The Role of the Central Nervous System as a Sanctuary Site for HIV due to Limited Penetration of Antiretroviral Drugs

Thesis submitted in accordance with the requirements of the University of Liverpool for the Degree of Doctor in Philosophy by Sam Nightingale

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Liverpool or any other University or Institute of learning.

Signature: _____

Date:_____

Statement of work performed

Collection of patient samples

I was the lead principal investigator (PI) for the PARTITION study (chapters 4, 5, 6, 8 and 9) which was funded by a personal MRC Fellowship. In addition I was the local PI at the Royal Liverpool Hospital and hence directly responsible for recruiting patients and performing lumbar punctures at that site. The CSF and plasma biomarker data in chapter 7 was from CHARTER study, led by Dr Scott Letendre. The Vietnamese cohort of patients with tuberculous meningitis described together with PARTITION data in chapter 8 was from a study led by Dr Estee Torok. I was not involved in study design or the recruitment of patients in CHARTER or the Vietnamese study.

Lab work

I was responsible for processing, aliquotting and cataloging all samples in the PARTITION biobank. DNA extraction was performed by myself and Adeniyi Olagunju. Genotyping for *CYP2B6*, *CYP2A6* and *ABCB1* (chapters 8 and 9) were performed by myself with the supervision and assistance of Dr Dan Carr in the Wolfson Centre for Personalised Medicine. Mass spectrometry antiretroviral drug level quantification in chapters 4, 5, 8 and 9 were performed by myself and others and others in the Department of Clinical Pharmacology and Therapeutics at the University of Liverpool (Dr Victoria Watson, Dr Alieu Amara, Dr Laura Else, Dr Dierdre Egan). I was involved in the mass spectrometry assay development; this work was primarily conducted by Dr Laura Else, Dr Victoria Watson and others in the Department of Clinical Pharmacology and Therapeutics at the University of Liverpool. HIV-1 RNA quantification (standard and sensitive assays) and genotypic resistance

characterisation in the PARTITION study (chapters 4 and 5) was performed by Dr Stacey King and Dr Apostolos Beloukas in Prof Anna Maria Geretti's HIV virology group in the Institute of Infection and Global health at the University of Liverpool. Quantification of CSF:serum albumin ratios (chapters 8 and 9) were performed by me. Measurement of CSF biomarkers by cytometric bead array in chapter 6 was performed with Dr Benedict Michael. Measurement of plasma and CSF biomarkers in chapter 7 were performed by investigators in the CHARTER group.

Statistical analysis

CYP2B6 linkage disequilibrium analysis in chapter 8 (*Figure 18*) was performed with Dr Dan Carr. All other statistical analysis was performed by me under the supervision of Dr Steven Lane in the Department of Biostatistics.

Abstract

The role of the central nervous system as a sanctuary site for HIV due to limited penetration of antiretroviral drugs, Dr Sam Nightingale.

Introduction. HIV-associated neurocognitive disorders appear to remain common despite access to antiretroviral therapy (ART). Penetration of ART into the central nervous system (CNS) is highly variable between drugs and between individuals. Cerebrospinal fluid (CSF) concentrations of many antiretroviral medications fall below the minimum inhibitory concentration for wild type virus. HIV-1 RNA can be detected in the CSF at greater levels than in plasma (CSF/plasma discordance), however the clinical significance of this is unclear, and the degree of difference considered pathological varies. Whether the CNS can act as a sanctuary site leading to persistent HIV detection in plasma is not known.

Methods. The PARTITION study recruited HIV positive adults from 13 UK clinical sites. Paired CSF and plasma was collected from patients undergoing LP for clinical indication (group A) and subjects with unexplained viraemia despite ART (group B). The study aimed to determine a) the prevalence of CSF/plasma discordance and factors associated with this occurrence, and b) the prevalence of HIV-1 RNA detection in CSF in those with HIV-1 RNA persistence in plasma. A sensitive assay detected HIV-1 RNA below 50 copies/ml in a subgroup and a cytometric bead array determined CSF biomarkers. A matrix of clinical features and CSF/plasma biomarkers was related to cognitive decline in subjects from the CHARTER study. Drug concentrations in CSF and plasma were measured by mass spectrometry assays and related to host genetic factors in subjects in the PARTITION study and a Vietnamese cohort with tuberculous meningitis.

Results. CSF/plasma HIV discordance occurred in 13% of this cohort and was associated with nadir, but not current, CD4 cell count. CSF/plasma discordance occurred in 7 of 40 (18%) of subjects with ongoing viral detection in plasma vs. 0 of 39 of those without. Residual HIV-1 RNA detection below 50 copies/ml was also associated with CSF HIV-1 RNA detection. Resistance associated HIV mutations were detected in CSF of subjects with CSF/plasma discordance. CSF/plasma discordance above $0.5\log_{10}$ was associated with raised profiles of inflammatory CSF proteomic biomarkers compared to those without discordance. In the CHARTER cohort, cognitive decline over 18 months was associated with lower concentrations of CSF TNFa and plasma IGF1/2. CSF concentrations of efavirenz were associated with the *CYP2B6* c.516G>T single nucleotide polymorphism. Efavirenz metabolites were mainly glucuronidated in CSF, were present at neurotoxic levels, and were related to degree of blood brain barrier permeability. Host genetic factors were did not relate to CSF DRV concentrations.

Conclusions. CSF/plasma discordance is a frequent occurrence, likely related to processes established during advanced immunosuppression not fully reversed by ART. It is associated with CSF HIV resistance and raised CSF biomarkers, even at levels $0.5\text{-}1\log_{10}$ which have been considered non-significant in some studies. Potentially neurotoxic CSF concentrations of efavirenz relate to host genetic factors.

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Vietnam for providing the CSF and plasma samples to efavienz metabolite analysis in chapter 8. I would like to thank all my co-authors on the Lancet Neurology Review article entitled “Controversies in HIV-Associated Neurocognitive Disorders” that forms the basis for the introduction chapter; Drs Alan Winston, Scott Letendre, Benedict Michael and Justin McArthur and Professors Saye Khoo and Tom Solomon.

I would particularly like to thank all those that worked on the PARTITION study across the thirteen clinical sites. The Principle Investigators Drs Alan Winston, Steve Taylor, Andy Ustianowski, Jonathan Ainsworth, Mark Nelson, Edmund Ong, Richard Gilson, Lewis Haddow, Jane Minton, Clare Van Halsema, Jo Baxter, Dr Balajhi, Frank Post and Professors Martin Fisher and Clifford Lean. All the Research Nurses that worked on the project, including Helen Rogers, Scott Mullaney, David Stacey, Sheila Morris, Anele Waters, Laura Phillips, Ashley Moyes, Lorna Murray, James Calderwood, Lucy Campbell, Gerry Gilleran, Wendy Hadley, Yvonne Clowes, Karen Martin and Scott Barclay. I am indebted to all the patients enrolled in PARTITION and other studies for providing the clinical samples which made this work possible.

Finally I would like to thank my family for their love and support. This PhD is dedicated to my father who retired this year; his passion for neurology and commitment to patients remains a constant inspiration to me.

Abbreviations

ART – antiretroviral therapy

ATZ – atazanavir

BHIVA – British HIV Association

CCL – chemokine (CC-motif) ligand

CMV - cytomegalovirus

CNS – central nervous system

CPE – CNS penetration effectiveness

CSF – cerebrospinal fluid

CT – computed tomography

CXCL10 - inducible protein 10

CYP – cytochrome p450

DNA – Deoxyribonucleic acid

DRV – darunavir

EACS - European AIDS Clinical Society

EBV – Ebstein Barr virus

EFZ - efavirenz

ETV – etravirine

FTC – emtricitabine

G-CSF - granulocyte-colony stimulating factor

GM-CSF - granulocyte-macrophage-colony stimulating factor

HIV – human immunodeficiency virus

ICAM - intracellular adhesion molecule

IFN – interferon

IL – interleukin

IL1RA – interleukin-1 receptor antagonist

IQR – interquartile range

IVDU – intravenous drug user

LP – lumbar puncture

LPV – lopinavir

MMP - matrix metalloproteinases

MPO – myeloperoxidase

MRC – Medical Research Council

MRI – magnetic resonance imaging

MSM – men who have sex with men

MVC – maraviroc

NRTI – nucleos(t)ide reverse transcriptase inhibitor

NNRTI – non-nucleos(t)ide reverse transcriptase inhibitor

NVP – nevirapine

7OH EFZ – 7 hydroxy efavirenz

8OH EFZ – 8 hydroxy efavirenz

PARTITION - Penetration of AntiRetroviral Therapy InTO the Nervous system study

PI – protease inhibitor

PK - pharmacokinetics

RAL – raltegravir

RNA – ribonucleic acid

RPV – rilpivarine

RT – reverse transcriptase

RTV – ritonavir

SNP - single nucleotide polymorphism

TB – tuberculosis

TBM – tuberculous meningitis

3TC – lamivudine

TDF – tenofovir

TNF - tumour necrosis factor

TNFR - tumour necrosis factor soluble receptor

TRAIL - tumour necrosis factor alpha related apoptosis inducing ligand

UGT – UDP glucuronosyltransferases

VCAM - vascular cell adhesion molecule

VEGF - vascular endothelial growth factor

VZV – Varicella Zoster virus

WCC – white cell count

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1 Introduction

1.1 Background

Worldwide, approximately 34 million people are chronically infected with Human Immunodeficiency Virus (HIV) Type 1.[1] In the UK over 90,000 people are infected, a quarter of whom are unaware of their diagnosis, and numbers continue to rise.[2] Antiretroviral therapy (ART) has revolutionised the treatment of HIV disease. Many now live healthily for many decades while on treatment and life expectancy for those with access to modern treatment now approaches that of uninfected cohorts.[3] HIV enters the brain early in disease via migrating myeloid and lymphoid cells and establishes productive infection in perivascular macrophages and microglia. Restricted infection also occurs in astrocytes.[4] Before the widespread use of ART, severe cognitive impairment was common affecting up to 50% of HIV infected individuals prior to death.[5] In countries where ART has become widely available, the incidence of HIV-associated dementia (HAD) has dramatically declined along with other Acquired Immune Deficiency Syndrome (AIDS)-defining conditions.[6] However in this era of more potent ART we continue to observe cognitive disorders. This has several possible underlying pathogenic mechanisms. Distribution of ART into the CNS can be poor, and ART concentrations in cerebrospinal fluid (CSF) fall below the inhibitory concentration for wild type virus for several drugs.[7] This may have clinical consequences since some patients have detectable HIV ribonucleic acid (RNA) in the CSF even when it is undetectable in blood.[8, 9] Other potential mechanisms include a legacy effect of CNS damage sustained prior to ART initiation, persistent immune and glial activation, ART neurotoxicity, and indirect effects of co-morbid conditions associated with HIV disease. The impact of this pathology may be evidenced by the finding that HIV-associated neurocognitive disorders (HAND), particularly milder

forms, persist in cohorts with access to treatment.[10, 11] Some studies have suggested that as many as half of HIV-seropositive people in Europe and the USA may have cognitive impairment, which in many cases appears to be either asymptomatic or does not cause functional incapacity.[12]

However, controversy exists in the field regarding several of these points. HAND prevalence estimates vary depending on the population and assessment methods.[13-19] Several guidelines now recommend screening for HAND, although the most appropriate populations to target and the best tools to use have not been determined for every clinical environment.[20-22] The clinical significance of identifying asymptomatic impairment is not fully understood and there are uncertainties around the most suitable investigation and management of patients identified as cognitively impaired.[23-25] Although persistently detectable HIV RNA in the CSF may herald progressive CNS damage, reports have not found that it consistently relates to cognitive functioning.[26] Evidence suggests that some antiretroviral drugs are more effective in the CNS than others,[7] but this conclusion depends on how effectiveness is estimated. Reports focusing on cognitive functioning have not consistently found differences between antiretrovirals, although methods vary substantially between studies and few randomised trials have been performed.[27-29] This introduction chapter reviews these important clinical issues and provides approaches for resolving key issues of debate.

1.2 Antiretroviral therapy

Modern ART typically comprises a combination of at least three drugs.[30]

Nucleos(t)ide reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) inhibit reverse transcriptase. Reverse transcriptase (RT) is an HIV-specific DNA polymerase used to generate complimentary DNA from an RNA template. NRTIs were the first antiretroviral drugs to be discovered. Purine and pyrimidine nucleosides are required for proviral DNA elongation. NRTIs are structurally similar to DNA nucleoside bases. They become incorporated into the proviral DNA preventing incorporation into the host cell genome. NRTIs are prodrugs and undergo phosphorylation by intracellular kinases.[31] Most antiretroviral regimens involve two NRTIs in combination to form an NRTI "backbone" (*Table 1*). NNRTIs non-competitively bind the p66 subunit at a site distant from the active site of the enzyme. This induces a conformational change in RT and alters the active site. NNRTIs are metabolised by the cytochrome P450 system via a number of specific isoenzymes (eg, *CYP2B6, CYP2A6, CYP3A4, CYP2C9*), and via uridine diphosphate glucuronosyltransferase (UGT) enzymes.[32] Significant induction and inhibition occurs and can result in the potential for drug interactions (*Table 2*).

Protease inhibitors (PIs) prevent cleavage of polypeptides to form functional subunits for viral capsid formation by competitively binding to HIV protease, a 99-amino-acid, aspartate protease that is responsible for maturation of HIV virus particles. PIs undergo significant first pass metabolism by *CYP3A4* and *3A5*. They are also substrates for the p-glycoprotein efflux transporter.[33] This results in significant variability in PI pharmacokinetics between and within individuals. Ritonavir blocks intestinal and hepatic *CYP3A* metabolism. It is frequently co-administered at low-dose (100-200mg) with other PIs to improve pharmacokinetic availability and "boost" their effect, thus decreasing the variability in pharmacokinetics.[33]

Raltegravir is the most commonly used integrase inhibitor. Integrase inhibitors competitively inhibit the strand transfer reaction of proviral integration. This prevents or inhibits the binding of the pre-integration complex to host cell DNA, terminating the integration step of HIV replication.[34]

HIV entry inhibitors include chemokine receptor antagonists and fusion inhibitors. The chemokine receptor 5 (CCR5) antagonist maraviroc selectively binds the CCR5 co-receptor, preventing fusion of cell membranes following binding of HIV to the CD4 receptor. As such it is only effective for virus that uses the CCR5 co-receptor (termed CCR5 tropic virus) rather than CXCR4 tropic or dual/mixed tropic virus.[35]

The fusion inhibitor enfuvirtide acts extracellularly to prevent the fusion of HIV to the CD4 cell by binding to the HR1 region of glycoprotein 41 (gp41) resulting in a conformational change.[36] Enfuvirtide requires twice daily sub-cutaneous injection.

	Preferred	Alternative
NRTI	Tenofovir and emtricitibine	Abacavir and lamivudine [1]
3rd agent	Atazanivir/ritonavir	Fosamprenavir/ritonavir
	Darunavir/ritonavir	Lopinavir/ritonavir
	Efavirenz	Nevirapine [2]
	Raltegravir	Rilpivarine [3]

Table 1. Recommended first line antiretroviral therapy in UK

*[1] Abacavir is contraindicated if HLA-B*57:01 positive.*

[2] Nevirapine is contraindicated if baseline CD4 cell count is greater than 250/400 cells/mL in women/men.

[3] Use recommended only if baseline VL <100 000 copies/mL: rilpivirine as a third agent, abacavir and lamivudine as non-nucleoside reverse transcriptase inhibitor (NRTI) backbone.

Adapted from the British HIV Association guidelines for the treatment of HIV-1-positive adults with antiretroviral therapy 2012, updated Nov 2013.[30]

Class/drug	Metabolism	Induces	Inhibits	Transport	Protein binding	Plasma half life	CSF:plasma ratio
Nucleoside reverse transcriptase inhibitors							
ABC	ADH, UGT	None observed	BRCP, MRP1, MRP2	P-gp, BCRP1	~49%	1.5 h	30-44%
FTC	Limited hepatic metab	Possibly CYP450	MRP1, MRP2, MRP3	None	<4%	~10 h	43%
3TC	Hepatic metab low (5-10%)	na	MRP1, MRP2, MRP3	Possibly MRP4, MRP8	<36%	5-7 h	~12%
TDF	No P450 involvement.	na	MRP1, MRP2, MRP3	OATP 1 and 3, MRP4	<0.7%	~12-18 h	na
d4T	Unknown	na	Not P450	Unknown	Negligible	1.3-2.3 h	39 ± 6%
ddI	Xanthine oxidase	na	na	na	<5%	~1.4 h	~21%
AZT	UGT. No P450 involvement.	na	BCRP	BCRP1	34-38%	1.1 h	50%
Non-nucleoside reverse transcriptase inhibitors							
EFZ	CYP3A4, CYP2B6	CYP3A4	CYP2C9, CYP2C19, CYP3A4; BCRP; MRP1, MRP2, MRP3	Unknown	>99%	40-55 h	0.26-1.19%
ETV	CYP3A4, CYP2C9, CYP2C19	CYP3A4	CYP2C9, CYP2C19	Unknown	99.9%	41 h	na
NVP	CYP3A4, CYP2B6	CYP3A4 +/- CYP2B6	BCRP; MRP1, MRP2, MRP3	Unknown	~60%	25-30 h	45 ± 5%
RPV	CYP 3A +/- CYP2A19.	na	Possibly P-gp	Not by P-gp	99.7%	~50 h	na
Protease inhibitors							
ATV/r	CYP3A4	P-gp, MRP1	CYP3A4, UGT1A1, CYP2C8, BCRP, P-gp, MRPs, OATPs	P-gp, MRPs, BCRP	~86%	8.6 h	0.21- 2.26%
DRV/r	CYP3A4	CYP2C9, CYP2C19, CYP2C8	CYP3A4, CYP2D6, P-gp, OATPs	P-gp	~95%	15 h	na
FOS/r	CYP3A4	Possibly CYP3A4	CYP3A4, BCRP, P-gp, MRP1, OATPs	P-gp	~90%	15-23 h	na
IND/r	CYP3A4	na	CYP3A4, P-gp, MRP1, OATP-C	P-gp, MRP1	~60%	1.8 ± 0.4 h	14%
LPV/r	CYP3A	na	CYP3A, BCRP	P-gp, MRP1, MRP2, OATPs	98-99%	5-6 h	0%
RTV	CYP3A, CYP2D6	CYP1A2, CYP2C8, CYP2C9, CYP2C19, MRP1	CYP3A, CYP2D6, P-gp, MRP1, OATP-C, BCRP	P-gp, MRP1	98-99%	~5 h (100 mg bd/od)	0-52%
Fusion/entry/integrase inhibitors							
MVC	CYP3A4	na	Possibly CYP2D6	P-gp	~ 76%	13.2 h	na
RAL	UGT1A1. No CYP450	na	na	na	~83%	~9 h	na
T20	No CYP450	na	na	na	92%	~3.8 h	na

Table 2. Key pharmacokinetic parameters of the major antiretroviral drugs[37]

BCRP, breast cancer resistance protein; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein; ADH, alcohol dehydrogenase; UGT, uridine 5'-diphospho-glucuronosyltransferase; CYP, cytochrome P450; OATP, organic anion transporting polypeptides; na, not available.

1.3 Are some antiretroviral drugs more effective than others in the CNS?

Evidence supports the concept that ART generally protects the CNS: current regimens suppress CSF HIV RNA in the majority of adherent patients, have reduced the incidence of HAD, and have improved histopathological findings in brain tissue of patients dying with HIV disease.[38, 39] A central question is whether some ART drugs are more effective in the brain than others.

Several drug characteristics influence the distribution of ART into the CNS including molecular weight, protein binding, fat solubility, and influx/efflux transporters on brain endothelium.[40] As a result there is a wide range of CSF ART concentrations across different ART classes and individual drugs within a class. A hierarchy of drug distribution into the CNS has been proposed based on drug chemical properties, pharmacokinetics, and effectiveness in clinical studies.[7] The CNS Penetration Effectiveness (CPE) score assigns each drug an integer between one and four (*Table 3*), with one indicating below average estimated effectiveness and four indicating much above average estimated effectiveness. The CPE score is the sum of all antiretrovirals in a regimen. The score was devised as a research tool but has been used by some to guide prescribing in those with neurological complications of HIV. However the score has several limitations, as described below, and the benefits of its use in the clinic are unproven. A consensus report from the Mind Exchange Working Group, a group of 66 specialists from 30 countries, suggests that for patients with persistent or worsening neurocognitive impairment and detectable CSF HIV RNA, modifying treatment according to CPE score may be an option when other factors (e.g., poor adherence, virological resistance and comorbidities) have been

considered.[20] The CPE score is not part of the European AIDS Clinical Society (EACS) guidelines for the treatment of cognitive impairment – these guidelines refer instead to use of "CNS-active" ART; an approach to categorisation that has some overlap with drugs that are "above average" using the CPE approach.[22] The current British HIV Association (BHIVA) guidelines expressly advise against use of the CPE method to guide prescribing.[41]

	4	3	2	1
NRTIs	Zidovudine	Abacavir	Didanosine	Tenofovir
		Emtricitabine	Lamivudine	Zalcitabine
			Zalcitabine	
			Stavudine	
NNRTIs	Nevirapine	Delavirdine	Etravirine	
		Efavirenz		
PIs	Indinavir-r	Darunavir-r	Atazanavir-r	Nelfinavir
		Fosamprenavir-r	Atazanavir	Ritonavir
		Indinavir	Fosamprenavir	Saquinavir-r
		Lopinavir-r		Saquinavir
				Tipranavir-r
Fusion/Entry Inhibitors		Maraviroc		Enfuvirtide
Integrase Inhibitors		Raltegravir		

4 = much above average, 3 = above average, 2 = average, 1 = below average; NNRTIs = non nucleoside reverse transcriptase inhibitors; NRTIs = nucleoside reverse transcriptase inhibitors; PIs = protease inhibitors

Table 3. CNS Penetration Effectiveness (CPE) 2010 ranking scale.[7]

CPE score is sum of the individual scores of all drugs in regime.

In cohort studies, the CPE score has been statistically significantly associated with CSF HIV RNA; the greater the regimen CPE score the lower the proportion of patients with detectable CSF HIV RNA.[24, 42] To date the evidence linking higher CPE scores to improved cognitive performance has been mixed.[27-29] A systematic review using rigorous methods concluded that antiretrovirals with higher estimated CNS penetration were effective in improving cognitive function and decreasing CSF HIV RNA levels, but only two of the included studies were adequately statistically powered and all were observational.[43] The only randomised trial to date of better CNS penetrating ART for treatment of HAND used a "CNS-targeting" approach that took CPE into account when selecting a new ART regimen. This trial found no difference in the neurocognitive outcome 16 weeks after the ART change, although it was underpowered, enrolling only 49 of the planned 120 subjects.[44] A planned subgroup analysis of subjects who entered the trial with suppressed plasma HIV RNA did identify a trend towards neurocognitive benefit in the CNS-targeted arm. Subjects in the CNS-targeted arm, however, also trended towards poorer plasma virologic suppression at 16 weeks, supporting the idea that prescribing solely on the basis of estimates of CNS penetration, rather than proven efficacy, may lead to unacceptable clinical outcomes.

One theory is that antiretrovirals with the lowest CNS concentrations, as estimated by CSF drug levels or CPE score, are more likely to develop resistance mutations in the CNS than in the periphery. The findings in the literature to date are mixed,[45, 46] although a high viral genetic diversity suggesting autonomous replication in the CNS

has been associated with low CPE score.[47] An adjusted CPE score has been proposed, taking into account virological resistance mutations in the CSF.[48]

One criticism is that the CPE score is heavily reliant on pharmacokinetic data from CSF, not brain tissue itself. Although CSF is commonly used as a surrogate for brain events, drug concentrations in CSF may not accurately reflect drug concentrations in brain tissue. Drug concentrations in CSF and brain tissue can differ for several reasons, including the differing characteristics of the blood-brain and blood-CSF barriers.[49] Indeed drug concentrations can differ between the brain tissue and CSF,[50] as well as between different brain regions.[51] While currently impossible to ascertain in living people, the concentration that may best predict ART effectiveness in the brain is that within perivascular macrophages, a critical target cell of HIV in the brain.[4] NRTIs and NNRTIs are not generally effective in chronically infected macrophages and the 90% inhibitory concentration (IC90) at which PIs inhibit replication is higher in macrophages than in T lymphocytes.[52, 53]

Direct comparisons of individual antiretroviral drugs is challenging since this requires administration of monotherapy, a practice that is uncommon in most clinics. In a 1993 report, zidovudine showed efficacy against HAD.[54] Since then, few drugs have been assessed in isolation in controlled trials. Recently simplifying maintenance treatment to a single PI has been investigated as an approach that might reduce toxicity while maintaining full viral suppression. Concern exists that PI monotherapy may not control HIV replication in the brain since PI drug concentrations in the CSF can be low, perhaps because many are substrates for drug efflux transporters.[55] Despite this

concern, PI monotherapy was not associated with cognitive impairment when compared with triple drug ART in a recent observational study of 191 subjects.[56]

1.3.1 Neurotoxicity

Evidence is mounting that at least some modern antiretroviral drugs can cause neurotoxicity.[25, 57, 58] The relationship between the drug concentration required to inhibit HIV replication in the CNS and the concentration required to cause neurotoxicity likely differs by drug. As such it does not necessarily follow that the drugs that have the highest distribution into the CNS will also be the most neurotoxic. The ultimate goal is to identify which drugs best balance efficacy and toxicity, maintaining concentrations in the so-called "therapeutic window".

In addition to direct injury of neurons, ART may also indirectly alter brain functioning via immune, glial or endothelial effects. One potential mechanism is a low-grade immune reconstitution inflammatory syndrome (IRIS) in the absence of underlying lesion leading to chronic lymphocyte activation and persistent inflammation in the CNS in those on ART.[26, 59, 60] Such events present challenges for neurocognitive recovery and eradication of HIV from the CNS.

1.4 What is the clinical significance of HIV RNA detection in the CSF?

Systemic HIV replication is a risk factor for cognitive impairment and HIV RNA in blood has been independently associated with cognitive impairment.[12] Cognition typically improves following virological control with effective ART, even if the subject was not functionally impaired at baseline.[61] This has been elegantly

demonstrated in animals: Rhesus macaques experimentally infected with simian immunodeficiency virus (SIV) had only 70% the locomotor activity of those uninfected. Following effective ART, locomotor activity returned to pre-infection levels.[62]

Only 19% of all HIV infected individuals in the US are estimated to achieve suppression of HIV in blood;[63] only 80% are aware of their diagnosis and fewer still have been linked to HIV care, stay in HIV care, receive ART, and are able to adhere to their treatment. While aviraemic patients taking ART may represent a minority of the total HIV-infected population, the prevalence of HAND in this population is substantially lower than in those who are taking either no, or ineffective, ART. A UK cross-sectional study of 100 asymptomatic subjects, all aviraemic on effective ART, found cognitive impairment in just 19% overall, similar to that expected in an HIV negative population.[64, 65]

Plasma HIV RNA levels can strongly correlate with those in CSF. Whether HIV replication within or outside the nervous system, or both, leads to CNS injury remains unknown.

1.4.1 CSF/plasma discordance

Modern ART can control systemic viraemia well but concern has arisen that it may not fully control HIV replication in the CNS where drug levels can be much lower. Markers of neuroinflammation and neurodegeneration remain raised in the CSF of ART treated patients. Such markers include beta-2-microglobulin (a marker of

cytotoxic T-lymphocytes in the CNS), neopterin (a marker of macrophage/monocyte activation), S100B (a marker of astrocytosis), neurofilament light chain (a major structural protein of neurons) and a number of pro-inflammatory modulators including interleukins, interferons, chemokines and tumor necrosis family proteins.[26] Neuroimaging studies of ART treated patients have shown reduced brain volume, changes in brain metabolites on magnetic resonance spectroscopy and reduced white matter structural integrity on diffusion tensor imaging.[66] Neuropathologic changes also persist in ART treated patients and correlate to cognitive dysfunction.[67]

CSF/plasma discordance describes HIV RNA at higher levels in CSF than in plasma. There is currently no consensus on the degree of discordance between CSF and plasma HIV-1 RNA that should be considered clinically significant. Some studies have defined discordance as CSF HIV-1 RNA $1\log_{10}$ greater than the corresponding plasma sample,[68] while others have used a lower cut off of $0.5\log_{10}$.[8] In those with unquantifiable HIV-1 RNA in plasma (typically <50 copies/ml) some studies have described the detection of CSF virus at any level above 50 copies/ml (termed "CSF viral escape"),[9] while others have excluded those with CSF HIV-1 RNA below 200 copies/ml.[8, 68]

Sensitive testing can now detect HIV-1 RNA below the traditional threshold of 50 copies/ml. A proportion of patients have CSF/plasma discordance detected on these assays that would not otherwise be classified as discordant if tested with standard assays.[39, 69] The source and significance of CSF/plasma discordance at this low level remains controversial.[20, 70] Sensitive testing is currently a research tool. However assays with a lower limit of quantification of 20 copies/ml are now being used

routinely in many clinical centres and consequently CSF/plasma discordance below 50 copies/ml is an increasingly recognised phenomenon in clinical practice.

Depending on the definition used, CSF/plasma discordance occurs in approximately 10% of individuals undergoing lumbar puncture in clinical practice.[8, 9, 39, 71] Studies in which a lumbar puncture was performed solely for research purposes in asymptomatic individuals found the prevalence of CNS/plasma discordance to be lower.[9, 39] Although common, the degree to which CSF/plasma discordance leads to CNS injury and HAND remains uncertain. CSF/plasma discordance has been associated with a number of fulminant syndromes in case series including reduced conscious level, dementia and status epilepticus, but these appear to be uncommon in clinical practice.[48, 68] In untreated subjects in the pre-ART era, greater levels of HIV RNA in the CSF were associated with HAD,[72-75] however the association between CSF HIV RNA and cognitive function in treated subjects in the era of potent ART is less clear.[26, 73, 76]

CSF analysis from the large US National Institute of Mental Health-funded CNS HIV AntiRetroviral Therapy Effects Research (CHARTER) cross-sectional cohort study showed that the absolute level of CSF HIV RNA was not related to cognitive performance but the presence of discordant virus was.[39] However this association was present using an assay that can detect HIV RNA at 2 copies/ml, a standard that is much more sensitive than currently used in clinical practice. Other studies have demonstrated no association between CSF HIV RNA levels and cognitive performance but few have used such sensitive assays.[77] The Mind Exchange consensus report recommends using a sensitive assay to quantify HIV RNA to 1-2.5

copies/mL in those with HIV RNA less than 50 copies/ml in both CSF and plasma, who have cognitive impairment confirmed on neuropsychological testing without alternative explanation.[20] This recommendation is based on two reports at international conferences that have not yet been published in peer-reviewed journals;[39, 69] the merits of this approach require further investigation.

One explanation for the weakness of association between CSF HIV RNA and cognitive functioning is that CSF HIV RNA levels are only an imperfect proxy for HIV replication in brain tissue. While regional brain levels correlate to some extent with CSF levels, this correlation is not always strong, hence levels of HIV RNA in the CSF may not always be an accurate surrogate for production of HIV virions (and viral proteins) within the brain.[78, 79] Another explanation relates to the pathogenesis of cognitive impairment in HIV; if this is a result of immune dysregulation rather than virus-related cytopathy, then CSF HIV RNA may not be the most useful biomarker. In fact, in large cohorts, cognitive impairment appears to correlate more strongly with severity of prior immunosuppression than with CSF HIV RNA.[80] One cohort study showed that 21% of 203 patients with AIDS progressed to HAD despite being aviraemic with undetectable HIV RNA in CSF.[77] Based on this and other published findings, a combination of biomarkers reflecting neuronal injury and glial or immune activation in combination with CSF HIV RNA may be more useful than CSF HIV RNA alone.[26, 81] However, the clinical validity, reproducibility, and cost-effectiveness of these biomarkers have not been evaluated.

1.4.2 Can antiretroviral drug resistance develop in the CNS?

If HIV can continue to replicate in the CNS in the presence of sub-therapeutic levels of ART, drug resistance could develop. Studies have found mutations conferring resistance to several classes of ART in the CSF when they were not present in blood.[82-84] In two separate case series of patients with new onset neurocognitive impairment or other neurological symptoms, and CSF/plasma discordance, the majority had resistance-associated mutations detected in CSF.[48, 68] Altering ART based on drug resistance mutations in CSF led to clinical improvement and decrease in CSF HIV RNA in both series.

In addition to differences in the drug resistance patterns between CSF and plasma, HIV may differ in terms of pathogenicity, tropism, and co-receptor use. These differences may be due to adaptation of the virus to CNS target cells, or to differences in exposure to ART in the CNS compartment.[85, 86] The degree of compartmentalisation is associated with duration of infection, past immune suppression, and risk of HAD. Of note, indicators of compartmentalisation do not differ between those with mildly impaired cognitive function and unimpaired patients, supporting the hypothesis that the impact of HIV genetics on neuropathogenesis differs between milder and more severe forms of HAND.[87, 88] One reason may be that the most severe form of HAND, HAD, is associated with the most advanced immunosuppression, which may allow increased viral replication and accelerate viral evolution in the CNS.

