The Role of Inflammation, Oxidative Stress and the Apolipoprotein E Genotype in HIV-associated Cognitive Impairment: A clinical, biochemical and neuro-imaging study

by

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ABSTRACT

The Role of Inflammation, Oxidative Stress and Apolipoprotein E Genotype in HIV-associated Cognitive Impairment: A clinical, biochemical and neuromaging study.

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Background: Twenty percent of HIV-infected patients develop HIV-associated neurocognitive disorders (HAND), a spectrum of neurological diseases which includes HIV-associated dementia (HAD), a debilitating sub-cortical dementia. HIV-related cognitive impairment is a primary neurological complication of HIV infection, as opposed to neurological diseases secondary to opportunistic infections in the immune compromised host. Neurodegeneration in HAD is attributed to indirect injury mediated by an inflammatory process. The mechanisms underlying HAD have not previously been studied in a Sub-Saharan population.

Aims: Our study investigated the role of systemic markers of inflammation and oxidative stress, inflammatory changes in the brain on magnetic resonance spectroscopy (MRS) and the role of the APOE E4 allele in HIV-associated dementia (HAD) in a South African population. We hypothesized that cognitive impairment and brain inflammation would correlate with systemic markers of inflammation and oxidative stress; with an inverse relation to anti-oxidant capacity. We investigated the APOE E4 allele and its relation to markers of systemic and brain inflammation in HAD.

Methods: We recruited 35 HIV-infected, HAART-naïve subjects between the ages of 18 and 35 years, and 10 HIV-negative age- and population-matched controls. Subjects were classified into three groups according to degree of cognitive impairment evaluated by a composite score of the neuropsychological test battery: HIV-infected subjects

without cognitive impairment (NP 0), HIV- infected subjects with minor cognitive impairment (NP 1) and HIV- infected subjects with dementia (NP 2).

Inflammatory markers tested in serum included tumour necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β) and transforming growth factor beta (TGF-β). Oxidative stress was measured with conjugated dienes (CD), lipid hydroperoxides (LOOH), thiobarbituric acid reactive substances (TBARS) and the total anti-oxidant capacity was measured with the Oxygen Radical Absorbance Capacity (ORAC). Inflammatory changes in the brain were assessed with MRS using N-acetyl aspartate (NAA) and myo-inositol (MI) as markers of viable neurons and glial proliferation respectively. APOE genotyping was performed to determine the relation of the APOE E4 allele to cognitive impairment in HIV-infected subjects.

Results: Systemic markers of inflammation, the pro-inflammatory cytokine IL-1β in particular, correlated significantly with HIV-associated cognitive impairment, oxidative stress and MRS measures of inflammation. Markers of systemic oxidative stress correlated significantly with brain inflammation on MRS in patients with dementia (NP2). We found no relation between the APOE E4 allele and inflammation in HAD, but the APOE E2 allele was less frequent in the HIV-infected participants compared to the population allelic frequency.

Conclusion: We found significant associations between systemic inflammatory markers, systemic oxidative stress, inflammatory markers in the brain and cognitive impairment in HIV-positive subjects. Systemic infection, with associated systemic inflammation, oxidative stress and depletion of anti-oxidant defenses, may all play an important role in the development of central inflammation and neurodegeneration in HAD. Monitoring of this underlying process of inflammation, with markers of systemic inflammation, oxidative stress and metabolites on MRS, as explored in this study, may open new therapeutic and preventative strategies in the management of HAD.

TITLE PAGE

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A clinical, biochemical and neuro-imaging study

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DECLARATION

I, Anna Cecilia Mahne, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work or part of it has been, is being, or is to be submitted for another degree in this or any other university.

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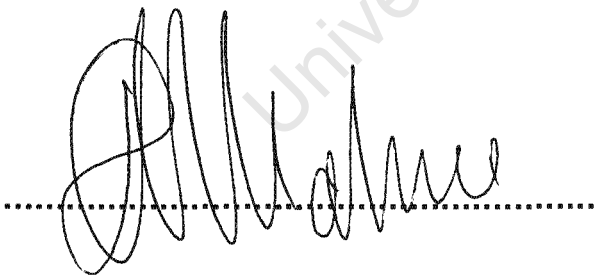

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My family: With your support the impossible becomes a possibility.

And my husband, Arnold: Your love inspires me.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ADL	activities of daily living
AIDS	Acquired Immunodeficiency Syndrome
AMPA	alpha–amino-3-hydroxy-5methyl-4-isoxalopropionate
ANI	asymptomatic neurocognitive impairment
APOE E4	apoliprotein E4
APP	amyloid precursor protein
ATP	adenosine triphophate
BACE1	beta site of the amyloid precursor protein cleaving enzyme 1
BBB	blood brain barrier
BHT	butylated hydroxyl toluene
cAMP	adenosine 3', 5'-cyclic monophosphate
CCR5	chemokine co-receptor 5
CD	conjugate dienes
$CD4^+$	clusters of differentiation 4^+
$CD8^+$	clusters of differentiation 8^+
Cho	choline
CNS	central nervous system
Cr	creatine
CRP	C-reactive protein
CSF	cerebrospinal fluid
CSI	chemical shift imaging
CUBIC	Cross University Brain Imaging Centre
CXCR4	CXC-chemokine receptor 4
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease

dNTPs	deoxyribonucleotide triphosphate
DTI	diffusion tensor imaging
DWI	diffusion weighted imaging
EIA	enzyme immunometric assay
Fe^{2+}	ferrous
Fe^{3+}	ferric
FID	free induction decay
FOX	ferric-xylenol orange
GABA	gamma aminobutyric acid
gp41	glycoprotein 41
gp120	glycoprotein 120
gp160	glycoprotein 160
GSH	mitochondrial glutathione
GSSH	oxidized glutathione disulfide
HAART	highly active antiretroviral treatment
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorders
HDS	HIV Dementia Scale
^{1}H-isotope	hydrogen isotope
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus type-1
HLP	hyperlipoproteinaemia
4-HNE	4-hydoxynonenal
HNRC	HIV Neurobehavioral Research Centre
HOCl	hypochlorous acid
H_2O_2	hydrogen peroxide

