation in an implicit feature space without having to transform data into a higher dimensional '*feature space*.'²⁴⁸

For the purposes of classification, models were computed with support vector machines (SVM) and Gaussian processes classification (GPC) for cognitive impairment defined using the Frascati criteria, global deficit score and multivariate normative comparison as previously described. To test the predictive ability of different imaging modalities, for each definition of cognitive impairment a separate model was computed with spatially normalised: grey matter

volume; white matter volume and skeletonised fractional anisotropy (in similarity matrix form). Furthermore, to take advantage of the complementary functions of grey and white matter, additional models with concatenated, spatially normalised grey and white matter volumes (for CHARTER participants as diffusion data was not available) and grey matter volume and skeletonised fractional anisotropy for each subject (for COBRA participants) were also computed.

To assess the ability of structural imaging data to predict cognitive function in a continuous nature, the global T-score was defined as the dependent variable with the multivariate imaging data (in similarity matrix form) as the independent variables. Separate models using Gaussian processes regression (GPR) and kernel ridge regression (KRR) were calculated using different imaging modalities in a similar nature to that used for classification above.

Assessing models

Multivariate techniques are prone to overfitting by their nature which limits their generalisability and application to new data. Cross-validation of a model is therefore essential to assess model performance. This is usually accomplished by partitioning the data into a training set to fit the model with performance assessed on a separate testing set. To obtain an approximately unbiased estimate of the true error of a model the data can be repeatedly repartitioned into different training and test sets. Leave one out cross validation trains the model on all but one subject's data (i.e. $n-1$), which the model is then tested on. This is then repeated exhaustively so that each subject is excluded from training the model. This method of cross-validation was used for all models. The training set was used to subtract the voxelwise mean from each data vector to mean centre features as recommended in the PRoNTTo manual (http://www.mlnl.cs.ucl.ac.uk/pronto/prt_manual.pdf).

For classification, class (i.e. cognitively impaired or not) accuracy and predictive value were calculated for each model. Additionally, the area under the receiver operator curve (AUC) and the balanced accuracy (mean of accuracies across classes) were calculated. This is important if groups are unbalanced and gives an estimation of the utility of the model with new data. For regression, model accuracy was quantified with the correlation (Pearson's r), total variance explained (R^2), mean absolute error (MAE) and root mean squared error (RMSE). P-values were calculated using permutation testing with 1,000 repetitions.

Multivariate prediction of brain age

As an alternative method of multivariate analysis, prediction of brain age using structural imaging data was performed and deviation from normal was determined. For the purposes of brain age prediction, a dataset of healthy volunteers was used to train a model of ‘normal’ brain ageing. This algorithm was developed by Dr James Cole of the Division of Brain Sciences, Imperial College London and I am grateful for his help in implementing this algorithm to the CHARTER data.

Independent training set to build normative model of brain ageing

T1-weighted scans for 2,001 healthy individuals (mean [standard deviation] age: 37.0 [18.1]; range: 18-90; males: 1016 [50.8%]) were acquired from publicly available data repositories (see *Table 2-D* for details) for the purposes of establishing a training dataset. Each study had ethical approval and participants were screened according to local study protocols. Data sharing was verified for each data repository.

Image pre-processing

All structural images were pre-processed using SPM12 as per section 2.3.2. Segmented grey and white matter were then non-linearly registered to a custom template, based on the training dataset, and then to MNI152 space using DARTEL as previously described. As before, tissue volumes were modulated to retain their volumetric information and smoothed with a 4mm full width half maximum smoothing kernel.

Multivariate brain age prediction

A multivariate model of brain ageing based on structural image data and subsequent brain age prediction was performed on CHARTER subjects with the Pattern Recognition for Neuroimaging Toolbox (PRoNT v2.0)⁸⁹ using a previously described method.¹⁷⁶ Briefly, a model of ‘normal’ brain ageing was developed using the independent training dataset (n=2,001, see *Table 2-D* for further details). A Gaussian processes regression model was calculated with age as the dependent variable and the similarity matrix of imaging data as the independent variables. Model accuracy and statistical significance were quantified as previously described. Cross-validation in the training set indicated that the Gaussian processes regression model

accurately predicted chronological age in the normative training set ($r = 0.94$, $R^2 = 0.88$, mean absolute error 5.01, root mean squared error 6.31, $p = 0.001$). An additional calibration step was added to remove the influence of a proportional bias in the training model, whereby chronological age correlated with prediction error ($r = -0.25$, $p < 0.01$). Calibration parameters were based on the slope and intercept of a linear regression model of age against brain age.

Subsequently, data from subjects participating in the CHARTER study ($n = 139$) were then entered into the model yielding a predicted brain age for each subject. Predicted brain age difference (PBAD) was then calculated for each subject by subtracting their chronological age from their predicted brain age. Therefore, a positive PBAD represents an ‘older’ appearing brain relative to chronological age.

Table 2-D. Data sources for normative healthy brain age sample used to develop predictive model of brain ageing.

Cohort	N	Age mean (SD)	range	Sex male/female	Scanner (Field strength)	Scan
ABIDE	184	25.9 (6.66)	18-48	161/23	Various (all 3T)	MPRAGE
Beijing Normal University	179	21.3 (1.92)	18-28	72/107	Siemens (3T)	MPRAGE
Berlin School of Brain & Mind	49	31.0 (7.08)	20-60	24/25	Siemens Tim Trio (3T)	MPRAGE
CADDementia	12	62.3 (6.26)	55-79	9/3	GE Signa (3T)	3D IR- FSPGR
Cleveland Clinic	31	43.6 (11.1)	24-60	11/20	Siemens Tim Trio (3T)	MPRAGE
ICBM	322	24.8 (5.14)	24-60	177/145	Siemens Magnetom (1.5T)	MPRAGE
IXI	561	48.6 (16.5)	20-86	250/311	Philips Intera (3T); Philips Gyroscan Intera (1.5T); GE Signa (1.5T)	T1-FFE; MPRAGE
MCIC	93	32.5 (12.0)	18-60	64/29	Siemens Sonata/Trio (1.5/3T); GE Signa (1.5T)	MPRAGE; SPGR
MIRIAD	23	69.7 (7.18)	58-85	12/11	GE Signa (1.5T)	3D IR- FSPGR
NEO2012 (Adelstein, 2011)	39	29.6 (8.38)	20-49	18/21	Siemens Allegra (3T)	MPRAGE
NKI / Rockland	160	41.5 (18.1)	18-85	96/64	Siemens Tim Trio (3T)	MPRAGE
OASIS	288	44.1 (23.0)	18-90	106/188	Siemens Vision (1.5T)*	MPRAGE
WUSL (Power, 2012)	24	23.0 (1.42)	20-24	4/20	Siemens Tim Trio (3T)	MPRAGE
TRAIN-39	36	22.7 (2.56)	18-28	11/25	Siemens Allegra (3T)	MPRAGE
Training set total	2001	37.0 (18.1)	18-90	1016/985	-	-

Abbreviations: ABIDE: Autism Brain Imaging Data Exchange; COINS: Collaborative Informatics and Neuroimaging Suite (<http://coins.mrn.org/>); ICBM: International Consortium for Brain Mapping; INDI: International Neuroimaging Data-sharing Initiative (http://fcon_1000.projects.nitrc.org/); IXI: Information eXtraction from Images (<http://biomedic.doc.ic.ac.uk/brain-development>; LONI: Laboratory of Neuro Imaging Image & Data Archive (<https://lida.loni.usc.edu/>); MCIC: MIND Clinical Imaging Consortium; MIRIAD: Minimal Interval Resonance Imaging in Alzheimer's Disease (<https://www.ucl.ac.uk/drc/research/miriad-scan-database>); NKI: Nathan Kline Institute; OASIS: Open Access Series of Imaging Studies (<http://www.oasis-brains.org/>)

Chapter 3

The prevalence of HIV-associated cognitive impairment and its associations with patient reported measures of mental and physical health

Table of contents

CHAPTER 3 THE PREVALENCE OF HIV-ASSOCIATED COGNITIVE IMPAIRMENT AND ITS ASSOCIATIONS WITH PATIENT REPORTED MEASURES OF MENTAL AND PHYSICAL HEALTH		89
3.1	INTRODUCTION	91
3.2	METHODS.....	92
	Participants	92
	Cognitive testing and other measures	92
	Statistical analysis	92
3.3	RESULTS	93
	Participants	93
	Cognitive function	93
	Agreement between the different definitions of cognitive impairment	96
	Association between definitions of cognitive impairment and self-reported cognitive problems.....	97
	Other patient reported outcome measures.....	98
3.4	DISCUSSION	102
	Conclusion.....	105

Figures

FIGURE 3-A. BOXPLOTS OF DEMOGRAPHICALLY ADJUSTED COGNITIVE T-SCORES BY HIV SEROSTATUS.	95
FIGURE 3-B. VENN DIAGRAM OF HIV-POSITIVE INDIVIDUALS WITH COGNITIVE IMPAIRMENT BY DIAGNOSTIC CRITERIA.....	97
FIGURE 3-C. MENTAL AND PHYSICAL HEALTH SUMMARY SCORES FOR THOSE WITH VS. WITHOUT COGNITIVE IMPAIRMENT BY DIAGNOSTIC CRITERIA.	101

Tables

TABLE 3-A. BASELINE CHARACTERISTICS OF THE POPPY COHORT.	94
TABLE 3-B. PREVALENCE OF COGNITIVE IMPAIRMENT BY RECREATIONAL DRUG.	96
TABLE 3-C. COGNITIVE DOMAIN T-SCORES BY RECREATIONAL DRUG USE IN THE PAST 6 MONTHS (HIV-POSITIVE GROUP ONLY) ...	96
TABLE 3-D. LEVELS OF AGREEMENT BETWEEN DIFFERENT DIAGNOSTIC CRITERIA.....	97
TABLE 3-E. SELF-REPORTED COGNITIVE SYMPTOMS AND THEIR ASSOCIATIONS WITH COGNITIVE IMPAIRMENT.	98
TABLE 3-F. ASSOCIATIONS OF FOUR DEFINITIONS OF COGNITIVE IMPAIRMENT AND PATIENT-REPORTED OUTCOME MEASURES.....	99
TABLE 3-G. OPTIMAL GLOBAL T-SCORE THRESHOLD WITH ASSOCIATED STATISTICS FOR EACH PATIENT REPORTED OUTCOME MEASURE.	100

3.1 Introduction

As discussed in the detail in the chapter 1, cognitive impairment is frequently reported in HIV-positive cohorts despite antiretroviral therapy. Also discussed were the different diagnostic classification systems currently in use and their possible limitations. Previously, cognitive impairment has been associated with impairment of objective measures of everyday functioning. However, this was in historic cohorts with lower CD4 cell counts and rates of antiretroviral prescriptions that would not be typical in clinical practice today.^{41,42} Furthermore, the prevalence of HIV-associated dementia, has fallen precipitously and is uncommonly reported in contemporary cohorts.^{15,243} It is unclear whether the milder forms of cognitive impairment prevalent today are associated with the same degree of functional impairment or other symptoms as part of a syndrome of general ill health or frailty. It is often assumed that patients with cognitive impairment are more likely to report symptoms, such as memory complaints or attention difficulties.¹² As such, regular screening for cognitive impairment in HIV-positive patients was previously recommended in European guidelines although this is no longer the case in the most recent version.^{68,208} Screening for cognitive impairment is a controversial area regardless of HIV-serostatus and is not routinely recommended.²⁴⁹⁻²⁵¹ Another issue is that while previous associations of cognitive impairment with other symptoms may be statistically significant differences between those with and without impairment were small (i.e. the effect size was small). This makes the predictive power of symptoms to discern those who do and don't have cognitive impairment weak and vice versa.

In this chapter, I test my first hypothesis that cognitive impairment remains more prevalent in well-treated patient cohorts even when compared to an appropriate control group. Furthermore, I compare the commonly used definitions of cognitive impairment to see if they identify the same people. In addition, I assess how different diagnostic criteria, including novel ones, affect the association of cognitive impairment with other patient reported symptoms/outcomes. This was to test the hypothesis that the milder forms of cognitive impairment, the predominant phenotype in the antiretroviral era, would be associated with other measures of mental and physical dysfunction as part of a syndrome of general ill health.

3.2 Methods

Participants

HIV-positive and demographically comparable HIV-negative participants were recruited into the Pharmacokinetic and Clinical Observations in People Over Fifty (POPPY) study from seven sites across the UK and Ireland. Further details including inclusion and exclusion criteria are described in section 2.2.1 of the methods.

Cognitive testing and other measures

Participants completed the CogState computerised cognitive battery testing six cognitive domains as described in section 2.2.1 of the methods. Raw scores were transformed and then converted to demographically-adjusted T-scores (with a mean of 50 and a standard deviation of 10) using the HIV-uninfected control group as the reference population as described. These adjusted T-scores took into account age, level of education, gender and ethnicity as appropriate. A single domain T-score was calculated for each of the six cognitive domains by averaging individual T-scores within each domain. A global T-score was also obtained by calculating the mean of the six individual domain T-scores. For all T-scores, higher scores represented better cognitive function.

All participants answered the previously recommended European AIDS Clinical Society (EACS) cognitive complaints screening questions,²⁰⁸ Short Form Health Survey²⁰⁹ (SF-36); activities of daily living with the Lawton Instrumental Activities of Daily Living²¹⁰ (IADL); depression, using the Patient Depression Questionnaire²¹¹ (PHQ-9) and the Centres for Epidemiologic Studies Depression scale²¹² (CES-D). Additionally, falls and sexual desire were assessed and outcomes were then dichotomised as described in section 2.2.1 of the methods.

Statistical analysis

Baseline characteristics between groups were assessed using Fisher's exact or Chi-squared for categorical data and independent sample t-tests or Wilcoxon rank-sum tests, as appropriate (further details are provided in section 2.8.1 of the methods). Associations between and classification performance of different definitions of cognitive and patient-

reported outcome measures (as dichotomised questionnaire results) were assessed using the concordance (or 'c') statistic (further details are provided in section 2.8.3 of the methods).

Agreement between different diagnostic criteria of HIV-associated cognitive impairment were assessed using Cohen's κ statistic²³⁴ and interpreted following Landis and Koch guidelines²³⁵ where 0.4-0.6 represents moderate agreement and >0.8 is regarded as excellent. The asymptotic test was used to reject the null hypothesis that there was no agreement between diagnostic criteria (i.e. Cohen's $\kappa=0$) other than what would be expected by chance.²³⁶ Further details are described in section 2.8.2 of the methods. All analyses were using R v3.2.1. Only p-values <0.05 (two-tailed) were considered statistically significant.

3.3 Results

Participants

387 participants enrolled in the POPPY study between January 2013 and September 2014, of whom 290 were HIV-positive and 97 were matched HIV-negative controls (see Table 3-A for their baseline characteristics). The HIV-positive group was representative of older patients attending for care in the UK with a median (IQR) age of 57 (53-62) years and CD4 cell count of 610 (479-780) cells/ μ L with 80% receiving antiretroviral therapy. HIV-positive patients and HIV-negative controls were well matched in terms of demographics such as age, ethnicity and level of educational attainment, however there was a slight preponderance of females in the control group. Recreational drug use was more frequent in the patients; however, alcohol consumption and smoking rates were similar.

Cognitive function

HIV-positive participants exhibited poorer cognitive function with higher rates of cognitive impairment compared to the HIV-negative control group. Using the Frascati criteria, the prevalence of cognitive impairment was: 30.0% vs. 16.5%, odd ratio (OR) 2.17 (95% confidence interval: 1.20 – 3.92, $p=0.01$), with GDS: 34.5% vs. 14.4%, OR 3.12 (1.69 – 5.78, $p<0.01$); and with MNC 22.1% vs. 7.2%, OR 3.64 (1.61 – 8.24, $p<0.01$) for HIV-positive vs. HIV-negative groups respectively.

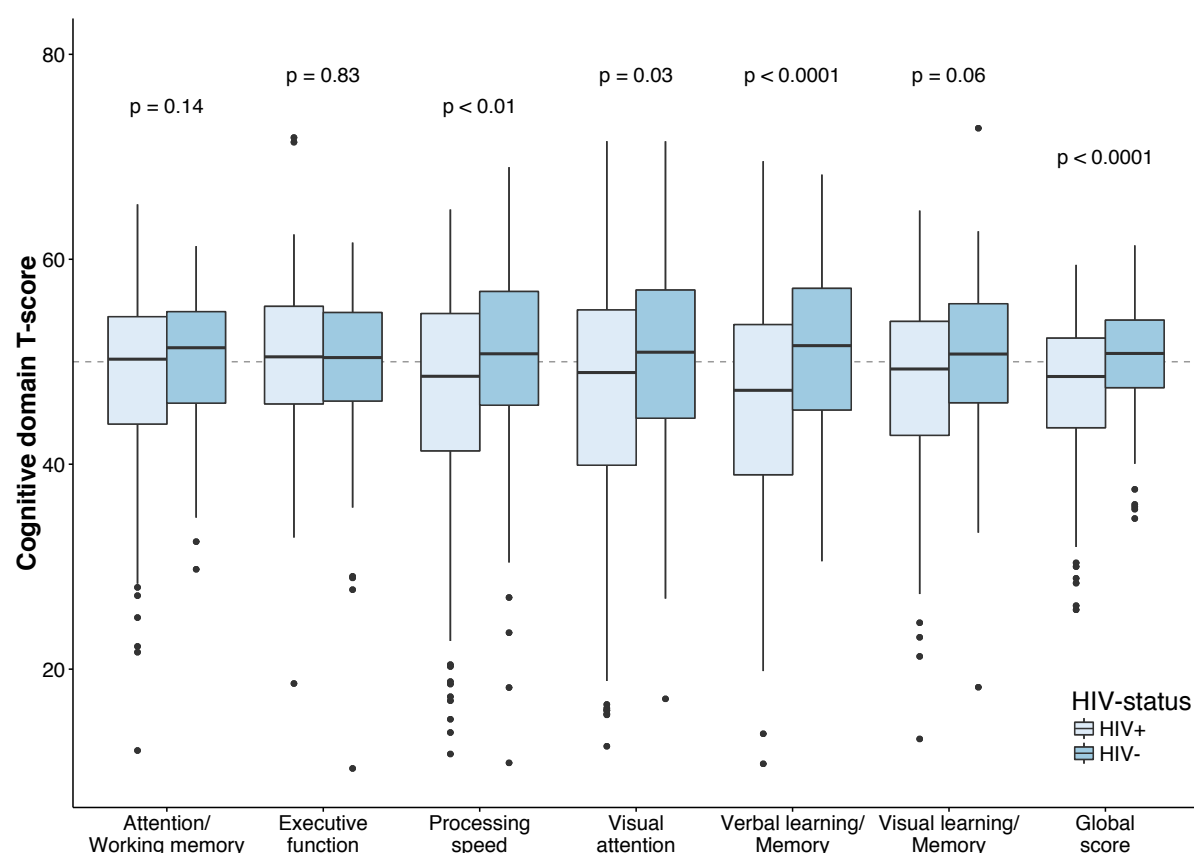
Table 3-A. Baseline characteristics of the POPPY cohort.

	HIV-positive (n=290)	HIV-negative (n=97)	p-value
Age (years), median (IQR)	57 (53-62)	58 (54-62)	0.22
Gender , n (%)			<0.01
Female	34 (11.7%)	33 (34.0%)	
Male	256 (88.3%)	57 (66.0%)	
Ethnicity , n (%)			0.46
Black-African	37 (12.8%)	9 (9.3%)	
White	253 (81.2%)	88 (90.7%)	
Education , n (%)			0.17
No qualifications	31 (10.9%)	4 (4.2%)	
O levels/GCSEs (or equivalent at age 16)	42 (14.7%)	18 (18.9%)	
A levels (or equivalent qualifications at age 18)	41 (14.4%)	20 (21.1%)	
University degree or above	138 (48.4%)	45 (47.4%)	
Other	23 (8.1%)	4 (4.2%)	
Unknown	10 (3.5%)	4 (4.2%)	
Smoking status , n (%)			0.79
Current smoker	70 (24.1%)	20 (20.6%)	
Ex-smoker	108 (37.2%)	37 (38.1%)	
Never smoked	111 (38.3%)	39 (40.2%)	
Not known	1 (0.3%)	1 (1.0%)	
Years of smoking (current/past smokers), median (IQR)	32 (20-40)	33 (21-40)	0.86
Alcohol consumption , n (%)			0.13
Current consumption	227 (78.3%)	83 (85.6%)	
Previous consumption only	40 (13.8%)	6 (6.2%)	
Never consumed alcohol	23 (7.9%)	8 (8.3%)	
Recreational drugs in past 6 months , n (%)	83 (27.9%)	12 (12.4%)	<0.01
Cardiovascular conditions , n (%)			
Hypercholesterolemia	62 (21.5%)	18 (18.8%)	0.79
Hypertension	46 (16.0%)	11 (11.6%)	0.51
Myocardial infarction	14 (4.9%)	3 (3.2%)	0.62
Other cardiovascular disorders	57 (19.7%)	15 (15.4%)	0.36
Neurological conditions , n (%)			
Peripheral neuropathy	104 (35.9%)	7 (7.5%)	<0.01
CVA/TIA	14 (4.8%)	1 (1.1%)	0.13
Epilepsy	9 (3.1%)	0 (0.0%)	0.12
Encephalitis	3 (1.0%)	1 (1.1%)	1.00
Parkinson's	2 (0.7%)	1 (1.1%)	0.57
Other central nervous system disorders	27 (9.3%)	5 (5.3%)	0.22
CD4+ count (cells/ μ L), median (IQR)	610 (479-780)	N/A	
Nadir CD4+ count (cells/ μ L), median (IQR)	180 (84-270)	N/A	
Duration of known HIV infection (years), median (IQR)	16.8 (10.2-22.9)		
Likely route of HIV transmission , n (%)		N/A	
MSM	229 (79.0%)		
Heterosexual sex	61 (21.0%)		
Receiving antiretrovirals , n (%)	281 (96.9%)	N/A	
Plasma HIV RNA <50 copies/ml , n (%)	263 (90.7%)	N/A	

Abbreviations: MSM – men who have sex with men, IQR – interquartile range.

Using a global T-score threshold of <45 to define cognitive impairment, the prevalence was 31.0% vs. 16.5%, OR 2.28 (1.29 – 4.24, $p < 0.01$) for the patient vs. control groups respectively. In general, cognitive impairment was mild with only 2% of the HIV-positive cohort fulfilling the definition of HIV-associated dementia. The cognitive domains with the biggest difference between groups were processing speed and verbal learning/memory with little differences seen in the domains of executive function and attention/working memory (see **Figure 3-A** for a graphical illustration).

Figure 3-A. Boxplots of demographically adjusted cognitive T-scores by HIV serostatus.



P-values calculated using the Wilcoxon rank-sum test.

There was no correlation between duration of known HIV-infection and cognitive function (for the global T-score: $\rho = 0.01$, $p = 0.81$). Immune function, quantified by the absolute CD4+ cell count was similar between those taking or not taking antiretrovirals (median CD4+ cell count 607 vs 610 cells/ μ L, $p = 0.75$).

Recreational drug use was not associated with impaired cognitive function. In the HIV-positive group, cognitive impairment was more common in patients who did not report recreational

drug use in the past six months (Table 3-B). However, the differences in T-scores for each cognitive domain were small (see Table 3-C for further details) and only significantly different (with no correction for multiple comparisons) for executive function, where those who had used recreational drugs in the last six months had higher T-scores.

Table 3-B. Prevalence of cognitive impairment by recreational drug.

Criteria, n (%)	No Rec. drug (n=207)	Rec. drug (n=83)	p-value
Frascati	68 (32.9%)	19 (22.9%)	0.09
GDS	79 (38.2%)	21 (25.3%)	0.04
MNC	48 (23.2%)	16 (19.3%)	0.47

Drug use in the past six months (HIV-positive group only).

Abbreviations: GDS: global deficit score; MNC: multivariate normative comparison.

Table 3-C. Cognitive domain T-scores by recreational drug use in the past 6 months (HIV-positive group only)

Cognitive domain	T-score, median (IQR)		p-value
	No rec. drug (n=207)	Rec. drug (n=83)	
Attention/working Memory	49.3 (42.8-54.4)	50.8 (45.7-54.4)	0.16
Executive Function	49.8 (44.8-54.9)	52.1 (48.6-55.7)	0.04
Processing speed	47.5 (39.1-54.2)	50.5 (43.3-55.2)	0.14
Visual Attention	48.0 (38.3-54.5)	50.2 (43.1-56.7)	0.10
Verbal Learning/memory	47.4 (39.0-54.7)	46.7 (37.7-52.5)	0.33
Visual Learning/memory	49.5 (42.9-54.2)	49.3 (42.4-52.8)	0.73
Global T-score	48.0 (42.6-52.3)	49.0 (45.3-52.2)	0.18

Agreement between the different definitions of cognitive impairment

Overall 169 (58.3%) HIV-positive individuals did not meet any of the three definitions of cognitive impairment while 41 (14.1%) were classified as cognitively impaired by all three definitions (see *Figure 3-B* for a graphical illustration). Agreement between the Frascati criteria and the GDS was good ($\kappa=0.74$, $p<0.01$). Whereas, the agreement between MNC and either the Frascati criteria or the GDS was moderate ($\kappa=0.42$ and $\kappa=0.48$, respectively; $p<0.01$ for each). Agreement was higher in the control group (see *Table 3-D*).

Figure 3-B. Venn diagram of HIV-positive individuals with cognitive impairment by diagnostic criteria.

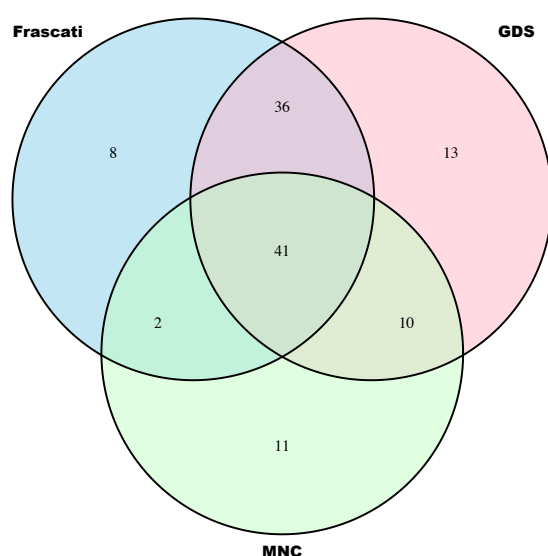


Table 3-D. Levels of agreement between different diagnostic criteria.

Diagnostic criteria	Cohen's κ (95% confidence intervals)	
	HIV-positive	HIV-negative
Frascati vs. GDS	0.74 (0.66-0.82)	0.84 (0.69-0.99)
Frascati vs. MNC	0.42 (0.31-0.54)	0.57 (0.32-0.81)
GDS vs. MNC	0.48 (0.38-0.59)	0.53 (0.26-0.79)

Abbreviations: GDS: global deficit score; MNC: multivariate normative comparison.

Association between definitions of cognitive impairment and self-reported cognitive problems

The numbers of patients who did not have complete data (either no stated answer or missing data) were 17 (5.9%), 14 (4.8%), 15 (5.2%) and for attention, memory loss and reasoning respectively. Among patients with complete information, 79/273 (28.9%) reported problems with attention, 79/276 (28.6%) reported frequent memory loss, and 105/275 (38.2%) reported problems with reasoning. Of the three self-reported cognitive symptoms only memory loss was significantly associated with cognitive impairment (Table 3-E). This was irrespective of the definition of cognitive impairment used.

Table 3-E. Self-reported cognitive symptoms and their associations with cognitive impairment.

	Attention, n (%)			Memory loss, n (%)			Reasoning, n (%)		
	No	Yes	<i>p</i>	No	Yes	<i>p</i>	No	Yes	<i>p</i>
Frascati			0.59			0.02			0.77
Impaired	55 (69%)	25 (31%)		50 (62%)	31 (38%)		49 (60%)	32 (40%)	
Not impaired	139 (72%)	54 (28%)		147 (75%)	48 (25%)		121 (62%)	73 (38%)	
GDS			0.07			<0.01			0.24
Impaired	59 (64%)	33 (36%)		56 (60%)	37 (40%)		53 (57%)	40 (43%)	
Not impaired	135 (75%)	46 (25%)		141 (77%)	42 (23%)		117 (64%)	65 (36%)	
MNC			0.28			0.02			0.42
Impaired	40 (66%)	21 (34%)		36 (59%)	25 (41%)		35 (57%)	26 (43%)	
Not impaired	154 (73%)	58 (27%)		161 (75%)	54 (25%)		135 (63%)	79 (37%)	

Other patient reported outcome measures

In the patient group, data were incomplete or missing for II (3.8%) for IADL, 27 (9.3%) and 29 (10%) for PHQ-9 and CES-D depression scores respectively, 10 (3.4%) for falls and 13 (4.5%) for sexual function. Of those with complete data 40/729 (14.3%) were not fully independent, 76/263 (28.9%) and 102/261 (39.1%) were depressed by PHQ-9 and CES-D scoring respectively, 50/280 (17.9%) reported falls in the last 28 days, and 125/277 (45.1%) reported minimal sexual desire over the preceding 4 weeks.

In general, the associations between cognitive impairment and patient reported outcome measures were weak regardless of the method of identification of cognitive impairment used (see Table 3-F for further details): mean c-statistics were 0.543 (GDS), 0.530 (MNC) and 0.519 (Frascati). Using an alternative definition of cognitive impairment, based on a global T-score cut-off of <45, associations were not dramatically improved (mean c-statistic 0.560, see Table 3-F for associations by measure). Even using the combined measure of cognitive impairment where participants had to have impairment using all three criteria (14.1% of HIV-positive individuals and 6.2% of HIV-negative individuals), the associations were weak (mean c-statistic 0.534). The strongest associations between cognitive impairment and symptoms were consistently seen with memory loss and in general the weakest were with sexual desire.

Defining cognitive impairment using the global T-score and adjusting the threshold by maximising both the sensitivity and specificity per outcome yielded optimum thresholds to

define impairment ranging from 41.3-47.6 (see Table 3-G for details). The mean T-score cut-off was 45.6 yielding a mean c-statistic of 0.582.

Summary health scores (SF-36) were lower in those with cognitive impairment vs. without cognitive impairment, for both mental health and physical health (see Figure 3-C for a graphical illustration) but only significantly so for cognitive impairment diagnosed with MNC. There were weak, positive correlations between the global T-score and SF-36 summary measures of mental and physical health ($\rho=0.166$, $p<0.001$; and $\rho=0.179$, $p<0.01$ respectively).

Table 3-F. Associations of four definitions of cognitive impairment and patient-reported outcome measures.

Outcome (n)	Frascati			GDS			MNC			T-score <45		
	SENS (%)	SPEC (%)	c- statistic	SENS (%)	SPEC (%)	c- statistic	SENS (%)	SPEC (%)	c- statistic	SENS (%)	SPEC (%)	c- statistic
Cognitive symptoms:												
Attention (273)	31.7	71.7	0.516	41.8	69.6	0.557	26.6	79.4	0.530	40.5	74.2	0.574
Memory loss (276)	39.2	74.6	0.569	46.8	71.6	0.592	31.7	81.7	0.567	43.0	75.1	0.591
Reasoning (275)	40.5	71.2	0.508	38.1	68.8	0.535	24.8	79.4	0.521	39.1	75.3	0.522
Lawton IADL (279)	35.0	71.1	0.531	42.5	67.4	0.549	30.0	78.7	0.543	42.5	71.1	0.568
Depression:												
PHQ-9 (263)	35.5	73.3	0.544	43.4	71.1	0.573	30.3	80.2	0.552	40.8	74.9	0.578
CES-D (261)	29.4	71.1	0.502	38.5	71.1	0.548	22.9	79.6	0.513	33.0	74.3	0.537
Falls (280)	32.0	70.9	0.514	36.0	66.5	0.513	26.0	78.7	0.523	38.0	71.3	0.547
Sexual desire (277)	26.4	67.8	0.471	31.2	63.8	0.475	20.8	77.6	0.492	32.0	63.2	0.479

Associations between four definitions of cognitive impairment as well as the optimal global T-score cut-off for patient-reported measures of cognitive function (EACS questions), independence (Lawton IADL), depression (PHQ-9, CES-D), falls and sexual desire.

Abbreviations: CES-D: Centres for Epidemiologic Studies Depression scale; GDS: global deficit score; IADL: instrumental activities of daily living; MNC: multivariate normative comparison; PHQ-9: Patient Depression Questionnaire; SENS: sensitivity; SPEC: specificity.

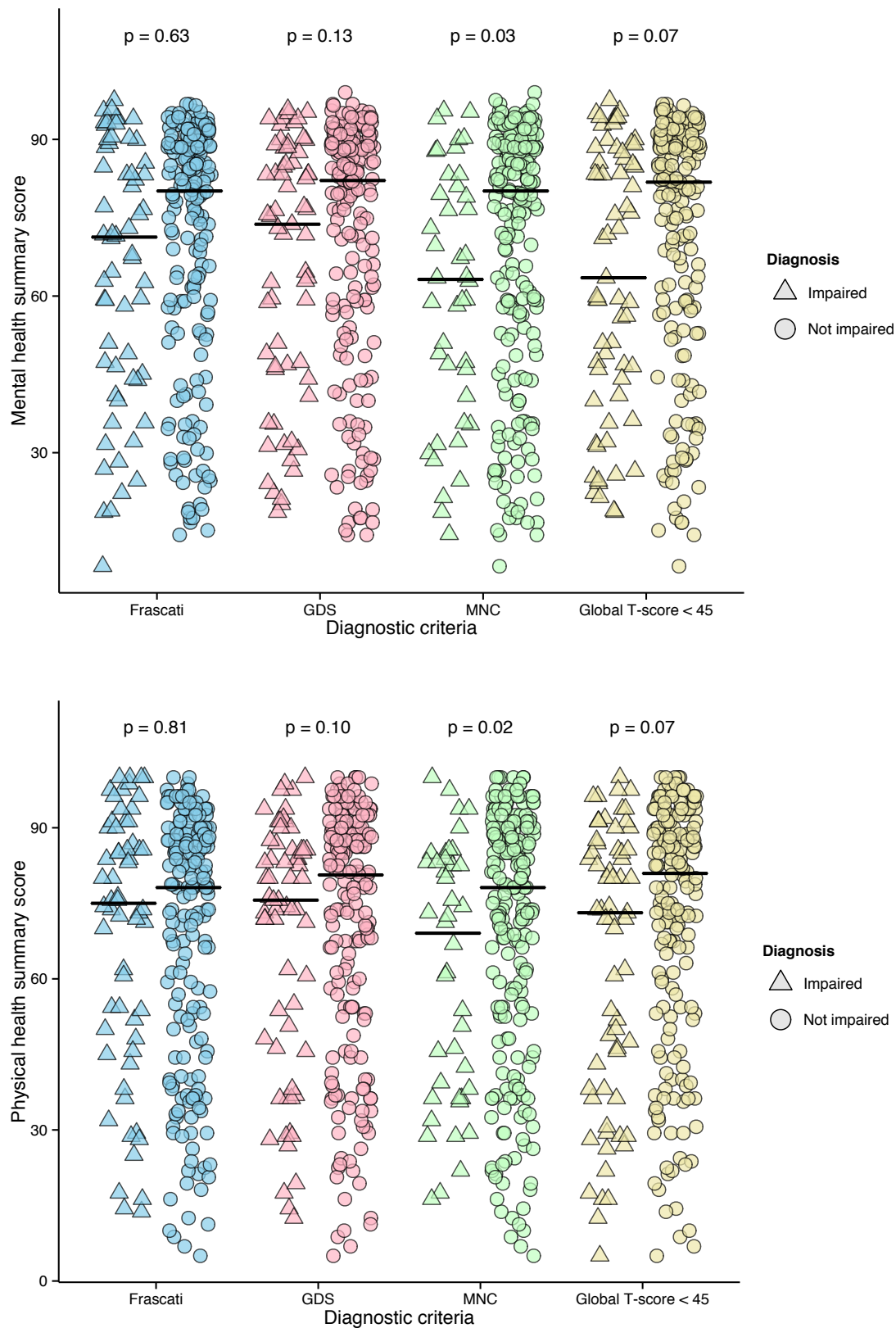
Table 3-G. Optimal global T-score threshold with associated statistics for each patient reported outcome measure.

Outcome (n)	Threshold ¹	Sensitivity	Specificity	c-statistic
Cognitive symptoms				
Attention (273)	46.8	53.16	64.95	0.591
Memory loss (276)	46.6	53.16	68.02	0.606
Reasoning (275)	45.3	41.90	75.29	0.586
Lawton IADL (279)	41.3	35.00	84.94	0.600
Depression:				
PHQ-9 (263)	47.6	59.21	62.57	0.609
CES-D (261)	47.2	48.62	63.82	0.562
Falls (280)	43.9	36.00	77.39	0.567
Sexual desire (277)	46.2	68.00	38.16	0.531

¹Threshold that maximises the c-statistic (i.e. same weight to sensitivity and specificity).

Abbreviations: CES-D: Centres for Epidemiologic Studies Depression scale; GDS: global deficit score; IADL: instrumental activities of daily living; MNC: multivariate normative comparison; PHQ-9: Patient Depression Questionnaire.

Figure 3-C. Mental and physical health summary scores for those with vs. without cognitive impairment by diagnostic criteria.



Black lines denote medians. P-values calculated with the Wilcoxon rank-sum test. Abbreviations: GDS: global deficit score; MNC: multivariate normative comparison.

3.4 Discussion

Supporting my first hypothesis, well-treated HIV-positive patients had poorer cognitive function compared to a demographically comparable HIV-negative control group. However, the differences in T-scores between the two groups were small and less than 5, a difference that may be not considered *clinically* significant.²¹⁴ Cognitive impairment was more prevalent reflecting a skew towards lower scores in the patient group. Again, this was generally mild with only 2% of the patient group fulfilling the criteria for HIV-associated dementia. Interestingly, there were no associations between recreational drug use and impaired cognitive function. In general, those who had used recreational drugs in the last six months had higher T-scores.

As expected and in concordance with previous studies,^{13,22} the prevalence of cognitive impairment differed according to the applied diagnostic criteria. What has not been assessed previously is whether they identify the same individuals as impaired. Agreement was moderate, which was likely to reflect the ability of each method to exclude cognitive impairment in the same subjects. As expected, due to similarities in their methods, agreement was higher between the Frascati and GDS criteria than either with MNC. The lack of agreement is of some concern if this is used as the basis for trying to determine the underlying pathophysiology of cognitive impairment and its associations with other outcomes.

If the diagnosis of cognitive impairment is uncertain and potentially incorrect subsequent associations will also be affected. For example, if half of those identified as having cognitive impairment using the Frascati criteria were in fact just at the lower end of the normal distribution then the pathophysiological mechanisms responsible for impairment in the other half are likely to remain elusive. This may well be the case, as Gisslen *et al*²², using a simplified model of cognitive function testing across five domains, have suggested that 20% of a *normative* population would be ‘diagnosed’ with cognitive impairment (see section 1.3.2 for fuller discussion of the issue). Clearly this is problematic as this criterion may not distinguish between those with pathological impairment and normal variation with any degree of certainty. It is unclear whether the global deficit score approach is similarly prone to classifying a large proportion of a *normative* population as impaired. In Chapter 4 I will explore this issue in more depth using simulated data and an *in silico* model informed with cognitive data from

the POPPY study. Furthermore, I describe and test a novel multivariate method of defining cognitive impairment which may allow more control over the type I error (false positive) rate of diagnosis.

Regardless of the definition used, cognitive impairment correlated poorly with symptomology with predictive power little better than tossing a coin for most outcomes. There are several possible explanations for these observations. Firstly, a lack of a 'gold-standard' method of diagnosing cognitive impairment may limit subsequent associations as discussed above. To mitigate against this, I tested several diagnostic criteria for cognitive impairment as well as to assessing different global T-score thresholds for each outcome measure separately. Secondly, over-reporting of symptoms, whereby patients both with and without cognitive impairment report high rates of symptomatology makes finding associations challenging. Thirdly, the subjectivity of some questions regarding mental state (e.g. memory) makes it difficult to establish a clear relationship between subjective experience and objective measures of cognitive function. Lastly, there may only be a weak relationship between subjective experience and objective measures, particularly in those with only mild impairment. Similar reasons may also explain the weak associations with other patient reported measures. These findings contrast with previous studies that reported poorer functional outcomes in those with cognitive impairment.^{41,42} However, these differences may be explained by differences in the patient populations studied. As discussed previously, historic cohorts had poorer coverage of antiretroviral therapy. As such, prior AIDS events and immunosuppression were more common⁴² (e.g. mean CD4+ cell count 360 vs. 664 cells/ μ L) and the degree of cognitive impairment was more severe⁴² (prevalence of HIV-associated dementia: 34% vs. 2% observed here). Therefore, it is likely that these historic patients had genuine pathological cognitive impairment with much greater disability accumulated from greater HIV-exposure, subsequent immunosuppression and AIDS defining illnesses. This would therefore strengthen the relationship between cognitive function and other patient reported outcomes. However, the associations between cognitive impairment and patient reported measures remained weak even using a combined definition of cognitive impairment, which only captured the most severely impaired. This suggests the link between mild cognitive impairment and symptomology is weak at best.

The lack of clear associations between cognitive impairment and depression was interesting although the relationship between the two is clearly complicated. Depression is commonly

reported in HIV-positive cohorts and it occurred in approximately 30-40% of this cohort depending on the diagnostic criteria used. However, it occurred distinctly from cognitive impairment judged by the poor concordance between the two. Recent work suggests that depression has a significant neuroinflammatory component with changes to indoleamine 2,3-dioxygenase activity and subsequent perturbations of tryptophan metabolism implicated in its aetiology.²⁵² In HIV-positive individuals, depression has been associated with higher plasma HIV RNA with a lower prevalence reported in those on antiretroviral therapy. Both of these findings support a potential aetiological role for HIV.²⁵³ It may be therefore that depression is a manifestation of HIV-associated neuroinflammation in some, whereas in others it occurs distinctly and is associated with more traditional risk factors, such as social isolation. In common with cognitive impairment, the aetiology of depression is complicated and it is likely that there are distinct and potentially multiple pathological processes occurring in different groups that get 'lumped together' into the same disease category. Therefore, further study of those that have co-existing depression and cognitive impairment in HIV-positive patients may reveal common aetiological mechanisms and therapeutic targets. In chapter 6 I will explore this in more detail.

Limitations

This work has several limitations, primarily that as this is a cross-sectional analysis establishing causation is impossible. Although great effort was made to recruit a comparable control population, measured and unmeasured differences between the groups unrelated to HIV-infection exist, e.g. the proportion of females was greater in the control group. As such, not all the observed differences in cognitive function may be attributable to HIV-infection. However, it is unlikely that differences in gender between the two groups explain different rates of cognitive impairment and this was accounted for statistically when standardising cognitive function test results. Another potentially confounding difference between the two groups was the prevalence of recreational drug use, which occurred more commonly in the HIV-positive group. Interestingly, cognitive domain T-scores were generally higher in patients who reported recreational drug use in the last 6 months. Therefore, it is very unlikely that this explains the observed differences in cognitive function. To maximise recruitment and generalisability, exclusion criteria were kept to a minimum. As such, cognitive impairment may have been caused by other degenerative neurological diseases. However, the prevalence of neurological diagnoses did not differ significantly between the groups therefore rates of

cognitive impairment should not be biased towards one group. Furthermore, given the age range of this cohort, neurodegenerative diseases such as Alzheimer's disease or vascular dementia would be uncommon.²⁵⁴

Given the minimal exclusion criteria, the cohort presented here represents a 'real-world' sample. As subjects were recruited prior to the publication of the INSIGHT START study,²⁵⁵ not every patient was receiving suppressive antiretroviral therapy. However, antiretroviral prescribing was in line with national guidelines at the time of enrolment and patients not receiving antiretroviral therapy had a median CD4+ cell count of 664 cells/mm³. Whilst, these patients may have had poorer cognitive function, it is unlikely that this tiny minority are responsible for the differences observed between the groups. Additionally, given the poor concordance of cognitive impairment with other patient reported outcome measures it is unlikely excluding a small percentage of this sample would dramatically change the results.

Missing data may in itself be an 'answer' to a question (e.g. not stating an answer to the question about attention may be a sign of poor attention). However, given that data were complete in over 90% it is unlikely that these results are skewed by this small population of individuals.

Conclusion

Cognitive impairment was more common in HIV-positive patients compared to an appropriate control group. However, the degree of impairment was generally mild and its associations with patient reported measures of physical and mental health were generally weak. This was irrespective of the method used to identify those with cognitive impairment and suggests cognitive impairment remains a distinct co-morbidity and is not part of a general syndrome of ill health associated with chronic HIV-infection. These findings provide support for my first hypothesis and justifies further study into the pathophysiology of cognitive impairment, which I will investigate over the subsequent chapters.

Chapter 4

Medicalising normality? Using synthetic data and simulation to assess the performance of different diagnostic criteria.

Table of contents

CHAPTER 4 MEDICALISING NORMALITY? USING SYNTHETIC DATA AND SIMULATION TO ASSESS THE PERFORMANCE OF DIFFERENT DIAGNOSTIC CRITERIA.		106
4.1	INTRODUCTION	108
4.1.1	<i>A novel, less biased method of determining cognitive impairment</i>	<i>109</i>
4.2	METHODS.....	110
4.2.1	<i>A novel, potentially less biased method of determining cognitive impairment.....</i>	<i>114</i>
	Determining the multivariate distance from normality	115
	Determining the critical value	115
4.3	RESULTS	118
	Prevalence of cognitive ‘impairment’ in a ‘normal’ population.....	118
	Agreement between the Frascati criteria and the GDS	121
	Expected prevalence of cognitive impairment in the POPPY study	123
4.4	DISCUSSION	127
	Conclusion	130

Figures

FIGURE 4-A. VISUALISATION OF A HYPOTHETICAL CORRELATION MATRIX FOR SIX COGNITIVE DOMAINS	110
FIGURE 4-B. VISUALISATION OF A HYPOTHETICAL CORRELATION MATRIX FOR SIX COGNITIVE DOMAINS EACH COMPRISING OF TWO TESTS.	112
FIGURE 4-C. DENSITY PLOTS SHOWING THE EFFECT OF SKEWING THE SYNTHETIC DATA.	114
FIGURE 4-D. CONVERGENCE OF THE CRITICAL VALUE TO DETERMINE COGNITIVE IMPAIRMENT AS THE SAMPLE SIZE INCREASES....	118
FIGURE 4-E. PREVALENCE OF COGNITIVE IMPAIRMENT FOR A SYNTHETIC POPULATION (SIX DOMAIN MODEL)	119
FIGURE 4-F. PREVALENCE OF COGNITIVE IMPAIRMENT FOR A SYNTHETIC POPULATION (SIX DOMAINS, TWO TESTS PER DOMAIN MODEL)	120
FIGURE 4-G. AGREEMENT BETWEEN THE FRASCATI CRITERIA AND GLOBAL DEFICIT SCORE FOR THE TWO MODELS.....	122
FIGURE 4-H. COMPARISON OF AGREEMENT BETWEEN THE GLOBAL DEFICIT SCORE AND FRASCATI CRITERIA BY MODEL.	122
FIGURE 4-I. VISUALISATION OF THE INTER-DOMAIN CORRELATION MATRIX FOR THE HIV-POSITIVE AND HIV-NEGATIVE PARTICIPANTS IN THE POPPY STUDY.....	123
FIGURE 4-J. EXPECTED PREVALENCE OF COGNITIVE IMPAIRMENT BY DIAGNOSTIC CRITERIA FOR A HYPOTHETICAL STUDY POPULATION.	124
FIGURE 4-K. EXPECTED PREVALENCE OF COGNITIVE IMPAIRMENT BY DIAGNOSTIC CRITERIA FOR A HYPOTHETICAL STUDY POPULATION	126

Tables

TABLE 4-A. MAXIMUM PREVALENCE OF EXPECTED COGNITIVE IMPAIRMENT FOR A SYNTHETIC POPULATION BY DIFFERENT DIAGNOSTIC CRITERIA (SIX DOMAINS).....	119
TABLE 4-B. MAXIMUM PREVALENCE OF EXPECTED COGNITIVE IMPAIRMENT IN FOR A SYNTHETIC POPULATION BY DIFFERENT DIAGNOSTIC CRITERIA (SIX DOMAINS WITH TWO TESTS PER DOMAIN).....	121
TABLE 4-C. BOOTSTRAPPED MEAN AND 95% CONFIDENCE INTERVALS FOR THE EXPECTED PREVALENCE OF COGNITIVE IMPAIRMENT BY DIAGNOSTIC CRITERIA.	125
TABLE 4-D. BOOTSTRAPPED MEAN AND 95% CONFIDENCE INTERVALS FOR THE EXPECTED PREVALENCE OF COGNITIVE IMPAIRMENT BY DIAGNOSTIC CRITERIA	126

4.1 Introduction

As previously discussed, it is likely the prevalence of HIV-associated cognitive impairment has been overstated due to methodological issues surrounding the common diagnostic criteria for assessing cognitive impairment (namely the Frascati criteria and the global deficit score). This is especially problematic if a study has no control group to compare the patient group to (e.g. The CHARTER study⁸). A particular method may ‘diagnose’ 30% of a patient group with cognitive impairment and without a control group to compare this value to, this sounds high. If the same method also happens to ‘diagnose’ 20% of a control group too, 30% starts to look less impressive.

The Frascati criteria defines cognitive impairment in those who score at least one standard deviation below the normative mean in two or more cognitive domains.²⁵ For one test, approximately 16% of a normally distributed population would fall below one standard deviation (and therefore 84% score above this threshold). For two tests, it is more complicated and the correlation between the tests determines the overall probability of impairment. At the extremes of correlation, the mathematics is simpler. Where there is no correlation between tests, they are truly independent. Assuming there are six cognitive domains, in this case, the probability of cognitive impairment is:

= 1 – (the probability of not having impairment - i.e. *not* meeting the Frascati criteria)

This is equivalent to:

= 1 – (the probability of scoring either one standard deviation (SD) below the mean in only one domain or not scoring below one SD in all tests)

This is equivalent to:

= 1 – (the probability of scoring one SD below the mean in one test x the number of possible combinations this can occur + [the probability of not scoring below one SD in all tests])

Which approximates to:

$$\approx 1 - \left(\frac{6!}{1!(6-1)!} (0.84^5 \times 0.16) + 0.84^6 \right) \approx 0.25 \text{ or } 25\%$$

If the tests are perfectly correlated the probability (or prevalence) of cognitive impairment in a normal population is the same as if there was only one test, i.e. 0.16 or 16%. The Frascati method makes no correction for multiple comparisons and sets no upper limit of tests/domains specified. A better method may be to only define the bottom 5% of the normal

population as impaired (which is analogous to a one-sided $\alpha=0.05$). For a normal distribution, this is equivalent to scoring approximately 1.64 standard deviations below the mean (for one test). Using the same logic as before, assuming six domains, if they are perfectly correlated the probability of impairment would be ≈ 0.05 or 5% and for fully independent tests it would be ≈ 0.03 or 3%.

To be classified as cognitively impairment by the global deficit score, the mean ('global') deficit score must be ≥ 0.5 . If the cognitive tests are perfectly correlated, to have a global deficit score ≥ 0.5 , the score must be at least one standard deviation below the mean to score a point (no half points are awarded) and therefore the probability of impairment in this scenario is ≈ 0.16 or 16%. If the tests are not correlated, then it is much more complicated as there are many ways to achieve a global deficit score ≥ 0.5 (e.g. having a deficit score of 3 in one domain only or having 3 domains with a deficit score of only 1).

4.1.1 A novel, less biased method of determining cognitive impairment

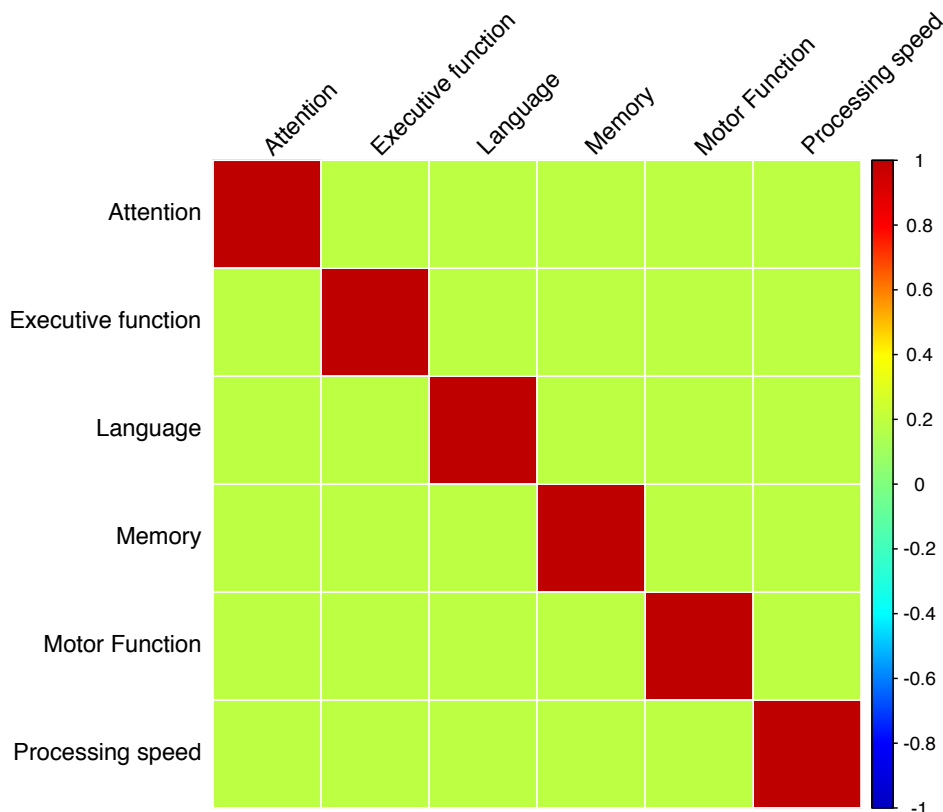
Both the global deficit score and the Frascati criteria are essentially methods of outlier detection with a fixed threshold used to determine impairment for all populations. This allows simplicity and ease of application at the price of accuracy. They are not stringent and therefore are prone to overcalling impairment in a normal population (as demonstrated above). Furthermore, as they do not take into account the correlations between different cognitive domains and that these correlations will be different depending on the tests used and the population studied, the 'one size fits all' approach of defining a threshold is inappropriate. A better method of outlier detection, taking account of the covariance between tests, is to use the Mahalanobis distance. This statistic can be thought of as a multivariate standard deviation.²⁵⁶ If one does not have a control group, then the Mahalanobis distance can be computed from the centre of a hypothetical normal population with same inter-domain correlations as the study population. A synthetic population, similar in size to the study population, can then be used to determine a critical value corresponding to the bottom 5th percentile, so that the probability of impairment is fixed at 0.05 (i.e. one tailed $\alpha=0.05$). Alternatively, the critical value can be determined using probability theory with either the β

or F distributions (outlined later in 4.2.1). If a subject's Mahalanobis distance exceeds this critical value, then they are defined as impaired. This method is not biased by the number of tests performed and is robust to varying correlations between tests. In this chapter, I tested the performance of this method using against other diagnostic criteria using synthetic data and simulation as well as real patient data.

4.2 Methods

To compare different diagnostic criteria, I firstly created a synthetic 'normal' population of 10,000 individuals' cognitive tests. This was accomplished using the 'mvrnorm' command in the MASS package,²⁵⁷ with which I created random data representing 6 cognitive domains with a population mean of 50 and standard deviation of 10 (i.e. a T-score). These data followed a multivariate normative distribution allowing me to control the correlation coefficients between different variables. Figure 4-A displays the correlation matrix of six cognitive domains with an inter-domain correlation coefficient of 0.3.

Figure 4-A. Visualisation of a hypothetical correlation matrix for six cognitive domains

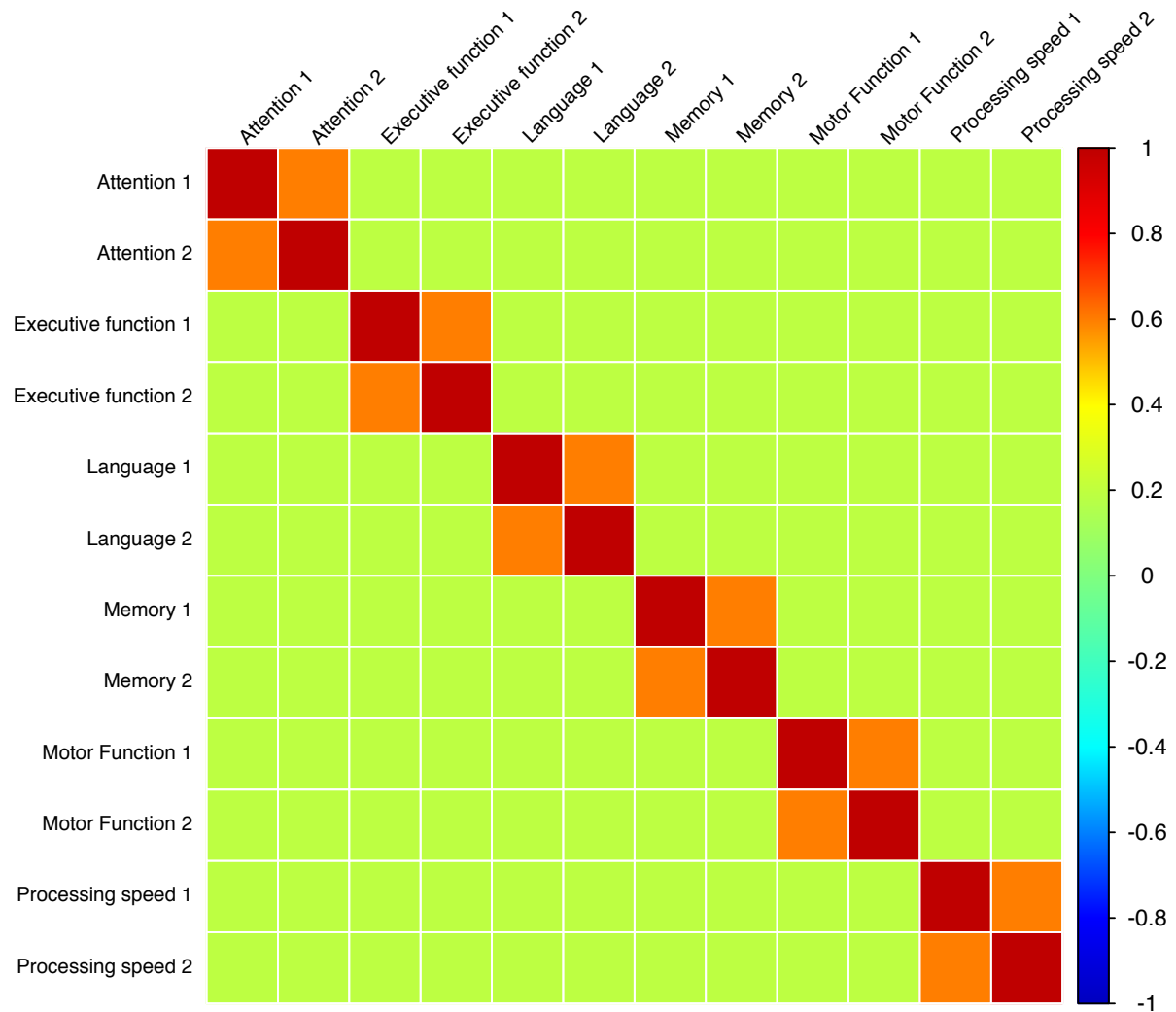


Colour scale determined by Pearson's r (scale to the right of the figure).