Although drug resistance testing of CSF is not currently recommended for the routine management of asymptomatic patients, it may be useful in those with neurocognitive impairment and other neurological disorders and forms part of the current EACS, BHIVA and Mind Exchange guidelines.[20, 22, 41] What is not clear, however, are the implications of viral resistance in the CNS for HIV treatment failure systemically. The goal of antiretroviral therapy (ART) is sustained suppression of HIV-1 RNA in plasma, commonly interpreted as a viral load <50 copies/ml.[41] Some ART treated individuals do not maintain viral suppression below this level; HIV-1 RNA above 50 copies/ml may be intermittent "blips" or persistent low-level viraemia. HIV-1 RNA levels between 50 and 400 copies/ml have not been associated with long-term virological failure in most studies,[89] however it is not known whether some cases of HIV-1 detection at this level may represent ongoing replication in sanctuary sites such as the brain. It is not clear whether such patients should be offered a lumbar puncture or have changes made to ART based on estimated CNS penetration; indeed clinical practice varies in this regard. More work is needed to determine the clinical applications of CSF HIV resistance testing and the implications of drug resistant mutations in the CSF for systemic HIV control.

1.5 How common are HIV-associated neurocognitive neurocognitive disorders in the antiretroviral era?

HAND is defined by the widely used research classification, the Frascati criteria (*Figure 1*). The Frascati criteria divide HAND into asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND) and HIV-associated dementia (HAD). CNS problems in HIV infection remain a major public health

problem in resource poor settings, however in this section I will focus on the problem of HAND in populations with access to potent ART. Cross-sectional studies indicate that HAND remains common in industrialised nations in the era of widespread ART availability (*Figure 2*). The CHARTER study reported cognitive impairment in 53% of 1555 HIV-seropositive patients.[12] This cohort was designed to reflect the clinic population and, as such, it includes all causes of cognitive impairment occurring in people with HIV. Among the 843 people of this cohort who had minimal neuropsychiatric confounding conditions (i.e., no conditions, other than HIV disease accounting for reduced neurocognitive functioning), 40% met criteria for cognitive impairment. Multiple other studies estimate HAND prevalence between 20 and 50%,[13-19] with some finding that HAND could occur in as much as 69% of the HIV-seropositive population despite adequate ART.[27] This wide range of prevalence estimates has resulted in uncertainty about the prevalence that best describes cognitive impairment due to HIV pathology in populations with access to ART.

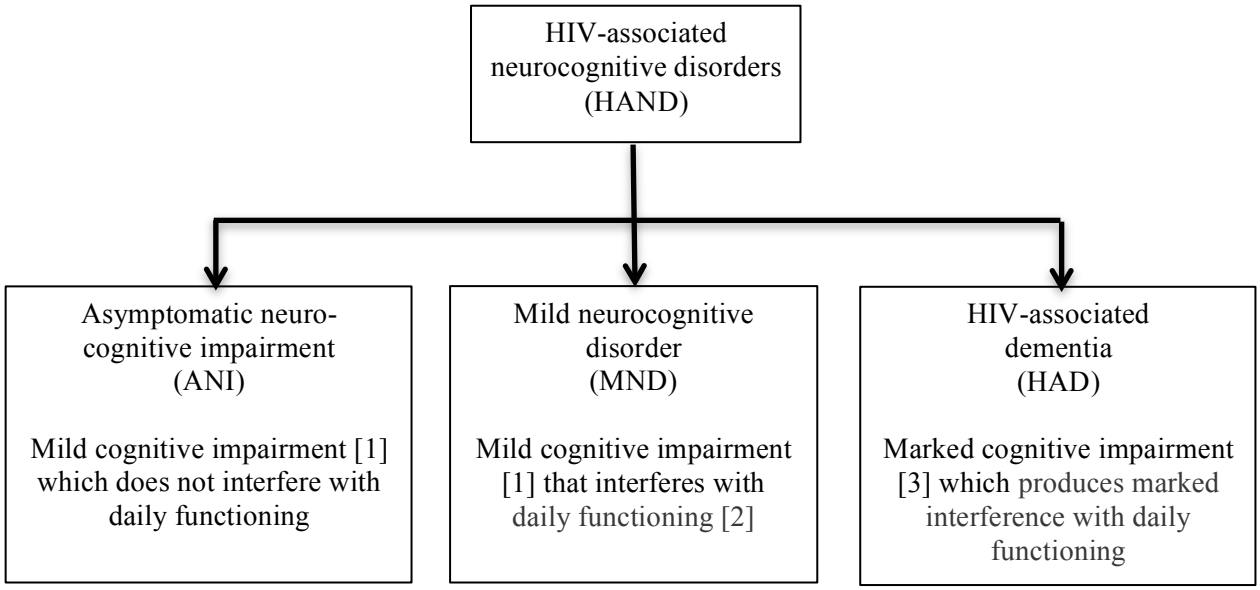

Figure 1. Summary of Frascati criteria for HIV-associated neurocognitive disorders.[90]

[1] Performance on neuropsychological tests of at least 1 standard deviation below age-education corrected norms in at least two cognitive domains. The assessment must survey at least the following domains, if possible with at least two test measures per domain: verbal/language; attention/working memory; abstraction/executive; memory (learning; recall); speed of information processing; sensory-perceptual, motor skills. Impairment must be acquired, cannot be explained by other comorbidities, and there is no evidence of delirium or preexisting cause.

[2] Daily functioning can be formally assessed by Lawton & Brody's modified Activities of Daily Living scale and/or the Patient's Assessment of Own Functioning Inventory.[91, 92]

[3] Performance on neuropsychological tests of at least 2 standard deviations below demographically corrected mean for norms in at least two cognitive domains. Typically the impairment is in multiple domains, especially in learning of new information, slowed information processing, and defective attention/concentration.

The pattern of cognitive impairment does not meet criteria for delirium and there is no evidence of another, preexisting cause for the dementia.

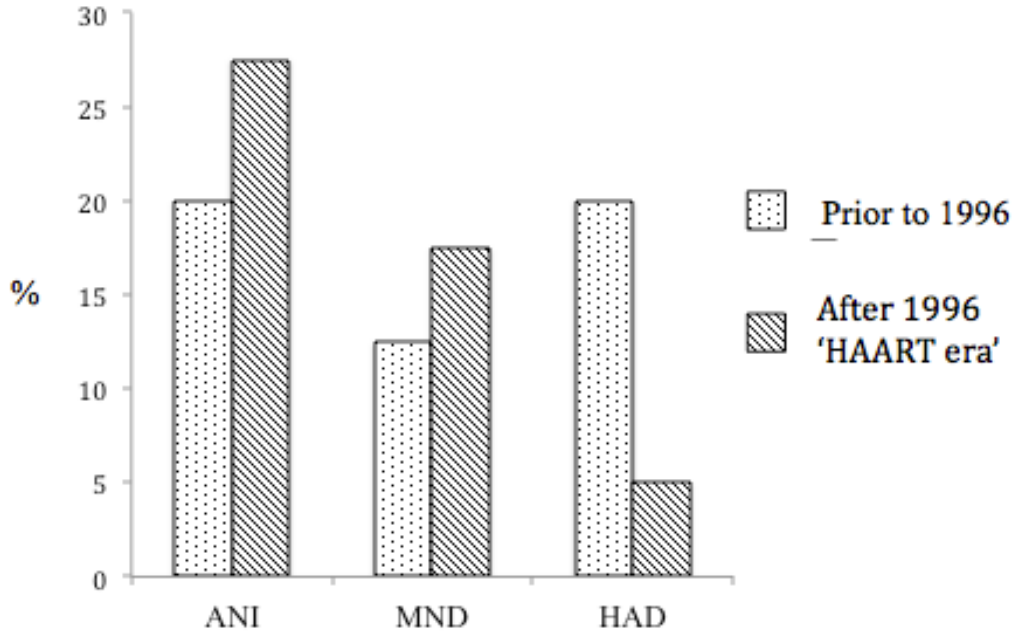

Figure 2. Changes in the US prevalence of HAND in the era before (HNRC study, n=678) and after (CHARTER study, n=843) the widespread use of potent ART.[10-12]

ANI, asymptomatic neurocognitive impairment; MND, Mild neurocognitive impairment; HAD, HIV-associated dementia; HAART, highly active antiretroviral therapy.

1.5.1 Co-morbid conditions also influence neurocognitive functioning

There are multiple factors that may predispose to cognitive impairment in an HIV infected individual; these are detailed in *Figure 3*. The extent to which each factor contributes to the prevalence of cognitive impairment in different populations is unclear. Although many co-morbid factors are linked to reduced cognitive functioning, strictly speaking they do not cause HAND as the Frascati criteria state the diagnosis is only possible if cognitive impairment is not explained by co-morbid conditions (*Figure 1*). Considering co-morbid conditions and HIV risk factors is important in studies of HAND and the assessment of HIV-seropositive patients with

cognitive impairment. Some, such as low nadir CD4+ T-cell count, are potentially preventable risk factors for HAND, while others, such as depression and systemic HIV replication may be amenable to treatment.

Many such cofactors were evident in the CHARTER cohort; those with cognitive impairment were more likely to have hepatitis C co-infection or AIDS, had a lower nadir CD4+ T-cell count, had more comorbidities, were more likely to have substance abuse or be depressed, and were older and more poorly educated than those without cognitive impairment. Overall 52% had cognitive impairment, however this varied from 83% in those with severe neuropsychiatric comorbidities to 40% in those with minimal neuropsychiatric co-morbidities. Even in the latter group with the least comorbidities, 71% had a diagnosis of lifetime substance use (16% had recent use demonstrated by positive breathalyzer or urine drug screen), 16% had current depression or psychotic disorder, 60% had AIDS, and 60% had detectable HIV RNA in plasma (not all were taking ART).[12] This diverse population might generalise well to the HIV clinical population in the U.S. but not to the ideal patient who is taking long-term suppressive ART that was begun at an early stage of disease and who has good immune recovery. Differences in the prevalence and severity of these factors explains some of the observed variation in estimates of HAND prevalence between studies. Based on these findings, multiple conditions should be considered in the assessment of HIV-seropositive patients with cognitive impairment; a careful clinical assessment is essential to identify the most important.

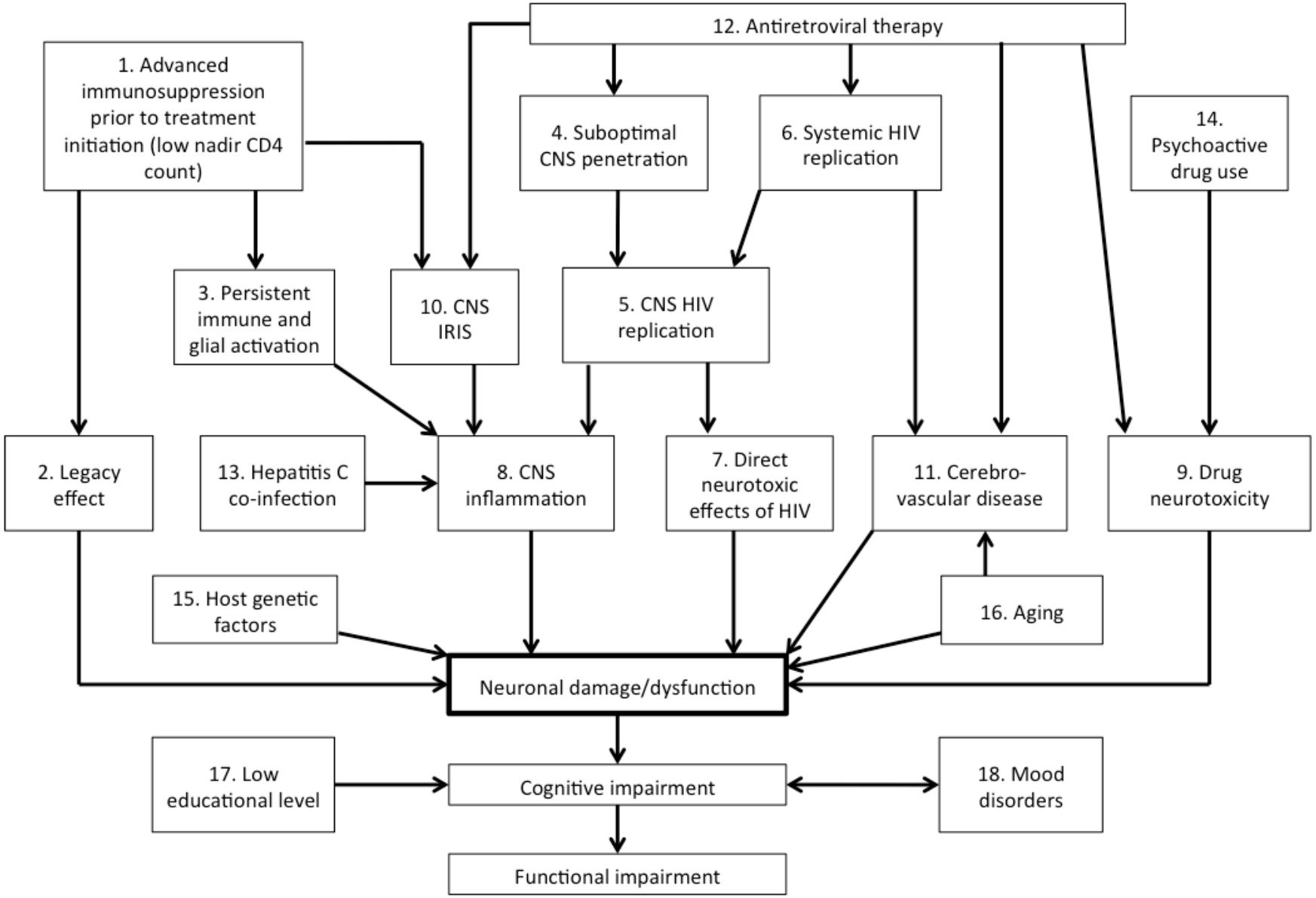
12. Antiretroviral therapy
1. Advanced immunosuppression prior to treatment initiation (low nadir CD4 count)
4. Suboptimal CNS penetration
6. Systemic HIV replication
14. Psychoactive drug use
3. Persistent immune and glial activation
10. CNS IRIS
5. CNS HIV replication
2. Legacy effect
13. Hepatitis C co-infection
8. CNS inflammation
7. Direct neurotoxic effects of HIV
11. Cerebro-vascular disease
9. Drug neurotoxicity
15. Host genetic factors
16. Aging
Neuronal damage/dysfunction
17. Low educational level
Cognitive impairment
18. Mood disorders
Functional impairment

Figure 3. Overview of proposed pathological mechanisms underlying HIV-associated neurocognitive disorders

Advanced immunosuppression prior to treatment initiation, as estimated by the nadir CD4+ T lymphocyte count, is strongly associated with cognitive impairment [1].[12, 16] *This may be due to a degree of irreversibility in pre-treatment CNS injury, termed a "legacy effect". Alternatively there may a process of immune or glial activation set up during advanced immunosuppression which persists following treatment and immune recovery [3].*[4] *In some patients variation in CNS distribution of ART may be important; suboptimal levels [4] may lead to failure to suppress HIV replication in the CNS [5].*[24, 42] *CNS HIV replication may also be driven by systemic HIV control [6].*[12] *Direct neurotoxicity occurs from the HIV virus and its proteins, such as the envelope glycoprotein gp120 and the regulatory protein Tat [7], which may be increased for different HIV clades. Chronic sustained immune activation in the CNS leads to the production of neurotoxic products such as nitric oxide, arachidonic acid and pro-inflammatory cytokines [8].*[93] *Some antiretroviral drugs may be directly neurotoxic [9].*[25] *Immune reconstitution inflammatory syndrome (IRIS) is an alternative mechanism by which antiretroviral therapy may damage the CNS; an insidious low-grade immune IRIS in the absence of pathogens other than HIV may lead to chronic lymphocyte activation and persistent inflammation in the CNS [10].*[26, 59, 60] *This may be more severe in those with advanced immunosuppression at treatment initiation [1].*[12, 16] *Cerebrovascular disease [11] can result from the metabolic and systemic effects of HIV disease [6] and its treatment [12] on endothelial function and cardiovascular risk factors.*[94, 95] *These mechanisms may become increasingly important as the HIV-seropositive population ages [16], and the CNS can be affected by so called "premature aging" associated with HIV infection.*

Hepatitis C infection [13] is associated with cognitive dysfunction independently of HIV,[96] and this effect is compounded by HIV disease. HIV and hepatitis C co-infected patients may be almost twice as likely to have cognitive impairment as HIV-seropositive individuals without HCV infection.[97, 98] The use of psychoactive drugs [14], in particular methamphetamine, has a deleterious effect on cognition which is more pronounced in combination with HIV infection.[99, 100]

Host genetic factors [15], in particular the apolipoprotein E4 allele, have been associated with HAD, but the association with milder impairment is inconsistent.[101, 102] Cognitive changes related to ageing [16] may be compounded by HIV infection,[103] and low educational level [17] can contribute to poor cognitive performance. Mood disorders [18] may masquerade as, or be caused by, cognitive impairment.[104]

1.5.2 Prevalence estimates vary with demographic characteristics

HAND is defined by deviation from normative neuropsychological testing data that are derived from a control population that is matched to the target population for at least age and education (referred to as "norms", see *Figure 1*). These criteria therefore depend on how accurately the control population reflects the tested population. The socioeconomic, ethnic, and educational diversity of the typical HIV population in Europe and the North America makes using appropriately matched control data essential for determining cognitive impairment. Demographic variation can affect the proportion classified as impaired, as evidenced by clinical trials that did not use appropriate normative data. For example variation in cognitive status has been demonstrated between countries studied,[105] and between ethnic groups within a country.[106] Although these differences could potentially be due to disease-related factors (e.g., HIV clade), other causes include the educational, cultural, and social differences that vary by geographical region and clinical settings.[107]

Perhaps the best opportunity to study a representative control group was provided by a cohort of Chinese plasma donors.[14, 108] Farmers in a rural area of China supplemented their income by donating plasma to a local laboratory. Several hundred became infected with HIV due to the use of unsterilised needles. This tragic situation provided a rare opportunity to study a well-matched control group by comparing infected subjects with those attending the same clinic who did not become infected. Cognitive assessment of this population demonstrated impairment in 34-39% of 203 HIV-seropositive subjects (depending on hepatitis C co-infection) compared to 13% of 198 subjects without HIV.[14] When followed over one year, 28% of those with HIV deteriorated compared to only 5% of those without HIV.[108] Of note however, the HIV

population in this study was not ideal: less than half of patients who took ART had undetectable plasma HIV RNA, 93% had hepatitis C co-infection, and the majority had previously progressed to advanced immune suppression or AIDS, which are known risk factors for HAND.

Studies using comprehensive neuropsychological testing, stringent criteria for the definition of HAND and well-matched control data, have found HAND prevalence to be high.[12] However, the challenge is generating the most appropriate normative data for each clinical setting.

1.5.3 Possible diagnostic overestimation: The role of the HAND definition

Debate about the current HAND classification approach is ongoing. Concerns have been raised that this approach will overestimate HAND prevalence by falsely classifying a proportion of the target population as impaired solely as a consequence of the method, not because they have a medical illness (i.e., they have a 'false positive' diagnosis). A purely statistics-focused approach using the criterion of two impaired tests per domain will identify 8-13% of the normative population – and therefore the target population as well – as having impaired performance.[109] Some studies have used the less conservative criterion of one impaired test per domain;[12] in this situation the 'false positive' frequency rises to 16-21%. The former figure is considered by some to be more accurate than the latter and is similar to the 14% of HIV seronegative controls classified as impaired in the normative population of some studies.[90] Proponents of this reasoning raise the concern that adoption of these criteria into clinical practice will result in misdiagnosing approximately 14% of patients as

having HAND, which could lead to costly and potentially unsafe clinical interventions as well as anxiety and stigma among misdiagnosed patients. The counterargument is based on at least two facts: worse performance on neuropsychological testing reflects worse functioning regardless of the population (HIV-seropositive or negative) and HAND is not diagnosed solely on the basis of neuropsychological testing results. HIV negative populations that are evaluated as normative 'controls' have a spectrum of functioning that ranges from much worse-than-average to much better-than-average. The individuals in the normative population who have the worst test performance are, by definition, the lowest functioning. Thus, individuals in the target population who perform at a level similar to the lowest functioning normative controls are themselves also low functioning. Requiring that individuals perform poorly in at least two cognitive abilities (as the Frascati criteria do) is intended, in part, to reduce the risk of misdiagnosis. Importantly, HAND diagnosis does not depend solely on cognitive testing but includes additional criteria: the impairment must not precede HIV transmission and severely confounding diagnoses (e.g., psychosis and active recreational drug use) and delirium must be absent at the time of diagnosis. Diagnostic confidence is further strengthened when the impaired performance is new, i.e. a decline has occurred.

In summary, some experts are concerned that the current approach to diagnosing impaired neuropsychological test performance will lead to false positive diagnoses of HAND with attendant financial and emotional costs. Others point out that HAND diagnosis is not based solely on neuropsychological test results and that below-average performance on properly normed neuropsychological tests is reason for concern and further evaluation regardless of the cause.

1.6 What is the clinical significance of asymptomatic neurocognitive impairment; should the HIV-seropositive population be screened for cognitive difficulties?

People with ANI have poor performance on cognitive testing but by definition do not have symptoms or diminished daily functioning.[90] Estimates of prevalence vary but ANI may occur in 33% to 60% of HIV-seropositive people despite ART.[12, 27] Although ANI is a research classification, both symptomatic and asymptomatic cognitive impairment have been associated with poorer quality of life, unemployment, worse medication adherence and lower driving ability.[110] Even mild cognitive impairment has been associated with neuropathologic evidence of HIV-encephalitis in some patients.[67] In the CHARTER cohort, patients with ANI were at significantly higher risk of progressing to symptomatic or functional impairment (termed mild neurocognitive disorder or MND) than those with normal cognitive function.[111] This has led to questions about the value of routine screening for ANI in the HIV-seropositive population.

The Mind Exchange consensus report advocates screening for HAND in all patients, regardless of symptoms or risk factors, using sensitive screening tools.[20] It suggests this be done early in disease and every 6-24 months thereafter. BHIVA also state that screening should be performed annually – however do not give details of which methods should be used or which populations should be targeted.[21] Guidance from EACS recommends restricting formal neuropsychological testing to patients who are symptomatic on a screening questionnaire.[22]

Several important issues should be considered regarding screening for ANI. For a screening test to be clinically useful the disease must have a well-defined early stage that would progress to a more severe stage without intervention. An effective intervention should be available and the distress and subsequent investigation of a positive screening result must be outweighed by the benefit of early treatment.[112] Since no effective therapy for ANI other than ART has yet been identified, the benefits of screening asymptomatic patients who have normal daily functioning and are already taking suppressive ART are debatable, particularly since no single screening method appears to be adequately sensitive and specific for ANI in all clinical settings. In many cases ANI is not progressive; for instance around half of people with any form of HAND fluctuate in cognitive functioning over time, with improvement from HAND to an unimpaired classification occurring as frequently as deterioration.[15] This variation may be due to true fluctuations in the pathological process, perhaps reflecting varying degrees of HIV replication in the CNS, or may reflect limitations in neurocognitive testing methods. Importantly, whether the poorer outcomes reported in those classified as ANI are driven primarily by HIV, by comorbid disorders, or both is unclear.

There may be some merit in identifying patients who have early impairment so that risk factors can be controlled, treatment adjusted and progression monitored. Changing ART based on distribution into the CNS, efficacy in monocytes, or neurotoxicity has been posited as a treatment for ANI, but no randomised clinical trials have yet clearly supported these or other interventions, such as exercise or cognitive rehabilitation. As a result no strong consensus exists on the best course of

action for patients who are taking adequately suppressive ART and are identified as having ANI, and there is no widely accepted framework for changing ART based on differences between drugs in estimated effectiveness or toxicity in the CNS.[23-25] A remaining important consideration is the impact of the screening process on patients; informing an asymptomatic individual that they have cognitive impairment could cause emotional distress. For these and other reasons, routine cognitive screening for ANI has not yet been widely adopted in the clinic.

HIV-Associated Neurocognitive Disorders are Common in the Antiretroviral Era.

For	Against
Multiple, well powered, observational studies have demonstrated cognitive impairment in up to 60% of populations with access to antiretroviral therapy. Most subjects typically have asymptomatic disease.[12-19, 27]	Approximately 15% of normative populations will be classified as impaired. Functioning at the lower end of the normative population may be concerning but HIV-related neuropathology is not necessarily the cause.
Some studies that found high prevalence used a thorough neurocognitive evaluation and matched control data to key demographic variables.[12]	Prevalence estimates depend on use of appropriate normative data (norms). Most studies did not use norms appropriate to all demographic groups in their cohort.
Prevalence remains high in sub-populations with few neuropsychiatric co-morbidities.[12]	Prevalence estimates derive from mixed populations who have other conditions that can affect cognition. Prevalence has been similar to HIV uninfected controls in ideal HIV patients who are taking durably suppressive ART and who do not have these other conditions.[64, 65]

People with HIV Should be Screened for Asymptomatic Neurocognitive Impairment.

For	Against
As ANI is typically more common than MND or HAD, most impairment would go unrecognised without screening.	No ANI screening tools perform well in all clinical settings.
ANI has been associated with poorer quality of life, worse medication adherence, and unemployment.[110]	There is no consensus on the management of ANI. Screening could lead to diagnostic procedures that may be unnecessary, costly and invasive.
ANI may be associated with increased risk for progressive neurocognitive disease.[111]	Worse outcomes in ANI may be related to associated comorbidities in this group.
A negative screen may reassure patients who are aware of the high prevalence of ANI.	A positive screen may upset patients
	Screening for ANI uses clinical resources which are limited.

HIV RNA in CSF is a Useful Clinical Tool in the Assessment of HIV Patients.

For	Against
In the era before antiretroviral therapy, higher HIV RNA in CSF was associated with HAD in people who had advanced immune suppression.[72-75]	In people successfully treated with ART, cognitive impairment may be caused by ongoing inflammation within the CNS and/or comorbidities, rather than ongoing HIV replication.
Case series have linked improvements in CNS symptoms to declines in HIV RNA in CSF.[48, 68]	HIV RNA levels in CSF may not accurately reflect HIV replication in brain parenchyma.
One antiretroviral era study found that people with greater HIV RNA in CSF than in blood were more likely to have neurocognitive impairment.[73]	Most studies have failed to show an association between HIV RNA in CSF and neurocognitive status in the antiretroviral therapy era.
Persistent HIV RNA in CSF during antiretroviral therapy may increase the risk of antiretroviral resistance and virologic failure.[82-84]	No longitudinal studies have demonstrated that people who have CSF/plasma discordance are more likely to develop antiretroviral resistance or progress to virologic failure.

Some Antiretroviral Drugs are More Effective in the CNS than Others.

For	Against
Concentrations of some antiretrovirals in CSF do not exceed the inhibitory concentration for wild-type HIV.	Using routine HIV RNA assays, CSF/plasma discordance is uncommon during therapy with any antiretroviral combination.[12]
Drugs with poorer estimated CNS effectiveness have been associated with higher HIV RNA in CSF.[24]	Some observational studies have not found an association between drugs with better estimated CNS effectiveness and neurocognitive functioning.[12, 27-29, 113]
Some studies have identified that drugs with higher estimated CNS effectiveness are associated with better neurocognitive performance.[43] Studies have also identified that some antiretrovirals are neurotoxic.[25]	Estimates of CNS effectiveness are largely based on antiretroviral pharmacokinetics in CSF, which may not accurately reflect antiretroviral pharmacokinetics in infected glial cells or brain macrophages.
Some observational studies have reported HIV RNA declines in CSF and neurocognitive improvement following ART changes based on estimated CNS effectiveness.[62, 63]	

Table 4. Summary of arguments for and against clinical statements associated with controversy in the field of HIV-associated neurocognitive disorders.

HIV, human immunodeficiency virus; ART, antiretroviral therapy; ANI, asymptomatic neurocognitive impairment; MND, mild neurocognitive disorder; HAD, HIV-associated dementia; RNA, ribonucleic acid; CSF, cerebrospinal fluid; CNS, central nervous system.

1.7 Conclusions and future directions

A summary of the arguments surrounding the key areas of controversy in the field of HIV-associated neurocognitive disorders is given in *Table 4*.

1.7.1 ART effectiveness in the CNS

Large, prospective, well-stratified, randomised trials are required to determine the association between cognitive performance and the CNS distribution of different ART regimens. Cross-sectional studies may no longer be reliable since at least some prescribers may tend to select more "CNS active" regimens for their patients who have evidence of CNS problems.[114] Given the number of ART combinations in use and the length of follow up required, evaluating each regimen in this manner is a major undertaking.

One alternative strategy is to monitor improvements in cognitive functioning following ART initiation. In most individuals there is improvement in cognitive function following commencement of effective ART,[115, 116] which is detectable as early as three months,[117] and may continue beyond a year.[118] This effect has been compared for different ART regimes. Treatment with ART that has greater estimated distribution into the CNS has been associated with better cognitive performance in some studies, however results are not consistent.[61] This strategy requires further evaluation in randomised clinical trials with larger sample sizes.

There are currently no data on the use of ART to prevent, rather than treat, HAND. Cognitive impairment may develop soon after HIV transmission and whether a milder

degree of irreversible impairment can occur at higher CD4+ T-cell counts is not known.[119] A study of US military personnel who were diagnosed and managed early during the course of HIV disease showed a low prevalence of cognitive impairment, comparable to matched HIV uninfected persons.[65] The question of whether to start ART early to prevent cognitive impairment is being investigated in a sub-study of the Strategic Timing of AntiRetroviral Treatment (START) study, in an NIH-funded clinical trial in China (http://clinicaltrials.gov/ct2/show/NCT01340950), and in other projects.

Although evidence is increasing that at least some modern antiretroviral drugs can cause neurotoxicity, whether this can lead to persistent cognitive impairment has not been systematically studied. The relationship between effective and toxic drug concentrations, the mechanisms of neurotoxicity, and approaches to identify patients who are suffering neurotoxicity in the clinic requires further investigation. Given the large degree of inter-individual variability in antiretroviral concentrations in CSF, and considerable variation in effectiveness vs. toxicity in the CNS observed between individuals, host genetic factors relating to ART distribution and metabolism in the CNS require further study.

1.7.2 CSF/plasma discordance

The clinical significance of CSF/plasma discordance is not known. To determine the extent to which this accounts for neurocognitive disease risk longitudinal studies are required to follow those with CSF/plasma discordance to determine the impact on cognitive function and the consequences for the development of ART resistance in the

CNS. Studies are needed to assess CSF and serum proteins and neuroimaging markers of inflammation to determine whether CSF/plasma discordance is associated with evidence of inflammatory up-regulation and neuroglial injury.

In addition to associations of CSF/plasma discordance with HAND, studies are needed to determine whether on-going HIV replication in the CNS is associated with ART treatment failure systemically. At present the best approach to identify which patients would benefit from a lumbar puncture for measurement of HIV RNA in CSF is unclear. More work is needed to determine the clinical applications of CSF HIV resistance testing and the implications of drug resistant mutations in the CSF for systemic HIV control.

The definition of CSF/plasma discordance should be standardised across studies and in clinical practice. Studies should determine the lowest level of CSF/plasma discordance associated with CNS inflammation and/or the detection of resistant virus in CSF. As increasingly sensitive HIV RNA assays become available for clinical use, the significance of discordance at lower levels also requires further study.

1.7.3 HAND incidence and prevalence

Whether patients who initiate suppressive ART early in disease are at risk of developing HAND is unclear from existing data. Studies of avireamic patients have produced HAND prevalence estimates ranging from 19-64%.[27, 64] These discrepant findings may reflect differences in comorbidities and the severity of immune suppression prior to initiating ART. More work is needed to identify the most

influential risk factors in ideal patients who meet current clinical goals, i.e., they began ART at higher CD4+ T-cell counts, have durable aviraemia and few neuropsychiatric comorbidities.

Future studies describing the incidence or prevalence of HAND should include at least two key elements: identification of the impact of co-morbid factors on neurocognitive functioning and close matching of normative control populations to the diversity of the target HIV population. Ideally, a core set of methods should be standardised to allow for comparison between studies. More research should be performed comparing the Frascati criteria with the more conservative approach that requires two impaired tests per cognitive domain to reduce the risk of misclassification.[109] Authors of studies of HAND should be encouraged to publish the demographic characteristics of the normative control population used.

The current approach to identifying cognitive impairment is focused on developing a research classification, not on clinical diagnosis. The 2007 Frascati criteria should be updated to provide practical guidance for clinicians; for example how to interpret cognitive functioning in the context of demographics and co-morbid factors. Further work is needed in this area to develop clinically useful methods of distinguishing HAND due to HIV disease (and therefore potentially amenable to earlier ART initiation, more potent ART, or ART directed at the CNS) from cognitive impairment related to other factors.