HRP	horseradish peroxidase
H_2SO_4	sulphuric acid
HSV1	herpes simplex virus type-1
HVLT	Hopkins verbal learning test
IFN	interferon
IHDRS	International HIV Dementia Rating Scale
IL	interleukin
IL-1β	interleukin-1 beta
IL-6	interleukin-6
ILGFBP-2	Insulin-like growth factor binding protein – 2
iNOS	inducible nitric oxide synthetase
IP 10	interferon inducible protein 10
IP_3	tri-phosphate
IP3	inositol triphosphate
L-cysteine	L-enantiomer of cysteine
LDL	low density lipoprotein
LDLR	low density lipoprotein receptors
LOOH	lipid hydoperoxides
LPS	lipopolysaccharide
MCD	mild cognitive deficit
MCMD	minor cognitive and motor disorder
MCP-1	monocyte chemoattractant protein-1
MDM	macrophages derived from monocytes
MI	myoinositol
MMSE	mini-mental state examination
MNGC	multinucleated giant cells
MRC	Medical Research Council

MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MSK	Memorial Sloan-Kettering
M-tropic	macrophage tropic
NAA	N-acetyl aspartate
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NEAD	Northeast AIDS Dementia Consortium
Nef	negative replication factor
NF-κβ	nuclear factor kappa beta
NINDS	National Institute of Neurological Diseases and Stroke
NMDA	N-methyl-D-aspartate
$NO^{\cdot}$	nitric oxide
NO	nitric oxide
NPI	Neuropsychiatric Inventory
NK	natural killer
O_2^{-}	superoxide
OH^{-}	hydroxyl
$ONOO^{-}$	peroxynitrite
ONOOH	peroxynitrous acid
^{-}OOH	hydroperoxide
ORAC	oxygen radical absorbance capacity
PAF	platelet activating factor
PAOF	patient's assessment of own functioning
PAOFI	patient's assessment of own functioning index
PCR	polymerase chain reaction

PET	positron emission tomography
PG	prostaglandin
PUFA	polyunsaturated fatty acids
RANTES	"regulated upon activation, normal T-cell expressed, and presumably excreted"
Rev	rev protein
RF	radio frequency
RNA	ribonucleic acid
RNOS	reactive nitrous oxygen species
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT	reverse transcriptase
SVS	single voxel spectroscopy
SPSS	Statistical Package for the Social Sciences
Tat	transactivator of transcription
TBA	thiobarbituric acid
TBARS	thiobarbituric reactive substances
TE	echo times
TGF	transforming growth factor
TGF-β	transforming growth factor beta
TNF-α	tumour necrosis factor alpha
TPP	triphenylphosphine
UCT	University of Cape Town
UNAIDS	The Joint United Nations Programme on HIV/AIDS
UVB	ultraviolet B
VLDL	very low density lipoproteins

Vpr	viral protein R
WASI	Wechsler Abbreviated Scale of Intelligence
XO	xylenol orange

1. INTRODUCTION

1.1 Epidemiology

The Joint United Nations Programme on HIV/AIDS (UNAIDS) reported in 2008 that 33.2 million people worldwide were infected with HIV by the end of 2007, of which 22 million (67% of the world prevalence) were living in Sub-Saharan countries. Human Immunodeficiency Virus (HIV)-associated dementia (HAD) affects 40% of HAART-naïve HIV-infected patients (Boisse, 2008). While most published work on HIV-associated neurocognitive disorders (HAND) is derived from North American and European studies, a recent report by Wong *et al.* (Wong, 2007) suggested that the prevalence of cognitive disorders in an HIV clinic in Uganda was similar to that reported in U.S. studies. The prevalence of HAD and minor cognitive motor disorder (MCMD) in the Uganda study was 31% and 45% respectively (Sacktor, 2007; Ellis, 2009), quantifying the extent of HAD in Sub-Saharan Africa.

1.2 The indirect mechanism of neuronal damage

HIV-associated dementia (HAD) is a primary complication of HIV-1 infection. HAD develops due to the direct effects of HIV-1 infection, independent of underlying opportunistic infections. As the virus is unable to infect neurons directly, neurotoxicity is mediated through an indirect mechanism (Power, 2009). HIV-1 displays neurovirulence indirectly through two pathological mechanisms: firstly, through activation of microglia, macrophages and astrocytes, with release of inflammatory mediators and through oxidative species stress (Boisse, 2008), and secondly, through oxidative damage mediated by viral proteins, including transactivator of transcription (tat) and glycoprotein 120 (gp120).