Secondly, cognitive impairment was defined according the Frascati criteria²⁵, global deficit score²⁶, a combination of both of these and using a global T-score threshold <45 as previously described (in section 2.2 of the methods). As a comparator, the method using the multivariate Mahalanobis distance (outlined above and detailed later) was also tested using a critical value of -3.26 to determine impairment. Thirdly, as cognitive tests are correlated to some degree and reflect underlying intelligence (see introduction for further discussion), steps one and two were repeated to create synthetic populations over the range of possible inter-domain correlations (Pearson's $r=0-1$). Finally, agreement between cognitive impairment defined using the Frascati criteria and global deficit score across the range of inter-domain correlation coefficients was assessed as described previously using Cohen's κ statistic. These simulations were performed in R v3.2.4 using the 'MASS' package v7.3-45. The code for these simulations is included in Appendix 4.

Next a more complicated synthetic population was created. This time instead of 6 cognitive domains, synthetic data was created for 6 domains each comprised of the mean of 2 tests. In this case, the inter-domain and intra-domain correlations could be controlled. Although more complicated, this model is a closer approximation to the sort of data that is routinely collected in clinical studies. Furthermore, it allows one to test the most liberal interpretation of the Frascati criteria,¹³ namely, defining impairment as scoring one standard deviation below the normative mean in two or more tests (rather than domains). As before, the different definitions of cognitive impairment were applied and the process was repeated for the complete range of inter-domain correlations. As tests within a domain should be more correlated than between domains, the intra-domain correlation coefficient was set at $r=0.6$, which represents the likely upper end of cognitive test re-test reliability.^{60,61} When the simulated inter-domain correlation coefficient exceeded 0.6 the intra-domain correlation was set to be the same as the inter-domain correlation. An example correlation matrix for the 12 cognitive tests with an inter-domain correlation coefficient of 0.3 as before but this time with an intra-domain correlation coefficient of 0.6.

Figure 4-B. Visualisation of a hypothetical correlation matrix for six cognitive domains each comprising of two tests.



Colour scale determined by Pearson's r (scale to the right of the figure).

These models are obviously a simplification as they have fairly simple correlation structures. However, as the correlation structure of the synthetic population of these models can be controlled, it is possible to generate synthetic data with the same inter-domain correlations as any given study population. This then allows the estimation of the prevalence of cognitive impairment in hypothetical study population sampled from a synthetic normal population by various methods. As a study population invariably involves sampling a subset from the entire population, the sample prevalence of cognitive impairment only approximates the population prevalence when n is large. The largest study of HIV-associated cognitive impairment is the CHARTER study⁸ ($n=1,555$). Many other studies of HIV-associated cognitive impairment have much smaller sample sizes, which therefore increases the error associated with their estimates of the population prevalence of cognitive impairment. Although this sampling error

should be unbiased it may be subject to publication bias, whereby a study reporting a high prevalence of cognitive impairment may be more likely to be published than one with a lower prevalence. With synthetic data, one can estimate prevalence and 95% confidence intervals one would expect to find with bootstrapping. As an example, the HIV-positive group (n=290) from the POPPY study in the previous chapter was used as follows. Firstly, the inter-domain correlation coefficients were calculated. Secondly, a synthetic normal population of 100,000 individuals' cognitive data was created assuming a multivariate normal distribution using the correlation matrix calculated in step one. Next, a sample (n=290) was drawn from this synthetic population. The Frascati and global deficit score criteria were then applied as previously described to calculate the sample prevalence of cognitive impairment. Bootstrapping was performed, whereby this sampling step was then repeated 10,000 times (with replacement). The mean prevalence of cognitive impairment and 95% confidence intervals from these bootstrapped samples were then calculated. To illustrate the distributions of the 'measured' prevalences of cognitive impairment, probability density plots were computed with a Gaussian kernel.

As a further, exploratory step, the synthetic normal data was transformed so that it had a negative skew. The purpose of this was to more closely simulate a patient group, where one would hypothesise that HIV-disease (or any other pathological process) may result in poorer cognitive function. This model may also approximate an older 'healthy' population where the distribution may be negatively skewed by the ageing process and occult neurodegenerative disease.²⁵⁸ To transform the data, a skewing function was devised: the natural logarithm of each data point in the original synthetic data was calculated and this was then raised to the power 0.01 (equivalent to taking the 100th root):

$$f(x) = \sqrt[100]{\ln x}$$

This latter value was chosen following a 'tuning' exercise whereby values over a range were assessed and compared to the measured skewness and kurtosis from the HIV-positive patients from the POPPY study. The median skew and kurtosis for these data were -1.02 and 2.49 and for the transformed synthetic data -0.99 and 2.53. Following this transformation, the data was scaled to ensure the standard deviation and median (chosen over the mean because the data

was now skewed) remained comparable to the original data and data from other populations (i.e. mean & median 50 and standard deviation of 10):

$$f(x) = \frac{\sigma(x - \tilde{x})}{s} + \mu$$

Where:

μ = population mean (in this case 50)

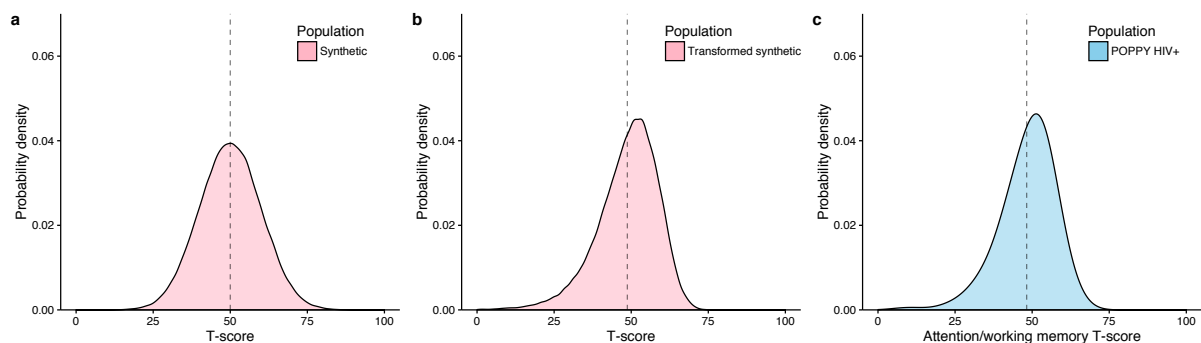
σ = population standard deviation (in this case 10)

$\tilde{x}$ = skewed synthetic population median

s = skewed synthetic population standard deviation

The similarities between real patient data and the transformed synthetic data are illustrated graphically in Figure 4-C. As before, the sample prevalence of cognitive impairment was determined for each method and this step was repeated 10,000 times to obtain mean and 95% confidence interval estimates of the prevalence of cognitive impairment, given the POPPY study data.

Figure 4-C. Density plots showing the effect of skewing the synthetic data.



Density plots of synthetic cognitive data (panel a), skewed synthetic cognitive data (panel b) and for comparison the attention/working memory domain for the HIV-positive group from the POPPY study (panel c). Dotted vertical lines denote the mean.

4.2.1 A novel, potentially less biased method of determining cognitive impairment

In addition to the standard criteria for assessment of HIV-associated cognitive impairment, I propose a new method of assessment that is less prone to type I errors and hence over diagnosis. This method is related to the method of Huizenga *et al*,⁴⁰ however it does not necessarily require a control group. As outlined above this method uses the multivariate

Mahalanobis distance to measure deviation from the norm in a multivariate manner. This is then compared to a critical value and if it exceeds this the subject is then defined as impaired.

Determining the multivariate distance from normality

Firstly, a matrix of correlation coefficients of the study sample's cognitive data is calculated. Next, the Mahalanobis distance is calculated from a hypothetical normal population. This requires the means and covariance of this normal population to be known. For T-scores, the normative mean is 50 and standard deviation is 10 for each domain. The population covariance is estimated from the study sample (or ideally a control group) using the previously calculated correlation matrix. This is then converted into a covariance matrix – this is very straightforward for a T-score as each element of the correlation matrix is multiplied by 100 (10^2). Now the Mahalanobis distance is calculated for each subject of the study. As the Mahalanobis distance has magnitude and not direction, it cannot be assumed that larger values correspond to more severe cognitive impairment (for a normal distribution those above and below the norms would be equal). To provide direction, the sign of the difference between the global T-scores (i.e. the means of the domains) of the subject and the population mean are given to the Mahalanobis distance, so that positive values represent scores in general that are above the mean and negative values represent scores below (i.e. impairment). To determine impairment each subject's signed Mahalanobis distance is then compared to a critical value.

Determining the critical value

There are 3 potential methods for determining the critical value to decide the threshold of impairment. For a mathematically defined critical value, the critical value can be determined in two ways, depending on whether the study sample is used to estimate the comparator covariance or if this is determined from an independent reference population (i.e. a control group). In the first instance, the distribution of the squared Mahalanobis distance from the independent multivariate mean approximates the F distribution and the following formula can be used:

$$critical\ value = -\sqrt{\frac{F_{\alpha; p, n-p} \cdot p(n-1)(n+1)}{n(n-p)}}$$

Where:

n = the number of subjects

p = the number of domains/tests

F = the critical value from the F distribution with p and $n-p$ degrees of freedom with $\alpha=0.05$ (i.e. the bottom 5%)

If no control group is present, or if the assessment of impairment of a member of the control group was to be performed, then each study participant's Mahalanobis distance will be calculated from a hypothetical normal population. To accomplish this, the study observations are used to estimate the covariance for the normal population. In this case, the squared Mahalanobis distance approximates the β distribution and the following formula can be used:

$$critical\ value = -\sqrt{\frac{(n-1)^2}{n} \cdot \beta_{\alpha; \frac{p}{2}, \frac{(n-p-1)}{2}}}$$

Where:

n = the number of subjects

p = the number of domains/tests

β = the critical value from the β distribution with parameters $\frac{p}{2}$ and $\frac{(n-p-1)}{2}$ with $\alpha=0.05$ (i.e. the bottom 5%)

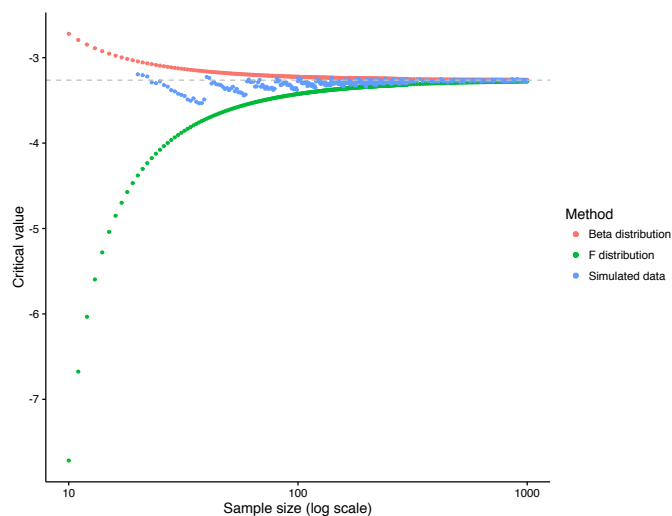
These formulae are derived from the equations described by Maesschalck *et al.*²⁵⁶ taking account of the signing process to determine direction of deviation from the norm and that the base formulae relate to the squared Mahalanobis distance. It should be noted that the first equation using the F distribution is very similar to that described for the multivariate normative comparison between two groups described by Huizenga *et al.*⁴⁰ Further, they are only appropriate in the context of a multivariate normal distribution (i.e. not skewed/transformed).

Lastly, the value can be determined by simulation. To achieve this, firstly, the correlation coefficients of the study sample need to be calculated. Next, a synthetic normal population equal to the sample size of the study is created, as before, based on these correlations with means and standard deviations appropriate to the norm required (e.g. for T-scores a mean of 50 and standard deviation of 10) – as outlined above. Next the Mahalanobis distance is calculated for each 'subject' of this synthetic population. To provide direction, the sign of the difference between the global T-scores (i.e. the means of the domains) of the 'subject' and the population mean (50 for a T-score) are given to the Mahalanobis distance, so that positive values represent scores in general that are above the mean and negative values represent

scores below. These signed distances are ranked in order and the critical value corresponding to the bottom 5th percentile is determined. As the synthetic population has a degree of randomness, this process is repeated 10,000 times and the mean critical value can then be used as a threshold to be applied to real world data. One advantage of this method over using a critical value derived from the F distribution is that it is potentially more robust to skewed distributions as it does not make assumptions about the normality of the data. This could be useful if the model here is extended to include comparison with non-normal control populations. The disadvantage is the inherent variability in using synthetic data is that in each replication a different normal population is created. This leads to potentially imprecise estimates of the critical value. However, given that the population covariance is unknown and is estimated from the data this imprecision is likely to be irrelevant and is minimised by taking the mean from a number of replicates.

Note, where the number of subjects (n) greatly exceeds the number of tests (p) and is large (>200) these three methods converge, e.g. for $n=100,000$, the critical values are -3.2626, -3.2628 and -3.2623 using the β distribution, F distribution and simulated data respectively. This is illustrated graphically in Figure 4-D. It should be noted that for each value of n the critical value determined using simulation for this figure only used 100 replicates (rather than 10,000) to reduce the computation time substantially. It is not anticipated this would change the result significantly. Additionally, where n is small (<100) the bottom 5th percentile cannot really be determined. For example, the bottom 5th percentile where $n=40-59$ corresponds to the 2nd ordered value, however, when $n=60$ it is the 3rd. This explains the observed saw tooth distribution of the data where $n<100$. However, it should be noted that all these values fall between the values calculated using either the β or F distributions and that for studies with a 'good' sample size (say >100) this issue is largely irrelevant.

Figure 4-D. Convergence of the critical value to determine cognitive impairment as the sample size increases.



This model assumes a six test/domain model. The critical value from simulated data was the mean of 100 replicates.

4.3 Results

Prevalence of cognitive ‘impairment’ in a ‘normal’ population

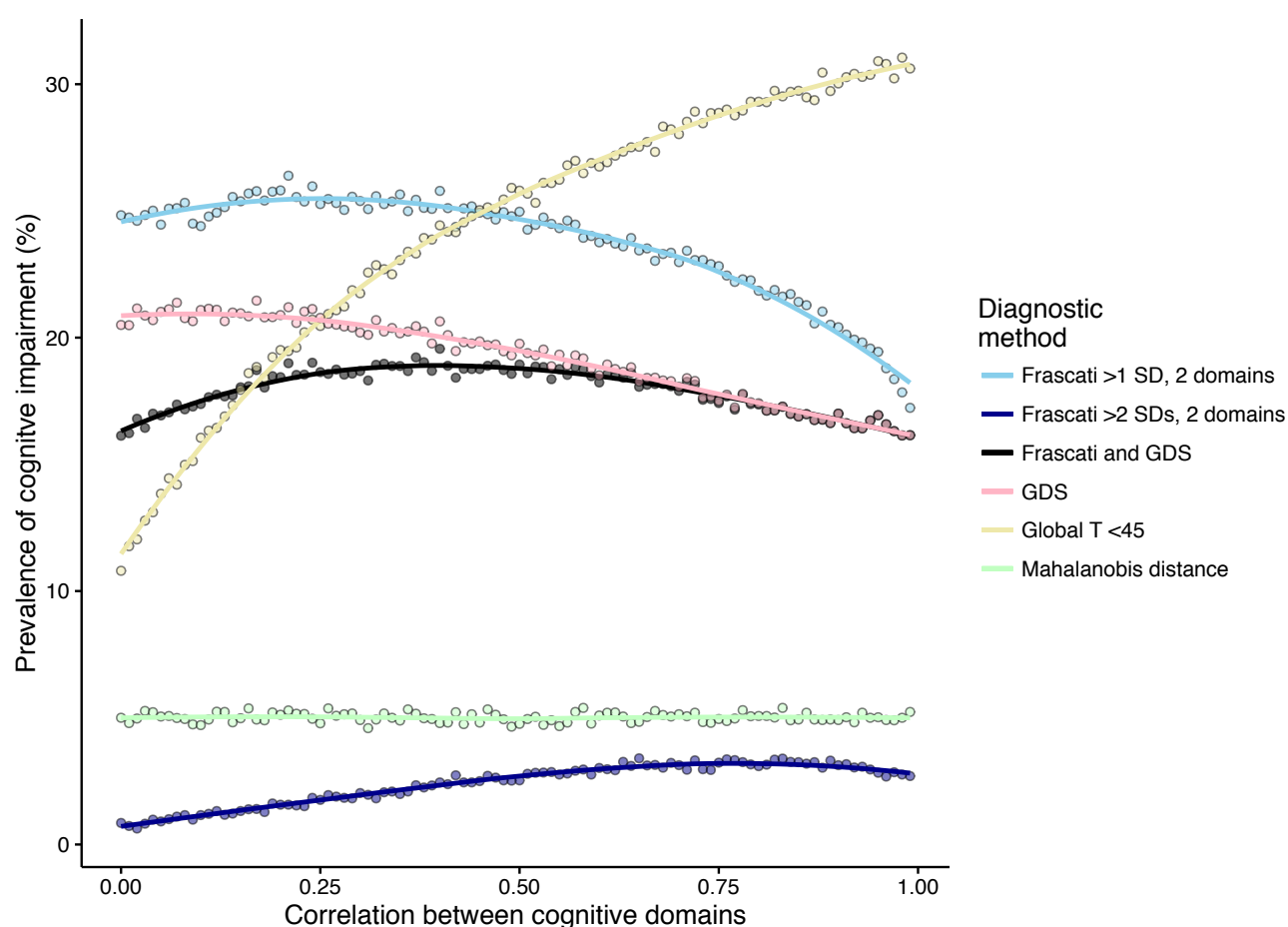
With a six domain model of cognitive function, the prevalence of cognitive impairment in a normal population varies according to the inter-domain correlation coefficient (Figure 4-E). Using the standard definition of the Frascati criteria, cognitive impairment was diagnosed in approximately a quarter of individuals over the range of inter-domain correlation coefficients that are likely in real world data (0.1-0.5). As theory would predict this decreases to approximately 16% as the inter-domain correlation coefficient approaches one. A similar pattern, albeit with lower prevalences over the entire range of inter-domain correlations, was seen for the global deficit score. There was little difference in the prevalence of impairment if the Frascati and global deficit score were combined over the global deficit score alone. The opposite pattern was seen if a global T-score threshold of 45 is used to define impairment, whereby the prevalence of impairment increases from 12% to 31% as the inter-domain correlation coefficient increases from zero to one. The Frascati criteria define HIV-associated dementia present if an individual scores greater than two standard deviations below the normative mean in two or more tests.²⁵ Using this criterion, the maximum expected prevalence of cognitive impairment in a normal population was 3.4%.

In contrast to the other methods, using the multivariate Mahalanobis distance as a measure of deviation from normality, the prevalence of cognitive impairment remained stable at approximately 5% across the entire range of inter-domain correlation coefficients. Table 4-A shows the maximum expected prevalence of cognitive impairment by diagnostic criteria.

Table 4-A. Maximum prevalence of expected cognitive impairment for a synthetic population by different diagnostic criteria (six domains)

Diagnostic criteria	Maximum prevalence (%)	Correlation coefficient (r)
Frascati (>1 SD, 2 domains)	26.4	0.21
Frascati (>2 SDs, 2 domains)	3.4	0.65
Global deficit score (GDS)	21.5	0.17
Frascati and GDS	19.6	0.40
Global T-score <45	31.1	0.98
Mahalanobis distance	5.4	0.58

Figure 4-E. Prevalence of cognitive impairment for a synthetic population (six domain model)



Cognitive function assessed over six domains by different diagnostic criteria across the range of inter-domain correlation coefficients. Abbreviation: GDS: global deficit score.

Using a more complicated model, whereby cognitive function was tested over six domains with two domains per test, similar high prevalences of cognitive impairment were seen for the majority of diagnostic methods (Figure 4-F). Applying the Frascati criteria in its most liberal form, where only two abnormal tests are needed (irrespective of the number administered), cognitive impairment was apparent in over 50% of the synthetic normal population at the lower values of the inter-domain correlation coefficient. Applying the Frascati criteria to cognitive domains rather than individual tests reduced the prevalence of impairment substantially (see Table 4-B for a comparison of the maximum prevalences by diagnostic method). Again, using the multivariate Mahalanobis distance method resulted in a stable prevalence of cognitive impairment across the range of inter-domain correlation coefficients. The prevalence of HIV-associated dementia was higher than in the earlier model but remained fairly stable at approximately 5% over the range of inter-domain correlation coefficients.

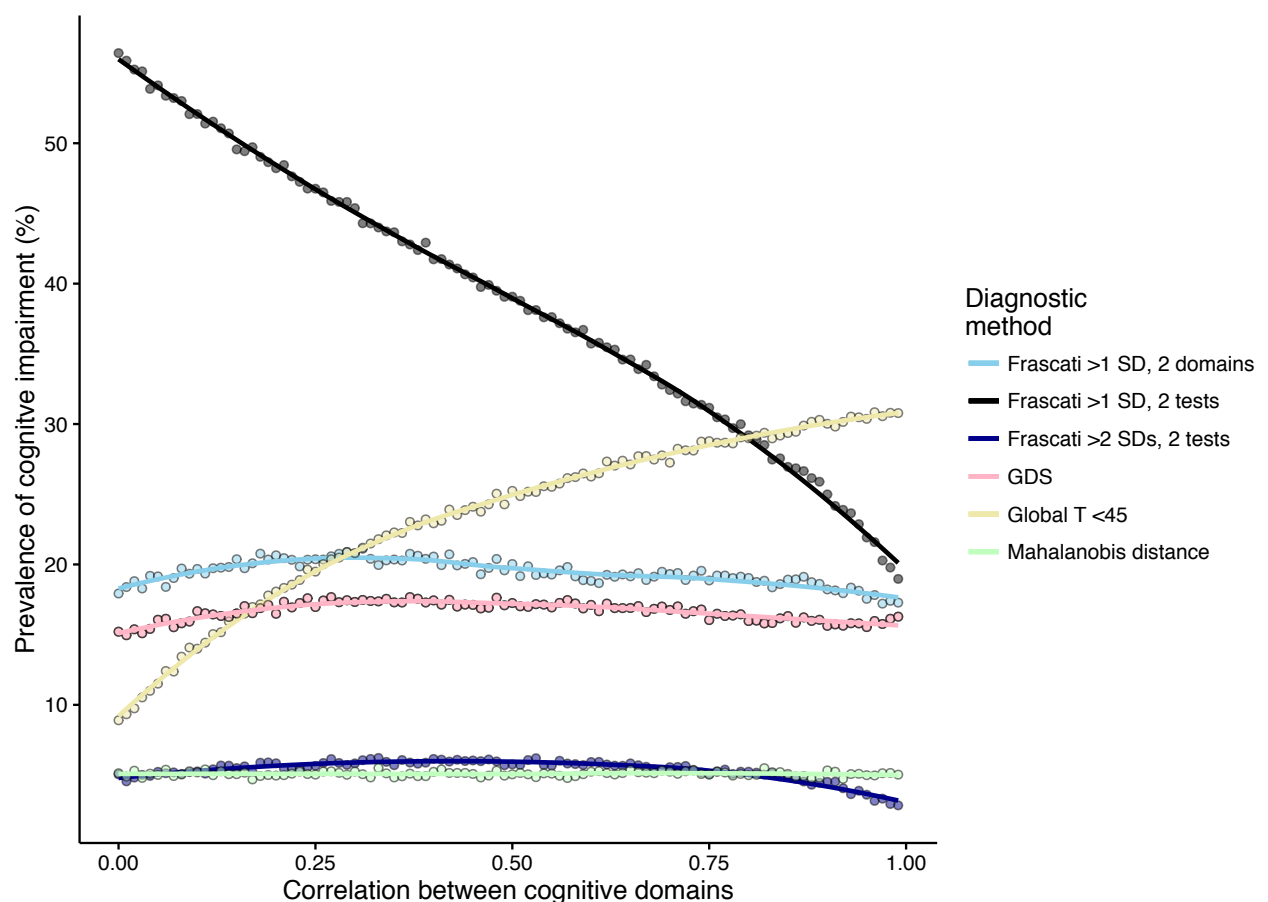


Figure 4-F. Prevalence of cognitive impairment for a synthetic population (six domains, two tests per domain model)

Cognitive function assessed over six domains, with two tests per domain, by different diagnostic criteria across the range of inter-domain correlations coefficients. Abbreviation: GDS: global deficit score.

Table 4-B. Maximum prevalence of expected cognitive impairment in for a synthetic population by different diagnostic criteria (six domains with two tests per domain)

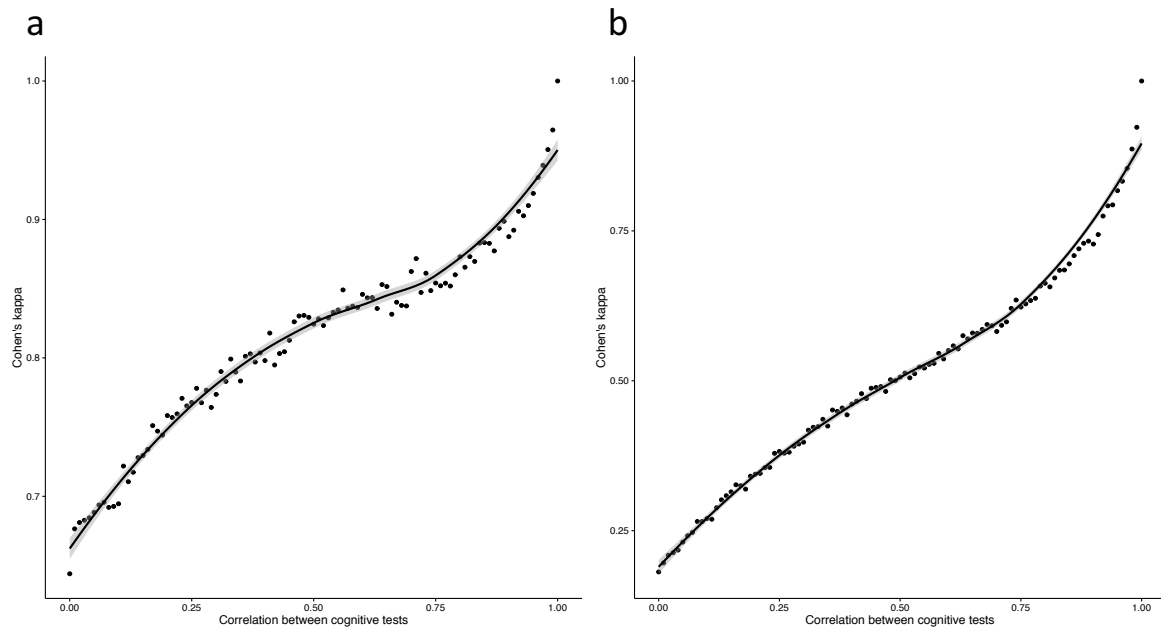
Diagnostic criteria	Maximum prevalence (%)	Correlation coefficient (r)
Frascati (>1 SD, 2 tests)	56.4	0.00
Frascati (>1 SD, 2 domains)	21.1	0.31
Frascati (>2 SDs, 2 tests)	6.2	0.33
Global deficit score (GDS)	17.7	0.27
Global T-score <45	30.8	0.96
Mahalanobis distance	5.5	0.33

Agreement between the Frascati criteria and the GDS

Given that the Frascati criteria and global deficit scores diagnose cognitive impairment in a normal population between 18-56% depending on the model used, I next sought to answer the obvious question: do they identify the same people as impaired? Using the simple six cognitive test/domain model it can be seen that agreement is generally good, but by no means perfect, across the range of inter-test correlation coefficients (Figure 4-G panel a). It can be seen that agreement is poorest between inter-test correlations of 0.0-0.5, the range that would be expected with real world data. Using the more complicated model of cognitive function testing, with six domains each comprising of two tests, a similar relationship between agreement and the inter-test correlation coefficient was seen (Figure 4-G panel b). However, the level of agreement was substantially lower and was poor in the range expected with real world data.

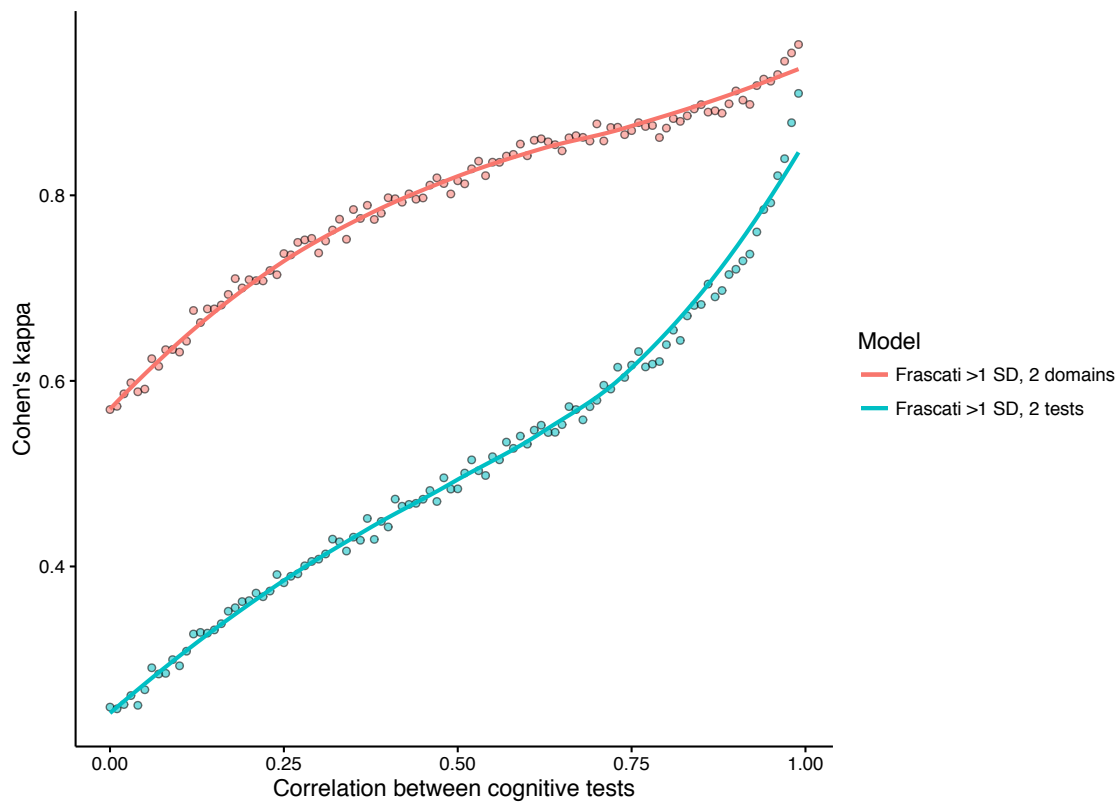
It should be noted that this latter comparison is between the global deficit score and the original definition of the Frascati criteria, requiring only two abnormal tests, rather than two abnormal domains. Using the more conservative definition of the Frascati criteria, requiring two abnormal domains rather than two tests to define impairment, resulted in substantially better agreement (Figure 4-H).

Figure 4-G. Agreement between the Frascati criteria and global deficit score for the two models.



Level of agreement (quantified with Cohen's κ) between the Frascati criteria and the global deficit score for the six tests/domains model of cognitive function testing (panel a) and the six domains, two tests per domains model (panel b).

Figure 4-H. Comparison of agreement between the global deficit score and Frascati criteria by model.

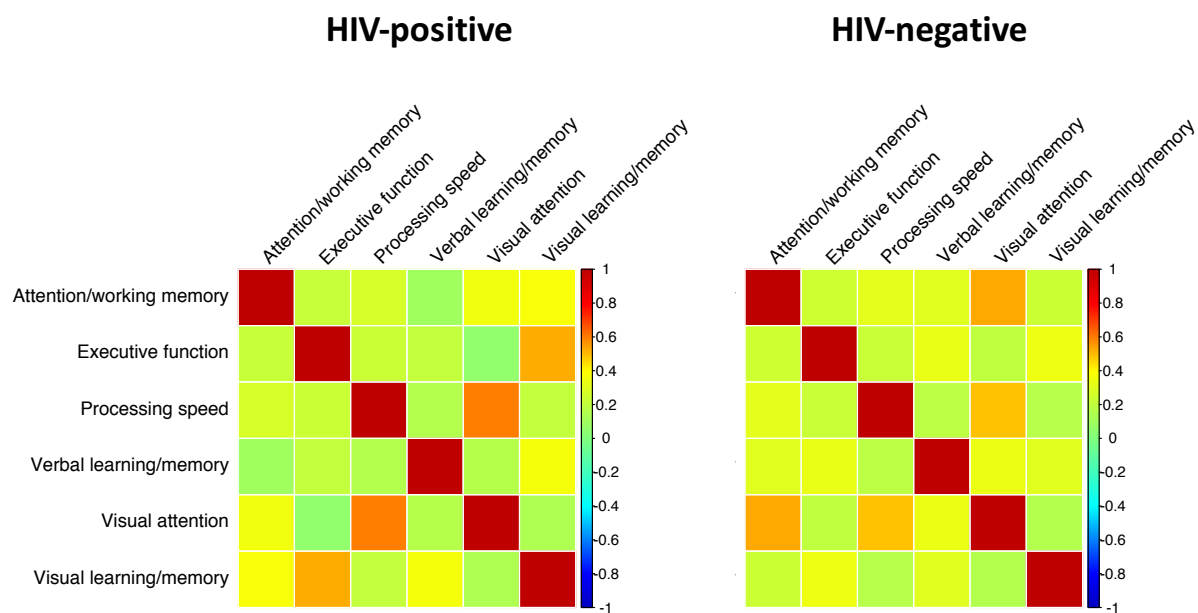


Level of agreement (quantified with Cohen's κ) between the Frascati criteria and the global deficit score for a six domain (two tests per domain) model of cognitive function testing where the Frascati criteria are applied to individual tests (blue line) or individual domains (red line).

Expected prevalence of cognitive impairment in the POPPY study

The simulated data from the synthetic population demonstrated that both the Frascati criteria and global deficit score methods would classify a significant proportion of a normal population as cognitively impaired. However, this was shown using simplistic models of testing cognitive function. Are these estimates relevant to real world data? To investigate this, I used the cognitive data from the HIV-positive participants from the POPPY study ($n=290$, see previous chapter and methods for details). Firstly, the inter-domain correlation coefficients were calculated. These ranged from $r=0.05$ for processing speed and verbal learning/memory to $r=0.60$ for executive function and processing speed (illustrated graphically in Figure 4-I with the correlations for the HIV-negative group shown for comparison).

Figure 4-I. Visualisation of the inter-domain correlation matrix for the HIV-positive and HIV-negative participants in the POPPY study.



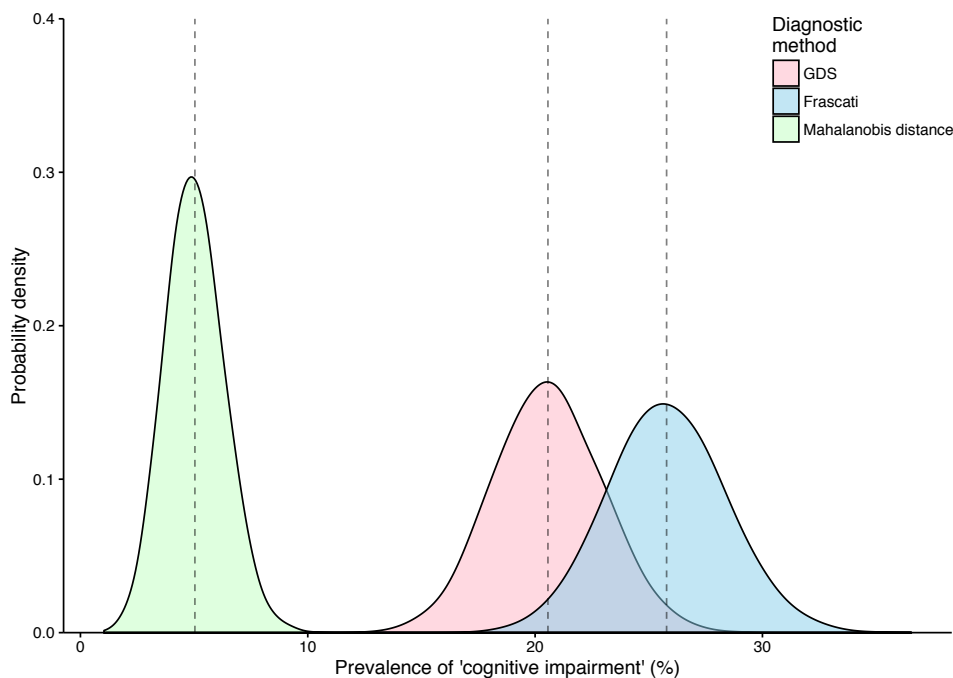
Colour scale determined by Pearson's r (scale to the right of the figure).

Next, a synthetic normative sample of 100,000 individuals' cognitive scores across 6 domains was created with identical inter-domain correlations but with a mean T-score of 50 and standard deviation of 10. Using the method of Steiger²⁵⁹ the inter-domain correlation matrices did not differ significantly between the HIV-positive and HIV-negative control groups ($\chi^2_{15}=19.5$, $p=0.19$) and the synthetic population and the HIV-positive POPPY participants are

shown to be the same ($\chi^2_{36}=0, p=1$). To determine the distribution of expected prevalences of cognitive impairment for the POPPY participants, a sample of 290 subjects were chosen at random, without replacement, from the synthetic population. The standard Frascati and global deficit score criteria were then applied to determine the prevalence of impairment in this sample. Furthermore, the novel definition of cognitive impairment based on the Mahalanobis distance was also applied using a critical value of -3.26 (see the methods for how this threshold was defined). This process was then repeated 10,000 times.

Using the Frascati or global deficit score methods of classifying impairment, on average over 20% of a normal sample would be classified as impaired but with significant variability (Figure 4-J and Table 4-C). In contrast, the Mahalanobis distance method resulted in a lower estimated prevalence of impairment of 5% (95% CIs: 3.1-7.2%).

Figure 4-J. Expected prevalence of cognitive impairment by diagnostic criteria for a hypothetical study population.



Prevalence estimates for a hypothetical study population (n=290) sampled from a synthetic normal population (n=100,000) with 10,000 replicates. Abbreviation: GDS: global deficit score.

Table 4-C. Bootstrapped mean and 95% confidence intervals for the expected prevalence of cognitive impairment by diagnostic criteria.

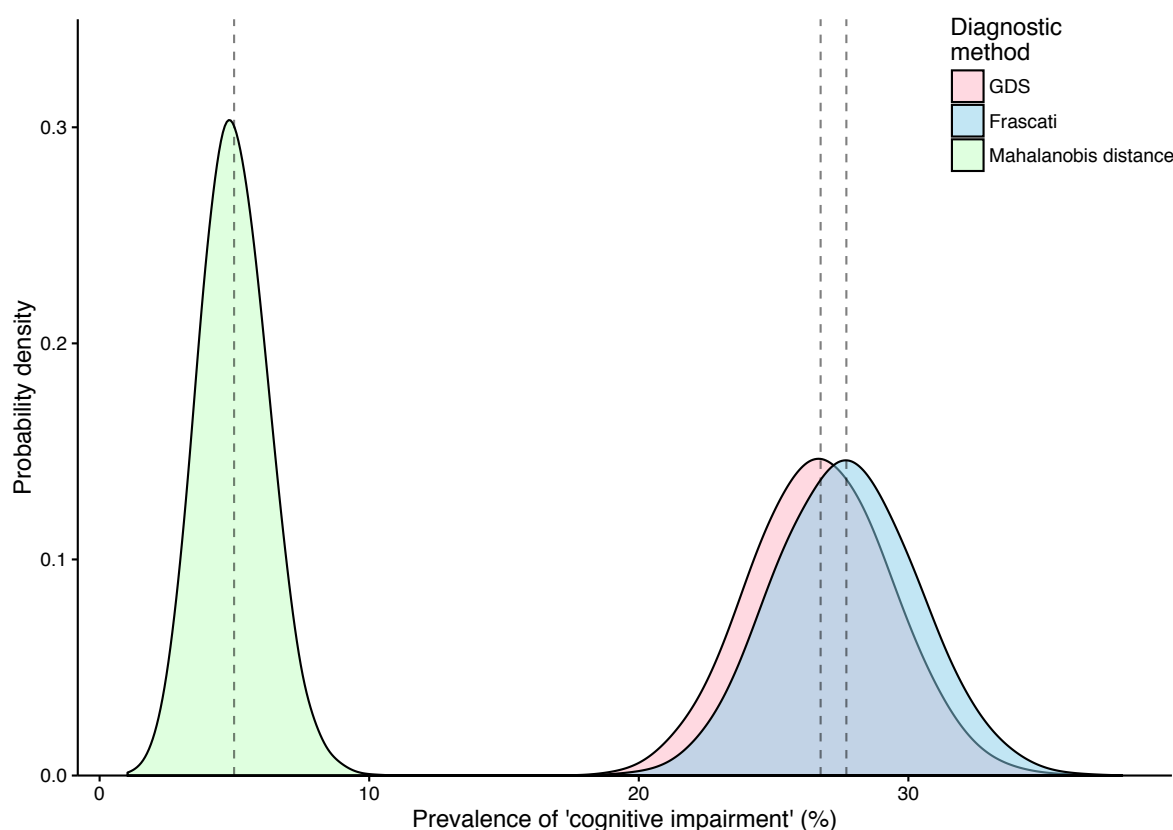
Criteria	Mean prevalence (%)	95% CIs (%)
Frascati	25.8	21.7-30.0
GDS	20.6	16.9-24.5
Mahalanobis distance	5.0	3.1-7.2

Prevalence estimates for a hypothetical study population (n=290) sampled from a synthetic normal population (n=100,000) with 10,000 replicates. Abbreviations: CI: confidence intervals; GDS: global deficit score.

For reference purposes, the prevalence of cognitive impairment in the HIV-negative control group in the POPPY study using the Frascati criteria was 16.5% and for the global deficit score was 14.4%, both lower than would be expected by chance.

As an exploratory step, the same method was then applied to the skewed, synthetic data (median [IQR] T-score 50.0 [43.2-55.7] for each domain), which may be more representative of an older ‘normal’ population (median skew and kurtosis for the POPPY cohort: -1.10 and 2.71; for the synthetic population 0.00 and -0.01; for the skewed synthetic population: -1.01 and 2.96). As before, the Frascati and global deficit score methods diagnosed a significant proportion of this ‘normal’ population as cognitively impaired (over 26% on average, Figure 4-K and Table 4-D). Again, and in contrast to the other two methods, the Mahalanobis distance method, this time with the critical value determined by simulation ($=-3.86$) resulted in a much lower estimated prevalence of impairment of 5% (95% CIs: 3.1-7.2%).

Figure 4-K. Expected prevalence of cognitive impairment by diagnostic criteria for a hypothetical study population



Prevalence estimates for a hypothetical study population ($n=290$) sampled from a skewed synthetic population ($n=100,000$) with 10,000 replicates.

Abbreviation: GDS: global deficit score.

Table 4-D. Bootstrapped mean and 95% confidence intervals for the expected prevalence of cognitive impairment by diagnostic criteria

Criteria	Mean prevalence (%)	95% CIs (%)
Frascati	27.7	23.4-32.1
GDS	26.7	22.4-31.0
Mahalanobis distance	5.0	3.1-7.2

Prevalence estimates for a hypothetical study population ($n=290$) sampled from a skewed synthetic population ($n=100,000$) with 10,000 replicates. Abbreviations: CI: confidence intervals; GDS: global deficit score.

As a final step, the Mahalanobis distance method of defining cognitive impairment outlined here was applied to the POPPY participants from Chapter 3. Using this method, the prevalence of impairment was 15.8% in the patient group and 6.3% in the control group (odds ratio 2.80 [1.15-6.80], $p=0.03$). Next, the same method as before (see section 3.2) was used to assess the relationship between subjective cognitive symptoms and objective cognitive

impairment. However, using this new diagnostic definition, HIV-positive individuals identified as cognitively impaired did not report significantly greater symptoms of attentional problems, memory loss or reasoning difficulties than those who were not impaired (30.1% vs. 27.1%, $p=0.34$; 37.3% vs. 24.1%, $p=0.09$; 38.6% vs 36.7%, $p=1.0$ respectively).

4.4 Discussion

In this chapter, I have shown the utility of using synthetic data to test the underlying assumptions of two commonly used methods of defining HIV-associated cognitive impairment. The expected prevalence of cognitive impairment changes depending on the correlation between individual cognitive tests. However, in the range that would be expected with real-world cognitive data the Frascati and global deficit score methods would classify over 20% of a 'normal' population as impaired. Furthermore, as all studies rely on sampling from a population this figure may be further enlarged due to the inherent error in sampling. Using data from the POPPY study (chapter 3), it can be shown that for a sample of equivalent size ($n=290$), up to 30% may be labelled as cognitively impaired. This confirms my hypothesis that these methods are prone to over diagnosis. This overestimation can be mitigated by using a multivariate approach, limiting the prevalence (or type I error) of cognitive impairment in the normal population to any desired level. The value of 5% was chosen for study here due to tradition ($\alpha=0.05$), to balance sensitivity and specificity and for the purposes of study I am only interested in impairment and not supranormal cognitive performance, hence the bottom 5% (i.e. one-sided $\alpha=0.05$ which has been used in previous studies of HIV-associated cognitive impairment¹³). However, I have outline here how any desired threshold can be calculated.

As discussed in the introduction, it is not unsurprising given the methodology of both the Frascati criteria and global deficit score that such a large proportion on a normal population are classified as impaired by chance. Standard deviations are useful when dealing with one dimensional, normally distributed data. With correlated multivariate data, it is less useful and arguably inappropriate. This is particularly true if there is no stipulated maximum number of tests that a given criterion is applied to. This was demonstrated by the expected prevalence of cognitive impairment using the Frascati criteria applied to six tests (model 1) or 12 tests (model 2) where the maximum prevalence increased from 26% to 56%. This compares to two recent studies that reported rates of impairment in the HIV-negative control group of 36%¹³

and 29%.¹⁵ Similarly, McDonnell *et al*¹⁵ reported impairment in 27% of the HIV-negative control group they studied using the global deficit score. As I have demonstrated, these are similar to what would be expected by chance. This is clearly problematic for several reasons. Firstly, without a control group to compare to (e.g. the CHARTER study⁸), the prevalence of impairment may be over interpreted. The headline figure of 52% of HIV-positive individuals having cognitive impairment does not seem quite as dramatic considering what would be expected by chance alone. This may be interpreted as being ‘caused’ by HIV but in fact the attributable proportion is much lower. Secondly, if approximately a quarter of a normal population are labelled as impaired by chance it is likely that a similar proportion of a patient group would be too. Therefore, within the impaired patient group only some will have a pathological process resulting in low test scores. Interestingly, patients in CHARTER with nadir CD4+ cell counts >200 cells/ μ L, on antiretrovirals with ‘undetectable’ plasma HIV RNA had a rate of cognitive impairment of 30%,⁸ which is at the upper end of what may be expected by chance. If impairment is subsequently used to then explore the pathophysiology of disease by associations with other measures of interest (e.g. CSF biomarkers), statistical power will consequently be reduced, which may lead to false conclusions. Thirdly, if these methods are used to decide entry into a clinical trial or used to decide the success of treatment they again are likely to reduce the power to detect changes, given that some of the impaired group will be labelled as such by chance and will not have a pathological process amenable to treatment. Finally, if such methods are used in clinical practice they may cause unnecessary anxiety to patients and may prompt unnecessary further investigations or treatment. Hence the need for a multivariate method to define cognitive impairment, which is robust to the distribution of data and can control the false discovery rate.

With the shortcomings of these methods demonstrated here it is worth considering the reasons why we would want to diagnose cognitive impairment. Scientific curiosity aside, the purpose of diagnosis is generally to aid the treatment of disease. At some point a yes/no or impaired/not impaired decision must be made and the simplicity of dichotomised cognitive function data is desirable. The motivation for updating the HIV-associated neurocognitive disorders classification, to create the so-called Frascati criteria, was to improve standardisation by introducing clear definitions of what was considered abnormal and to include a category of asymptomatic impairment.²⁵ This category of mild impairment, without deficit in performance in everyday activities, was thought to be a possible precursor to more severe impairment. Consequently, identification of patients in this group was considered

important as subsequent intervention may prevent further decline. However, the evidence that these mild forms of cognitive impairment progress is mixed and the data that do suggest an increased risk of functional impairment longitudinally were in populations with inadequate suppression of HIV-replication.^{11,59,260,261} It is clear from these simulation data that only requiring deviation of one standard deviation from the norm in two or more tests is not a stringent enough criterion, resulting in many patients being labelled as impaired by chance alone. The global deficit score method represents a slight improvement, but still labels approximately 20% as impaired by chance. Using the more stringent criteria of requiring deviation of two standard deviations in two or more tests substantially reduces the false positive rate. However, the extent to which it does this is dependent on the number of tests performed and how performance correlates between them. A better, albeit more complicated, method using the multivariate Mahalanobis distance is detailed here, which allows the false positive rate to be controlled at any desired level (5% in this work).

These findings have potentially important implications. Although the Frascati and global deficit scores are research methods of defining HIV-associated cognitive impairment their use may not be limited to academic studies. Research classification and scoring systems often find their way into clinical practice and the Frascati criteria are no exception.²⁶² The data presented here provide strong evidence that a significant proportion of a *normal* population would be defined as ‘cognitively impaired’ using either the Frascati or global deficit score methodologies. If these criteria were used to screen otherwise healthy HIV-positive individuals they are likely to lead to substantial proportion being labelled as ‘cognitively impaired’. This is problematic as it is likely to lead to psychological distress for the patient and may lead to unnecessary investigation and treatment of what may well be *normal* variation. The multivariate method outlined and tested here is less prone to type I error and can easily be adapted to reduce this from the 5% level presented and tested here. Therefore, it offers considerable advantages over the Frascati and global deficit score methods of defining impairment and is more likely to identify patients with a genuine pathological process which may be more amenable to treatment. Further study of this novel definition of cognitive impairment is warranted – in particular investigation of whether patients identified using this method differ in terms of biomarker evidence of brain injury. This is especially pertinent given the lack of association reported between HIV-associated cognitive impairment and brain structure.¹²⁷⁻¹³⁰ Additionally, its use should be considered in controlled treatment trials where more lenient definitions of

cognitive impairment, which may have resulted in recruitment of patients simply at the lower end of the *normal* distribution, may have contributed to the negative results in the past.^{214,263}

As outlined earlier, dichotomising continuous data is desirable to make decisions. However, for the purposes of research it may be better to take advantage of the continuous nature of cognitive function testing data. For the purposes of statistical analysis, dichotomising data into two groups has many drawbacks.²⁶⁴ Firstly, given the distribution of most cognitive data, the assumption there are two clear groups is probably not valid. Furthermore, the difference between those with and without impairment at the margin, is greatly exaggerated. Secondly, as up to a third of information is lost,²⁶⁴ statistical power is reduced which may lead to false conclusions being drawn. For the purposes of determining the pathophysiology of HIV-associated cognitive impairment in subsequent chapters I will therefore use continuous measures of cognitive function.

Conclusion

The commonly used diagnostic methods of HIV-associated cognitive impairment are too lenient and label a significant proportion of a normal population as cognitively impaired by chance alone. These findings have serious implications for future research. More stringent methods of diagnosis should be implemented if decisions regarding care are to be made, preferably using multivariate techniques. Where possible, continuous rather than dichotomised measures of cognitive function should be used, particularly in the research setting.

Chapter 5

Structural brain abnormalities and their association with cognitive function in well-treated HIV-disease

Table of contents

CHAPTER 5 STRUCTURAL BRAIN ABNORMALITIES AND THEIR ASSOCIATION WITH COGNITIVE FUNCTION IN WELL-TREATED HIV-DISEASE.....		137
5.1	INTRODUCTION	133
5.2	METHODS.....	134
	Participants	134
	Cognitive function tests	135
	Neuroimaging acquisition (at 3 Tesla).....	135
	Image processing	135
	Statistical analyses	136
5.3	RESULTS	137
	Participants	137
	Cognitive function	139
	Brain tissue volumes	140
	Diffusion tensor imaging	141
	Cognitive function and grey matter volume	141
	Cognitive function and fractional anisotropy.....	145
	Cluster analysis.....	148
5.4	DISCUSSION	150
	Conclusions	155

Figures

FIGURE 5-A. GRAPHICAL OVERVIEW OF METHODS.	140
FIGURE 5-B. BOXPLOTS OF DEMOGRAPHICALLY ADJUSTED COGNITIVE DOMAIN T-SCORES BY HIV-STATUS.	146
FIGURE 5-C. GREY MATTER VOXEL-BASED MORPHOMETRY GROUP COMPARISON.	147
FIGURE 5-D. WHITE MATTER TRACT BASED SPATIAL STATISTICS GROUP COMPARISON.	148
FIGURE 5-E. GREY MATTER VOLUME POSITIVELY CORRELATES WITH GLOBAL COGNITIVE FUNCTION.....	150
FIGURE 5-F. GREY MATTER VOLUME POSITIVELY CORRELATES WITH EXECUTIVE FUNCTION.	150
FIGURE 5-G. SPATIAL CORRESPONDENCE BETWEEN HIV-ASSOCIATED GREY MATTER ATROPHY AND GREY MATTER REGIONS CORRELATED WITH COGNITIVE FUNCTION.....	151
FIGURE 5-H. FRACTIONAL ANISOTROPY POSITIVELY CORRELATES WITH GLOBAL COGNITIVE FUNCTION.....	153
FIGURE 5-I. FRACTIONAL ANISOTROPY POSITIVELY CORRELATES WITH EXECUTIVE FUNCTION.	153
FIGURE 5-J. SPATIAL CORRESPONDENCE BETWEEN HIV-ASSOCIATED WHITE MATTER MICROSTRUCTURAL INJURY AND WHITE MATTER TRACTS CORRELATED WITH COGNITIVE FUNCTION.	154
FIGURE 5-K. CLUSTER ANALYSIS SPLITS PARTICIPANTS INTO TWO GROUPS ON THE BASIS OF VOLUMETRIC AND DIFFUSION DATA. .	156

Tables

TABLE 5-A. BASELINE CHARACTERISTICS OF THE COBRA COHORT.	145
TABLE 5-B. BRAIN VOLUME ADJUSTED MEANS BY HIV-STATUS AND 95% CONFIDENCE INTERVALS (CI).	147
TABLE 5-C. DIFFUSION MEASURE ADJUSTED MEANS BY HIV-STATUS AND 95% CONFIDENCE INTERVALS (CI).	148
TABLE 5-D. MULTIPLE LINEAR REGRESSION ESTIMATES FOR GREY MATTER VOLUME BY COGNITIVE DOMAIN.....	149
TABLE 5-E. GREY MATTER VOLUME LEAST SQUARES MEANS FOR BRAIN REGIONS POSITIVELY CORRELATED WITH COGNITIVE DOMAIN T-SCORES.	152
TABLE 5-F. MULTIPLE LINEAR REGRESSION ESTIMATES FOR GREY MATTER VOLUME AND FRACTIONAL ANISOTROPY BY COGNITIVE DOMAIN.....	152
TABLE 5-G. MEAN FRACTIONAL ANISOTROPY LEAST SQUARES MEANS FOR BRAIN REGIONS POSITIVELY CORRELATED WITH COGNITIVE DOMAIN T-SCORES.....	155
TABLE 5-H. K-MEANS CLUSTER VALIDATION STATISTICS.....	155

5.1 Introduction

Despite dramatic improvements in life expectancy because of antiretroviral therapy³ cognitive impairment remains prevalent, reportedly affecting up to 50% of HIV-positive individuals.^{10,243} However, the reported prevalence of HIV-associated cognitive impairment varies substantially,¹³ which is likely to be due in part to the methodological issues exposed in the previous chapter. Furthermore, there remains uncertainty about its aetiology in well-treated populations.