1.7.4 Screening for HAND

Screening for asymptomatic impairment requires further evaluation. At present, few systematic studies have evaluated the progression of ANI to MND; well-powered longitudinal studies are required to define the natural history of HAND. Despite a substantial body of evidence evaluating several HAND screening tools, no single, affordable, easy-to-perform screening test has proven adequately sensitive and specific across all clinical settings.[20] The most appropriate techniques to screen for HAND remain to be determined. Computerised cognitive assessments require further evaluation in people living with HIV as these could potentially be administered quickly and inexpensively in clinic.

While comprehensive neuropsychological assessment is appropriate for some patients, it is unlikely to be cost-effective for widespread adoption as a screening tool. Restricting such testing to those reporting cognitive symptoms on a screening questionnaire is an approach suggested by EACS,[22] however self-report of symptoms can be unreliable in patients with cognitive difficulties.[120] The approach of targeting symptomatic patients for further screening also warrants prospective investigation.

Other tests have been evaluated for their diagnostic and prognostic potential for HAND. For example, soluble and cellular biomarkers in CSF or blood may be abnormal in patients with HIV suppression, however the implications for the development of cognitive impairment are not well understood.[26] Many of these biomarkers correlate with cognitive performance at testing and also at follow-up,[81] but none have yet been developed for clinical use. Advanced neuroimaging techniques such as magnetic resonance spectroscopy, amyloid positron emission topography

scanning, and functional magnetic resonance imaging may also be useful in some cases, and are the subject of further study.[121, 122] Biomarkers and neuroimaging techniques require evaluation as screening tools, as well as to monitor response to ART and as endpoints in clinical trials. As the HIV-seropositive population ages, such techniques should be evaluated in older patients to distinguish HAND from other causes of cognitive impairment, such as Alzheimer's disease.

2 Hypotheses

CSF/plasma discordance is a common determinant of viral detection in plasma (addressed in chapter 5).

Low CSF antiretroviral drug concentrations are an important determinant of ART failure in the CNS and lead to CSF/plasma discordance and resistance (addressed in chapters 4 and 5).

Biomarkers of neuroinflammation are associated with CSF/plasma discordance and neurocognitive impairment (addressed in chapters 6 and 7).

Pharmacogenetic factors such as *CYP2B6* and *ABCB1* genotype impact the distribution of antiretroviral drugs into the CSF (addressed in chapters 8 and 9).

3 General methods

3.1 The PARTITION Study (chapters 4, 5, 6, 8 and 9)

The Penetration of AntiRetroviral Therapy InTO the Nervous system (PARTITION) study was a multicentre UK study.

3.1.1 Setting

Patients were recruited from 13 centres in the United Kingdom: Royal Liverpool and Broadgreen University Hospitals NHS Trust, Brighton and Sussex University Hospitals NHS Trust, North Middlesex University Hospitals NHS Trust, Chelsea and Westminster Hospitals NHS Trust, NHS Lothian, Newcastle Upon Tyne Hospitals NHS Foundation Trust, Leeds Teaching Hospitals NHS Trust and Kings College Hospital NHS Foundation Trust. Sites recruiting to PARTITION A only were Imperial College Healthcare NHS Trust, Walton Centre NHS Foundation Trust, Central and North West London NHS Foundation Trust - Camden Provider Services (Mortimer Market Centre/University College Hospital), Heart of England NHS Foundation Trust and Pennine Acute Hospitals NHS Trust.

3.1.2 Recruitment

Subjects were prospectively recruited from two groups.

3.1.2.1 PARTITION group A

In PARTITION group A CSF and plasma samples were collected at the time of a clinically indicated lumbar puncture.

Inclusion Criteria

- HIV positive adults (≥16 years) undergoing a lumbar puncture for any clinical indication.

Exclusion Criteria

- Children under 16 years.
- Emergency lumbar puncture where there is insufficient time to discuss the study and obtain informed consent.

3.1.2.2 PARTITION group B

In PARTITION group B lumbar puncture was performed for research purposes.

Inclusion Criteria

- HIV positive adults (≥16 years), with at least 1 episode of unexplained HIV-1 RNA detection ≥50 copies/ml within 12 months.

Exclusion Criteria

- Children and adolescents younger than 16 years.
- Patients with a contraindication to lumbar puncture such as space occupying lesion, coagulopathy, low platelet count ($<50x10^9$/L), recent history of seizure, focal neurological signs, papilloedema on fundoscopy or infected skin over the needle entry site.
- Patients for whom there is a clear alternative explanation for treatment failure, such as non-adherence (self reported adherence <95% on Modified Medication Adherence Self Report Inventory (MASRI) scale).
- Patients lacking the capacity to consent for lumbar puncture.

3.1.3 Definitions

CSF/plasma discordance

CSF/plasma discordance was defined as CSF HIV-1 RNA levels >0.5 $\log_{10}$ higher than those measured in the simultaneous plasma sample, or >0.5 $\log_{10}$ higher than the lower limit of quantification of the standard HIV-1 assay (40 copies/ml) in those with unquantifiable HIV-1 RNA on standard testing or <40 copies/ml on sensitive testing.

Discordance on sensitive testing

CSF HIV RNA not >0.5log10 the lower limit of quantification of the standard HIV-1 assay (40 copies/ml), ie. not meeting criteria for discordance given above. However when plasma HIV RNA is quantified by sensitive testing, discordance >0.5$\log_{10}$ is demonstrated. An example would be a subject with 20 copies/ml in plasma, and 100 copies/ml in CSF.

High discordance (chapter 6)

CSF HIV-1 RNA >1$\log_{10}$ plasma.

Low discordance (chapter 6)

CSF HIV-1 RNA 0.5-1$\log_{10}$ plasma or discordance only detected on sensitive testing. This group represents subjects that would not be considered significant by some definitions of discordance.

CSF viral escape

Plasma HIV-1 RNA less than LLQ for the standard assay (ie 40 copies/ml), and CSF greater than 40 copies/ml.

Residual HIV-1 RNA detection

HIV RNA detection between 1 and 49 copies/ml.

Although the lower limit of quantification of the standard assay used in PARTITION was 40 copies/ml, the goal of ART is persistent suppression of HIV RNA, commonly interpreted as a viral load <50 copies/ml.[41]

Ongoing HIV viraemia despite treatment

At least 1 episode of unexplained HIV-1 RNA detection ≥50 copies/ml within 12 months

CNS infection excluded, suspected or confirmed

On the basis of clinical findings and results of lumbar puncture the treating clinician was asked to determine whether CNS infection had been excluded. Those without "CNS infection excluded" were categorised as "CNS infection suspected" (abnormal CSF parameters without definite diagnosis) or "CNS infection confirmed" (a CNS pathogen identified).

3.1.4 Data collection

Written informed consent was taken from all patients enrolled. A separate section included a request for additional specific consent to store DNA for future studies, in line with published guidelines.[123] Demographic and clinical information was obtained from the patient records by a study investigator or research nurse. Basic information (that could not be completed in retrospect, such as time since last dose of antiretroviral medication) was collected on a paper based CRF by the clinician at the time of lumbar puncture. Subsequently this information was added to other data, such as CD4, HIV viral load and lumbar puncture results, onto a secure online pseudo-anonymised, password-protected CRF using OpenClinica™ software. Each subject was assigned a unique identification code which was anonymously linked to information in the following areas:

- Demographic information
    - Age, gender, ethnic group.
- Medical information
    - Date of HIV diagnosis.
    - Mode of infection
    - History of CNS infection.
    - Indication for lumbar puncture
- Medication information
    - Current antiretroviral regimen, dose and time last taken.
- Immunological markers
    - CD4 (current and nadir), HIV viral load.
- The results of clinically indicated CSF tests (cell count, protein, glucose, culture)

Consent was obtained such that if future studies required information on disease progression, participants could be contacted or their notes consulted.

3.1.5 Sample collection

Group A

- **CSF –** A separate CSF sample of up to 3-5ml was taken. Surplus CSF left over after clinically indicated tests had been performed was collected.
- **Plasma –** Approximately 10ml of whole blood centrifuged and plasma frozen in EDTA

Group B

- **CSF –** Up to 8-12ml collected
- **Plasma –** Approximately 15ml of whole blood centrifuged and plasma frozen in EDTA.

All subjects

- **Whole blood –** Approximately 5ml of whole blood frozen in EDTA

  Sample used for genetic analysis.

- **Serum** – Approximately 5ml of whole blood centrifuged and serum frozen

  To measure biomarkers and albumin for CSF:serum albumin ratios.

- **PAXgene** – Approximately 2.5ml of whole blood frozen in PAXgene RNA preservative.

  Vacutainers containing PAXgene were provided for isolation and purification of intracellular RNA.

Samples were frozen at site at ≤ -20℃ before being transferred to a biobank at the department of Pharmacology and Therapeutics at the University of Liverpool where they were held at -80℃. I undertook site visits and applied data checks.

3.1.6 Adherence

Medication adherence was assessed using the Modified Medication Adherence Self Report Inventory (MASRI) questionnaire. This is an abbreviated version of the full MASRI questionnaire which has been validated against HIV viral load.[124]

3.1.7 Cognitive and functional assessment

Subjects in Group B underwent assessment with the following tools. Testing was not done in Group A as results could be confounded by intercurrent illness.

Cognitive symptoms were assessed by asking subjects to answer whether they had problems with memory, reasoning and attention, as described by Simioni *et al.*[27] Subjects were considered to have an abnormal result when answering "yes, definitely" on at least 1 of the 3 questions.

Subjects were screened for cognitive impairment using the International HIV Dementia scale. This scale uses 3 brief tests to assess motor speed, psychomotor speed and memory-recall. A score of less than 10 was considered abnormal.[125]

Functional impairment was measured using the Instrumental Activities of Daily Living Scale.[20, 91] The maximum score of 8 means no impairment in the following activities; telephoning, shopping, preparing food, housekeeping, laundry, travel, medications and finances. A score of 7 or less indicates impairment.

Mood disorders were assessed using the Hospital Anxiety and Depression scale, addressing depressive (HAD-D) and anxious (HAD-A) symptoms separately. Patients were considered depressed or anxious if the HAD-D or HAD-A subscale score was at least 10 out of 21.[126, 127]

3.1.8 Safety

Lumbar puncture is a safe and well tolerated procedure that does not cause excessive discomfort to the patient when performed effectively.[128] Full informed consent was taken and a detailed patient information leaflet was provided explaining the potential risks of the procedure. The clinical team excluded contraindications to lumbar puncture such as raised intracranial pressure, coagulopathy or focal neurological signs. All patients received full blood count, coagulation screen and ophthalmoscopy prior to procedure. Brain imaging (CT/MRI) was not required prior to LP in the absence of reduced conscious level or focal neurological signs.[129] The lumbar puncture was performed by an experienced clinician taking the usual precautions of site location, local anaesthesia and aseptic technique.

Headache is the most common complication following lumbar puncture. This is usually mild and self-limiting.[130] The use of the Sprotte atraumatic needle significantly reduces headache risk[131] and these needles were provided for use in recruiting centres.

3.1.9 Ethics

Informed consent was obtained from all participants. The study was approved by the North Wales Research Ethics Committee (Central and East). The study was co-sponsored by the University of Liverpool and the Royal Liverpool and Broadgreen University Hospitals NHS Trust.

3.2 Other cohorts (chapters 7 and 8)

Other clinical cohorts include subjects from the CNS HIV AntiRetroviral Therapy Effects Research (CHARTER) study in the USA[12] and patients with HIV and tuberculous meningitis from a study of early vs. late antiretroviral therapy in Vietnam.[132, 133] These cohorts are described in more detail in chapters 7 and 8 respectively.

3.3 Laboratory testing

3.3.1 Quantification of antiretroviral concentration in CSF and plasma; assay development (chapters 4, 5, 8 and 9)

Antiretroviral concentrations were determined in CSF and plasma by tandem liquid chromatography-mass spectrometry (LC-MS). The analysis of compounds by LC-MS involves first producing gas phase charged molecular ions in the H-ESI source by application of a heat assisted electrical potential. The precursor (parent) ions are then selected according to their mass-to-charge ratio (m/z) in the first mass analyser (quadrupole 1) and pass into a collision cell (quadrupole 2) where they are bombarded with gas molecules (nitrogen) resulting in the formation of positively charged fragment (daughter) ions. Finally, the fragment ions are focused into a second mass analyser (quadrupole 3) and the intensity of each fragment ion is measured resulting in a chromatogram. The intensity of the fragment ions produced is proportional to the concentration of the analyte. Concentrations can be determined by comparison to the relevant calibrator of known concentration. An internal standard solution is included in the sample extraction

Drugs were extracted by protein precipitation (plasma) or directly injected (CSF). Standard curves were made up in artificial CSF (Harvard Apparatus, Holliston, MA, USA), and matrix effects assessed by post column injection. Assays were validated against repeated runs of quality controls (inter and intra-assay variability) and EQA standards. All runs were performed in duplicate.

3.3.1.1 Plasma assays

Concentrations of 11 antiretrovirals in plasma (protease inhibitors: atazanavir, amprenavir, darunavir, lopinavir, ritonavir and saquinavir; non-nucleoside reverse transcriptase inhibitors: etravirine, nevirapine and rilpivirine; CCR5 receptor antagonist maraviroc; integrase inhibitor raltegravir) were simultaneously quantified in a combined assay as described elsewhere.[134] The analytical ranges of this assay are given in *Table 5.*

The nucleoside reverse transcriptase inhibitors lamivudine, tenofovir and emtricitabine were measured in plasma separately by two assays with different sensitivities. The analytical ranges are given in *Table 6.*

3.3.1.2 CSF assay

Concentrations of 10 antiretrovirals in CSF (protease inhibitors: lopinavir, ritonavir, amprenavir, atazanavir, darunavir; non-nucleoside reverse transcriptase inhibitors: rilpivirine, etravirine, nevirapine; integrase inhibitor raltegravir; CCR5 entry inhibitor maraviroc) were simultaneously quantified in a combined assay. The analytical ranges of this assay are given in *Table 7.*

	Maraviroc (ng/mL)	Raltegravir (ng/mL)	Rilpivarine (ng/mL)	Nevirapine (ng/mL)	Etravarine (ng/mL)
Lower limit of quantification	13.2	5	24.3	25.6	13.1
Lower quality control	48	15	140	140	79
Medium quality control	392	350	1522	1499	589
Higher quality control	691	3500	7222	7372	3777

	Darunavir (ng/mL)	Atazanavir (ng/mL)	Ritonavir (ng/mL)	Lopinavir (ng/mL)	Amprenavir (ng/mL)	Saquinavir (ng/mL)
Lower limit of quantification	43.9	25.5	14.6	46.6	25.9	25.2
Lower quality control	230	132	87	247	154	152
Medium quality control	3948	1518	797	3978	1537	1543
Higher quality control	11887	7433	3848	11802	7795	7075

Table 5. Analytical ranges for combined plasma 11 ART assay.

	Emtricitabine, Tenofovir, Lamivudine. Low conc assay (ng/mL)	Emtricitabine, Tenofovir, Lamivudine. High conc assay (ng/mL)
Lower limit of quantification	0.5	5
Lower quality control	1.5	5
Medium quality control	3	200
Higher quality control	8	4000
Upper limit of quantification	10	5000

Table 6. Analytical ranges for low/high concentration NRTI assay.

	Lopinavir, Maraviroc, Ritonavir (ng/mL)	Raltegravir, Amprenavir, Atazanavir, Rilpivarine, Etravirine, Darunavir (ng/mL)	Nevirapine (ng/uL)
Lower limit of quantification	0.78	1.95	19.53
Lower quality control	2	5	50
Medium quality control	10	25	250
Higher quality control	50	125	1250
Upper limit of quantification	100	250	2500

Table 7. Analytical ranges for combined CSF 10 ART assay.

3.3.1.3 Assay for the quantification of efavirenz and major metabolites (free and glucuronidated) in plasma and CSF (chapter 8)

These methods relate to results presented in chapter 8. Efavirenz was charecterised in 2 cohorts (PARTITION and a Vietnamese TB co-infected population), with quantification of the major metabolites and determination of the effect of glucuronidation. Freshly prepared standards and quality control samples (prepared in artificial CSF) and clinical samples (100uL) were transferred into labeled 7uL stoppered glass tube to which 100mL of acetonitrile was added. The samples were the evaporated to dryness at room temperature in a stream of nitrogen. The samples were then incubated at 37^{o}C for 2h with 400mL of a solution containing 200 units of b-glucuronidase from *H. pomatia* in 0.2M sodium acetate buffer (pH = 5). The samples were subsequently alkalinised with 20mL of potassium carbonate buffer (0.1M, pH = 9.4) and extracted with 3 mL of a mixture of organic solvents ethylacetate:hexane (60:40 v/v). After centrifugation, the organic phase was evaporated to dryness, the residue reconstituted in 100uL of a mobile phase (50/50 v/v ACN/H_2O in 1mM ammonium Acetate) and 20uL of this solution was analysed directly by LC-MS/MS on a Thermo Access Triple Quadrupole mass spectrometer. Hexobarbital was used as internal standard. Gradient elution was on a reverse-phase C_{18} column using 1mM ammonium acetate in water and acetonitrile. Quantification was by selective reaction monitoring in negative ionisation mode. A 1 mg/mL solution of efavirenz, 8-hydroxy-efavirenz, 7-hydroxy-efavirenz and internal standard solution was separately infused directly into the mass spectrometer at a flow rate of 8 µL/min in MS–MS scan mode using a syringe. This generated product ions of specific masses that can be used to characterise each compound. The fragmentation patterns and precursor ions ([M-H]-)

were monitored in the negative ion mode and these characteristic fragments were used for quantification of efavirenz, its metabolites and internal standard (*Table 8*).

Analytes	QC	Nominal Concentration (ng/mL)	Mean Measured Concentration (ng/mL)	Accuracy %Bias (SD)	Precision (%CV) Intra assay
EFV	**LQC**	7.30	6.96	-4.60 (0.52)	7.51
	MQC	17.40	17.70	1.70 (1.55)	8.74
	HQC	34.90	36.16	3.61 (6.39)	6.39
8-OH EFV	**LQC**	8.10	7.87	-2.87 (0.60)	7.63
	MQC	16.30	15.19	-6.80 (1.17)	7.69
	HQC	35.00	32.81	-6.27 (2.04)	6.23
7-OH EFV	**LQC**	4.50	4.63	2.84 (0.38)	8.19
	MQC	9.00	9.09	0.96 (0.25)	2.79
	HQC	19.00	19.68	3.60 (0.57)	2.91

Table 8. Mass spectrometry assay for the quantification of efavirenz and major metabolites in plasma and CSF.

EFZ, efavirenz; 8-OH EFZ, 8-hydroxy-efavirenz; 7-OH EFZ, 7-hydroxy-efavirenz; QC, quality control; SD, standard deviation; CV, coefficient of variability; LQC, low quality control; MQC, medium quality control; HQC, high quality control.

3.3.2 CNS penetration effectiveness (CPE) score

The revised CPE score (2010) was calculated by assigning a predefined point to each component of the ART regimen, as proposed by Letendre et al.,[24, 135] whereby a higher score corresponds to increased effectiveness. The score reflects the physic-chemical, pharmacokinetic, and pharmacodynamic properties of a drug combined with clinical and virological data. See *Table 3* in chapter 1.

3.3.3 Quantification of HIV-1 RNA in CSF and plasma

HIV-1 RNA levels were measured by the Abbott RealTime HIV-1 assay (Maidenhead, UK). The assay quantifies the viral load over the range of 40–10,000,000 copies/mL (1mL input) and reports qualitative RNA detection >40 copies/mL. The manufacturer indicates detection of 30, 20, and 10 RNA copies/mL in 96%, 88%, and 68% of cases, respectively.

3.3.3.1 Sensitive HIV-1 RNA assay (chapter 5)

A modified assay was used for sensitive HIV-1 RNA testing in a subset of subjects with unquantifiable HIV-1 RNA levels on standard testing. Briefly, following venous and lumbar puncture for collection of EDTA blood and CSF respectively, paired plasma and CSF were ultra-centrifuged at 40,000rpm for 15 minutes and the pellet was resuspended in 1ml Basematrix (Alere, Stockport, UK). HIV-1 RNA was measured using automated RNA extraction (Abbott m24sp) and quantification (Abbott m2000), as previously described.[136]

3.3.4 Resistance testing (chapter 4)

Discordant samples underwent sequencing of reverse transcriptase (RT, amino acids 1 to 335) and protease (amino acids 1 to 99) to detect drug resistance mutations, as described elsewhere.[137] Mutations were evaluated according to the Stanford HIV Drug Resistance Database.[138]

3.3.5 Genetic testing (chapters 8 and 9)

Genomic DNA was purified from stored samples using standard phenol-chloroform extraction methods. Allelic discrimination by real-time PCR was performed for individual single nucleotide polymorphisms (SNPs). *CYP2A6* copy number was determined by real-time PCR.

3.3.5.1 Haplotype structure

In order to map haplotype structure across the *CYP2B6* locus in chapter 8, I undertook a combined approach of genotyping known functional SNPs with tagging SNPs identified through HapMap (hapmap.ncbi.nlm.nih.gov). SNPs with a minor allele frequency (MAF) of >0.05 were identified within dbSNP (http://www.ncbi.nlm.nih.gov/SNP) at intervals of approximately 1 kb across the *CYP2B6* locus at chr19q13.2 and an additional 5 kb up- and downstream of the gene. Where no SNPs with validated mean allele frequencies were available, the nearest positional SNP was included. A total of 196 individuals were genotyped for 16 SNPs using the MALDI-TOF-based Sequenom iPLEX system (Sequenom Inc., San Diego,

CA, USA) according to the manufacturer's protocol. The following SNPs were included: rs1041983, rs1045642, rs11882450, rs1523130, rs2032582, rs2099361, rs2307424, rs2472677, rs28399433, rs3745274, rs7250601, rs763645, rs776746, rs8192711, rs8192712. Multiplex assays were designed using the software available at https://mysequenom.com/default.aspx. SNPs with mean allele frequency <0.05, call rate <90% or Hardy Weinberg p-value <0.0001 were omitted.

3.3.6 Testing performed at clinical sites

In the PARTITION study CSF cell count, protein and glucose measurements, microbiological investigations, and CD4 cell counts were performed as indicated in the centres of care by routine methods. Ethical permission was granted for CSF testing (including CSF white cell count, CSF HIV-1 RNA quantification and other tests) to be performed at local sites in clinical laboratories only when felt to be clinically indicated by the treating clinician. As the significance of these tests in asymptomatic patients was unclear it was felt that knowledge of these results by the clinician could lead to changes to management that would not have occurred had they not been enrolled in a research study. As CSF white cell count can only be accurately performed on fresh samples this parameter was not available for all subjects in group B.

3.3.7 Measurement of cytokine and chemokine levels (chapter 6)

Thirty-seven mediators (cytokines, chemokines and associated co-mediators) were assessed using a commercial cytometric bead array assay system (Procarta ®

Immunoassay kit, Panomic Solutions, Affymetrix ® Milano, Itay) in accordance with the manufacturers' instructions. The mediators assessed were chosen from reviewing previous literature: granulocyte-colony stimulating factor [G-CSF], granulocyte-macrophage-colony stimulating factor [GM-CSF], myeloperoxidase [MPO], CCL2 [monocyte chemotactant protein 1], CCL3 [C-C motif ligand 3, monocyte inflammatory protein 1a], CCL4, CCL5 [regulated on activation normal T cell expressed and secreted], CXCL10 [inducible protein 10], vascular cell adhesion molecule [VCAM], intracellular adhesion molecule [ICAM], vascular endothelial growth factor [VEGF]a, and matrix metalloproteinases [MMP] 1, 2, 3, 7, 8, 9, 12 and 13, interferon [IFN] a, b, o, g, tumour necrosis factor [TNF]a and its soluble receptors [TNFR1 and TNFR2] and TNFa-related apoptosis inducing ligand [TRAIL], IL1a, 1b, 2, 4, 6, 8, 10 and 17a, IL17f, and IL1-receptor antagonist [IL1RA]. Analytes were assessed for all patient groups on 1 plate for MMPs and 1 plate for all other mediators. Fluorescence intensity was determined using a Bio-Rad platform (BioPlex Manager 4.1, Bio-Rad Laboratories ©, Hertfordshire, UK). Standards and samples were analysed in duplicate and the mean value used in analysis. Standard curves were adjusted at the points of fluorescence intensity saturation, to generate a sigmoid standard curve with 6-8 fluorescence intensity points. To avoid undetectable levels of mediators or missing data biasing the analysis, only mediators that were detected in at least 80% of the cohort were included in the analyses.[139, 140]

3.4 Statistical analysis

All analysis was undertaken using SPSS version 22. In all chapters continuous clinical and demographic variables were compared by Student's t-test if normally distributed, or by Mann-Whitney *U* if non-parametrically distributed. Visual checks were applied to determine normality. Categorical data was analysed using Chi squared or Fisher's exact test.

In chapters 4 and 5, median HIV RNA levels and drug concentrations were compared with the Mann-Whitney *U* test. Variables demonstrated to be significant in univariate analysis went forward for multivariate logistic regression with a forced entry method. A p value of 0.05 was used to determine statistical significance.

In chapter 6 mediator concentrations between groups were compared with the Mann-Whitney *U* test. Within cases, individual CSF mediator concentrations were correlated with CSF HIV-1 RNA using Spearman's r. Multivariate analysis of mediator concentrations in this chapter is described in detail below (section 3.4.1). Statistical techniques employed in chapter 7 are described within the chapter.

In chapter 8 the geometric mean of $\log_{10}$ drug/metabolite concentrations were compared between studies and between genotypes by Student's t test 1 way ANOVA respectively. Pearson r was used to determine the correlation between continuous variables. In chapter 9, effects of ABCB1 genotype on drug concentration were assessed using the Kruskal-Wallis test.

3.4.1 Two-Way Unsupervised Sample Clustering (chapter 6)

CSF mediators significantly ($p<0.01$) associated with CSF/plasma discordance in

univariate analysis were included in a 2-way unsupervised hierarchical clustering based on the patterns of relative mediator concentrations. This approach offered an opportunity to examine the synergistic interactions of mediators and allow patients to be classified by their overall host mediator response. Subjects and then mediators were hierarchically clustered using the program Cluster (version 3.0), using the uncentered correlation score as the similarity metric and the average branch linkage method. Results were viewed as heat-maps using Tree View software (http://rana.lbl.gov/EisenSoftware.htm).

4 The PARTITION study; factors associated with CSF/plasma discordance of HIV

4.1 Aims

The aims of this study were to determine the prevalence of CSF/plasma discordance in a clinical cohort, and determine the factors associated with this phenomenon.

4.2 Methods

Subjects were recruited from the PARTITION study in 2 groups (see section 3.1).

4.3 Results

A total of 153 ART treated subjects were recruited, 113 (74%) from group A and 40 (26%) from group B (*Figure 4*). Overall CSF and plasma HIV-1 RNA levels were directly correlated (Spearman r=0.510; p<0.0001). Median HIV-1 RNA level was 20 copies/ml in both CSF and plasma. HIV-1 RNA was below 50 copies/ml in 113 (74%) plasma and 115 (75%) CSF measurements. CSF/plasma discordance was detected in 20 (13%); in these subjects median HIV-1 RNA levels were 501 copies/ml (IQR 210,2016) in CSF vs. 20 copies/ml (IQR 20,20) in plasma (Figure 5).

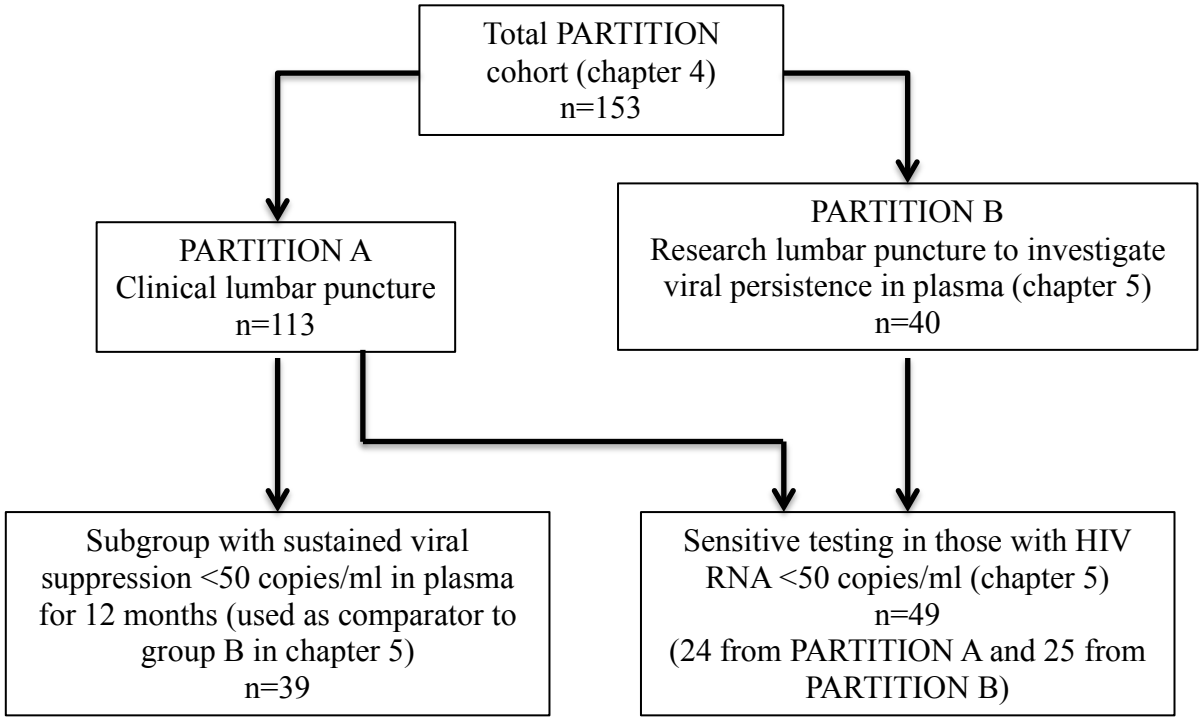

Figure 4. Subgroups from the PARTITION study

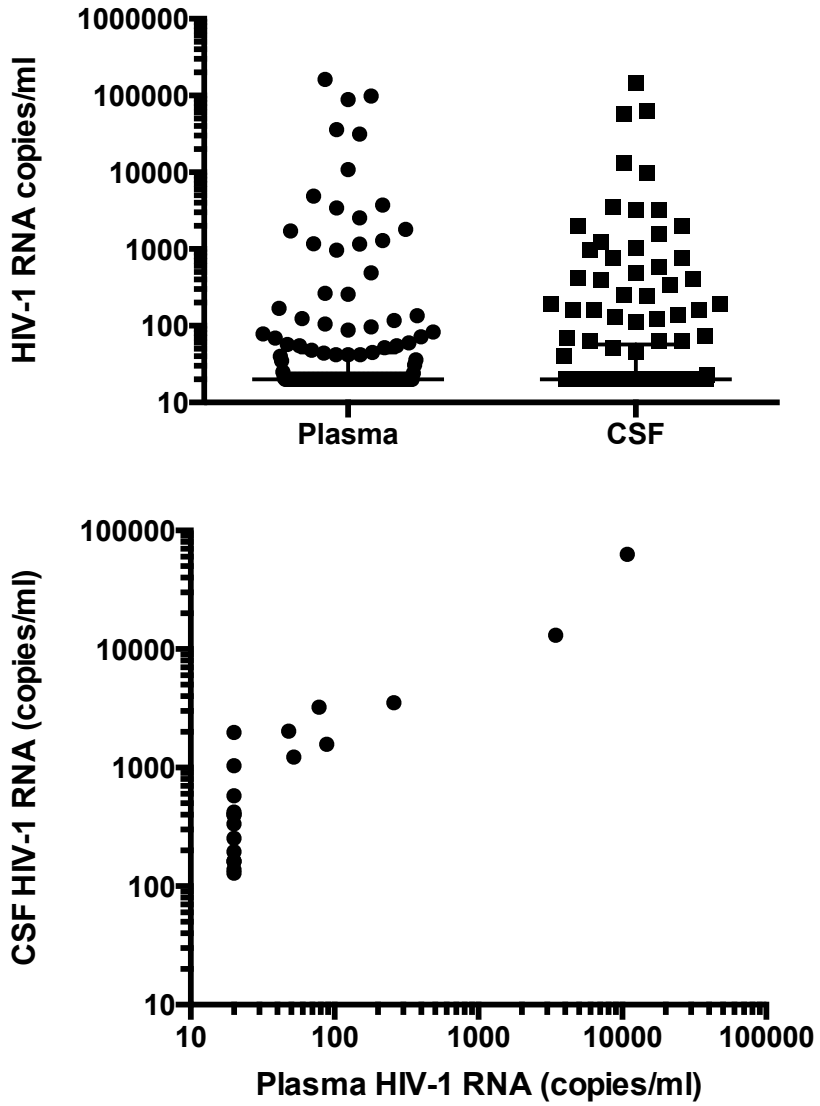

Figure 5. HIV-1 RNA in plasma and CSF

a. HIV-1 RNA in plasma and CSF in groups A and B combined; n=153

b. Paired HIV-1 RNA in plasma and CSF in the 20 subjects with CSF/plasma discordance.