1.3 Clinical features of HIV-associated dementia

Clinical features of HIV-associated dementia develop as a result of neuronal injury mediated by the HIV infection. HAD affects mainly the basal ganglia and deep white matter resulting in a sub-cortical dementia with neurocognitive impairment, motor deficits and emotional disturbances (See Table 1 for Clinical features). (Boisse, 2008; McArthur, 2005; Power, 2009; Nath, 2008)

TABLE 1: Clinical features of HIV-associated neurocognitive disorders (HIV-associated dementia and minor neurocognitve disorder)

- Neurocognitive dysfunction: Memory impairment, poor concentration, psychomotor slowing.
- Emotional disturbances: Apathy and social withdrawal, which can be mistaken for depression. Also irritability, mental inflexibility, and decreased sex drive.
- Motor abnormalities: Weakness, ataxia, clumsy gait, slowing motor skills, tremor, diffuse increase in tone, hyper reflexia, spasticity, abnormal eye movements and parkinsonism. Frontal release signs and myoclonus in advanced stages of disease.
- Brain atrophy and abnormal subcortical white matter signal on magnetic resonance imaging and computerized tomography scanning.
- Pleocytosis, increased protein and high viral load in cerebrospinal fluid.
- Abnormal neuropsychological testing.

1.4 Classification of HAD

HIV-associated cognitive impairment is defined as a neurocognitive disorder associated with HIV-1 infection in the absence of delirium or another explanation for the cognitive impairment. It is classified according to the revised American Academy of Neurology criteria. HIV-associated neurocognitive disorders (HAND) incorporates three symptom complexes based on the severity of the disease, namely asymptomatic neurocognitive impairment (ANI), minor neurocognitive disorder (MND) and HIV-associated dementia (HAD). The revised criteria take the asymptomatic form of the disease into account and focus on the neurocognitive impairment rather than motor, emotional and personality aspects, as do previous classifications.

Neurocognitive impairment is graded on a neuropsychiatric score of less than 1 standard deviation below the norm in two or more domains of at least five domains tested. The patient is then further classified according to the presence of symptoms as asymptomatic or mildly symptomatic. The disease is classified as severe, or dementia, if the neuropsychiatric score is more than 2 standard deviations below the demographically ascertained norm in two or more of the cognitive domains tested. The latter is associated with marked difficulty of everyday function.

We devised a similar neuropsychiatric scoring system to evaluate cognitive impairment in combination with functional assessment tools to assess symptom severity.

TABLE 2: The National Institute of Neurological Diseases and Stroke (NINDS)
Classification of HIV-associated neurocognitive disorder (HAND) into three broad groups
1. Asymptomatic Neurocognitive Impairment (ANI)
• mild impairment of at least 1 Standard Deviation below the norm on neuropsychological testing in two or more domains of at least five cognitive domains tested • without a clear effect on everyday functioning
2. HIV-associated Mild Neurocognitive Disorder (MND)
• mild impairment of at least 1 Standard Deviation below the norm on neuropsychological testing in two or more domains of at least five cognitive domains tested • a mild effect on everyday functioning
3. HIV–associated dementia (HAD)
• moderate to severe impairment of at least 2 Standard Deviations on neuropsychological testing in two or more domains of at least five cognitive domains tested • substantial effect on everyday functioning • patient is unable to practice an occupation and is often unable to live independently

(Antinori, 2007)

Table Revised research criteria for HIV-associated neurocognitive disorders (HAND) (modified from HIV Neurobehavioral Research Center criteria[24])

HIV-associated asymptomatic neurocognitive impairment (ANI)*

1. Acquired impairment in cognitive functioning, involving at least two ability domains, documented by performance of at least 1.0 SD below the mean for age-education-appropriate norms on standardized neuropsychological tests. The neuropsychological assessment must survey at least the following abilities: verbal/language, attention/working memory, abstraction/executive, memory (learning, recall), speed of information processing, sensory-perceptual, motor skills.
2. The cognitive impairment does not interfere with everyday functioning.
3. The cognitive impairment does not meet criteria for delirium or dementia.
4. There is no evidence of another preexisting cause for the ANI.†

*If there is a prior diagnosis of ANI, but currently the individual does not meet criteria, the diagnosis of ANI in remission can be made.

†If the individual with suspected ANI also satisfies criteria for a major depressive episode or substance dependence, the diagnosis of ANI should be deferred to a subsequent examination conducted at a time when the major depression has remitted or at least 1 month after cessation of substance use.

HIV-1-associated mild neurocognitive disorder (MND)*

1. Acquired impairment in cognitive functioning, involving at least two ability domains, documented by performance of at least 1.0 SD below the mean for age-education-appropriate norms on standardized neuropsychological tests. The neuropsychological assessment must survey at least the following abilities: verbal/language, attention/working memory, abstraction/executive, memory (learning, recall), speed of information processing, sensory-perceptual, motor skills.

   Typically, this would correspond to an MSK scale stage of 0.5 to 1.0.
2. The cognitive impairment produces at least mild interference in daily functioning (at least one of the following):
   a) Self-report of reduced mental acuity, inefficiency in work, homemaking, or social functioning.
   b) Observation by knowledgeable others that the individual has undergone at least mild decline in mental acuity with resultant inefficiency in work, homemaking, or social functioning.
3. The cognitive impairment does not meet criteria for delirium or dementia.
4. There is no evidence of another preexisting cause for the MND.†

*If there is a prior diagnosis of MND, but currently the individual does not meet criteria, the diagnosis of MND in remission can be made.