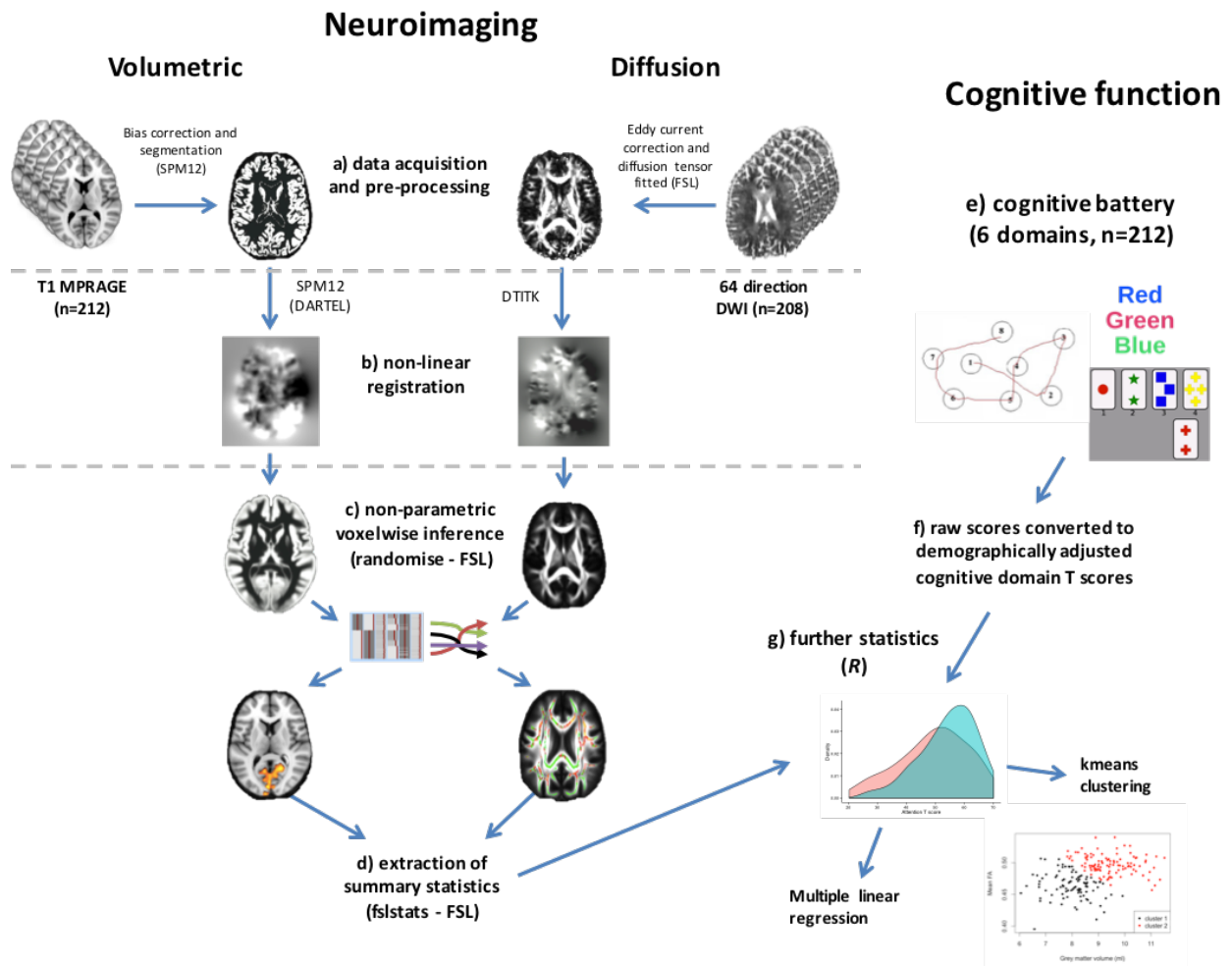
One possibility is that cognitive impairment results from structural damage to the brain regions that support cognition. Earlier work describing grey and white matter abnormalities in HIV-positive individuals provide support for this hypothesis as detailed in chapter one. For example, HIV-associated grey matter atrophy has been described in numerous locations.¹²²⁻¹²⁷ Less extensive HIV-associated white matter atrophy has also been reported, most consistently in the corpus callosum.^{124,127} Diffusion weighted imaging is more sensitive at revealing white matter abnormalities in HIV-positive individuals.^{129,130,147-150} However, the majority of data are from cohorts of patients with treatment that would be considered suboptimal by today's standards. Moreover, the relationships between imaging abnormalities and cognitive function remain uncertain, particularly in well-treated patients, with several studies reporting no associations.¹²⁷⁻¹³⁰

Given the advances in HIV-medicine, the study of exclusively virally suppressed HIV-positive individuals is necessary. Additionally, comparison with demographically matched HIV-negative control group is necessary to reduce confounding due to lifestyle factors that may impact cognitive function. Using detailed data from the COBRA cohort, this chapter will address two overarching hypotheses of this thesis, firstly that cognitive impairment remains more prevalent in well-treated HIV-positive individuals compared to comparable controls. And secondly that cognitive impairment will be associated with neuroimaging biomarkers, specifically grey and white matter abnormalities. These hypotheses were tested using advanced methods of data acquisition and analysis including a 'machine-learning' approach to explore structural abnormalities in HIV-infection in a data-driven way. This multivariate approach allowed the combination of diffusion and volumetric data into a single model taking advantage of their complementary information regarding brain structure.

5.2 Methods

The study methods are summarised in *Figure 5-A*.

Figure 5-A. Graphical overview of methods.



Participants

In total, 134 HIV-positive and 79 demographically comparable HIV-negative participants were recruited to the COBRA study from sites in London (n=88, 59 HIV-positive) and Amsterdam (n=125, 75 HIV-positive). Eligible participants were aged >45 with no significant neurological conditions, substance or alcohol abuse or moderate/severe depression (see methods, section 2.2.2 for exclusion criteria and other details). All HIV-positive participants were required to be on combination antiretroviral therapy and to have had an undetectable plasma HIV RNA (<50 copies/mL) recorded for at least 12 months prior to enrolment.

Cognitive function tests

Subjects completed a comprehensive battery of neuropsychological tests assessing attention, executive function, language, memory, speed of information processing and motor function (see methods, section 2.2.2 and table 2.2 for further details). The domains and individual tests were chosen according to previously published international guidelines²⁵ and due to their sensitivity for detecting HIV-associated cognitive impairment in previous studies.^{8,10,13} A harmonised study protocol, standardised training and supervision of research staff ensured consistent testing and scoring of neuropsychological tests across sites. Raw cognitive function test scores were converted to standardised T-scores accounting for age and level of education with higher T-scores representing better cognitive function. Three common classification methods, the HIV-associated neurocognitive disorder or ‘Frascati’ criteria, the global deficit score and multivariate normative comparison were then applied to domain T-scores according to published methods (detailed in methods, section 2.3).^{25,26,40}

Neuroimaging acquisition (at 3 Tesla)

A harmonised neuroimaging protocol was used across the two study sites within given hardware and vendor constraints. In London, images were acquired using a Siemens Verio scanner (n=88) and in Amsterdam initially with a Philips Intera (n=66) and then using a Philips Ingenia (n=59, both Philips Healthcare, Best, the Netherlands) due to a scanner upgrade. High-resolution 3D T1-weighted structural images and diffusion weighted images along 64 non-collinear directions were acquired at both sites (see methods, section 2.4.1 for further details of acquisition parameters).

Image processing

3D T1 images were pre-processed (Figure 5-A step a) using SPM12 (University College London, London, UK) as described in the methods, section 2.4.2. Briefly, images were bias corrected, segmented into grey matter, white matter and cerebrospinal fluid (CSF) and volumes calculated. Segmented images were then registered to a custom template and normalised (Figure 5-A step b) to standard Montreal Neurological Institute (MNI) space using the DARTEL algorithm.²¹⁷

Diffusion data were pre-processed (Figure 5-A step a) using FSL v5.0.6 (FMRIB, University of Oxford) as described in section 2.4.2. Briefly, images were corrected for eddy currents and head motion by rigid-body registration to each subject's initial B0 image. Non-brain tissue was deleted⁴⁵ and the diffusion tensor model was fitted at every voxel, using weighted least squares. These were then registered to a custom template and standard space, using DTI-TK v2.3.1 (step b). This method uses the full information of the diffusion tensor which allows more accurate registration than the standard FSL pipeline used in two recent diffusion studies in HIV-disease.^{130,145,265} Fractional anisotropy, axial, mean and radial diffusivity maps for each participant were then 'skeletonised' using FSL and thresholded using fractional anisotropy ≥ 0.2 to exclude areas with considerable inter-individual variability prior to performing *tract based spatial statistics* (TBSS). This method confers several advantages over previous approaches and aims to improve sensitivity and reliability of multi-subject diffusion studies.¹⁴⁶

Statistical analyses

Group comparisons

Group comparisons of baseline characteristics, cognitive domain T-scores and cognitive impairment were assessed using chi-square, Fisher's exact, Wilcoxon rank-sum and t-tests (two-tailed) as appropriate. Least squares means, a potentially better estimate of the true population mean adjusted for age, intracranial volume and scanner, and 95% confidence intervals were calculated from a linear model for each summary imaging measure and compared across groups. These analyses are described in more detail in section 2.8.4. Only p-values < 0.05 considered as statistically significant.

Voxelwise inference testing

Voxelwise comparisons allow localised changes in brain structure to be delineated within the framework of a general linear model. Group differences in grey matter volume, white matter volume and diffusion measures (Figure 5-A step c) were calculated using non-parametric permutation testing¹⁶⁹ as described in section 2.8.4. Correction for multiple comparisons was accomplished using threshold-free cluster enhancement (TFCE).¹⁷¹ Corrected p-values < 0.05 were considered significant.

Combining imaging and cognitive data

To investigate the relationship between brain structure and measures of cognitive function, for each cognitive domain, a multiple linear regression models and voxelwise regressions were performed as described in section 2.8.5.

Cluster analysis

Volumetric and diffusion data provide complementary information regarding underlying brain structure. T1-weighting provides good contrast between grey and white matter for volumetric assessment. Whereas, diffusion-weighting provides information about white matter microstructure. Whilst their independent associations with cognition are important, data from both modalities were combined to better understand grey and white matter pathology at the individual level and to test for the presence of sub-groups of individuals defined on their neuroimaging characteristics. This was accomplished using the method described in section 2.8.6.

5.3 Results

Participants

All HIV-positive participants (n=134) had an undetectable plasma HIV RNA with a median CD4+ cell count of 618 cells/ μ L and duration of antiretroviral therapy of 12.5 years. The HIV-negative group (n=79) was highly comparable to the patient group in terms of age and gender with similar levels of education, cardiovascular risk factors, smoking and recreational drug use, although there was a small imbalance in ethnicity with the HIV-positive group having a greater proportion of Black-Africans (table 1). One participant did not complete the full cognitive battery. One participant did not have useable T1 data and four participants did not have complete diffusion data due to incomplete acquisition (one participant) or excessive movement (three participants).

Table 5-A. Baseline characteristics of the COBRA cohort.

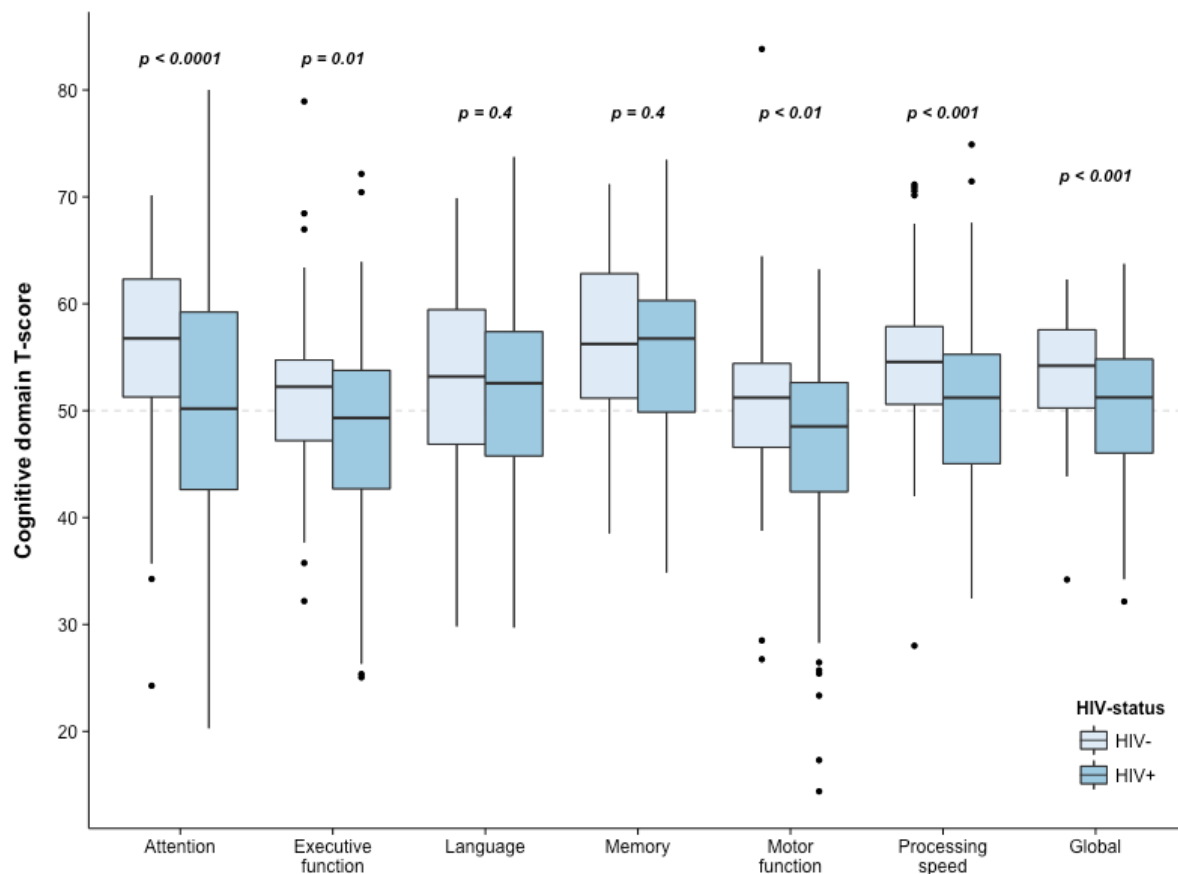
	HIV-positive (n=134)	HIV-negative (n=79)	p-value
Age (years), median (IQR)	55 (51, 62)	57 (52, 64)	0.24
Gender , n (%)			0.79
Female	9 (6.7%)	6 (7.6%)	
Male	125 (93.3%)	73 (92.4%)	
Ethnicity , n (%)			0.03
Black-African	16 (12.0%)	2 (2.6%)	
White	117 (88.0%)	76 (97.4%)	
Sexuality , n (%)			0.45
MSM	104 (77.6%)	59 (74.7%)	
Bisexual	10 (7.5%)	4 (5.1%)	
Heterosexual	18 (13.4%)	16 (20.2%)	
Years of education , median (IQR)	14 (13-16)	16 (14-17)	0.23
Cardiovascular disease , n (%)			
Hypertension	56 (42.1%)	30 (38.5%)	0.66
Myocardial infarction	2 (1.5%)	3 (3.8%)	0.36
Type 1 diabetes	0 (0%)	0 (0%)	N/A
Type 2 diabetes	10 (7.5%)	5 (6.3%)	1.00
BMI (kg/m ²), median (IQR)	24.6 (22.6-27.4)	24.6 (23.3-28.4)	0.30
Total cholesterol (mmol/L), median (IQR)	5.28 (5.11-5.45)	5.33 (5.11-5.55)	0.51
HDL cholesterol (mmol/L), median (IQR)	1.26 (1.07-1.50)	1.30 (1.07-1.57)	0.51
LDL cholesterol (mmol/L), median (IQR)	2.99 (2.83-3.16)	3.14 (2.83-3.16)	0.30
Triglycerides (mmol/L), median (IQR)	1.70 (1.15-2.53)	1.51 (1.07-2.25)	0.20
Smoking status , n (%)			0.24
Current smoker	40 (29.9%)	20 (25.3%)	
Ex-smoker	58 (43.2%)	29 (36.7%)	
Never smoked	36 (26.9%)	30 (38.0%)	
Alcohol consumption , n (%)			0.04
Current drinker	104 (77.6%)	71 (89.9%)	
Previous drinker	18 (13.4%)	3 (3.8%)	
Never drunk	12 (9.0%)	4 (5.1%)	
Use of recreational drugs in past 6 months , n (%)	44 (32.8%)	18 (22.8%)	0.16
CD4+ count (cells/ μ L), median (IQR)	618 (472-806)	900 (692-1174)	<0.01
CD4+:CD8+ cell count ratio , median (IQR)	0.84 (0.60-1.12)	2.01 (1.44-2.64)	<0.01
Nadir CD4+ count (cells/ μ L), median (IQR)	180 (90-250)	N/A	
Years since HIV diagnosis , median (IQR)	15.0 (9.1-20.0)	N/A	
Duration of antiretroviral therapy (years), median (IQR)	12.5 (7.4-16.9)	N/A	
HIV RNA viral load < 200 copies/mL , n (%)	134 (100%)	N/A	
Prior clinical AIDS , n (%)	42 (31.3%)	N/A	
Likely route of HIV transmission , n (%)		N/A	
MSM	115 (85.8%)		
Heterosexual sex	15 (11.2%)		
IVDU/Blood product	1 (0.8%)		
Unknown	3 (2.2%)		

P-values calculated using the chi-squared test, Fisher's exact and Wilcoxon rank-sum tests as appropriate. Hypertension was defined by either: three measurements of systolic BP ≥ 140 mmHg; three measurements of diastolic BP ≥ 90 mmHg or use of blood pressure lowering medication. Abbreviations: HDL – high-density lipoprotein, LDL – low-density lipoprotein, MSM – men who have sex with men, IQR – interquartile range.

Cognitive function

Cognitive impairment was more prevalent in the HIV-positive group. This was true regardless of the method used: global deficit score: 18.0% vs. 3.8% (odds ratio [95% confidence interval] 5.58 [1.86-24.1], $p < 0.01$); Frascati criteria: 18.0% vs. 3.8% (5.58 [1.86-24.1], $p < 0.01$); multivariate normative comparison: 19.5% vs. 2.5% (9.36 [2.68-59.2], $p < 0.001$). The HIV-positive group scored lower than the control group in the domains of attention, executive function, motor function and processing speed (Figure 5-B, $W_{210} > 6300$ and $p \leq 0.01$ for all). No significant group differences were found in language and memory T-scores.

Figure 5-B. Boxplots of demographically adjusted cognitive domain T-scores by HIV-status.



P-values calculated using the Wilcoxon rank-sum test.

Brain tissue volumes

HIV-positive individuals had smaller total grey matter volume than controls (Table 5-B). As expected, there was a significant negative correlation between age and grey matter volume ($r=-0.31$, $p<0.0001$) but there was no significant age and HIV-serostatus interaction ($t_{205}=0.45$, $p=0.65$).

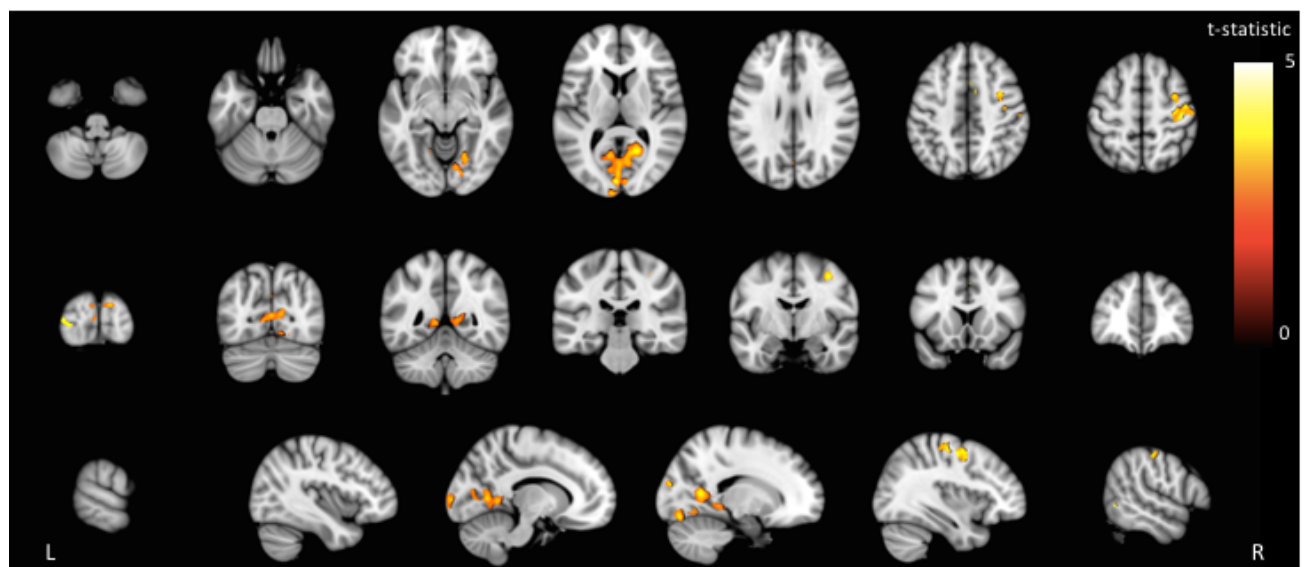
Table 5-B. Brain volume adjusted means by HIV-status and 95% confidence intervals (CI).

Imaging measure	Volumetric measures			t-statistic ₂₀₆	p-value
	HIV-positive	HIV-negative	Difference (95% CI)		
Grey matter volume (mL)	659	673	13.7 (2.3-25.1)	2.37	0.02
White matter volume (mL)	479	476	2.4 (-6.4-11.2)	-0.54	0.59

Subscript indicates degrees of freedom for the t-statistic. Adjusted for age, intracranial volume and scanner type.

Voxelwise analysis demonstrated grey matter volume reductions in the HIV-positive group, located principally in the intracalcarine and supracalcarine cortices, lingual gyrus, occipital pole, precuneus, occipital fusiform gyrus and the posterior cingulate cortex (**Figure 5-C**). In contrast, total white matter volume was not significantly different between the groups, nor were there any significant voxelwise differences.

Figure 5-C. Grey matter voxel-based morphometry group comparison.



Areas with significantly ($p<0.05$) lower grey matter volume coloured by t-statistic (red/yellow) - corrected for multiple comparisons and adjusted for age, intracranial volume and scanner. Significant differences are overlaid on the Montreal Neurological Institute 152 T1 brain image.

Diffusion tensor imaging

Despite the lack of white matter atrophy, the HIV-positive individuals had lower fractional anisotropy and higher mean and radial diffusivity than controls (Table 5-C). There was no significant difference between the groups in axial diffusivity. There were age-associated changes to all diffusion metrics ($r=-0.36, 0.45, 0.45$ and 0.40 for fractional anisotropy, mean, radial and axial diffusivity respectively, $p<0.0001$ for all) but there were no interactions between age and HIV-status ($p>0.5$ for all).

Table 5-C. Diffusion measure adjusted means by HIV-status and 95% confidence intervals (CI).

Imaging measure	Diffusion measures			t-statistic ₂₀₂	p-value
	HIV-positive	HIV-negative	Difference (95% CI)		
Fractional anisotropy	0.477	0.484	0.007 (0.002-0.012)	2.92	<0.01
Mean diffusivity (mm ² /s)	705	696	8.5 (1.2-15.8)	-2.27	0.02
Radial diffusivity (mm ² /s)	503	493	10.3 (2.7-17.9)	-2.66	<0.01
Axial diffusivity (mm ² /s)	1,108	1,103	4.9 (-2.5-12.3)	-1.31	0.19

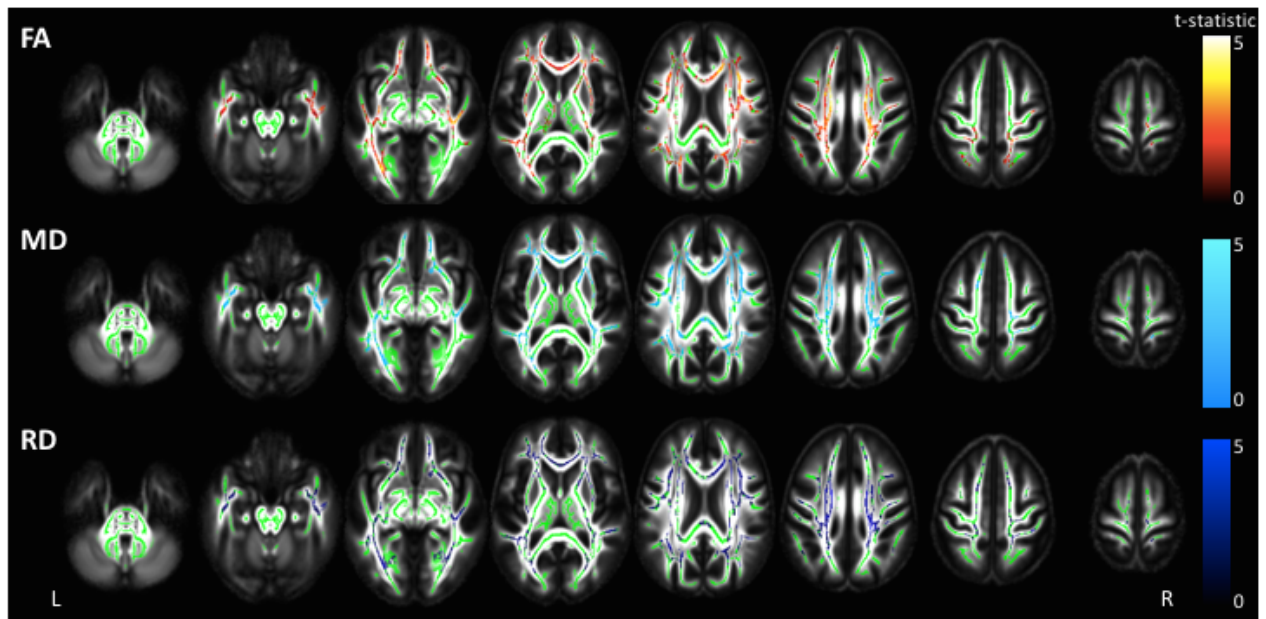
Subscript indicates degrees of freedom for the t-statistic. Adjusted for age, intracranial volume and scanner type.

Using a voxelwise approach, widespread abnormalities in fractional anisotropy, mean and radial diffusivity were detected in HIV-positive individuals. A similar pattern to the whole brain diffusion metrics was seen i.e. lower fractional anisotropy with higher mean and radial diffusivity but with no difference in axial diffusivity (Figure 5-D). These abnormalities occurred predominantly in: the right superior fronto-occipital fasciculus, right and left anterior corona radiata and genu of the corpus callosum.

Cognitive function and grey matter volume

Across patients and controls, larger grey matter volumes were associated with better cognitive performance. Total grey matter volume was positively correlated with executive function, motor function and global T-scores (Table 5-D). There were no significant interactions between HIV-status and cognitive test scores, although the interaction between HIV-status and attention approached significance ($t_{203}=1.91$, $p=0.06$).

Figure 5-D. White matter tract based spatial statistics group comparison.



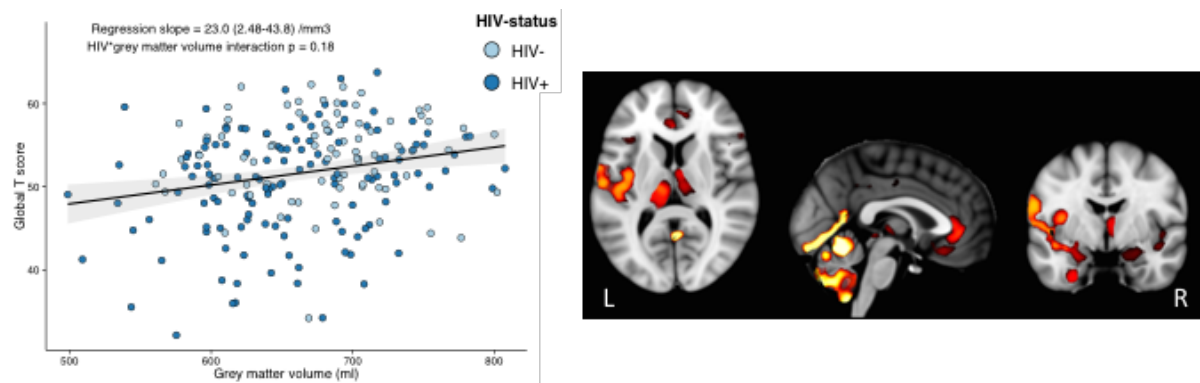
Areas of significantly ($p < 0.05$) lower fractional anisotropy (FA), higher mean diffusivity (MD) and higher radial diffusivity (RD) are coloured red-yellow, light blue and dark blue by t-statistic respectively - corrected for multiple comparisons and adjusted for age, intracranial volume and scanner. Significant differences overlaid on the white matter skeleton (green) and the mean fractional anisotropy image (greyscale).

Table 5-D. Multiple linear regression estimates for grey matter volume by cognitive domain.

Cognitive domain	Grey matter volume regression estimate (T-score/mL)			HIV-grey matter interaction	
	Estimate	(95% CI)	p-value	t ₂₀₃	p-value
Attention	0.035	(-0.001-0.072)	0.06	1.91	0.06
Executive function	0.041	(0.013-0.068)	<0.01	0.75	0.45
Memory	0.010	(-0.016-0.037)	0.43	-0.67	0.50
Motor function	0.029	(0.001-0.057)	0.04	0.69	0.49
Processing speed	0.017	(-0.010-0.043)	0.21	0.76	0.45
Global	0.026	(0.006-0.046)	0.01	1.34	0.18

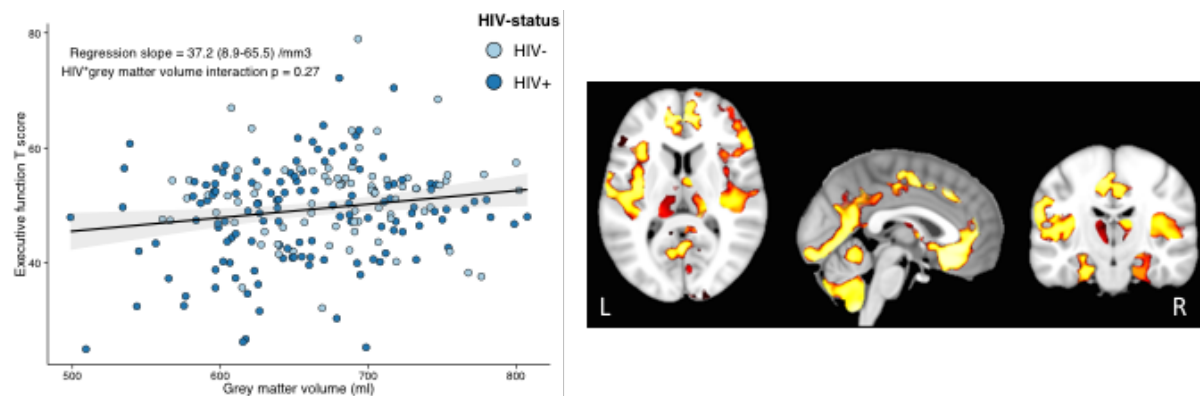
Data adjusted for age, intracranial volume and scanner type. In addition, HIV-status and neuroimaging measure interaction statistics for each cognitive domain.

Voxelwise analyses were then performed to allow regional differences in the relationship between brain structure and cognition to be explored. There was a positive correlation of global T-scores with grey matter volume in a number of locations including: the anterior and posterior cingulate cortices; left and right thalami; right and left hippocampi and the insular cortex (Figure 5-E). In these regions, the HIV-positive group had smaller grey matter volume of borderline significance (difference 1.72 mL, $t_{206} = 1.8$, $p = 0.08$).

Figure 5-E. Grey matter volume positively correlates with global cognitive function.

Left: scatterplot of global T-score vs. total grey matter volume. Right: voxelwise analyses of grey matter volume depicting voxels that were positively correlated with global T-scores across both groups - corrected for multiple comparisons and adjusted for age, intracranial volume, HIV-status and scanner ($p < 0.05$). Significant regions of grey matter (red/yellow) overlaid on Montreal Neurological Institute 152 T1 brain image (greyscale).

Executive function positively correlated with grey matter volume in many of the same locations as global T-scores (Figure 5-F). There was a small amount of HIV-associated atrophy present in this executive network as the HIV-positive group had smaller grey matter volume compared to controls (difference 2.67 mL, $t_{206} = 2.02$, $p = 0.04$).

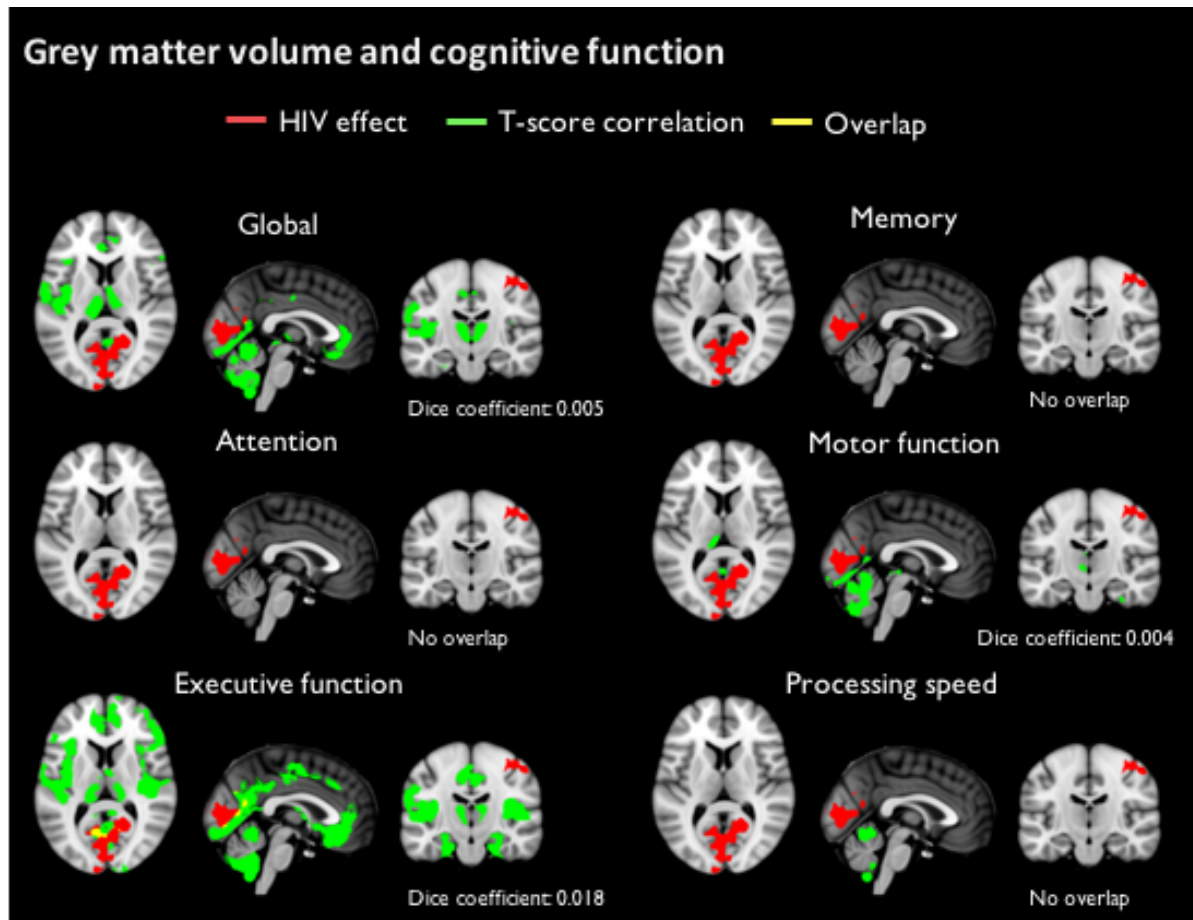
Figure 5-F. Grey matter volume positively correlates with executive function.

Left: scatterplot of executive function T-score vs. total grey matter volume. Right: voxelwise analyses of grey matter volume depicting voxels that were positively correlated with executive function T-scores across both groups - corrected for multiple comparisons and adjusted for age, intracranial volume, HIV-status and scanner ($p < 0.05$). Significant regions of grey matter (red/yellow) overlaid on Montreal Neurological Institute 152 T1 brain image (greyscale).

Spatial correspondence between HIV-associated grey matter atrophy and brain regions correlated with cognitive function are illustrated in Figure 5-G. Voxelwise analyses revealed positive correlations of motor function and processing speed T-scores with grey matter

volume in similar locations, predominantly in the cerebellum. Only small volumes of grey matter, primarily in the temporal fusiform gyrus, were associated with memory and attention T-scores. There were no voxelwise associations between language T-scores and grey matter volume. There were no significant differences in grey matter volume between the HIV-positive and HIV negative groups in any of these networks (Table 5-E).

Figure 5-G. Spatial correspondence between HIV-associated grey matter atrophy and grey matter regions correlated with cognitive function.



Voxel-wise analysis depicting regions of grey matter volume significantly lower in the HIV-positive group vs. HIV-negative group (red), positively correlated with cognitive function T-scores (green) and overlap (yellow) - corrected for multiple comparisons and adjusted for age, intracranial volume and scanner. Statistical images overlaid on MNI 152 T1.

Table 5-E. Grey matter volume least squares means for brain regions positively correlated with cognitive domain T-scores.

Cognitive domain	Grey matter volume (mL)			t_{206}	p -value
	HIV-positive	HIV-negative	Difference (95% CI)		
Attention	0.72	0.72	0.00 (-0.02-0.03)	0.43	0.67
Executive function	135.83	138.51	2.67 (0.06-5.28)	2.02	0.04
Memory	6.05	6.11	0.057 (-0.09-0.21)	0.75	0.45
Motor function	52.02	52.85	0.83 (-0.26-1.93)	1.50	0.13
Processing speed	14.50	14.69	0.20 (-0.16-0.55)	1.10	0.27
Global	98.82	100.54	1.72 (-0.20-3.65)	1.77	0.08

Data adjusted for age, intracranial volume and scanner type. P -value and t -statistic (degrees of freedom as subscript) calculated using multiple linear regression. There were no voxelwise correlations between grey matter volume and language T-scores and there were no voxelwise correlations between fractional anisotropy and memory or language T-scores.

Cognitive function and fractional anisotropy

Across both groups, higher fractional anisotropy was associated with improved cognitive performance. Fractional anisotropy averaged across the main white matter tracts was positively correlated with attention, executive function, processing speed and global T-scores (table 2). There were no interactions between HIV-status and cognitive test scores although the interaction with attention approached significance ($t_{199} = -1.68$, $p = 0.09$).

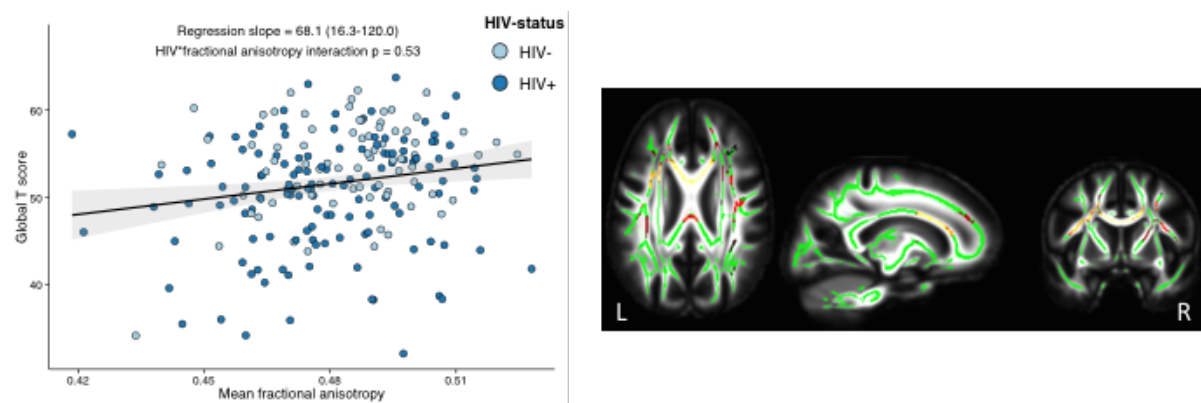
Table 5-F. Multiple linear regression estimates for grey matter volume and fractional anisotropy by cognitive domain.

Cognitive domain	Fractional anisotropy regression estimate (T-score/unit FA)			HIV-fractional anisotropy interaction	
	Estimate	(95% CI)	p -value	t_{199}	p -value
Attention	0.099	(0.013-0.187)	0.03	-1.67	0.09
Executive function	0.073	(0.008-0.139)	0.03	-0.62	0.54
Memory	0.035	(-0.028-0.097)	0.27	-0.53	0.60
Motor function	0.079	(0.013-0.144)	0.02	1.25	0.21
Processing speed	0.092	(0.031-0.152)	<0.01	-0.41	0.68
Global	0.062	(0.014-0.110)	0.01	-0.63	0.53

Data adjusted for age, intracranial volume and scanner type. In addition, HIV-status and neuroimaging measure interaction statistics for each cognitive domain.

Across both groups, using a voxelwise approach, fractional anisotropy was positively correlated with global T-scores in several locations (Figure 5-H). These included the body and genu of the corpus callosum; left and right, anterior and superior divisions of the corona radiata and the right and to a lesser extent left anterior limb of the internal capsule. These were many of the same white matter tracts with reduced fractional anisotropy in the HIV-positive group. Furthermore, the HIV-positive group had lower mean fractional anisotropy in these tracts (difference 0.012, $t_{202}=3.33$, $p=0.001$).

Figure 5-H. Fractional anisotropy positively correlates with global cognitive function.



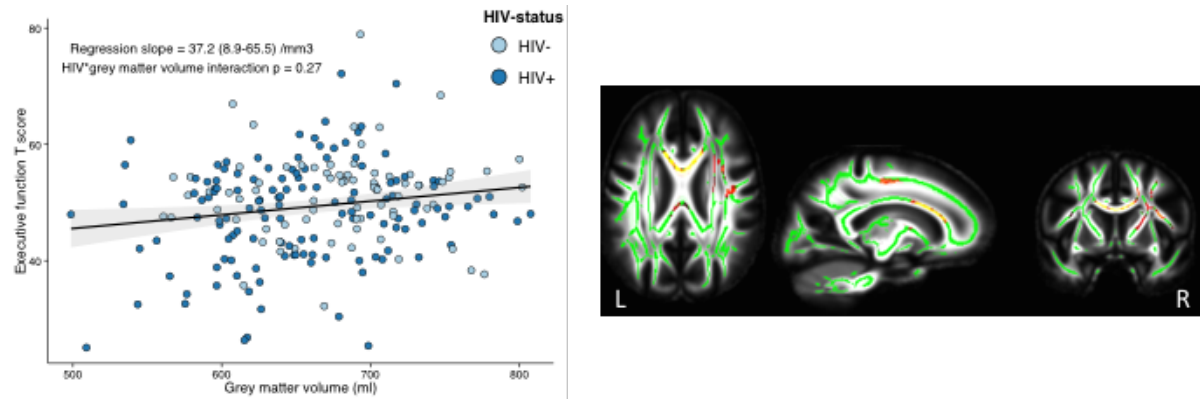
Left: scatterplots of global T-score vs. mean fractional anisotropy. Right: voxelwise analyses of fractional anisotropy depicting voxels that were positively correlated with global T-scores across both groups - corrected for multiple comparisons and adjusted for age, intracranial volume, HIV-status and scanner ($p<0.05$). Significant white matter tract voxels (red/yellow) overlaid on the white matter skeleton (green) and the mean fractional anisotropy image (greyscale).

The voxelwise relationships between fractional anisotropy and executive function were similar to those observed with global T-scores with many white matter tracts positively correlated with executive function T-scores (Figure 5-J). These included the body and genu and to a lesser extent the splenium of the corpus callosum and the right internal and external capsules and superior corona radiata. As before, the HIV-positive group had lower fractional anisotropy in these regions (difference 0.013, $t_{202}=3.49$, $p<0.001$).

Spatial correspondence between HIV-associated white matter microstructural injury and white matter tracts correlated with cognitive function are illustrated in **Figure 5-J**. Voxelwise analyses revealed positive correlations of fractional anisotropy with motor function and processing speed T-scores, in similar regions, primarily in the body and genu of the corpus callosum. Attention T-scores positively correlated with fractional anisotropy primarily in the body of the corpus callosum and the right superior fronto-occipital fasciculus. As with the other cognitive domains, the HIV-positive group had significantly lower fractional anisotropy

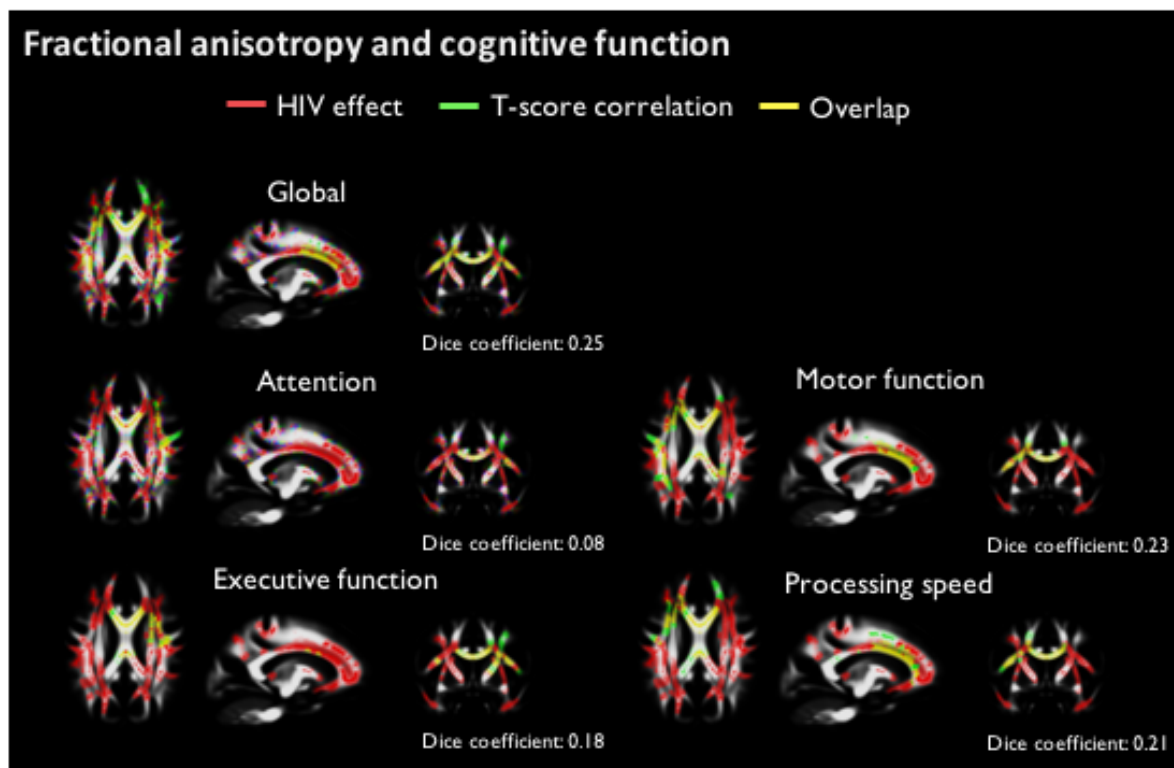
in these regions (Table 5-A). There were no voxelwise associations between language or memory T-scores and fractional anisotropy.

Figure 5-I. Fractional anisotropy positively correlates with executive function.



Left: scatterplots of executive function T-score vs. mean fractional anisotropy. Right: voxelwise analyses of fractional anisotropy depicting voxels that were positively correlated with executive function T-scores across both groups - corrected for multiple comparisons and adjusted for age, intracranial volume, HIV-status and scanner ($p < 0.05$). Significant white matter tract voxels (red/yellow) overlaid on the white matter skeleton (green) and the mean fractional anisotropy image (greyscale).

Figure 5-J. Spatial correspondence between HIV-associated white matter microstructural injury and white matter tracts correlated with cognitive function.



Voxel-wise analyses depicting regions of fractional anisotropy significantly lower in the HIV-positive group vs. HIV-negative group (red), positively correlated with cognitive function T-scores (green) and overlap (yellow) - corrected for multiple comparisons and adjusted for age, intracranial volume and scanner. Statistical images overlaid on the mean fractional anisotropy image (greyscale).

Table 5-G. Mean fractional anisotropy least squares means for brain regions positively correlated with cognitive domain T-scores.

Cognitive domain	Fractional anisotropy			t_{202}	p -value
	HIV-positive	HIV-negative	Difference (95% CI)		
Attention	0.471	0.482	0.011 (0.003-0.019)	2.57	0.01
Executive function	0.548	0.561	0.013 (0.006-0.021)	3.49	<0.001
Motor function	0.549	0.559	0.011 (0.003-0.018)	2.79	<0.01
Processing speed	0.503	0.515	0.012 (0.005-0.019)	3.36	<0.001
Global	0.490	0.502	0.012 (0.005-0.019)	3.33	0.001

Data adjusted for age, intracranial volume and scanner type. P -value and t -statistic (degrees of freedom as subscript) calculated using multiple linear regression. There were no voxelwise correlations between grey matter volume and language T-scores and there were no voxelwise correlations between fractional anisotropy and memory or language T-scores.

Cluster analysis

Grey and white matter measures were then combined to take advantage of their complementary information regarding brain structure in order to test whether participants could be divided into subgroups with different clinical features based on multi-modal neuroimaging data. The correlation structure between different brain regions differed between patients and controls ($\chi^2_{5886}=7231$, $p<0.0001$). This is illustrated graphically in Figure 5-K. Using k-means clustering, two groups were identified that were stable with resampling (mean Jaccard coefficient 0.99). Other cluster models (i.e. $k>2$) were much less stable (mean Jaccard coefficient <0.7). The Duda-Hart test confirmed the two cluster solution was superior to no cluster structure ($L_1=0.94$, $p<0.0001$). Table 5-H displays the cluster validation statistics.

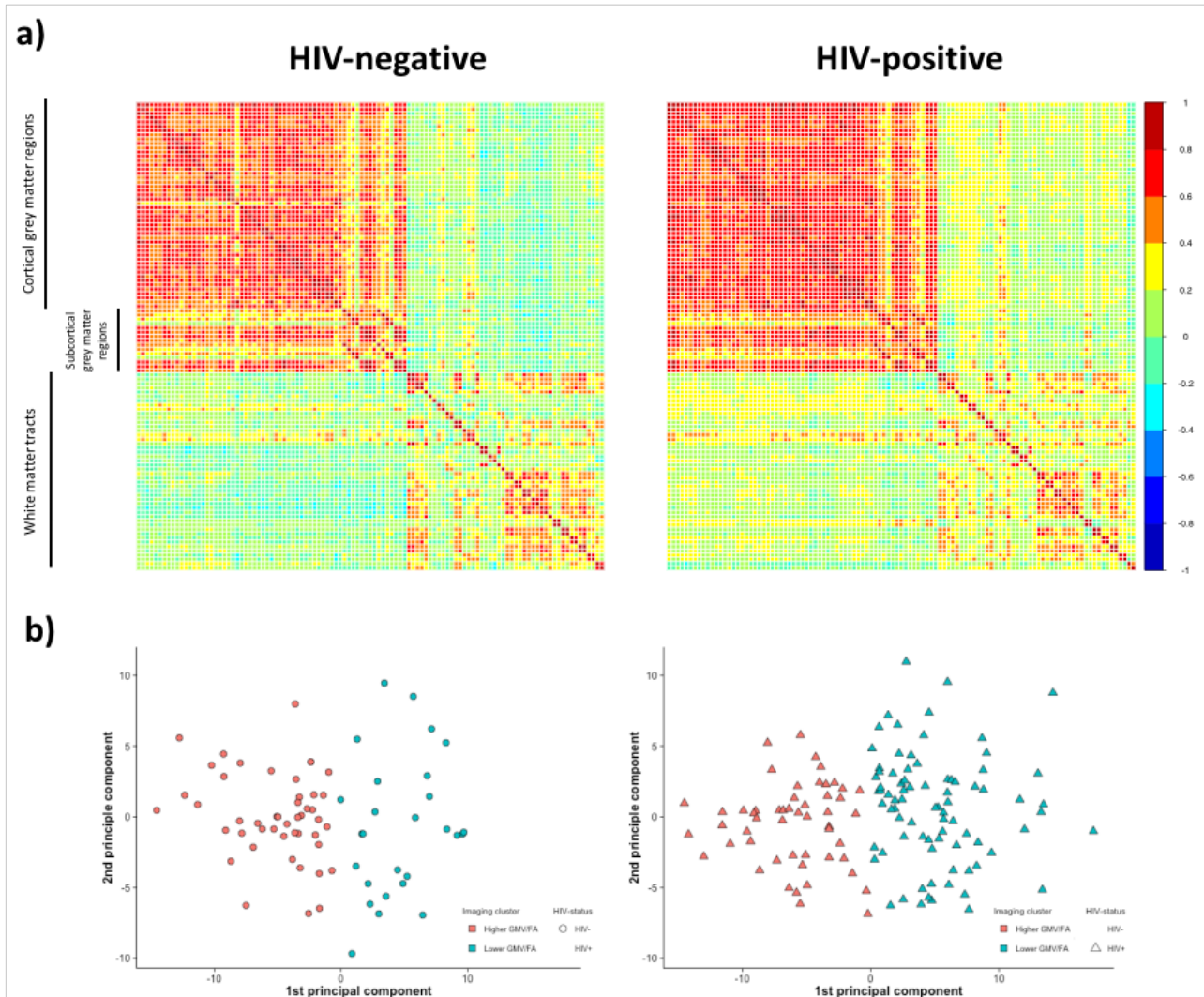
Table 5-H. K-means cluster validation statistics.

Number of clusters	Calinski-Harabasz index	Average silhouette width	Jaccard bootstrap mean
2	72.1	0.21	0.99
3	48.3	0.16	0.67
4	39.0	0.12	0.62
5	33.3	0.09	0.62
6	28.6	0.08	0.61
7	25.4	0.08	0.55
8	22.8	0.08	0.58
9	21.1	0.08	0.58
10	19.6	0.07	0.56

The Jaccard bootstrap mean is the mean of each cluster's Jaccard similarity measure following resampling (with 10,000 resamples) and indicates the stability of cluster membership to resampling. Values >0.85 are representative of highly stable clusters.

With a two cluster model, cluster one (n=99) had higher grey matter volume and higher mean fractional anisotropy in all regions whereas cluster two (n=109) had lower grey matter volume and lower mean fractional anisotropy in all regions. HIV-positive individuals were more likely to be members of cluster two, 61.2% vs. 36.4% (odds ratio 2.74 [1.53-4.98], $p < 0.001$).

Figure 5-K. Cluster analysis splits participants into two groups on the basis of volumetric and diffusion data.



a) Visualisation of the correlation matrices for the regions of cortical and subcortical grey matter volume (Harvard-Oxford atlas) and white matter fractional anisotropy (ICBM DTI-81 white matter labels) used in the k-means clustering analysis for the HIV-positive and HIV-negative groups. The correlation coefficient is represented by the colour of the box (scale to the right of the figure).

b) Principle component plot showing the separation of the clusters based on the k-means clustering analysis of parcellated grey matter and mean fractional anisotropy data for the HIV-negative controls on the left (circles) and HIV-positive participants on the right (triangles). Cluster one (orange) had higher grey matter volume and higher fractional anisotropy for each region whereas cluster two (green) had lower grey matter volume and lower fractional anisotropy for each region. HIV-positive individuals were more likely to be members of the lower grey matter volume and lower fractional anisotropy cluster (odds ratio [95% confidence intervals]: 2.74 [1.53-4.98]).

Furthermore, subjects in cluster two were older (median 59.3 vs 54.6 years, $W_{206}=3634$, $p<0.001$) and had significantly poorer cognitive function in the domains of attention, executive function, motor function and processing speed (Fig. 6, $W_{205}\geq 6307$, $p\leq 0.03$ for all). Importantly, these cognitive differences between members of clusters one and two were not due to demographic differences, as the cognitive T-scores are adjusted for age and level of education.

Associations with clinical parameters

In the HIV-positive group, grey and white matter volumes and mean fractional anisotropy were not associated with known duration of untreated infection, current or nadir CD4+ cell counts or CD4+:CD8+ cell count ratio ($p>0.15$ for all). There were trends for patients with prior AIDS to have lower grey matter volume (difference 14.3 mL, $t_{128}=1.9$, $p=0.06$) and lower mean fractional anisotropy (difference 0.0059, $t_{128}=1.7$, $p=0.08$). Although there were no significant differences in current and nadir CD4+ cell counts ($p=0.10$ and 0.17), HIV-positive individuals in cluster two had lower CD4+:CD8+ ratios (0.82 vs. 1.06, $W_{132}=2666$, $p=0.01$) and were older (58.6 vs 53.2 years, $W_{132}=1380$, $p<0.001$) than those in cluster one.

5.4 Discussion

Confirming my first hypothesis, HIV-positive individuals had evidence of cognitive impairment, despite successful treatment and in comparison to a demographically comparable control group. Furthermore, grey matter atrophy and widespread white matter microstructural abnormalities were also evident in the patient group. Generally, HIV-associated grey matter atrophy was modest and did not occur in regions correlated with cognitive function. In contrast, HIV-associated white matter abnormalities were widespread and found in many of the white matter tracts correlated with cognitive function, providing evidence for my second hypothesis.

These findings extend previous work that generally had not linked structural brain abnormalities with cognitive impairment in chronic HIV-infection.¹²⁷⁻¹³⁰ My findings suggest that the pathophysiology of cognitive impairment in treated HIV-positive individuals is predominantly due to white matter microstructural injury and support the use of fractional anisotropy as a biomarker to identify HIV-associated brain injury.