4.3.1 Factors associated with CSF/plasma discordance

Differences in measured variables among subjects with and without CSF/plasma discordance are shown in *Table 9*. Nadir CD4, MSM risk group and black ethnicity were included in a multivariate logistic regression model; although the model was significantly associated with CSF/plasma discordance (p=0.013), no single variable made a significant contribution to the model suggesting these variables explain the same variation in the outcome. There was significant correlation between predictor variables: only 5 (5%) subjects in the MSM risk group were black, vs. 87 (81%) white (p<0.0001). Nadir CD4 was higher in white-MSM, median 173 cells/mm^3 (IQR 48,292) vs. 110 cells/mm^3 (IQR 35,242), p=0.029. Raltegravir use was more frequent in discordant subjects and CSF white cell count was higher in discordant subjects.

	CSF/plasma discordance		
	Yes (n=20)	**No (n=133)**	**p value**
Age, years (IQR)	46 (43,50)	46 (41,54)	0.913
Gender male, n (%)	15 (75)	117 (88)	0.156
Risk group, n (%)			
MSM	8 (40)	90 (68)	0.024
Heterosexual	9 (45)	31 (23)	0.054
IDU	0 (0)	3 (2)	1.000
Vertical tx	1 (5)	2 (2)	0.345
Not disclosed	2 (10)	7 (5)	0.334
Ethnicity, n (%)			
White	11 (55)	98 (74)	0.111
Black	8 (40)	21 (18)	0.027
Asian	1 (5)	5 (4)	0.575
Other/mixed	0 (0)	7 (5)	0.595
Years since HIV diagnosis, median (IQR)	10 (6.5,15.5)	9 (5,16)	0.833
CD4, median cells/mm^3 (IQR)			
Nadir	32 (21,256)	159 (49,281)	0.030
Current	374 (190,613)	464 (310,707)	0.163
ART at sampling, n (%)			
PI/r	15 (75)	77 (58)	0.220
NNRTI	5 (25)	51 (38)	0.323
NRTI	18 (90)	114 (86)	1.000
RAL	6 (30)	15 (11)	0.035
MVC	4 (20)	8 (6)	0.053
CPE, median (IQR)	7 (7,9.5)	7 (6,8)	0.250
CSF WCC*, median cells/mm^3 (IQR)	11 (0,21)	0 (0,3)	0.018
Confirmed/suspected CNS infection, n (%)	3 (15.0)	14 (10.5)	0.469

Table 9. Factors associated with CSF/plasma discordance.

**Tested in 24 (60%) of subjects in group B (see section 3.3.6).*

MSM, men who have sex with men; IVDU, intravenous drug user; IQR, interquartile range; ART, antiretroviral therapy; PI/r, ritonavir boosted protease inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside/tide reverse transcriptase inhibitor; CCR5 antag, CCR5 receptor antagonist; CPE, CNS penetration effectiveness score 2010; CSF WCC, cerebrospinal fluid white cell count.

Within group A 13 (12%) subjects had CSF/plasma discordance. There were no differences in indication for lumbar puncture between those with and without CSF/plasma discordance (p=0.972). The most frequent indications for LP in group A were to investigate cognitive symptoms, (n=50, 44%), and headache (n=20, 18%). Other indications for lumbar puncture were as follows: abnormal neurological examination (n=7), follow up CNS infection (n=8), seizure (n=5), measure CSF HIV RNA (n=3), in PIVOT (Protease Inhibitor Versus Ongoing Triple-therapy) clinical trial (n=3), peripheral neuropathy (n=2), suspected neurosyphilis (n=7), suspected encephalitis (n=2), suspected multiple sclerosis (n=1), suspected CNS malignancy (n=1), acute confusion (n=1), HTLV-1 infection (n=1), sepsis unknown source (n=1) and staging for CNS lymphoma (n=1). Based on the LP results CNS infection was confirmed or suspected in 3 (15%) patients with CSF/plasma discordance, and 14 (11%) without; this difference was not statistically significant (p=0.469). In group A, self reported adherence was <95% in 2 (15%) of those with, and 12 (12%) of those without CSF/plasma discordance (p=0.361). Adherence was not reported in 19 (17%) subjects. Self reported adherence ≥95% was an inclusion criteria for group B.

4.3.2 Pharmacokinetics

Differences in measured antiretroviral concentrations in CSF and plasma are shown in *Table 10.* In the total cohort (n=153) time of sampling post ART was not significantly different between those with and without CSF/plasma discordance, median 15 hours (IQR 5.5,20) vs. 13 hours (IQR 5,16.5) respectively (p=0.238).

There were no significant differences observed between groups in measured concentrations of the remaining antiretroviral drugs in plasma, or with any drugs in

CSF either in univariate analysis (*Table 10*) or when combined with time post dose in a multivariate model. No significant differences between groups were observed when time post dose was incorporated with CSF/plasma drug levels in a multivariate model.

Median plasma darunavir concentrations were significantly lower in discordant subjects (p=0.023). Median time post darunavir dose was significantly longer for discordant vs. non-discordant subjects; median 18 hours (IQR 10.5,24) vs. median 7 hours (IQR 3,16) respectively, p=0.007. Plasma darunavir levels were negatively correlated with time post dose (Spearman r=-0.413, p=0.002) (*Figure 6*).

A logistic regression analysis was performed including time post dose and plasma darunavir concentration. Overall the model was significantly associated with CSF/plasma discordance (p=0.003), however neither parameter made a unique statistically significant contribution to the model (time post dose p=0.063, plasma darunavir concentration p=0.160) suggesting these variables explain the same variation in the data, ie. plasma darunavir concentrations were lower in discordant subjects as they were sampled later post dose.

There was a non-significant trend to lower plasma lamivudine concentrations in discordant subjects (p=0.059), however this trend was lost when combined with time post dose in a multivariate model (p=0.509) as time post dose tended to be longer in discordant subjects receiving lamivudine (median 15 hours [IQR 8,22]) vs. non-discordant subjects receiving lamivudine (median 7 hours [IQR 5,15]), p=0.085.

	CSF HIV-1 RNA >0.5log$_{10}$ plasma				
	Yes (n=20)		No (n=133)		
	n	Median (IQR)	n	Median (IQR)	p value
Plasma					
ATZ	2	1459 (305.1,2614)	17	1038 (366.5,1672)	NS
DRV	11	2168 (1196,2732)	44	3535 (2147,5915)	*0.023
ETV	1	362.7	3	362.5 (72.51,724.6)	NS
LPV	0		3	8731 (8206, 8807)	NS
MVC	3	169.2 (117.9,178.5)	5	232.4,88.06,523.4)	NS
NVP	0		10	4351 (2416,5004)	NS
RAL	2	680.1 (122.8,1237)	9	736.4 (155.4,1465)	NS
RPV	0		4	79.07 (45.99,95.07)	NS
RTV	0		15	192.7 (69.04,282.5)	NS
EFZ	3	3231 (714.8,6103)	24	1709 (1049,2480)	NS
TDF	8	72.07 (34.89,106.5)	54	98.06 (63.19,180.3)	0.116
FTC	7	122.8 (45.54,248.9)	58	260.8 (93.42,610.5)	0.367
3TC	5	240.5 (46.71,632.3)	19	714.8 (136.3,1596)	**0.059
CSF					
ATZ	2	23.87 (1.950,45.80)	18	11.17 (3.857,17.18)	NS
DRV	10	26.79 (19.63,93.75)	44	42.37 (19.69,86.54)	0.754
LPV	0		3	9.871 (6.386,15.02)	NS
MVC	3	47.58 (16.61,78.03)	6	7.079 (2.296,9.282)	NS
NVP	0		12	1497 (1183,2041)	NS
RAL	3	19.82 (18.04,170.6)	11	21.30 (15.27,44.95)	NS
EFZ	2	38.87 (28.06,49.68)	24	13.45 (9.07,20.97)	NS

Table 10. Plasma and CSF antiretroviral drug concentrations in those with and without CSF/plasma discordance.

*P values were calculated for drugs with 6 or more measurements in each group. * not significant when combined with time post dose in a multivariate model (p=0.160). ** trend towards significance lost when combined with time post dose in a multivariate model (p=0.509)*

NS, not significant; ATZ, atazanavir; DRV, darunavir; ETV, etravirine; LPV, lopinavir; MVC, maraviroc; NVP, nevirapine; RAL, raltegravir; RPV, rilpivarine; RTV, ritonavir; EFZ, efavirenz; TDF, tenofovir; FTC, emtricitabine; 3TC, lamivudine; IQR, interquartile range.

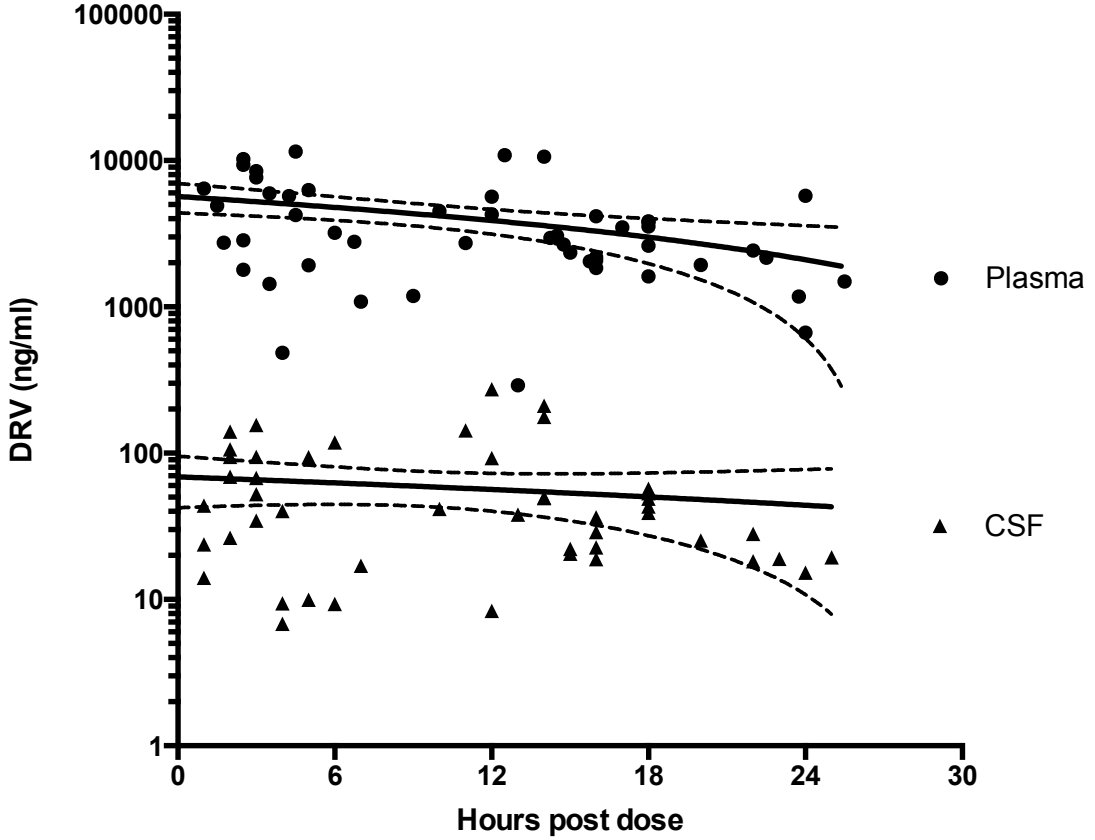

Figure 6. Plasma and CSF darunavir concentration over time post dose

Darunavir concentration was negatively correlated with time post dose in plasma (Spearman r=-0.413, p=0.002) but not CSF (Spearman r=-0.186, p=0.186).

Solid line linear regression, dashed lines 95% confidence intervals.

4.3.3 CSF resistance

CSF from discordant subjects underwent sequencing (*Table 11*). Four CSF samples did not amplify despite repeated attempts. Two subjects had insufficient volume of CSF remaining for resistance testing. Protease inhibitor (PI) major resistance mutations were detected in 2 of 9 (22%) CSF samples; both were taking PIs at sampling. Resistance to NNRTI was detected in 4 of 9 (44%) CSF samples; 1 subject was receiving NNRTIs at sampling and 2 had historic NNRTI use. Resistance to NRTIs, including the M184V/I mutation associated with high-level resistance to lamivudine and emtricitabine and increased susceptibility to tenofovir, was detected in 9 of 12 (75%) CSF samples. Seven subjects were receiving either lamivudine or emtricitabine at sampling. One subject (subject 16) was receiving tenofovir at sampling and had received lamivudine until 7 years prior to sampling. One subject (subject 17) was not taking NRTIs at sampling but had received emtricitabine and tenofovir until 1 year prior to sampling. Resistance to integrase inhibitors was detected in all 4 CSF samples from subjects receiving this drug at sampling.

		HIV-1 RNA copies/ml			CSF resistance associated mutations			
Subject	**Group**	**Plasma**	**CSF**	**ART at sampling**	**PI**	**NRTI**	**NNRTI**	**INT**
1	A	40	1038	TDF/FTC/DRV/r	None	-	-	-
2	A	52	1231	TDF/FTC/RAL/MVC	-	-	-	-
3	A	3443	13088	3TC/AZT/EFZ	-	None	**V108I, E138A**	
4	A	10817	63010	TDF/FTC/RAL	-	None	None	**L74I**
5	A	<40	400	ABC/3TC/DRV/r/RAL	-	-	-	-
6	A	<40	422	TDF/FTC/ATZ/r	-	**T69S, M184I**	-	-
7	A	<40	335	TDF/FTC/DRV/r	-	-	-	-
8	A	<40	162	3TC/AZT/DRV/r/MVC/ETR	-	-	-	-
9	A	<40	129	DRV/r	-	-	-	-
10	A	<40	579	ATZ/r/TDF/FTC	**V82AV**	**M184V, T215I**	-	-
11	A	<40	1981	DRV/r/RAL/MVC/3TC	None	***M184V***	V108I	***N155H***
12	A	<40	195	EFZ/TDF/FTC	-	-	-	-
13	A	<40	405	LPV/r/AZT/RAL	None	None	None	**N155H**
14	B	<40	138	TDF/FTC/DRV/r	-	***M184I***	-	-
15	B	<40	254	EFZ/TDF/FTC	-	-	-	-
16	B	48	2028	DRV/r/TDF/RAL	None	***D67N, K70R, L74V, M184V, T215Y, K219E***	*L100IL, K103KN*	**T66I, Y143C**
17	B	78	3234	DRV/r/MVC/ETR	None	*A62V, K65R, M184V*	None	-
18	B	88	1569	TDF/FTC/DRV/r	None	***M184I***	None	-
19	B	<40	162	TDF/FTC/DRV/r	V82A	***D67N, M184V, T215Y, K219Q***	V108I	-
20	B	258	3518	ABC/3TC/DRV/r	None	**L74LV, *M184V***	None	-

Table 11. Viral detection and CSF resistance in subjects with CSF/plasma discordance.

RAMs in bold are to ARVs taken at the time of sampling. RAMs in italics have been previously detected in plasma. The symbol "-" represents samples not tested or unable to amplify; 5 CSF samples did not amplify despite repeated attempts (subjects 2, 5, 7, 9 and 12), 1 sample only amplified for PI RAMs (subject 1), and 2 subjects had insufficient volume of CSF remaining for resistance testing (subjects 8 and 15). Subject 2 showed CCR5 tropic virus in CSF. Subject 4 had plasma testing showing no NRTI or NNRTI RAMs.

UA, unable to amplify; RM, resistance mutation; PI, protease inhibitor; NRTI, nucleoside/tide reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse

transcriptase inhibitor; INT, integrase inhibitor; ATZ, atazanavir; DRV, darunavir; LPV, lopinavir; ETV, etravirine; MVC, maraviroc; NVP, nevirapine; RAL, raltegravir; RPV, rilpivarine; r, ritonavir; EFZ, efavirenz; TDF, tenofovir; FTC, emtricitabine; 3TC, lamivudine; AZT, zidovudine.

4.4 Discussion

CSF/plasma discordance was present in 13% of the study population overall which is consistent with most other reports,[8, 9] although the CNS HIV Anti-Retroviral Therapy Effects Research (CHARTER) cohort reported that less than 2% of 1555 HIV infected subjects had CSF/plasma discordance.[12] Resistance mutations to multiple ART classes were demonstrated in CSF of discordant subjects. Of note, CSF ART resistance mutations were demonstrated in all 12 samples successfully tested, including NRTI RAMs in 8 of 10 on NRTIs at sampling and raltegravir RAMs in all 4 subjects on raltegravir at sampling. PI RAMs were less frequent, occurring in 2 or 9 samples tested. In discordant group B subjects, in whom viraemia was generally too low to perform resistance testing in plasma, CSF examination revealed RAMs to the current ARVs in 5 of 6 samples tested. Analysis of resistance in most plasma samples was not possible due to low viral load. Selected plasma samples are currently undergoing digital PCR as part of an ongoing study on compartmentalisation (see *section 10.2*). No follow up data was obtained to determine whether those subjects developed resistance in plasma, however *Table 11* shows that in many cases there was a past history of resistance in plasma.

An association between CSF/plasma discordance and nadir CD4 has been demonstrated in in other studies,[68] and is confirmed here, suggesting the presence of HIV in the CNS relates to a longer duration of viral replication and a lower immune status. Cognitive impairment has also been associated with nadir CD4;[12, 16] it is not clear whether this is due to a 'legacy effect' of CNS damage sustained in advanced disease, or whether it represents an ongoing CNS inflammatory process and/or

seeding of virus during advanced immunosuppression. These data would suggest the latter as discordant viral detection is detected in those with a history of low CD4 many years after the nadir, following immune reconstitution and plasma viral control. Whereas the former can be prevented by earlier diagnosis and ART initiation, the latter is potentially amenable to treatment with CNS targeted therapy. CSF white cell count was higher in discordant subjects as previously described suggesting a neuroinflammatory process in these subjects.[26] Further work to characterise CSF inflammatory biomarkers in those with CSF/plasma discordance at different levels is reported in chapter 6.

There is no universally accepted definition for CSF/plasma discordance; some definitions exclude those with $<1\log_{10}$ difference between CSF and plasma HIV-1 RNA, [68] and others exclude those with a CSF viral load <200 copies/ml.[8] Five subjects (subjects 8, 9, 12, 14 and 19) would be excluded by these criteria. Subject 19 has extensive multiclass resistance in CSF despite a CSF HIV-1 RNA of 162 copies/ml. Two subjects with CSF HIV-1 RNA 100-200 copies/ml (subjects 14 and 19) had resistance-associated mutations including M184V/I which is associated with impaired replicative capacity. The significance of CSF/plasma discordance at low levels is unknown.

Drug sampling was random which limited the ability to demonstrate associations with discordance. Pharmacokinetic modeling to calculate Cmin would be an alternative approach but was not undertaken as the sparse data was not rich enough for most drugs. When time post dose was taken into account with multivariate modeling there were no significant differences in measured antiretroviral concentrations between

groups in either plasma or CSF. The reasons why CSF drug levels did not correlate with discordance are not known. One explanation is that these measured concentrations may not reflect levels within brain parenchyma,[49, 50] or levels within perivascular macrophages, a critical target of HIV in the brain.[4] Although these data do not support measuring antiretroviral concentration in CSF in clinical practice, whether CSF antiretroviral drug concentrations relate to clinical outcomes (eg. cognitive functioning) or to neurotoxic side effects, was not examined. For many antiretroviral drugs measured, there were too few subjects receiving this medication at sampling to determine whether a statistically significant association with CSF/plasma discordance was present. This was due to the unselected nature of the cohort and the wide variety of antiretroviral regimen commonly used in clinical practice. The revised version of the CPE score has been associated with better HIV-1 RNA suppression in CSF relative to regimens scoring lower values.[141] In this study there was no association of CPE score with the occurrence of CSF/plasma discordance, however this study was not set up to look for such an association. Prescriber bias has been demonstrated; patients with CNS problems may be more likely to receive an ART regimen with a higher CPE score.[114] Subjects in group B were significantly more likely to be receiving raltegravir due the increased prescription of this treatment in patients with incomplete HIV-1 RNA suppression in plasma. In this study there was very little variability in CPE scores; 44% had a CPE score of 7 and median CPE score was 7 in all groups. This would further limit the ability of this study to determine differences between ART regimens with different CPE scores. Increased use of raltegravir in discordant subjects was unexpected. This may represent a history of plasma viral detection in these patients as is the case in group B (see chapter 5). Of note raltegravir resistance was detected in all 4 samples tested. These data suggest

that integrase inhibitors may be suboptimal when used to treat viral persistence in CSF, unlike the use of this class to treat viral persistence in plasma.

4.5 Conclusions

CSF/plasma discordance with drug resistance in CSF is not uncommon in subjects undergoing lumbar puncture for a clinical indication or to investigate unexplained HIV viraemia. This phenomenon is associated with nadir, but not current, CD4 count and did not appear to relate to ART concentrations in plasma or CSF. Definitions for CSF/plasma discordance excluding those with 0.5-1$\log_{10}$ difference may increase the chance of type 2 error.

5 PARTITION group B; CSF HIV-1 RNA detection in subjects with incomplete viral suppression in plasma

5.1 Introduction

Whether ongoing replication of HIV in a CNS sanctuary site can lead to failure of sustained suppression in blood (commonly interpreted as a viral load <50 copies/ml[41]) is not known (see section 1.4.2). Sensitive assays can now detect HIV-1 RNA below the 50 copies/ml threshold in a proportion of stably treated patients, however the source and significance of residual plasma HIV-1 RNA detection at this level remains controversial.[70]

5.2 Aims

The aim of this study was to investigate HIV-1 RNA levels and ART concentrations in the CSF in those with a) intermittent or persistent low-level plasma HIV-1 RNA detection above 50 copies/ml despite ART, and b) residual HIV-1 RNA detection below 50 copies/ml.

5.3 Methods

Subjects were recruited from the PARTITION study in 2 groups (see section 3.1). Forty cases of ongoing intermittent or persistent HIV-1 RNA detection in the last 12 months were prospectively recruited in group B. This study was designed to allow selection of subjects from group A with durable virological suppression (plasma HIV RNA <50 copies for ≥1 years, no evidence of CNS infection) to act as controls. A

total of 39 subjects from group A were selected. Forty-nine subjects (24 group A, 25 group B) with HIV-1 RNA <50 copies/ml underwent sensitive HIV-1 RNA quantification. See *Figure 4* outlining substudy groups.

5.4 Results

5.4.1 PARTITION group B; CSF HIV-1 RNA detection in subjects with incomplete viral suppression in plasma

In group B, 33 (83%) subjects had intermittent low-level viraemia in the preceding 12 months, while the remaining 7 (17%) had persistent low-level viraemia, median 144 copies/ml (IQR 90,279). Median CSF HIV-1 RNA was 20 copies/ml in both groups. Seven (18%) subjects in group B had CSF/plasma discordance which was significantly more than the comparison group in which there were no discordant subjects (p=0.012) (*Figure 7*).

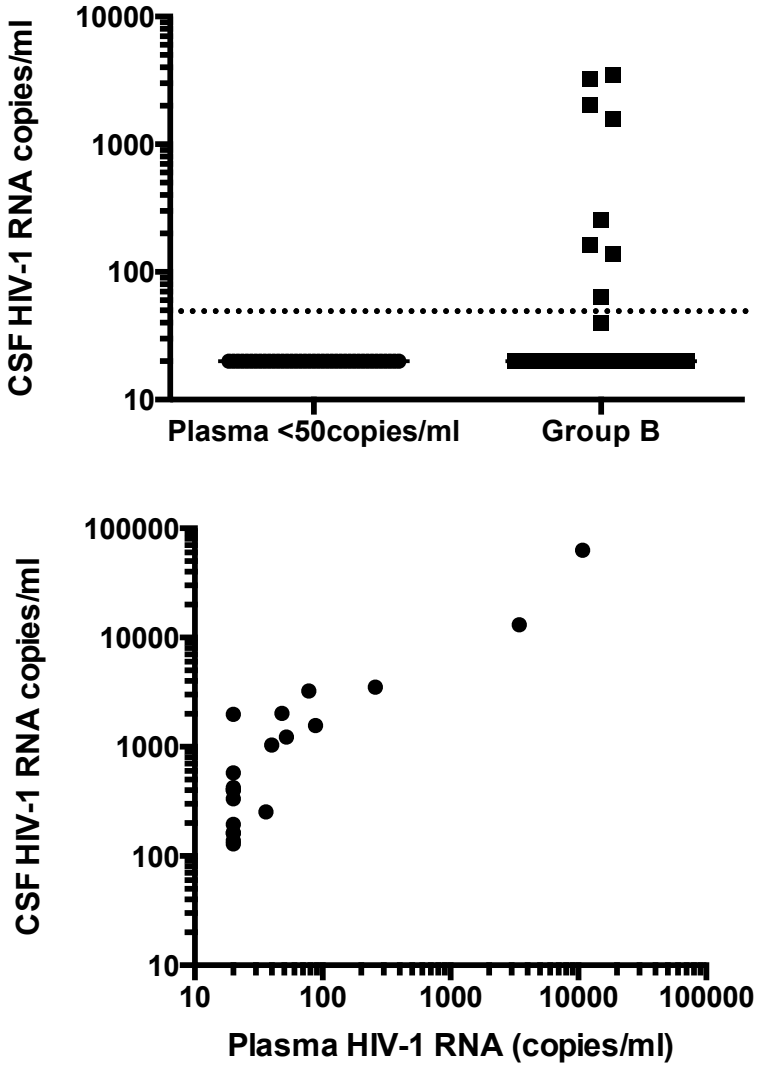

Figure 7. CSF HIV-1 RNA in subjects with and without sustained plasma HIV-1 RNA suppression

a. CSF HIV-1 RNA in 40 subjects with intermittent or persistent HIV-1 RNA ≥50 copies/ml (group B) vs. 39 subjects with consistent HIV-1 RNA suppression <50 copies/ml. Dashed line is 50 copies/ml.

b. Paired plasma and CSF HIV-1 RNA in the 7 subjects with CSF/plasma discordance.

Covariates which were significantly associated with plasma HIV-1 RNA detection ≥50 copies/ml in the preceding 12 months were compared between patients with detectable HIV-1 RNA (n=40) and controls (n=39). Significant associations were observed in CSF/plasma discordance and years since HIV diagnosis (*Table 12*). No significant differences were observed with age, gender, ethnicity, nadir/current CD4 count or CPE score. ART regimen was not associated with a history of plasma viraemia, apart from increased raltegravir use in patients with detectable viraemia.

CSF/plasma discordance was unable to be fitted to a logistic regression model as there is no variability in one of the groups (ie. no subjects with CSF/plasma discordance in the aviraemic group). A logistic regression model containing the 2 remaining significant variables from univariate analysis was significantly associated with HIV-1 RNA detection ≥50 copies/ml within 12 months (p=0.006). Both variables: years since HIV diagnosis and treatment with integrase inhibitor, remained significant in the model (p=0.042 and 0.022 respectively) showing they were independently associated with the outcome. The model as a whole explained between 12% (Cox and Snell R square) and 16% (Nagelkerke R squared) of the variance in discordance, and correctly classified 67% of cases. The strongest predictor of outcome was treatment with integrase inhibitor, reporting an odds ratio of 0.192, indicating that treatment with an integrase inhibitor was associated with an 81% increase in HIV-1 RNA detection within 12 months. This is likely to reflect the use of integrase inhibitors to intensify ART in patients with ongoing viraemia.

	Plasma HIV-1 RNA detection ≥50 copies/ml in past 12 months		
	Yes (n=40)*	**No (n=39)****	**p value**
CSF/plasma discordance, n (%)	7 (18)	0 (0.0)	0.012
Age, median years (IQR)	46 (40,52)	48 (41,57)	0.337
Male gender, n (%)	35 (88)	35 (90)	1.000
Risk group, n (%)			
MSM	28 (72)	27 (68)	
Heterosexual	9 (23)	9 (23)	0.557
IDU	0 (0)	1 (3)	
Vertical	1 (3)	0 (0)	
Not disclosed	1 (3)	3 (8)	
Ethnicity, n (%)			
White	34 (85)	27 (68)	
Black	5 (13)	8 (20)	0.214
Asian	1 (3)	1 (3)	
Mixed/other	0 (0)	3 (8)	
Years since HIV diagnosis, median (IQR)	9.6 (4.7,17.4)	12.1 (9.1,20.3)	0.039
CD4, median cells/mm3 (IQR)			
Nadir	132 (43,325)	196 (88,289)	0.314
Current	520 (395,729)	523 (354,770)	0.936
ART, n (%)			
PI	29 (73)	22 (56)	0.162
NNRTI	12 (30)	17 (44)	0.248
NRTI	33 (83)	34 (87)	0.756
RAL	10 (25)	1 (3)	0.007
MVC	1 (3)	2 (5)	0.615
CPE, median (IQR)	7 (6,7)	7.0 (6,8)	0.923

Table 12. Factors associated with plasma HIV-1 RNA detection ≥50 copies/ml within 12 months.

**Group B. **Subset from group A, excluding those with CNS infection, with persistent HIV-1 RNA suppression below 50 copies/ml for 12 months.*

MSM, men who have sex with men; IVDU, intravenous drug user; IQR, interquartile range; ART, antiretroviral therapy; PI/r, ritonavir boosted protease inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside/tide reverse transcriptase inhibitor; CCR5 antag, CCR5 receptor antagonist; CPE, CNS penetration effectiveness score 2010.

5.4.2 Cognitive and functional testing in Group B

One subject (3%) had an abnormal International HIV Dementia Scale (score 9.5/12) consistent with modern prevalence data on HIV-associated dementia[12]. This subject had CSF/plasma discordance. One subject had abnormal Instrumental Activities of Daily Living scale; this subject also had CSF/plasma discordance but was a different subject to that with abnormal International HIV-Dementia Scale. Ten subjects (25%) were considered anxious, and 2 subjects (6%) were considered depressed. Eight subjects (20%) recorded abnormal responses to cognitive symptoms questionnaire. There were no statistically significant differences between subjects with and without CSF/plasma discordance in any of these tests.

5.4.3 Pharmacokinetics

No significant differences were observed between the groups in measured antiretroviral concentrations in plasma or CSF (*Table 13*). Trends to lower plasma darunavir and higher CSF efavirenz in group B were lost when combined with time post dose in a multivariate model (p=0.174 and 0.624 respectively).

	Plasma HIV-1 RNA ≥50copies/ml in past 12 months				
	Yes (n=40)		No (n=39)		
	n	Median (IQR)	n	Median (IQR)	p value
Plasma					
ATZ	3	393.3 (202.3,1678)	7	1038 (713.8,1665)	NS
DRV	20	2050 (1450,5710)	11	3838 (3489,6291)	*0.079
ETV	2	543.5 (362.5,7724.6)	1	72.51	NS
LPV	1	8206	2	8769 (8731,8807)	NS
MVC	1	178.5	2	236.0 (42.19,429.7)	NS
NVP	2	13319 (4916,21723)	3	4870 (4468,5268)	NS
RAL	6	863.0 (162.7,1360)	0		NS
RPV	1	89.04	1	97.08	NS
RTV	0		6	257.1 (65.44,287.4)	NS
EFZ	6	1709 (1377,3586)	10	1327 (923.4,3433)	0.426
TDF	18	92.21 (47.80,174.1)	17	99.09 (67.35,181.8)	0.726
FTC	19	276.7 (55.31,527.5)	18	201.8 (72.60,709.8)	0.980
3TC	2	66.77 (35.11,98.42)	9	714.8 (120.8,1678)	NS
CSF					
ATZ	4	5.09 (2.337,17.93)	7	12.32 (1.95,14.84)	0.930
DRV	22	38.58 (25.42,93.12)	9	53.26 (20.50,93.23)	0.893
LPV	1	9.871	2	10.70 (6.386,15.02)	NS
MVC	1	16.61	2	8.491 (5.627,11.35)	NS
NVP	2	3870 (1880,5860)	4	1429 (1183,1977)	NS
RAL	9	19.82 (16.51,48.31)	0	21.30 (15.27,44.95)	NS
EFZ	6	23.95 (16.11,30.13)	10	13.10 (9.986,21.60)	**0.073

Table 13. Plasma and CSF antiretroviral drug concentrations in group B vs. a suppressed comparison group.

*P values were calculated for drugs with 6 or more measurements in each group. Differences remained non-significant when combined with time post dose in a multivariate model; *p=0.174, **p=0.624.*

NS, not significant; ATZ, atazanavir; DRV, darunavir; ETV, etravirine; LPV, lopinavir; MVC, maraviroc; NVP, nevirapine; RAL, raltegravir; RPV, rilpivarine; RTV, ritonavir; EFZ, efavirenz; TDF, tenofovir; FTC, emtricitabine; 3TC, lamivudine; IQR – interquartile range.