†If the individual with suspected MND also satisfies criteria for a severe episode of major depression with significant functional limitations or psychotic features, or substance dependence, the diagnosis of MND should be deferred to a subsequent examination conducted at a time when the major depression has remitted or at least 1 month after cessation of substance use.

HIV-1-associated dementia (HAD)*

1. Marked acquired impairment in cognitive functioning, involving at least two ability domains, typically the impairment is in multiple domains, especially in learning of new information, slowed information processing, and defective attention/concentration. The cognitive impairment must be ascertained by neuropsychological testing with at least two domains 2 SD or greater than demographically corrected means. (Note that where neuropsychological testing is not available, standard neurological evaluation and simple bedside testing may be used, but this should be done as indicated in algorithm, see below).

   Typically, this would correspond to an MSK scale stage of 2.0 or greater.
2. The cognitive impairment produces marked interference with day to day functioning (work, home life, social activities).
3. The pattern of cognitive impairment does not meet criteria for delirium (e.g., clouding of consciousness is not a prominent feature), or, if delirium is present, criteria for dementia need to have been met on a prior examination when delirium was not present.
4. There is no evidence of another, preexisting cause for the dementia (e.g., other CNS infection, CNS neoplasm, cerebrovascular disease, preexisting neurologic disease, or severe substance abuse compatible with CNS disorder).†

*If there is a prior diagnosis of HAD, but currently the individual does not meet criteria, the diagnosis of HAD in remission can be made.

†If the individual with suspected HAD also satisfies criteria for a severe episode of major depression with significant functional limitations or psychotic features, or substance dependence, the diagnosis of HAD should be deferred to a subsequent examination conducted at a time when the major depression has remitted or at least 1 month has elapsed following cessation of substance use. Note that the consensus was that even when major depression and HAD occurred together, there is little evidence that pseudodementia exists and the cognitive deficits do not generally improve with treatment of depression.

FIGURE 1: Revised HAND Classification (Antinori, 2007)

After the initiation of highly active antiretroviral treatment (HAART) the incidence of HIV-associated dementia decreased, but prolonged survival rates of patients on HAART resulted in an increase in prevalence of mild neurocognitive disorder (MND). MND develops in 20% of patients despite HAART (McArthur, 2005) and, in contrast to the decline in HAD, 30-50% of patients on HAART suffer from MND (Ghafouri, 2006; Ellis, 2009).

HAART attenuates the disease course of HAD and new subtypes of HAD have been described as a result.

- Sub acute progressive dementia develops in patients who are not on HAART and disease progress is similar to HAD in the pre-HAART era. develop
- Chronic active dementia develops in patients with poor adherence or viral resistance, associated with progression of disease.
- Chronic inactive dementia is seen in patients with good adherence and response to HAART with stable neurological disease.
- Reversible dementia is recognized on clinical presentation in patients with good HAART adherence (Nath, 2008).

1.5 Clade diversity

A diversity of clades, or HIV subtypes, exists. 10% of HIV-1 infections worldwide are caused by clade B, mostly affecting European and North American populations, while Clade C is responsible for 50 % of the HIV-1 infections in the world (Ellis, 2009; Boisse, 2008; Nath, 2008). Most studies on HAD have been performed in Western countries with a clade B predominance while clade C is most prevalent in Africa. This difference in clades may influence the phenotypical expression of HIV associated dementia. Information on clade-specific differences between clade B and C is limited. Further research may

reveal differences in the clinical presentation of HAD in clade C compared with clade B.

1.6 Aims of the study and Hypotheses

Currently the prevalence of HAD in South Africa is unknown, but our clinical experience suggests that the burden of HIV-associated dementia is significant. The aim of this study was to contribute to the understanding of mechanisms underlying HAD. We investigated systemic inflammation, systemic oxidative stress and inflammation in the brain and their role in HAD. We focused on two postulated mechanisms of neuronal damage in HIV infection. Firstly, indirect neuronal damage caused by cytokines released by activated microglial cells during inflammatory processes in the nervous system. The second mechanism involves damage caused indirectly by viral proteins through increased oxidative stress in a setting of impaired anti-oxidant defenses. We hoped to attain additional knowledge on the pathogenesis of HAD, studied in a Sub-Saharan African population. Information which might lead to new forms of treatment, earlier diagnoses and possibly even measures to prevent the advent of HAD.

We investigated possible relationships between systemic inflammation, systemic oxidative stress, peripheral anti-oxidant defenses, inflammation in the brain and the APOE genotype and HIV-associated cognitive impairment. We measured the cytokines, TNF-α, IL-1β and TGF-β, in the peripheral blood as markers of systemic inflammation. We studied serum levels of conjugate dienes (CD), lipid hydoperoxides (LOOH) and Thiobarbituric Reactive Substances (TBARS) to assess systemic oxidative stress. The Oxygen Radical Absorbance Capacity (ORAC) measured total anti-oxidant capacity. Magnetic resonance spectroscopy peaks of N-acetyl aspartate (NAA) and myo-inositol (MI), using chemical shift imaging (CSI) of the lentiform nuclei of the basal ganglia, were used to assess neuronal integrity

and inflammation in the brain. We investigated the role of the APOE E4 allele in inflammation and HIV-associated cognitive impairment.

We formulated the following hypotheses regarding the associations of systemic inflammatory markers, markers of oxidative stress, markers of inflammation in the brain on MRS and the APOE E4 allele with HIV-associated cognitive impairment based on evidence in current literature. The rationale of these hypotheses will be addressed in the relevant chapters.