The prevalence of cognitive impairment in well-treated HIV-positive patients is uncertain. Although early studies reported high rates of cognitive impairment in chronically treated patients,^{8,10,243} some recent studies have failed to observe this.¹⁵ I analysed data from a large multi-centre cohort, where great care was taken to recruit an appropriate control group. This allowed comparison of cognitive performance between HIV-positive individuals and controls well matched in other respects. Poorer cognitive performance was observed regardless of the method used, with a consistent prevalence of impairment of approximately 19%. Given the low prevalence of cognitive impairment, this finding is likely to be a reliable estimate for the true prevalence of cognitive impairment in well-treated older HIV-positive populations. This figure is lower than previous reports,^{8,10} which is probably due to the fact that all the HIV-positive participants studied had sustained suppression of viraemia on antiretroviral therapy and that they were compared to a comparable control group.

Total grey matter volume was reduced in the HIV-positive group with atrophy occurring primarily in the intracalcarine and supracalcarine cortices. Previous studies in populations with variable suppression of HIV replication have described more widespread grey matter volume reductions.^{122,124,126} However, the location of atrophy is consistent with regions of greatest neuronal loss¹¹⁸ and atrophy¹²⁵ previously associated with untreated HIV-infection. Together with the trend for those with prior AIDS to have lower grey matter volume, my findings suggest that these regions are the earliest affected after primary HIV-infection and that further atrophy may be prevented with successful treatment. Additionally, a more demographically comparable control population, such as the one I have studied, potentially reduces confounding due to lifestyle factors that may exaggerate the differences between HIV-positive and HIV-negative groups given the high rates of alcohol consumption, smoking and substance misuse.

Although diffusion imaging showed widespread evidence of white matter microstructural abnormalities, white matter volume did not differ between the groups. Diffusion abnormalities in the absence of white matter atrophy have previously been reported on one occasion in HIV-positive individuals, albeit in a much smaller sample.¹²⁹ Although this study population were not all treated with antiretroviral therapy they did have relatively preserved immunity at the time of testing (mean CD4+ 613 cells/uL) suggesting that white matter atrophy may be a late complication of inadequately treated HIV-infection. Atrophy is common in diseases such as traumatic brain injury²⁶⁶ where diffusion abnormalities are also commonly found.¹³⁸ The

decoupling of atrophy and diffusion abnormalities in well-treated chronic HIV-disease, suggests that subtle alteration in the microstructure of axons or glial cells occurs rather than frank neurodegeneration. The latter would be expected to result in atrophy, which I did not observe.

Supporting my second hypothesis, white matter abnormalities correlated with cognitive function. Strong relationships were seen across both groups, with the white matter tracts showing abnormalities in the HIV-positive group also correlating with cognitive function. In contrast, regions of grey matter atrophy in the HIV-positive group mainly did not overlap with those correlating with cognitive function. Although this could reflect the cognitive battery not specifically testing function in these regions, my findings suggest that white matter abnormalities may be more important than atrophy in determining cognitive impairment in treated HIV-disease. White matter abnormalities of this type have been shown to correlate with cognitive function in many other disease states.^{138,139} This type of microstructural change is thought to affect the efficiency of neural transmission which disrupts the synchronised functioning of large-scale distributed brain networks that cognition depends on.⁵⁶ Network dysfunction secondary to axonal injury after traumatic brain injury predicts cognitive impairment²⁶⁷ and has also been hypothesised as a mechanism of HIV-associated cognitive impairment.²⁶⁸ Therefore, damage to these tracts may produce many of the cognitive impairments seen in chronic HIV-infection through alteration of brain network function. This could be tested by further study assessing functional connectivity using resting-state functional MRI.

Neuropathological data are rare in the antiretroviral era, so the histopathological changes associated with HIV-associated cognitive impairment in treated individuals are uncertain.⁵³ However, HIV-positive subjects showed increased radial but not axial diffusivity, a disparity that may indicate white matter demyelination.¹⁴³ This is consistent with histopathological reports from the pre-antiretroviral era, where myelin damage was found in the central white matter, associated with multinucleated giant cells.^{51,52} No association was observed with duration of untreated infection, which probably reflects the fact that duration of *known* infection is a poor proxy for duration of *actual* infection. However, there was a trend for the most severe abnormalities occurring in patients with prior AIDS, suggesting that the neuroimaging changes may reflect historical damage prior to initiation of antiretroviral treatment and that patients who are adequately treated may not have an *active* process causing

further damage. Antiretroviral therapy results in durable suppression of HIV replication in the overwhelming majority in both plasma and CNS compartments.^{30,31} Therefore, one interpretation of these results is that antiretroviral therapy prevents progressive neurodegeneration without resolving the grey matter atrophy and demyelination that predates treatment. As this is cross-sectional data, I am unable to resolve the issue of whether these imaging changes reflect historical damage or an on-going pathological process. However, the lack of interactions between HIV-serostatus, ageing and neuroimaging biomarkers lends further evidence to *static* rather than *active* brain injury hypothesis in well-treated patients. This contrasts with a recent diffusion study by Seider *et al*²⁶⁵ which reported steeper declines in fractional anisotropy with age in HIV-positive individuals. This may be explained by differences in patient populations, as almost one third of the patients in this study had detectable plasma HIV RNA. Therefore, some patients are likely to have ongoing active brain injury accumulating with time and therefore greatest in older individuals. Longitudinal data, assessing rates of atrophy and changes in diffusion measures in patients and controls, are needed to definitively distinguish between active and static brain injury in well-treated patients.

Using a multivariate approach to examine individual differences in volumetric *and* diffusion data simultaneously, I identified a brain phenotype associated with ageing, cognitive dysfunction, and HIV-serostatus. Furthermore, this phenotype was associated with lower CD4+:CD8+ ratios. This biomarker of immune activation has previously been associated with other age-related comorbidities in treated HIV-positive cohorts but not with brain injury.²⁶⁹ Together, this suggests that persistent immune activation may also have adverse effects on the CNS - possibly driven by the premature development of age-related changes, or that persistent immune activation and CNS injury share a common aetiology. Normalisation of this ratio has also been associated with the earlier initiation of antiretroviral treatment,²⁷⁰ which supports my hypothesis of the protective effects of early initiation of antiretroviral therapy in the CNS. Again, longitudinal study is needed to determine whether individuals in these clusters have divergent 'brain ageing' trajectories. If this is the case, identification may allow targeted intervention strategies to minimise further decline.

Limitations

This study has a number of possible limitations. Whilst the patient cohort is not representative of the *entire* HIV-positive population, it is representative of older HIV-positive adults receiving

care in a Northern European setting. Cohort studies are potentially limited by unmeasured differences confounding group comparisons. This is particularly an issue when studying HIV-positive populations given high rates of smoking, alcohol and recreational drug use. This was limited by the great efforts made to recruit a comparable HIV-negative control population. This therefore overcomes one of the methodological issues of some previous studies, but differences between the groups unrelated to HIV-infection may exist. As such, not all of the observed changes can be fully attributed to HIV-infection or its treatment.

One potential advantage of multisite studies such as the one presented in this chapter, is that the findings may be more generalisable as well as potentially improving statistical power due to recruitment from a larger pool of eligible participants. However, scanner differences, particularly those between different manufacturers, may introduce variation and potentially bias if patient and control groups are scanned on different scanners. While this may be an issue for multi-centre studies such as COBRA, data from reliability studies suggest that these effects are relatively small for both volumetric and diffusion studies.^{238,239,271} Furthermore, the HIV-positive and HIV-negative participants were balanced across the different scanner systems. To account for variance potentially associated with different scanners, scanner was covaried as a three-level factor in both the multiple regression models of whole brain measures and in the general linear model used to analyse the data voxelwise. This approach has been used before in multisite studies of HIV-positive individuals.^{122,128} While this simple categorical approach may not account for all possible scanner-related variance, the HIV-association remained after including this potential confounder in the models, which provides confidence that the observed differences are not due to an artefact of using different scanner systems.

Partial volume effects, which diffusion methods may be particularly sensitive to, may artificially lower fractional anisotropy due to contamination of white matter voxels with CSF. These effects were mitigated with my analysis pipeline where I used a TBSS approach where voxelwise analyses were only performed in the centre of white matter tracts common to all participants.¹⁴⁶ In addition, I utilised DTI-TK and DARTEL image registration tools to minimise registration errors, as these errors may be more pronounced in atrophied brains. These methods have been shown to be superior to the standard analysis pipelines.^{145,272} Additionally, as macroscopic white matter atrophy was not present, it is likely the TBSS results are particularly robust to misregistration errors. Cluster analysis may split the participants

into potentially arbitrary groups and the ‘good’/‘bad’ brain phenotype may, in reality, be more of a continuum. In common with other methods, k-means clustering, has several assumptions namely that all variables have the same importance and that clusters are of similar size and sum-of-squares error. Although exploratory in nature, I found excellent internal validity demonstrated by a high level of cluster stability to resampling and good external validity by the associations of cluster membership with age, HIV-serostatus, cognitive function and markers of immune activation.

Conclusions

To conclude, I found that despite successful antiretroviral therapy HIV-positive individuals exhibited poorer cognitive function when compared to an appropriate HIV-negative control population. HIV-infection was associated with both grey matter atrophy and white matter microstructural injury. While HIV-associated white matter injury tended to occur in regions associated with cognitive function, the same was not true of HIV-associated grey matter atrophy. Using a multivariate approach, I have shown that grey and white matter abnormalities tend to occur together in the same individuals, suggesting a common aetiology for HIV-associated brain injury. This may be related to prolonged untreated infection and systemic immune activation, in common with other non-AIDS comorbidities. These results shed light on the pathophysiology of cognitive impairment in well-treated individuals and confirm two of my overarching hypotheses, namely, that HIV-positive individuals have evidence of cognitive impairment despite successful antiretroviral therapy and that cognitive function is associated with neuroimaging biomarkers of grey matter atrophy and white matter microstructural injury. Furthermore, I have demonstrated the utility of using a multimodal approach to explore the complex relationships between brain injury, structure and function.

Chapter 6

Blood, cerebrospinal fluid and central nervous system biomarkers and their association with cognitive function in treated HIV-disease

Table of contents

CHAPTER 6 BLOOD, CEREBROSPINAL FLUID AND CENTRAL NERVOUS SYSTEM BIOMARKERS AND THEIR ASSOCIATION WITH COGNITIVE FUNCTION IN TREATED HIV-DISEASE.....	163
6.1 INTRODUCTION	165
6.2 METHODS.....	166
Participants	166
Cognitive function tests	166
Blood and CSF biomarkers	167
Magnetic resonance spectroscopy (MRS)	167
Statistical analyses	167
6.3 RESULTS	169
Immune activation and neuroinflammation	170
Neuronal injury	174
Scanner differences in metabolite concentrations	175
Putamen and frontal white matter cerebral metabolites	177
Grey matter cerebral metabolites.....	180
Blood & CSF biomarkers and cerebral metabolites.....	182
Blood & CSF biomarkers and structural imaging measures	185
6.4 DISCUSSION	187
Conclusion	190

Figures

FIGURE 6-A. EXAMPLE FRONTAL WHITE MATTER SPECTRA BY SCANNER DEMONSTRATING THE DIFFERENCES IN SIGNAL TO NOISE RATIO.	162
FIGURE 6-B. JITTERPLOTS OF MARKERS OF SYSTEMIC IMMUNE ACTIVATION BY HIV-STATUS.	163
FIGURE 6-C. SCATTERPLOTS OF GLOBAL T-SCORES AND PLASMA SOLUBLE CD14 (SCD14, PANEL A) AND SOLUBLE CD163 (SCD163, PANEL B) BY HIV-STATUS SHOWING THE SIGNIFICANT INTERACTION.	164
FIGURE 6-D. JITTERPLOTS OF CSF CONCENTRATIONS OF SOLUBLE CD14 (SCD14, PANEL A), SOLUBLE CD163 (SCD163, PANEL B), NEOPTERIN (PANEL C) AND THE KYNURENINE:TRYPTOPHAN RATIO (PANEL D) BY HIV-STATUS.	165
FIGURE 6-E. SCATTERPLOTS OF AGE WITH THE CD4+:CD8+ RATIO (PANEL A) CSF SOLUBLE CD14 (SCD14, PANEL B) AND SOLUBLE CD163 (SCD163, PANEL C) BY HIV-STATUS.	166
FIGURE 6-F. JITTERPLOTS OF CSF CONCENTRATIONS OF TOTAL-TAU (T-TAU, PANEL A), PHOSPHORYLATED-TAU (P-TAU, PANEL B), A β 42 (PANEL C) AND NEUROFILAMENT LIGHT (NFL, PANEL D) PROTEINS BY HIV-STATUS.	167
FIGURE 6-G. BOXPLOTS OF FRONTAL WHITE MATTER CEREBRAL METABOLITE RATIOS BY HIV-STATUS.	170
FIGURE 6-H. BOXPLOTS OF PUTAMEN CEREBRAL METABOLITE RATIOS BY HIV-STATUS.	171
FIGURE 6-I. SCATTERPLOTS OF PUTAMEN METABOLITE CONCENTRATIONS WITH GLOBAL T-SCORES BY HIV-STATUS.	172
FIGURE 6-J. BOXPLOTS OF ANTERIOR CINGULATE CORTEX (ACC) CEREBRAL METABOLITE RATIOS BY HIV-STATUS.	173
FIGURE 6-K. BOXPLOTS OF ANTERIOR CINGULATE CORTEX (ACC) CEREBRAL METABOLITE CONCENTRATIONS BY HIV-STATUS.	174
FIGURE 6-L. SCATTERPLOTS OF ANTERIOR CINGULATE CORTEX (ACC) MYO-INOSITOL CONCENTRATION AND RATIO WITH AGE BY HIV-STATUS.	175
FIGURE 6-M. VISUALISATION OF THE CORRELATION MATRIX OF SOLUBLE BIOMARKERS AND CEREBRAL METABOLITES.	176
FIGURE 6-N. VISUALISATION OF THE CORRELATION MATRIX OF SOLUBLE BIOMARKERS AND STRUCTURAL IMAGING MEASURES.	177
FIGURE 6-O. VISUALISATION OF THE CORRELATION MATRIX OF STRUCTURAL IMAGING MEASURES AND CEREBRAL METABOLITES.	178

Tables

TABLE 6-A. CORRELATIONS BETWEEN MARKERS OF SYSTEMIC IMMUNE ACTIVATION AND AGE.	163
TABLE 6-B. CORRELATIONS BETWEEN MARKERS OF INTRATHECAL IMMUNE ACTIVATION AND AGE.	164
TABLE 6-C. CORRELATIONS BETWEEN MARKERS OF NEURONAL INJURY AND AGE.	166
TABLE 6-D. SCANNER DIFFERENCES FOR WHITE MATTER METABOLITE CONCENTRATIONS.	168
TABLE 6-E. SCANNER DIFFERENCES FOR PUTAMEN METABOLITE CONCENTRATIONS.	169
TABLE 6-F. CORRELATIONS BETWEEN MARKERS LEFT FRONTAL WHITE MATTER METABOLITE CONCENTRATIONS AND AGE.	170
TABLE 6-G. CORRELATIONS BETWEEN MARKERS PUTAMEN METABOLITE CONCENTRATIONS AND AGE.	171
TABLE 6-H. CORRELATIONS BETWEEN MARKERS ANTERIOR CINGULATE CORTEX METABOLITE CONCENTRATIONS AND AGE.	173

6.1 Introduction

Due to the huge advances HIV-medicine, life expectancy of HIV-positive individuals now approaches normal.³ As such, few die and histopathological samples in well-treated patients are rare. Therefore, the study central nervous system biomarkers are essential given the obvious impracticalities of obtaining brain tissue for histopathological examination in living patients.

Plasma and cerebrospinal fluid biomarkers provide complementary information to the neuroimaging biomarkers described here and in earlier chapters. Broadly speaking, cerebrospinal fluid biomarkers can be thought of in two categories, those associated with neuronal injury and those relating to neuroinflammation. Reductions in CSF A β 42 concentrations have been described in those with the most severe HIV-associated cognitive impairment although this finding is not consistent.^{185,191,192} Elevations of total Tau (t-Tau) but not phosphorylated Tau (p-Tau) are generally described in HIV-disease, with the greatest abnormalities observed in those with HIV-associated dementia.^{185,190,191,199} Elevated CSF NFL concentrations have been consistently documented in chronically infected individuals, with the highest concentrations found in those with HIV-associated dementia and the lowest in those on antiretroviral therapy.¹⁸⁵⁻¹⁸⁷ This makes NFL a potentially attractive biomarker for distinguishing between active and quiescent HIV-associated brain injury. Elevated plasma sCD14 and sCD163, suggestive of systemic immune activation, have both been associated with HIV-associated cognitive impairment.^{199,200} Another neuroinflammatory consequence of HIV-infection is the metabolism of tryptophan via the kynurenine pathway.^{202,203} This can be quantified by the kynurenine:tryptophan ratio. Metabolites from this pathway have been associated with the AIDS dementia complex in untreated patients.²⁰⁴

Magnetic resonance spectroscopy allows the non-invasive quantification of central nervous system metabolites. These provide data regarding both neuronal injury and neuroinflammation impairment. Many studies have documented a reduction in N-acetylaspartate and elevated concentrations of both myo-inositol and choline in HIV-positive individuals, including those on suppressive antiretroviral therapy, with the most marked reductions in those with HIV-associated dementia.^{33,153-157}

Many of these biomarker abnormalities have been reported in either untreated HIV-infection or in patient cohorts without universal antiretroviral therapy. The extent to which they differ between well-treated patients and well matched controls is largely unknown. In addition, if and how they are related to cognitive function in treated cohorts are unclear. In this chapter, I explored the differences in cerebral metabolites and soluble biomarkers between well-treated patients and controls as well as the relationships between these markers and cognitive function. This was to test my second hypothesis that these biomarkers would be associated with cognitive function.

6.2 Methods

Participants

HIV-positive and demographically comparable HIV-negative participants were recruited into the CO-morBidity in Relation to HIV/AIDS (COBRA) study from sites in London and Amsterdam. Eligible participants were aged >45 with no significant neurological conditions, substance or alcohol abuse or moderate/severe depression (see methods, section 2.2.2 for exclusion criteria and other details). All HIV-positive participants were required to be on combination antiretroviral therapy and to have had an undetectable plasma HIV RNA (<50 copies/mL) recorded for at least 12 months prior to enrolment.

Cognitive function tests

Subjects (n=213) completed a comprehensive battery of neuropsychological tests assessing attention, executive function, language, memory, speed of information processing and motor function (see methods, section 2.2.2 and table 2.2 for further details).

Blood and CSF biomarkers

Blood and cerebrospinal fluid was collected in participants as described in section 2.5 of the methods. Plasma and CSF biomarkers of neuronal injury and neuroinflammation were quantified as described in section 2.7 of the methods.

Magnetic resonance spectroscopy (MRS)

Spectra were acquired at 3 Tesla (details of the specific pulse sequences at each site are found in section 2.4.1). Single voxel spectroscopy was performed with 15 mm x 15mm x 15 mm voxels placed in the left frontal white matter, left putamen and the anterior cingulate cortex (participants in London only, n=79). Absolute metabolite concentrations of choline containing compounds (Cho), creatine (Cr); glutamate and glutamine (as Glx); myo-inositol (ml), N-acetyl-aspartate (NAA) were quantified using the water unsuppressed data as a reference signal (as described in section 2.4.2 of the methods). In addition, metabolite ratios using creatine as a reference were also calculated. All fitted spectra were visually inspected for quality control. Additionally, spectra were rejected if the signal to noise ratio was <4 or the metabolite full width half maximum of >0.1ppm as per Ernst *et al.*²²⁶

Statistical analyses

As most blood and CSF biomarkers were positively skewed, group comparisons were performed using the Wilcoxon rank-sum test and correlations with Spearman's *rho*. For cerebral metabolites correlations were assessed using Pearson's *r* due to their normal or near normal distribution. To investigate differences in individual cerebral metabolites between the scanners, ANOVA was performed with Tukey's honest significant difference used to perform post-hoc tests. This method accounts for differences in sample size to give more representative 95% confidence intervals as well as controlling for the inherent multiple testing. Given the differences in sequence acquisition parameters across sites, group comparisons of metabolites and metabolite ratios were calculated from multiple linear regression models with age $\pm$ scanner (for the frontal white matter and putamen) as covariates in a similar procedure to the one described in the methods, section 2.7.4.

Multiple linear regression models

Most biomarkers relating to the brain, in particular biomarkers of neuronal injury, are associated with age. As such, correlations between biomarkers may be confounded by age. Therefore, to assess the independent relationships between biomarkers multiple linear regression models were used. Blood and CSF biomarkers were first natural log transformed due to their skewed distributions to improve the model fit. To assess the associations with age, a separate multiple linear regression model was created with the biomarker of interest

as the dependent variable with age and HIV-status as independent variables. To test whether HIV-infection modified the relationship between the biomarker and age, another model with an additional age and HIV-status interaction term was also tested. Scanner was also entered as a factor as an additional covariate for white matter and putamen metabolite models as described in the methods, section 2.7.4.

The relationship between biomarkers and brain injury was assessed in two ways. Firstly, the relationships with cognitive function were determined using multiple linear regression models with global T-score as the dependent variable and the biomarker of interest as the independent variable. Age and HIV-status were entered as covariates, as was scanner for the white matter and putamen metabolites. Separate models with an HIV-status and transformed biomarker interaction term were also tested to assess whether HIV-infection modified the relationship between the biomarker of interest and global cognitive function. Secondly, a similar process was followed to assess the relationship between blood/CSF biomarkers (independent variables) and cerebral metabolites (dependent variable) with age, HIV-status $\pm$ scanner as covariates.

For the correlation analysis between all the biomarkers, multiple comparisons were corrected by controlling the false discovery rate (5%) and only corrected p-values <0.05 were considered significant. Whereas for group comparisons and descriptive correlations there was no correction for multiple comparisons and p-values <0.05 (two-tailed) were considered significant. All analyses were performed using R v3.2.1.

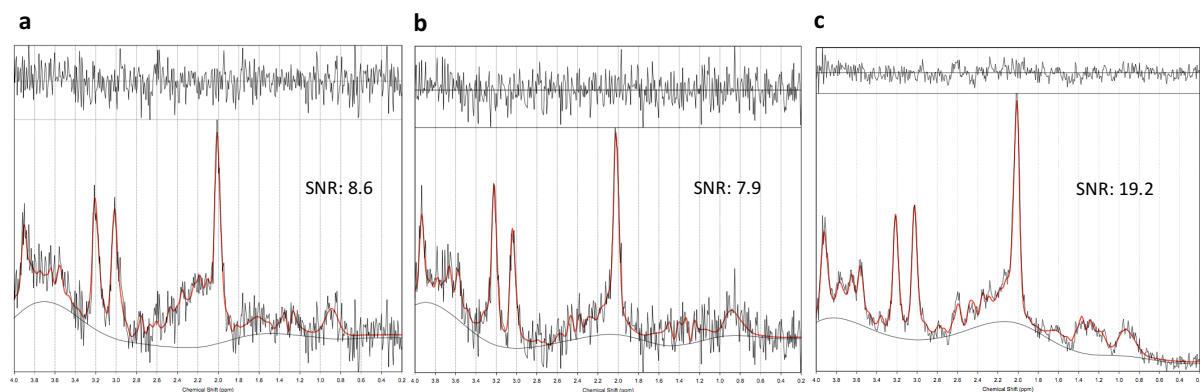
6.3 Results

Demographic details of COBRA cohort are presented in table I-A of the previous chapter. In total, plasma and CSF soluble biomarker data was available for 132 HIV-positive participants and 78 HIV-negative controls (one patient had a medical contraindication to lumbar puncture, another developed lymphoma during the study so did not have a lumbar puncture and lumbar puncture was unsuccessful in one control).

Magnetic resonance spectroscopy (MRS) data was acquired at both sites for the left frontal white matter and putamen in all but one control. Anterior cingulate cortex spectra were

acquired in London only in all but one control. After initial processing, MRS data of the sufficient quality to permit further analysis was available in the left frontal white matter voxel for: 124 patients and 68 controls; putamen: 88 patients and 45 controls; anterior cingulate cortex: 56 patients and 26 controls. The signal to noise ratio (of all acquired spectra, pre-quality control) was lower for spectra acquired in Amsterdam compared with London and in the putamen compared to frontal white matter or anterior cingulate cortex (Amsterdam: 5.0 vs 9.1; London: 8.4 vs. 17.6 and 18.8 for putamen vs. white matter and anterior cingulate cortex (London only) respectively, $p < 0.0001$ for all comparisons). There was a small difference in the signal to noise ratio in the putamen between the two scanners used in Amsterdam but not for the frontal white matter (difference [95% confidence intervals] 0.81 [0.05-1.57], $p_{adj} = 0.03$; -0.49 [-1.98-1.00], $p_{adj} = 0.72$ for scanner 2 vs. scanner 1 for the putamen and frontal white matter respectively. Example spectra are illustrated in Figure 6-A. Further details of scanner differences in metabolites are detailed later in Table 6-D and Table 6-E.

Figure 6-A. Example frontal white matter spectra by scanner demonstrating the differences in signal to noise ratio.



Spectra from scanner 1 (Philips Intera, panel a), scanner 2 (Philips Ingenia, panel b) in Amsterdam and scanner 3 (Siemens Verio, panel c) in London. Signal (black line), modelled signal (red line), residuals (upper black line).

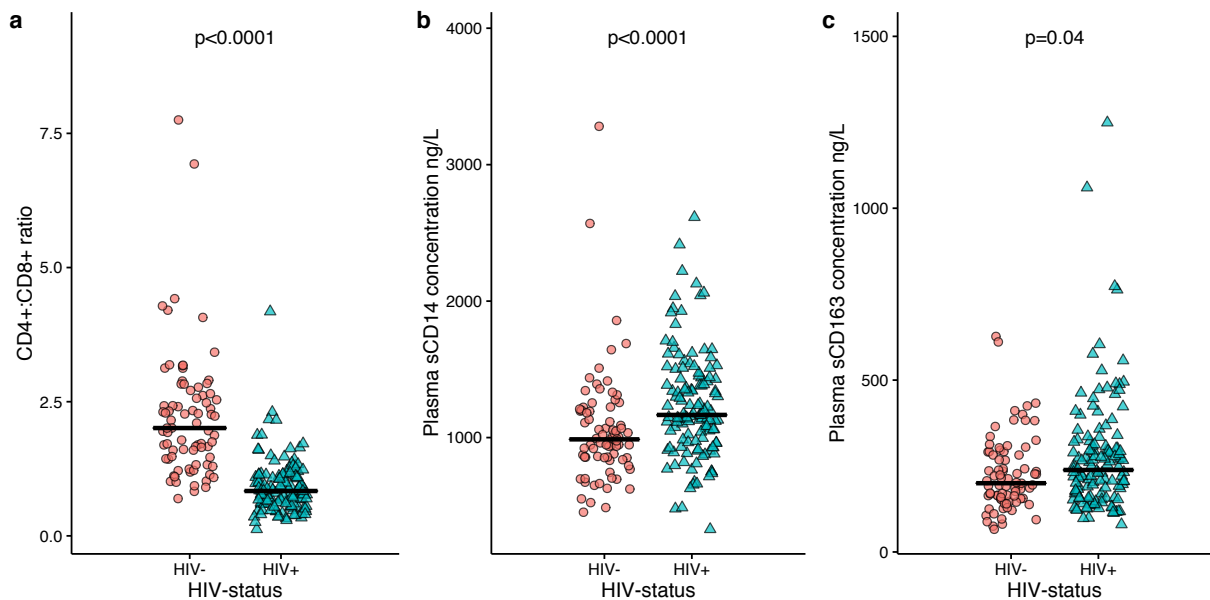
CSF HIV RNA was detectable (>40 copies/mL) in only two patients (1.6%). One had 59 copies/mL and the other 1,043 copies/mL.

Immune activation and neuroinflammation

HIV-positive participants had lower CD4⁺:CD8⁺ ratios (median 0.84 vs. 2.06, $W=9561$, $p < 0.0001$) as well as greater concentrations of soluble CD14 and CD163 (sCD14 and sCD163, **Figure 6-B**) than the HIV-negative control group. There were no associations of

systemic markers of immune activation with age (Table 6-A). However, there was a significant interaction between age, HIV-status and CD4+:CD8+ ratios (model adjusted $R^2=0.41$, $F_{3,208}=49.1$, $p=0.001$; interaction: $t_{208}=-2.67$, $p<0.01$), whereby the CD4+:CD8+ ratio declined with age in patients but not controls (illustrated graphically in Figure 6-E). Using a multiple linear regression model, there was no association between CD4+:CD8+ ratios and global T-scores ($p=0.87$), nor an interaction with HIV-status ($p=0.78$). However, there were significant interactions between HIV-status and plasma sCD14 and sCD163 (model adjusted $R^2=0.08$, $F_{4,202}=5.78$, $p<0.001$; interaction $t_{202}=2.42$, $p=0.02$; and model $R^2=0.06$, $F_{4,206}=4.41$, $p=0.001$; interaction $t_{206}=2.40$, $p=0.02$). These are illustrated graphically in Figure 6-C.

Figure 6-B. Jitterplots of markers of systemic immune activation by HIV-status.



Black lines denote medians. P-values calculated with the Wilcoxon rank-sum test.

Table 6-A. Correlations between markers of systemic immune activation and age.

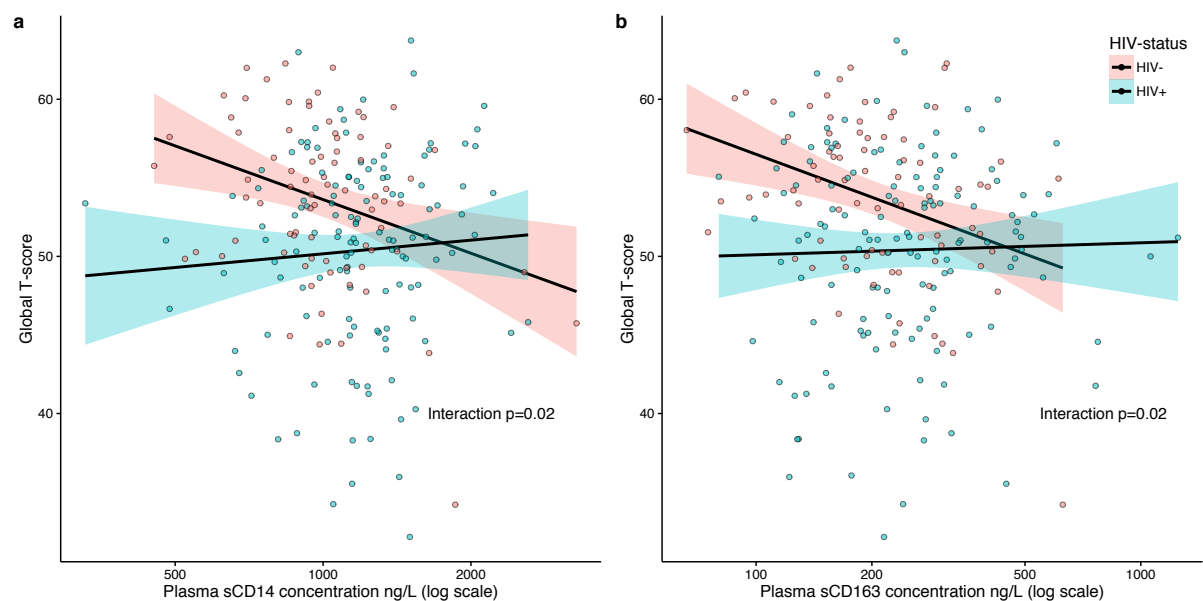
Plasma biomarker	Spearman's rho	p-value	HIV-status * age interaction p-value
CD4+:CD8+ ratio	0.03	0.63	<0.001
Soluble CD14	0.01	0.85	0.54
Soluble CD163	-0.01	0.85	0.67

Interaction p-value calculated from a multiple linear regression model.

Although, CSF concentrations of sCD14 and sCD163 did not differ between the groups, HIV-positive participants had greater CSF kynurenine:tryptophan ratio and CSF neopterin

concentrations (Figure 6-D). There were moderate correlations of sCD14, sCD163 and neopterin with age (Table 6-B) and significant interactions with HIV-status for CSF sCD14 and sCD163 concentrations (model adjusted $R^2=0.05$, $F_{3,207}=4.34$, $p<0.01$; interaction $t_{207}=2.18$, $p=0.03$; and model adjusted $R^2=0.07$, $F_{3,207}=6.55$, $p<0.001$; interaction $t_{207}=2.33$, $p=0.02$). These interactions are illustrated graphically in Figure 6-E. There were no associations between CSF biomarkers of immune activation and global cognitive function ($p>0.15$ for all) nor were there any significant interactions with HIV-status ($p>0.1$ for all).

Figure 6-C. Scatterplots of global T-scores and plasma soluble CD14 (sCD14, panel a) and soluble CD163 (sCD163, panel b) by HIV-status showing the significant interaction.

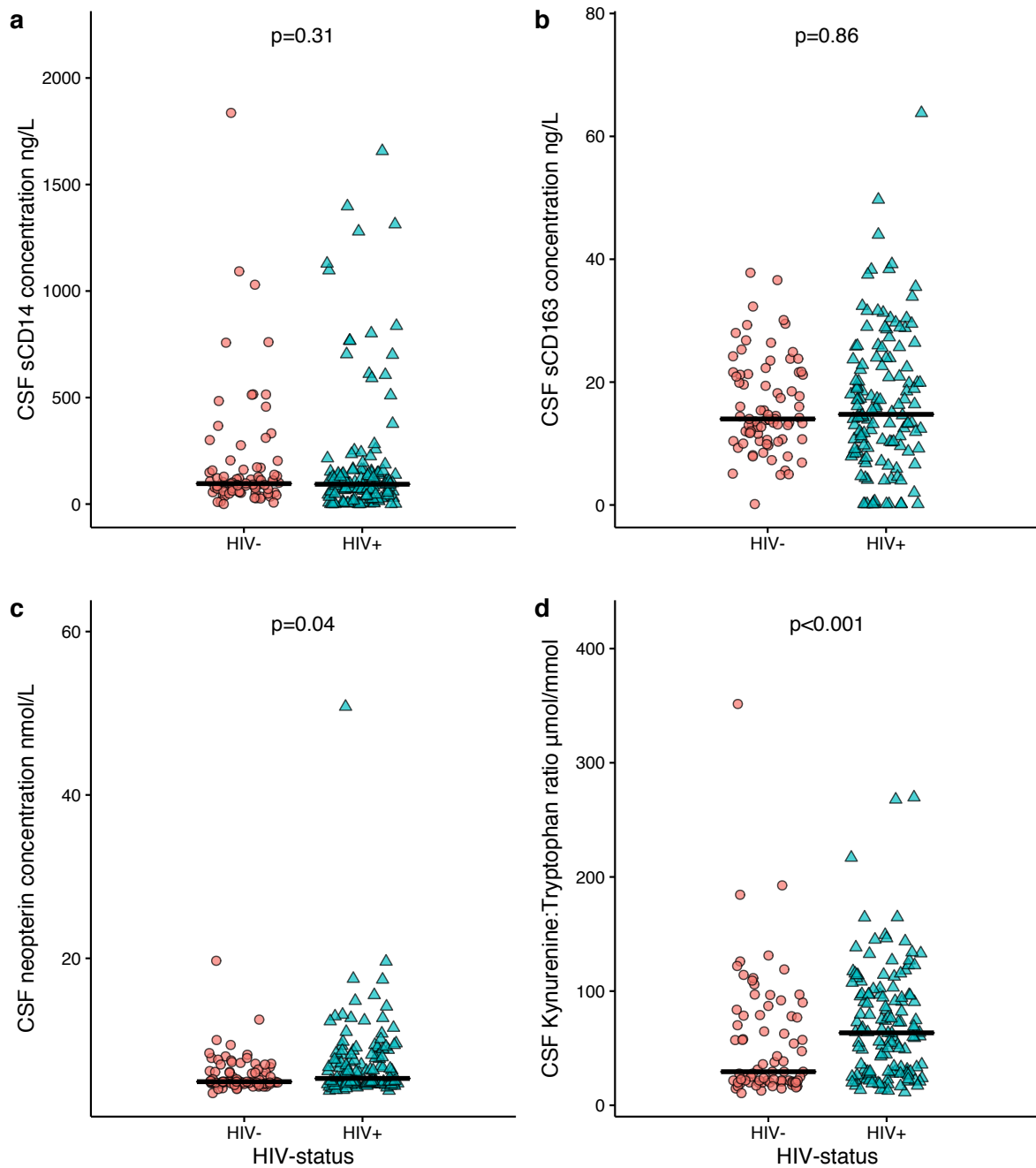


Black lines denote fit lines with 95% confidence intervals. P-values calculated from multiple linear regression models (also adjusted for age).

Table 6-B. Correlations between markers of intrathecal immune activation and age.

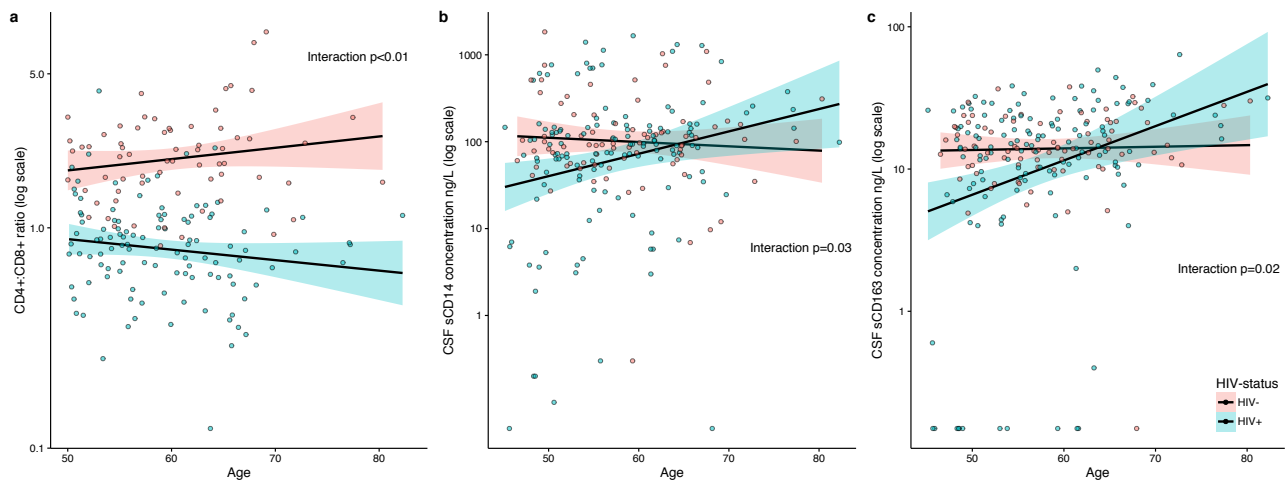
CSF Biomarker	Spearman's rho	p-value	HIV-status * age interaction p-value
Soluble CD14	0.15	0.03	0.03
Soluble CD163	0.22	0.001	0.02
Neopterin	0.31	<0.001	0.55
Kynurenine:tryptophan ratio	-0.01	0.92	0.76

Figure 6-D. Jitterplots of CSF concentrations of soluble CD14 (sCD14, panel a), soluble CD163 (sCD163, panel b), neopterin (panel c) and the kynurenine:tryptophan ratio (panel d) by HIV-status.



Black lines denote medians. *P*-values calculated with the Wilcoxon rank-sum test.

Figure 6-E. Scatterplots of age with the CD4+:CD8+ ratio (panel a) CSF soluble CD14 (sCD14, panel b) and soluble CD163 (sCD163, panel c) by HIV-status.



Black lines denote fit lines with 95% confidence intervals. P-values calculated from multiple linear regression models.

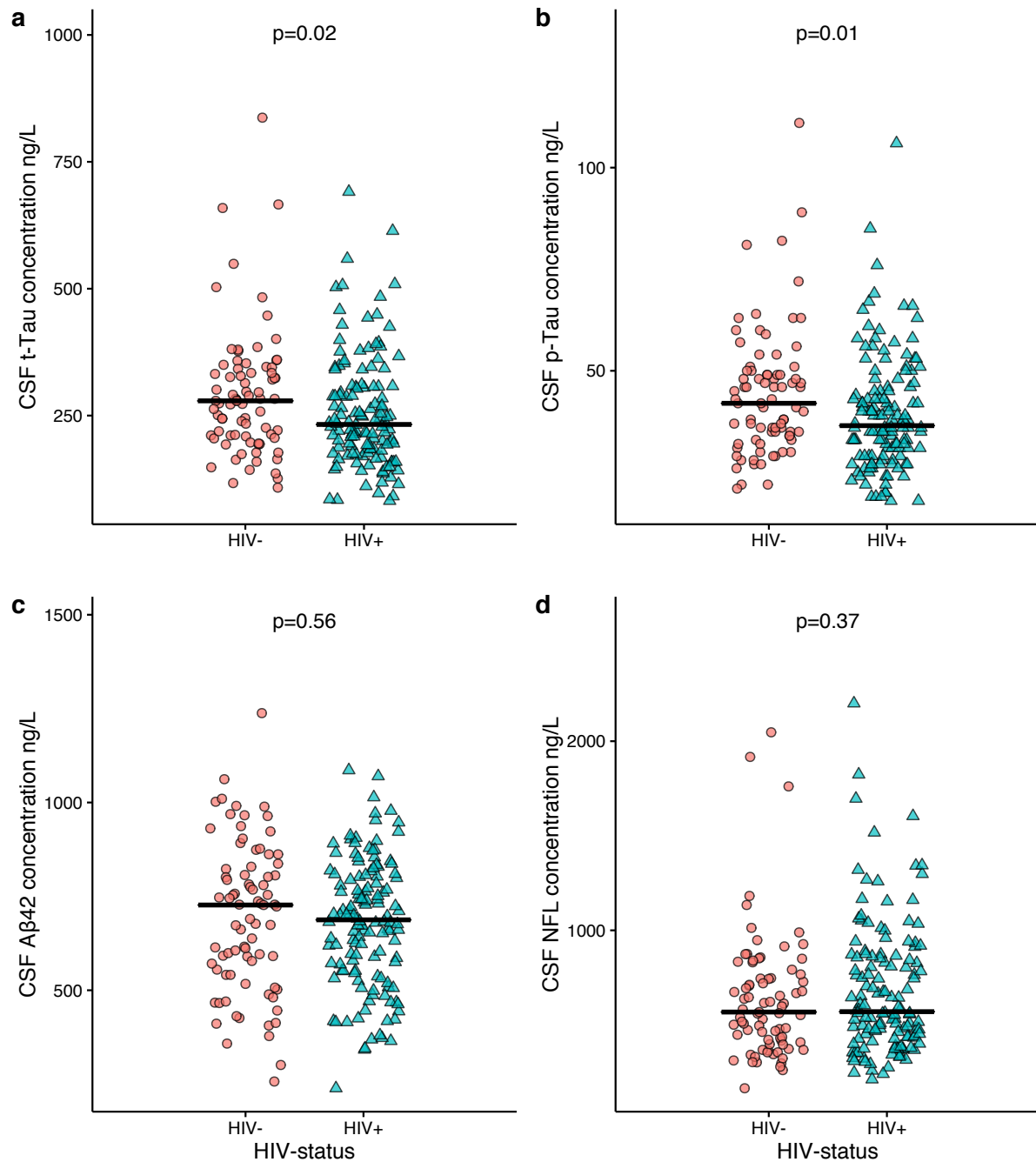
Neuronal injury

The HIV-positive group had lower CSF concentrations of the neuronal markers t-Tau and p-Tau than the HIV-negative control group (Figure 6-F). There were no differences in CSF concentrations of A β 42 or NFL between the groups. All the CSF biomarkers of neuronal injury correlated with age (Table 6-C), with an interaction with HIV-status present for A β 42 ($t_{207}=2.22$, $p=0.04$) but this model was not significant overall (adjusted $R^2=0.02$, $F_{3,207}=2.61$, $p=0.05$). There were no associations between CSF biomarkers of neuronal injury and global cognitive function ($p>0.1$ for all), nor were there any significant interactions with HIV-status ($p>0.5$ for all). However, there was a trend for higher T-scores in those with greater CSF concentrations of t-Tau (model adjusted $R^2=0.07$, $F_{3,207}=6.33$, $p<0.001$, estimate=0.93, $t_{207}=1.77$, $p=0.08$).

Table 6-C. Correlations between markers of neuronal injury and age.

CSF Biomarker	Spearman's rho	p-value	HIV-status * age interaction p-value
Total tau	0.39	<0.0001	0.35
Phosphorylated tau	0.40	<0.0001	0.42
A β 42	0.17	0.01	0.04
Neurofilament light (NFL)	0.58	<0.0001	0.94

Figure 6-F. Jitterplots of CSF concentrations of total-Tau (t-Tau, panel a), phosphorylated-Tau (p-Tau, panel b), A β 42 (panel c) and neurofilament light (NFL, panel d) proteins by HIV-status.



Black lines denote medians. P-values calculated with the Wilcoxon rank-sum test.

Scanner differences in metabolite concentrations

There was a significant scanner effect for all metabolites. In the frontal white matter, participants scanned in London had lower Cho, Cr, Glx and NAA but greater MI (Table 6-D). There were no significant differences between the two scanners used in Amsterdam although the difference in choline approached significance (difference [95% confidence intervals]: 0.13

[0.00-0.27] mM, $p=0.05$). Accordingly this led to greater metabolite ratios in data acquired in Amsterdam for Cho:Cr, Glx:Cr and NAA:Cr (0.05 [0.03-0.08] and 0.06 [0.04-0.09]; 0.47[0.26-0.68] and 0.39 [0.18-0.61]; 0.30 [0.20-0.39] and 0.26 [0.17-0.35] for Cho:Cr, Glx:Cr and NAA:Cr at scanner 1 vs. 3 and 2 vs. 3 respectively $p_{adj}<0.0001$ for all) and lower MI:Cr (0.09 [0.02-0.16], $p_{adj}=0.01$ and 0.13 [0.05-0.20], $p_{adj}<0.001$ for scanner 3 vs. 1 and 3 vs. 2 respectively).

Table 6-D. Scanner differences for white matter metabolite concentrations.

Metabolite	Scanner comparison	Difference (mM)	95% CIs	p_{adj}
Choline	1 vs. 2	0.13	0.00-0.27	0.05
	1 vs. 3	0.48	0.35-0.60	<0.0001
	2 vs. 3	0.34	0.21-0.47	<0.0001
Creatine	1 vs. 2	0.23	-0.09-0.54	0.20
	1 vs. 3	0.43	0.14-0.73	<0.01
	2 vs. 3	0.20	-0.10-0.51	0.26
Glutamate/glutamine	1 vs. 2	0.60	-0.70-1.90	0.52
	1 vs. 3	3.05	1.86-4.24	<0.0001
	2 vs. 3	2.45	1.21-3.69	<0.0001
Myo-inositol	1 vs. 2	0.26	-0.21-0.72	0.39
	3 vs. 1	0.28	-0.14-0.69	0.25
	3 vs. 2	0.54	0.10-0.97	0.01
N-acetyl aspartate	1 vs. 2	0.05	-0.36-0.47	0.95
	1 vs. 3	1.95	1.56-2.34	<0.0001
	2 vs. 3	1.90	1.50-2.30	<0.0001

95% family-wise confidence intervals (CIs) take account of differences in numbers in each group and p -values adjusted for multiple comparisons using Tukey's honest significant difference.

In the putamen, a similar pattern of scanner differences was observed with participants scanned in London having lower Cho, Cr, Glx and NAA but greater MI (Table 6-E). However, for the metabolite ratios there were no significant differences in Cho:Cr or NAA:Cr ($p_{adj}>0.1$ for all comparisons) but there were for Glx:Cr (difference [95% family-wise confidence intervals]: 0.73 [0.26-1.21], $p_{adj}=0.001$; 1.21 [0.62-1.80], $p_{adj}<0.0001$ for scanner 1 vs. 3 and 2 vs. 3 respectively) and for MI:Cr (difference 0.21 [0.04-0.38], $p_{adj}=0.01$). It should be noted that these differences do not account for age differences or potential HIV-effects between sites, hence why age, scanner (where appropriate) and HIV-status were included in all the multiple regression models.

Table 6-E. Scanner differences for putamen metabolite concentrations.

Metabolite	Scanner comparison	Difference (mM)	95% CIs	p_{adj}
Choline	1 vs. 2	0.02	-0.19-0.23	0.97
	1 vs. 3	0.31	0.11-0.53	0.001
	2 vs. 3	0.30	0.10-0.49	0.001
Creatine	1 vs. 2	0.14	-0.50-0.79	0.85
	1 vs. 3	1.96	1.31-2.60	<0.0001
	2 vs. 3	1.82	1.22-2.41	<0.0001
Glutamate/glutamine	2 vs. 1	0.69	-5.12-6.51	0.96
	3 vs. 1	9.83	5.90-13.8	<0.0001
	2 vs. 3	10.5	5.63-15.4	<0.0001
Myo-inositol	2 vs. 1	0.89	-0.78-2.55	0.41
	1 vs. 3	1.85	0.39-3.31	<0.01
	2 vs. 3	2.73	1.63-3.83	<0.0001
N-acetyl aspartate	2 vs. 1	0.24	-0.41-0.89	0.66
	1 vs. 3	0.97	0.32-1.62	0.001
	2 vs. 3	1.21	0.62-1.81	<0.0001

95% family-wise confidence intervals (CIs) take account of differences in numbers in each group and p -values adjusted for multiple comparisons using Tukey's honest significant difference.

Putamen and frontal white matter cerebral metabolites

Left frontal white matter cerebral metabolite ratios did not differ significantly between well-treated HIV-positive patients and controls (Figure 6-G). Similarly, considering the metabolites separately (i.e. as absolute concentrations and not ratios), there were no significant differences in concentrations between the groups ($p > 0.1$ for). There were moderate negative correlations with age for absolute metabolite concentrations (Table 6-F) but not ratios nor were there any interactions with HIV-status ($p \geq 0.1$). There were no associations between these cerebral metabolite ratios and global T-scores ($p > 0.3$ for all ratios), nor were there any interactions between HIV-status, metabolite ratios and global T-scores ($p > 0.1$ for all).

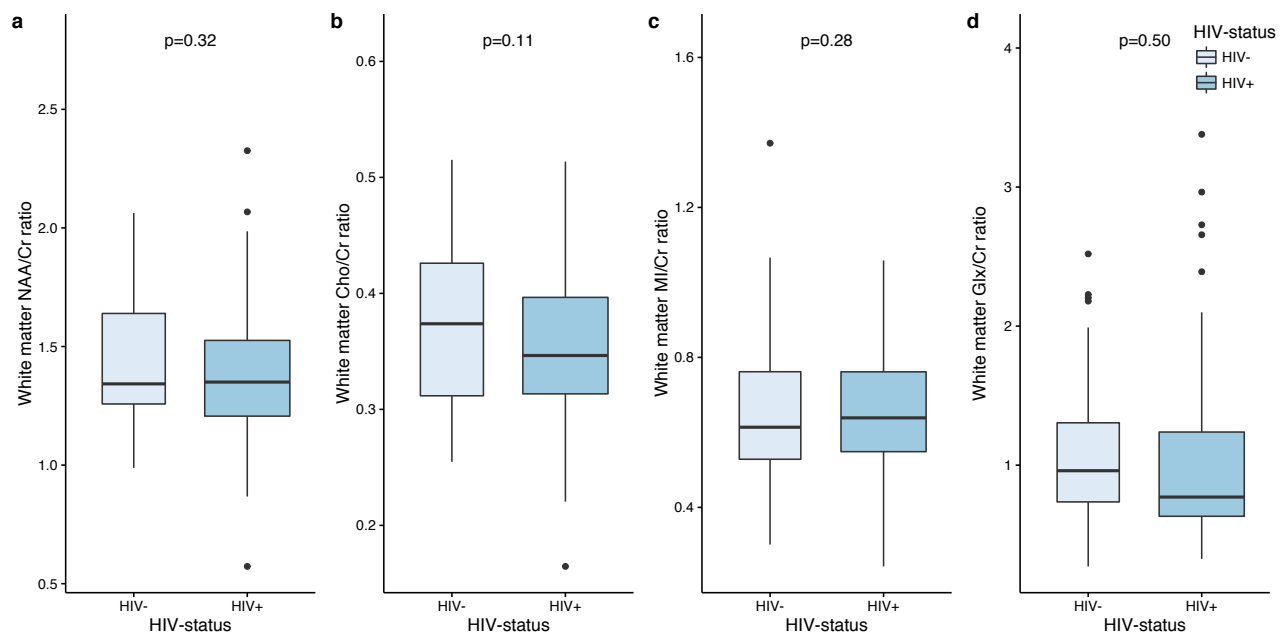
Considering the sites separately, there were no significant differences in any of the frontal white matter metabolite ratios or concentrations between patients and controls from the data collected in Amsterdam ($p > 0.1$ for all). However, in London, patients ($n=51$) had lower frontal white matter Glx concentrations and Glx:Cr ratios than controls ($n=22$; median 3.72 vs 4.50 mM, $W=765$, $p=0.01$, $p_{\text{adj}}=0.04$; median 0.69 vs 0.79, $W=746$, $p=0.03$, $p_{\text{adj}}=0.05$ respectively; $p > 0.1$ for all other white matter metabolites and ratios).

In the left putamen, the HIV-positive group had greater Glx:Cr ratios than controls with a trend towards greater MI:Cr ratios (Figure 6-H). Considering the metabolites separately, there were no significant differences between the groups, although there was a trend for higher glutamate/glutamine concentrations in patients (model adjusted $R^2=0.47$, $F_{4,57}=14.8$, $p<0.0001$, estimate 2.36, $p=0.09$; $p>0.1$ for all other metabolites).

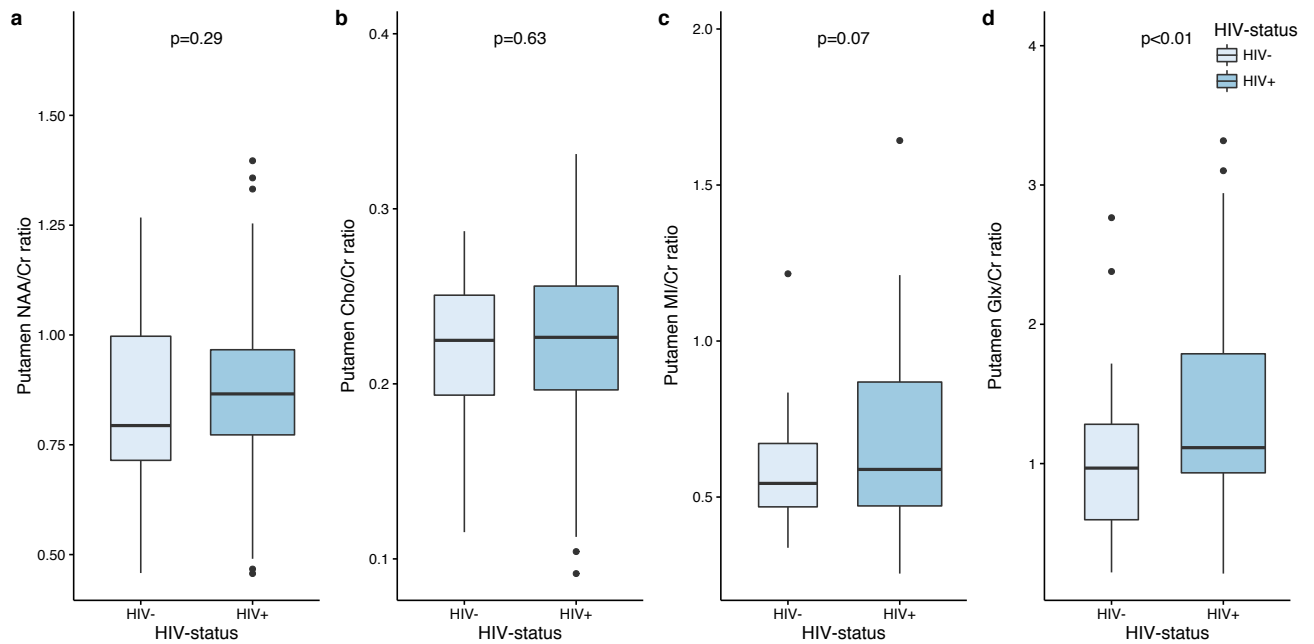
Table 6-F. Correlations between left frontal white matter metabolite concentrations and age.

White matter metabolite	Pearson's <i>r</i>	p-value	HIV-status * age interaction p-value
Choline	-0.33	0.0001	0.60
Creatine	-0.36	<0.0001	0.88
Glutamate/glutamine	-0.26	0.04	0.88
Myo-inositol	-0.09	0.46	0.36
N-acetyl-aspartate	-0.30	<0.001	0.15

Figure 6-G. Boxplots of frontal white matter cerebral metabolite ratios by HIV-status.



Abbreviations: Cho: choline; Cr: creatine; Glx: glutamate and glutamine; MI: myo-inositol; NAA: N-acetyl-aspartate. P-values calculated from a multiple linear regression model adjusted for age and scanner. Box width proportional to the number in each group.

Figure 6-H. Boxplots of putamen cerebral metabolite ratios by HIV-status.

Abbreviations: Cho: choline; Cr: creatine; Glx: glutamate and glutamine; MI: myo-inositol; NAA: N-acetyl-aspartate. P-values calculated from a multiple linear regression model adjusted for age and scanner. Box width proportional to the number in each group.

Age was negatively correlated with absolute metabolite concentrations (Table 6-G) but not with metabolite ratios nor were there any interactions with HIV-status ($p>0.2$ for all).