5.4.4 CSF resistance

The CSF resistance profiles of discordant subjects in group B (subjects 14-20) are given in *Table 11* in chapter 4. All discordant CSF samples sequenced showed resistance mutations in CSF; one subject had insufficient CSF sample for sequencing. Five of 6 subjects showed resistance to drugs in use at sampling. PI major resistance mutations were detected in 1 subject on darunavir at sampling (subject 19). Resistance to NNRTIs was detected in 2 subjects. Neither were receiving NNRTIs at sampling but both had historic NNRTI use; subject 16 received nevirapine between 1996 and 1997 and subject 19 received efavirenz between 2000 and 2001. Resistance to NRTIs, including the M184V/I mutation associated with high-level resistance to lamivudine and emtricitabine and increased susceptibility to tenofovir, was detected in all 6 CSF samples. Three subjects were receiving emtricitabine (subjects 14, 18 and 19), 1 subject was receiving lamivudine (subject 20), and 1 subject was receiving tenofovir (subject 16) at sampling. Subject 16 had received lamivudine until 7 years prior to sampling. One subject with NRTI resistance (subject 17) was not receiving NRTIs at sampling but had received emtricitabine and tenofovir until 1 year prior to sampling. Resistance to integrase inhibitors was detected in the 1 subject on raltegravir at sampling (subject 16).

One subject (subject 16) receiving tenofovir, raltegravir and ritonavir-boosted darunavir 600/100 mg twice daily showed multiple mutations comprising thymidine analogue mutations (TAMs: D67N, K70R, T215Y, K219E), L74V and M184V for the NRTIs, L100I and K103N for the NNRTIs, and Y143C for the integrase inhibitors. The simultaneous plasma sample was 49 copies/ml. The same RT resistance profile observed in the discordant CSF sample had been detected 10 years

previously in plasma virus sequenced after virological failure of zidovudine, lamivudine plus abacavir followed by abacavir, lamivudine plus efavirenz. The patient then started PI/r-based ART and between 2002 and 2008 and experienced 5 consecutive HIV-1 RNA measurements between 50 and 542 copies/ml; three of these viremic samples were sequenced and two, collected while the patient was receiving tenofovir, atazanavir, fosamprenavir, and ritonavir, showed the protease mutation V32I but no RT mutations. In 2008 the patient started tenofovir, raltegravir and ritonavir-boosted darunavir (600/100 mg twice daily) and between 2008 and 2011 experienced two further episodes of viremia (50 and 117 copies/ml respectively); one of the two samples was sequenced with no mutations detected in RT, protease or integrase.

5.4.5 Sensitive HIV-1 RNA testing in the PARTITION study

Median volume of plasma tested was 4.5ml (IQR 3,6); median volume of CSF tested was 4ml (IQR 3,7). There was no correlation between volume of sample tested and virus detection (Spearman r=-0.119, p=0.242). Overall significantly more CSF samples had no HIV-1 RNA detected (n=39) than plasma (p<0.0001).

Thirty-two (65%) subjects had plasma HIV-1 RNA detection between 1-49 copies/ml, median 13 copies/ml (IQR 6,24). Seventeen (35%) subjects had no HIV-1 RNA detected in plasma; in these subjects the median plasma volume tested was 5ml (IQR 3.5,5.75) giving a median sensitivity of detection of 7 copies/ml (IQR 5,7).

All subjects with no HIV-1 RNA detection in plasma on sensitive testing, also had no virus detection in CSF on sensitive testing. Median CSF volume tested for

undetectable samples was 4ml (IQR 3,7) giving a median sensitivity of detection of 7 copies/ml (IQR 3,7). Sensitivity of detection was calculated by the group that developed and applied the assay, details will be presented elsewhere.[142] Ten (31%) subjects with residual HIV-1 RNA detection in plasma had HIV-1 RNA detection in CSF, median 19 copies/ml (IQR 4,167) (*Figure 8*). Viral detection in CSF was significantly more frequent in this group than in those without plasma viral detection (p=0.009). Three (9%) of these subjects met study criteria for CSF/plasma discordance (*Table 11*; subjects 14, 15 and 16). One further subject (group B) had CSF/plasma discordance only detected on sensitive testing; 4 copies/ml in plasma and 40 copies/ml in CSF. The discordance rate was not significantly different between groups (p=0.542).

Covariates which were significantly associated with residual plasma HIV-1 RNA detection below 50 copies/ml were compared between patients with undetectable HIV-1 RNA (n=17) and with those with 1-49 copies/ml (n=32) (*Table 14*). Time since HIV diagnosis was significantly longer in those with no residual HIV-1 RNA detection in plasma, however when combined with nadir CD4 in a multivariate logistic regression model, neither variable remained significant (p=0.159, 0.219 respectively). It was not possible to include CSF HIV-1 RNA detection in the model due to lack of variability in one of the groups (ie no CSF HIV-1 RNA detection in the group without residual plasma HIV-1 RNA detection). No significant differences were observed with age, gender, ethnicity, nadir/current CD4 count or CPE score.

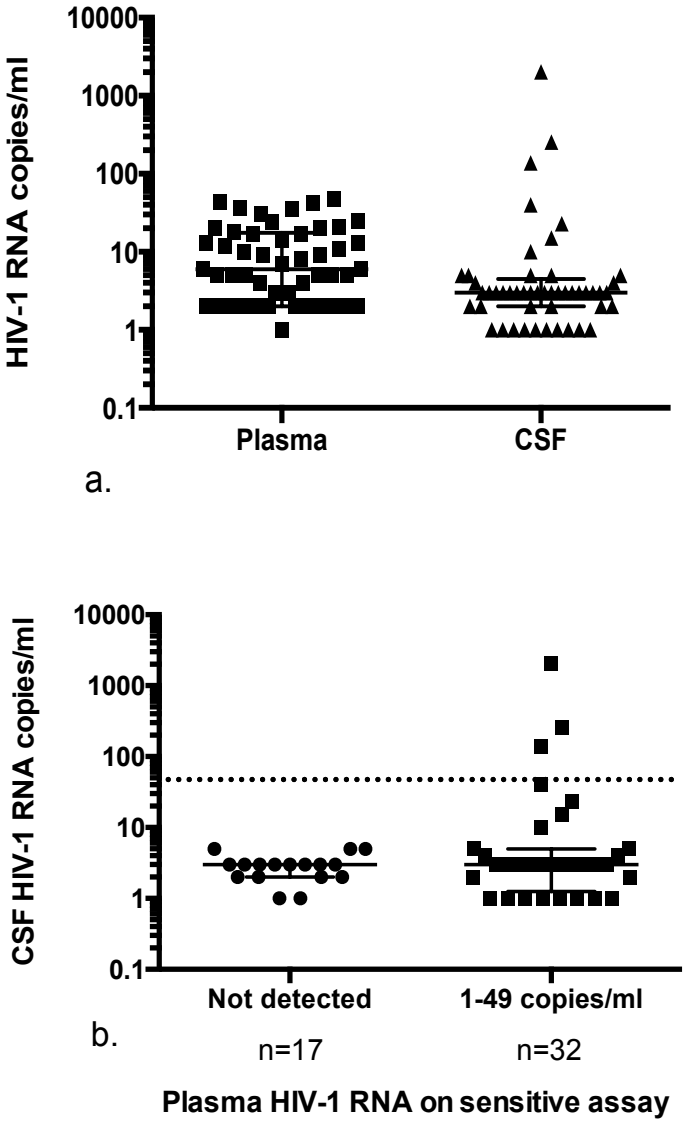

Figure 8. Low level HIV-1 RNA detection in CSF and plasma by sensitive assay

a. CSF and plasma HIV-1 RNA in 49 subjects undergoing HIV-1 RNA quantification with sensitive assay

b. CSF HIV-1 RNA in 17 subjects with no residual plasma HIV-1 RNA detected vs. 32 subjects with plasma HIV-1 RNA detection between 1 and 49 copies/ml. Dashed line is at 50 copies/ml.

	Plasma HIV-1 RNA		
	<LLQ US assay (n=17)	**1-49 copies/ml (n=33)**	**p value**
HIV-1 detection in CSF, n (%)	0 (0)	11 (33)	**0.009
CSF HIV-1 RNA, median copies/ml (IQR)	-	23 (4,254)	
Age, years (IQR)	53 (47,59)	48 (41,55)	0.107
Male gender, n (%)	14 (82)	31 (94)	0.322
Risk group, n (%)			0.281
MSM	10 (59)	23 (74)	
Heterosexual	6 (35)	8 (26)	
IVDU	1 (5.9)	0 (0.0)	
Ethnicity, n (%)			0.779
White	14 (82)	26 (79)	
Black	3 (18)	5 (15)	
Asian	0 (0)	1 (3)	
Mixed	0 (0)	1 (3)	
Years since HIV diagnosis, median (IQR)	17 (7.5,27.0)	8 (3.5,17.0)	*0.041
CD4, median cells/mm3 (IQR)			
Nadir	82 (32,230)	178 (55,338)	*0.092
Current	334 (102,414)	540 (379,841)	0.197
ART, n (%)			
PI/r	14 (82)	20 (61)	0.2
NNRTI	6 (35)	12 (36)	1.000
NRTI	14 (82)	29 (88)	0.677
INI	1 (6)	6 (18)	0.398
CCR5 antag	0 (0)	3 (9)	0.542
CPE, median (IQR)	7.0 (3.5,7.5)	7.0 (3.0,8.0)	0.233

Table 14. Factors associated with residual HIV-1 RNA detection.

**Variables were taken forward into a multivariate model. Neither nadir CD4 or years since HIV diagnosis remained significant in this model. **HIV-1 RNA detection in CSF could not be included in multivariate regression model due to lack of variability in one of the groups.*

MSM, men who have sex with men; IVDU, intravenous drug user; IQR, interquartile range; ART, antiretroviral therapy; PI/r, ritonavir boosted protease inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside/tide reverse transcriptase inhibitor; CCR5 antag, CCR5 receptor antagonist; CPE, CNS penetration effectiveness score 2010.

5.4.6 Pharmacokinetics

There were no significant differences in measured antiretroviral concentrations in plasma or CSF between the two groups (*Table 15*). No significant differences between groups were observed when time post dose was incorporated in a multivariate model.

	Plasma HIV-1 RNA				
	<LLQ US assay (n=17)		1-49 copies/ml (n=33)		
	n	Median (IQR)	n	Median (IQR)	p value
Plasma					
ATZ	3	1312 (871.3,1665)	2	1611 (47.01,3174)	NS
DRV	10	3920 (2329,10692)	14	4425 (1759,5726)	0.400
ETV	1	362.5	0		NS
MVC	0		1	617	NS
NVP	2	3838 (2408,5268)	4	4692 (4222,17521)	NS
RAL	0		4	863.0 (289.6,1155)	NS
RPV	1	97.08	1	89.04	NS
RTV	6	111.4 (42.48,272.7)	2	207.3 (169.8,244.9)	NS
EFZ	2	2356 (1934,2778)	6	2359 (1723,3586)	NS
TDF	9	156.2 (93.85,226.0)	12	97.20 (68.86,157.1)	0.128
FTC	8	605.7 (461.6 (922.5)	13	275.9 (144.8,751.0)	0.140
3TC	2	2003 (1783,2224)	5	610.2 (282.1,1605)	NS
CSF					
ATZ	3	14.69 (12.32,19.01)	3	3.50 (1.95,25.28)	NS
DRV	10	40.46 (16.21,94.13)	14	39.11 (25.95,81.02)	0.910
MVC	0		2	5.567 (2.602,8.531)	NS
NVP	2	958 (751.8,1165)	4	2199 (1612,5024)	NS
RAL	1	24.69	6	18.43 (14.21,27.78)	NS
EFZ	2	22.92 (20.29,25.56)	6	24.22 (18.62,30.13)	NS

Table 15. Plasma and CSF antiretroviral drug concentrations in those with and without residual HIV-1 RNA detection in plasma on sensitive testing.

P values were calculated for drugs with 6 or more measurements in each group.

NS, not significant; ATZ, atazanavir; DRV, darunavir; ETV, etravirine; LPV, lopinavir; MVC, maraviroc; NVP, nevirapine; RAL, raltegravir; RPV, rilpivarine; RTV, ritonavir; EFZ, efavirenz; TDF, tenofovir; FTC, emtricitabine; 3TC, lamivudine; IQR – interquartile range.

5.5 Discussion

Importantly, discordance was observed in 18% of patients with intermittent or continuous low-level viraemia (group B) compared with no discordance in HIV-positive patients with durably and optimally suppressed plasma viral loads. This suggests that a significant proportion of HIV-positive patients with low-level viraemia despite ART have suboptimal control of virus replication within the CNS. The clinical significance of this finding remains unclear, but studies are currently investigating the link between ongoing replication during ART and immune activation, cardiovascular risk, and virological rebound. One interpretation of these findings could be that ongoing replication of HIV in a CNS sanctuary site and the development of ART resistance in this compartment is responsible for viral detection in plasma in a proportion of patients. In 7 subjects at least part of the resistance detected in CSF virus was consistent with the previous history of failure and older resistance tests in plasma. This combined with the observed impact of low nadir CD4, suggest that advanced immune suppression may allow the establishment of a viral reservoir in the CNS that is only partially responsive to ART. This virus may then continue to evolve and develop resistance to some ARVs, for example raltegravir, and thus become less susceptible to suppression than virus in peripheral blood. Low-level viraemia with a genetically different virus may originate from a similar process of incomplete suppression at other sites such as lymphoid tissue. Alternatively, intermittent reseeding of virus into the CNS during periods of systemic replication due to another cause (eg. non-declared poor adherence) may have established compartmentalised CNS infection.

All discordant CSF samples sequenced showed resistance mutations in CSF. This suggests that the CNS may be the source of plasma virus in patients with low-level treatment failure. Subject 16 appears to demonstrate seeding of virus into the CNS early in infection, which has persisted despite changes to antiretroviral therapy. This phenomenon is currently being examined further with digital PCR and phylogenetic analysis of virus in CSF and plasma in subject 16 and other discordant subjects at the Center for Disease Control and Prevention (CDC) as part of an MRC Early Career Award (see section 10.2.1).

Of note, most subjects had HIV viral loads in plasma too low to perform resistance testing. Determining the presence of resistant virus, and hence the ability to make changes to antiretroviral treatment with the knowledge of resistance profile, would only be possible by performing lumbar puncture in otherwise asymptomatic individuals. These data suggest that performing lumbar puncture to quantify CSF HIV-1 RNA and determine viral resistance in this compartment may be a useful strategy in those with blips, or systemic treatment failure.

The source and significance of residual plasma HIV-1 RNA detection <50 copies/ml remains unclear; this may be due to release of latent virus from resting memory T-cells and therefore not capable of replication, or be due to ongoing HIV-1 replication in sanctuary sites such as the brain. Patients with a viral load <50 copies/ml monitored with the Abbott RealTime assay, plasma HIV-1 RNA levels between 40 and 49 copies/ml and to a lesser extent qualitative RNA detection below 40 copies/ml have been associated with a markedly increased risk of viral load rebound above 50 copies/ml and above 400 copies/ml over 12 months of follow-up.[137] Recent analyses

have confirmed these initial findings.[143, 144] These observations indicate that a subset of patients with plasma HIV-1 RNA detection below the 50 copies/ml threshold may be experiencing on-going virus replication. In this study residual HIV-1 RNA detection below 50 copies/ml on sensitive testing was significantly associated with CSF HIV-1 RNA detection, suggesting that in some cases the CNS may be the source of HIV-1 detection in the plasma. This has implications for targeting antiretroviral treatment to the CNS and the eradication of HIV from sanctuary sites. Determining low level viraemia in this setting may represent a clinical use for sensitive HIV-1 RNA assays, currently only available as a research tool in most centres. Three subjects had CSF/plasma discordance, however this was not significantly different to the absence of CSF/plasma discordance in 17 without HIV-1 detection in plasma. In 1 additional case discordance was detected only on sensitive testing (plasma 4 copies/ml, CSF 40 copies/ml). The significance of discordance detected at this level remains controversial and is examined further in chapter 6.

This study has several limitations. Prospectively recruiting persistently suppressed patients would have been methodologically preferable than selecting the control population from group A, but this was not felt to be ethically justified. However, as discordance is more frequent in clinically indicated lumbar punctures[8, 9, 39, 71] than those performed for research purposes,[9, 39] this would be more likely to lead to an increase in CSF/plasma discordance rates in the control group and an underestimation of the difference with group B; hence a type 1 error would be more likely than type 2.

This study has shown association, rather than causality. Ongoing phylogenetic analysis will help to address this by determining the genetic origins of virus in CSF

(section 10.2.1). It is intended that the effect of treating CSF/plasma discordance on viral persistence in plasma will be addressed in a longitudinal follow-on study (section 10.2.2).

Sensitive HIV-1 RNA testing remains a research tool. The variability in viral detection at very low levels over time within a single individual has not been determined. Whether CSF/plasma discordance is associated with viral detection in plasma <50 copies/ml was not shown in this study and requires clarification in larger cohorts.

5.6 Conclusions

The CNS may be the source of ongoing HIV-1 replication in a proportion of patients that fail to suppress below 50 copies/mL in plasma, as well as those with residual plasma HIV-1 RNA detection between 1-49 copies/mL. This has implications for the management of low-grade viraemia and 'blips', both in terms of stratifying patients for lumbar punctures and tailoring treatment for effective suppression in the CNS.

6 CSF/plasma HIV-1 RNA discordance above 0.5log$_{10}$ associated with raised expression of CSF biomarkers compared with non-discordant subjects

6.1 Introduction

The clinical significance of CSF/plasma discordance remains to be determined (see section 1.4.1). There is currently no consensus on the degree of discordance between CSF and plasma HIV-1 RNA that should be considered clinically significant.[8, 9, 68] In addition, sensitive testing can now detect HIV-1 RNA below the traditional threshold of 50 copies/ml. However the source and significance of CSF/plasma discordance at this low level remains controversial.[20, 70] Pro-inflammatory mediators have been shown to be raised in the CSF of some HIV positive individuals and in some cases this has been linked to cognitive impairment, however no single mediator has a strong enough correlation with cognitive status to be a clinically useful biomarker alone and a combination of biomarkers may be more useful.[81]

6.2 Aims

This nested case-control study aimed to use a cytometric bead array system to determine whether a panel of CSF cytokines, chemokines and associated mediators differed in subjects with CSF/plasma discordance compared with non-discordant subjects. Furthermore this study aimed to examine the relationship between mediator profile and CSF/plasma discordance at different levels.

6.3 Methods

6.3.1 Study population

Subjects were recruited from the PARTITION study in 2 groups (see section 3.1). Subjects with confirmed or suspected CNS infection were excluded.

6.3.2 Selection criteria

Discordance was defined as HIV-1 RNA >0.5$\log_{10}$ a simultaneous plasma sample, or in those with unquantifiable plasma HIV-1 RNA >0.5$\log_{10}$ the lower limit of quantification (ie. 40 copies/ml). Subjects were categorised as "high discordance" if CSF HIV-1 RNA was >1$\log_{10}$ plasma. Subjects with 0.5-1$\log_{10}$ discordance, or with discordance only detected on sensitive assay, were categorised as "low discordance". Low discordance represents a group that would be excluded by some definitions of discordance. CSF viral escape was defined as plasma HIV-1 RNA less than lower limit of quantification for the standard assay (ie 40 copies/ml), and CSF greater than 40 copies/ml.

For controls, we looked among all participants in the partition study who did not have discordance, and selected those with a plasma HIV RNA-1 which most closely matched that of subjects with discordance.

6.4 Results

Discordant subjects: Nineteen cases of CSF/plasma discordance were identified in 18 subjects (1 subject had 2 discordant results in CSF collected at an interval of more than 2 years). Eleven (58%) had high discordance and 8 (42%) had low discordance;

of those with low discordance, 3 had CSF/plasma discordance only detectable on sensitive testing - plasma and CSF HIV-1 RNA 4, <7, <10 and 40, 74, 51 copies/ml respectively. Median HIV-1 RNA in plasma was 20 copies/ml (IQR 10,52) and in CSF was median 422 copies/ml (IQR 138,1981).

Non-discordant subjects: Twenty-one non-discordant subjects were selected. Median HIV-1 RNA in plasma was 20 copies/ml (IQR 8,50) which was not significantly different to discordant subjects (p=0.655). Twelve (57%) subjects had low CSF HIV-1 RNA demonstrated on sensitive assay; median HIV-1 RNA in CSF was 4 copies/ml (IQR 4,20). HIV-1 RNA in CSF was lower than plasma in 15 (71%) subjects, the same as plasma in 5 (24%) subjects, and greater than plasma in 1 (5%) subject (plasma <40, CSF 69 copies/ml).

6.4.1 Subject characteristics

Demographic, clinical and laboratory parameters did not differ between discordant and non-discordant subjects, with the exception of CSF pleocytosis which was more frequent in those with discordance (*Table 16*).

	Discordant (n=19)	Non-discordant (n=21)	p value
Age, median years (IQR)	47 (44,57)	47 (41,53)	0.5773
Gender male, n (%)	15 (78.9)	15 (71.4)	1.0000
Ethnicity, n (%)			0.3265
White	12 (63.2)	15 (71.4)	
Black	6 (31.5)	3 (14.3)	
Other	1 (5.3)	3 (14.3)	
Risk group, n (%)			0.2353
MSM	9 (47.4)	14 (70.0)	
Heterosexual	9 (47.4)	4 (19.0)	
IVDU	0 (0.0)	1 (4.8)	
Other/unknown	1 (5.3)	2 (9.5)	
CD4			
Current, median cells/ul (IQR)	380 (319,490)	464 (359,741)	0.1888
Nadir [1], median cells/ul (IQR)	31 (27,79)	112 (34,283)	0.1019
Time since HIV diagnosis, median years (IQR)	13.0 (9.3,18.0)	8.0 (4.5,16.5)	0.0869
Plasma HIV-1 RNA			
<40 copies/ml, n (%)	12 (63.2)	14 (66.7)	1.000
≥40 copies/ml, median (IQR)	78 (48,88)	83 (45,117)	0.6865
Median copies/ml (IQR) using LLQ	40 (40,52)	40 (9,50)	0.2998
CSF HIV-1 RNA, median copies/ml (IQR)	422 (138,1981)	7 (6,10)	**< 0.0001**
ART at sampling, n (%)			0.3057
PI/r	17 (89.5)	13 (61.9)	
NNRTI	2 (10.5)	7 (33.3)	
NRTI	17 (89.5)	18 (85.7)	
RAL	5 (26.3)	2 (9.5)	
MVC	2 (10.5)	3 (14.3)	
CPE score (2010), median (IQR)	7 (7,8)	7 (6,9)	0.9030
Self reported adherance, n (%)			0.9875
≥95%	15 (78.9)	17 (81.0)	
<95%	2 (10.5)	2 (9.5)	
Unknown	2 (10.5)	2 (9.5)	
Indication for LP, n (%)			0.0587
Investigate cognitive symptoms	5 (26.3)	2 (9.5)	
Headache	0 (0.0)	3 (14.3)	
Follow up CNS infection [2]	3 (15.8)	0 (0.0)	
Measure CSF HIV viral load	0 (0.0)	2 (9.5)	
Investigate abnormal neurology	1 (5.3)	4 (19.0)	
Confusion	1 (5.3)	0 (0.0)	
? neurosyphilis	1 (5.3)	0 (0.0)	
? Encephalitis	0 (0.0)	2 (9.5)	
Research LP	8 (42.1)	8 (38.1)	

Table 16. Subject characteristics in 19 cases of CSF/plasma discordance >0.5log$_{10}$ vs. 21 without discordance.

IQR, interquartile range; MSM, men who have sex with men; IVDU, intravenous drug user; LP, lumbar puncture; CPE, CNS penetration effectiveness score (2010); ART, antiretroviral therapy; PI/r, ritonivir boosted protease inhibitor; NNRTI, non nucleoside reverse transcriptase inhibitor; RAL, raltegravir; MVC, maraviroc.

CSF white cell count was significantly higher in the discordant group (median 4.5 cells/ul [IQR 0,18]) than in the non-discordant group (median 0 cells/ul [IQR 0,1]); p=0.003. CSF white cell count was recorded in 10 of 16 (63%) subjects in group B (not all group B subjects had CSF white cell count measured as this was not a condition of the study protocol (see *section 3.3.6*). Eight (53%) of 15 discordant subjects had normal CSF white cell count (ie. ≤4cells/ul); of these 6 (40%) had no measurable white cells in CSF (ie. <1cell/ul). All subjects in the non-discordant group had normal CSF white cell count; 14 (74%) had no measureable cells (*Figure 9*).

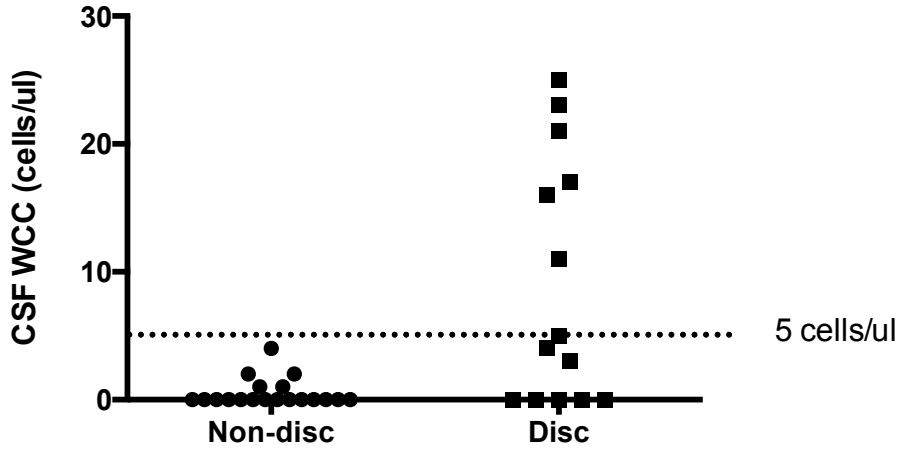

Figure 9. CSF white cell count (WCC) in subjects with and without CFS/plasma discordance

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6.4.2 CSF mediators

The following mediators had measurements at or below the lower limit of quantification in ≥80% of measurements (ie. ≥30 samples) and hence were excluded from further analysis (see *section 3.3.7*): GCSF, GM CSF, IFNa2, IFNb, IL17a, IL17, IL2, IL4, TNFa, VEGFa, MMP1, 3, 8, 9, 12 and 13.

6.4.2.1 Univariate analysis

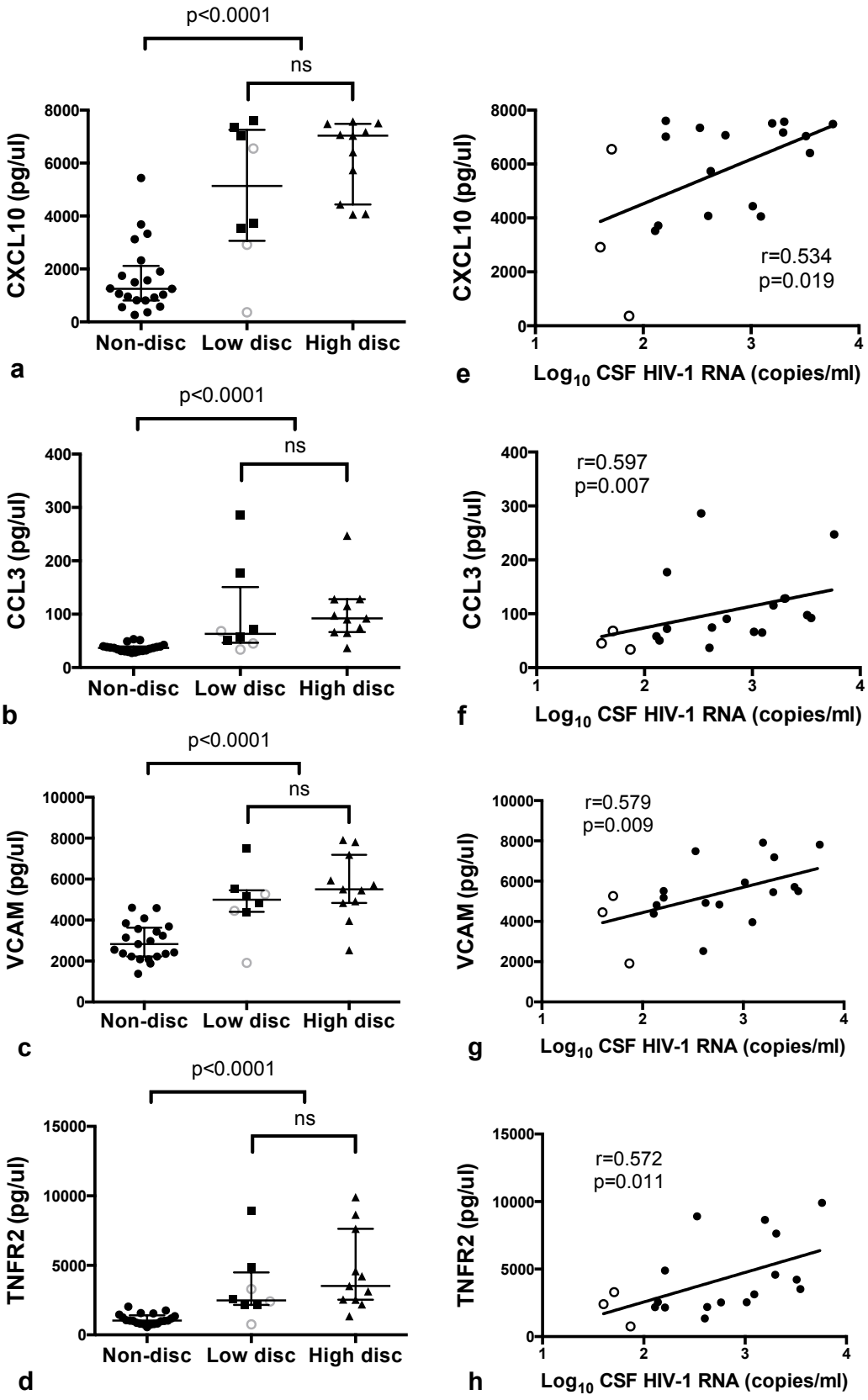
Nineteen (90%) of 21 CSF mediators were significantly higher in discordant than non-discordant subjects (*Table 17*); these broadly pro-inflammatory mediators included chemokines (CXCL10, CCL3, CCL4, CCL5), interferons (IFNy), interleukins (IL1a, IL1b, IL6, IL8, IL10) and their receptor antagonists (IL1RA), tumour necrosis factors (TNFb) with their receptors (TNFR1, TNFR2) and the related apoptosis inducing ligand (TRAIL), myeloperoxidase enzyme (MPO), adhesion molecules (ICAM, VCAM) and metalloproteinases (MMP2). There were no significant differences between CSF mediator concentrations in subjects with high vs. low discordance. CSF mediator concentrations significantly correlated with CSF HIV-1 RNA for 9 mediators; IL10, IL1a, CXCL10, CCL3, CCL4, CCL5, VCAM, TNFR2 AND TRAIL. The 4 CSF mediators with the most significant correlation with CSF HIV-1 RNA (CXCL10, CCL3, VCAM and TNFR2) are shown in *Figure 10*.