1.6.1 Raised inflammatory markers in the peripheral blood are associated with cognitive impairment in HIV-infected patients.

1.6.2 Inflammatory markers in the peripheral blood relate to markers of oxidative stress in the peripheral blood of HIV-infected patients with cognitive impairment. Higher levels of inflammatory markers are associated with higher levels of markers of oxidative stress.

1.6.3 Inflammatory markers in the peripheral blood relates to inflammatory markers on MRS in HIV-infected patients with cognitive impairment. Higher levels of inflammatory markers are associated with higher levels of inflammation markers on MRS.

1.6.4 Raised markers of oxidative stress in the peripheral blood are associated with greater cognitive impairment in HIV-infected patients.

1.6.5 Raised markers of oxidative stress in the peripheral blood are associated with raised levels of inflammatory markers on MRS in HIV infected patients with cognitive impairment.

1.6.6 Anti-oxidant defenses are inversely related to markers of oxidative damage in participants.

1.6.7 Anti-oxidant defenses are lower in HIV-infected patients with cognitive impairment.

1.6.8 Levels of anti-oxidant defenses are inversely related to markers of inflammation on MRS in the CNS.

1.6.9 Higher levels of inflammatory markers on MRS are associated with cognitive impairment in HIV-infected patients.

1.6.10 The APOE E4 allele is associated with greater cognitive impairment in HIV-infected patients in a dose-dependent manner.

1.6.11 The APOE E4 allele is associated with higher levels of inflammatory markers in HIV-infected patients in a dose-dependent manner.

1.6.12 The APOE E4 allele is associated with higher levels of markers of anti-oxidant stress in HIV-infected patients in a dose-dependent manner.

1.6.13 The APOE E4 allele is associated with increased levels of inflammatory markers on MRS in HIV-infected patients in a dose-dependent manner.

1.6.14 Low CD4+ count is associated with greater cognitive impairment in HIV-infected patients.

1.6.15 Low hemoglobin values are associated with greater cognitive impairment in HIV-infected patients.

2. METHODS

2.1 Recruitment of participants

We performed a cross-sectional study on HIV-infected participants and HIV negative controls between the ages of 18 and 35 years. We recruited 35 HIV infected participants and 10 controls. Both participants and controls were recruited from the same population. Recruitment took place at the Voluntary Counselling and Testing services of three peripheral clinics, namely Khayelitsha Site C Clinic, Mitchell's Plain Clinic and the Woodstock Community Clinics, Western Cape, South Africa. The participants were predominantly from a lower socio-economic class, consisting of unskilled manual workers, domestic workers or they were unemployed. They had a minimum of six years of education. All participants were HAART naive on recruitment and investigations were completed prior to initiation of HAART.

Participants were provided with information regarding the study, what participation involved and the extent of testing procedures. The participants gave written informed consent after counselling. Specific counselling for blood sampling and imaging was conducted and informed consent for these procedures taken on separate forms. Participants were booked for a neuro-cognitive assessment at the Department of Psychiatry, Groote Schuur Hospital, Cape Town, South Africa.

The exclusion criteria included:

- Presence of an uncontrolled medical condition, e.g. poorly controlled diabetes, epilepsy with ongoing seizures, active tuberculosis requiring admission or non-standard treatment. The above-mentioned conditions were

excluded with a detailed medical history performed as part of the initial screening of participants.

- Presence of an identified central nervous system neurological condition, such as lymphoma, untreated neurosyphilis or cryptococcal infection were excluded with detailed questioning of relevant symptoms during the medical questionnaire and syphilis screening on blood. The absence of gross pathology was confirmed on structural magnetic resonance imaging (MRI), which was performed at the same time as the MRS.
- Participants who have abused alcohol or psycho-active substances within the preceding month.
- Participants who in the investigator's opinion have a neurocognitive disorder NOT primarily due to HIV infection.

2.2 The neuropsychological battery

We performed a battery of neuropsychological tests on each participant, focusing on the sub-cortical symptom complex associated with HAD (McArthur, 2005). Psychomotor speed, memory and executive function are preferentially affected on the neuropsychological examination of patients with HAD (Nath, 2008). Motor involvement result in gait disturbances with ataxia, bradykinesia, spasticity and loss of manual dexterity. Agitation and apathy are prevalent in HAD but psychiatric symptoms are diverse and include mania and psychosis (Boisse, 2008; McArthur, 2005; Power, 2009; Nath, 2008). The home languages of the participants included Xhosa, English and Afrikaans. However, if the participants were fluent in English, the neuropsychological battery was performed in English. If the participants were not fluent in English, testing was performed in their mother tongue, either Xhosa or Afrikaans.