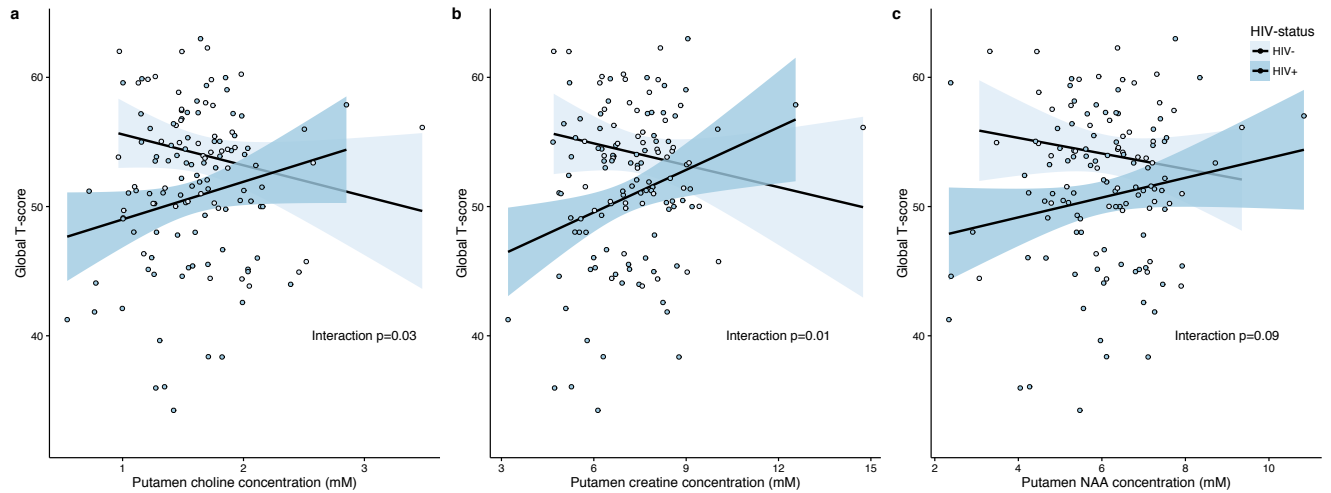
Table 6-G. Correlations between putamen metabolite concentrations and age.

Putamen metabolite	Pearson's r	p-value	HIV-status * age interaction p-value
Choline	-0.15	0.04	0.14
Creatine	-0.08	0.27	0.69
Glutamate/glutamine	0.00	0.99	0.61
Myo-inositol	0.13	0.10	0.43
N-acetyl-aspartate	-0.25	<0.001	0.33

There were no associations between putamen cerebral metabolite ratios and global T-scores ($p>0.6$ for all ratios), nor were there any significant interactions between HIV-status, metabolite ratios and global T-scores ($p>0.4$ for all ratios). Considering the metabolites separately, there were no associations between absolute concentrations and global T-scores. However, there were interactions between HIV-status and concentrations of choline, creatine with global T-scores (model adjusted $R^2=0.11$, $F_{6,126}=3.60$, $p<0.01$, interaction $t_{126}=2.57$, $p=0.01$; model adjusted $R^2=0.07$, $F_{6,126}=2.75$, $p=0.02$, interaction $t_{126}=2.16$, $p=0.03$) and a trend

for NAA (model adjusted $R^2=0.06$, $F_{6,126}=2.52$, $p=0.02$, interaction $t_{126}=1.71$, $p=0.09$). These are illustrated graphically in Figure 6-I.

Figure 6-I. Scatterplots of putamen metabolite concentrations with global T-scores by HIV-status.



Black lines denote fit lines with 95% confidence intervals. P-values calculated from multiple linear regression models. Abbreviations: NAA: N-acetyl-aspartate.

Considering the sites separately, there were no significant differences in any of the putamen metabolite ratios or concentrations between patients and controls from the data collected in Amsterdam ($p>0.1$ for all). However, in London patients ($n=33$) had increased Glx concentrations and Glx:Cr ratios than controls ($n=13$; median 6.63 vs. 4.55 mM, $W=115$, $p=0.01$, $p_{adj}=0.02$; median 0.69 vs 0.79, $W=106$, $p<0.01$, $p_{adj}=0.01$ respectively; $p>0.05$ for all other putamen metabolites and ratios).

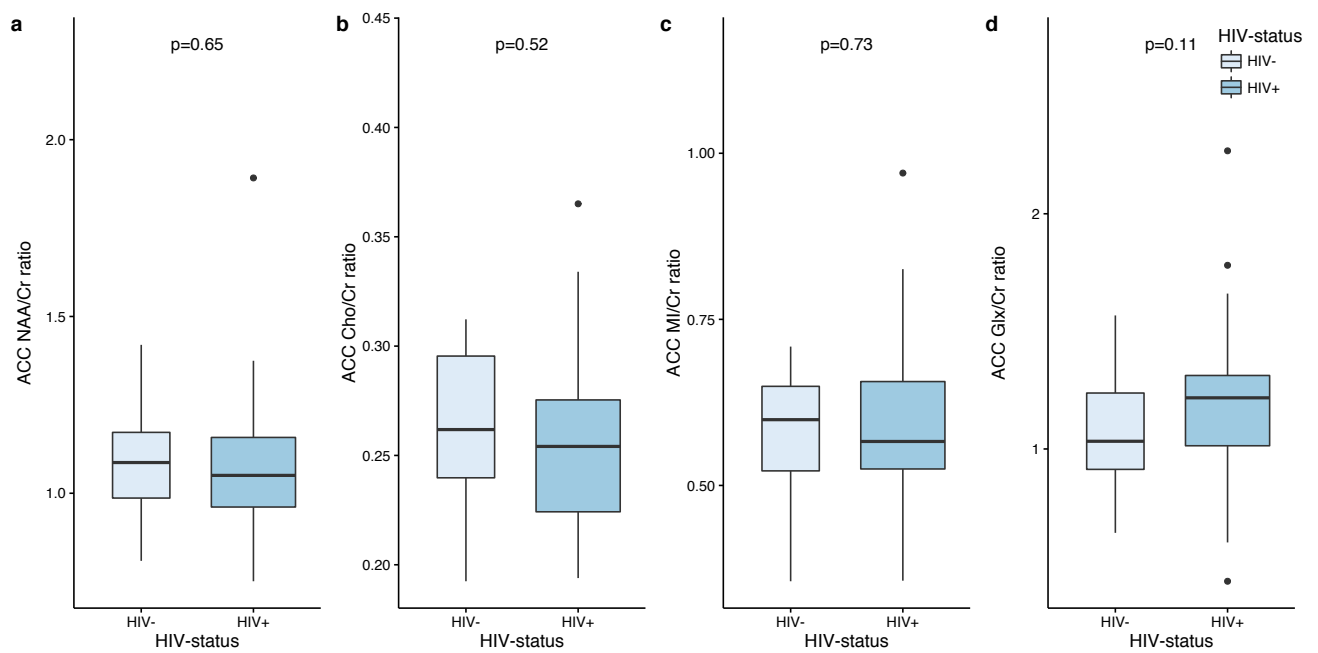
Grey matter cerebral metabolites

In the anterior cingulate cortex, there were no significant differences between the patients and controls for any of the cerebral metabolite ratios (Figure 6-J). However, when considered separately, the HIV-positive group had lower NAA, choline and creatine concentrations (Figure 6-J). ml:Cr ratios negatively correlated with age ($\rho=-0.28$, $p=0.01$), and there was a significant interaction with HIV-status (model adjusted $R^2=0.15$, $F_{3,78}=5.66$, $p=0.001$; interaction $t_{78}=-3.03$, $p<0.01$). The other metabolite ratios did not correlate with age, nor were there any interactions with HIV-status ($p>0.2$ for all). In contrast, all the anterior cingulate cortex metabolite concentrations were negatively correlated with age (Table 6-H) but there was only a significant interaction between HIV-status and myo-inositol (model adjusted $R^2=0.22$, $F_{3,78}=8.64$, $p<0.0001$; interaction $t_{78}=-2.05$, $p=0.04$). This and the interaction

between the absolute concentration of myo-inositol, HIV-status and age are illustrated in Figure 6-L.

There were no associations between anterior cingulate cortex cerebral metabolite ratios and global T-scores ($p>0.6$ for all ratios), nor were there any significant interactions between HIV-status, metabolite ratios and global T-scores ($p>0.4$ for all ratios).

Figure 6-J. Boxplots of anterior cingulate cortex (ACC) cerebral metabolite ratios by HIV-status.

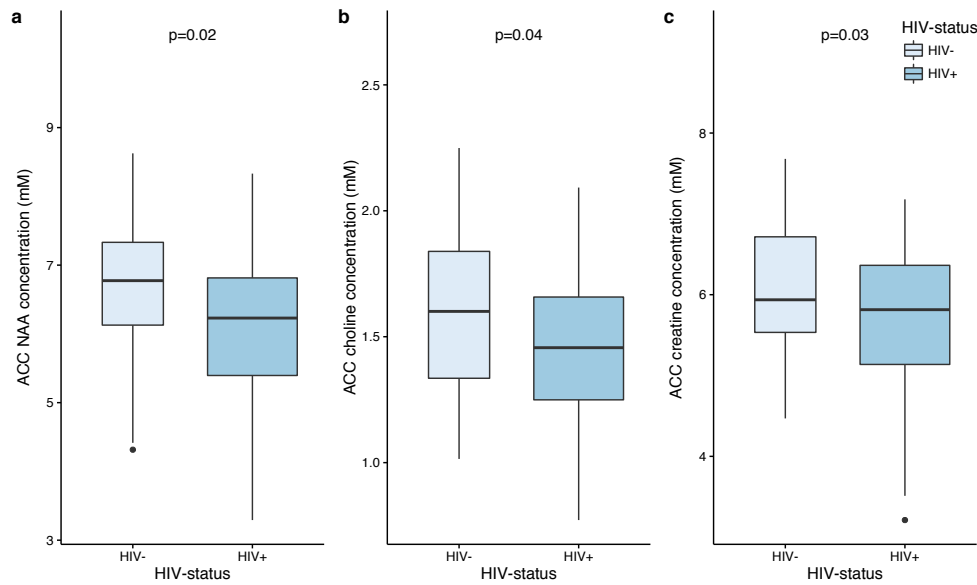


Abbreviations: ACC: anterior cingulate cortex; Cho: choline; Cr: creatine; Glx: glutamate and glutamine; MI: myo-inositol; NAA: N-acetyl-aspartate. P-values calculated from a multiple linear regression model adjusted for age. Box width proportional to the number in each group.

Table 6-H. Correlations between markers anterior cingulate cortex metabolite concentrations and age.

Metabolite	Pearson's <i>r</i>	p-value	HIV-status * age interaction p-value
Choline	-0.35	0.001	0.47
Creatine	-0.43	<0.0001	1.00
Glutamate/glutamine	-0.29	<0.01	0.19
Myo-inositol	-0.44	<0.0001	0.04
N-acetyl-aspartate	-0.42	<0.0001	0.56

Figure 6-K. Boxplots of anterior cingulate cortex (ACC) cerebral metabolite concentrations by HIV-status.



Abbreviations: ACC: anterior cingulate cortex; Cho: choline; Cr: creatine; Glx: glutamate and glutamine; MI: myo-inositol; NAA: N-acetyl-aspartate. P-values calculated from a multiple linear regression model adjusted for age. Box width proportional to the number in each group.

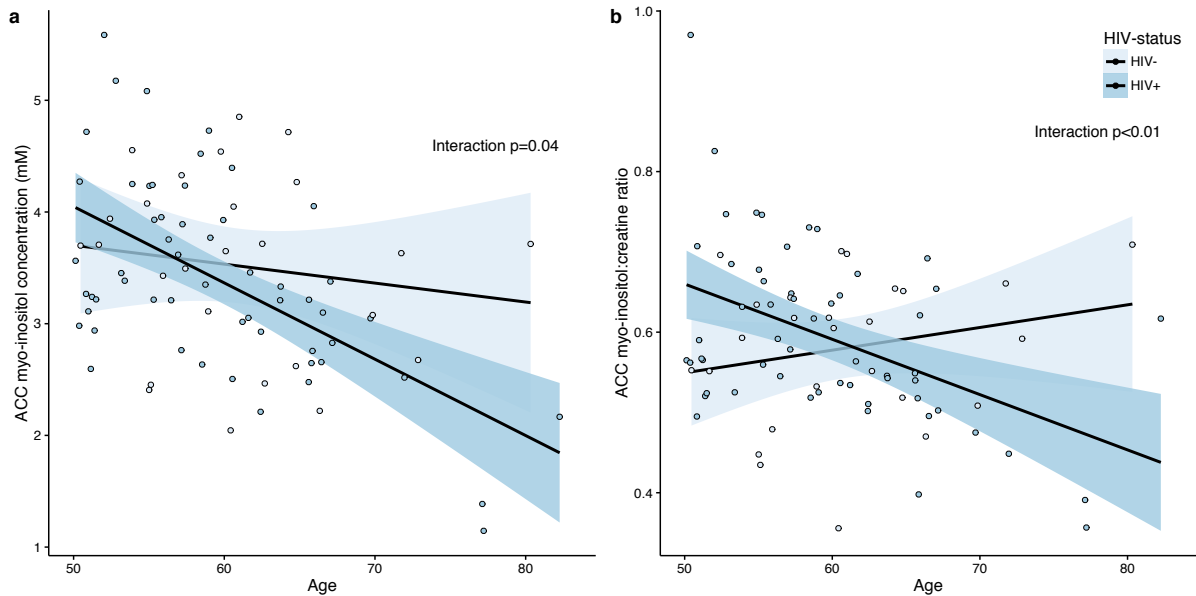
Blood & CSF biomarkers and cerebral metabolites

To further assess the relationships between blood and CSF biomarkers and sub-clinical brain injury correlation and multivariable linear regression were performed. Generally, the correlations were weak between plasma and CSF biomarkers of neuronal injury and inflammation and cerebral metabolites quantified with MRS (mean absolute $\rho < 0.2$ except for CSF neopterin where mean absolute $\rho = 0.21$). This is illustrated graphically in Figure 6-M.

After adjusting for age, HIV-status and scanner plasma sCD14, sCD163 and CD4+:CD8+ ratios were not associated with any of the white matter and putamen metabolites or ratios ($p \geq 0.1$ for all). However, in the anterior cingulate cortex, plasma sCD14 was associated with anterior Glx:Cr (overall model adjusted $R^2 = 0.07$, $F_{3,74} = 2.82$, $p = 0.02$, estimate 0.30, $t_{74} = 2.44$, $p = 0.02$) with a trend for a positive association with Glx concentration (overall model adjusted $R^2 = 0.09$, $F_{3,74} = 3.56$, $p = 0.02$, estimate 1.25, $t_{74} = 1.96$, $p = 0.05$). CD4+:CD8+ ratios were negatively associated with anterior cingulate cortex NAA concentrations (overall model adjusted $R^2 = 0.26$, $F_{3,77} = 10.2$, $p < 0.0001$, estimate -0.50, $t_{77} = -2.10$, $p = 0.04$) and there was a

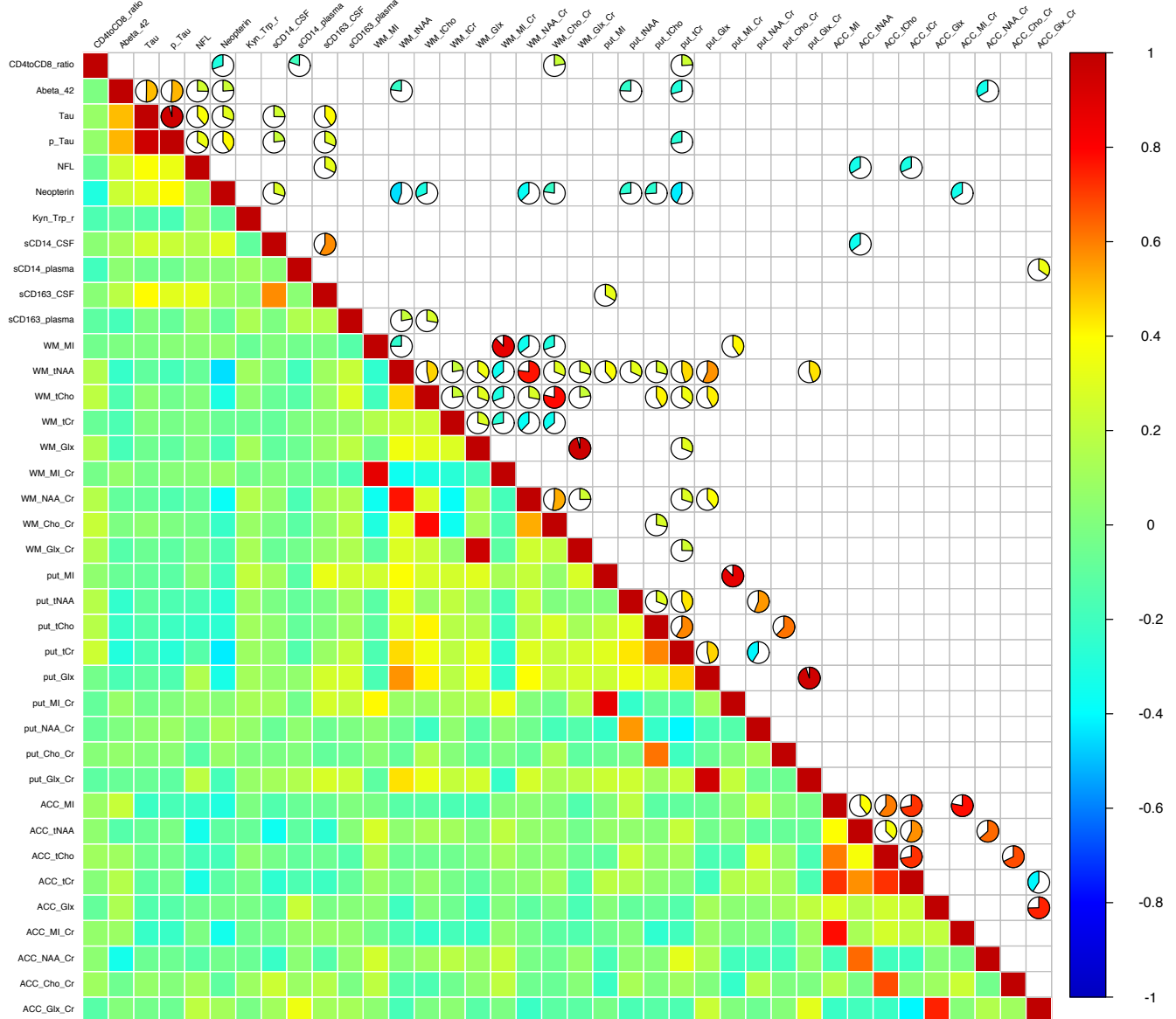
negative trend with creatine concentrations (overall model adjusted $R^2=0.25$, $F_{3,77}=9.68$, $p=0.08$, estimate -0.34 , $t_{77}=1.78$, $p=0.08$); all other metabolites and ratios $p>0.1$).

Figure 6-L. Scatterplots of anterior cingulate cortex (ACC) myo-inositol concentration and ratio with age by HIV-status.



Black lines denote fit lines with 95% confidence intervals. P-values calculated from multiple linear regression models. Abbreviations: NAA: N-acetyl-aspartate.

After adjusting for age, HIV-status and scanner, CSF sCDI4 concentration was not associated with any of the white matter or putamen metabolite concentrations nor ratios although there was a trend for a negative association with white matter Glx:Cr ratio (overall model adjusted $R^2=0.17$, $F_{5,174}=8.23$, $p<0.0001$, estimate -0.048 , $t_{76}=-1.85$, $p=0.07$; $p>0.1$ for the rest). Similarly, CSF sCDI63 concentration was not associated with any of the white matter or putamen metabolites concentrations nor ratios, although there was a trend for a negative relationship with white matter NAA:Cr (adjusted $R^2=0.27$, $F_{5,185}=15.3$, $p<0.0001$, estimate -0.024 , $t_{76}=-1.75$, $p=0.08$; $p>0.1$ for the rest). After adjustment for age and HIV-status, CSF sCDI4 was negatively associated with anterior cingulate cortex NAA concentration (adjusted $R^2=0.25$, $F_{3,76}=10.2$, $p<0.0001$, estimate -0.57 , $t_{76}=-2.56$, $p=0.01$; $p>0.1$ or overall model $p<0.05$ for the rest). CSF sCDI63 was not associated any anterior cingulate cortex metabolite concentrations or ratios although there was a trend for an association with MI:Cr ratios (overall model adjusted $R^2=0.08$, $F_{3,76}=3.4$, $p=0.02$, estimate 0.043 , $t_{76}=1.87$, $p=0.07$).

Figure 6-M. Visualisation of the correlation matrix of soluble biomarkers and cerebral metabolites.

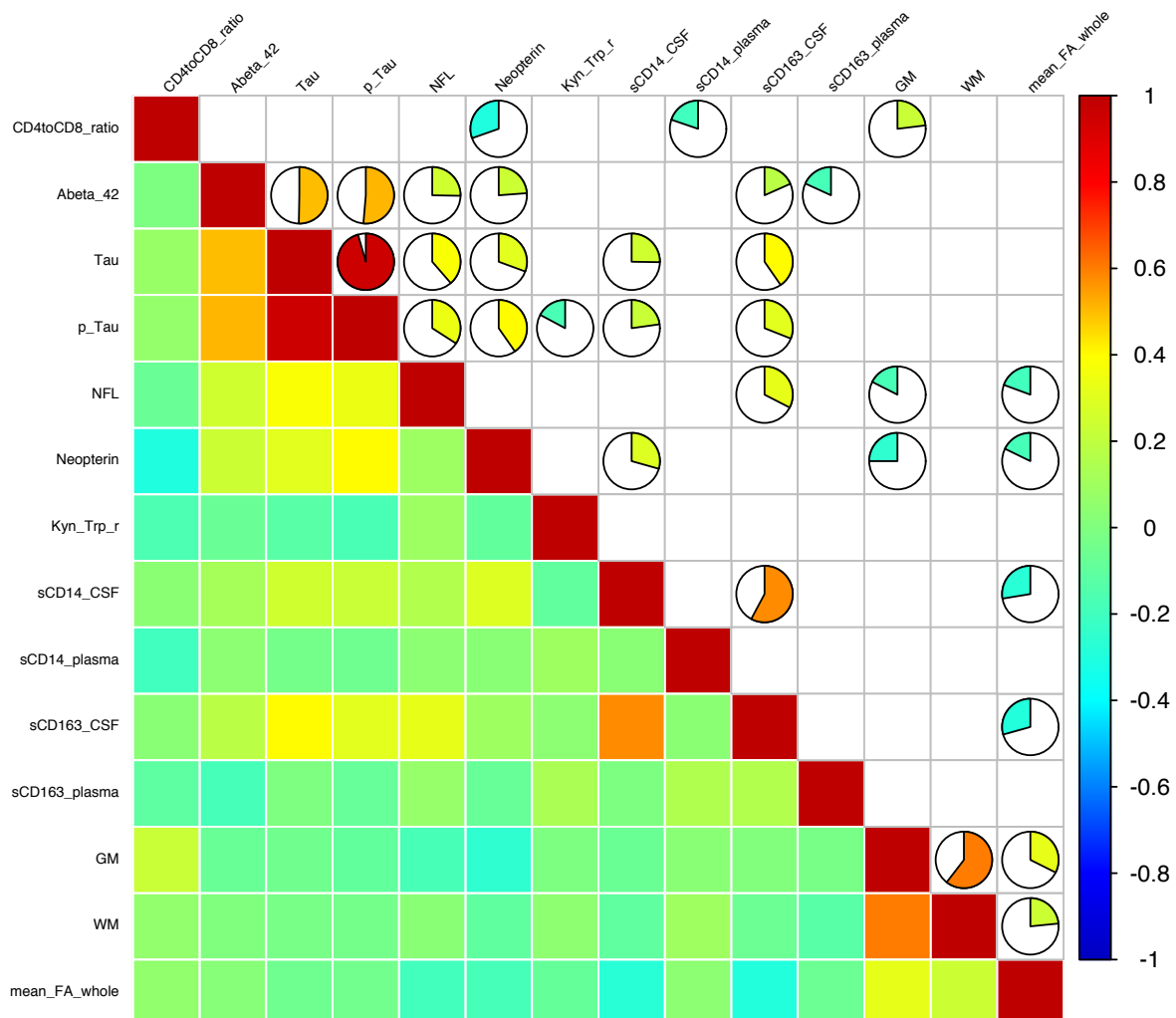
Lower half: Spearman's correlation coefficients coloured according to the scale on the right. Top half: significant correlations (Spearman's rho) depicted as pie charts (false discovery rate corrected p -values < 0.05).

CSF neopterin concentration was negatively associated with anterior cingulate cortex MI concentration (overall model adjusted $R^2=0.26$, $F_{3,76}=10.1$, $p<0.0001$, estimate -0.54 , $t_{76}=-2.86$, $p<0.01$) and MI:Cr (overall model adjusted $R^2=0.15$, $F_{3,76}=5.8$, $p=0.001$, estimate -0.078 , $t_{76}=-3.20$, $p<0.01$) but no other metabolites or ratios ($p \geq 0.1$ for all). The CSF kynurenine:tryptophan ratio was not associated with any of the cerebral metabolites nor ratios ($p > 0.1$ for all), nor was there a correlation with either the Beck depression inventory or PHQ-9 score ($\rho = -0.01$, $p = 0.87$; $\rho = -0.01$, $p = 0.91$).

Blood & CSF biomarkers and structural imaging measures

Next the associations between the total grey matter volume, total white matter volume and mean fractional anisotropy, determined in the previous chapter, and the blood and CSF biomarkers were determined. The correlations are displayed graphically in Figure 6-N. After controlling for the false discovery rate, total grey volume positively correlated with the CD4+:CD8+ ratio, and negatively with CSF NFL and neopterin concentrations. In contrast, total white matter volume was not significantly correlated with any of the blood nor CSF biomarkers after accounting for multiple comparisons. Mean skeleton fractional anisotropy was negatively correlated with CSF concentrations of NFL, neopterin, sCD14 and sCD163.

Figure 6-N. Visualisation of the correlation matrix of soluble biomarkers and structural imaging measures.

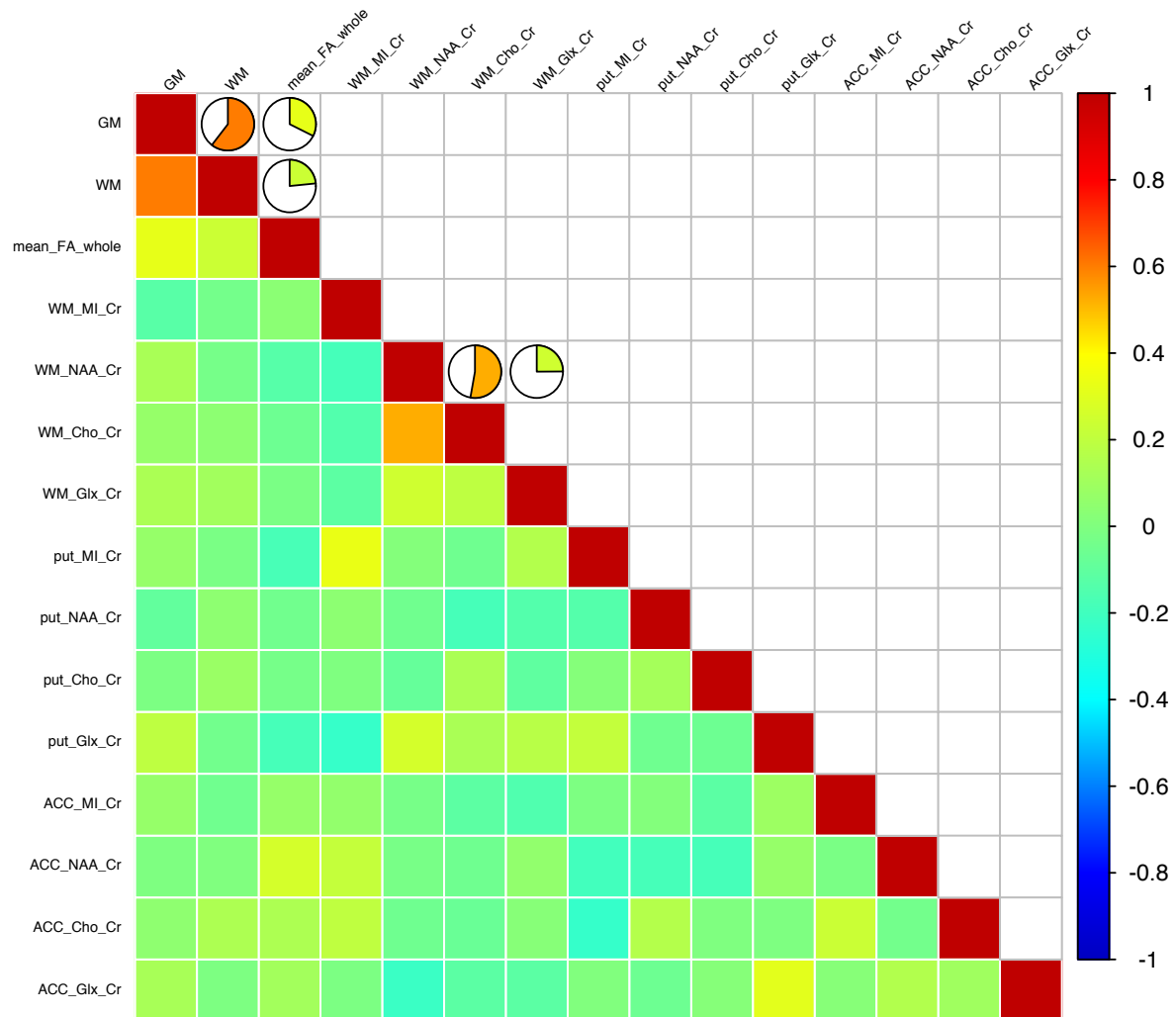


Lower half: Spearman's correlation coefficients coloured according to the scale on the right. Top half: significant correlations (Spearman's rho) depicted as pie charts (false discovery rate corrected p-values < 0.05).

However, after accounting for the potential confounders of age, intracranial volume, HIV-status and scanner (as previously described in Chapter 5) only the negative relationship of mean skeleton fractional anisotropy with CSF sCD14 and sCD163 remained significant (CSF sCD14: overall model adjusted $R^2=0.26$, $F_{6,198}=13.2$, $p<0.0001$, estimate -0.0019, $t_{198}=-2.29$, $p=0.02$; CSF sCD163: adjusted $R^2=0.27$, $F_{6,199}=13.4$, $p<0.0001$, estimate -0.0025, $t_{199}=-2.44$, $p=0.02$; $p>0.1$ for the rest).

There were no significant associations between total grey and white matter volume or mean skeleton fractional anisotropy with any of the cerebral metabolite ratios after correcting for multiple comparisons (Figure 6-O).

Figure 6-O. Visualisation of the correlation matrix of structural imaging measures and cerebral metabolites.



Lower half: Spearman's correlation coefficients coloured according to the scale on the right. Top half: significant correlations (Spearman's rho) depicted as pie charts (false discovery rate corrected p-values < 0.05).

6.4 Discussion

In this chapter, I demonstrated that compared to demographically comparable controls, well-treated HIV-positive patients have clear evidence of persistent systemic immune activation. This was present despite suppression of HIV RNA in both the plasma and CSF compartments in the overwhelming majority. Intrathecally, the differences between the groups were less marked, with only half the neuroinflammatory markers associated with microglial activation elevated in the HIV-positive group. Importantly there was no evidence of active neuronal injury in the patient group. Furthermore, cortical and subcortical grey matter and frontal white matter metabolite concentrations were also similar between the two well-treated patients and controls. Additionally, there were no clear signals of an adverse interaction between HIV-infection and age related changes to cerebral metabolite or biomarker concentrations. In contrast to my second hypothesis, there were no clear associations of any of these biomarkers with cognitive function.

The finding of persistent immune activation in treated patients is not a new finding. A low CD4+:CD8+ ratio has previously been described in treated populations and associated with many non-AIDS comorbidities.^{269,270,273} The finding of reduced anterior cingulate cortex NAA concentrations in those with greater CD4+:CD8+ ratios is therefore surprising as the opposite effect may be expected. NAA concentrations are thought to reflect neuronal health and as has been previously reported, the HIV-positive patients studied here had lower concentrations than controls. The pathophysiological explanation for this finding is unclear, however, given the number of comparisons performed it may represent type I error. Alternatively, it may reflect the complicated relationship between systemic markers of immune dysfunction and CNS markers of brain injury. The disparity between plasma and CSF concentrations of sCD14 and sCD163 have been described previously.^{197,199} This may reflect distinct immunological activation pathways in the plasma and CNS compartments. In the modern treatment era where sustained plasma suppression of HIV replication is the norm, the contribution of HIV both directly and indirectly to the activation of monocytes/macrophages in the peripheries and microglia in the CNS is diminished.

Persistent immune activation is hypothesised to be caused by microbial translocation resulting from compromised gastrointestinal mucosa as a result of HIV-infection.²⁷⁴ Using PET imaging,

Vera et al⁶² recently showed that microbial translocation may also be associated with microglial activation in well-treated patients. However, there was no association between microglial activation and the CSF biomarkers eotaxin, MIP-1b, and IL-8 suggesting a possible decoupling between CNS and CSF measures of inflammation. In the CSF data presented here, patients had elevated kynurenine:tryptophan ratios and neopterin concentrations but not sCD14 or sCD163 concentrations. The disparity may reflect activation of different inflammatory pathways or that CSF kynurenine:tryptophan ratios and neopterin are more sensitive markers of HIV-disease or remain elevated in the presence of injury. However, the lack of association between these markers and cerebral metabolites, cognitive function or structural imaging measures suggests the sequelae of these elevations may be limited. In contrast, CSF concentrations of sCD14 and sCD163 were negatively associated with fractional anisotropy across both groups. Fractional anisotropy is a measure of the degree that water diffusion is restricted by white matter structure²⁷⁵ and reductions may be associated with injury and inflammation with a concomitant increase in the free diffusion of water molecules. Therefore, these findings suggest that elevation of these neuroinflammatory markers may be associated with white matter microstructural injury or oedema irrespective of HIV-status. However, as the CSF concentrations did not differ between patients and controls this pathway is unlikely to explain the differences in cognitive function described in section 5.3.

NFL has previously been described as a sensitive biomarker of HIV-associated brain injury.¹⁸⁵⁻¹⁸⁷ Its temporal relationship to brain injury, well documented in the context of traumatic brain injury,¹⁸⁴ is especially useful for assessing the effectiveness of treatment in HIV-disease. In untreated individuals CSF concentrations correlate with plasma HIV RNA and CSF concentrations of sCD14 and sCD163.²⁷⁶ Longitudinal studies of CSF NFL in patients initiating antiretroviral treatment are sparse. However, elevated concentrations have been reported despite treatment in chronic infection, albeit in a small sample of patients.¹⁸⁶ However, this study did not have an appropriate HIV-negative control group to compare the patient group to and therefore relied on normative data. This may potentially introduce bias due to differences in lifestyle factors that may also affect brain health between patients and normative controls. Here, there were no differences in CSF NFL concentration between well-treated patients and demographically comparable controls. Furthermore, there was no relationship with cognitive function. Taken together, these findings suggest that successful antiretroviral therapy prevents further significant HIV-associated brain injury. Further supporting this

hypothesis is the finding of lower CSF total- and phosphorylated-Tau concentrations in the HIV-positive group compared to controls. Elevation of cerebrospinal fluid Tau concentration is described in many neurodegenerative diseases as well as normal ageing.¹⁸⁹ In HIV-disease, elevated t-Tau but not p-Tau has been reported but concentrations are also reported to be similar to controls in antiretroviral treated patients.^{185,190,191} The findings presented here are therefore surprising and need further work to elucidate.

Across the entire cohort, there were few differences between the groups in white matter and putamen metabolite concentrations. This contrasts with previous work, including in treated individuals, where generally HIV-infection has been associated with a reduction in *N*-acetylaspartate and elevated concentrations of both myo-inositol and choline.^{33,153-157} This may reflect comparison to an appropriate control group as well as the protective effects of successful treatment. Cerebral metabolites provide dynamic information about brain health and HIV-associated abnormalities have been reported to improve with treatment.^{106,277} Therefore, a lack of difference between the groups may represent the absence of significant ongoing HIV-associated brain injury. This needs to be tested by longitudinal study however.

Unlike the volumetric and diffusion measures in the previous chapter, metabolite quantification seems particularly vulnerable to scanner effects. In the data presented in this chapter, the differences between scanners was often larger than the difference between study groups. Whilst the HIV-negative and HIV-positive participants were balanced across scanners, which should ensure that scanner related differences did not confound group comparisons, scanner associated variance in absolute metabolite concentration of this magnitude greatly reduces the power to detect differences between study groups. It should be noted that the data from the putamen and from Amsterdam were of poorer quality relative to the spectra acquired in white matter and from London respectively. The signal to noise ratio in the putamen from spectra acquired in Amsterdam in particular were poor, leading to many failing quality control and being rejected from further analysis. This is likely to reflect the relatively low number of averages (64) used during data acquisition at this site, which was a compromise to shorten scanning time to allow the acquisition of other MRI modalities. Furthermore, there were other qualitative differences in spectra acquired between the sites which probably represents different shimming algorithms used by the different scanner manufacturers. As in the previous chapter, an attempt to account for the variance associated with different scanners was made by covarying scanner in the multiple regression models (as a three-level

factor) and by using metabolite ratios, a method that has previously been used in multi-site studies of HIV-disease.^{96,106} However, it is likely that these methods do not fully account for all the variance associated with scanner differences across sites (certainly using ratios did not remove the large differences associated with scanner) and highlights a drawback of performing multi-site neuroimaging studies. The differences in scanner protocol for spectroscopy acquisition predated my involvement with the COBRA project as data collection began two years before London in Amsterdam. Due to time constraints and the poor quality data, a decision was made to not collect putamen data at both sites for the longitudinal part of the COBRA study (not reported in this thesis). If data collection was to be repeated, I would advise on collecting more averages (ideally at least 124) and having the same number at each site. This would allow a better signal to noise ratio and therefore more precise modelling of the signal and consequent improvements in quantifying absolute metabolite concentrations. Whilst this is unlikely to remove all the variance associated with scanner differences it is likely to increase the number of useable spectra, increase the precision of the results and therefore allow more complete modelling of scanner associated effects.

Considering the sites separately, group differences in Glx concentration and Glx:Cr ratios were present in the participants from London only. However, the direction of these differences contrasted between the frontal white matter, where they were reduced in patients, and the putamen where they were increased. A similar disparity has been reported previously in HIV-disease,²²⁶ albeit with reductions described in grey matter rather than white matter. These regional differences may be explained by spatially varying effects of HIV on the brain. However, these findings should be interpreted with caution given that, firstly, they represent a sub-group analysis; secondly, the number of subjects in each group was low, particularly for the putamen due poor quality data acquisition; thirdly there were no differences in the other metabolites; and finally, as the results have not been corrected for multiple comparisons. Anterior cingulate cortex data were acquired in London only. In this voxel, group differences were seen in absolute concentrations but not metabolite ratios. There are several potential reasons for this disparity. Firstly, it may reflect differing HIV-associated pathologies across the brain. Secondly, greater atrophy in the anterior cingulate cortex in the HIV-positive group may cause reduced metabolite concentrations (but not ratios) as this voxel may also include CSF. However, this would be mitigated somewhat by the inclusion of age in the multivariable models. Another explanation is that if the absolute concentration of creatine is affected by HIV-infection then its use as a denominator in ratios

is questionable and may obscure differences in other metabolite concentrations. Absolute values may therefore be preferable,²⁷⁸ provided scanner related differences can be accounted for. However, despite these group differences in metabolite concentrations there were no associations with cognitive function. This suggests the impact of these changes are likely to be modest at most. It should also be noted that with the number of comparisons presented here, type I errors are inevitable and therefore small group differences and borderline *p*-values should be interpreted with caution.

Conclusion

Despite evidence of increased systemic immune activation, well-treated HIV-positive individuals did not have clear CSF or MRS biomarker evidence of active brain injury. In contrast to my second hypothesis, these biomarkers were not associated with cognitive function. These findings contrast with previous work in less successfully treated patients and may reflect the biomarker panel used and problems with spectroscopy acquisition. Another explanation is that they are dynamic biomarkers of brain injury and if significant active injury is no longer present the correlation between them and more static measures like cognitive function may be lost.

Chapter 7

Plasma and CSF non-nucleoside reverse transcriptase inhibitor exposure and their relationship with cognitive function.

Table of contents

CHAPTER 7 PLASMA AND CSF NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR EXPOSURE AND THEIR RELATIONSHIP WITH COGNITIVE FUNCTION.....	192
7.1 INTRODUCTION	186
7.2 METHODS.....	188
Participants	188
Cognitive function tests	188
Antiretroviral pharmacokinetics	188
Blood and CSF biomarkers of neuronal injury and neuroinflammation.....	189
Neuroimaging.....	189
Statistical analyses	190
7.3 RESULTS	191
Plasma efavirenz concentrations and cognitive function	191
Efavirenz exposure and structural neuroimaging measures.....	192
Plasma efavirenz concentration and CSF biomarkers	194
Nevirapine exposure and cognitive function	195
Nevirapine exposure and structural neuroimaging measures	199
Nevirapine exposure and CSF biomarkers	201
7.4 DISCUSSION	201
Conclusion	207

Figures

FIGURE 7-A. PATIENTS RECEIVING EFAVIRENZ HAVE POORER COGNITIVE FUNCTION THAN HIV-NEGATIVE CONTROLS.	192
FIGURE 7-B. PLASMA EFAVIRENZ CONCENTRATIONS NEGATIVELY CORRELATED WITH ATTENTION AND GLOBAL T-SCORES	193
FIGURE 7-C. PATIENTS RECEIVING NEVIRAPINE HAVE LOWER COGNITIVE T-SCORES THAN HIV-NEGATIVE CONTROLS.	196
FIGURE 7-D. PLASMA AND CEREBROSPINAL FLUID NEVIRAPINE CONCENTRATIONS ARE HIGHLY CORRELATED.....	197
FIGURE 7-E. PLASMA AND CEREBROSPINAL FLUID CONCENTRATIONS OF NEVIRAPINE BY ETHNICITY.....	197
FIGURE 7-F. PLASMA NEVIRAPINE CONCENTRATIONS NEGATIVELY CORRELATED WITH COGNITIVE DOMAIN T-SCORES.....	198
FIGURE 7-G. CSF NEVIRAPINE CONCENTRATIONS NEGATIVELY CORRELATED WITH COGNITIVE DOMAIN T-SCORES	199
FIGURE 7-H. REGIONS OF GREY MATTER THAT CORRELATE NEGATIVELY WITH PLASMA NEVIRAPINE CONCENTRATION.....	200

Tables

TABLE 7-A. DEMOGRAPHICS OF HIV-POSITIVE INDIVIDUALS RECEIVING EFAVIRENZ.....	192
TABLE 7-B. CORRELATIONS BETWEEN DURATION OF EFAVIRENZ USE AND PLASMA CONCENTRATIONS WITH COGNITIVE DOMAIN T-SCORES.	192
TABLE 7-C. CORRELATIONS BETWEEN PLASMA EFAVIRENZ CONCENTRATION AND CSF BIOMARKERS OF NEURONAL INJURY AND NEUROINFLAMMATION.	194
TABLE 7-D. DEMOGRAPHICS OF HIV-POSITIVE INDIVIDUALS RECEIVING NEVIRAPINE.....	195
TABLE 7-E. CORRELATIONS BETWEEN DURATION OF NEVIRAPINE USE, PLASMA AND CSF NEVIRAPINE CONCENTRATIONS WITH COGNITIVE DOMAIN T-SCORES.	198
TABLE 7-F. CORRELATIONS BETWEEN PLASMA AND CSF NEVIRAPINE CONCENTRATIONS AND CSF BIOMARKERS OF NEURONAL INJURY AND NEUROINFLAMMATION.....	201

7.1 Introduction

Antiretroviral therapy has hugely improved the life expectancy of HIV-infected individuals.³ The improvements in mortality have been mirrored by reductions in AIDS related comorbidity.²⁴ In tandem with the drop in incidence central nervous system opportunistic infections, the incidence and prevalence of HIV-associated dementia has declined precipitously.^{4,7} However, the prevalence of milder forms of cognitive impairment remains stubbornly at a similar level to that reported in the pre-antiretroviral era.^{8,10,243} Methodological issues of diagnosis notwithstanding,¹³ the possible aetiological role of antiretroviral central nervous system neurotoxicity has yet been elucidated.³⁴ This is despite the well-recognised phenomenon of peripheral nerve toxicity, particularly with some of the older de-dideoxy-nucleoside reverse transcriptase inhibitors⁷⁷ and the well documented psychiatric manifestations of efavirenz use.²⁷⁹ Determining the potential for toxicity is important given the dramatic improvements in life expectancy for people living with HIV and the consequent exposure to decades of antiretrovirals. Furthermore, given the plethora of antiretrovirals available today, it is potentially easily modifiable if one drug or a particular class were found to be toxic. Potentially much easier than other modifiable risk factors for cognitive impairment such as cardiovascular and lifestyle factors. This potential for toxicity over the long term is one reason why study of these effects has been difficult. Randomised clinical trials of central nervous system antiretroviral toxicity would be impractical and prohibitively expensive as participants are likely to be needed to be followed up for years to see if different combinations of drugs have divergent toxicity profiles (see introduction *** for a graphical model of the long term consequences of potential toxicity).

In vitro, neuronal toxicity has been demonstrated for many of the antiretrovirals in widespread use today.⁸⁷ Animal experiments have also demonstrated toxicity for the non-nucleoside reverse transcriptase inhibitors (NNRTIs) efavirenz and nevirapine, two drugs that form the cornerstone of combination therapy for millions worldwide.^{113,114} Human studies specifically investigating central nervous system antiretroviral toxicity have been sparse. However, one study investigating antiretroviral interruption showed an improvement in cognitive function with the cessation of therapy, in contrast to the original study hypothesis and in contrast to other non-AIDS comorbidities.⁹⁸ Additionally, efavirenz, a drug well long recognised to have psychiatric side-effects, has recently been associated with an increased rate of suicide.²⁸⁰

Furthermore, its use has also been associated with poorer cognitive function in some but not all studies.^{14,104,110} Nevirapine, the other non-nucleoside reverse transcriptase inhibitor prescribed to millions worldwide, has also been previously associated with poorer cognitive function.¹⁴ However, as these studies did not investigate the dose response relationships of these drugs, rather the associations with their use, they are subject to potential channelling bias and other unmeasured confounders.

Studying pathogenic mechanisms of antiretroviral central nervous system toxicity is difficult *in vivo* without histopathological correlates, which makes the study of biomarkers essential. Soluble biomarkers of neuronal injury and neuroinflammation, neuroimaging biomarkers of brain structure and function and tests of cognitive function all provide complementary information about central nervous system health and permit the investigation of antiretroviral neurotoxicity *in vivo*. Furthermore, the simultaneous study of these parameters in the same individuals allows potential pathophysiological mechanisms to be determined, as abnormalities to brain structure may underpin observed cognitive impairment. To date, reports of the associations of blood and CSF antiretroviral pharmacokinetics and biomarkers of brain health are sparse. Higher plasma efavirenz concentrations have previously been associated with neuropsychiatric symptoms.¹⁰³ However, the relationship between efavirenz with more objective measures of brain health, such as brain tissue volumes or cognitive function have not been determined.

In this chapter, I tested the hypothesis that greater non-nucleoside reverse transcriptase inhibitor exposure would be associated with evidence of central nervous system toxicity, measured by poorer cognitive function and abnormal neuroimaging and CSF biomarkers of brain health. The study of these drugs in particular is justified given their use by millions of patients worldwide. Additionally, their widespread prescription permits sufficient numbers of participants to be recruited and studied in observational cohorts.

7.2 Methods

Participants

HIV-positive patients receiving either nevirapine (n=30) or efavirenz (n=30) and demographically comparable HIV-negative controls (n=79) were recruited from CO-morBidity in Relation to HIV/AIDS (COBRA) study from sites in London and Amsterdam. The HIV-negative participants were included for comparative purposes only to potentially highlight cognitive domains that differed significantly from controls. Eligible participants were aged >45 with no significant neurological conditions, substance or alcohol abuse or moderate/severe depression (see methods, section 2.2.2 for exclusion criteria and other details). All HIV-positive participants were required to be on combination antiretroviral therapy and to have had an undetectable plasma HIV RNA (<50 copies/mL) recorded for at least 12 months prior to enrolment.

Cognitive function tests

Subjects completed a comprehensive battery of neuropsychological tests assessing attention, executive function, language, memory, speed of information processing and motor function (see methods, section 2.1.2 and Table 2-B for further details).

Antiretroviral pharmacokinetics

Blood and cerebrospinal fluid was collected in participants as described in section 2.4 *** of the methods. Plasma and cerebrospinal fluid antiretroviral concentrations were assayed using ultra high-performance liquid chromatography (HPLC) at the Department of Clinical Pharmacy of Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands as described in section 2.5*** of the methods. Due to the long half-lives of efavirenz ($t_{1/2}$ =40-55 hours) and nevirapine ($t_{1/2}$ =30 hours), any differences between sampling time and time of drug intake between patients is not relevant as CSF concentrations will vary only to a small extent over a dosing interval. Therefore, absolute values can directly be used for correlation with other outcome measures.

Blood and CSF biomarkers of neuronal injury and neuroinflammation

Plasma and CSF biomarkers of neuronal injury and neuroinflammation were quantified as described in 2.5 of the methods.

Neuroimaging

Acquisition

A harmonised neuroimaging protocol was used across the two study sites within given hardware and vendor constraints. In London, images were acquired using a Siemens Verio scanner (n=13, n=9 and n=29 for the efavirenz, nevirapine and control groups respectively) and in Amsterdam initially with a Philips Intera (n=10, n=13 and n=20) and then using a Philips Ingenia (n=7, n=8, n=30), both Philips Healthcare, Best, the Netherlands) due to a scanner upgrade. High-resolution 3D T1-weighted structural images and diffusion weighted images along 64 non-collinear directions were acquired at both sites (see methods, section 2.4.1 for further details of acquisition parameters).

Image processing

3D T1 images were pre-processed as described in the methods, section 2.4.2. Briefly, images were bias corrected, segmented into grey matter, white matter and cerebrospinal fluid (CSF) and volumes calculated. Segmented images were then registered to a custom template and normalised to standard Montreal Neurological Institute (MNI) space using the DARTEL algorithm.²¹⁷

Diffusion data were pre-processed using FSL v5.0.6 (FMRIB, University of Oxford) as described in section 2.4.2. Briefly, images were corrected for eddy currents and head motion by rigid-body registration to each subject's initial B0 image. Non-brain tissue was deleted⁴⁵ and the diffusion tensor model was fitted at every voxel, using weighted least squares. These were then registered to a custom template and standard space, using DTI-TK v2.3.1. Fractional anisotropy maps for each participant were then 'skeletonised' using FSL and thresholded ≥ 0.2 to exclude areas with considerable inter-individual variability prior to performing *tract based spatial statistics* (TBSS).¹⁴⁶

Statistical analyses

Group comparisons and correlations

Group comparisons of baseline characteristics, cognitive domain T-scores using chi-square, Fisher's exact, and Wilcoxon rank-sum (two-tailed) as appropriate as described in section 2.8.4. Due to their skewed distribution, drug concentration data were natural log transformed prior to analysis. Following this, correlations with cognitive T-scores were assessed using Pearson's r due to their normal or near normal distribution following transformation. Given the relatively small sample size, this increases the power to detect associations over a non-parametric method. However, as most blood and CSF biomarkers were more positively skewed, log transformation was not as successful and therefore correlations were assessed using Spearman's ρ . Similarly, data pertaining to duration of therapy was not normally distributed and was not improved significantly by log transformation. Therefore, correlations with drug duration were assessed using Spearman's ρ . Interpatient variability in drug concentration was expressed as a coefficient of variation, which given the log-normal distribution of data²⁸¹ $= \sqrt{e^{s_{ln}^2} - 1}$ where s_{ln} = standard deviation of the data of natural log transformed data. Two-tailed p-values <0.05 were considered statistically significant.

Multiple linear regression models

To assess the independent relationships between efavirenz and nevirapine concentrations with brain tissue volumes and mean fractional anisotropy multiple linear regression models were used. The neuroimaging marker of interest was the dependent variable with the natural log transformed drug concentration as the independent variable with age, intracranial volume and scanner as covariates. Only p-values <0.05 were considered as statistically significant.

Voxelwise inference testing

Voxelwise comparisons were performed to investigate localised changes in brain that were related to NNRTI exposure using non-parametric permutation testing¹⁶⁹ as described in section 2.8.4 accounting for age, intracranial volume and scanner. Correction for multiple comparisons was accomplished using threshold-free cluster enhancement (TFCE).¹⁷¹ Corrected p-values <0.05 were considered significant.

7.3 Results

7.3.1 Efavirenz

Thirty patients from the COBRA study were receiving efavirenz (Table 7-A). All had plasma and CSF HIV RNA <40 copies/mL. There were no differences in age, level of education, gender or ethnicity between patients receiving efavirenz or the HIV negative controls (demographics reported in chapter 5 table 1, $p>0.2$ for all). Patients receiving efavirenz had poorer global cognitive function (median global T-score 51.2 vs. 54.2, $W=1527$, $p=0.02$) and significantly lower T-scores in the domains of motor function and processing speed with a trend for lower scores in attention (illustrated graphically in Figure 7-A). However, there were no differences in any of the cognitive domain T-scores for HIV-positive patients receiving efavirenz compared to those not receiving efavirenz ($p>0.3$ for all)

Table 7-A. Demographics of HIV-positive individuals receiving efavirenz.

Demographic	n=30
Age (years), median (IQR)	59 (53-63)
Male, n (%)	30 (100%)
Ethnicity, n (%)	
White	27 (90%)
Black-African	3 (10%)
Weight (kg), median (IQR)	79.5 (74.1-83.7)
Duration of HIV-infection (years), median (IQR)	12.8 (10.5-17.4)
Current CD4 (cells/ μ L), median (IQR)	642 (548-789)
Nadir CD4 (cells/ μ L), median (IQR)	150 (73-198)
Duration of efavirenz (years), median (IQR)	9.2 (5.2-11.4)
Efavirenz plasma concentration (mg/L), geometric mean (95%CI)	2.12 (1.95-2.31)
Efavirenz plasma concentration coefficient of variation	0.69

Plasma efavirenz concentrations and cognitive function

Geometric mean efavirenz concentration and coefficient of variation are reported in . There was no association between age and plasma efavirenz concentration ($r=-0.12$, $p=0.53$) but there was a trend for patients of Black-African ethnicity to have higher plasma efavirenz concentrations (median plasma efavirenz concentrations 4.16 vs. 1.80 mg/L, $W=66$, $p=0.08$).

Plasma efavirenz concentration was not associated with weight ($r=0.15$, $p=0.44$) nor height ($r=-0.13$, $p=0.48$) but was negatively correlated with intracranial volume ($r=-0.39$, $p=0.04$). Accounting for the latter is therefore imperative in subsequent analyses of brain structure as this could confound relationships between plasma exposure and brain volumes.

To assess whether efavirenz exposure was associated with impaired cognitive function and which domains were affected, correlations of duration of therapy and plasma concentrations of efavirenz with global T-scores and subsequently domain T-scores were calculated. There were trends for poorer global cognitive function and motor function with longer duration of efavirenz exposure (Table 7-B). Stronger relationships were observed for plasma efavirenz concentrations, which was negatively correlated with all T-scores but significantly so for attention and global T-scores (Table 7-B). These are illustrated graphically in Figure 7-B.

Table 7-B. Correlations between duration of efavirenz use and plasma concentrations with cognitive domain T-scores.