	All subjects			Discordant subjects			Correlation with CSF HIV-1 RNA	
	Discordant (n=19)	Non-discordant (n=21)	p value	Low discordant (n=8)	High discordant (n=11)	p value	Spearman r	p value
IFNγ	40.0 (32.0,48.3)	29.5 (24.5,34.9)	**0.0014**	39.9 (32.1,46.1)	41.5 (32.0,53.3)	0.7624	0.2328	0.3376
IL10	26.5 (22.5,33.0)	17.5 (16.5,19.5)	**< 0.0001**	25.8 (21.3,28.1)	31.0 (22.5,37.8)	0.4546	**0.4706**	**0.042**
IL1a	53.0 (39.8,62.0)	20.5 (18.0,30.3)	**< 0.0001**	47.1 (36.6,60.9)	53.0 (43.5,63.0)	0.3615	**0.4783**	**0.0383**
IL1b	9.0 (8.0,11.0)	7.0 (7.0,7.8)	**< 0.0001**	8.8 (8.0,9.9)	9.5 (8.0,14.5)	0.4528	0.2694	0.2647
IL1RA	15.0 (13.8,17.5)	13.5 (12.5,14.0)	**0.0008**	15.0 (13.5,15.5)	16.0 (14.3,24.8)	0.2642	0.3481	0.1442
IL6	9.0 (8.0,10.5)	7.3 (6.6,8.3)	**0.0048**	8.5 (7.9,10.8)	9.0 (7.9,10.5)	0.9528	0.2191	0.3674
IL8	125.5 (96.0,169.5)	63.0 (53.0,79.4)	**< 0.0001**	119.4 (79.9,145.4)	128.5 (104.0,117.3)	0.6364	0.3221	0.1787
CXCL10	6553 (4058,7342)	1249 (812.8,2114)	**< 0.0001**	5136 (3067,7260)	7038 (4439,7480)	0.2318	**0.5336**	**0.0186**
CCL2	1271 (872.8,1582)	944.5 (792.9,1322)	0.3048	1139 (691.6,1562)	1271 (878.8,1678.0)	0.753	0.2835	0.2396
CCL3	74.5 (58.0,128.0)	36.8 (30.8,39.6)	**< 0.0001**	63.0 (46.6,150.8)	92.0 (66.5,128.0)	0.2654	**0.5968**	**0.007**
CCL4	23.5 (16.0,59.0)	14.5 (13.0,18.8)	**0.0041**	17.8 (15.3,48.9)	26.5 (22.5,66.5)	0.2014	**0.5055**	**0.0273**
MPO	23.0 (17.5,67.8)	16.0 (13.0,17.6)	**0.0006**	21.6 (16.4,27.8)	29.5 (21.3,117.0)	0.134	0.4139	0.0781
CCL5	57.5 (25.0,190.5)	14.5 (11.5,22.3)	**< 0.0001**	33.8 (22.1,157.3)	85.8 (31.5,281.5)	0.2816	**0.5233**	**0.0215**
ICAM	296.0 (176.8,622.5)	71.5 (62.3,93.8)	**< 0.0001**	286.0 (161.2,525.7)	325.0 (204.0,774.0)	0.5278	0.4493	0.0536
VCAM	5263 (4458,5935)	2222 (2831,3629)	**< 0.0001**	4995 (4400,5452)	5508 (4835,7190)	0.2318	**0.5792**	**0.0094**
TGFb	82.8 (65.5,99.8)	55.0 (47.0,65.1)	**0.0031**	91.9 (68.6,134.1)	82.8 (64.0,94.0)	0.2363	-0.0974	0.6916
TNFR2	3131 (2195,4891)	1032 (815.8,1402)	**< 0.0001**	2477 (2159,4492)	3514 (2526,7629)	0.3018	**0.5722**	**0.0105**
TNFR1	11788 (8992,14874)	7761 (7461,9346)	**0.0006**	11267 (9256,13491)	11788 (8444,18161)	0.6364	0.4239	0.0705
TRAIL	42.5 (36.5,51.5)	26.9 (29.4,31.8)	**< 0.0001**	40.3 (35.4,46.3)	45.3 (37.3,52.0)	0.3413	**0.4800**	**0.0375**
MMP7	35.1 (26.7,46.0)	34.1 (22.1,39.5)	0.6343	34.8 (28.8,43.7)	35.5 (25.7,46.9)	0.9537	0.2654	0.2721
MMP2	1234 (959.6,1335)	941.4 (757.7,1154)	**0.0136**	1186 (910.6,1308)	1263 (959.6,1485)	0.429	0.4370	0.0613

Table 17. CSF biomarker concentrations

Nineteen of 21 CSF mediators were significantly higher in discordant than non-discordant subject in univariate analysis. There were no significant differences between CSF mediator concentrations in subjects with high (CSF HIV-1 RNA >1log$_{10}$ plasma) vs. low (0.5-1log$_{10}$ plasma or discordant only on sensitive testing) discordance. Nine CSF mediator concentrations correlated with CSF HIV-1 RNA.

Values are median pg/ul (IQR). P values are by Mann-Whitney-U or Spearman r correlation. CSF, cerebrospinal fluid; RNA, ribonucleic acid.


p<0.0001
ns
CXCL10 (pg/ul)
8000
6000
4000
2000
0
Non-disc
Low disc
High disc
a
CCL3 (pg/ul)
400
300
200
100
b
VCAM (pg/ul)
10000
c
TNFR2 (pg/ul)
15000
d
r=0.534
p=0.019
e
r=0.597
p=0.007
f
r=0.579
p=0.009
g
r=0.572
p=0.011
h
1
2
3
4
Log10 CSF HIV-1 RNA (copies/ml)

.

Figure 10. CXCL10, CCL3, VCAM and TNFR2 concentrations in high/low discordance vs. non-discordant subjects, and correlation with HIV-1 RNA.

CXCL10, CCL3, VCAM and TNFR2 concentration (a-d respectively) were significantly higher in discordant vs. non-discordant subjects. No significant differences were observed between subjects with high (CSF >1log $_{10}$ plasma) vs. low (CSF 0.5-1log$_{10}$ plasma or discordant only on sensitive testing) degrees of discordance. Subjects discordant only on sensitive testing are represented with an open circle. Mediator concentrations correlated with CSF HIV-1 RNA (e-h).

Non-disc, non-discordant; Low-disc, low-discordant; High-disc, high-discordant; ns, not significant; CXCL10, inducible protein 10; VCAM, vascular cell adhesion molecule; CCL3, C-C motif ligand 3 AKA monocyte inflammatory protein 1a; TNFR2, tumour necrosis factor receptor 2.

6.4.2.2 Cluster analysis of subjects

The subjects grouped into 2 main clusters (*Figure 11*). These clusters corresponded to CSF/plasma discordance ($p<.0001$, Fisher's exact test). In cluster 1 all mediators had relatively high abundance; this cluster included 18 discordant subjects and 3 non-discordant subjects. Grouped in cluster 1 were all subjects with CSF HIV-1 RNA >1$\log_{10}$ plasma, all with CSF HIV-1 RNA 0.5-1$\log_{10}$ plasma, and 2 of the 3 with CSF/plasma discordance only on sensitive testing. In cluster 2 all mediators had relatively low abundance; this cluster included 18 non-discordant subjects and 1 discordant subject; this subject was only discordant on sensitive testing (CSF HIV-1 RNA 74, plasma <7 copies/ml). Two of the 3 subjects with CSF viral escape not meeting criteria for CSF/plasma discordance were in cluster 2.

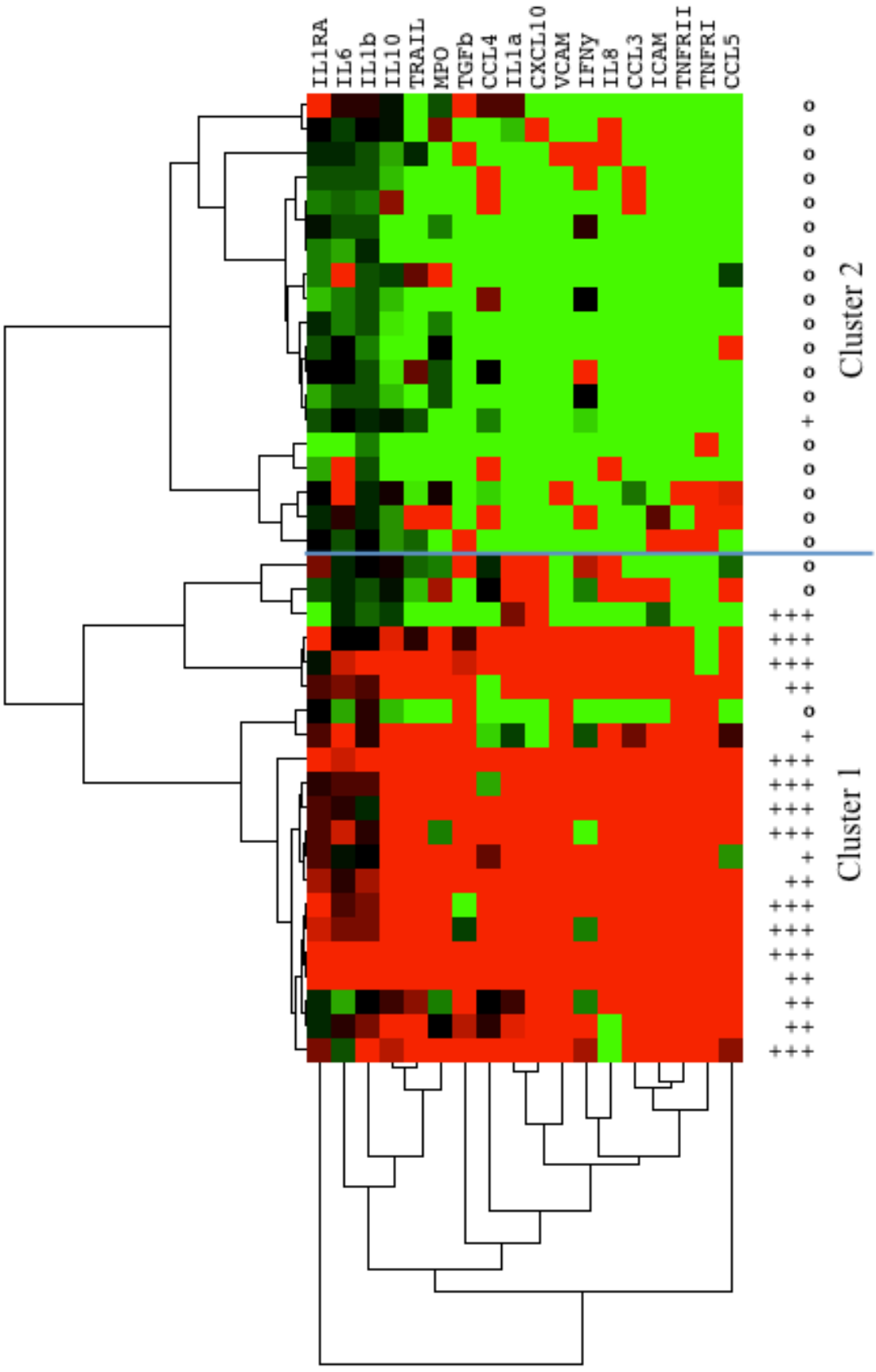

IL1RA
IL6
IL1b
IL10
TRAIL
MPO
TGFb
CCL4
IL1a
CXCL10
VCAM
IFNy
IL8
CCL3
ICAM
TNFRII
TNFRI
CCL5
Cluster 2
Cluster 1

Figure 11. Heat map displaying the relative concentration of mediators in subjects with and without CSF/plasma discordance.

Samples and mediators were organised via an unsupervised hiarachical clustering algorithm along the horizontal and vertical axes respectively. Twenty-one subjects without discordance are labeled on the x-axis with the symbol "o". Nineteen subjects with discordance are indicated with the symbol "+++/++/+"; discordant subjects with CSF HIV-1 RNA >1log$_{10}$ plasma are labeled "+++" (n=11), CSF 0.5-1log$_{10}$ plasma are labeled "++" (n=5) and those with discordance only present on sensitive HIV-1 RNA testing are labeled "+" (n=3). Tile colour indicates relative mediator concentration: red, increased; green, decreased; black, at median concentration (for the samples). On the horizontal axis the subjects segregated into 2 main clusters, cluster 1 and 2, corresponding significantly to discordant and non-discordant subjects respectively (p<0.0001). Grouped in cluster 1 were all subjects with CSF HIV-1 RNA >1log$_{10}$ plasma, all with CSF HIV-1 RNA 0.5-1log$_{10}$ plasma, and 2 of the 3 with CSF/plasma discordance only on sensitive testing. One subject with discordance only on sensitive testing (CSF HIV-1 RNA 74, plasma <7 copies/ml) was grouped in cluster 2.

6.4.2.3 Cluster analysis of mediators

CSF mediators significant in univariate analysis clustered into 2 main sets with 2 mediators (IL1RA and CCL5) outlying these sets (*Figure 11*). The first set included IL6, IL1b, IL10, TRAIL and MPO; the second set included TGFb, CCL4, IL1a, CXCL10, VCAM, IFNy, IL8, CCL3, ICAM, TNFRII and TNFRI.

6.5 Discussion

These data demonstrate that CSF/plasma discordance is associated with the increased expression of CSF mediators. Clinical outcomes were not assessed in this study, however these mediators are broadly pro-inflammatory and risk leading to neuronal damage if chronically raised. Raised CSF concentrations of many of these have been shown to correlate with cognitive impairment in patients with HIV infection, although no single mediator has a strong enough correlation with cognitive status to be a clinically useful biomarker and a combination of biomarkers may be more useful.[81]. The associations of these mediators with CNS disease in HIV positive populations has been reviewed by Brew and Letendre and Morris et. al.[26, 81]

Interleukins produced by macrophages such as IL-1, IL-6, IL-8 and IL-10 have been associated with cognitive impairment in HIV infected adults and children, although most studies were in the pre/early-HAART era. TNFa is produced by macrophages and microglia and is involved with immune regulation and inflammation. CSF levels have been associated with brain injury and TNFa mRNA levels are elevated in the brain tissue of cognitively impaired HIV infected patients. Although >80% of TNFa measurements were below the limit of quantification in this analysis, levels of the

closely related proteins TNFR1, 2 and TRAIL were associated with discordance. CSF levels of interferons such as IFNa (excluded from analysis as >80% below limit of quantification), and to a lesser extent IFNg, have been associated with cognitive impairment in HIV. Levels of the interferon-inducible protein CXCL10 (IP-10) in CSF has been correlated with CSF HIV RNA and higher levels have been associated with adverse neurological outcomes. Chemokines CCL3, 4 and 5 (MIP1b, MIP1a and RANTES) bind CCR5 and are involved in monocyte chemotaxis and HIV entry into microglia and lymphocytes. They have been identified in brain tissue of a simian SIV-encephalitis model. The chemokine CCL2 (MCP-1) is associated with chemotaxis of monocytes across the blood-brain barrier and has been associated with cognitive impairment in several studies in HIV positive populations in the pre and post-HAART era; surprisingly in our study levels were not significantly associated with discordance. TGFb modulates the inflammatory responses of cytokines and macrophage activation and CSF levels are raised in HIV infected patients with cognitive impairment. Adhesion molecules ICAM and VCAM mediate adhesion of monocytes and lymphocytes to vascular endothelium and promote monocyte trafficking across the blood-brain barrier. CSF levels are associated with CNS inflammation, blood-brain barrier damage and are raised in HIV encephalopathy in the pre-HAART era.[26, 145]

CSF pleocytosis was more frequent in the discordant group, however the observed clustering is not purely an epiphenomenon of CSF pleocytosis as the majority of subjects did not have pleocytosis above 4 cells/ul and almost half had no measurable white cells in CSF.

The majority of subjects with low CSF discordance had raised CSF mediator concentrations in a similar pattern to those with high discordance. Only 1 low discordant subject had low CSF mediator concentrations and was grouped with the non-discordant cluster (this subject was discordant only on sensitive testing). This suggests that the criteria for CSF/plasma discordance used in some studies (ie. excluding subjects with $<1\log_{10}$ difference and/or <200 copies/ml in CSF) may risk type 2 error.[8, 68] These data suggest that the lower cut-off of $0.5\log_{10}$ should be considered, both in clinical practice and future research studies. This is consistent with our previous work demonstrating resistant HIV-1 in CSF, including M184V which is associated with decreased replicative capacity, in subjects with $0.5\text{-}1\log_{10}$ discordance and <200 copies/ml in CSF (*Table 11*).[142]

Subjects with higher degrees of CSF/plasma discordance trended to higher CSF mediator concentrations. This effect was not strong and the difference between low and high discordant groups was not statistically significant for any single mediator. This is consistent with data from the CNS HIV Antiretroviral Therapy Effects Research (CHARTER) study, the largest cohort in this area, which found that although the absolute level of CSF HIV RNA was not related to cognitive performance, the presence of discordant virus was.[39]

Reports have suggested that CSF HIV-1 RNA detection at levels below 50 copies/ml may be associated with cognitive impairment.[39, 69] In this study, 2 of the 3 subjects with CSF/plasma discordance only detected on sensitive testing had raised CSF mediator profiles grouped with other discordant subjects. Although the numbers here are small, these data support the MIND exchange consensus report recommendations

that sensitive HIV-1 RNA testing should be considered in those with neurological or cognitive symptoms and less than 50 copies/ml in CSF and plasma.[20] Subjects with CSF viral escape not meeting criteria for CSF/plasma discordance did not consistently have raised CSF mediator profiles. Such patients should also be considered for sensitive testing in plasma to determine the extent of discordance between the 2 compartments.

Some studies have demonstrated no association between CSF HIV RNA levels and cognitive performance.[77] This may be due to the multifactorial nature of cognitive impairment in HIV rather than indicating that CSF HIV-1 detection does not have potential consequences for the brain.[146] These data demonstrate that CSF/plasma discordance is associated with neuroinflammation and supports performing CSF HIV-1 RNA quantification in clinical practice. In this study the majority of discordant subjects had plasma HIV-1 RNA levels that were unquantifiable on standard testing (median plasma HIV RNA <40 copies/ml, IQR <40,52) and would have been classified as successfully treated on plasma testing alone. No subject had plasma HIV-1 RNA above 400 copies/ml, the level associated with long-term virological failure.[89] CSF discordance, even at low levels, should be investigated and treated; the indications for performing lumbar puncture solely to measure CSF HIV-1 RNA, and strategies for managing those patients found to be discordant, remain an important topic for future research.[146]

7 Identification of a prognostic model of clinical features and cerebrospinal fluid and plasma biomarkers for HIV associated neurocognitive impairment

7.1 Introduction

A number of clinical and laboratory parameters, and biomarkers in CSF and plasma, have been associated with the occurrence of HAND. It is unclear whether these parameters herald on-going damage and the progression of cognitive impairment over time.

Tools to identify those at risk of developing HAND are required clinically to determine those patients that would benefit from more intensive monitoring of cognitive function and/or changes in antiretroviral therapy (ART) targeted at the CNS. Such tools could also be of use as endpoints in clinical trials characterising the effectiveness of ART in the CNS.

7.2 Aims

To assess the association of plasma/CSF biomarkers and clinical/laboratory parameters with changes in neurocognitive function over time in the CNS HIV Anti-Retroviral Therapy Effects Research (CHARTER) cohort.

7.3 Methods

I performed a focused analysis of the combined biomarker data arising from the CHARTER study.

7.3.1 Patients

The US National Institute of Mental Health-funded CHARTER cross-sectional cohort study recruited 1555 HIV-seropositive patients. Subjects were drawn from 6 participating university centers: Johns Hopkins University (Baltimore, MD); Mt. Sinai School of Medicine (New York, NY); University of California at San Diego (San Diego, CA); University of Texas Medical Branch (Galveston, TX); University of Washington (Seattle, WA); and Washington University (St. Louis, MO). Subject recruitment began in September 2003 and ended in August 2007.

The cohort was unselected and represented the clinic population at recruiting centres. The reported prevalence of cognitive impairment in this cohort was 53%.[12] Subjects with ANI were at significantly higher risk of progressing to symptomatic or functional impairment than those with normal cognitive function.[111]

7.3.2 Procedures

All subjects completed a venipuncture, neuromedical assessment, comprehensive neuropsychological testing, detailed substance use history, structured psychiatric interviews for detecting lifetime and current diagnoses of substance use disorders and affective disorders, a measure of current mood, and self-reported assessments of cognitive symptoms, vocational functioning, and independence with instrumental activities of daily living. For details of testing procedures see the original CHARTER

publication.[12] For those who consented (n=1,205), CSF was withdrawn by lumbar puncture.

All participants completed a comprehensive neurocognitive test battery, covering 7 cognitive domains known to be commonly affected by HIV-associated CNS dysfunction (administration time = 2–2.5 hours). The best available normative standards were used, which correct for effects of age, education, sex, and ethnicity, as appropriate. Test scores were automatically converted to demographically corrected standard scores (t scores) using available computer programs.

7.3.3 Analysis

Clinical features, including demographic and routine laboratory parameters, and a matrix of plasma and CSF biomarkers were compared with the subsequent change in neurocognitive function. Multiple linear regression analysis was employed with cognitive change score as the outcome variable.

As many biomarkers have not been measured in the same patients at the same time-point, multiple comparisons between biomarkers was not possible for the full data set. The following methods were employed to identify a matrix with the greatest number of biomarkers measured at the same time points in the same patients with the same duration of follow up.

7.3.3.1 Biomarker selection

Biomarkers with less than 20 measurements were excluded. In this analysis it was not possible to distinguish between biomarkers with undetectable levels due to assay

failure and those with levels truly falling below quantification threshold, therefore biomarkers with skewed data due to a large proportion of the measurements falling below the lower limit of quantification were removed. The data was converted to a binary outcome (measured/not measured) and a hierarchical Euclidian cluster analysis (simple matching method), using the value of a measured time-point as the similarity metric, was performed in SPSS 22 to determine a matrix of biomarkers measured in the same patients at the same time-point. Overlapping clusters were then compared to determine the greatest number of biomarkers assessed at the greatest number of time points in the greatest number of patients.

7.3.3.2 Time point selection

Neurocognitive function was measured every 6 months for a variable duration up to a maximum of 8 years. As HAND develops slowly it was considered that follow up of less than 12 months is likely to reflect variation in testing rather than true neurocognitive decline. To determine the most appropriate follow up period I calculated the number of biomarker measurements with neurocognitive data available at each time point after 12 months and selected the longest follow-up period for which the greatest proportion of the biomarker data remained available.

7.4 Results

7.4.1 Biomarkers

Thirty-two CSF and 23 plasma biomarkers were measured a median of 206 times each (IQR 145,367). Seven-hundred and ninety subjects had at least one biomarker measured at at least one time point. Preliminary analyses were conducted to ensure no

violation of the assumptions of normality, linearity, multicollinearity and homoscedasticity. Ten biomarkers (9 CSF, 1 plasma) were removed due to skewed measurements. Ten biomarkers (7 CSF, 3 plasma) had less than 20 measurements and were also removed (CSF GM-CSF, IFN-g, IL-17, IL-4, MIP-1a, MIP-1B and RANTES; plasma GM-CSF, IL-4 and sCD163).

Figure 12 demonstrates 5 clusters of biomarkers measured at similar time points (clusters 1-5). Clusters and individual biomarkers were examined to determine overlap and give the most extensive biomarker/patient matrix. Clusters 1 and 3 could be combined with biomarkers CSF IL1b, CD40L, IF10 and MCP1 to give 25 biomarkers (10 CSF, 15 plasma) measured together 138 times in 138 subjects (ie. no more than 1 measurement per subject).

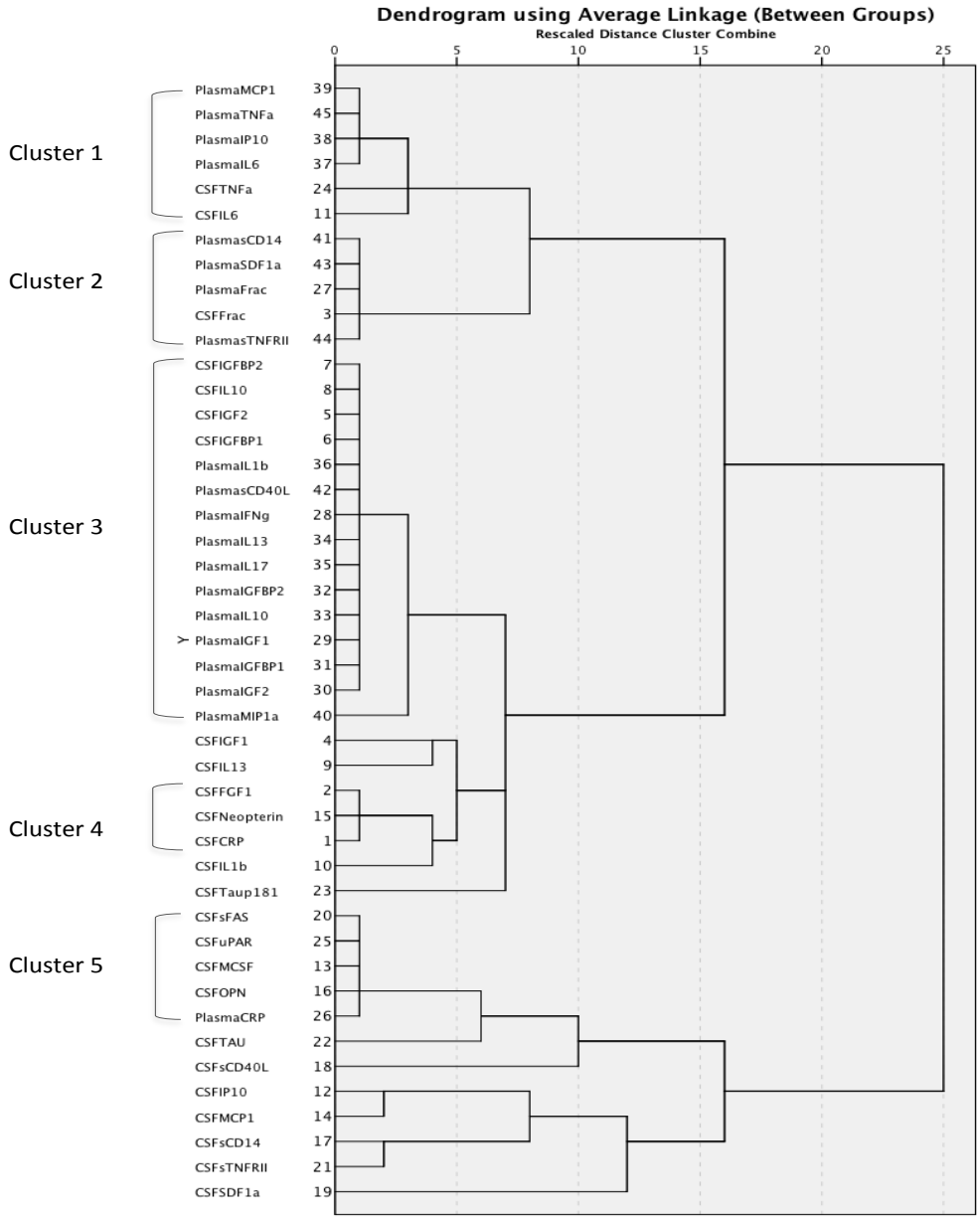


Figure 12. Euclidian hierarchical cluster analysis to determine biomarkers measured at common time points.

Patient biomarker data converted to binary outcome (measured/not measured) underwent hierarchical Euclidian cluster analysis (simple matching method). This determined 5 clusters of biomarkers measured at similar time points (clusters 1-5). Overlapping clusters were then compared to determine the greatest number of biomarkers assessed at the greatest number of time points in the greatest number of patients.

7.4.2 Time-point selection

Of subjects with the biomarkers measured in the clusters described above, 93 had neurocognitive data at 12 months follow-up. Extending the follow up period from 12 to 18 months resulted in only 6% loss of measurements. Extending to 24, 30 and 36 months resulted in much higher losses of 23%, 34% and 42% respectively (illustrated by changes in observations in total cohort over time in *Figure 13*). Based on this an 18-month follow up was selected for analysis.

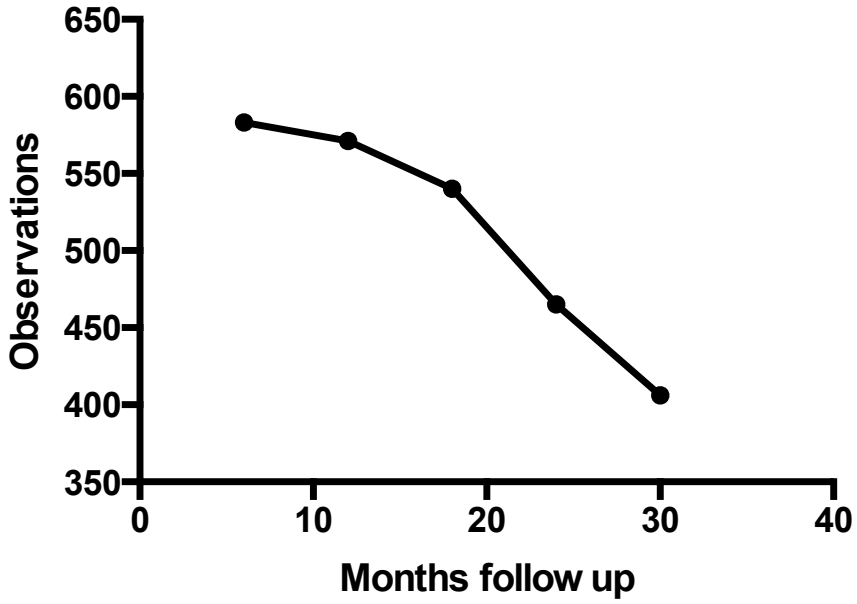


Figure 13. Number of cognitive assessments (observations) performed at different lengths of follow up.

Only subjects with at least one biomarker measured are included.

7.4.3 Patients

Demographic and laboratory variables of the 88 subjects included in the model (ie. those with the clustered biomarkers measured and 18 month cognitive follow-up data available) are given in *Table 18.*

		Median (IQR) or n (%)
Age (years)		43 (39,49)
Gender	Male	76 (86.4)
Education		13 (12,16)
	White	48 (54.5)
	Black	28 (31.8)
	Hispanic	10 (11.4)
	Other	2 (2.3)
Estimated duration of HIV infection (months)		145 (48,195)
AIDS diagnosis		56 (63.6)
Comorbidities		
	Incedental	62 (70.5)
	Contributing	26 (29.5)
Cognitive status impaired at baseline		43 (48.9)
CD4 T-lymphocyte count (cells/mm3)		
	At sampling	428 (251,552)
	Nadir	166 (43,284)
Log10 HIV-1 RNA		
	Plasma	2.6 (1.7,4.0)
	CSF	1.7 (1.7,2.4)
HIV-1 RNA <LLQ*		
	Plasma	38 (43.2)
	CSF	56 (63.6)
Discordant plasma/CSF HIV-1 RN		2 (2.3)
Antiretroviral therapy		
	NRTI	64 (72.7)
	NNRTI	25 (28.4)
	PI	41 (46.6)
	Not on ART	22 (25.0)
CPE score		7 (6,8.75)

Table 18. Demographic and laboratory variables in 88 patients selected for analysis

*All figures are at biomarker sampling. *1.7 = lower limit of quantification. ** CSF > 0.5log10 plasma.*

Variable	Parameter	Univariate
Comorbidities	Clinical	0.36
Age	Clinical	0.798
Education	Clinical	*0.044
Ethn1	Clinical	0.304
Eth2	Clinical	*0.068
Gender	Clinical	*0.059
AIDS status	Clinical	0.975
Est duration of	Clinical	0.836
CD4	Clinical	0.269
Nadir CD4	Clinical	0.567
Plasma HIV	Clinical	0.76
<LLQ plasma	Clinical	0.482
CSF HIV	Clinical	0.968
<LLQ CSF	Clinical	0.862
CSF-plasma	Clinical	0.672
On ART	Clinical	0.255
CPE	Clinical	0.107
Cog impairment	Clinical	0.233
IGF-2	CSF	0.215
IGFBP-1	CSF	0.749
IGFBP-2	CSF	0.378
IL-10	CSF	0.267
IL-1b	CSF	0.179
IL-6	CSF	0.24
IP-10	CSF	0.219
MCP-1	CSF	0.575
sCD40L	CSF	0.483
TNFa	CSF	*0.004
IFNg	Plasma	0.48
IGF-1	Plasma	*0.008
IGF-2	Plasma	*<0.0001
IGFBP-1	Plasma	0.112
IGFBP-2	Plasma	*0.073
IL-10	Plasma	0.825
IL-13	Plasma	0.115
IL-17	Plasma	0.477
IL-1b	Plasma	0.445
IL-6	Plasma	0.881
IP-10	Plasma	0.438
MCP-1	Plasma	0.784
MIP-1a	Plasma	0.912
sCD40L	Plasma	0.485
TNFa	Plasma	0.801

Table 19. Univariate regression of clinical features and plasma/CSF biomarkers compared with change in neurocognitive status at 18 months follow up.

Star indicates parameters taken forward into multivariate model. White, black or other ethnicity was transformed to binary outcomes; ethn1 and ethn2. Cog impairment is presence of HAND determined by Frascatti criteria.*[90]

7.4.4 Linear regression

The linear regression analysis was carried out in two stages. The first stage of the analysis was to investigate the association between each of the biomarkers independently and the change in cognitive score (*Table 19*). Those biomarkers shown to be significantly associated with change in cognitive score ($p<0.1$) were then taken forward to the multivariate analysis. From this initial analysis three clinical variables: education, ethnicity and gender were identified as being significantly related to change in cognitive score. Four biomarkers, CSF tumour necrosis factor alpha (TNFa), plasma insulin-like growth factor-1 (IGF-1), plasma insulin-like growth factor-2 (IGF-2) and insulin-like growth factor binding protein-2 (IGFBP-2) were also identified as being significantly related to change in cognitive score and were also carried forward for the multivariate analysis. The second stage of the analysis was to incorporate the seven clinical variables and biomarkers into a multivariate model. This was done using a forward stepwise process, adding one variable at a time, starting with the two with the smallest p values, into the model. A p value of 0.05 was used to determine statistical significance and a backward stepping approach with the same p value was used to test the consistency of the model. This stage of the analysis identified that the biomarkers plasma IGF-1 and IGF-2 were correlated with each other (Pearson's $r=0.664$, $p<0.0001$) and were identifying the same proportion of the variability in the model. Effectively two similar models could be constructed containing either plasma IGF-1 or IGF-2 along with CSF TNFa. The first model is represented in *Table 20* - r squared 0.230, $p<0.0005$.

	Standard error of the coefficient	Unstandardised beta coefficient	p value
Constant*	0.194		0.001
CSF TNFa	0.023	0.225	0.026
Plasma IGF-2	<0.001**	0.374	<0.001

Table 20. Results of multivariate linear regression of clinical factors and biomarkers against change in cognitive status at 18 month follow-up.