The following domains formed part of the cognitive assessment:

- verbal/language
- attention/working memory
- abstract thinking
- memory (learning, recall)
- speed of information processing
- sensory-perceptual
- motor skills

2.3 Summary of neuropsychological tools

The following battery of tests was performed on the participants:

TABLE 3: Neuropsychological Assessment Dementia Screening Tests		
International HIV dementia scale (IHDS) (Sacktor, N. 2005; Boisse, 2008; Power, 2009)	Screening tool for HIV-associated dementia	Validated for use in developing countries
Mini Mental State Examination (MMSE) (Folstein, 1975)	Screening tool for cognitive impairment	
a) Functional Assessment Tools		
HIV Neurobehavioral Research Centre (HNRC) Activities of Daily Living (ADL)	Assessment of general functioning	
The Patient's Assessment of Own Functioning (PAOF)	Self-assessment tool to assess general functioning	
Neuropsychiatric Inventory (NPI) (Cummings, 1994)	Screening for psychopathology	
A quality of life instrument (Antinori, 2007)	Self reported tool on quality of life	
b) Motor Speed And Coordination		
Grooved Pegboard (non-dominant)	Testing manual dexterity and co-ordination for the dominant and non-dominant hand and motor speed	25 pegs are rapidly inserted in a slotted form board with each hand individually
Finger tapping (non-dominant)	Motor speed and motor control	The test is performed bilaterally; five consecutive 10 second trials of repetitions are timed
Timed gait	Motor speed	Walking speed over specific distance timed

TABLE 3: Neuropsychological Assessment Dementia Screening Tests		
c) Memory	Learning and retrieval are impaired in HAD Working, semantic and long term memory are preserved	
The Hopkins Verbal Learning Test Recall (HVLT)	HVLT is a brief assessment of verbal learning and memory	Based on recognition and recall of various figures
The Brief Visuospatial Memory Test Recall	Visuospatial and recall ability	Reproduction of the features and spatial placement of two dimensional geometric figures
The Rey Osterreith Complex Figure Copy (Corwin, 1993)	Various cognitive domains are involved including visiospatial, attention, planning and working memory	Copying and drawing a complex figure from memory
Wechsler Memory Scale (Wechsler, 1997)	Most sensitive tool in early HAD	Memorizing of stories
The Mental Alternation Test	Sequencing and category switching	Patients count up to 20, then recite the alphabet, thereafter they must alternate numbers and letters chronologically in a set time

TABLE 3: Neuropsychological Assessment Dementia Screening Tests		
d) Psychomotor speed and Attention		
Trail-Making (Reitan, 1958)	Visual attention, task switching and mental flexibility	A connect-the-dot task Trail Making A : consecutive numbers
Digit Symbol Coding (McArthur, 2005; Grant, 2008)	Assessing processing speed Close correlation with global impairment (Bottiggi, 2007)	Consist of digit-symbol pairs. The digit is then given and the patient must write the symbol as fast as possible
Color Trails 1&2 (D'Elia, 1996)	Visual attention, task switching and mental flexibility Recommended in subjects who speak English as second language	The test does not require knowledge of the alphabet, using colors instead of letters. Trails 1 involves connecting consecutive numbers, Trail 2 has two color number pairs, the colors must be alternated during connection of the numbers in chronological order

TABLE 3: Neuropsychological Assessment Dementia Screening Tests		
e) Executive functions	abstract reasoning, planning and decision making	
The Stroop Colour Word Test (Stroop, 1953)	Executive functions, assessing speed of processing, selective attention and cognitive flexibility	The Stroop effect is obtained by printing names of colours in conflicting colours. Reading speed slows when compared to reading of names printed in black
The Wisconsin Card-Sorting Test (Nelson, 1976)	Executive functions, especially frontal lobe functioning, including attention, set-shifting, modulating impulsive responses and goal directed action	The test employ shapes, colours and quantities, with change in instruction to assess the ability to recognize sets
Trail-Making (Grant, 2008)	Visual attention, task switching and mental flexibility	A connect-the-dot task Trail Making B: alternating numbers and letters, assessing task switching abilities.
f) Verbal Language Skills	Fluency deteriorates with impaired word generation	
Verbal fluency tests • Category Fluency Animals • Category Fluency Fruit And Vegetables (Lezak, 1995)	Language especially fluency with impaired spontaneous generation of words (Grant, 2008)	Naming tests of animals and fruit or vegetables
g) Cognitive Functioning and Intellectual Ability		
Wechsler Abbreviated Scale of Intelligence (WASI) (Weschler, 1997; Pilgrim, 1999)	Quick tool to evaluate cognitive functioning and intellectual ability	We applied the four subsets of vocabulary, block design, similarities and matrix reasoning

2.4 Neuropsychiatric Score (NP Score)

No validated normative data are available for our population. Z-scores were calculated from control data and used to calculate the degree of impairment. Scoring was based on the updated American Academy of Neurology Criteria (Joska, 2010; Antinori, 2007).

TABLE 4: Neuropsychiatric Score (NP Score)

NP score	Staging	Requirement
2	Moderate to severe cognitive impairment (HAD)	Participants with a performance of two standard deviations below the mean in two cognitive domains
1	Mild cognitive impairment	Participants whose performance scored at least one standard deviation below the mean in two domains of cognitive function
0	No cognitive impairment	Participants with less than one standard deviation from the mean across all domains tested

(Joska, 2010)

2.5 Composite score

Participants were classified into three groups using data obtained from the self reported tests, neuromedical examination and neuropsychological battery. A neurological raw score was compiled interpreting neurological symptoms on a semi-quantitative scale.

The Patient's Assessment of Own Functioning Index (PAOFI) was used to score functional impairment. Scores were rated on a scale from 0 to 2 to calculate a composite score similar to the approach used in the Northeast Acquired

Immunodeficiency Syndrome (AIDS) Dementia (NEAD) Consortium study with the Memorial Sloan-Kettering (MSK) scale (Marder, 2003). The neuropsychological test battery and the composite scoring system were developed in collaboration with Dr John Joska and the team at the Neuropsychology Unit at the Department of Psychiatry (Joska, 2010).