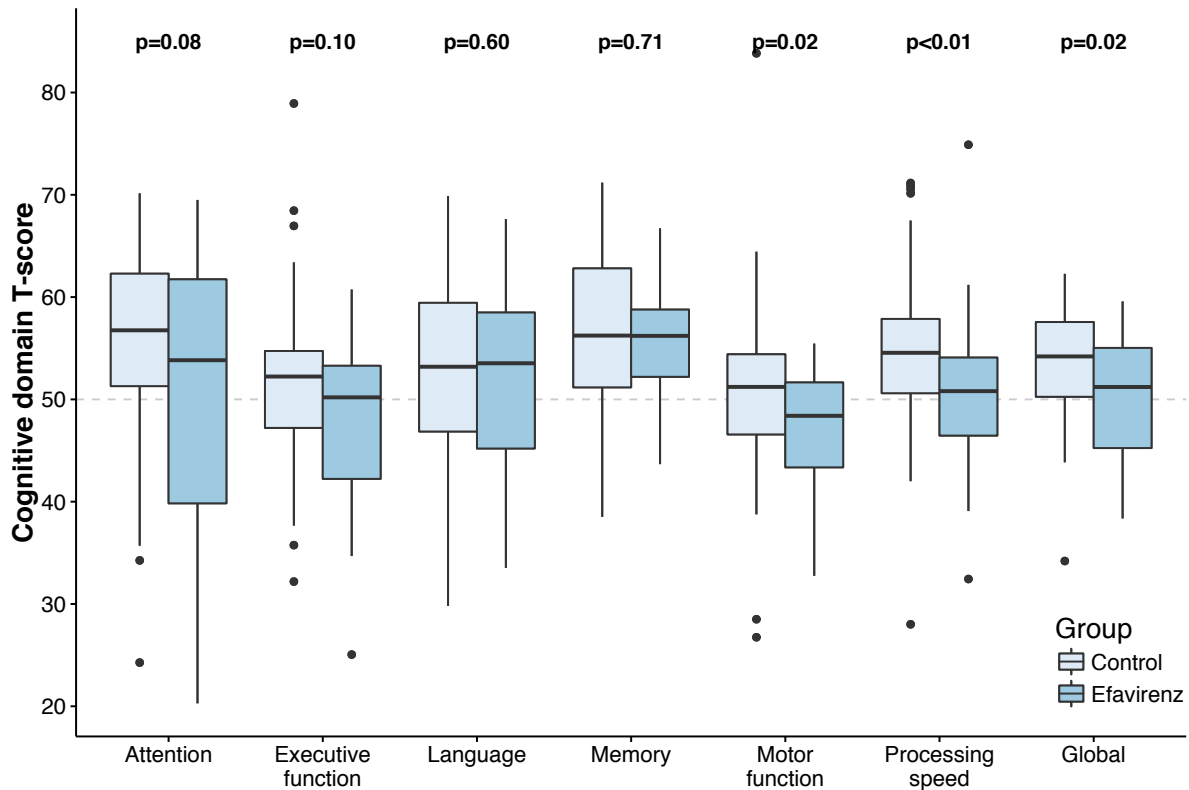
Cognitive domain	Duration		Plasma concentration	
	Spearman's ρ	p-value	Pearson's r	p-value
Attention	-0.14	0.27	-0.45	0.01
Executive function	-0.12	0.34	-0.28	0.13
Language	-0.10	0.45	-0.26	0.16
Memory	-0.03	0.80	-0.16	0.39
Processing speed	-0.19	0.13	-0.19	0.31
Motor function	-0.21	0.10	-0.22	0.25
Global	-0.23	0.08	-0.43	0.02

Efavirenz exposure and structural neuroimaging measures

To explore the pathogenesis of possible efavirenz associated central nervous system toxicity, the relationship between plasma efavirenz exposure and brain structure was determined. Using multiple linear regression models accounting for age, intracranial volume and scanner differences, plasma efavirenz concentration was not associated with total grey matter volume, white matter volume or mean fractional anisotropy (model adjusted $R^2=0.64$, $F_{5,24}=11.2$, $p<0.0001$, estimate=-0.064, $t_{24}=-0.73$, $p=0.47$; model adjusted $R^2=0.70$, $F_{5,24}=14.4$, $p<0.0001$, $t_{24}=-0.50$, $p=0.62$; model adjusted $R^2=0.06$, $F_{5,24}=1.37$, $p=0.27$, $t_{24}=-0.44$, $p=0.68$ respectively).

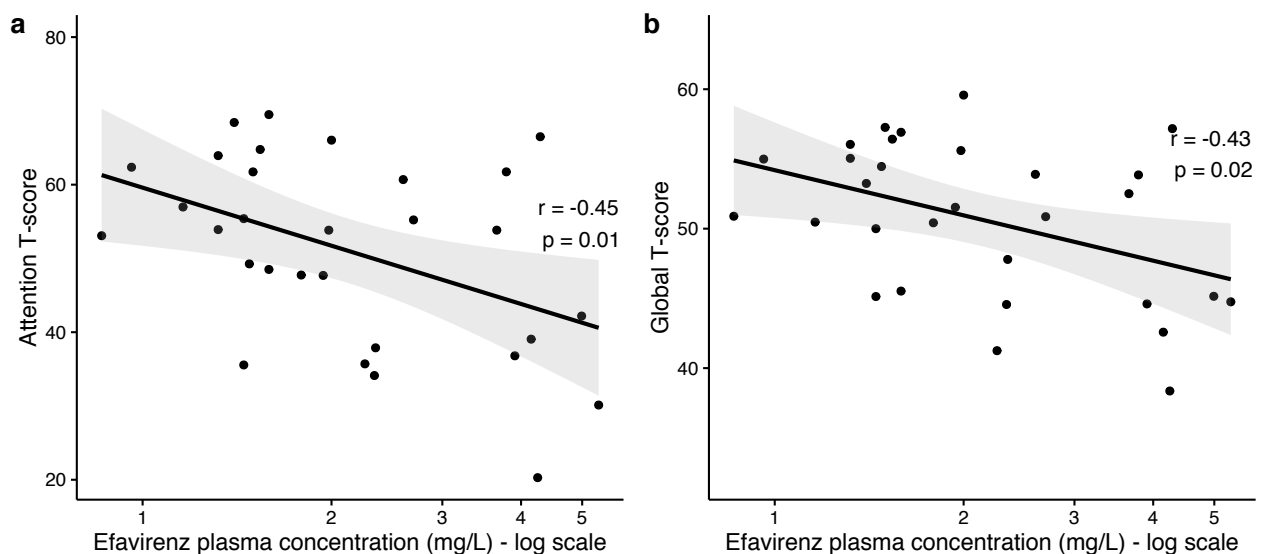
Furthermore, there were no significant voxelwise associations between plasma efavirenz concentration and grey matter volume, white matter volume or fractional anisotropy.

Figure 7-A. Patients receiving efavirenz have poorer cognitive function than HIV-negative controls.



Boxplots of demographically adjusted cognitive T-scores by domain and group. P-values calculated with the Wilcoxon rank-sum test.

Figure 7-B. Plasma efavirenz concentrations negatively correlated with attention and global T-scores



Scatterplots of plasma efavirenz concentrations against attention (panel a) and global T-scores.

Plasma efavirenz concentration and CSF biomarkers

To further delineate the central nervous system effects of efavirenz the relationships of efavirenz exposure with CSF biomarkers of neuronal injury and neuroinflammation were determined. Plasma efavirenz concentrations were not associated with any of these biomarkers (Table 7-C).

Table 7-C. Correlations between plasma efavirenz concentration and CSF biomarkers of neuronal injury and neuroinflammation.

CSF Biomarker	Spearman's <i>rho</i>	p-value
Total tau	-0.17	0.36
Phosphorylated tau	-0.17	0.36
A β 42	-0.27	0.15
Neurofilament light (NFL)	-0.17	0.37
Soluble CD14	0.03	0.87
Soluble CD163	-0.09	0.65
Neopterin	-0.04	0.82
Kynurenine:tryptophan ratio	0.29	0.12

7.3.2 Nevirapine

Thirty patients from the COBRA cohort were receiving nevirapine. All had plasma and CSF HIV RNA <40 copies/mL. There were no differences in age, level of education or gender between patients receiving efavirenz or the HIV negative controls ($p>0.2$ for all). However, there was a greater proportion of patients of Black-African ethnicity who were receiving nevirapine than in the control group (17.2 vs. 2.6%, $\chi^2=5.24$, $p=0.02$).

Patients receiving nevirapine had lower attention and motor function T-scores than controls (median T-score 49.2 vs. 56.8, $W=1662$, $p=0.001$; 49.2 vs. 51.2, $W=1481$, $p=0.04$ respectively) and a trend for global cognitive function (51.2 vs. 54.2, $W=1442$, $p=0.08$). The differences in cognitive domain T-scores in those taking nevirapine compared to controls is illustrated graphically in Figure 7-C. There were no differences in T-scores between HIV-positive patients receiving nevirapine compared to those were not for all domains except processing

speed, where there was a trend for higher T-scores in those receiving nevirapine (median T-score 53.4 vs. 50.9, $W=1848$, $p=0.08$).

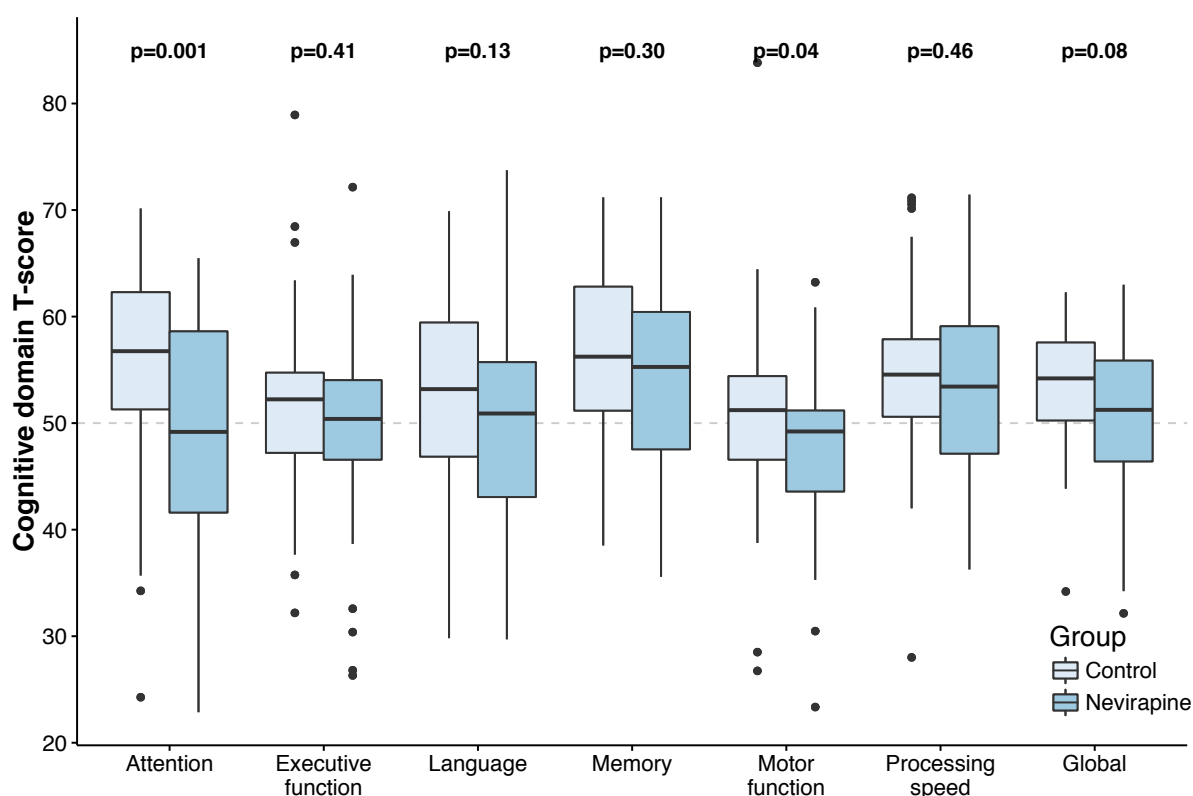
Table 7-D. Demographics of HIV-positive individuals receiving nevirapine.

Demographic	n=30
Age (years), median (IQR)	55 (50-61)
Male , n (%)	28 (93.3%)
Ethnicity , n (%)	
White	24 (82.8%)
Black-African	5 (17.2%)
Weight (kg), median (IQR)	81.2 (71.8-88.9)
Duration of HIV-infection (years), median (IQR)	16.3 (14.0-20.1)
Current CD4 (cells/ μ L), median (IQR)	677 (500-823)
Nadir CD4 (cells/ μ L), median (IQR)	215 (85-270)
Duration of nevirapine (years), median (IQR)	11.5 (8.3-14.3)
Nevirapine plasma concentration (mg/L), geometric mean (95% CIs)	5.75 (5.31-6.22)
Nevirapine plasma concentration coefficient of variation	0.68
Nevirapine CSF concentration (mg/L), geometric mean (95% CIs)	2.43 (2.25-2.62)
Nevirapine CSF concentration coefficient of variation	0.67

Nevirapine exposure and cognitive function

Plasma and CSF geometric mean nevirapine concentrations and coefficients of variation are reported in Table 7-D. Plasma nevirapine concentrations were highly correlated with CSF concentrations ($r=0.96$, $p<0.0001$, Figure 7-D). There was no association between age and either plasma or CSF nevirapine concentrations ($r=0.09$, $p=0.65$; $r=0.19$, $p=0.33$ respectively). Patients of self-identified Black-African ethnicity had higher plasma and CSF nevirapine concentrations than those of white ethnicity (median plasma nevirapine concentration: 5.00 vs. 9.73 mg/L, $W=97$, $p=0.03$; CSF concentration: 2.35 vs. 3.63 mg/L, $W=97$, $p=0.04$). These differences are illustrated graphically in Figure 7-E. Plasma and CSF nevirapine concentrations were not associated with weight ($r=-0.26$, $p=0.17$; $r=-0.26$, $p=0.17$) but both were negatively correlated with height and intracranial volume (height: $r=-0.59$, $p<0.001$; $r=-0.59$, $p<0.001$; intracranial volume: $r=-0.47$, $p<0.01$; $r=-0.48$, $p<0.01$).

Figure 7-C. Patients receiving nevirapine have lower cognitive T-scores than HIV-negative controls.



Boxplots of demographically adjusted cognitive T-scores by domain and group. P-values calculated with the Wilcoxon rank-sum test.

As before, to assess whether nevirapine exposure was associated with impaired cognitive function and which domains were affected, correlations between measures of nevirapine exposure with global T-scores and subsequently domain T-scores were calculated. There was a trend for lower attention T-scores with longer duration of therapy, but no other associations with cognitive function (Table 7-E). In contrast, plasma nevirapine concentrations were negatively correlated with all T-scores (Table 7-E), but only significantly so for the domains of attention, processing speed and motor function. These are illustrated graphically in Figure 7-F. Similar, but stronger relationships were seen with CSF nevirapine concentration and cognitive T-scores (Table 7-E and Figure 7-G). Even after excluding the patient with the greatest nevirapine concentration, the associations with attention T-scores remained ($r=-0.47$, $p=0.01$; $r=-0.53$, $p<0.01$ for plasma and CSF concentrations respectively) and there was a negative trend of CSF concentration with processing speed T-scores ($r=-0.34$, $p=0.07$).

Figure 7-D. Plasma and cerebrospinal fluid nevirapine concentrations are highly correlated

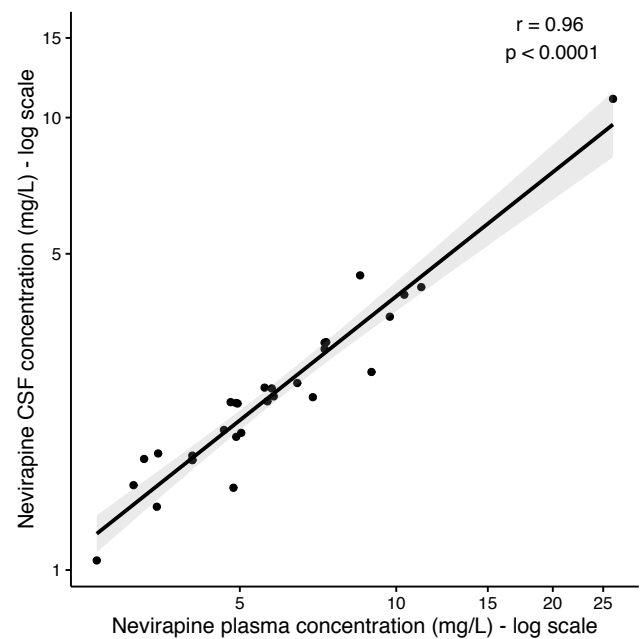
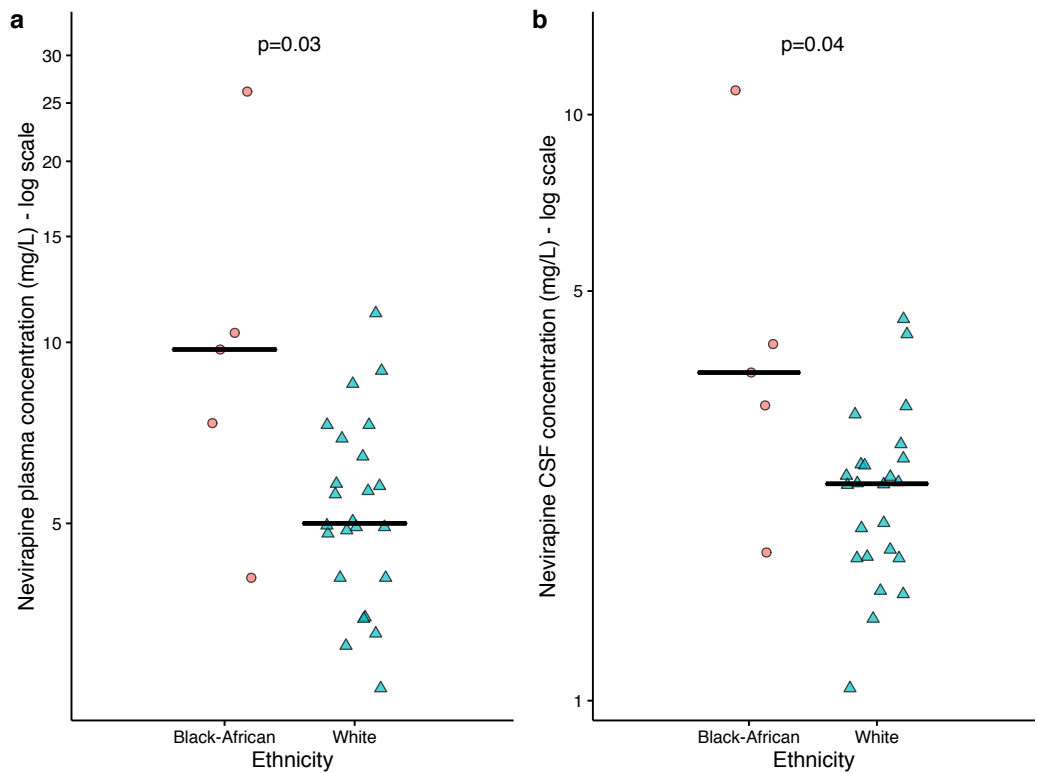


Figure 7-E. Plasma and cerebrospinal fluid concentrations of nevirapine by ethnicity.

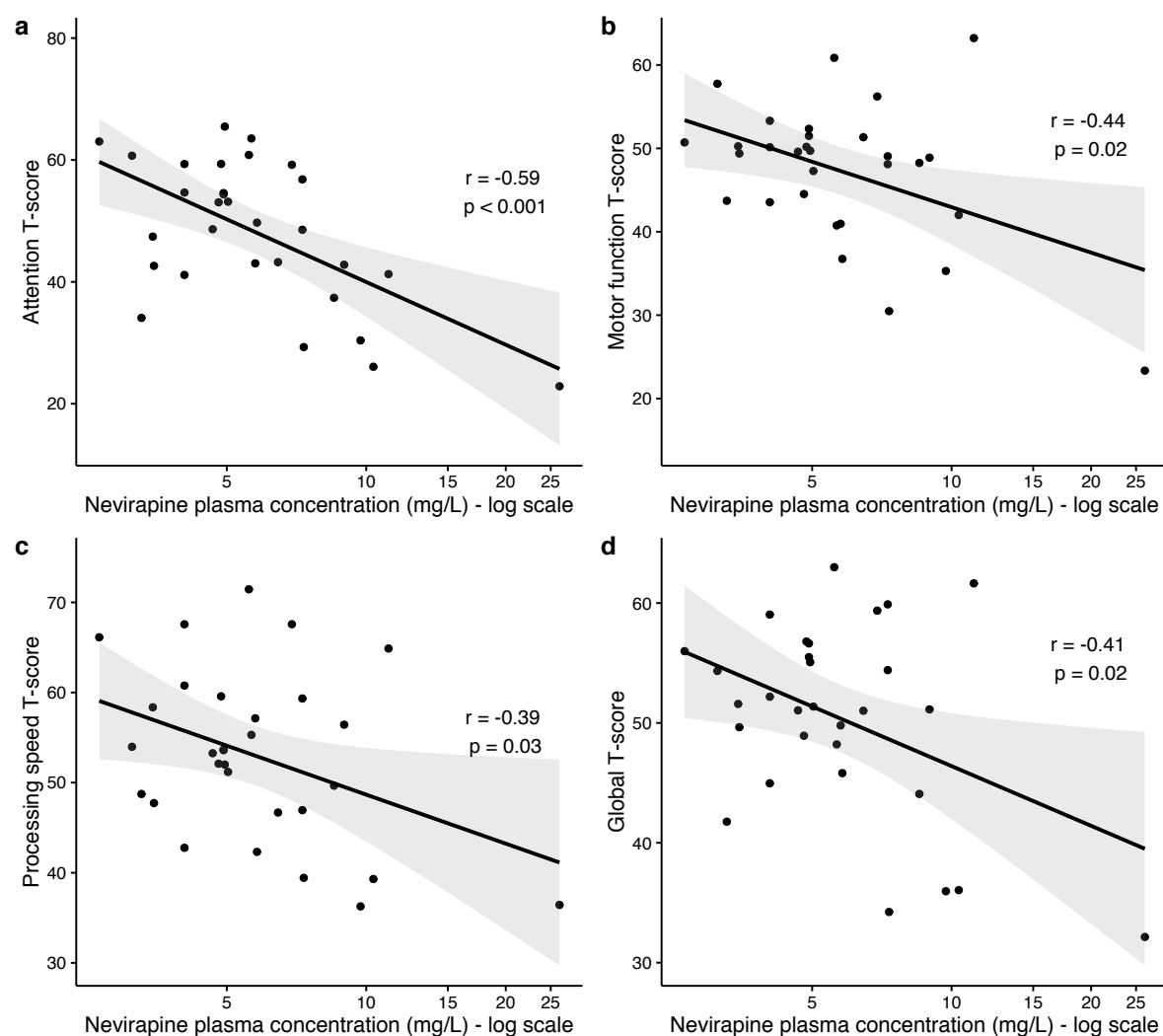


Black lines denote medians. P-values calculated with the Wilcoxon rank-sum test.

Table 7-E. Correlations between duration of nevirapine use, plasma and CSF nevirapine concentrations with cognitive domain T-scores.

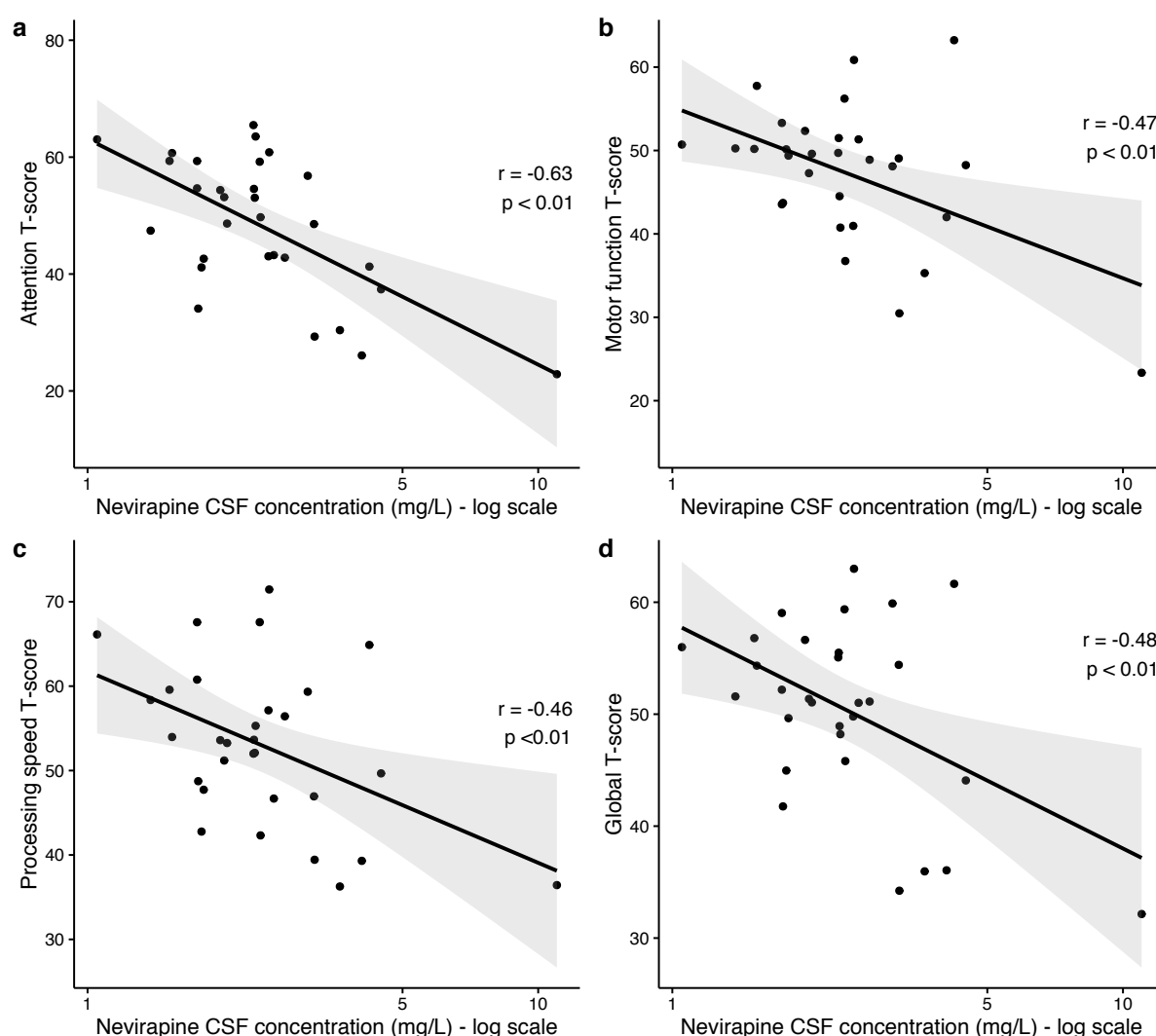
Cognitive domain	Duration		Plasma concentration		CSF concentration	
	Spearman's ρ	p-value	Pearson's r	p-value	Pearson's r	p-value
Attention	-0.24	0.06	-0.59	<0.001	-0.63	<0.001
Executive function	0.12	0.37	-0.28	0.13	-0.29	0.12
Language	-0.07	0.61	-0.10	0.60	-0.23	0.22
Memory	0.05	0.73	-0.21	0.25	-0.25	0.18
Processing speed	0.12	0.35	-0.39	0.03	-0.46	<0.01
Motor function	-0.07	0.58	-0.44	0.02	-0.47	<0.01
Global	0.00	1.00	-0.41	0.02	-0.48	<0.01

Figure 7-F. Plasma nevirapine concentrations negatively correlated with cognitive domain T-scores



Scatterplots of plasma nevirapine concentration against attention (panel a), motor function (panel b), processing speed (panel c) and global T-scores (panel d).

Figure 7-G. CSF nevirapine concentrations negatively correlated with cognitive domain T-scores



Scatterplots of CSF nevirapine concentration against attention (panel a), motor function (panel b), processing speed (panel c) and global T-scores (panel d).

Nevirapine exposure and structural neuroimaging measures

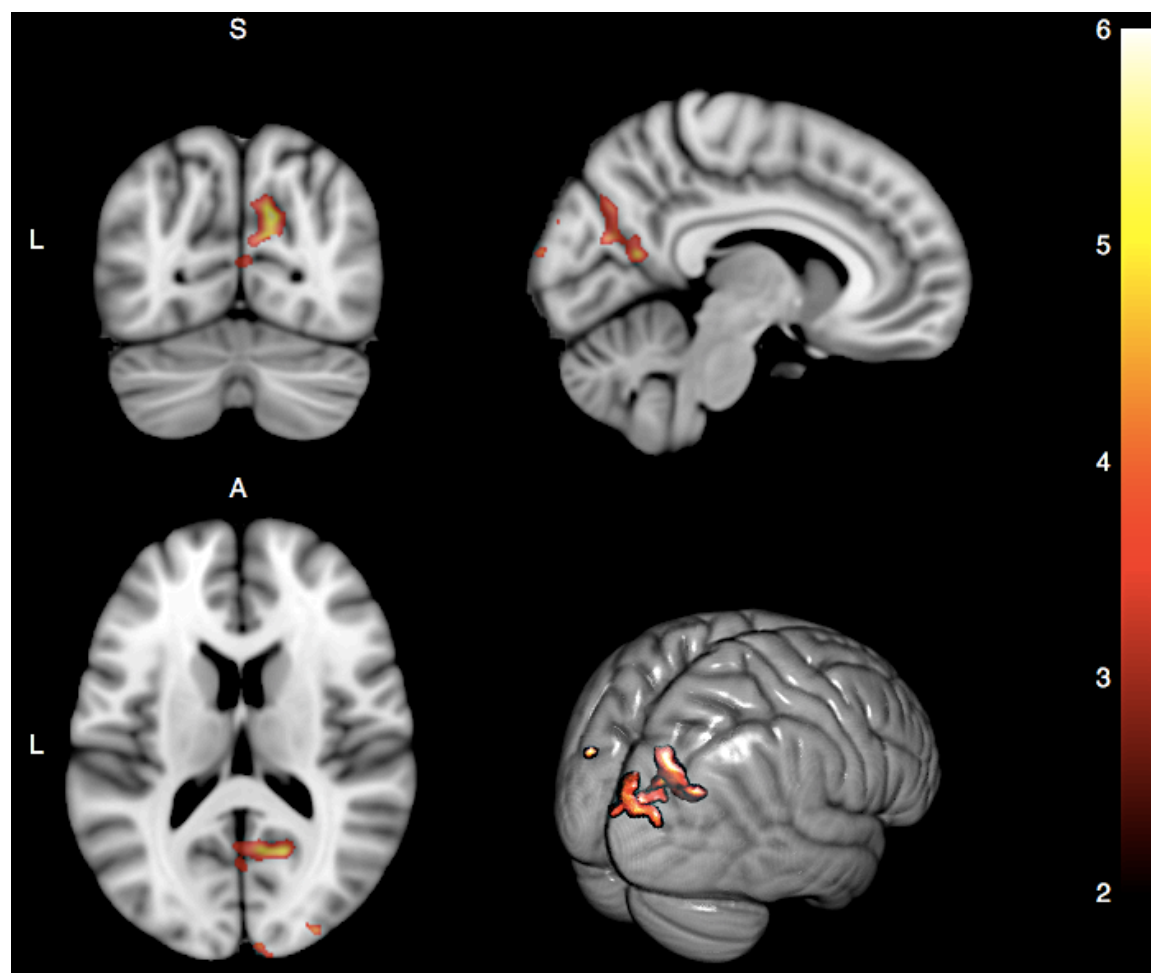
In a similar process to before, the independent relationship between nevirapine exposure and brain structure was determined. Plasma concentration of nevirapine was negatively associated with total grey matter volume but not total white matter volume or mean fractional anisotropy (overall model adjusted $R^2=0.68$, $F_{5,24}=13.1$, $p<0.0001$, $t_{24}=-2.92$, $p<0.01$; model adjusted $R^2=0.59$, $F_{5,24}=9.42$, $p<0.0001$, $t_{24}=0.49$, $p=0.63$; model adjusted $R^2=0.37$, $F_{5,24}=4.45$, $p<0.01$, $t_{24}=-0.02$, $p=0.99$ respectively).

There was a trend for a negative relationship between CSF nevirapine concentration and total grey matter volume but there were no associations with total white matter volume or mean

fractional anisotropy (overall model adjusted $R^2=0.62$, $F_{5,24}=10.4$, $p<0.0001$, $t_{24}=-1.90$, $p=0.07$; model adjusted $R^2=0.61$, $F_{5,24}=9.92$, $p<0.0001$, $t_{24}=1.05$, $p=0.30$; model adjusted $R^2=0.37$, $F_{5,24}=4.45$, $p<0.01$, $t_{24}=-0.10$, $p=0.92$ respectively).

To delineate localised regions of grey matter atrophy, voxelwise analysis was performed using voxel-based morphometry. Plasma nevirapine concentration was negatively correlated with grey matter volume in the principally in the precuneus, lingual gyrus, intracalcarine and posterior cingulate cortices (Figure 7-H). Similar relationships of CSF concentrations of nevirapine seen, however, these were above the threshold of significance (TFCE corrected $p>0.05$). There were no voxelwise associations of plasma or CSF nevirapine concentrations with white matter volume or fractional anisotropy.

Figure 7-H. Regions of grey matter that correlate negatively with plasma nevirapine concentration



Grey matter voxel-based morphometry of patients receiving nevirapine. Red-yellow areas depict regions of grey matter volume negatively correlated with plasma nevirapine concentration coloured by the t-statistic (scale to the right) - corrected for multiple comparisons (TFCE) and adjusted for age, intracranial volume and scanner. Overlaid on MNI 152 T1 brain image (greyscale). Abbreviations: A: anterior, L: left, S: superior.

Nevirapine exposure and CSF biomarkers

Aside from positive correlations with CSF A β 42 concentrations, plasma and CSF nevirapine concentrations were not associated with CSF biomarkers of neuronal injury or neuroinflammation (Table 7-F).

Table 7-F. Correlations between plasma and CSF nevirapine concentrations and CSF biomarkers of neuronal injury and neuroinflammation

CSF Biomarker	Plasma concentration		CSF concentration	
	Spearman's rho	p-value	Spearman's rho	p-value
Total tau	-0.35	0.06	-0.27	0.15
Phosphorylated tau	-0.29	0.12	-0.19	0.31
A β 42	0.42	0.02	0.41	0.03
Neurofilament light (NFL)	0.20	0.29	0.18	0.34
Soluble CD14	-0.23	0.21	-0.18	0.34
Soluble CD163	-0.25	0.20	-0.17	0.37
Neopterin	0.21	0.28	0.30	0.11
Kynurenine:tryptophan ratio	-0.19	0.53	-0.17	0.38

7.4 Discussion

In support of the third hypothesis of this thesis, I have shown using multimodal analysis efavirenz and nevirapine exposure are associated central nervous system toxicity. Greater exposure to both drugs, was associated with poorer cognitive function. Furthermore, in the case of nevirapine, greater exposure was associated with grey matter atrophy – providing a potential pathophysiological mechanism for the observed associations with cognitive function. Confirming the first overarching hypothesis of this thesis, well-treated patients had evidence of poorer cognitive function despite successful treatment with suppressed plasma and CSF HIV RNA. In support of the second overarching hypothesis, plasma and CSF drug concentrations are potentially useful biomarkers, with greater concentrations associated with poorer cognitive function. These findings build on earlier work that reported poorer cognitive function in patients receiving these drugs^{14,104} and confirm the observed toxicity in rodents may also be present in humans.^{113,114} These findings are important as if they are replicated in other cohorts and confirmed prospectively treatment guidelines advocating their use may need to be revised.

It should be noted that the differences in cognitive function between patients and controls presented here were mainly for illustrative purposes and it is not possible to determine the exact cause of these differences. Whilst some of the differences are likely to be related to the direct or indirect toxicity of HIV itself, the associations of greater plasma concentrations with poorer cognitive function does provide evidence for an independent NNRTI central nervous system toxicity effect. Long term efavirenz use has been associated with neuropsychiatric side effects since its introduction. However, the relationship with more objective measures of cognitive function have been found in some^{104,110} but not all studies.^{14,282} An important source of bias in these studies may be ‘channelling’, whereby in patients in whom there may be a concern of a higher risk of neuropsychiatric side effects will be prescribed nevirapine or another alternative in the belief that these drugs are safer. While this may explain the one previous report of poorer cognitive function in patients receiving nevirapine¹⁴ it would not necessarily explain the associations with efavirenz. A potential explanation for these findings is that patients receiving comparator drugs, such as protease inhibitors, in these analyses may be more treatment experienced and have potentially higher rates of drug resistance, comorbidities and greater exposure to unsuppressed HIV replication, which may confound comparisons of cognitive function. However, these potential sources of bias would not explain the association with drug concentrations described here. Furthermore, in a randomised control trial setting patients treated with 48 weeks of efavirenz had less improvement in cognitive function compared with one of the comparator arms.⁹⁶ Similar results have been presented, but not yet unpublished, from a larger randomised control trial of efavirenz,²⁸³ These data are suggestive of toxicity, at least in the short term, and may evolve into the changes observed here associated with chronic use.

The negative association of plasma efavirenz concentrations with cognitive function is perhaps not a surprise given clinical experience of using the drug and pre-clinical data suggesting central nervous system toxicity. Robertson *et al*⁸⁷ described toxicity for many of the antiretrovirals currently in use using an *in vitro* model. The greatest toxicities were observed with efavirenz where extensive loss of dendrites was observed at the highest concentrations. Further work by Tovar-y-Romo *et al*⁸⁸ has suggested that the 8-hydroxy metabolite of efavirenz may exhibit neuronal toxicity an order of magnitude greater than the parent drug. The mechanism of this toxicity may well be explained by acute mitochondrial dysfunction, a phenomenon that neuronal cells may be particularly vulnerable to.⁹² These effects may explain the negative

correlation of cognitive T-scores with increasing plasma efavirenz concentration. Although there was a trend for lower global and motor function T-scores in those that had been treated the longest, there were no clear associations with the other domain scores. This suggests that if there is a genuine neurotoxic effect, the concentration of efavirenz has to exceed a certain threshold before its effects are appreciable. It should be noted that the efavirenz concentrations recorded here are similar to previous reports and not abnormally high.³⁰ The lack of associations with brain tissue volumes, white matter microstructure and CSF biomarkers of neuronal injury suggests that the pathological correlate of efavirenz toxicity is not frank neuronal loss. This is supported by *in vitro* work, which suggest that neuronal loss only occurs at the highest concentrations and this is unlikely given observed *in vivo* pharmacokinetics.⁸⁷ The volumetric measures used here are likely to not be sensitive to the dendritic beading that may occur as opposed to overt loss of neurons. This could be further investigated using new, state of the art diffusion imaging techniques that allow neurite density to be quantified in grey and white matter.²⁸⁴ Another explanation is that efavirenz affects cognitive function without permanently affecting brain structure, in effect acting more like an intoxicant. This is supported by the cessation of symptoms when switching efavirenz for another drug in a double-blind randomised controlled trial¹¹¹ as well as its primary effect on the domain of attention, which may be particularly vulnerable to intoxicant effects.²⁸⁵ Switching studies assessing the changes in cognitive function longitudinally are needed to assess this possibility. However, to date these have not been performed. Another possible method to assess this would be to assess brain network function using functional MRI. Again, these studies have not yet been performed.

The suggestion of neurotoxicity associated with long-term nevirapine use is interesting. Concerns were raised about possible neuropsychiatric side effects in 2002 in a small (n=4) case series.²⁸⁶ However, concern amongst clinicians and researchers has largely focused its fellow NNRTI efavirenz. However, long-term nevirapine use has been associated with poorer cognitive function previously.¹⁴ Although this finding may be influenced by the sources of bias previously discussed. The findings presented here suggest that this effect is likely to represent some degree of neurotoxicity. Perhaps this should not come as a surprise given that in the seminal work by Robertson *et al*⁸⁷ nevirapine had a greater estimated toxicity risk given its pharmacokinetics and readiness to cross the blood brain barrier. CSF nevirapine concentration is ~15% of plasma concentration²⁸⁷ compared with ~0.7% for efavirenz³⁰. Furthermore, Gibbs *et al*²⁸⁸ demonstrated (in guinea pigs) that nevirapine readily crosses the

blood brain barrier and accumulates in the central nervous system. This may explain why stronger negative correlations with cognitive function were observed with plasma and CSF nevirapine concentrations compared with efavirenz. Again, the lack of clear associations of duration of nevirapine therapy with poorer cognitive function suggests a threshold effect of toxicity whereby nevirapine concentrations must exceed a certain threshold for a given duration to exert an appreciable toxic effect. Again it should be noted that the plasma nevirapine concentrations were comparable to previous reports in stable patients and were not abnormally high.²⁸⁷ The finding of a negative association between plasma nevirapine concentrations and loss of grey matter volume is novel and suggests a possible pathophysiological correlate of the observed negative association between nevirapine exposure and poorer cognitive function. Streck *et al*^{113,114} demonstrated two mechanisms resulting in perturbations of energy homeostasis in the cortex, hippocampus and striatum from chronic nevirapine in a mouse model. Together with the findings of Robertson *et al* and the findings presented here it is likely that nevirapine exerts a toxic effect, above a certain threshold, resulting in disrupted brain energy metabolism leading to neuronal loss and subsequent cognitive dysfunction. The location of grey matter atrophy is consistent with the observed effects on cognitive function. Whilst the battery employed may not have tested the function of all the brain regions showing atrophy, the precuneus and posterior cingulate cortex are a core hub of the default mode network and crucial for normal cognition.²⁸⁹ Injury to these regions in traumatic brain injury results in attentional deficits.²⁹⁰ The largest effects of nevirapine were observed in the domain of attention with strong negative correlations observed for both plasma and CSF concentrations. Furthermore, attention was the cognitive domain where a trend for poorer cognitive function in those treated longest was observed. It should be noted that the discrepancy between blood and CSF results in terms of grey matter volume may reflect a lack of statistical power. There was a trend for a negative association of CSF nevirapine concentration with total grey matter volume in a multiple regression model and, with a greater p-value threshold, localised atrophy in similar regions to those observed with plasma concentrations. This may also reflect the complicated nature of the relationship between plasma, CSF and central nervous system drug exposure. Further study in a larger sample is therefore justified.

The lack of association between plasma and CSF nevirapine concentrations and CSF biomarkers of neuronal injury and neuroinflammation is interesting given the strong negative associations with cognitive function. Again, this may suggest that nevirapine exerts its central

nervous system toxicity effect in part as an intoxicant, as hypothesised with efavirenz, especially given its primary effect on the domain of attention. Another possibility is that these biomarkers are not sensitive to the type of brain injury that nevirapine exposure may cause. As CSF neurofilament light (NFL) is a sensitive marker of axonal injury,¹⁸² elevations may not be expected if toxicity is exclusive to grey matter. Interestingly, both plasma and CSF nevirapine concentrations were positively correlated with CSF A β 42. Decreased concentrations of this protein is associated with Alzheimer's disease and have also been reported, albeit inconsistently, in the most severe HIV-associated cognitive impairment.^{185,191,192} Interestingly, reductions have also been observed in those with other central nervous system infections^{191,192} suggesting that reductions in this biomarker may represent immune activation/inflammation as well as amyloid pathology. One explanation for the relationship described here is that increased central nervous system nevirapine exposure results in greater suppression of HIV-replication with a consequent reduction in microglial activation and neuroinflammation. This is consistent with the direction of effect with the inflammatory markers sCD14, sCD163 and the kynurenine:tryptophan ratio. This potentially highlights the dichotomy between greater central nervous system antiretroviral exposure having beneficial effects in terms of suppressing HIV replication but conversely increasing the risk of neurotoxicity.

The observation of increased plasma and CSF nevirapine concentrations in those of Black-African ethnicity is not new finding. There was a trend for increased plasma concentrations of efavirenz in those of Black-African ethnicity. However, there were only 3 patients of Black-African ethnicity, which limits the statistical power to detect differences, which are likely to be genuine.³⁰ This disparity is likely to be related to ethnic differences in the prevalence of genetic polymorphisms in cytochrome P450 enzymes, particularly CYP2B6.²⁹¹⁻²⁹³ If NNRTIs are associated with central nervous system toxicity, these genetic differences may go some way to explain some of the previously observed ethnic differences in cognitive function in treated HIV-disease.^{14,59,104} Although it should be noted that these differences may largely be explained by ethnic differences in normative scores.^{38,39} Another potential explanation for ethnic differences is that in less well developed healthcare systems HIV-infection may be diagnosed at a later stage, which may also be associated with brain injury. This could confound ethnic differences in measures of cognitive function if groups were not balanced in terms of disease stage. However, these effects would not explain the observed relationships between drug concentration and cognitive function. Nor would ethnic differences in HIV clade. The

numbers of patients of Black-African ethnicity were small in this study but there were no significant differences in nadir CD4 count or prevalence of prior AIDS in those of Black-African compared with white ethnicity.

As a cross-sectional study, the findings reported here need to be interpreted with some caution. Efavirenz and nevirapine are prescribed less frequently now in high-resource settings such as the UK, largely because they are no longer recommended as the preferred third agent of an antiretroviral regimen.^{67,68} However, the largest burden of HIV is in sub-Saharan Africa where the WHO treatment guidelines,²⁹⁴ which are the usual starting point for any local antiretroviral programme, recommend efavirenz as the preferred third agent with nevirapine as an alternative. If chronic efavirenz and nevirapine use are associated with cognitive impairment the potential burden of morbidity and accompanying economic costs are great given that millions are prescribed these drugs worldwide. Clearly, the results presented here are by no means definitive given the small sample size and cross-sectional nature of the data. However, the consistent negative correlations between efavirenz and nevirapine exposure and cognitive function are concerning and warrant further characterisation. Future work should consist of examining the relationships between drug exposure and other neuroimaging measures to better understand the pathophysiological mechanisms. Resting state functional MRI may be useful to determine if drug exposure is related to brain network connectivity. Metabolic effects could be examined by determining the relationship between drug exposure and cerebral metabolite concentrations quantified using spectroscopy. Longitudinal study of cognitive function and neuroimaging measures, in particular rates of grey and white matter atrophy, and their relationship with drug exposure would provide more convincing evidence of central nervous system toxicity. Lastly, sufficiently powered, blinded, randomised controlled trials switching patients off efavirenz or nevirapine to an alternative third agent should be performed, using cognitive and neuroimaging (to potentially detect sub-clinical changes) measures as primary endpoints. If further study confirms the central nervous system toxicity of efavirenz and nevirapine, in tandem with evidence demonstrating that switching off them proves favourable, treatment guidelines should be reviewed.

Limitations

The cross-sectional nature of study makes establishing causation impossible and the results are only suggestive of NNRTI associated central nervous system toxicity. The differences in

cognitive function between the HIV-positive patients and controls, presented mainly for illustrative purposes to highlight cognitive domains that differed between patients and controls, may be confounded by other unmeasured factors and may not be solely driven by HIV or its treatment. However, great care was taken to recruit a demographically appropriate control group so these differences were minimised. As this was not a specific pharmacokinetic study, blood and CSF sampling was timed in relation to study visits where MRI scanning or cognitive function testing was performed rather than at a fixed time post dose. As such, variability exists between the timing of sampling post dose between subjects. However, this is unlikely to have biased these results given that this should have no bearing on tests of cognitive function. Similarly, the effects were consistent across both drugs which strengthens a hypothesis of a possible class effect of central nervous system toxicity. Furthermore, given the long plasma half-lives (>24 hrs) of both drugs, and stability of concentrations over time,^{287,295} differences between participants of a few hours in the timing of sampling post dose are unlikely to be significant and further modelling of extrapolated trough concentrations are unnecessary. Furthermore, for the purposes of this thesis only the effects of the NNRTIs efavirenz and nevirapine were studied and CSF concentrations were only available for nevirapine. The scope of these analyses was originally envisaged to be broader with the study of other drug classes. However, there were significant delays of over 18 months in measurement of drug concentrations after shipping the samples to the lab, which prohibited further analysis within the timescale of my PhD. Moreover, quantification of CSF concentrations of efavirenz and its major metabolites was not possible and these samples are now in the process of being analysed at the University of Liverpool. Future work to assess the effects of other antiretrovirals is warranted given the findings presented here.

Conclusion

These data indicate that greater efavirenz and nevirapine exposure are associated with central nervous system toxicity. It is likely that the toxic effects are as a result of intoxication, but in the case of nevirapine it may also be associated with grey matter atrophy. Further work to delineate the pathophysiology of the observed effects is justified. Repetition of these novel findings in larger cohorts and longitudinal study are necessary before treatment guidelines should be reviewed.

Chapter 8

Multivariate prediction of HIV-associated cognitive impairment

Table of contents

CHAPTER 8 MULTIVARIATE PREDICTION OF HIV-ASSOCIATED COGNITIVE IMPAIRMENT.....	208
8.1 INTRODUCTION	210
8.2 METHODS.....	212
Participants	212
Cognitive function	212
Neuroimaging.....	213
Multivariate analysis	214
8.3 RESULTS	217
8.3.1 COBRA.....	217
Classification	217
Regression	221
8.3.2 CHARTER	221
Cognitive function	222
Multivariate prediction of cognitive function	224
Multivariate brain age prediction	225
Predicted brain age difference and cognitive function	227
8.4 DISCUSSION	229
Conclusion	232

Figures

FIGURE 8-A. HYPOTHETICAL MODEL OF BRAIN AGEING.....	211
FIGURE 8-B. GRAPHICAL OVERVIEW OF PREDICTING BRAIN AGE.	216
FIGURE 8-C. MULTIVARIATE CLASSIFICATION OF COGNITIVE IMPAIRMENT USING NEUROIMAGING DATA DEFINED USING MULTIVARIATE NORMATIVE COMPARISON.	219
FIGURE 8-D. SMOOTHED DENSITY PLOT SHOWING THE DIFFERENCES BETWEEN FRACTIONAL ANISOTROPY SKELETONS BETWEEN SCANNERS.	220
FIGURE 8-E. BOXPLOTS OF DEMOGRAPHICALLY ADJUSTED COGNITIVE DOMAIN T-SCORES FOR THOSE WITH VS. WITHOUT IMPAIRMENT.....	223
FIGURE 8-F. SPAGHETTI PLOT SHOWING STABILITY OF GLOBAL COGNITIVE FUNCTION OVER TIME.	224
FIGURE 8-G. JITTERPLOT OF PREDICTED BRAIN AGE DIFFERENCE BY COMORBIDITY STATUS.	226
FIGURE 8-H. BOXPLOT OF PREDICTED BRAIN AGE DIFFERENCE BY SCANNER	227
FIGURE 8-I. JITTERPLOT OF PREDICTED BRAIN AGE DIFFERENCE BY THOSE WITH VS. WITHOUT CLINICALLY SIGNIFICANT DECLINE IN COGNITIVE FUNCTION.	228
FIGURE 8-J. RECEIVER OPERATOR CHARACTERISTICS (ROC) CURVE OF PREDICTED BRAIN AGE DIFFERENCE (BPAD) SHOWING THE OPTIMUM CUT-OFF FOR PREDICTING CLINICALLY SIGNIFICANT LONGITUDINAL COGNITIVE DECLINE.	229

Tables

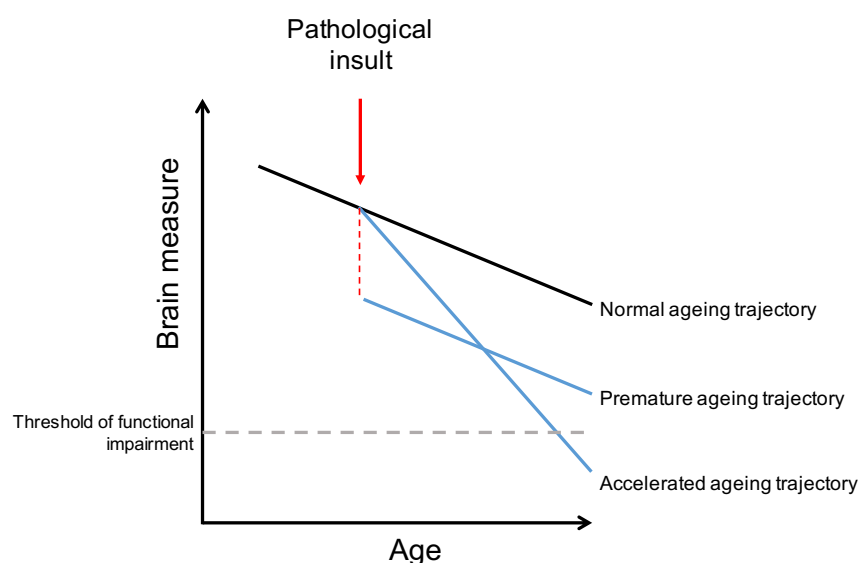
TABLE 8-A. PREDICTION PERFORMANCE OF COGNITIVE IMPAIRMENT DEFINED BY THE FRASCATI CRITERIA BY MODEL.	217
TABLE 8-B. PREDICTION PERFORMANCE OF COGNITIVE IMPAIRMENT DEFINED BY THE GLOBAL DEFICIT SCORE BY MODEL.	218
TABLE 8-C. PREDICTION PERFORMANCE OF COGNITIVE IMPAIRMENT DEFINED BY MULTIVARIATE NORMATIVE COMPARISON BY MODEL.	219
TABLE 8-D. MULTIVARIATE PREDICTION PERFORMANCE OF THE GLOBAL T-SCORE USING STRUCTURAL IMAGING DATA BY MODEL.	221
TABLE 8-E. BASELINE DEMOGRAPHICS OF THE CHARTER PARTICIPANTS.....	222
TABLE 8-F. PREDICTION PERFORMANCE USING STRUCTURAL IMAGING DATA FOR COGNITIVE IMPAIRMENT DEFINED BY THE GLOBAL DEFICIT SCORE BY MODEL.....	225
TABLE 8-G. MULTIVARIATE PREDICTION PERFORMANCE OF THE GLOBAL T-SCORE USING STRUCTURAL IMAGING DATA BY MODEL.	225
TABLE 8-H. CORRELATIONS AND MULTIPLE LINEAR REGRESSION ESTIMATES SHOWING THE RELATIONSHIPS.....	228

8.1 Introduction

Care for HIV-positive individuals has changed markedly over the past three decades. Successfully treated HIV-infection is now the norm in Northern European settings, with over 90% of patients receiving antiretroviral therapy in the UK with over 90% of them having sustained suppression of plasma HIV RNA.²⁴ This trend for universal treatment is clear following the publication of the INSIGHT START study²⁵ and with the World Health Organisation's ambitious 90:90:90 goal whereby 90% of HIV-positive individuals are diagnosed, 90% receive antiretroviral therapy and 90% of them achieve suppression of plasma viraemia. As such, treatment of long term comorbidities is likely to make up a larger burden of care than opportunistic infections. Cognitive impairment has been reported to remain prevalent in the era of modern antiretrovirals.^{8,10} However, data from exclusively well-treated patients is lacking, which provided the motivation for this thesis. In chapter 3 and 5 I have described the prevalence in well-treated patients in some detail. The prevalence of impairment is sensitive to the method used but is approximately 20% when compared appropriately to a demographically comparable HIV-negative control population using statistical techniques that take account of multiple comparisons. It is unclear whether virally suppressed patients experience further significant brain injury or if any injury present is static and a legacy of previous untreated infection. The evidence from chapters five, six and seven would suggest the latter. However, caution is needed when interpreting these findings given these data are cross-sectional. Longitudinal data are needed to answer these important questions. Longitudinal changes in cognitive function in well-treated populations remain sparse. Data from the Multicenter AIDS Cohort Study (MACS) and Central nervous system HIV Anti-Retroviral Therapy Effects Research (CHARTER) cohorts suggests the majority (>60%) of HIV-positive individuals remain stable cognitively over time.^{11,59} Approximately 10% experienced a decline in cognitive function, which was associated with ineffective antiretroviral therapy or treatment interruption. It should be noted that antiretroviral therapy was not universal in these cohorts and effective suppression of plasma HIV RNA was only evident in 70% and 41% respectively, which makes drawing conclusions for successfully treated cohorts difficult. Here, I describe the longitudinal cognitive function of exclusively virally suppressed patients from the CHARTER cohort.

The ability to accurately predict longitudinal changes in cognitive function would potentially allow targeted interventions to prevent cognitive decline. Multivariate machine learning techniques have yielded accurate classification of Alzheimer's disease¹⁷⁴ and white matter injury following traumatic brain injury¹⁷⁵ as well as other neurological and psychiatric diseases.¹⁷² An alternative approach to building a model to predict a specific disease is to model atrophy associated with ageing. Deviation of brain age from chronological age can either represent accentuated (premature) or accelerated ageing depending on the trajectory of deviation (illustrated graphically in Figure 8-A). This may result from neurodegeneration from multiple different pathologies that share a final common pathological pathway of atrophy. The accurate prediction of age in healthy individuals using just neuroimaging data is possible, and accelerated ageing has been reported following traumatic brain injury.¹⁷⁶ Neuroimaging studies of HIV-infected individuals have suggested both accentuated and accelerated ageing,²⁹⁶ of which the later may be associated with untreated disease. These multivariate approaches have yet to be applied in HIV-disease but conceptually HIV-associated cognitive impairment may be accurately classified on neuroimaging data alone. Whilst diagnosis of impairment or prediction of cognitive function solely from an MRI scan is potentially useful clinically, the ability to be able to predict longitudinal changes has the most utility. However, being able to predict current cognition is the first step before being able to predict future changes. I tested the second hypothesis of this thesis, namely that biomarkers can be used to predict impairment and longitudinal changes in cognition.

Figure 8-A. Hypothetical model of brain ageing.



Following a pathological insult ageing may occur in an accelerated fashion (i.e. with a steeper gradient than normal) or in a premature fashion (i.e. a similar gradient to normal but with a lower starting value) or in combination.

8.2 Methods

Participants

For the predicative analyses, HIV-positive (n=134) and HIV-negative participants (n=79) were enrolled into the COBRA study as described in section 2.1.2. Eligible participants were aged >45 with no significant neurological conditions, substance or alcohol abuse or moderate/severe depression (see methods, section 2.2.2 for exclusion criteria and other details). All HIV-positive participants were receiving combination antiretroviral therapy and had an undetectable plasma HIV RNA (<50 copies/mL) recorded for at least 12 months prior to enrolment. Due to the low number of COBRA participants with cognitive impairment both HIV-positive patients and controls from COBRA with impairment were grouped together to maximise the sample size.

For the predictive and longitudinal analyses, HIV-positive participants from the CHARTER cohort with plasma HIV RNA <50 copies/ml the time of baseline neuroimaging data (n=139). The CHARTER cohort is described in more detail in the methods, section 2.1.3 and in Heaton *et al.*⁸ Longitudinal data was available for a subgroup of patients (n=111).

Cognitive function

Cognitive function for the COBRA participants was tested with of a comprehensive battery of neuropsychological tests assessing attention, executive function, language, memory, speed of information processing and motor function described in detail in the methods, section 2.2.2 and table 2.2.

Cognitive function for the CHARTER participants was measured using published methods across seven domains with cognitive impairment as described in section 2.1.3 and in Heaton *et al.*⁸ Cognitive impairment was defined using the global deficit score (GDS) as the CHARTER cohort had no HIV-negative control group permitting multivariate normative comparison. The GDS was used in preference to the Frascati criteria because the latter is likely to overcall impairment (see chapter 4 for simulation data to show this) especially as the number of domains increases.