**The value of the y intercept is -0.651.*

***The standard error of the coefficient for plasma IGF-2 is very low due to the scale of the variable,(ie the numerator (change in cognitive score) is small and, the denominator (plasma IGF-2) is relatively large) and the slope is not steep.*

In the second model plasma IGF-1 was substituted for IGF-2. The model remained significant but explained less of the variance in the outcome (r squared of 0.165, p=0.001). Both plasma IGF-1 and CSF TNFa made significant unique contributions to the model (p=0.010 and 0.005 respectively). Euclidian hierarchical cluster analysis of the 25 biomarkers in the model demonstrated a dominant closely related cluster of 18 biomarkers (*Figure 14*). This was represented in the model by CSF TNFa. Both IGF1 and 2 were outside this cluster.

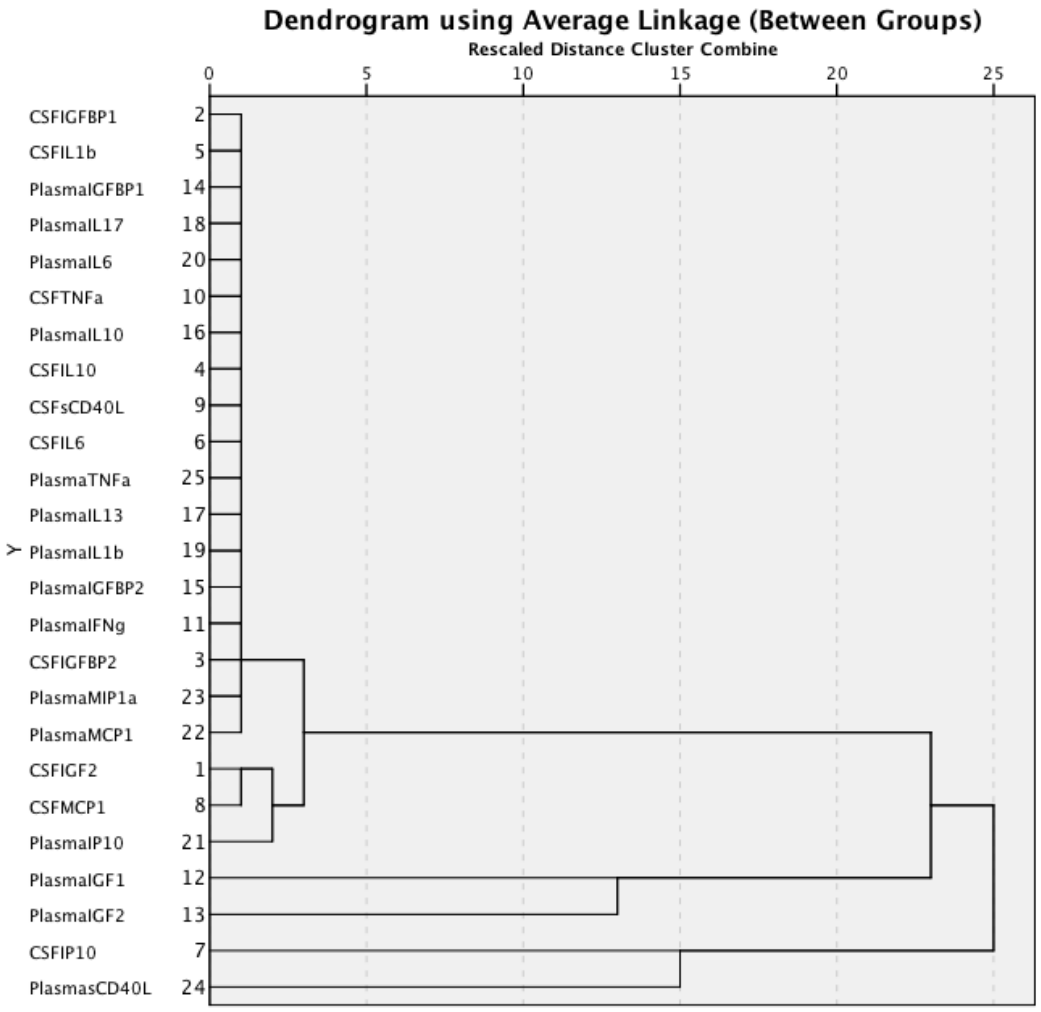


Figure 14. Euclidian hierarchical cluster analysis of biomarkers in regression model.

A dominant closely related cluster of 18 biomarkers was represented in the model by CSF TNFa

7.5 Discussion

In this study, plasma IGF-1 and 2 and CSF TNFa had a protective effect on neurocognitive functioning over 18 months. The amount of the variance explained by the models may be insufficient for use clinically as prognostic indicators, however it is interesting that these biomarkers were able to predict cognitive change where standard clinical and laboratory parameters, in particular CSF/plasma HIV-1 RNA, were not.

These findings were unexpected. Analysis of patients from the PARTITION study in chapter 6 demonstrated that higher concentrations of CSF mediators were associated with CSF/plasma discordance. Although detailed cognitive testing was not performed in PARTITION, it could be extrapolated that this would relate to more, rather than less, deterioration in neurocognitive functioning. Unfortunately these associations cannot be compared directly between the studies; IGFs were not measured in PARTITION and TNFa was excluded from analysis as levels were below the lower limit of quantification in over 80% of subjects. One explanation could be that CSF/plasma discordance was infrequent in the CHARTER cohort, occurring in less than 2% overall.[12] Unlike in PARTITION, CSF biomarkers in the CHARTER cohort may not be reflecting ongoing replication of HIV in the CNS, but alternative protective mechanisms in those without discordance.

IGFs have an important neuroprotective role in development, cell differentiation, plasticity, and neuronal survival/anti-apoptosis. They have been implicated in the pathogenesis of a number of neurodegenerative disorders.[147] IGF treatment has been

shown to have neuroprotective and trophic effects in vitro and in vivo.[148, 149] IGF-1 and 2 have been shown to increase neuronal survival in HIV-1 infected brain cell cultures,[150] and are protective in a murine model of HAND.[151]

TNFa has a protective, as well as pro-inflammatory, role in the nervous system by diminishing the production of nitric oxide and free radicals, alteration of excitatory amino acid neurotransmission, maintenance of neuronal calcium homeostasis and induction of the synthesis of neurotrophic factors.[152] Exposure to TNFa has been shown to render primary cortical neurons more resistant to excitotoxic cell death in vitro.[153] The role of TNFa in HAND is unclear and TNFa has been implicated in both neuronal injury and neuronal protection in HIV-1 infected subjects.[154] TNFa can enhance HIV-1 replication, but has also been shown to inhibit entry of HIV-1 into macrophages, the primary site of HIV-1 infection in the brain.[155, 156]

7.6 Conclusion

This analysis suggests a neuroprotective role for IGF-1 and 2 and TNFa in HIV infection. These biomarkers had greater predictive power for neurocognitive change than standard clinical and virological investigations. These cytokines require further investigation as prognostic indicators as well as potential therapeutic strategies for HAND.

8 Pharmacokinetics of Efavirenz and its major metabolites in cerebrospinal fluid, and relationship with *CYP2B6* c.516G>T genotype and perturbed blood brain barrier due to tuberculous meningitis

8.1 Introduction

Efavirenz (EFZ) is widely deployed within first-line combination HIV treatment regimens worldwide because of its superior effectiveness,[157, 158] established safety record and resilience to rifampicin enzyme induction in patients who require concomitant anti-tuberculous therapy. EFZ undergoes rapid absorption, maximum plasma concentration is reached in 3–6 hours and therapeutic levels are reached within a few days of commencing treatment.[159] However there is large interindividual variability in EFZ pharmacokinetics (PK),[160-163] placing patients with low plasma concentrations at risk of losing virological control and developing resistance, or else developing adverse effects such as CNS toxicity.[164, 165,10] EFZ is primarily metabolised by cytochrome P450 *CYP2B6*, to yield the most abundant metabolite 8-hydroxy-efavirenz (8-OH-EFZ). Comparatively minor alternative metabolic pathways are through *CYP2A6* (leading to the 7-OH-EFZ metabolite) and *CYP3A*,[166] with most circulating metabolites (7-OH and 8-OH-EFZ) in plasma conjugated as glucuronides.

EFV plasma concentrations relate strongly to genetic polymorphism in *CYP2B6* metabolism,[167-171] including the most commonly studied *CYP2B6* SNP c.516G>T (rs3745274), which encodes a Gln172His amino acid substitution. Clinical studies in

HIV-TB coinfected patients have consistently shown that these pharmacogenetic effects more than compensate for any reduction in EFV concentrations as a result of rifampicin enzyme induction, with some preliminary data suggesting that in *CYP2B6* poor metabolisers, *CYP2A6* represents the dominant route of elimination and may be impacted by enzyme inhibition through concomitant isoniazid administration.[172] Furthermore, in-vitro experiments have reported that 8-OH-EFZ was associated with cytotoxicity via stimulation of mitochondrial dysfunction and stimulation of stress activated signaling pathways.[173] Recently 8-OH-EFZ was shown to be neurotoxic in vitro at a concentration similar to those found in CSF.[58] The role of 8-OH-EFZ in EFV-associated CNS toxicity has not been elucidated.

8.2 Aims

This study aimed to characterise the disposition of EFV and its metabolites within CSF in HIV-infected patients with and without tuberculous meningitis (TBM), and to evaluate the impact of phamacogenetic variability on drug disposition.

8.3 Methods

8.3.1 Participants and sampling

Patients were recruited from Vietnam and the UK PARTITION study, referred to hereon in this chapter as studies A and B (not to be confused with groups A and B within the PARTITION study).

Study A: In Vietnam, HIV-positive patients aged over 15 years with newly diagnosed TBM (ISRCTN63659091) were randomly assigned to receive immediate (within 7

days) vs. deferred (after 2 months) initiation of antiretroviral therapy as previously described.[132, 133] EFZ was dosed at 800 mg if taken with rifampicin, or 600 mg if taken without rifampicin, together with zidovudine plus lamivudine in fixed-dose combination. Anti-tuberculous therapy comprised isoniazid (5mg/kg/day; maximum 300mg), rifampicin (10mg/kg/day; maximum 600mg), pyrazinamide (25mg/kg/day; maximum 2g), and ethambutol (20 mg/kg/day; maximum 1.2g) for 3 months followed by isoniazid plus rifampicin for 6 months. Unless contraindicated, all patients received dexamethasone as described elsewhere.[174] Ethical approval was obtained from the Oxford Tropical Research Ethics Committee and the Hospital for Tropical Diseases Scientific and Ethical Committee.

Study B: The UK based PARTITION study recruited subjects in 2 groups as described in section 3.1. All patients received 600mg of EFV once-daily.

8.3.2 Efavirenz and metabolite measurement

EFZ concentrations were determined in plasma and CSF samples taken from subjects receiving EFV at steady-state (>10 days)[175] sampled at approximately mid-dosing interval. EFZ metabolite concentrations were determined in a single paired CSF/plasma sample per subject; measurements were repeated with and without beta-glucuronidase and the ratio between albumin concentration in CSF and plasma/serum was determined. Genotyping was by real-time PCR, see *section 3.3.5*.

8.3.3 Albumin ratio

The ratio between albumin concentration in CSF and blood (plasma/serum) was determined by radial immunodiffusion (Bindarid^{TM}). Results are expressed in the

standard way using the units CSF albumin (mg/L) / blood albumin (g/L), hence the expressed ratio represents the true value x 10^3. CSF:blood albumin ratio indicative of a breach in integrity of the blood:brain barrier was taken as ≥6.8 for subjects less than 45 years old and ≥10.2 for subjects over 45 years.[176]

8.3.4 Neurotoxic concentrations

Measured plasma and CSF concentrations were compared to the following concentrations associated with neurotoxicity: plasma EFZ concentrations greater than 4000ng/mL associated with an increased risk of CNS side effects; plasma EFZ concentrations less than 1000ng/ml associated with virological failure;[164] concentrations of EFZ, 8OH-EFZ and 7OH-EFZ associated with dendritic spine damage *in vitro*: 100uM, 10uM and 100uM respectively.[58]

8.4 Results

8.4.1 Subjects

Study A: EFZ and metabolite concentrations with and without b-glucuronidase were measured in paired plasma and CSF samples in 47 subjects. Sampling was mean 97 days (95% CI 73-120) after commencing treatment. Mean age was 30 years and median CD4 at sampling was 88 cells/mm^3. All were of Southeast Asian ethnicity.

Study B: : EFZ and metabolite concentrations were measured in plasma and CSF in 27 subjects. Mean age was 45 years and median CD4 at sampling was 432 cells/mm^3. 21 (78%) were of white ethnicity, 4 (15%) were of black ethnicity and 2 (7%) were of Asian ethnicity. CNS infection was excluded in 25 (93%) subjects; 1 subject had

cryptococcal meningitis and another had neurosyphilis. No subject was receiving antituberculous therapy or other enzyme inducing medication at the time of sampling.

8.4.2 Efavirenz and metabolite concentrations in CSF and plasma with and without beta-glucuronidase

Differences in the geometric mean concentration of EFZ and 8OH-EFZ in plasma were not significantly different between studies A and B. Concentrations of both EFZ and 8OH-EFZ in CSF were significantly higher in study A than B (both p<0.0001). (*Figure 15*). CSF:plasma ratio of EFZ concentration was median 2.7% [IQR 1.3,5.6] in study A and median 1.0% [IQR 0.7,1.2] in study B (p<0.0001). Plasma EFZ concentrations correlated with CSF EFZ concentration in both studies, but there was no correlation of plasma EFZ with CSF total 8OH-EFZ (*Figure 16*).

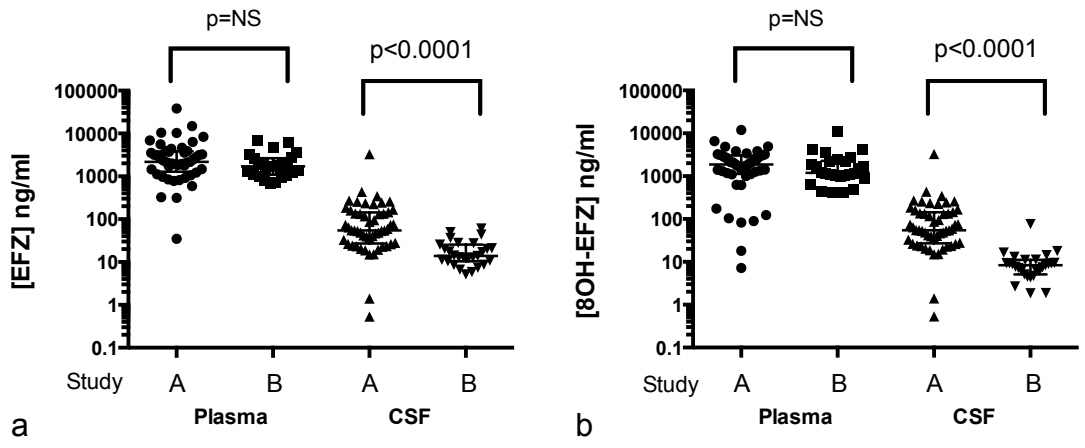


Figure 15. Concentrations of efavirenz (a) and 8OH-efavirenz (b) in CSF and plasma between studies A and B.

[EFZ], efavirenz concentrarion; NS, not significant.

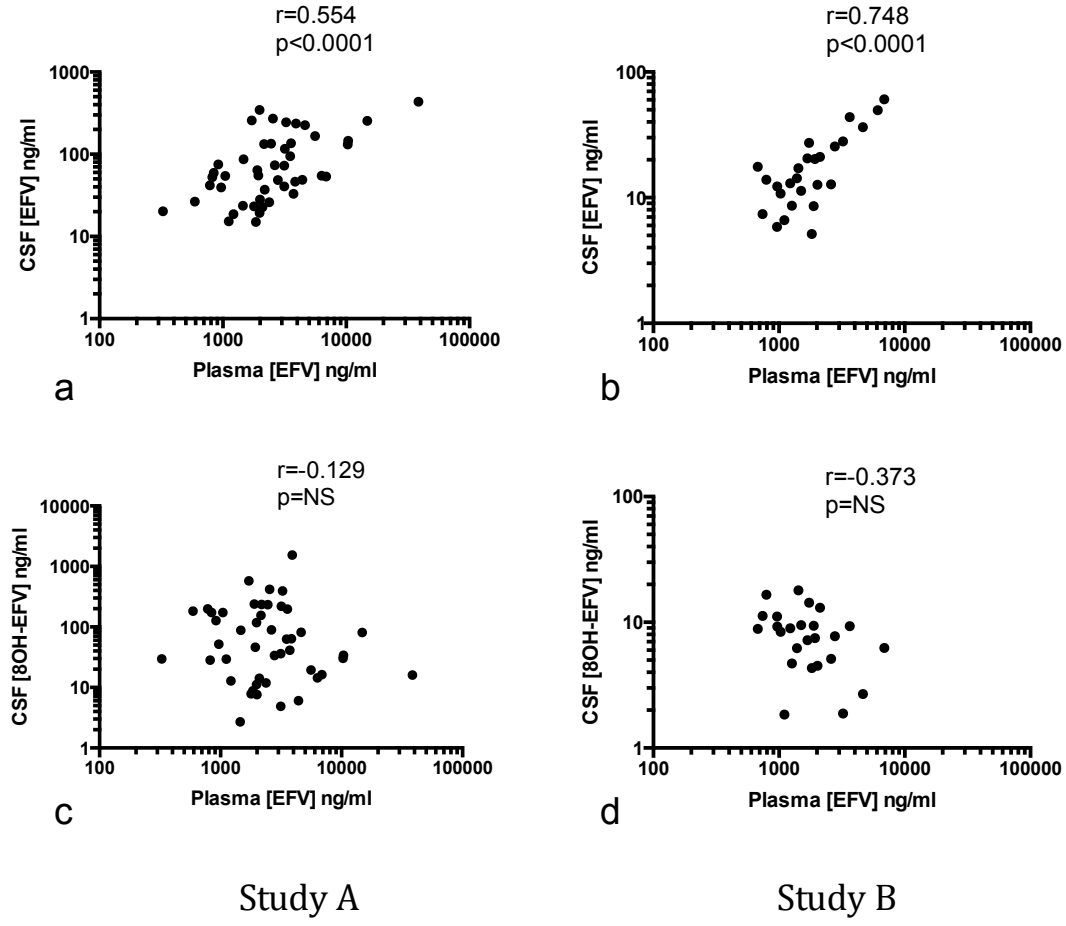


Figure 16. Relationship between plasma EFZ and CSF EFZ and 8OH-EFZ concentrations in studies A and B.

8OH-EFZ concentrations without b-glucuronidase were mean 87.3ng/mL (95% CI 63.8-122.5) in plasma and 3.7ng/mL (95% CI 2.7-5.7) in CSF. 8OH-EFZ concentrations in were mean 6.4% (95% CI 4.3-9.5) of total in plasma, and 7.5% (95% CI 5.1-11.1) in CSF. Without b-glucuronidase, 7-OH-EFZ levels were below the lower limit of quantification in CSF and plasma. Concentrations of EFZ were not significantly changed by the addition on b-glucuronidase. Metabolite levels without b-glucuronidase were too low for quantification in study B.

(*Figure 17*).

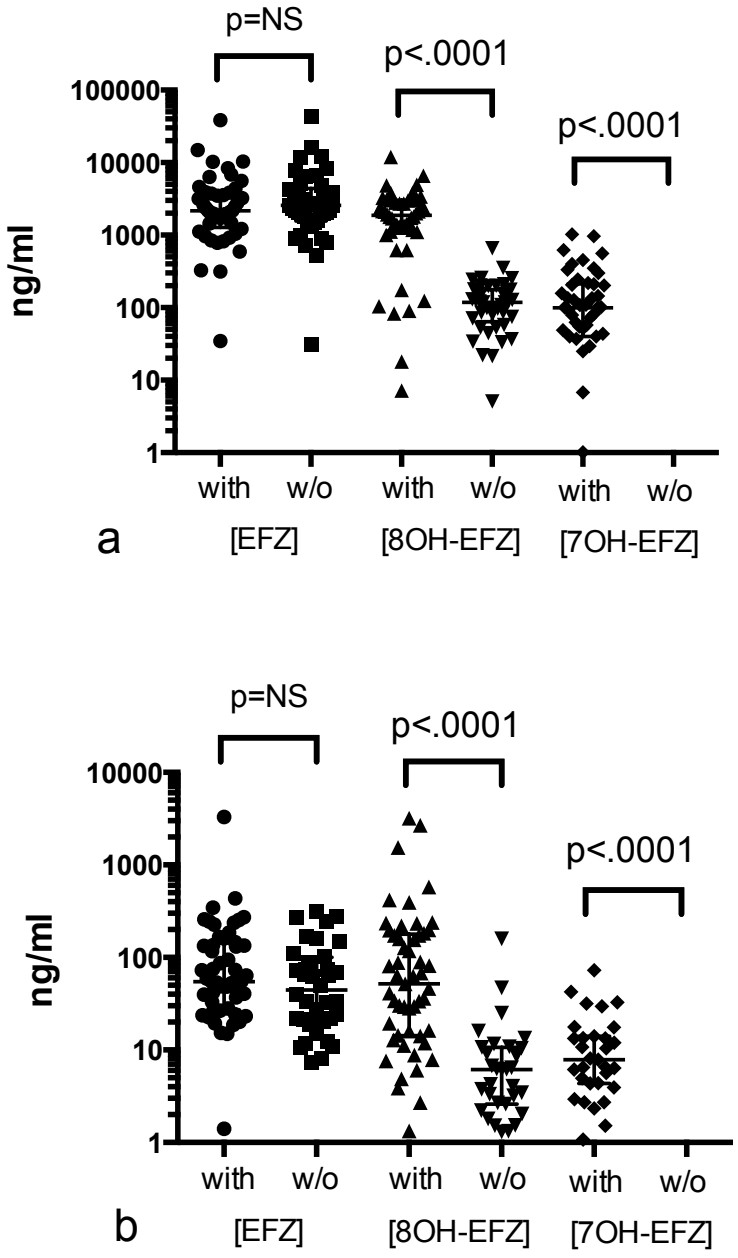


Figure 17. Study A; concentrations of EFZ, 8OH-EFZ and 7OH-EFZ in plasma (a) and CSF (b) with and without the addition of beta-glucuronidase.

w/o, without enzyme; [EFZ], efavirenz concentration; [8OH-EFZ], 8-hydroxyefavirenz concentration; [7OH-EFZ], 7-hydroxyefavirenz concentration; NS, not significant.

8.4.3 *CYP2B6* genotype

Forty-six samples in study A and 23 samples in study B were successfully genotyped for *CYP2B6* c.516G>T (call rates 98% and 85% respectively). Allele frequencies are given in *Table 21*. *CYP2B6* c.516G>T was in Hardy-Weinburg equilibrium in studies A and B (p=0.912 and 0.672 respectively). *CYP2B6* c.516G>T genotype related to the concentration of EFZ in CSF and plasma in both studies A and B (*Table 21*). This relationship was also present in CSF for EFZ, but not for the concentrations of EFZ metabolites. There was no difference in CSF:plasma EFZ ratio according to genotype. The effect of *CYP2B6* genotype on EFZ concentrations with respect to the estimated therapeutic range in plasma, and the *in vitro* toxic concentrations in CSF, are shown in *Figure 19*.

Given the high linkage disequilibrium between the *CYP2B6* c.516G>T functional polymorphism and other SNPs in the loci (*Figure 18*), it likely that this alone is the causal variant of the association with efavirenz concentrations and so haplotype analysis was not undertaken.

	Study	*CYP2B6* c.516G>T genotype			ANOVA
		GG	GT	TT	
Allele frequency, n (%)	A	23 (50.0)	20 (43.5)	3 (6.5)	
	B	10 (43.5)	11 (47.8)	2 (8.7)	
Plasma concentration, geometric mean (95% confidence interval)*					
[EFZ]	A	1694.3 (1297.2-2233.6)	3140.5 (1995.3-5081.6)	4852.9 (2287.2-6324.1)	0.015
	B	1264.7 (963.8-1674.9)	2202.9 (1482.5-3342.0)	3435.6 (3228.5-3655.9)	0.013
[8OH-EFZ]	A	1901.1 (1396.4-2630.3)	779.8 (269.8-2766.9)	666.8 (90.2-3069.0)	NS
	B	1559.6 (1002.3-2494.6)	1032.8 (632.4-1749.8)	687.1 (446.7-1086.4)	NS
[7OH-EFZ]	A	85.9 (62.5-120.8)	36.1 (5.0-2877.3)	316.2 (300.6-332.7)	NS
	B	162.3 (155.6,362.9)	261.3 (134.8,387.9)	NA	NS
CSF concentration, geometric mean (95% confidence interval)*					
[EFZ]	A	40.4 (29.4-57.0)	89.3 (61.5-134.0)	136.1 (54.7-237.1)	0.004
	B	11.5 (8.5-16.3)	17.0 (11.5-26.6)	34.8 (28.1-43.8)	0.037
[8OH-EFZ]	A	35.5 (19.1-74.8)	39.8 (21.6-82.8)	82.8 (14.4-1545.3)	NS
	B	7.8 (6.0-10.5)	5.3 (3.5-9.2)	3.3 (1.9-9.3)	NS
[7OH-EFZ]	A	0.3 (0.3,6.5)	4.5 (0.3,13.9)	7.4 (0.9,13.0)	NS
	B	0.5 (0.3,1.2)	0.8 (0.3,1.3)	3.3 (1.4,5.2)	NS

Table 21. CYP2B6 c.516G>T allele frequency and EFZ and metabolite concentrations in CSF and plasma in studies A and B.

**p values are Chi squared (allele frequencies) or Mann Whitney U test between GG with GT/TT.*

Concentrations are median ng/ml (IQR) or n (%). All concentrations are with enzyme. NP – not performed; EFZ – efavirenz. w/o – without.

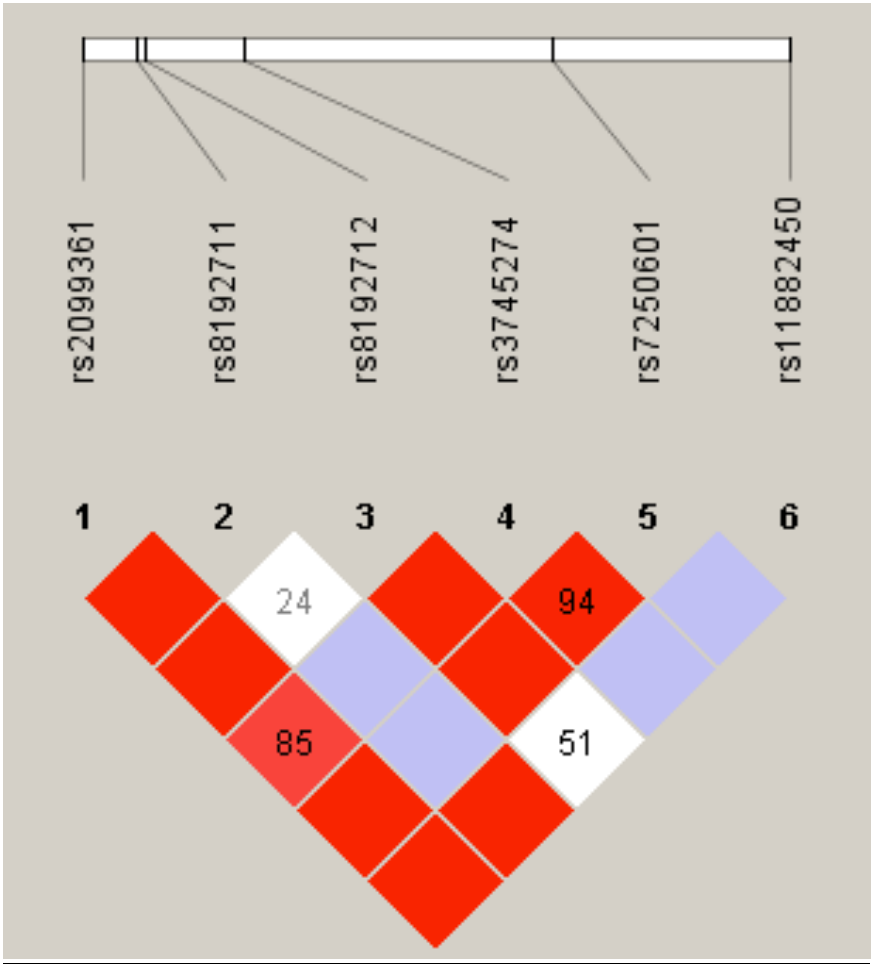


Figure 18. CYP2B6 linkage disequilibrium plot from 196 Vietnamese subjects (study A).

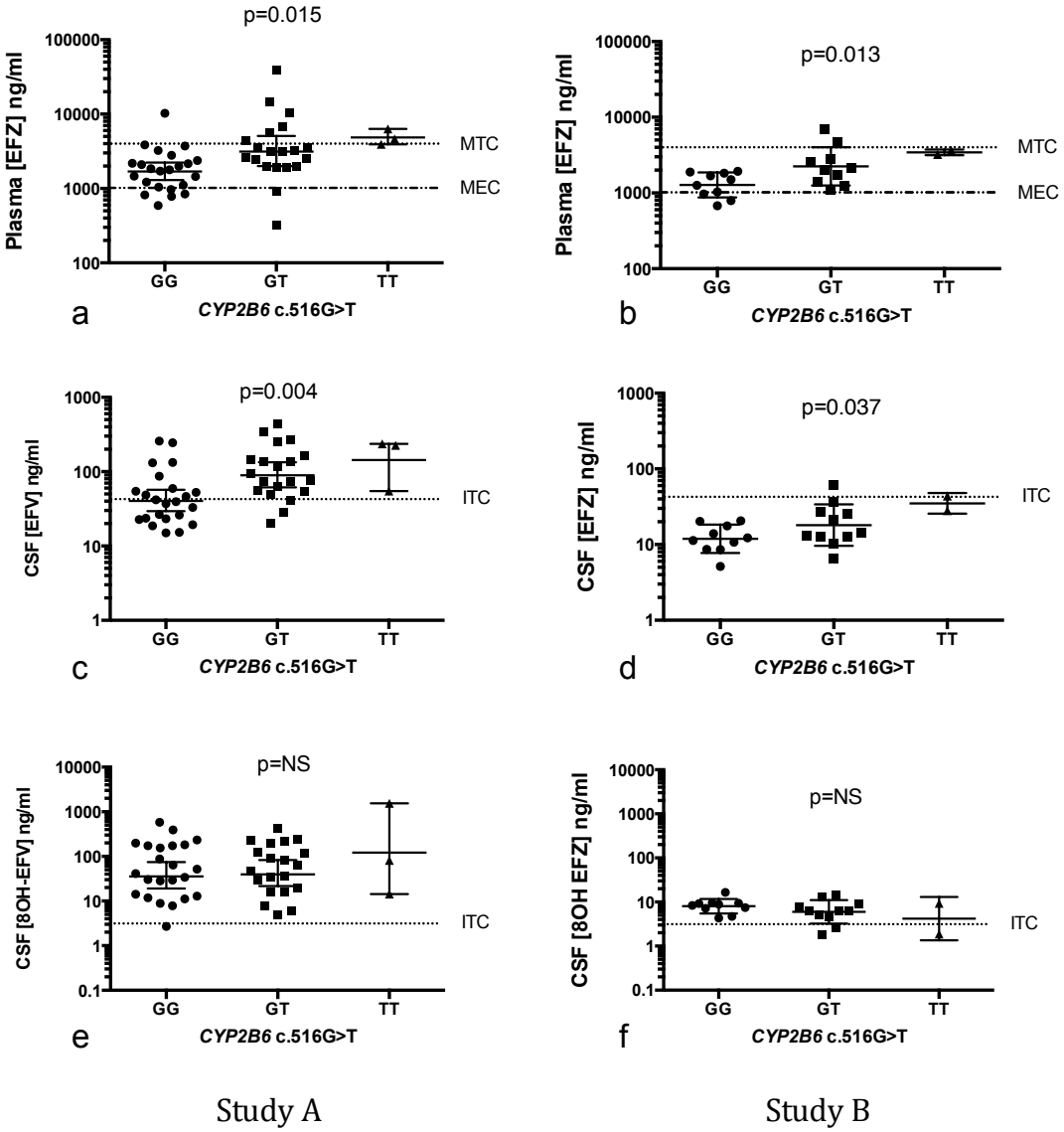


Figure 19. Effect of CYP2B6 c.516G.T genotype on estimated effective and toxic concentrations of efavirenz and total 8OH-EFZ concentrations in plasma and CSF.

Plasma efavirenz levels in the estimated neurotoxic range were related to CYP2B6 c.516G>T genotype (a and b). CSF EFZ above the in vitro toxic concentration was related to CYP2B6 c.516G>T genotype in study A (c), but not in study B where all but 1 subject had CSF efavirenz concentrations below this level (d). CSF 8OH-EFZ concentrations (with beta-glucuronidase) were above the in vitro toxic concentration

in most subjects in both studies; this was not related to CYP2B6 c.516G>T genotype (c and f).