2.6 The physical examination

The physical evaluation included a general physical examination and a neurological examination, which focused on the evaluation of gross and fine motor skills.

2.7 Blood specimens

A total volume of 30 millilitres of blood was obtained for performance of blood tests.

2.7.1 General markers

A general blood evaluation, including full blood count, HIV status, CD4 count, viral load and highly sensitive C-reactive protein (CRP), was obtained.

2.7.2 Method: Inflammatory markers

Obtained specimens for inflammatory markers and oxidative stress markers were immediately spun down. Serum was stored in a minus 70 degrees Celsius

freezer for later analyses. Inflammation markers included IL-1, TNF- α and TGF-β. Blood samples for inflammatory markers were processed at the immunology laboratory of the medicine department, University of Cape Town (UCT).

2.7.3. Methods: Oxidative Stress Markers

Oxidative stress markers included thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD), lipid hydro peroxides and anti-oxidant or total reductive defence capacity assessed with the Oxygen Radical Absorbance Capacity (ORAC) test.

Thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD) and lipid hydro peroxides (LOOH) were used to assess lipid peroxidation and the production of 4-hydoxynonenal (4-HNE) associated with oxidative stress and the Oxygen Radical Absorbance Capacity (ORAC) test was used to assess total anti-oxidative potential (Turchan, 2003). Assays for markers of oxidative stress were performed at the Lipid Laboratory, UCT.

2.8 Magnetic Resonance Spectroscopy (MRS)

A separate attendance was scheduled to perform imaging studies on participants. Imaging was performed at the Cross University Brain Imaging Centre (CUBIC) at Stellenbosch University Medical School in collaboration with the medical imaging unit at UCT. Transport for participants was arranged to the CUBIC and participants were reimbursed for personal expenses.

Magnetic Resonance Spectroscopy (MRS) was used as a non-invasive method for the assessment of metabolite levels in the brain and served as a measure of inflammation in the brain. In view of the importance of sub cortical involvement in the pathology of HIV-associated cognitive impairment, the voxel was placed to include the lentiform nucleus. MRS-related inflammatory markers were compared to peripheral measures of inflammation and oxidative stress in the blood. Metabolites were compared between groups with different stages of cognitive impairment, including HIV-infected subjects with cognitive impairment, HIV-infected subjects without cognitive impairment and HIV-negative control groups.

This enabled us to assess the relationship of systemic inflammation, markers of inflammation in the brain on MRS and HIV-related cognitive impairment.

The following metabolites were analyzed using creatine as reference:

- N-acetyl aspartate : Creatine
- Myo-inositol: Creatine
- Choline: Creatine
- Lactate: Creatine
- (Lipid+Lactate): Creatine

N-acetyl aspartate was used as marker of mature neurons, myo-inositol as marker of glial proliferation and lactate as marker of anaerobic glycolysis.

MRS metabolite values were also related to the APOE genotype to investigate genetic predisposition to inflammation and HIV associated cognitive impairment.

2.9 APOE genotyping

We assessed the relationship between the APOE genotype and inflammation, oxidative stress and metabolites of neuronal injury on spectroscopy respectively, to evaluate the role of APOE genotype in the development of HIV-associated cognitive impairment.

Blood samples of the subjects and controls were collected after informed consent. APOE genotyping on the samples was performed at the Department of Chemical Pathology (UCT Medical School). APOE genotyping was also performed on cord blood collected from 300 newborn babies to acquire a population allelic frequency for APOE E4 in the same population.

2.10 Statistical Analyses

The statistician at The Medicine Department of UCT was consulted to assist with statistical analyses. Analyses were performed with parametric (chi-square, analysis of variance (ANOVA), Tukeys HSD (Honestly Significantly Differences)) and non-parametric tests (Wilcoxon W.; Kruskal-Wallis Rank test, Mann Whitney U) in two-sided tests and multiple linear regression procedures. Statistical significance was declared at the 5% level. Analyses were performed using Statistical Package for the Social Sciences (SPSS) 14.