Clinically significant cognitive decline defined as a global T-score >5 points lower at follow-up than baseline (using the first and last available global T-scores). This value was chosen as a longitudinal T-score change of 5 (equivalent to a z-score of 0.5) has been used as an endpoint in clinical trials of HIV-associated cognitive impairment).²⁹⁷ The global T-score was used in preference to the global deficit score as the T-score allows more granularity of measurement.

Neuroimaging

Acquisition

For the COBRA participants, high-resolution 3D T1-weighted structural images and diffusion weighted images along 64 non-collinear directions were acquired at 3T in London (n=88) and Amsterdam (n=66 and n=59) as previously described in the methods, section 2.3.1.

For the CHARTER participants, data was collected with General Electric 1.5T scanners at Johns Hopkins University (n=30); Mt. Sinai School of Medicine (n=25); University of California at San Diego (n=47); University of Texas Medical Branch (n=29) and the University of Washington (n=8). Further details are described in the methods, section 2.3.1 and in Jernigan *et al.*¹²⁸

Image processing

3D T1 images were pre-processed as described in the methods, section 2.4.2. Briefly, images were bias corrected, segmented into grey matter, white matter and cerebrospinal fluid (CSF) and volumes calculated. Segmented images were then registered to a custom template and normalised to standard Montreal Neurological Institute (MNI) space using the DARTEL algorithm²¹⁷ prior to further multivariate analysis.

Diffusion data for the COBRA participants were pre-processed using FSL v5.0.6 (FMRIB, University of Oxford) as described in section 2.4.2. Briefly, images were corrected for eddy currents and head motion by rigid-body registration to each subject's initial B0 image. Non-brain tissue was deleted⁴⁵ and the diffusion tensor model was fitted at every voxel, using weighted least squares. These were then registered to a custom template and standard space,

using DTI-TK v2.3.1. Fractional anisotropy maps for each participant were then ‘skeletonised’ using FSL and thresholded ≥ 0.2 to exclude areas with considerable inter-individual variability prior further multivariate statistics.

Multivariate analysis

Supervised classification

Ultimately to make decisions about treatment a dichotomised outcome is desirable. Therefore, to assess the ability of a multivariate model to predict cognitive impairment, supervised classification was performed with the Pattern Recognition for Neuroimaging Toolbox (PRoNTo)⁸⁹ software package v.2.0 (Machine Learning and Neuroimaging Lab, University College London. For the purposes of classification, models were computed with support vector machines (SVM) and Gaussian processes classification (GPC) for cognitive impairment defined using the Frascati criteria, global deficit score and multivariate normative comparison as previously described. To test the predictive ability of different imaging modalities, for each definition of cognitive impairment a separate model was computed with spatially normalised: grey matter volume; white matter volume and skeletonised fractional anisotropy. Furthermore, to take advantage of the complementary functions of grey and white matter, additional models with concatenated spatially normalised grey and white matter volumes (for CHARTER participants as diffusion data was not available) and grey matter volume and skeletonised fractional anisotropy for each subject (for COBRA participants) were also computed. Fractional anisotropy was preferred over white matter volume as a measure of white matter function given the results from chapter 5 and its likely improved sensitivity to detect HIV-associated white matter pathology.^{129,130,147-150}

Regression

To assess the ability of structural imaging data to predict cognitive function in a continuous nature, the global T-score was defined as the dependent variable with the multivariate imaging data as the independent variables. Separate models using Gaussian processes regression (GPR) and kernel ridge regression (KRR) were calculated using different imaging modalities in a similar nature to that used for classification above. These analyses were performed using PRoNTo v2.0.

Model assessment

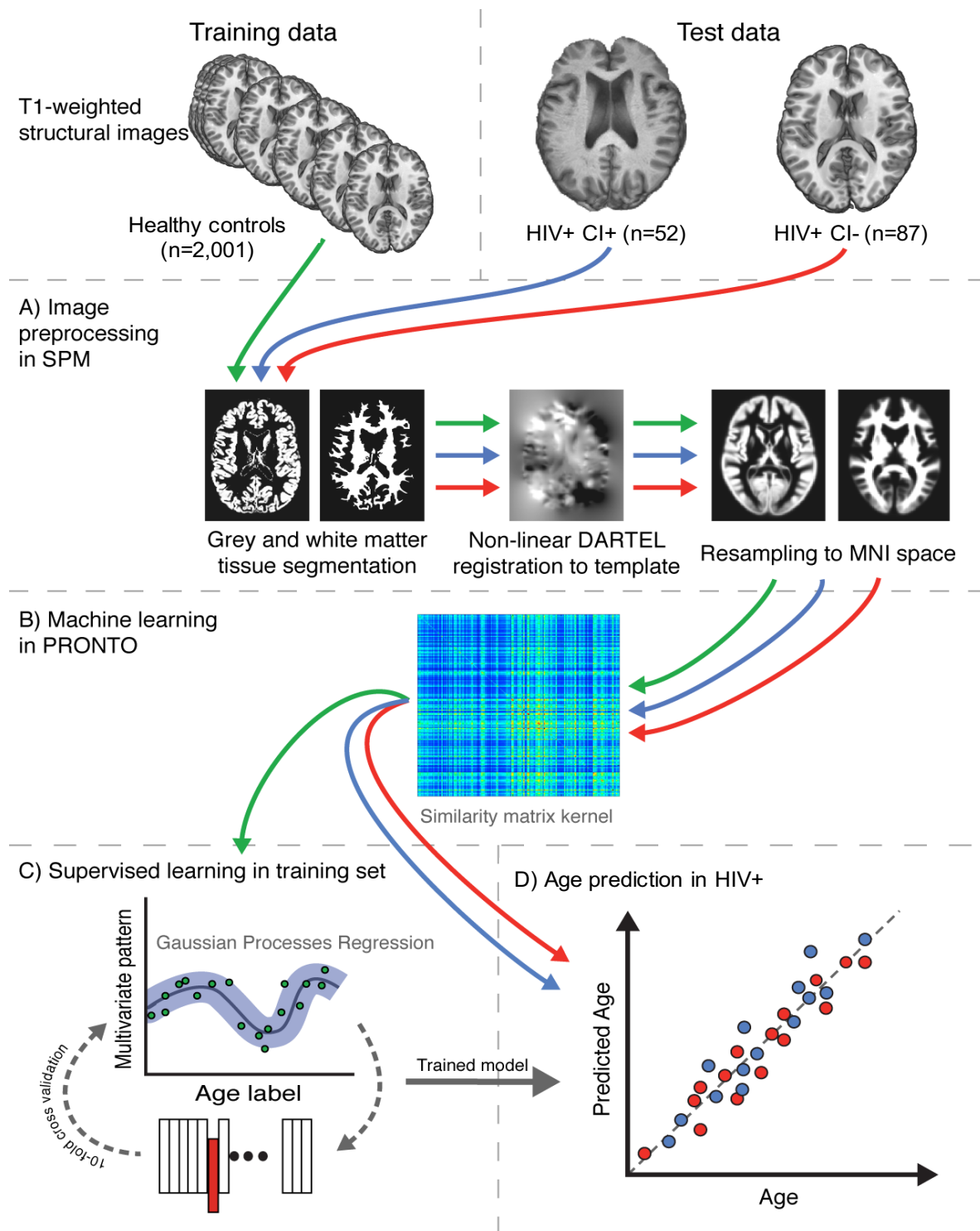
Predictive models were assessed using leave one out cross validation as described in section 2.7.6 of the methods. For classification, class (i.e. cognitively impaired or not) accuracy and predictive value were calculated for each model. Additionally, the area under the receiver operator curve (AUC) and the balanced accuracy (mean of accuracies across classes) were calculated. This is important if groups are unbalanced and gives an estimation of the utility of the model with new data. For regression, model accuracy was quantified with the correlation (Pearson's r), total variance explained (R^2), mean absolute error (MAE) and root mean squared error (RMSE). P-values were calculated with permutation testing with 1,000 repetitions.

Predicted brain age

As a different approach to multivariate analysis of structural imaging data, CHARTER participant's brain age was predicted using a validated algorithm, based on data from ~ 2,000 healthy subjects ranging from 18-90 years.¹⁷⁶ Further details of this algorithm and the training set used are detailed in section 2.7.6 and table 2.5 and the method is summarised graphically in Figure 8-B. Predicted brain age difference (PBAD), a measure of deviance from the normal ageing trajectory was calculated as follows: predicted brain age – chronological age so that positive scores represent brains that appear 'older' than expected. Group comparisons were performed with independent sample t-tests and in the case of comorbidity with ANOVA with post-hoc testing performed using Tukey's honest significant difference (see section 2.7.4 of the methods for further details). Relationships between cognitive domains and PBAD explored with multiple linear regression using a similar process as described in section 2.7.4 of the methods, accounting for age, scanner differences and comorbidity status.

All analyses were performed using R v3.2.1 and p-values <0.05 (two-tailed) were considered significant.

Figure 8-B. Graphical overview of predicting brain age.



Outline of the machine learning brain age prediction model. Training data: healthy individuals (n=2,001); testing data: CHARTER HIV-positive individuals (n=139) with and without CI (cognitive impairment defined using the global deficit score). A) T1 image data were pre-processed and segmented into grey and white matter. These segmented images were then normalised to a custom template using DARTEL for non-linear registration. B) Multivariate brain age prediction was performed with PRoNTto using data represented in kernel form. C) Supervised learning stage: data from the normative controls were used to define a model of healthy brain ageing with model accuracy assessed using ten-fold cross-validation. D) Test set prediction: the multivariate model was then used to predict brain age from the CHARTER HIV-positive group. Predicted brain age difference (BPAD) scores were defined by subtracting chronological age from predicted age. *** with thanks to James Cole for his help with this figure ***

8.3 Results

8.3.1 COBRA

Details of the COBRA participants and their cognitive function testing results are detailed in Chapter 5, section 5.3, Table 5-A and Figure 5-B. Cognitive impairment was more prevalent in the HIV-positive group. This was true regardless of the method used: global deficit score: 18.0% vs. 3.8% (odds ratio [95% confidence interval] 5.58 [1.86-24.1], $p<0.01$); Frascati criteria: 18.0% vs. 3.8% (5.58 [1.86-24.1], $p<0.01$); multivariate normative comparison: 19.5% vs. 2.5% (9.36 [2.68-59.2], $p<0.001$).

Classification

Due to the imbalance between the number of participants with and without cognitive impairment and the potential this has to bias classification, separate models with equal numbers of impaired and not impaired individuals were tested. To achieve this, a random sample of participants without impairment was chosen according to the number of with impairment by each classification criteria. There were no significant differences in age (median 56.0 vs. 55.5, $W=317$, $p=0.71$ for those with vs. without impairment) intracranial volume (1.46 vs 1.52 L, $W=243$, $p=0.08$) or scanner ($\chi^2_4=1.91$, $p=0.75$).

Using the Frascati criteria to define cognitive impairment yielded models with poor predictive performance (Table 8-A). None of the models predicted cognitive function better than chance although the combined grey matter volume and fractional anisotropy Gaussian processes model approached significance (accuracy 61.5%, $p=0.07$, area under the curve [AUC] 0.63).

Using the global deficit score to classify cognitive impairment yielded slightly improved classification accuracy (Table 8-B). Several models performed better than chance with a support vector machine using skeletonised fractional anisotropy data performing best (accuracy 75.0%, $p=0.001$, AUC 0.68).

Table 8-A. Prediction performance of cognitive impairment defined by the Frascati criteria by model.

Model	Not impaired (n=26)			Impaired (n=26)			Overall		
	Accuracy (%)	p-value	Predictive value (%)	Accuracy (%)	p-value	Predictive value (%)	Balanced accuracy (%)	p-value	AUC
SVM									
GM	57.7	0.23	57.7	57.7	0.22	27.7	57.7	0.18	0.61
WM	50.0	0.52	44.8	38.46	0.89	43.4	44.2	0.72	0.42
FA	61.5	0.13	64.0	65.4	0.07	63.0	36.5	0.05	0.71
GM+FA	57.7	0.22	57.7	57.7	0.22	57.7	57.7	0.17	0.60
GPC									
GM	65.4	0.06	60.7	57.7	0.21	62.5	61.5	0.08	0.63
WM	46.2	0.63	42.9	38.5	0.88	41.7	42.3	0.75	0.36
FA	46.2	0.63	54.6	61.5	0.09	53.3	53.9	0.18	0.67
GM+FA	69.2	0.02	60.0	53.9	0.30	63.6	61.5	0.07	0.63

P-values calculated with permutation testing (1,000 repetitions). Abbreviations: FA: fractional anisotropy skeleton; GM: grey matter volume; GPC: Gaussian process classification; SVM: support vector machine; WM: white matter volume.

Table 8-B. Prediction performance of cognitive impairment defined by the global deficit score by model.

Model	Not impaired (n=26)			Impaired (n=26)			Overall		
	Accuracy (%)	p-value	Predictive value (%)	Accuracy (%)	p-value	Predictive value (%)	Balanced accuracy (%)	p-value	AUC
SVM									
GM	65.4	0.07	60.7	57.7	0.22	62.5	61.5	0.08	0.60
WM	65.4	0.06	60.7	57.7	0.22	62.5	61.5	0.07	0.62
FA	80.8	0.001	72.4	69.2	0.03	78.3	75.0	0.001	0.68
GM+FA	65.4	0.07	60.7	57.7	0.19	62.5	61.5	0.08	0.60
GPC									
GM	69.2	0.02	62.1	57.7	0.20	65.2	63.5	0.05	0.61
WM	50.0	0.52	48.2	46.2	0.65	48.0	48.1	0.52	0.55
FA	73.1	0.01	65.5	61.5	0.11	69.6	67.3	0.01	0.66
GM+FA	69.2	0.02	60.0	53.9	0.33	63.6	61.5	0.06	0.62

P-values calculated with permutation testing (1,000 repetitions). Abbreviations: FA: fractional anisotropy skeleton; GM: grey matter volume; GPC: Gaussian process classification; SVM: support vector machine; WM: white matter volume.

In general, the performance of the multivariate models to distinguish between those with and without cognitive impairment defined using multivariate normative comparison was better than the other classification criteria (Table 8-C). The model with the best performance was achieved using Gaussian process classification on the combined grey matter volume and

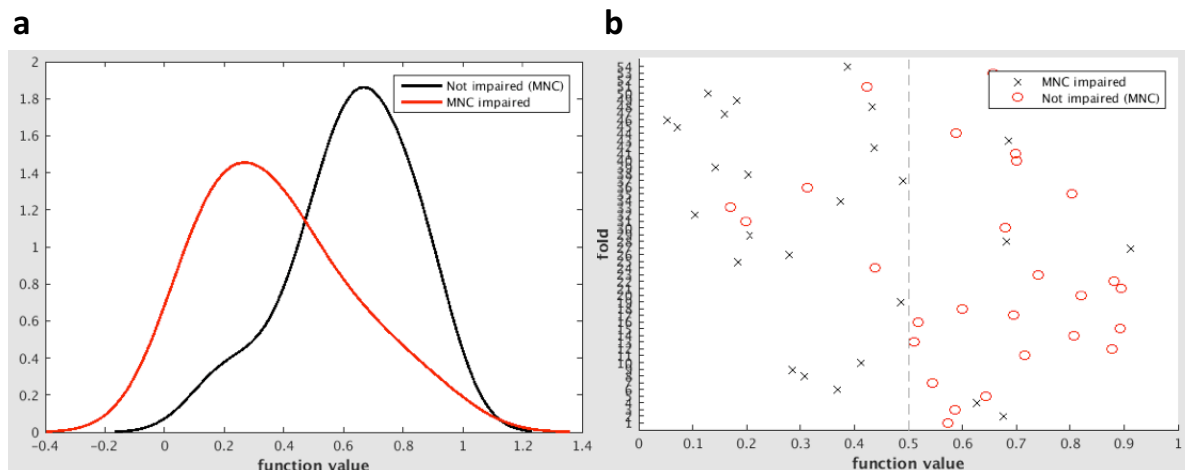
fractional anisotropy skeleton data, which had a balanced accuracy of 79.6% ($p=0.001$) and AUC of 0.80. Classification is illustrated graphically in Figure 8-C.

Table 8-C. Prediction performance of cognitive impairment defined by multivariate normative comparison by model.

Model	Not impaired (n=26)			Impaired (n=26)			Overall		
	Accuracy (%)	p-value	Predictive value (%)	Accuracy (%)	p-value	Predictive value (%)	Balanced accuracy (%)	p-value	AUC
SVM									
GM	70.4	0.02	73.1	74.1	<0.01	71.4	72.2	0.001	0.78
WM	70.4	0.02	73.1	74.1	<0.01	71.4	72.2	<0.01	0.79
FA	81.5	0.001	68.8	63.0	0.08	77.3	72.2	<0.01	0.72
GM+FA	70.4	0.02	73.1	74.1	0.001	71.4	72.2	0.001	0.78
GPC									
GM	81.5	0.001	75.9	74.1	<0.01	80.0	77.8	0.001	0.79
WM	70.4	0.02	67.9	66.7	0.05	69.2	68.5	0.01	0.72
FA	74.1	<0.01	66.7	62.3	0.08	70.8	68.5	<0.01	0.69
GM+FA	81.5	0.001	78.6	77.8	<0.01	80.8	79.6	0.001	0.80

P-values calculated with permutation testing (1,000 repetitions). Abbreviations: FA: fractional anisotropy skeleton; GM: grey matter volume; GPC: Gaussian process classification; SVM: support vector machine; WM: white matter volume.

Figure 8-C. Multivariate classification of cognitive impairment using neuroimaging data defined using multivariate normative comparison.

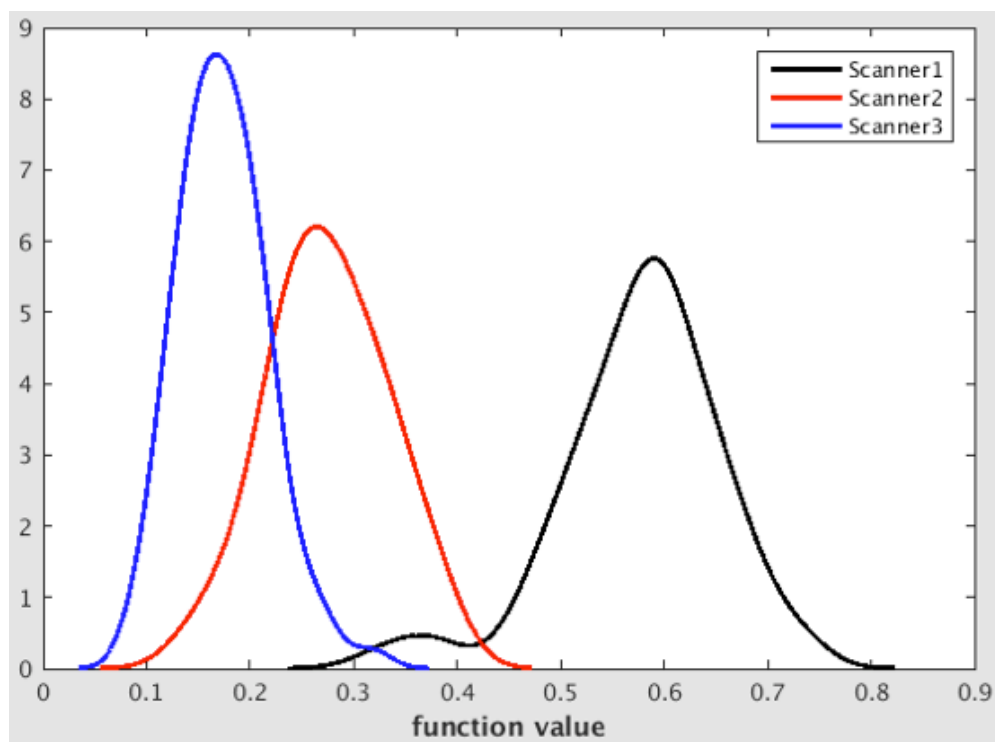


Smoothed density plot (panel a) and individual predictions for each subject (panel b). Function value computed with a Gaussian process classification model using concatenated grey matter volume and fractional anisotropy skeletons.

Due to the generally disappointing ability of these models distinguish between those with and without cognitive impairment, the ability of the models to detect scanner differences was assessed using a multi-class Gaussian process classification model (the support vector machine

model can only handle two classes, whereas there were three scanners). Using just segmented grey matter this yielded a balanced classification accuracy of 89.7% (85.9%, 84.2% and 98.9% for scanners 1,2 and 3 respectively). With segmented white matter the balanced accuracy was 81.2% (73.4, 77.2 and 93.1% for scanners 1,2 and 3 respectively). The accuracy was greatest for the fractional anisotropy skeletons, where the model had a balanced accuracy of 98.1% (95.3%, 100% and 98.9 % for scanners 1,2 and 3 respectively). The model's ability to distinguish between scanners is illustrated by the difference in model function values for each scanner Figure 8-D. With the combined grey matter volume and fractional anisotropy skeleton model balanced accuracy was 95.2% (93.8%, 93.0% and 98.9% for scanners 1,2 and 3 respectively).

Figure 8-D. Smoothed density plot showing the differences between fractional anisotropy skeletons between scanners.



Function value computed with a multi-class Gaussian process classification model.

Accurate classification of subjects by HIV-status just from an MRI scan was not possible using support vector machines or Gaussian (balanced accuracy 51.2%, 52.8%, 54.9% and 52.0% for segmented grey matter, segmented white matter, fractional anisotropy skeleton and the combination of segmented grey matter and fractional anisotropy).

Regression

Next, the ability of multivariate models to predict cognitive function was assessed in a continuous way. In general, the correlation between predicted global T-scores and actual global T-scores was poor (Table 8-D). Kernel ridge regression of skeletonised fractional anisotropy diffusion data performed best ($r=0.32$, $p=0.001$), however this model only explained ~10% of the variance in global T-scores ($R^2=0.10$, $p=0.001$). For comparison, a linear model of with global T-score as the dependent variable and the mean skeleton fractional anisotropy as the dependent variable only explains ~3% of the variance (model adjusted $R^2=0.03$, $F_{1,205}=7.32$, $p<0.01$) as the bivariate correlation $r=0.19$ ($p<0.01$). Similarly, the multivariate model outperformed total white matter volume ($r=0.16$, $p=0.02$). In contrast, total grey matter volume outperformed both multivariate grey matter volume models ($r=0.23$, $p<0.001$).

Table 8-D. Multivariate prediction performance of the global T-score using structural imaging data by model.

Model (n=207)	Correlation	p-value	R^2	p-value	MSE	p-value	Norm MSE	p-value
GPR								
GM	0.19	<0.01	0.04	0.73	36.0	<0.01	1.14	<0.01
WM	0.13	0.02	0.02	0.82	37.4	0.05	1.18	0.05
FA	0.30	0.001	0.09	0.06	34.0	0.001	1.08	0.001
GM+FA	0.19	<0.01	0.04	0.70	36.0	<0.01	1.14	<0.01
KRR								
GM	0.16	0.049	0.03	0.108	44.2	0.087	1.40	0.09
WM	0.28	0.002	0.08	0.002	40.8	0.003	1.29	<0.01
FA	0.32	0.001	0.10	0.001	34.0	0.001	1.08	0.001
GM+FA	0.16	0.041	0.03	0.100	42.4	0.066	1.34	0.07

P-values calculated with permutation testing (1,000 repetitions). Abbreviations: FA: fractional anisotropy skeleton; GM: grey matter volume; GPC: Gaussian process classification; KRR: kernel ridge regression; MSE: mean squared error; WM: white matter volume.

8.3.2 CHARTER

The baseline characteristics for the CHARTER participants (n=139) are described in Table 8-E, 47 (33.8%) of whom were CDC stage A, 37 (26.6%) stage B and 55 stage C (39.6%). Potentially confounding medical problems were common with 11.5% having ‘confounding’ and 25.2% having ‘contributing’ comorbidities as defined in Heaton *et al.*⁸ All participants had

suppressed plasma HIV RNA but two (1.4%) had detectable CSF HIV RNA. Of these, one had CSF HIV RNA of 780 copies/ml and the other at the limit of detection at 50 copies/mL.

Table 8-E. Baseline demographics of the CHARTER participants.

Demographic	n=139
Age (years), median (IQR)	44 (44-50)
Male, n (%)	110 (79.1%)
Ethnicity, n (%)	
White	64 (46.0%)
Black	61 (43.9%)
Hispanic	13 (9.4%)
Other	1 (0.7%)
Years of education, median (IQR)	13.0 (12.0-15.0)
Weight (kg), median (IQR)	77.7 (70.5-87.8)
Duration of HIV-infection (years), median (IQR)	12.2 (6.3-15.8)
Current CD4+ (cells/ μ L), median (IQR)	540 (353-698)
CD4+:CD8+ cell count ratio, median (IQR)	0.60 (0.42-0.91)
Nadir CD4+ (cells/ μ L), median (IQR)	121 (20-237)
Duration of antiretroviral therapy (years), median (IQR)	6.3 (2.6-9.4)
HIV RNA viral load <50 copies/mL, n (%)	139 (100%)
Prior clinical AIDS, n (%)	55 (39.6%)

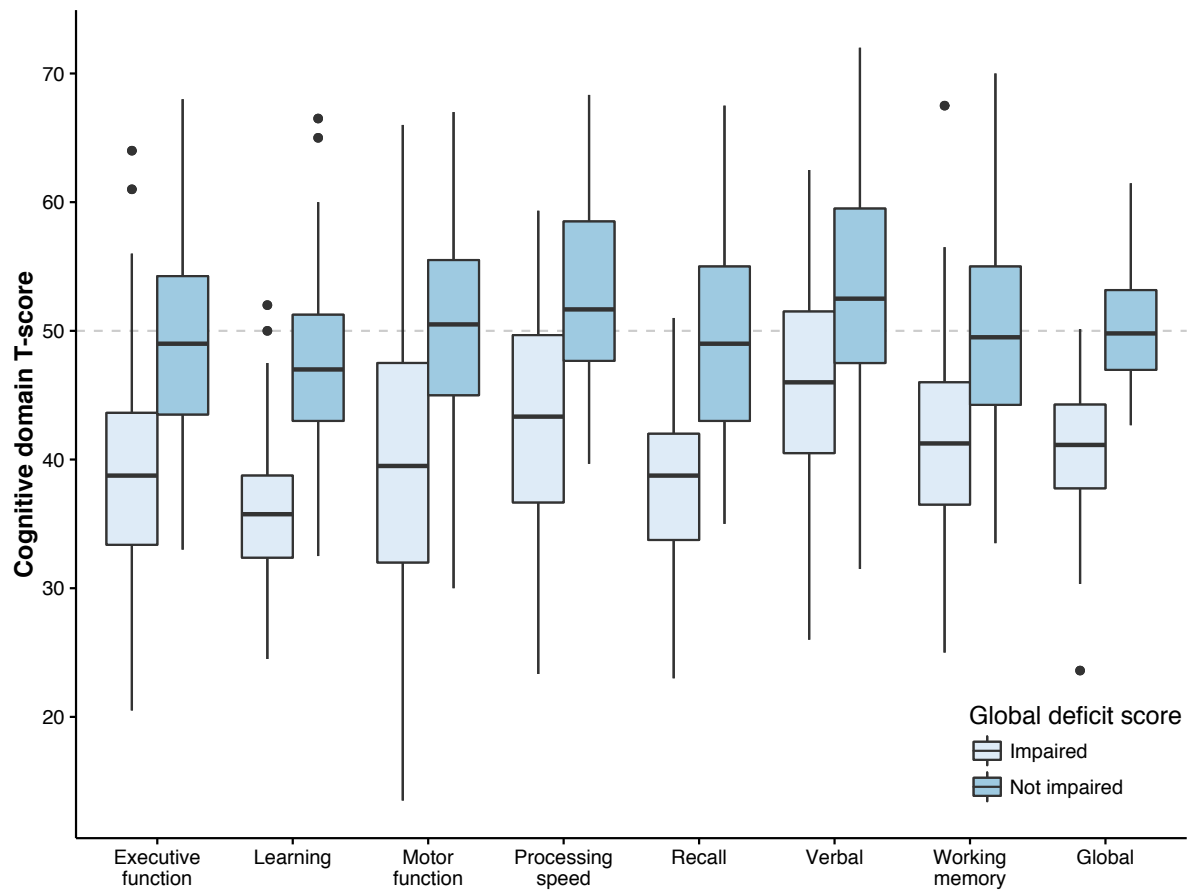
Cognitive function

Using the global deficit score, cognitive impairment was present in 52 (37.4%) well-treated patients from the CHARTER cohort at the time of MRI scanning. Despite this, median global T-score was 46.9, which was not significantly different from the mean of a normal population ($W=44$, $p=0.54$). Impairment at baseline which was more frequent in those with comorbidities (25/88 [28%] of those with ‘incidental’ comorbidities, 14/35 [40%] with ‘contributing’ and 13/16 [81%] with ‘confounding’, $\chi^2_2 = p<0.001$). Patients with cognitive impairment had poorer cognitive function in all domains (Figure 8-E). Using the multivariate technique, I described in detail in chapter 4, cognitive impairment was only present in 6 patients (4.3%).

The median (IQR) change in global T score was 0.47 (-1.63-2.93) over a median follow-up of 12 (6-18) months. Only five patients (4.5%) had clinically significant cognitive decline during the follow-up period (2/73 [2.7%] of those with ‘incidental’ comorbidities, 0/26 [0%] with

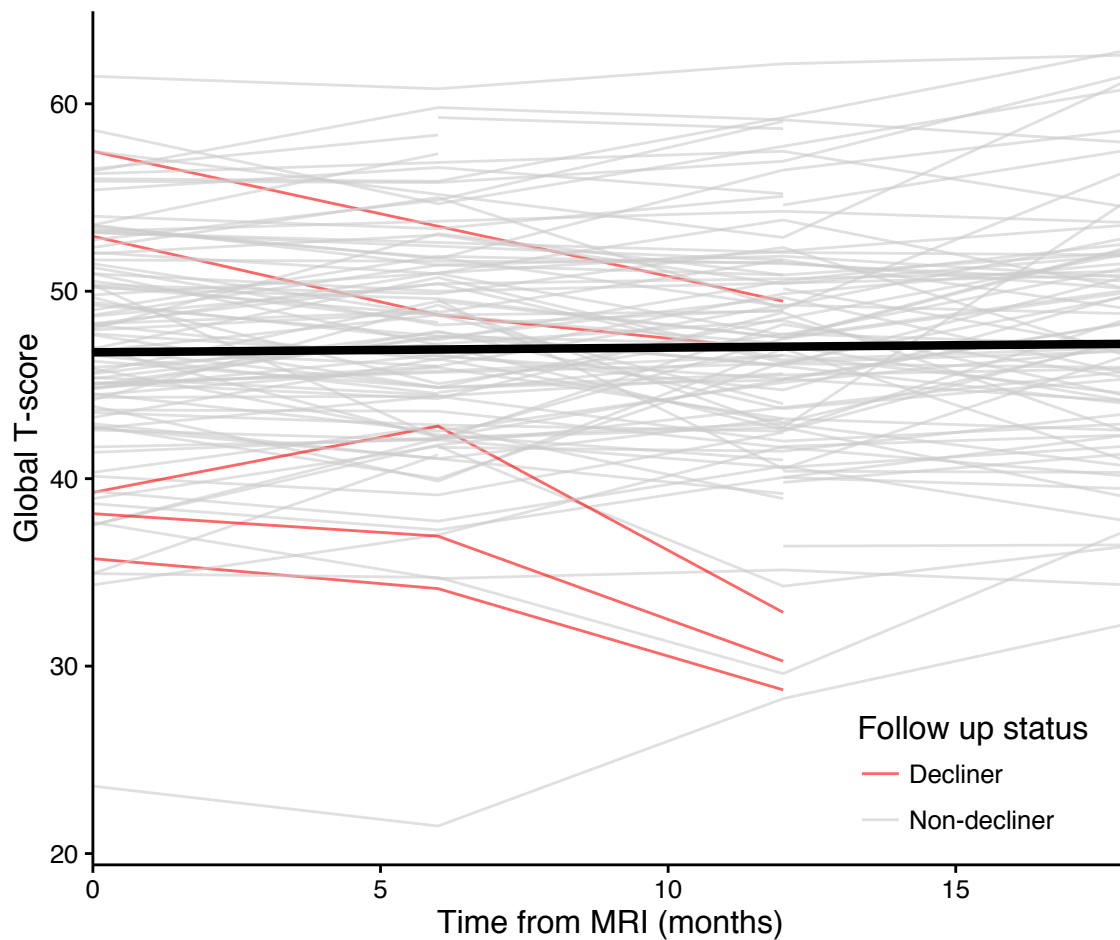
‘contributing’ and 3/12 [25%] with ‘confounding’, $\chi^2_2=13.5$, $p=0.001$). The trajectory of cognitive function is illustrated graphically in Figure 8-F. Neither of the patients with detectable CSF HIV RNA experienced clinically significant cognitive decline (change in T-score 2.3 and 1.8).

Figure 8-E. Boxplots of demographically adjusted cognitive domain T-scores for those with vs. without impairment.



P-values calculated with the Wilcoxon rank-sum test.

Figure 8-F. Spaghetti plot showing stability of global cognitive function over time.



Each line represents a participant's longitudinal cognitive function. Red lines represent those with clinically significant cognitive decline. The thick black line represents the linear fit line across the entire group.

Multivariate prediction of cognitive function

Multivariate classification performance using combined grey and white matter volume data from the CHARTER cohort was not better than chance for both predicting baseline cognitive impairment nor patients who would experience longitudinal decline (Table 8-F). Multivariate prediction of the global T-score using concatenated grey and white matter volume data yielded similar performance to the models presented earlier. The best performance was achieved using a kernel ridge regression model which explained ~8% of the variance in global T-scores. A comparable linear model using total grey and white matter volumes did not predict global T-scores (model adjusted $R^2=0.01$, $F_{2,136}=1.99$, $p=0.14$).

Using kernel ridge regression to predict longitudinal changes in cognitive function in a continuous manner was also unsuccessful ($r = -0.17$, $p = 0.87$, $R^2 = 0.03$, $p = 0.19$).

Table 8-F. Prediction performance using structural imaging data for cognitive impairment defined by the global deficit score by model.

Model	Accuracy (%)	p-value	PPV (%)	Accuracy (%)	p-value	PPV (%)	Balanced accuracy (%)	p-value
	Not impaired (n=87)			Impaired (n=52)			Overall	
SVM	72.4	0.33	65.0	34.6	0.25	42.9	53.5	0.22
GPC	77.0	0.56	65.7	32.7	0.10	46.0	60.4	0.14
	Non-declinors (n=106)			Decliners (n=5)			Overall	
SVM	100	0.87	95.5	0.0	1.00	0.00	50.0	0.87
GPC	100	0.92	95.5	0.0	1.00	0.00	50.0	0.92

P-values calculated with permutation testing (1,000 repetitions). Abbreviations: GPC: Gaussian process classification; PPV: positive predictive value; SVM: support vector machine.

Table 8-G. Multivariate prediction performance of the global T-score using structural imaging data by model.

Model (n=139)	Correlation	p-value	R ²	p-value	MSE	p-value	Norm MSE	p-value
GPR	0.27	0.001	0.07	0.28	37.7	0.001	1.00	0.001
KRR	0.28	<0.01	0.08	0.02	41.5	0.02	1.10	0.02

P-values calculated with permutation testing (1,000 repetitions). Abbreviations: GPC: Gaussian process classification; KRR: kernel ridge regression; MSE: mean squared error.

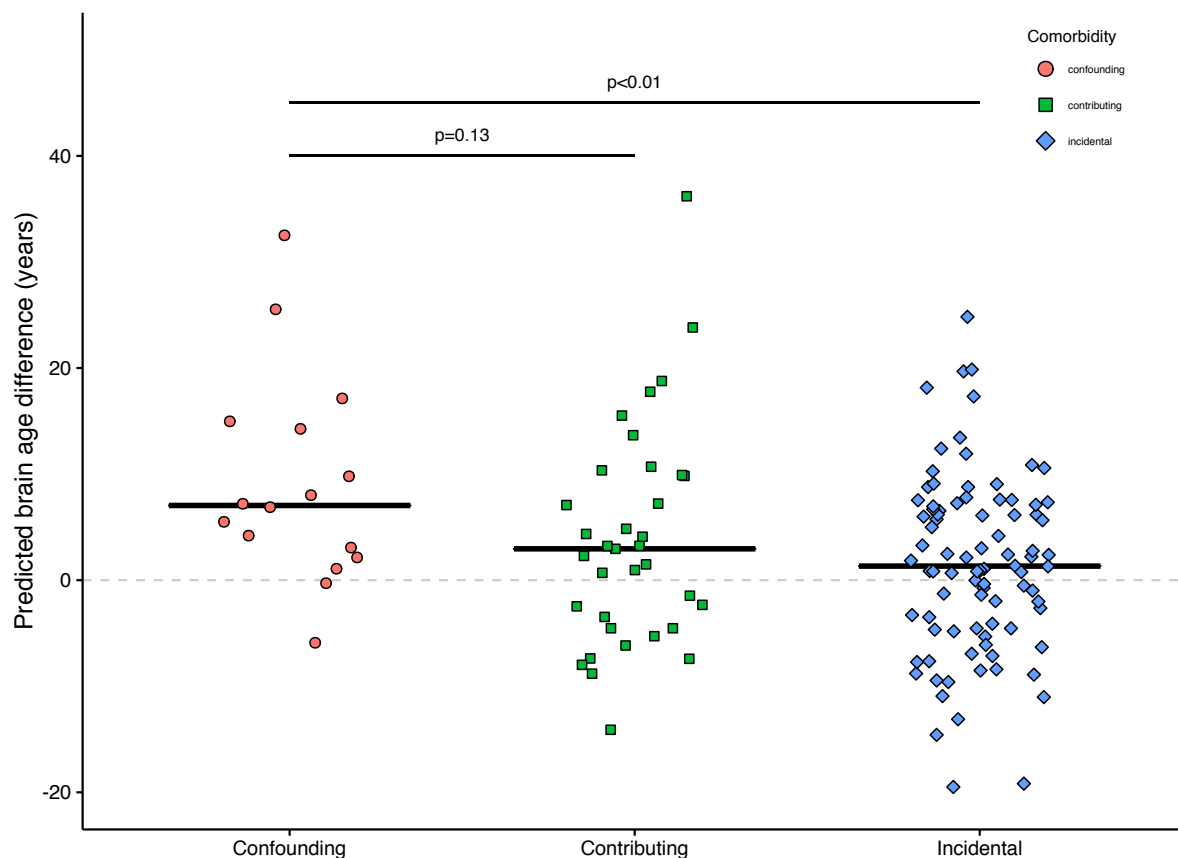
Multivariate brain age prediction

Next, a different approach to multivariate analysis of structural imaging data was performed. Using an accurate predicative model of brain ageing the difference between predicated and chronological age can be determined. Across all subjects predicted brain age exceeded chronological age by mean (95% confidence intervals) 2.94 (1.33-4.51) years, $p < 0.001$. For those with ‘incidental’ comorbidities the predicted brain age difference (PBAD) was elevated by 1.47 (-0.35-3.25) years, $p = 0.11$; for patients with ‘contributing’ comorbidities by 3.80 (0.21-7.36) years, $p = 0.04$; and for patients with ‘confounding’ comorbidities by 9.13 (3.87-14.4) years, $p < 0.01$. These differences are illustrated graphically in Figure 8-G. Differences in PBAD

between scanners were minimal (Figure 8-H), with the only significant difference occurring between scanner 1 and 2 (11.0 [0.84-21.4] years, $p_{\text{adj}}=0.03$; all other $p_{\text{adj}}>0.05$).

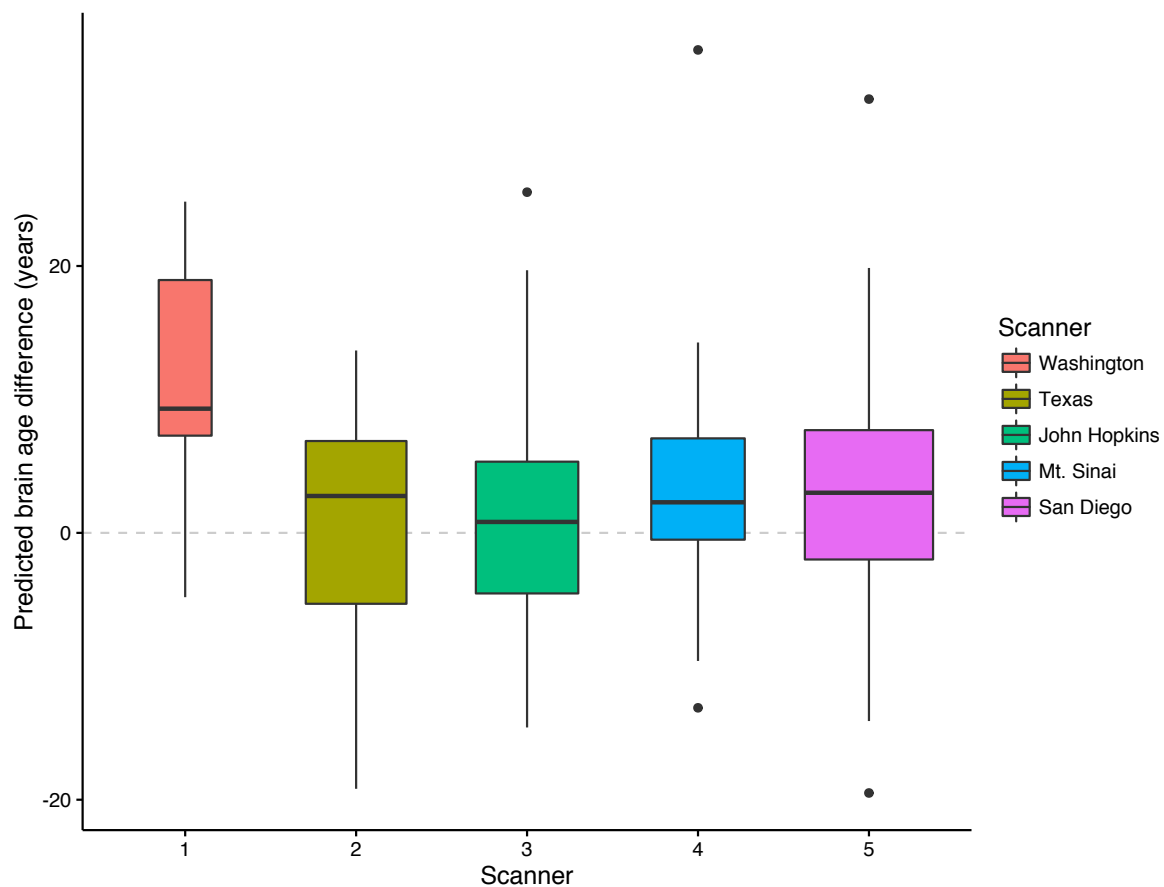
There was no suggestion of *accelerated* ageing associated with HIV-infection as there was no association between PBAD and age nor duration of known HIV-infection ($r=0.11$, $p=0.19$; $r=-0.01$, $p=0.95$ respectively). Additionally, there was no relationship between current or nadir CD4+ cell counts nor CD4:CD8 ratios and BPAD ($r=-0.01$, $p=0.94$; $r=-0.08$, $p=0.34$ and $r=-0.01$, $p=0.94$ respectively). PBAD did not differ between patients with compared to those without cognitive impairment defined using the global deficit score (mean PBAD 4.60 vs 1.95 years, $t_{108}=1.62$, $p=0.11$). Both the patients with detectable CSF HIV RNA had an elevated PBAD: 7.1 and 7.0 years.

Figure 8-G. Jitterplot of predicted brain age difference by comorbidity status.



Black lines represent the median for each group. Dotted line indicates predicted brain age difference in the control population. P-values for comparisons between groups calculated using Tukey's honest significant differences adjusted for multiple comparisons.

Figure 8-H. Boxplot of predicted brain age difference by scanner



Widths of boxes scaled to number in each group.

Predicted brain age difference and cognitive function

Increased PBAD was independently associated with greater global deficit scores (overall model $R^2=0.23$, $F_{8,130}=6.16$, $p<0.0001$, regression estimate 0.013 years, $t_{130}=2.88$, $p<0.01$) after adjusting for age, comorbidity status, and site/scanner. Increasing BPAD was negatively associated with motor function T-scores with negative trends for executive function, memory and global T-scores (Table 8-H) after adjusting for age, confounder status, and scanner differences.

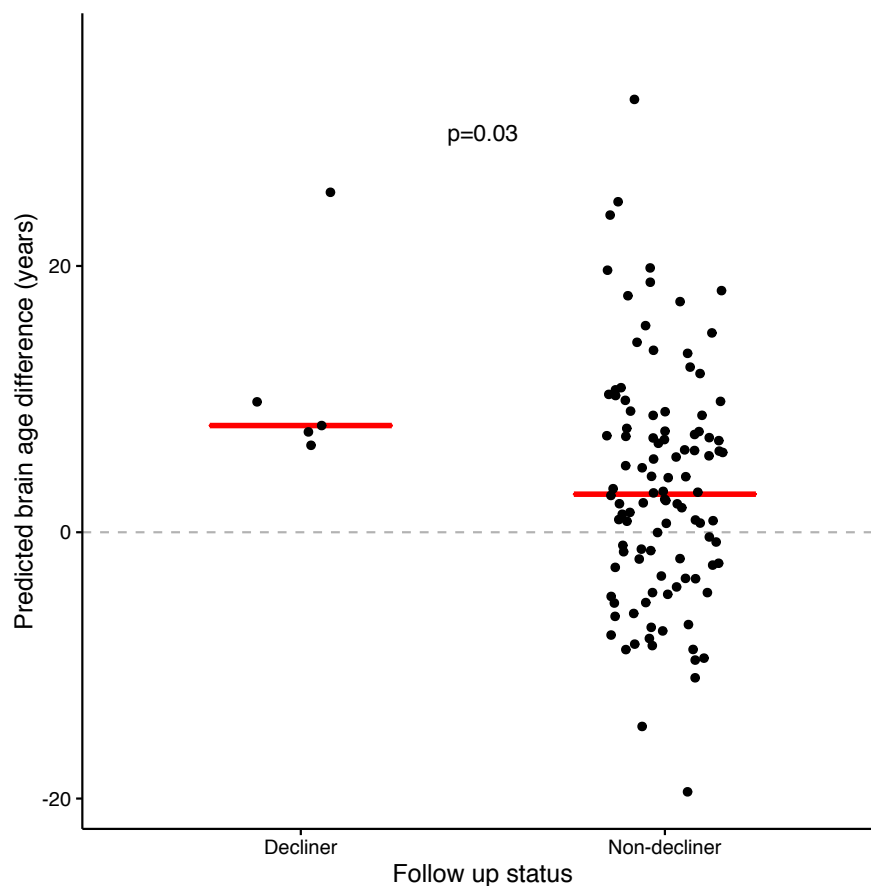
Median BPAD 8.01 vs. 2.86 years ($p=0.03$) for cognitive decliners vs. non decliners (Figure 8-I). Receiver operator characteristics analysis demonstrated that having a BPAD score of >6.2 years had a sensitivity of 100%, a specificity of 65.1% and an AUC of 0.78 for predicting a clinically significant deterioration in cognitive function with a negative predictive value of 100%. This is illustrated graphically in Figure 8-J.

Table 8-H. Correlations and multiple linear regression estimates showing the relationships between predicted brain age difference and cognitive function by domain.

Cognitive domain	<i>r</i>	p-value	Estimate	SE	<i>t</i> ₁₃₀	p-value
Attention/working memory	-0.07	0.40	-0.04	0.08	-0.58	0.65
Executive function	-0.14	0.11	-0.16	0.08	-1.85	0.07
Learning	-0.08	0.37	-0.07	0.08	-0.88	0.38
Motor function	-0.28	<0.001	-0.24	0.09	-2.55	0.02
Memory	-0.12	0.16	-0.14	0.08	-1.69	0.09
Speed of information processing	-0.12	0.17	-0.04	0.08	-0.51	0.61
Verbal fluency	-0.02	0.84	-0.01	0.09	-0.14	0.89
Global T score	-0.17	0.04	-0.10	0.05	-1.74	0.08

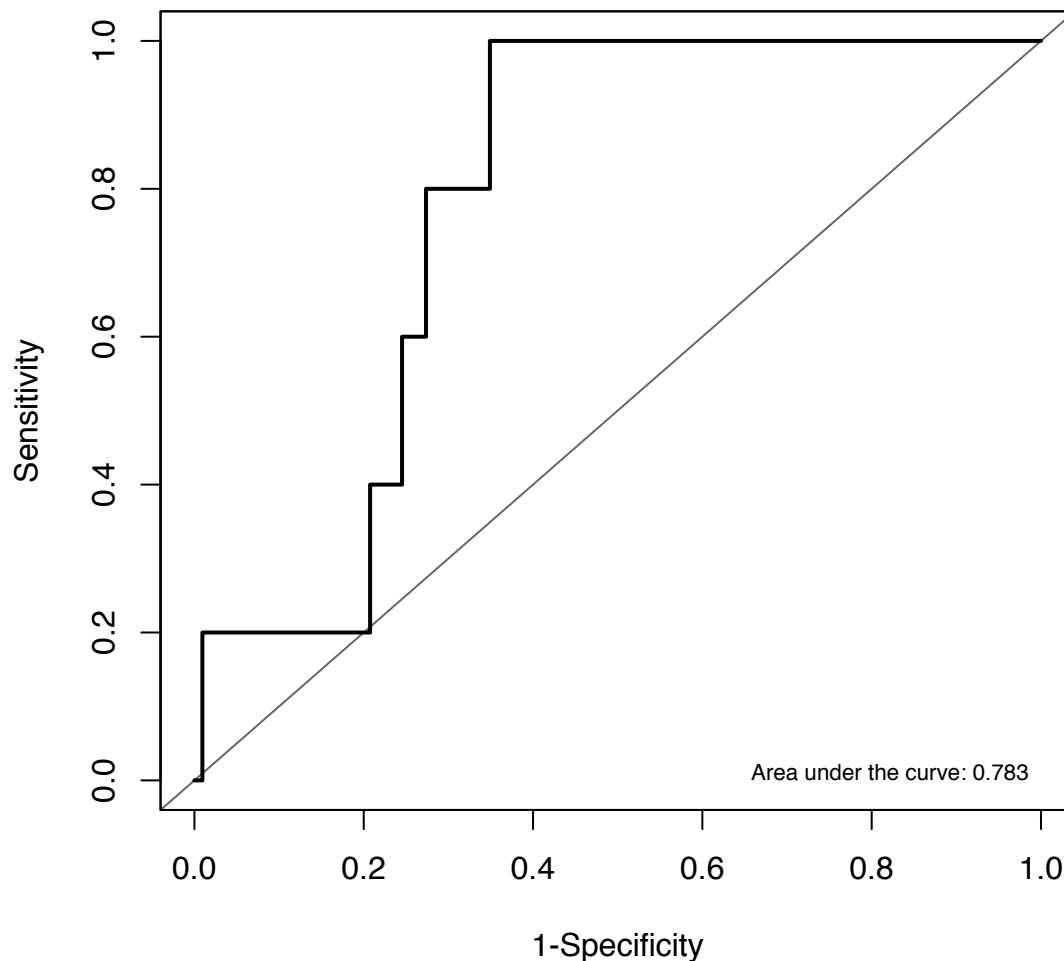
Regression estimates from multiple regression models adjusted for age, confounder status, and site/scanner. Subscript indicates degrees of freedom.

Figure 8-I. Jitterplot of predicted brain age difference by those with vs. without clinically significant decline in cognitive function.



Red lines represent the median for each group. Dotted line indicates PBAD in control population. P-value calculated using Wilcoxon ranked sum test.

Figure 8-J. Receiver operator characteristics (ROC) curve of predicted brain age difference (BPAD) showing the optimum cut-off for predicting clinically significant longitudinal cognitive decline.



8.4 Discussion

In this chapter, I tested my second hypothesis that neuroimaging biomarkers would be associated with cognitive function and could predict impairment and longitudinal changes in cognition. In support of this hypothesis I developed and tested a predictive model that could classify cognitive impairment with 80% accuracy using grey matter volume and fractional anisotropy data. Moreover, using a multivariate brain age prediction model based on 2,000 healthy subjects, I demonstrated that in a heterogeneous patient sample, deviance from chronological age was greater than expected and associated with cognitive function and longitudinal cognitive decline. I also show that cognitive decline is infrequent in well-treated patients.

The results of the multivariate predictive models are a little disappointing given the previous utility of similar models in Alzheimer's disease¹⁷⁴ where a diagnostic accuracy was 89% and in traumatic brain injury¹⁷⁵ where accuracy exceeded 90%. Multivariate models were generally an improvement on simpler univariate models but model performance was largely inadequate for clinical use. Using a combination of volumetric and diffusion data a machine learning classifier was trained that had accuracy at detecting cognitive impairment approaching 80%, providing evidence that my hypothesis may be correct regarding prediction of cognitive function. This is below the standard required in clinical practice given that one in five classifications would be incorrect. It should be remembered that this model was for cognitive impairment, not specifically HIV-associated cognitive impairment as ~10% of those with impairment were HIV-negative controls. However, as the models could not accurately distinguish between the groups it is unlikely this introduced significant bias.

To my knowledge, this is the first time that multivariate machine learning techniques have been applied to whole brain measures in HIV-disease and so the findings presented are genuinely novel. Recently, Zhang *et al*²⁹⁸ report a multivariate classification model that distinguished HIV-associated cognitive dysfunction from mild cognitive impairment. However, the number of patients with impairment in this study was small ($n=15$) and only 80% had undetectable plasma HIV RNA which limits the generalisability of these findings to well-treated cohorts. Additionally, it is not clear what the classification accuracy was between HIV-positive patients and controls and whether the controls for the HIV group were HIV-positive or HIV-negative. Using a random forest classifier on subcortical morphometry, Wade *et al*²⁹⁹ distinguished patients from controls with an AUC of 0.72. Accuracy was not reported but this is lower than the best performing model presented here with regards to classifying cognitive impairment. Conversely, the models presented here could not distinguish between patients and demographically comparable controls. However, only 38% of patients from this study had undetectable plasma viral loads, which is likely to accentuate differences with HIV-negative controls and limits the applicability of these findings to well-treated patients.

The limited classification performance may be reflective of the models not being sufficiently accurate. However, I used support vector machines (in addition to Gaussian processes classification), which have been used successfully other disease areas.¹⁷² Furthermore, the models could very accurately detect differences in scanners but not between patients and controls. Together with the cognitive results from these patients, the data presented here

suggests that HIV-associated cognitive impairment is generally mild and not associated with brain injury that is sufficient to be accurately detected using currently available neuroimaging acquisition and analysis techniques. It should be noted that the sample size used to train the classifiers was relatively small thus limiting the predictive power of the models. Given this, it was not possible to split participants into independent training and testing sets and thus the accuracy of the model is likely to be less when applied to independent data. However, leave one out cross validation technique does go some way to addressing this methodological shortcoming. Nevertheless, this method may be biased by significant outliers that may skew the training data, but given how few patients had impairment other validation schemes such as 5-fold cross validation may result in unstable models.

As less than 5% of well-treated patients experienced clinically significant cognitive decline, accurate identification using a trained classifier was not possible. Further study with a large sample size is necessary to determine whether predicting longitudinal cognitive decline in well-treated patients is possible.

The finding of older appearing brains in HIV-disease is novel. Deviations from chronological age using the same model have previously been reported following traumatic brain injury.¹⁷⁶ Increased markers of cellular⁷³ and mitochondrial ageing,⁷¹ associated with HIV-infection have been reported previously. The findings presented here complement those of Horvath *et al*⁷⁰ who elegantly described epigenetic changes suggestive of accelerated ageing in both the blood and brain of HIV-infected individuals. However, in contrast to the conclusion of that study there was no evidence of *accelerated* brain ageing as age and duration of infection were not associated with increased predicted brain age. This may reflect that the patients here were all successfully treated. The cause of this *accentuated* or premature ageing is not clear. It is likely to reflect brain injury from HIV replication in the central nervous system. However, it should be noted that comorbidity status was clearly associated with PBAD and drove most of the associations with cognitive function given the attenuations of the associations after adjustment. This may reflect a final common pathway of pathology with many different types of brain injury converging in atrophy mimicking the changes associated with age. Patients without comorbidities had the smallest deviance of the normal brain ageing trajectory. The increase of nearly three years is modest and less than that suggested by epigenetic changes.⁷⁰ Again, this may reflect differences in patient selection as all the patients presented here were well-treated which may attenuate further brain injury. An important limitation, is that this

model is designed to accurately detect the changes associated with ageing and therefore it may underestimate HIV-associated brain injury if it is distinct from the ageing process. However, the cohort of patients presented here did not have particularly severe cognitive impairment given that their global T-scores did not differ significantly from normal. Furthermore, despite the reportedly high prevalence of cognitive impairment according to the global deficit score, using the multivariate technique of assessment of cognitive function I described and tested in chapter 4, the prevalence of impairment was only 5%. More work is needed to accurately define cognitive impairment.

The pattern of associations with cognitive function was not entirely as predicted given that motor function was the only domain independently associated with BPAD. Language was preserved as expected, given its stability over the ageing process. Although there were trends for negative relationships between BPAD and executive function and memory, there was no relationship with processing speed, which are all cognitive domains described to decline with age.³⁰⁰ The other interesting finding was that patients who would not have cognitive decline could be predicated with a high degree of accuracy with a high degree of accuracy. All patients with PBAD <6 years did not experience cognitive decline. However, this should be interpreted with caution due to very small number of patients who did experience cognitive decline and that there were many patients who exceeded this threshold who did not deteriorate. If these findings are replicated in other studies this could be used as a potential biomarker to exclude those at risk of clinically significant cognitive deterioration.