MTC – minimum toxic concentration, MIC – minimum inhibitory concentration, ITC – in vitro toxic concentration.

8.4.4 *CYP2A6* copy number variation

Forty-six samples in study A were successfully genotyped for *CYP2A6* copy number (call rate 98%). The *CYP2A6* gene deletion occurred in 8 (17%) subjects and was in Hardy-Weinburg equilibrium (p=0.394). There was no association of *CYP2A6* copy number with the concentration of EFZ or metabolites in plasma or CSF either singly or in combination with *CYP2B6* genotype. A single subject had the *CYP2A6* gene deletion in combination with homozygous *CYP2B6* c.516G>T mutation; in this subject EFZ concentration was 6319.5 ng/ml in plasma and54.7 ng/ml in CSF.

8.4.5 Albumin ratio

CSF:serum/plasma albumin ratio was abnormal in 35 (90%) subjects in study A and 5 (24%) in study B (p<0.0001). In study A CSF:plasma albumin ratio was positively correlated with the CSF:plasma ratio of EFZ (r=0.537, p<0.001) and total 8OH-EFZ (r=0.408, p=0.014). In study B CSF:serum albumin ratio had no significant correlation with the CSF:plasma ratio of EFZ or 8OH-EFZ.

8.5 Discussion

This is the first report of EFZ metabolites in CSF of patients with TBM and, to my knowledge, the first report describing concentrations of the 7OH-EFZ metabolite in CSF. These data demonstrate that the *CYP2B6* c.516G>T polymorphism effects EFZ concentrations in CSF and well as plasma.

EFZ metabolites may have entered the CNS by crossing the blood brain barrier, or resulted from the CNS metabolism of EFV. The majority of metabolites in CSF were

present as glucuronide conjugate. This may be due to CSF trapping of plasma glucuronidated compound, however the percentage free compound was not significantly higher in CSF than plasma. Alternatively EFV metabolites may be conjugated within the CNS. A number of UDP-glucuronosyltransferases have been demonstrated to be present in human brain tissue.[177, 178] Functional *CYP2B6* and *CYP2A6* are present in the CNS and expression has been shown to be inducible and subject to genetic variation.[179-181]

CSF concentrations of EFZ and metabolites were higher in those with loss of blood brain barrier integrity due to TBM infection. These differences in between Study A and B could not have been explained by the higher doses of EFV used in Study A (800mg vs 600mg), since plasma exposures were comparable across both studies. Concentrations were highest in TBM patients with the greatest loss of blood brain barrier integrity as measured by CSF:plasma albumin ratio. EFZ is >99.75% protein bound in blood.[182, 183] Higher CSF EFZ concentrations may be due to leakage of free drug from plasma in those with loss of integrity of the blood brain barrier, or due to increased trapping of EFZ in those with higher albumin concentration in CSF.

CSF EFZ concentrations consistently exceeded *in vitro* neurotoxic concentrations in patients with a combination of TBM infection and *CYP2B6* c.516G>T mutation. In contrast CSF total 8OH-EFZ concentrations exceeded the *in vitro* neurotoxic concentration in the majority of subjects. Metabolite concentrations were independent of *CYP2B6* c.516G>T genotype but were related to blood brain barrier integrity. This has implications for neuronal damage in TBM which could contribute to the overall

neurological sequelae from this disease. The extent to which EFZ contributes to neurological sequelae in this situation is not known as any adverse neurological outcomes are likely to be attributed to TBM rather than drug neurotoxicity.

9 Effect of P-glycoprotein polymorphism on CSF darunavir concentration

9.1 Introduction

P-glycoprotein is a well charecterised ATP-dependent efflux pump with broad substrate specificity. P-plycoprotein is widely distributed and is expressed on brain capillary endothelium.[184] The protease inhibitors, including darunavir, have been shown to be substrates for P-glycoprotein[185] and it may be responsible for the low CSF levels observed in this class of ART.[186]

P-glycoprotein is encoded by the ATP-binding cassette sub-family B member 1 (*ABCB1)* gene. The *ABCB1* c.3435T>A/C (rs1045642) SNP has been shown to correlate with P-glycoprotein expression and function.[187, 188] *ABCB1* c.3435T>A/C TT homozygotes have significantly decreased duodenal *ABCB1* expression and increased P-glycoprotein substrate (digoxin) plasma concentrations.[189] P-glycoprotein expression has also been correlated with a promoter SNP, T-129C, and the non-synonomous SNP *ABCB1* c.2677T>A/G (rs2032582), which results in an Ala→Ser amino acid change at position 893 of the protein. *ABCB1* c.2677T>A/G has been shown to be in linkage disequilibrium with c.3435T>A/C.[190, 191]

The hypothesis of this study was that functional *ABCB1* single nucleotide polymorphisms would be associated with darunavir concentrations in CSF due to differences in the expression of P-glycoprotein on the blood brain barrier.

9.2 Aims

To determine the effect of *ABCB1* polymorphism and blood brain barrier permeability (as measured by CSF:serum albumin ratio) on the CSF:plasma DRV concentration ratio.

9.3 Methods

Paired CSF/plasma samples and genetic material were obtained from subjects in the PARTITION study (groups A and B). Drug assay and other methods are given in section 3.3.1.3.

9.4 Results

Paired plasma and CSF samples were analysed in 48 darunavir treated subjects.

9.4.1 Demographic and clinical variables

Median age was 48 years (IQR 43,55). 43 (90%) were male. 40 (83%) were white ethnicity, 8 (17%) were black. 36 (75%) were MSM risk group, 8 (17%) were heterosexual, 2 (4%) were intravenous drug users and in 2 (4%) the HIV acquisition risk was unknown. Adherence was ≥95% in 43 (90%) subjects. All subjects were taking darunavir, other antiretroviral drugs at sampling were: NRTI 38 (79%), NNRTI 4 (8%), raltegravir 6 (13%), maraviroc 2 (4%).

9.4.2 Indication for lumbar puncture

16 (33%) were from recruitment group B. Within group A the indications for lumbar puncture were as follows: to investigate cognitive symptoms (n=14), headache (n=4),

to investigate abnormal neurological examination (n=4), enrolled in PIVOT CSF substudy (n=2), follow up progressive multifocal leucoencephalopathy (n=1), to measure JC viral DNA (n=1), seizure (n=1), to measure CSF HIV RNA (n=1), possible neurosyphilis (n=1), acute confusion (n=1), suspected CMV encephalitis (n=1), suspected multiple sclerosis (n=1) and sepsis unknown source (n=1). Based on lumbar puncture results CNS infection was suspected in 3 (6%) subjects, although no pathogen was identified. One subject had probable viral radiculopathy, 1 had raised CSF opening pressure (40cm/H_2O) and 1 had CSF pleocytosis (13 cells/ul) of unknown cause.

9.4.3 Genotype analysis

ABCB1 c.3435T>A/C (rs1045642) and *ABCB1* c.2677T>A/G (rs2032582) polymorphisms were in linkage disequilibrium; D'=0.871.

No significant associations were found between CSF:plasma darunavir concentration and *ABCB1* SNPs, either singly or in combination (*Table 22 and 23*). No significant differences were observed in multivariate analysis including time post dose (*Figures 20 and 21*).

	ABCB1 c.3435T>A/C. rs1045642			
	CC	CT	TT	p value
Alelle frequence, n (%)	18 (37.5)	21 (43.8)	9 (18.8)	
CSF:plasma [DRV], median (IQR)	0.0135 (0.0085,0.0202)	0.0110 (0.0065,0.0185)	0.0140 (0.0100,0.0165)	NS
CSF [DRV], median (IQR)	29 (15,53)	42 (17,73)	53 (29,93)	NS
Plasma [DRV], median (IQR)	2676 (1372,5865)	3809 (2638,5466)	4163 (2123 (6135)	NS

Table 22. Association of the ABCB1 c.3435T>A/C polymorphism with CSF and plasma darunavir concentration.

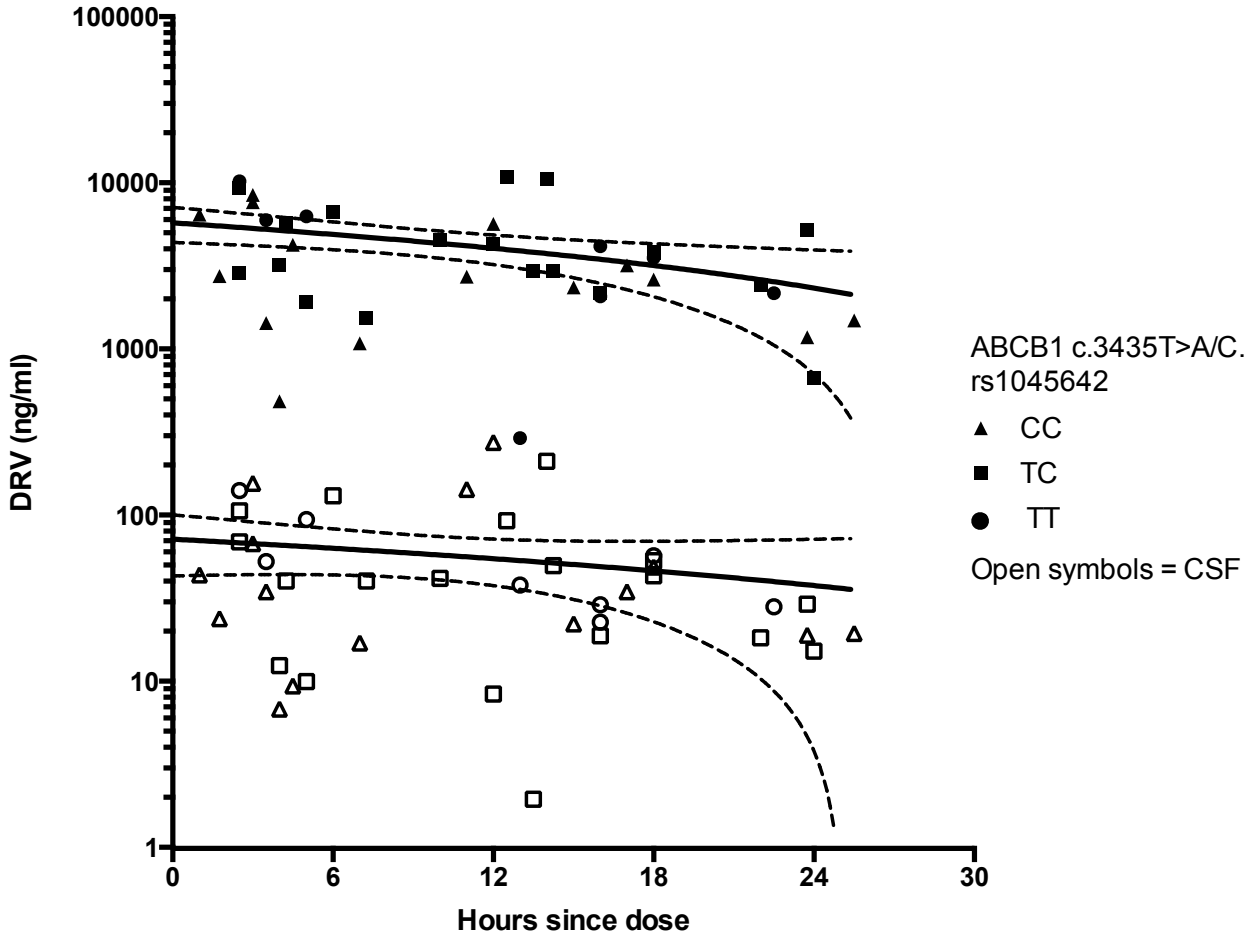


Figure 20. Darunavir concentration in plasma (filled symbols) and CSF (open symbols).

Darunavir concentrations in CSF and plasma were not associated with ABCB1 c.3435T>A/C (rs1045642) polymorphism in a multivariate model including time post dose.

	ABCB1 c.2677T>A/G. rs2032582					
	GG	GT	TT	GA	TA	p value
Alelle frequence, n (%)	20 (41.7)	17 (35.4)	7 (14.6)	3 (6.2)	1 (2.1)	
CSF:plasma [DRV], median (IQR)	0.0100 (0.0075,0.0190)	0.0110 (0.0065,0.0185)	0.0150 (0.0090,0.0170)	0.0180 (0.0070,0.0190)	0.012	NS
CSF [DRV], median (IQR)	35 (19,62)	23 (11,73)	53 (29,92)	49 (40,156)	44	NS
Plasma [DRV], median (IQR)	2973 (1500,6251)	2957 (2135,4489)	4163 (2168,5979)	5723 (2620,8472)	3521	NS

Table 23. Association of the ABCB1 c.2677T>A/G polymorphism with CSF and plasma darunavir concentration

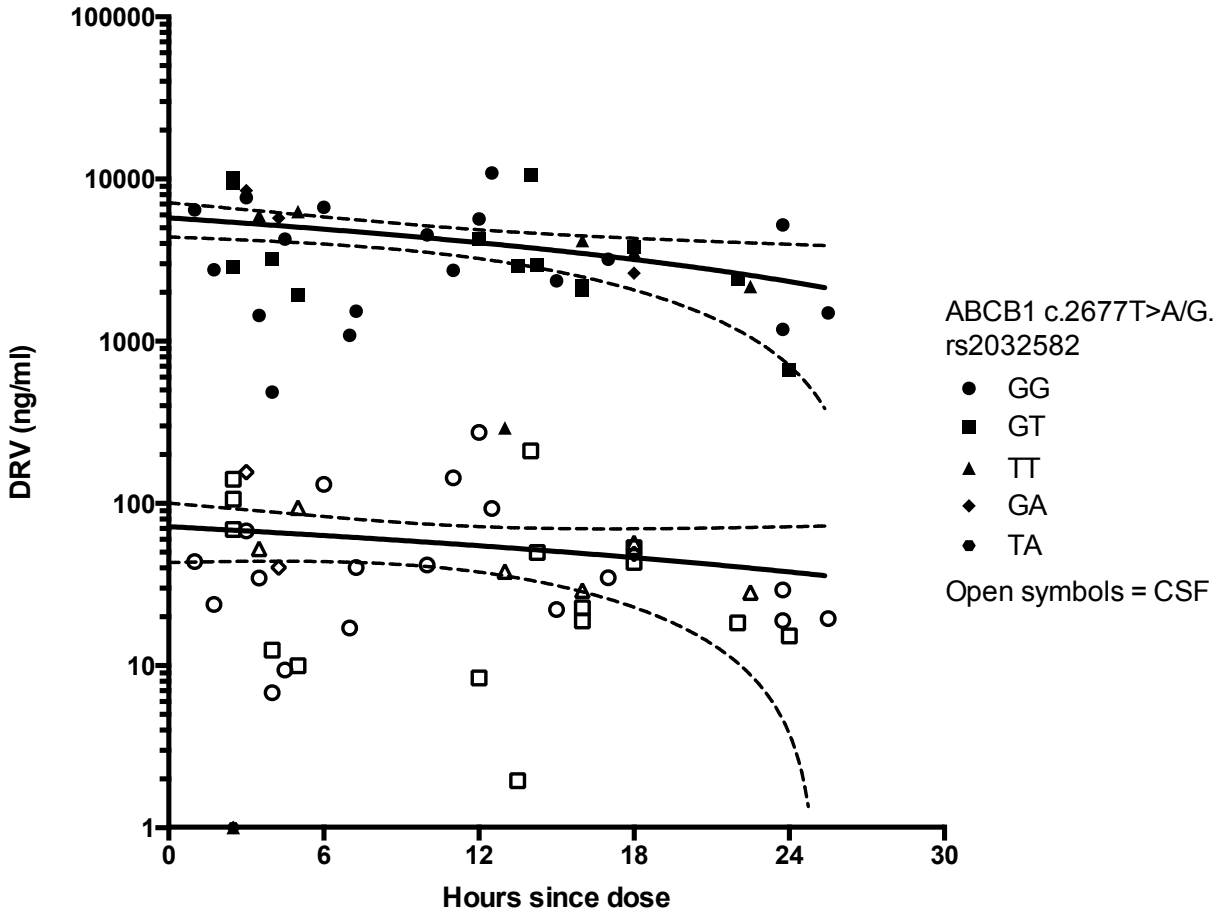


Figure 21. Darunavir concentration in plasma (filled symbols) and CSF (open symbols)

Darunavir concentrations in CSF and plasma were not associated with ABCB1 c.2677T>A/G (rs2032582) polymorphism in a multivariate model including time post dose.

9.4.4 CSF:serum albumin ratio

CSF:serum albumin ratio was measured in 23 subjects (48%). Median CSF:serum albumin ratio (expressed as mg/L:g/L) was 7.1 (IQR 5.3,8.4). CSF:serum albumin ratio was not correlated with CSF:plasma darunavir concentration (r=0.025, p=0.900); *Figure 22*.

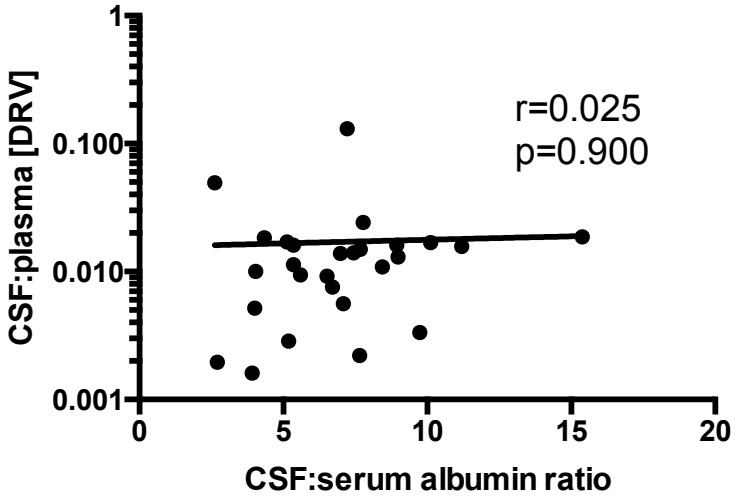


Figure 22. No significant correlation was found between CSF:serum albumin ratio (expressed as mg/L:g/L) and CSF:plasma darunavir concentration.

9.5 Discussion

There was considerable inter-individual variability in CSF darunavir concentrations, as has been previously observed.[192] In part this variability relates to random sampling. The variability was not related to differences in blood brain barrier permeability as measured by CSF:serum albumin ratio. This implies that active transport via brain endothelium influx/efflux transporters may be responsible for the significant observed inter-individual variation in CSF darunavir concentrations. In this study however, polymorphism of the measured *ABCB1* SNPs did not account for this variation.

The measured SNPs did not alter P-glycoprotein expression on the blood brain barrier to sufficient degree to cause observable changes in CSF darunavir concentration. *ABCB1* c.3435T>A/C has been shown to be the functional SNP in most, but not all tissues; for example in placenta the c.2677T>A/G SNP was more important.[190, 191] It may be that another SNP is functional in the expression of P-glycoprotein in brain endothelium. Alternatively, influx/efflux via membrane transporters other than P-glycoprotein may have a role. PIs have been shown to be substrates for other multi-drug resistance-associated protein (MRP) transporters and breast cancer resistance protein (BCRP),[193] and darunavir transport via organic anion transporting polypeptide (OATP) 1A2 and -1B1 has been reported.[194]

Kim *et al.* demonstrated dramatic (7-40 fold) increases in brain tissue concentrations of four different protease inhibitors in an *ABCB1* knockout mouse.[55] This difference was much greater in brain than for any other tissue (plasma, heart, lung, liver, spleen, kidney, small intestine, colon). Whether CSF darunavir levels measured in this study

reflected brain tissue levels is unclear. The blood brain barrier and blood CSF barrier differ in drug transporter expression, although P-glycoprotein is expressed on both.[195]

Several other factors effect P-glcoprotein expression, in particular induction and inhibition due to a number of drugs including many antiretroviral drugs coadministered with darunavir.[196] In addition P-glycoprotein expression on brain endothelium, astrocytes and microglia has also been shown to be affected by CNS inflammation in HIV and correlates with the histopathological degree of HIV-encephalitis.[197] Whether these factors were important in this cohort is not clear from these data.

10 General discussion

10.1 Overview

A review article based on the introduction chapter to this thesis was published in The Lancet Neurology.[198] It is the first major review article to directly address the controversies that have divided the field and suggest approaches to resolving key issues of debate. Its publication represents an important output from this thesis.

The significance of findings from this thesis are discussed in the individual chapter discussions. Below describes how overlapping aims and complimentary findings from the different chapters address the hypotheses laid out in chapter 2.

10.1.1 CSF/plasma discordance is a common determinant of viral detection in plasma

The PARTITION data suggest that lumbar puncture should be performed in subjects with incomplete viral suppression in plasma to measure CSF viral load and determine resistance profiles of HIV in the CSF. There is currently no consensus amongst clinicians as to whether this is appropriate management, and no guidance on the subject from professional bodies such as BHIVA or EACS. These data provide the first direct evidence upon which to inform this decision. Data presented in chapter 5 demonstrate that CSF/plasma discordance is a not uncommon occurrence in subjects with incomplete viral suppression in plasma, occurring in 18% of the cohort. This is a novel finding and PARTITION is the first study to address this issue. Data in chapter 4 shows that discordance is associated with history of immune suppression, suggesting the presence of HIV in the CNS relates to a longer duration of viral

replication and more advanced immune suppression. CSF resistance data presented in chapter 4 demonstrate that HIV resistance mutations can be identified from the CSF in patients with viral detection at levels too low for genotypic resistance testing in plasma. These findings have potential management implications for a large number of patients. Unexplained viral detection in plasma ≥50 copies/ml is a common clinical problem; in one study 122 (27%) of 448 subjects developed plasma blips ≥50 copies/ml over a median of 485 days. An additional 19 (4%) had persistent low-level viraemia between 50 and 400 copies/ml.[199] Given the large number of HIV infected individuals currently receiving ART,[2] this problem is likely to affect several thousand patients in the UK. I aim to prospectively assess the impact of providing lumbar puncture in this situation in a follow-up study - see section 10.2.2 below.

10.1.2 Biomarkers of neuroinflammation are associated with CSF/plasma discordance and neurocognitive impairment

Chapter 6 shows that CSF/plasma discordance is associated with raised expression of CSF biomarkers, suggesting potentially damaging CNS inflammatory processes in this group. Unexpectedly, data presented in chapter 7 found levels of TNFa and IGF1/2 were protective from deterioration in cognitive function. The significance of this is unclear. Despite the large number of biomarkers analysed, very few had significant associations with cognitive change. The model overall was poorly predictive and certainly not a clinically useful tool. One explanation for the apparently discrepant findings from chapters 6 and 7 is that CSF/plasma discordance was uncommon in the CHARTER cohort, described in just 2% of the population overall.[12] This implies that the progressive cognitive change may be due to causes other than ongoing HIV replication in the CNS and the potentially damaging neuroinflammatory

response shown in chapter 6 may not be an important factor here. As described in section 1.5.1, subjects in the CHARTER cohort had a large number of comorbidities which were linked to cognitive status. Comorbid conditions contributing to the observed cognitive decline may have limited the ability to detect an association with measured mediator concentrations.

Another important finding from this thesis is that an appropriate definition for CSF/plasma discordance should be >0.5$\log_{10}$. This is based on CSF resistance demonstrated in subjects with low discordance in chapter 4, and raised CSF mediator profiles in this group in chapter 6. Subjects with 0.5-1$\log_{10}$ discordance, or discordance but <200 copies/ml in CSF, should not be excluded, as has been the case in some studies.[8, 68] This is important for future studies in this area as use of this lower cut off reduces the risk of type 2 error. This finding is also important clinically as it guides management decisions in patients with CSF/plasma discordance detected at levels of previously uncertain significance. In addition the field should move away from using CSF escape when this does not meet the 0.5$\log_{10}$ level of discordance. Although used in many studies as well as in clinical practice, this criterion is prone to overestimate the extent of the problem. The definition of CSF viral escape (≥40 copies/ml in CSF and <40 copies/ml in plasma) would theoretically include a subject with 41 copies/ml in CSF and 39 copies/ml in plasma; such differences are likely to represent random biological and statistical variation or limitations of the assay, rather than CNS compartmentalised virus.[89] Of note, CSF viral escape has not been associated with cognitive impairment in large cross sectional studies.[12, 200] This may represent a situation where sensitive HIV RNA testing is appropriate to determine the true degree of CSF/plasma discordance; ie patients with CSF viral escape at levels not

meeting criteria for CSF/plasma discordance could be considered for sensitive testing in plasma to determine the true extent of discordance between the two compartments.

10.1.3 Low CSF antiretroviral drug concentrations are an important determinant of ART failure in the CNS and lead to CSF/plasma discordance and resistance

Although assays to measure CSF antiretroviral drug concentrations remain a research tool, requests are sometimes made by clinicians to perform CSF drug levels for individual patients and this practice may become more widespread with time. It is important to determine which patients are likely to benefit from CSF drug level testing, and in which clinical situations this is appropriate. Interestingly, data from chapters 4 and 5 do not support that antiretroviral drug concentrations in CSF are associated with ongoing replication of HIV within the CNS. This may in part be due to the random sampling and inability to accurately model Cmin for most drugs. This may also be due to differences between the concentration of drugs in the CSF and that within perivascular macrophages, a critical target cell of HIV in the brain.[4] Chapter 8 suggests that 8-hydroxy-efavirenz could be measured in CSF to assess neurotoxicity mediated by efavirenz metabolites. However as the majority of 8-hydroxy-efavirenz measurements were in the potentially neurotoxic range this may not be useful clinically. Data presented in chapters 4 and 5 show no association of CSF/plasma discordance with CPE score, however no firm conclusions can be drawn from this as the PARTITION study was not designed to assess the CPE score, was not randomised, and is prone to type 2 error due to prescriber bias (ie the demonstrated tendency for clinicians to prescribe regimens with a high CPE score to patients with CNS problems).[114]

10.1.4 Pharmacogenetic factors such as *CYP2B6* and *ABCB1* genotype impact the distribution of antiretroviral drugs into the CSF

Chapter 8 shows that CYP2B6 genotype is an important determinant of plasma and CSF concentrations of efavirenz. CYP2B6 was not associated with the neurotoxic metabolite 8-hydroxy-efavirenz concentrations in CSF. CSF 8-hydroxy-efavirenz concentrations were also independent of plasma efavirenz concentrations suggesting that neither genotyping nor therapeutic drug monitoring would be helpful in protecting patients from the harmful effects of efavirenz in the CNS. Chapter 9 found no association of ABCB1 polymorphism with darunavir concentrations in CSF. Together these chapters do not support genotyping to predict CSF concentrations, however there are several limitations to this analysis including that the subjects were randomly sampled and the data not modeled to predict Cmin. More work could be done in this area to further elucidate these mechanisms and the potential impact of pharmacogenetics on antiretroviral distribution in the CNS. However, chapters 4 and 5 show that CSF drug concentrations may not be a useful marker of ART efficacy in the CNS and hence CSF drug levels may not be the outcome of interest. I understand that investigators from the CHARTER study are planning to undertake a genome wide association study, looking at cognitive change as well as CSF drug concentrations. The results of this will be interesting, but may be limited by the effects of comorbidities on cognitive function, as discussed above.

The following outlines two areas of future study arising directly from this work. The first project has already been funded as part of an MRC Early Career Award, awarded in January 2014. Phylogentic analysis is currently underway at Centers for Disease Control and Prevention (CDC) in Atlanta. The second project takes forward findings from the PARTITION study; it is anticipated this will form part of an Intermediate Fellowship application.

10.2 Further research leading on from this work

10.2.1 Characterising viral divergence in PARTITION

The observation of the relationship between residual HIV-1 RNA detection in plasma and HIV-1 RNA levels in the CSF supports the hypothesis that the CNS serves as a site of on-going HIV replication during ART, and that CSF/plasma discordance can lead to persistence of HIV-1 RNA in plasma. To address this hypothesis and extend my current observations, I will characterise the HIV quasispecies in plasma and CSF of the subjects identified, using clonal analysis and next generation sequencing, followed by phylogenetic analysis to determine the genetic relatedness of viral strains. By sensitive sequencing methodologies, I will also determine whether HIV-1 RNA detection in the CNS is associated with the emergence of HIV resistance to the components of the ART regimen, and will assess the correlation between any drug resistant strain detected in the CSF and the drug susceptibility profile of plasma strains.

This work will addresses important clinical questions: Why is ART unable to eradicate HIV infection? Does HIV continue to replicate during seemingly successful ART? What is the site of the on-going HIV replication? Does it account for the detection of residual HIV-1 RNA in plasma? With this study I aim to provide conclusive evidence to support the hypothesis that HIV-1 replication continues in the CNS despite plasma HIV-1 RNA load suppression below the target level proposed by current guidelines (50 copies/ml), and that such replication causes residual HIV-1 RNA persistence in plasma.

10.2.2 PARTITION 2; the effect of treating CSF/plasma discordance on viral persistence in plasma

The PARTITION study demonstrated that CSF/plasma discordance is a frequent finding in subjects with incomplete viral suppression in plasma (ie. ≥50 copies/ml), however the current data do not conclusively show this association to be causal, or that treating viral persistence in CSF will improve viral detection in plasma. In the PARTITION 2 study I propose to charecterise a cohort of patients with incomplete HIV RNA suppression in plasma and follow them longitudinally for 2 years. Lumbar puncture will be performed at enrollment. In cases where CSF/plasma discordance is identified, antiretroviral treatment will be modified with respect to the genotypic resistance profile in CSF. If no resistance is identified in CSF, treatment will be adjusted according to estimated distribution into the CNS. Subjects identified to have CSF/plasma discordance will have a further lumbar puncture 6 months after treatment change, and thereafter every 6 months until CSF HIV-1 RNA is no longer discordant (using the $<0.5\log_{10}$ cutoff). Subjects without CSF/plasma discordance at enrollment

will be followed over the same period with sensitive plasma HIV RNA monitoring to act as a comparator group. Adherence to antiretroviral treatment will be closely monitored with pill counting and Medication Event Monitoring System (MEMS).

This project aims to conclusively answer the following important clinical questions: Should lumbar puncture be considered in all subjects with unexplained HIV RNA persistence in plasma? Does targeted treatment based on the CNS (ie. CSF resistance and/or estimated CNS penetration) lead to decreased HIV RNA in CSF in those with CSF/plasma discordance? Does treating CSF virus decrease viral detection in plasma? I intend to develop this proposal as part of an Intermediate Fellowship application.

Appendix

Publications arising from this thesis

Nightingale S, Winston A, Letendre S, Michael BD, McArthur J, Khoo S, Solomon T. Controversies in HIV-associated Neurocognitive Disorders. Review article. ***Lancet Neurology*** 2014 Nov;13(11):1139-1151.

Pozniak A, Rackstraw S, Deayton J, Barber T, Taylor S, Manji H, Melvin D, Croston M, Nightingale S, Kulasegaram R, Pitkanen M, Winston A. HIV associated neurocognitive disease: case studies and suggestions for diagnosis and management in different patient sub-groups. ***Antiviral Therapy*** 2014;19(1):1-13.

Nightingale S, Michael BD, Defrees S, Benjamin LA, Solomon S. Test them all; an easily diagnosed and readily treatable cause of dementia with life threatening consequences if missed. Editorial. ***Practical Neurology*** 2013 Dec;13(6):354-6.

Nightingale S, Geretti AM, Nelson M, *et al*. HIV-1 RNA detection in cerebrospinal fluid in those on antiretroviral therapy experiencing incomplete viral suppression in plasma; the PARTITION study. ***In preparation***

Nightingale S, Chau T, Fisher M, *et al*. Pharmacokinetics of efavirenz and its major metabolites in CSF of HIV-infected patients; influence of CYP2B6 G516T genotype and perturbed blood brain barrier due to tuberculous meningitis. ***In preparation***

Nightingale S, Michael BD, Ustianowski A, *et al*. CSF/plasma HIV-1 RNA discordance above $0.5\log_{10}$ associated with raised expression of CSF mediators compared with non-discordant subjects. ***In preparation***

Key presentations

Nightingale S, on behalf of the PARTITION investigators. HIV-1 RNA Detection in CSF in ART Treated Subjects with Incomplete Viral Suppression in Plasma. **Conference for Retrovirology and Opportunistic infection (CROI)** 2014, Boston Massachusetts.

Nightingale S. The CNS has an HIV reservoir (the PARTITION study). **18th Annual Resistance and Antiviral Therapy Meeting, Royal College of Physicians,** London. September 2014.

Nightingale S, Khoo S, Pirmohammed M, Solomon T. HIV and the Brain; Results from the PARTITION Study. **Malawi-Liverpool-Wellcome Conference.** Malawi. September 2013.

Nightingale S, Solomon T. Penetration of Antiretroviral Therapy into the Nervous System; Cognitive Impairment in HIV and the Role of the CNS as a Sanctuary Site. A Progress Report on the PARTITION Study. Poster presentation at **Association of British Neurologists** Annual Conference May 2012. Abstract in ***J Neurol Neurosurg Psychiatry*** 2012;83:Suppl 2

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