Other limitations of the work presented here are that in the CHARTER study subjects were scanned at six sites with varying protocols.¹²⁸ Although scanner differences were present they were not marked which probably reflects the fact that the brain age prediction model used training data from many different scanners and thus was mainly insensitive to scanner differences. Despite this, scanner was covaried in the multiple linear regression models to account for differences. These scanner differences may explain some of the poor classification performance of some of the multivariate models given the smaller sample size.

Conclusion

Supporting my second hypothesis, neuroimaging biomarkers were associated with cognitive function. However, their ability to predict cognitive impairment is limited to the present

rather than the future. Further work to refine these models is necessary before they have the potential for use in clinical practice.

Chapter 9

Conclusions

Table of contents

CHAPTER 9 CONCLUSIONS.....	234
9.1 FIRST HYPOTHESIS	235
9.2 SECOND HYPOTHESIS	237
9.3 THIRD HYPOTHESIS	240
9.4 OVERALL SUMMARY	241
9.5 CLINICAL IMPLICATIONS.....	241
9.6 FUTURE DIRECTION OF RESEARCH	244
9.6 CONCLUSION	246

Cognitive impairment is reportedly prevalent in HIV-positive despite antiretroviral therapy, which raises the possibility of toxicity from long-term use of these drugs. This thesis sought to test the following hypotheses:

1. Cognitive impairment remains more prevalent in HIV-positive individuals compared with demographically comparable controls, despite effective antiretroviral therapy.
2. Biomarkers will be associated with cognitive function and can be used to predict impairment and longitudinal changes in cognition.
3. Greater antiretroviral exposure will be associated with markers of central nervous system neurotoxicity.

9.1 First hypothesis

Cognitive impairment remains more prevalent in HIV-positive individuals compared with demographically comparable controls, despite effective antiretroviral therapy.

This hypothesis was tested in three different clinical populations. In chapter three, the prevalence of cognitive impairment was determined in the POPPY study cohort recruited from sites in the UK and Ireland. The prevalence of cognitive impairment was greater in HIV-positive individuals when compared to an appropriate control population. The prevalence was dependent on the method used to define impairment. Using a multivariate technique that may be more specific¹³ than other popular methods, cognitive impairment was present in 22% of HIV-positive patients. This was despite antiretroviral use in 97% with 91% achieving plasma HIV RNA <50 copies/ml. Similar results were seen in participants from the COBRA study, where again cognitive impairment was present in 20% of patients. All participants in this study had suppressed plasma HIV RNA and 98% had CSF HIV RNA <40 copies/ml. Finally, in a subgroup of patients from the CHARTER study, all of whom had suppressed plasma HIV RNA (and neuroimaging data) and 98% had suppressed CSF HIV RNA, 37% had cognitive impairment. It should be noted that in the latter case, cognitive impairment was defined using the global deficit score as there was no control group to compare them to. This is similar to

the prevalence of impairment defined using the global deficit score from the POPPY study where 35% had met this criterion. It should be noted however, that CHARTER is a North American cohort and enrolled patients over a decade ago (beginning in 2002), in contrast to POPPY and COBRA, which are comprised of patients cared for in Northern Europe and began enrolling participants in 2010. Therefore, differences in demographics and healthcare provision may confound direct comparisons between the studies (the prevalence of prior AIDS was 40% in the CHARTER cohort vs 31% in the COBRA cohort). The associations between objective cognitive impairment and subjective experience were generally weak, which may reflect that cognitive impairment in well-treated patients is generally mild. Given the lack of a gold standard it is imperative that prospective outcomes for patients diagnosed with each method are determined.

As I demonstrated in chapter four, the prevalence of cognitive impairment is sensitive to the method used to define it. Using simulation data, I demonstrated that over 25% of a normal population would be diagnosed as cognitively impaired according to the widely used Frascati criteria, making the case for having an appropriate control group and using a multivariate comparison technique. Furthermore, I outline a less biased method of determining cognitive impairment without the need for a control group using a multivariate technique, building on the work of Huizenga *et al.*⁴⁰ It should be noted that this method is suitable for other types of multivariate data and not just limited to the analysis of tests of cognitive function. Further work testing its utility in other disease areas is warranted.

Previous studies in exclusively well-treated patients are not common. Su *et al*¹³ recently reported the prevalence of cognitive impairment in well-treated Dutch patients of 17% who were demographically similar to the POPPY and COBRA cohorts using a multivariate comparison with an appropriate control population. This is similar to the prevalences described in this thesis in chapters three, five and eight for the POPPY, COBRA and CHARTER cohorts respectively. This is significantly less than the 40-50% reported in two widely cited papers that studied over 2,000 patients in the modern antiretroviral era.^{8,10} It should be noted that, firstly, neither of these studies recruited specific HIV-negative control groups and therefore relied on historical normative data for each neuropsychological test. Secondly, not all patients had achieved plasma HIV RNA <50 copies/ml and finally, the method used to diagnose impairment was likely to be overly sensitive and not specific. Interestingly, only 2% met the more stringent criteria for HIV-associated dementia in the CHARTER study.⁸

This is the same as I found in the POPPY study (chapter three) and as I demonstrated in chapter four is the prevalence you would expect to find by chance in a normal population. Taken together, my findings suggest that cognitive impairment is present in about a fifth of well-treated patients, is mild in a majority and importantly is not clearly associated with symptoms. Furthermore, with the longitudinal data I present in chapter eight, the majority of well-treated patients have stable cognitive function, at least over a follow-up period of two years.

9.2 Second hypothesis

Biomarkers will be associated with cognitive function and can be used to predict impairment and longitudinal changes in cognition.

In chapters five, six and eight I demonstrated that neuroimaging biomarkers in particular were associated with cognitive function. Using voxelwise comparisons, I demonstrated that well-treated patients had evidence of grey matter atrophy and widespread white matter microstructural abnormalities, suggestive of demyelination, compared to a demographically comparable control group. Furthermore, I demonstrated how brain structure was related to cognitive function and in particular how white matter microstructural abnormalities, evident using diffusion weighted imaging, were likely to be the major pathophysiological component of HIV-associated cognitive impairment in well-treated patients. The relationships between neuroimaging abnormalities and observed cognitive deficits had previously been uncertain, particularly in well-treated patients, with several studies reporting no associations between the two.¹²⁷⁻¹³⁰ The work presented here builds on recent reports of white matter abnormalities in well-treated patients¹³⁰ and contrasts with another recent report²⁶⁵, which reported an interaction between HIV-status and age suggesting HIV-infection may be responsible for accelerated ageing. One consistent finding from the structural neuroimaging data presented throughout this thesis was that HIV-status did not modify the normal age related changes to the brain in well-treated patients. In the recent work reported by Seider *et al*,²⁶⁵ not all patients were successfully treated, which makes it likely that the interaction they report is secondary to older patients having greater cumulative exposure to central nervous system HIV-replication than younger patients. The data presented in chapters five and eight suggest that HIV-infection results in premature or *accentuated* as opposed to

accelerated ageing. Furthermore, for the majority of well-treated patients, cognitive impairment is likely to be a legacy of previous untreated infection and that brain injury is a *static* rather than *active* phenomenon. CSF biomarkers of neuronal injury that have previously been associated with HIV-associated cognitive impairment, such as NFL,¹⁸⁷ did not differ between well-treated patients and a demographically comparable control group. Furthermore, biomarkers associated with microglial activation were not consistently elevated, despite clear evidence of persistent systemic immune activation. Neopterin and the kynurenine tryptophan ratio were both elevated in the patient group but they were not associated with cognitive function. The significance of these findings is unclear given the lack of other evidence for active neuronal injury and they may be a *consequence* of previous damage as opposed to a *cause* of further injury. Alternatively, given the noise associated with measuring cognitive function, cross-sectional analyses such as the ones presented here may lack power to detect subtle deleterious effects of persistent microglial activation and neuroinflammation. A recent PET study⁶² has suggested that persistent microglial activation, associated with microbial translocation, may be responsible for cognitive impairment in well-treated individuals. However, these findings were in patients without cognitive impairment so the long-term implications of these findings is unclear and longitudinal study is needed to tease out their significance. These findings, together with lack of white matter atrophy evident with volumetric imaging techniques and comparable metabolic imaging data in patients compared to controls, provide the basis of my static injury hypothesis. CSF biomarkers such as NFL and cerebral metabolites data provide dynamic information about brain injury,^{46,177,184} whereas volumetric changes like grey atrophy are irreversible as they represent neuronal loss. If the diffusion changes reported in chapter five were recent and ongoing, differences in these dynamic markers of central nervous system function would be expected. This was not the case. Further evidence for the static injury hypothesis is provided in chapter eight by the observed increase in predicted brain age relative to chronological age that was not associated with either age or duration of infection. This hypothesis of static brain injury in well-treated patients is further supported in chapter eight by the stability of cognitive function in well-treated patients over time. However, detailed longitudinal study of cognitive and neuroimaging data is necessary to test this hypothesis and the findings presented in this thesis should be viewed as suggestive rather than confirmative.

Another important caveat is that the data presented here is in highly selected patient groups. Participants were excluded from COBRA if they had a potentially confounding

neuropsychiatric illness. Therefore, they are not representative of all patients, many of whom may have comorbid conditions. Reassuringly, the CHARTER study did not have such exclusions and whilst enrolling in a detailed neurological study with MRI scanning and lumbar puncture clearly represents an element of self-selection bias, cognitive function remained stable in the majority. Additionally, virtually all the patients studied in this thesis had plasma HIV RNA suppressed below the standard level of detection. Whilst in the UK this is the norm,²⁴ it is not necessarily the case worldwide and care must be taken to extrapolate my findings to inadequately treated patients, in whom HIV-associated brain injury is much more likely and fully suppressive treatment must be the priority.

In chapter eight, I tested whether it was possible to predict cognitive impairment using structural neuroimaging data. Multivariate models were generally an improvement on simpler univariate models but model performance was largely disappointing. To my knowledge, the multivariate techniques presented here have not been applied in HIV-disease for the classification of cognitive impairment and so the findings presented are genuinely novel. Using a combination of volumetric and diffusion data, a machine learning classifier was trained that had an accuracy approaching 80% for detecting cognitive impairment. This compares less favourably with previous work in Alzheimer's disease¹⁷⁴ where a diagnostic accuracy of 89% was achieved and in traumatic brain injury¹⁷⁵ where accuracy exceeded 90%. Although this may reflect failure of the models themselves, I used support vector machines (in addition to Gaussian processes classification), which were used in both of these studies and have been successfully applied in other disease areas.¹⁷² Furthermore, the models could very accurately detect differences between scanners (not obvious to the naked eye) but not between patients and controls. Together with the cognitive results presented in chapters three, five and eight and the neuroimaging results from chapter five, these findings suggest that HIV-associated cognitive impairment is generally mild and not associated with brain injury that is easily detected using currently available techniques. It should be noted however, that the sample size of patients with impairment used to train the classifiers was relatively small thus limiting the models' predictive power. Given this, it was not possible to split participants into independent training and testing sets and therefore the accuracy of the model is likely to be less when applied to independent data. However, the leave-one-out cross-validation technique does go some way to addressing this methodological shortcoming. With so few well-treated patients experiencing clinically significant cognitive decline, training a model to predict this was not possible. However, using a multivariate model to predict brain age from

brain volume data it was possible to predict patients who would not experience significant longitudinal cognitive decline with a high degree of accuracy. All patients with a predicted brain age exceeding chronological age by less than six years did not experience cognitive decline. However, this should be interpreted with some caution due to very small number of patients who actually did experience cognitive decline and that there were many patients who exceeded this threshold of discrepant brain ageing who did not deteriorate.

9.3 Third hypothesis

Greater antiretroviral exposure will be associated with markers of central nervous system neurotoxicity.

This hypothesis was tested in chapter seven where I found evidence for toxicity for the NNRTIs efavirenz and nevirapine. Efavirenz has long been associated with neuropsychiatric side effects,²⁷⁹ however the *in vivo* evidence of central nervous system toxicity and impaired cognitive function has largely been absent with the exception of the work by Ciccarelli *et al.*¹⁰⁴ Therefore the findings I present in this thesis are the most comprehensive to date of clinically significant central nervous system toxicity. The negative correlations with cognitive domain T-scores but without evidence of structural imaging abnormalities or elevations in CSF biomarkers of neuronal injury described in chapter seven suggest that efavirenz's may exert an intoxicant effect that may therefore be reversible on cessation.

In contrast to efavirenz, nevirapine has been thought of clinically as a 'clean' drug with respect to the central nervous system and has been used successfully to reduce symptoms when switching from efavirenz.³⁰¹ However, it should be noted that the findings from this study should be interpreted with caution given it was non-randomised, uncontrolled and observational and therefore subject to significant bias. One previous clinical study¹⁴ has reported nevirapine use to be associated with poorer cognitive function. The data presented here builds on *in vitro* work by Robertson *et al.*⁸⁷ and mouse models of toxicity by Streck *et al.*^{113,114} In chapter seven, I describe the negative correlation of plasma nevirapine concentration with cognitive T-scores, particularly in the domain of attention, and grey matter atrophy in important hub regions of the brain that are essential for cognition. My findings

provide evidence for clinically significant central nervous system toxicity associated with long-term nevirapine use.

These findings should be interpreted with some caution given their cross-sectional nature. However, the use of pharmacokinetic measures limits channelling bias that may confound associations with usage in cohort studies. Furthermore, the comprehensive, multi-modal assessment of central nervous system health allowed the delineation of coherent pathophysiological pathways – for nevirapine in particular. The findings must be confirmed in other, larger cohorts, ideally prospectively, before changes to treatment guidelines or dosing can be considered. Furthermore, the toxicity of other commonly used antiretrovirals needs to be determined and I have outlined in this thesis a feasible model to assess toxicity *in vivo*.

9.4 Overall summary

In this thesis, I have demonstrated that well-treated HIV-positive patients do have evidence of cognitive impairment despite successful antiretroviral therapy and when compared to a demographically comparable control population. However, the prevalence is lower than previously reported, which may be related to methodological issues. Furthermore, impairment is generally mild and asymptomatic and for the majority remains stable over time. In well-treated patients, HIV-associated cognitive impairment is largely due to white matter microstructural injury that is most probably the legacy of damage occurring before sustained suppression of HIV-replication with antiretroviral therapy. Accentuated and not accelerated brain ageing is therefore the likely sequelae in well-treated patients. However, longitudinal studies are needed to confirm this, given I found some evidence suggesting persistent neuroinflammation despite successful antiretroviral therapy.

Neuroimaging biomarkers in particular are essential for the *in vivo* assessment of the central nervous system health in treated HIV-disease. Additionally, they may be useful for developing multivariate predictive models of cognitive function taking advantage of their inherent multidimensionality, although much further work is needed before they can be introduced into routine clinical practice.

Clinically significant central nervous system toxicity may be associated with long-term efavirenz and nevirapine exposure. Again, longitudinal study and replication are essential before these findings influence prescribing. However, given the long-term exposure of millions to these drugs, further study of their central nervous system effects is essential.

9.5 Clinical implications

Given the cross-sectional nature of most of this thesis and the relatively small sample sizes, particularly those of Chapter 7, the results presented here should be interpreted with a degree of caution. However, the consistent findings from the contemporary POPPY and COBRA cohorts were that although cognitive impairment was found more frequently compared to matched controls, the degree of impairment was mild. Together with the stability of cognitive function over time associated with sustained virological suppression demonstrated in the CHARTER cohort in Chapter 8, these findings should provide reassurance to patients and clinicians.

The lack of coherence between subjective cognitive symptoms and objective cognitive function reported in Chapter 3 in virologically suppressed patients is an important finding. Previously, the European AIDS Clinical society recommended regularly screening for HIV-associated cognitive impairment using ‘the three questions’.²⁰⁸ More recent guidance does not make formal screening recommendation,⁶⁸ which my findings would support. Given the poor performance of this limited battery of questions, a clinician should consider a more thorough assessment including formal neuropsychological testing with reference to an appropriate control group before considering further testing. The interpretation of these results should keep in mind the results of Chapter 4, where I demonstrated how the commonly used research definitions of cognitive impairment, the Frascati criteria and global deficit score, lack specificity. In my opinion, these definitions of impairment should not be used in clinical practice and should be used sparingly in the research setting, predominantly for comparative purposes and with a control group to provide context. If poorly discriminating questions are used to screen for impairment which is subsequently assessed using diagnostic criteria that are prone to over-diagnosis, then a significant proportion of patients will be labelled unnecessarily as having cognitive impairment. The implicit assumption of this diagnosis is that it is the result of a pathogenic process rather than simply an artefact of the methods used to interpret the data.

This could result in unnecessary investigations and treatment as well as distress on the part of the patient and clinician. The novel multivariate method of defining cognitive impairment needs to be formally tested in larger cohorts but it shows promise, particularly in the context where there is no control group to compare the study group to. As the prevalence of impairment defined using this method in a normative control population is known (5%) and can be defined *a priori*, the prevalence of impairment in the study group can be interpreted more easily than using the Frascati criteria or the global deficit score, where 22-30% and 17-25% of a *normal* population are identified as impaired respectively. This method is not specific to HIV-associated cognitive impairment and could be used with any multivariate cognitive data, with available norms, in any disease area. Furthermore, it has the potential to be used with other multivariate data, e.g. grey matter volumes from a number of regions of interest. However, further study is necessary to assess its performance.

The results of Chapter 5 and Chapter 6 strongly suggest that the predominant pathology of HIV-associated cognitive impairment in treated HIV-disease, white matter microstructural injury and demyelination, predates sustained virological suppression. Furthermore, it is likely to be a static phenomenon in virologically suppressed individuals rather than a progressive neurodegenerative disease, again supported by longitudinal cognitive data in Chapter 8. This highlights the importance of maintaining virological suppression in order to maintain brain health. Again, these data should be reassuring to clinicians and patients and provide additional motivation to maintain adherence to antiretroviral therapy and attendance in care to monitor virological suppression. Given these results, if a virologically suppressed patient does present with cognitive decline it is important to exclude CSF escape and HIV-associated brain injury with MRI and CSF examination (see Underwood and Winston³⁰² for further details of a suggested diagnostic algorithm). However, only 1.6% of patients in COBRA and 1.4% in CHARTER had detectable CSF HIV RNA, which suggests this is a rare finding in patients with sustained suppression of plasma HIV-viraemia. Moreover, only 4.5% of the CHARTER participants experienced a clinically significant decline. Together, these findings suggest that the *a priori* probability of HIV being responsible for the cognitive decline in the aforementioned patient is low. Therefore, in this context other potential diagnoses should be actively considered and excluded.

The findings of Chapter 7 need to be interpreted with care given the small sample size and cross-sectional nature of the data. However, they are suggestive of clinically significant central

nervous system toxicity of efavirenz and nevirapine. Given that they form the cornerstone of the WHO's antiretroviral treatment guidelines,²⁹⁴ millions are prescribed these drugs globally. Therefore, the implications of toxicity, if confirmed in larger, prospective studies, are potentially huge. However, given the limited access to antiretrovirals in most of the world and that these drugs are tremendously effective and responsible for preventing death in millions, evidence of harm would have to be certain and a long-term viable alternative would be needed before guidelines should be changes. Similarly, further study is needed before clinicians should consider switching off these drugs in resource rich settings, such as the UK. However, given wider access to several efficacious alternatives the burden of proof may prove to be lower. Nevertheless, with increasing cost-pressures, due to medical advances and an ageing population, a feature of modern medicine the risk-benefit calculation may change and definitive proof of toxicity may become more important.

In Chapter 8, I demonstrated how multivariate pattern analysis using machine learning techniques could be used to predict cognitive impairment in treated HIV-disease with up to 80% accuracy. This is likely to represent the upper bound of accuracy if this model was to be applied to new data given the low sample size of the training data I had available. Therefore, this method needs further research and development before being used in clinical practice. The possibility of being able to predict longitudinal decline in cognitive function remains elusive but I present some encouraging early data. Again, these models need further research and development, principally with a much larger training dataset and perhaps using more advanced techniques such as deep-learning, as well as rigorous testing and validation before being of any use in clinical practice.

9.6 Future direction of research

Given the findings presented here, where should the field go next? In my opinion, the universal rollout of antiretrovirals should be paramount. Given this is a global trend, incident HIV-associated cognitive impairment is likely to decline. Rather than describing the prevalence using methods that are not ideally suited to the data, researchers should instead focus on the patients who do not maintain stable cognitive function. It is likely that about 5% of otherwise well-treated patients will experience clinically significant cognitive decline. The likely aetiology is multifactorial and includes non-HIV related neurodegenerative diseases, vascular disease,

drug and alcohol misuse, antiretroviral toxicity, central nervous system HIV ‘escape’ and microglial activation in the face of persistent systemic immune activation. Additionally, depression, which is commonly reported in HIV-positive individuals, may complicate interpretation of cognitive function testing, as it may represent a confounder or be one of the manifestations of cognitive impairment in treated HIV-disease. Furthermore, given the ageing cohort the burden of age-associated comorbidities is likely to rise. Teasing out specific causes in individual patients will be difficult, if not impossible. From the HIV physician’s perspective, antiretroviral toxicity, central nervous system HIV ‘escape’ and the consequences of persistent microglial activation are particularly pertinent and warrant further study.

These three research strands will all benefit from high-fidelity longitudinal neuroimaging data. Describing the neurotoxic effects of antiretroviral drugs is essential to determine favourable combinations given decades of likely exposure. Research should include new drugs, including those that are considered ‘non-toxic’ in comparison to the drugs they are replacing. For example, unpublished data suggests high rates of discontinuation of dolutegravir,³⁰³ in contrast to randomised controlled trial data.³⁰⁴ The framework to assess *in vivo* toxicity presented in this thesis is suitable to assess the potential toxicity of other antiretrovirals. Furthermore, blinded, randomised controlled trials switching patients off potentially toxic drugs should be performed with cognitive and neuroimaging primary endpoints. Better understanding and non-invasive diagnostics are needed to identify those at risk of and those who have central nervous system HIV ‘escape’. More research into the consequences of persistent microglial activation is also needed, as well as strategies to treat this. For example, quantifying rates of atrophy in patients and its association with microglial activation would give useful data about the consequences of persistent abnormalities that are presented in this thesis and by others with PET imaging.⁶² Multi-modality, multivariate machine learning techniques have the potential to identify those at risk of future impairment and should be investigated further. One eye should also be kept on HIV-cure research field given that the “shock and kill” approach³⁰⁵ currently being trialled may have unintended inflammatory consequences in the central nervous system. Given the skull represents a fixed boundary, uncontrolled inflammation could, at least in theory, be catastrophic.³⁰⁶ Assessment of the central nervous system effects of cure strategies is therefore important, particularly considering that patients who are receiving these therapies are otherwise well and are likely to have a normal or near normal life expectancy.³ Finally, the beneficial central nervous system effects of initiating antiretroviral therapy in seroconversion

need to be determined as it this treatment strategy may prevent brain injury at the time of greatest HIV-replication.²³

9.7 Conclusion

To conclude, the beneficial effects of antiretroviral therapy greatly outweigh any potential central nervous system toxicity and this thesis should provide reassurance and encouragement to clinicians and patients alike. However, we must remain vigilant about the potential long-term toxicities to the brain from chronic antiretroviral exposure.

List of communications

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Appendix I

The CNS HIV Anti-Retroviral Therapy Effects Research (CHARTER) group

The CNS HIV Anti-Retroviral Therapy Effects Research (CHARTER) group is affiliated with the Johns Hopkins University, Mount Sinai School of Medicine, University of California, San Diego, University of Texas, Galveston, University of Washington, Seattle, Washington University, St. Louis and is headquartered at the University of California, San Diego and includes: Director: Igor Grant, M.D.; Co- Directors: J. Allen McCutchan, M.D., Ronald J. Ellis, M.D., Ph.D., Thomas D. Marcotte, Ph.D.; Center Manager: Donald Franklin, Jr.; Neuromedical Component: Ronald J. Ellis, M.D., Ph.D. (P.I.), J. Allen McCutchan, M.D., Terry Alexander, R.N.; Laboratory, Pharmacology and Immunology Component: Scott Letendre, M.D. (P.I.), Edmund Capparelli, Pharm.D.; Neurobehavioral Component: Robert K. Heaton, Ph.D. (P.I.), J. Hampton Atkinson, M.D., Steven Paul Woods, Psy.D., Matthew Dawson; Virology Component: Joseph K. Wong, M.D. (P.I.); Imaging Component: Christine Fennema-Notestine, Ph.D. (P.I.), Terry L. Jernigan, Ph.D., Michael J. Taylor, Ph.D., Rebecca J. Theilmann, Ph.D., John Hesselink, M.D.; Data Management Unit: Anthony C. Gamst, Ph.D. (P.I.), Clint Cushman; Statistics Unit: Ian Abramson, Ph.D. (P.I.), Florin Vaida, Ph.D.; Protocol Coordinating Component: Thomas D. Marcotte, Ph.D. (P.I.), Rodney von Jaeger, M.P.H.; Johns Hopkins University Site: Justin McArthur (P.I.), Mary Smith; Mount Sinai School of Medicine Site: Susan Morgello, M.D. (Co-P.I.) and David Simpson, M.D. (Co-P.I.), Letty Mintz, N.P., Cheuk Tang, Ph.D., and Thomas Naidich, M.D.; University of California, San Diego Site: J. Allen McCutchan, M.D. (P.I.), Will Toperoff, N.P.; University of Washington, Seattle Site: Ann Collier, M.D. (Co-P.I.) and Christina Marra, M.D. (Co-P.I.), Kenneth Maravilla, MD, KC Stegbauer, Ph.D.; Trudy Jones, M.N., A.R.N.P.; University of Texas, Galveston Site: Benjamin Gelman, M.D., Ph.D. (P.I.), Eleanor Head, R.N., B.S.N., Gregory Chaljub, M.D.; and Washington University, St. Louis Site: David Clifford, M.D. (P.I.), Muhammad Al-Lozi, M.D., Mengesha Teshome, M.D.

Funding/Support The CNS HIV Anti-Retroviral Therapy Effects Research (CHARTER) is supported by award N01 MH22005 from the National Institutes of Health.

Appendix 2

The Co-morBidity in Relation to Aids (COBRA) Collaboration

Academisch Medisch Centrum, Universiteit van Amsterdam - Department of Global Health and Amsterdam Institute for Global Health and Development (AIGHD): P. Reiss, F.W.N.M. Wit, J. Schouten, K.W. Kooij, R.A. van Zoest, B.C. Elsenga, F.R. Janssen, M. Heidenrijk, W. Zikkenheiner. Division of Infectious Diseases: M. van der Valk. Department of Experimental Immunology: N.A. Kootstra, A.M. Harskamp-Holwerda, I. Maurer, M.M. Mangas Ruiz, A.F. Girigorie. Department of Medical Microbiology: J. Villaudy, E. Frankin, A. Pasternak, B. Berkhout, T. van der Kuyl. Department of Neurology: P. Portegies, B.A. Schmand, G.J. Geurtsen, J.A. ter Stege, M. Klein Twennaar. Department of Radiology: C.B.L.M. Majoie, M.W.A. Caan, T. Su. Department of Cell Biology: K. Weijer. Division of Endocrinology and Metabolism: P.H.L.T. Bisschop. Department of Experimental neuroendocrinology: A. Kalsbeek. Department of Ophthalmology: M. Wezel. Department of Psychiatry: I. Visser, H.G. Ruhé.

Alma Mater Studiorum Universita di Bologna -Department of Experimental, Diagnostic and Specialty Medicine: C. Franceschi, P. Garagnani, C. Pirazzini, M. Capri, F. Dall'Olio, M. Chiricolo, S. Salvioli.

Erasmus Universitair Medisch Centrum Rotterdam - Department of Genetics: J. Hoeijmakers, J. Pothof.

GGD Amsterdam/Public Health Service Amsterdam -Cluster of Infectious Diseases, research department: M. Prins, M. Martens, S. Moll, J. Berkel, M. Totté, S. Kovalev.

Göteborgs Universitet - M. Gisslén, D. Fuchs, H. Zetterberg.

Imperial College of Science, Technology and Medicine - Department of Medicine, Division of Infectious Diseases: A. Winston, J. Underwood, L. McDonald, M. Stott, K. Legg, A. Lovell, O. Erlwein, N. Doyle, C. Kingsley. Department of Medicine, Division of Brain Sciences, The Computational, Cognitive & Clinical Neuroimaging Laboratory: D.J. Sharp, R. Leech, J.H. Cole.

Stichting HIV Monitoring - S. Zaheri, M.M.J. Hillebregt, Y.M.C. Ruijs, D.P. Benschop.

Stichting Katholieke Universiteit Nijmegen -D. Burger, M. de Graaff-Teulen.

Università degli studi di Modena e Reggio Emilia - Department of Medical and Surgical Sciences for Children & Adults: G. Guaraldi.

Universität Konstanz - Department of Biology: A. Bürkle, T. Sindlinger, M. Moreno-Villanueva, A. Keller.

University College London - Research Department of Infection and Population Health: C. Sabin, D. de Francesco.

Vlaams Instituut voor Biotechnologie - Inflammation research center: C. Libert, S. Dewaele.

Appendix 3

The Pharmacokinetic and Clinical Observations in People Over Fifty (POPPY) Study Group

POPPY Management Team:

Marta Boffito, Paddy Mallon, Frank Post, Caroline Sabin, Memory Sachikonye, Alan Winston

POPPY Scientific Steering Committee:

Jane Anderson, David Asboe, Marta Boffito, Lucy Garvey, Paddy Mallon, Frank Post, Anton Pozniak, Caroline Sabin, Memory Sachikonye, Jaime Vera, Ian Williams, Alan Winston

POPPY Sites and Trials Unit (alphabetical):

Caldecot Centre, King's College Hospital (Frank Post, Lucy Campbell, Selin Yurdakul, Sara Okumu, Louise Pollard)

Department of Infection and Population Health, University College London (Ian Williams, Damilola Otiko, Laura Phillips, Rosanna Laverick)

Elton John Centre, Brighton and Sussex University Hospital (Martin Fisher, Amanda Clarke, Jaime Vera, Andrew Bexley, Celia Richardson)

HIV Molecular Research Group, School of Medicine, University College Dublin (Paddy Mallon, Alan Macken, Bijan Ghavani-Kia, Joanne Maher, Maria Byrne, Ailbhe Flaherty)

Homerton Sexual Health Services, Homerton University Hospital (Jane Anderson, Sifiso Mguni, Rebecca Clark, Rhianon Nevin-Dolan, Sambasivarao Pelluri)

Ian Charleson Day Centre, Royal Free Hospital (Margaret Johnson, Nnenna Ngwu, Nargis Hemat, Martin Jones, Anne Carroll)

Imperial Clinical Trials Unit, Imperial College London (Andrew Whitehouse, Laura Burgess, Daphne Babalis)

St. Mary's Hospital London, Imperial College Healthcare NHS Trust (Alan Winston, Lucy Garvey, Jonathan Underwood, Matthew Stott, Linda McDonald)

St Stephen's Centre, Chelsea and Westminster Hospital (Marta Boffito, David Asboe, Anton Pozniak, Chris Higgs, Elisha Seah, Stephen Fletcher, Michelle Anthonipillai, Ashley Moyes, Katie Deats, Irtiza Syed, Clive Matthews)

POPPY Funders:

The POPPY study is funded from investigator initiated grants from BMS, Gilead Sciences, Janssen, Merck and ViiV Healthcare.

Other acknowledgements:

We acknowledge the use of the NIHR/Wellcome Trust Clinical Research Facility at King's College Hospital.

The research is supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the department of Health.

All the POPPY clinical sites in the UK are grateful for NIHR Clinical Research Network (CRN) support.

Appendix 4

simulated_CI_with_mahalanobis.R

```
# Simulated data to test the diagnostic criteria of HIV-associated cognitive impairment
# or why you should have an appropriate control group and use multivariate techniques to
reduce the false discovery rate
```

```
#
# Jonathan Underwood August 2016
require(MASS)
```

```
results_GDS = NULL
results_HAND = NULL
results_global = NULL
results_HAND3 = NULL
results_mahal_dist = NULL
```

```
# setup parameters for simulated data
n <- 10000 # number of 'subjects'
sd <- 10 # sd of simulated data
mean <- 50 # mean of simulated data
```

```
# generates data iteratively over a range of intervariable correlation coefficients
```

```
for(r in (seq(0.0,.99, 0.01))) {
```

```
  sigma <-matrix(c(1,r,r,r,r,r,
                   r,1,r,r,r,r,
                   r,r,1,r,r,r,
                   r,r,r,1,r,r,
                   r,r,r,r,1,r,
                   r,r,r,r,r,1), nrow = 6)
```

```
data.frame((mvrnorm(n, mu = rep(0,6), Sigma = sigma,empirical = TRUE) * sd) +mean) -> x
```

```
# checks for deficit scores using standard definition on T-score - see Carey (2004)
```

```
apply(x,2,function(x)
  ifelse(x>19,
    ifelse(x>24,
      ifelse(x>29,
        ifelse(x>34,
          ifelse(x>40,0,1),2),3),4),5)
  ) -> test
```

```
apply(test,1,function(x) mean(x)>=0.5) ->GDS # generates global deficit score
apply(test,1,function(x) sum(x>0)>1) -> HAND # Frascati (>1SD in 2 or more domains)
apply(x,1,function(x) sum(x<30)>1) -> HAND3 # Frascati (>2SD in 2 or more domains)
apply(x,1,function(x) mean(x))<45 -> global45
cbind(results_GDS, GDS )-> results_GDS
cbind(results_HAND, HAND) -> results_HAND
cbind(results_HAND3, HAND3) -> results_HAND3
cbind(results_global, global45) -> results_global
```

```
colMeans(x) -> means
cov(x) -> covar
```

```

apply(x,1, function(i) sign(mean(i)-mean)*sqrt(mahalanobis(i,center = means, cov = covar)))
-> mahal_dist
cbind(results_mahal_dist, -3.26 > mahal_dist) -> results_mahal_dist

}

results_GDS <- data.frame(results_GDS)
results_HAND <- data.frame(results_HAND)
results_HAND3 <- data.frame(results_HAND3)
results_global <- data.frame(results_global)
results_mahal_dist <- data.frame(results_mahal_dist)

HAND_prev = NULL
HAND3_prev = NULL
GDS_prev = NULL
HAND_GDS_prev = NULL
global_prev = NULL
mahal_prev = NULL

for(i in 1:100) {
  rbind(HAND_prev, sum(results_HAND[i]==T)/n * 100) -> HAND_prev
}
cbind(HAND_prev, seq(0.0, .99, 0.01)) -> HAND_prev
matplot(HAND_prev[,2], HAND_prev[,1], pch=20, xlab = "Correlation between cognitive tests",
ylab = "Prevalence of Frascati impairment 2 domains (%)")

for(i in 1:100) {
  rbind(HAND3_prev, sum(results_HAND3[i]==T)/n * 100) -> HAND3_prev
}
cbind(HAND3_prev, seq(0.0, .99, 0.01)) -> HAND3_prev
matplot(HAND3_prev[,2], HAND3_prev[,1], pch=20, xlab = "Correlation between cognitive
tests", ylab = "Prevalence of Frascati impairment 3 domains (%)")

for(i in 1:100) {
  rbind(GDS_prev, sum(results_GDS[i]==T)/n * 100) -> GDS_prev
}
cbind(GDS_prev, seq(0.0, .99, 0.01)) -> GDS_prev
matplot(GDS_prev[,2], GDS_prev[,1], pch=20, xlab = "Correlation between cognitive tests",
ylab = "Prevalence of GDS impairment (%)")

for(i in 1:100) {
  rbind(HAND_GDS_prev, sum(results_HAND[i] & results_GDS[i]==T)/n * 100) -> HAND_GDS_prev
}
cbind(HAND_GDS_prev, seq(0.0, .99, 0.01)) -> HAND_GDS_prev
matplot(HAND_GDS_prev[,2], HAND_GDS_prev[,1], pch=20, xlab = "Correlation between cognitive
tests", ylab = "Prevalence of Frascati and GDS impairment (%)")

for(i in 1:100) {
  rbind(global_prev, sum(results_global[i]==T)/n * 100) -> global_prev
}
cbind(global_prev, seq(0.0, .99, 0.01)) -> global_prev
matplot(global_prev[,2], global_prev[,1], pch=20, xlab = "Correlation between cognitive
tests", ylab = "Prevalence of global T<45 impairment (%)")
matplot(global_prev[,2], global_prev[,1], pch=20, xlab = "Correlation between cognitive
tests", ylab = "Prevalence of cognitive impairment (%)", col="green")
matplot(HAND_prev[,2], HAND_prev[,1], pch=20, add= T)
matplot(GDS_prev[,2], GDS_prev[,1], pch=20, col="red", add = T)

for(i in 1:100) {
  rbind(mahal_prev, sum(results_mahal_dist[i]==T)/n * 100) -> mahal_prev
}
cbind(mahal_prev, seq(0.0, .99, 0.01)) -> mahal_prev
matplot(mahal_prev[,2], mahal_prev[,1], pch=20, xlab = "Correlation between cognitive
tests", ylab = "Mahalanobis impairment (%)")

```

```

# combined graph using ggplot2
require(ggplot2)

qplot(x=HAND_GDS_prev[,2], y=HAND_GDS_prev[,1], xlab = "Correlation between cognitive
domains", ylab = "Prevalence of cognitive impairment (%)", shape="s", fill = "Frascati and
GDS", alpha=0.5)+
  scale_shape_manual(values = 21)+
  geom_point(aes(x=HAND_prev[,2], y= HAND_prev[,1], fill = "Frascati >1 SD, 2 domains"),
alpha = 0.5)+
  geom_smooth(mapping = aes(x=HAND_prev[,2], y= HAND_prev[,1], colour = "Frascati >1 SD, 2
domains"),se=FALSE)+
  geom_point(aes(x=HAND3_prev[,2], y= HAND3_prev[,1], fill = "Frascati >2 SDs, 2 domains"),
alpha = 0.5)+
  geom_smooth(mapping = aes(x=HAND3_prev[,2], y= HAND3_prev[,1], colour = "Frascati >2 SDs,
2 domains"),se=FALSE)+
  geom_smooth(mapping = aes(x=HAND_GDS_prev[,2], y=HAND_GDS_prev[,1], colour = "Frascati
and GDS"),se=FALSE)+
  geom_point(aes(x=global_prev[,2], y= global_prev[,1], fill = "Global T <45"), alpha =
0.5)+
  geom_smooth(mapping = aes(x=global_prev[,2], y= global_prev[,1],colour = "Global T
<45"),se=FALSE)+
  geom_point(aes(x=GDS_prev[,2], y=GDS_prev[,1], fill = "GDS"), alpha = 0.5)+
  geom_smooth(aes(x=GDS_prev[,2], y=GDS_prev[,1], colour = "GDS"),se=FALSE, alpha = 0.5)+
  geom_point(aes(x=mahal_prev[,2], y=mahal_prev[,1], fill = "Mahalanobis distance"), alpha
= 0.5)+
  geom_smooth(mapping = aes(x=mahal_prev[,2], y=mahal_prev[,1], colour = "Mahalanobis
distance"),se=FALSE)+
  theme_classic()+
  theme(
    axis.line.x = element_line(colour = 'black', size=0.5, linetype='solid'),
    axis.line.y = element_line(colour = 'black', size=0.5, linetype='solid')+
    scale_colour_manual(values=c("GDS" = "pink1", "Frascati >1 SD, 2 domains" =
"skyblue", "Frascati >2 SDs, 2 domains" = "darkblue", "Frascati and GDS" = "black", "Global T
<45" = "palegoldenrod", "Mahalanobis distance" = "darkseagreen1"), name=
"Diagnostic\\nmethod")+
    scale_fill_manual(values=c("GDS" = "pink1", "Frascati >1 SD, 2 domains" =
"skyblue", "Frascati >2 SDs, 2 domains" = "darkblue", "Frascati and GDS" = "black", "Global T
<45" = "palegoldenrod", "Mahalanobis distance" = "darkseagreen1"), name=
"Diagnostic\\nmethod")+
    guides(alpha=FALSE, fill=FALSE, shape=FALSE)

scale_shape_manual(values = 21)+
geom_point(aes(x=HAND_GDS_prev[,2], y=HAND_GDS_prev[,1], colour = "Frascati and GDS"),
alpha = 0.5)+
"skyblue", "pink1", "darkseagreen1", "palegoldenrod"

# peak prevalences
max(HAND_prev[,1]) # 25.9
HAND_prev[which(HAND_prev == max(HAND_prev[,1])),2] # 0.1
max(HAND3_prev[,1]) #
HAND3_prev[which(HAND3_prev == max(HAND3_prev[,1])),2] #
max(HAND_GDS_prev[,1]) #
HAND_GDS_prev[which(HAND_GDS_prev == max(HAND_GDS_prev[,1])),2] #
max(mahal_prev[,1]) #
mahal_prev[which(mahal_prev == max(mahal_prev[,1])),2] #
max(global_prev[,1]) # 31.17
global_prev[which(global_prev == max(global_prev[,1])),2] # 0.97
max(GDS_prev[,1]) # 21.8
GDS_prev[which(GDS_prev == max(GDS_prev[,1])),2] # 0.07

#### Cohen's K for Frascati and GDS ####
require(psych)

kappa = NULL

```

```

for(i in 1:101) {
  print((i/100 -0.01))
  cohen.kappa(table(results_GDS[i] == T, results_HAND[i] == T)) -> k
  rbind(kappa, k$confid[1,]) -> kappa
}

cbind(kappa, seq(0.0,1, 0.01)) -> kappa
data.frame(kappa) -> kappa

matplot(kappa[4], kappa[1], pch="-", xlab = "Correlation between cognitive tests", ylab =
"Cohen's kappa")
for(i in 1:101){
  segments(x0 = (i/100 -0.01), y0 = kappa[i,1], x1 = (i/100 -0.01), y1 = kappa[i,3], col =
"grey")
}
matplot(kappa[4], kappa[1], pch="-", add = T)
matplot(kappa[4], kappa[3], pch="-", add = T)
matplot(kappa[4], kappa[2], pch="20", add = T)

# graph using ggplot2
require(ggplot2)

qplot(x=kappa[4], y=kappa[2], xlab = "Correlation between cognitive tests", ylab = "Cohen's
kappa")+
  geom_smooth(colour = "black")+
  theme_classic()+
  theme(
    axis.line.x = element_line(colour = 'black', size=0.5, linetype='solid'),
    axis.line.y = element_line(colour = 'black', size=0.5, linetype='solid')) ->
plot.kappa6

sessionInfo()

require(corrplot)
require(colorRamps)
r=0.3
sigma <-matrix(c(1,r,r,r,r,r,
                r,1,r,r,r,r,
                r,r,1,r,r,r,
                r,r,r,1,r,r,
                r,r,r,r,1,r,
                r,r,r,r,r,1), nrow = 6)
data.frame(sigma)
colnames(sigma) <- c("Attention", "Executive function", "Language", "Memory", "Motor
Function", "Processing speed")
rownames(sigma) <- c("Attention", "Executive function", "Language", "Memory", "Motor
Function", "Processing speed")

```

with_extra_correlation_between_tests+_MD.R

```

# Simulated data to test the diagnostic criteria of HIV-associated cognitive impairment
# or why you should have an appropriate control group and use multivariate techniques to re
duce the false discovery rate
#
# Jonathan Underwood August 2016

require(MASS)

results_GDS = NULL
results_HAND = NULL
results_HANDdom = NULL

```

```

results_global = NULL
results_HAND3 = NULL
results_mahal_dist = NULL

# setup parameters for simulated data
n <- 10000 # number of 'subjects'
sd <- 10 # sd of simulated data
mean <- 50 # mean of simulated data
tests <- 12 # number of 'domains'/'tests'
offset <- 0.2 # additional correlation between tests of the same domain

# intradomain correlation = r2 - define how you like
#r2 <- r+offset
#ifelse(r2>1,r2<-1,r2)
r2 <-0.6
# generates data iteratively over a range of intervariable correlation coefficients

for(r in (seq(0.0,.99, 0.01))) {
  ifelse(r2<r,r2<-r,r2)
  sigma <-matrix(c(1,r2,r,r,r,r,r,r,r,r,r,r,
    r2,1,r,r,r,r,r,r,r,r,r,r,
    r,r,1,r2,r,r,r,r,r,r,r,r,
    r,r,r2,1,r,r,r,r,r,r,r,r,
    r,r,r,r,1,r2,r,r,r,r,r,r,
    r,r,r,r,r2,1,r,r,r,r,r,r,
    r,r,r,r,r,r,1,r2,r,r,r,r,
    r,r,r,r,r,r,r2,1,r,r,r,r,
    r,r,r,r,r,r,r,r,1,r2,r,r,
    r,r,r,r,r,r,r,r,r2,1,r,r,
    r,r,r,r,r,r,r,r,r,r,1,r2,
    r,r,r,r,r,r,r,r,r,r,r2,1), nrow = tests)

  data.frame((mvnrm(n, mu = rep(0,tests), Sigma = sigma,empirical = TRUE) * sd) +mean) ->
x

# checks for deficit scores using standard definintion on T-score - see Carey (2004)
apply(x,2,function(x)
  ifelse(x>19,
    ifelse(x>24,
      ifelse(x>29,
        ifelse(x>34,
          ifelse(x>40,0,1),2),3),4),5)
) -> test

apply(test,1,function(x) mean(x)>=0.5) ->GDS # generates global deficit score
apply(x,1,function(x) sum(x<40)>1) -> HAND # generates Frascati on each test
apply(x,1,function(x) sum(x<30)>1) -> HAND3 # generates HAD on each test
HANDdom=NULL
for(i in seq(from = 1, to = tests, by= 2)) {
  cbind(HANDdom,(rowSums(x[i:(i+1)])/2 <40)) -> HANDdom
}
# apply(HANDdom,1,function(x) sum(x>0)>2) -> HAND3 # Frascati (>1SD in 3 or more domain
s)
apply(HANDdom,1,function(x) sum(x>0)>1) -> HANDdom # generates Frascati on each domain
apply(x,1,function(x) mean(x)<45 -> global45
cbind(results_GDS, GDS )-> results_GDS
cbind(results_HAND, HAND) -> results_HAND
cbind(results_HANDdom, HANDdom) -> results_HANDdom
cbind(results_HAND3, HAND3) -> results_HAND3
cbind(results_global, global45) -> results_global

colMeans(x) -> means
cov(x) -> covar
apply(x,1, function(i) sign(mean(i)-mean)*sqrt(mahalanobis(i,center = means, cov = covar)

```

```

)) -> mahal_dist
  cbind(results_mahal_dist, -4.3 > mahal_dist) -> results_mahal_dist

}

results_GDS <- data.frame(results_GDS)
results_HAND <- data.frame(results_HAND)
results_HANDdom <- data.frame(results_HANDdom)
results_HAND3 <- data.frame(results_HAND3)
results_global <- data.frame(results_global)
results_mahal_dist <- data.frame(results_mahal_dist)

HAND_prev = NULL
HANDdom_prev = NULL
HAND3_prev = NULL
GDS_prev = NULL
HAND_GDS_prev = NULL
global_prev = NULL
mahal_prev = NULL

for(i in 1:100) {
  rbind(HAND_prev, sum(results_HAND[i]==T)/n * 100) -> HAND_prev
}
cbind(HAND_prev, seq(0.0, .99, 0.01)) -> HAND_prev
matplot(HAND_prev[,2], HAND_prev[,1], pch=20, xlab = "Correlation between cognitive tests",
ylab = "Prevalence of Frascati impairment - tests (%)")

for(i in 1:100) {
  rbind(HANDdom_prev, sum(results_HANDdom[i]==T)/n * 100) -> HANDdom_prev
}
cbind(HANDdom_prev, seq(0.0, .99, 0.01)) -> HANDdom_prev
matplot(HANDdom_prev[,2], HANDdom_prev[,1], pch=20, xlab = "Correlation between cognitive tests",
ylab = "Prevalence of Frascati impairment - 2 domains (%)")

for(i in 1:100) {
  rbind(HAND3_prev, sum(results_HAND3[i]==T)/n * 100) -> HAND3_prev
}
cbind(HAND3_prev, seq(0.0, .99, 0.01)) -> HAND3_prev
matplot(HAND3_prev[,2], HAND3_prev[,1], pch=20, xlab = "Correlation between cognitive tests",
ylab = "Prevalence of Frascati impairment - 3 domains (%)")

for(i in 1:100) {
  rbind(GDS_prev, sum(results_GDS[i]==T)/n * 100) -> GDS_prev
}
cbind(GDS_prev, seq(0.0, .99, 0.01)) -> GDS_prev
matplot(GDS_prev[,2], GDS_prev[,1], pch=20, xlab = "Correlation between cognitive tests",
ylab = "Prevalence of GDS impairment (%)")

for(i in 1:100) {
  rbind(HAND_GDS_prev, sum(results_HAND[i] & results_GDS[i]==T)/n * 100) -> HAND_GDS_prev
}
cbind(HAND_GDS_prev, seq(0.0, .99, 0.01)) -> HAND_GDS_prev
matplot(HAND_GDS_prev[,2], HAND_GDS_prev[,1], pch=20, xlab = "Correlation between cognitive tests",
ylab = "Prevalence of Frascati and GDS impairment (%)")

for(i in 1:100) {
  rbind(global_prev, sum(results_global[i]==T)/n * 100) -> global_prev
}
cbind(global_prev, seq(0.0, .99, 0.01)) -> global_prev
matplot(global_prev[,2], global_prev[,1], pch=20, xlab = "Correlation between cognitive tests",
ylab = "Prevalence of global T<45 impairment (%)")
matplot(global_prev[,2], global_prev[,1], pch=20, xlab = "Correlation between cognitive test

```

```

s", ylab = "Prevalence of cognitive impairment (%)", col="green")
matplot(HAND_prev[,2], HAND_prev[,1],pch=20, add= T)
matplot(GDS_prev[,2], GDS_prev[,1],pch=20, col="red", add = T)

for(i in 1:100) {
  rbind(mahal_prev, sum(results_mahal_dist[i]==T)/n * 100) -> mahal_prev
}
cbind(mahal_prev,seq(0.0,.99, 0.01)) -> mahal_prev
matplot(mahal_prev[,2], mahal_prev[,1],pch=20, xlab = "Correlation between cognitive tests"
, ylab = "Mahalanobis impairment (%)")

#### combined graph using ggplot2 ####

require(ggplot2)

qplot(x=GDS_prev[,2], y=GDS_prev[,1], xlab = "Correlation between cognitive domains", ylab =
"Prevalence of cognitive impairment (%)", shape=shape, fill = "GDS", alpha=0.5)+
  scale_shape_manual(values = 21)+
  geom_point(aes(x=HAND_prev[,2], y= HAND_prev[,1], fill = "Frascati >1 SD, 2 tests"), alph
a = 0.5)+
  geom_smooth(mapping = aes(x=HAND_prev[,2], y= HAND_prev[,1], colour = "Frascati >1 SD, 2
tests"),se=FALSE)+
  geom_point(aes(x=HANDdom_prev[,2], y= HANDdom_prev[,1], fill = "Frascati >1 SD, 2 domains
"), alpha = 0.5)+
  geom_smooth(mapping = aes(x=HANDdom_prev[,2], y= HANDdom_prev[,1], colour = "Frascati >1
SD, 2 domains"),se=FALSE)+
  geom_point(aes(x=HAND3_prev[,2], y= HAND3_prev[,1], fill = "Frascati >2 SDs, 2 tests"), a
lpha = 0.5)+
  geom_smooth(mapping = aes(x=HAND3_prev[,2], y= HAND3_prev[,1], colour = "Frascati >2 SDs,
2 tests"),se=FALSE)+
  geom_point(aes(x=global_prev[,2], y= global_prev[,1], fill = "Global T <45"), alpha = 0.5
)+
  geom_smooth(mapping = aes(x=global_prev[,2], y= global_prev[,1],colour = "Global T <45"),
se=FALSE)+
  geom_point(aes(x=GDS_prev[,2], y=GDS_prev[,1], fill = "GDS"), alpha=0.5)+
  geom_smooth(aes(x=GDS_prev[,2], y=GDS_prev[,1], colour = "GDS"),se=FALSE)+
  geom_point(aes(x=mahal_prev[,2], y=mahal_prev[,1], fill = "Mahalanobis distance"), alpha
= 0.5)+
  geom_smooth(mapping = aes(x=mahal_prev[,2], y=mahal_prev[,1], colour = "Mahalanobis dista
nce"),se=FALSE)+
  theme_classic()+
  theme(
    axis.line.x = element_line(colour = 'black', size=0.5, linetype='solid'),
    axis.line.y = element_line(colour = 'black', size=0.5, linetype='solid'))+
    scale_colour_manual(values=c("GDS" = "pink1", "Frascati >1 SD, 2 tests" = "black" , "Fr
ascati >1 SD, 2 domains" = "skyblue","Frascati >2 SDs, 2 tests" = "darkblue", "Global T <45
" = "palegoldenrod", "Mahalanobis distance" = "darkseagreen1"), name= "Diagnostic\nmethod")
+
    scale_fill_manual(values=c("GDS" = "pink1", "Frascati >1 SD, 2 tests" = "black" ,"Frasc
ati >1 SD, 2 domains" = "skyblue","Frascati >2 SDs, 2 tests" = "darkblue","Global T <45" =
"palegoldenrod", "Mahalanobis distance" = "darkseagreen1"), name= "Diagnostic\nmethod")+
    guides(alpha=FALSE, fill=FALSE, shape=FALSE)

geom_point(aes(x=HAND_GDS_prev[,2], y=HAND_GDS_prev[,1], colour = "Frascati and GDS"), alph
a = 0.5)+
  geom_smooth(mapping = aes(x=HAND_GDS_prev[,2], y=HAND_GDS_prev[,1], colour = "Frascati an
d GDS"),se=FALSE)+
  "Frascati and GDS" = "green",

# peak prevalences
max(HAND_prev[,1]) #
HAND_prev[which(HAND_prev == max(HAND_prev[,1])),2] #
max(HANDdom_prev[,1]) #
HANDdom_prev[which(HANDdom_prev == max(HANDdom_prev[,1])),2] #

```

```

max(HAND3_prev[,1]) #
HAND3_prev[which(HAND3_prev == max(HAND3_prev[,1])),2] #
max(GDS_prev[,1]) #
GDS_prev[which(GDS_prev == max(GDS_prev[,1])),2] #
max(HAND_GDS_prev[,1]) #
HAND_GDS_prev[which(HAND_GDS_prev == max(HAND_GDS_prev[,1])),2] #
max(mahal_prev[,1]) #
mahal_prev[which(mahal_prev == max(mahal_prev[,1])),2] #
max(global_prev[,1]) #
global_prev[which(global_prev == max(global_prev[,1])),2] #

#### Cohen's K for Frascati and GDS ####
require(psych)

kappa = NULL

for(i in 1:101) {
  print((i/100 -0.01))
  cohen.kappa(table(results_GDS[i] == T, results_HAND[i] == T)) -> k
  rbind(kappa, k$confid[1,]) -> kappa
}

cbind(kappa,seq(0.0,1, 0.01)) -> kappa
data.frame(kappa) -> kappa

matplot(kappa[4], kappa[1], pch="-", xlab = "Correlation between cognitive tests", ylab = "Cohen's kappa")
for(i in 1:101){
  segments(x0 = (i/100 -0.01), y0 = kappa[i,1], x1 = (i/100 -0.01), y1 = kappa[i,3], col = "grey")
}
matplot(kappa[4], kappa[1], pch="-", add = T)
matplot(kappa[4], kappa[3], pch="-", add = T)
matplot(kappa[4], kappa[2], pch="20", add = T)

# graph using ggplot2
require(ggplot2)

qplot(x=kappa[4], y=kappa[2], xlab = "Correlation between cognitive tests", ylab = "Cohen's kappa")+
  geom_smooth(colour = "black")+
  theme_classic()+
  theme(
    axis.line.x = element_line(colour = 'black', size=0.5, linetype='solid'),
    axis.line.y = element_line(colour = 'black', size=0.5, linetype='solid'))

# with the frascati criteria applied to domains

kappa2 = NULL

for(i in 1:101) {
  print((i/100 -0.01))
  cohen.kappa(table(results_GDS[i] == T, results_HANDdom[i] == T)) -> k
  rbind(kappa2, k$confid[1,]) -> kappa2
}

cbind(kappa2,seq(0.0,1, 0.01)) -> kappa2
data.frame(kappa2) -> kappa2

for(i in 1:101){
  segments(x0 = (i/100 -0.01), y0 = kappa2[i,1], x1 = (i/100 -0.01), y1 = kappa2[i,3], col = "red")
}

```

```

matplot(kappa2[4], kappa2[1], pch="-", add = T)
matplot(kappa2[4], kappa2[3], pch="-", add = T)
matplot(kappa2[4], kappa2[2], pch=20, add = T, col="red")

require(reshape)
names(kappa)[2] <- "Frascati >1SD, 2 tests"
names(kappa2)[2] <- "Frascati >1SD, 2 tests"

qplot(x=kappa[4], y=kappa[2], xlab = "Correlation between cognitive tests", ylab = "Cohen's
kappa", shape=shape, alpha =0.5, fill="Frascati >1 SD, 2 tests") +
  scale_shape_manual(values = 21)+
  geom_smooth(mapping=aes(x=kappa[4], y=kappa[2], colour="Frascati >1 SD, 2 tests"), se=FAL
SE)+
  geom_point(aes(y=kappa2[2], fill = "Frascati >1 SD, 2 domains"))+
  geom_smooth(mapping=aes(x=kappa[4], y=kappa2[2], colour="Frascati >1 SD, 2 domains"), se=
FALSE)+
  scale_fill_discrete()+
  scale_color_discrete(name="Model")+
  theme_classic()+
  theme(
    axis.line.x = element_line(colour = 'black', size=0.5, linetype='solid'),
    axis.line.y = element_line(colour = 'black', size=0.5, linetype='solid'))+
  guides(shape=F, alpha=F, fill=F)

```