2.11 Ethics

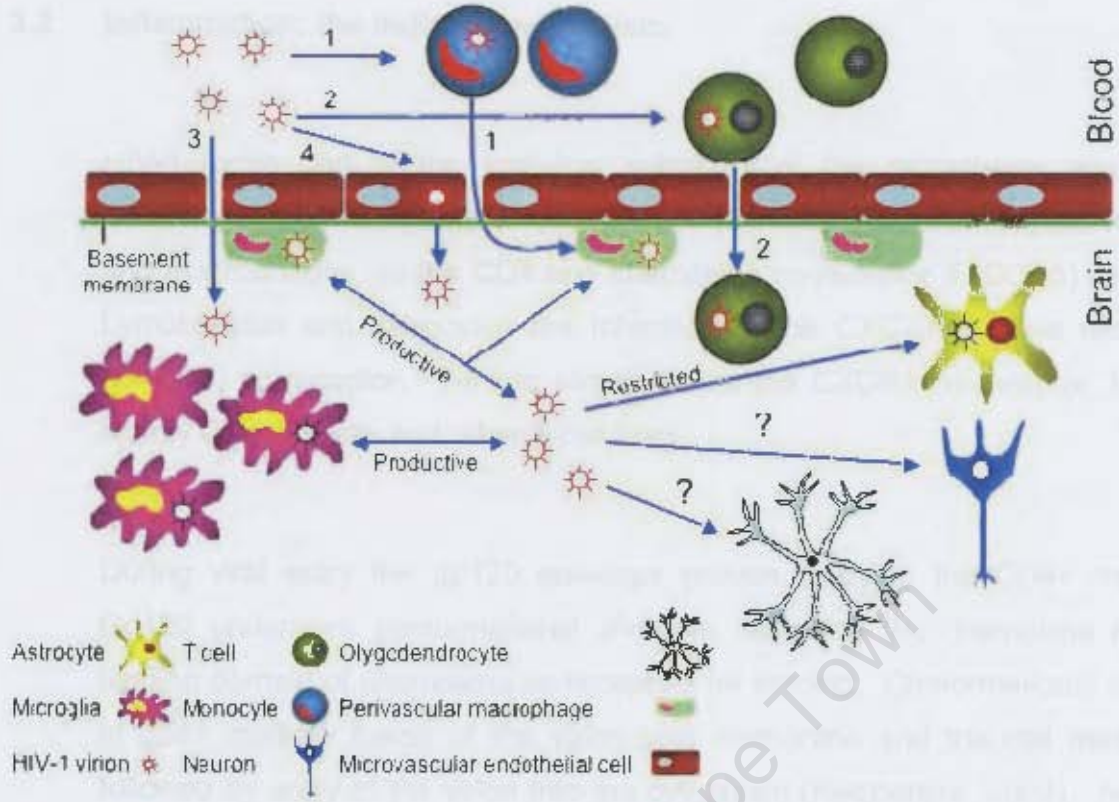
The study is a collaboration between Assoc Prof Marc Combrinck from the Neurology Division of the Department of Medicine, and Dr John Joska, from the Department of Psychiatry, and has been approved by the Research Ethics Committee of the Faculty of Health Sciences of the University of Cape Town (REC 263/2007). The genotyping of the 300 newborn babies for the determination of the population allelic frequency was done with ethical approval of the Research Ethics Committee of the Faculty of Health Sciences of the University of Cape Town (REC 093/2002) on cord blood previously collected by Prof Henderson from the Department of Chemical Pathology, UCT, as part of an international collaborative project. The study was conducted according to the ethical guidelines and principles of the International Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

3. INFLAMMATION

3.1 HIV entry into the Central Nervous System

The blood brain barrier (BBB) is selectively permeable due to tight junctions which adhere endothelial cells and astrocytes in close opposition. Upregulation of cytokines and nitric oxide (NO) during HIV infection increases the permeability of the BBB with an increase in traversing of immune factors (Hult, 2008; Ellis, 2009). Several factors accelerate entry into the CNS, impairment of tight junctions lead to increased penetration of HIV through the blood brain barrier, TNF-α mediates BBB breakdown through increased expression of adhesion molecules, and an increase in production of chemokines augments infiltration of HIV-infected monocytes and macrophages into the brain (Kedzierska, 2004).

HIV utilizes several routes to enter the CNS across the impaired blood brain barrier. HIV may infect endothelial cells with subsequent invasion of the parenchyma. A paracellular route could provide direct entry to HIV without intermediate cell involvement. HIV may cross the blood brain barrier intracellularly carried in monocytes, macrophages and clusters of differentiation 4^+ ($CD4^+$) cells, often referred to as the Trojan horse mechanism (Ghafouri, 2006).



HIV-1 neuroinvasion. 1) According to the "Trojan Horse hypothesis" entry of HIV-1 into the brain takes place by the migration of infected monocytes which different ate into perivascular macrophage. 2) The passage of infected CD4+ T cells can be another source of infection in the brain. Other probable causes of CNS infection might be: 3) the direct entrance of the virus or 4) entrance of HIV-1 by transcytosis of brain microvascular endothelial cells. Once the virus is in the brain it infects productively macrophages and microglia. Astrocyte infection is known to be restricted. The infection of oligodendrocytes and specially neurons is questionable.

(Ghafouri, 2006)

FIGURE 2: HIV-1 Neuroinvasion: The Trojan Horse Mechanism

3.2 Inflammation: the indirect mechanism

HIV-1 forms part of the lentivirus subfamily of the retroviruses and shows neurotropism. Macrophage tropic (M-tropic) viruses infect monocytes, microglia and macrophages via the CD4 and chemokine co-receptor 5 (CCR5) receptors. Lymphocytes and astrocytes are infected via the CXC-chemokine receptor 4 (CXCR4) co-receptor. T-tropic strains utilize the CXCR4 co-receptor, infecting mainly CD4+ T-cells and other T-cell lines.

During viral entry the gp120 envelope protein binds to the CD4+ molecule. Gp120 undergoes conformational changes, exposing the chemokine receptor binding domain of chemokine co-receptors for binding. Conformational changes in gp41 mediate fusion of the virion lipid membrane and the cell membrane, followed by entry of the virion into the cytoplasm (Kedzierska, 2002). Neurons, oligodendrocytes and epithelial cells are not productively infected by HIV (Kedzierska, 2002) and with limited evidence of the intracellular presence of HIV in neurons, direct infection of neurons remains controversial (Boisse, 2008; Ghafouri, 2006). Viral replication in astrocytes is highly restricted (Anderson, 2002). HIV mRNA is efficiently transcribed, but translation into structural HIV proteins is incomplete, with production of only HIV associated proteins (Gorry, 2003). Replication-competent viral genome persists, creating a viral reservoir.

Astrocytes act as a sanctuary site against viral eradication, harbouring HIV genome which maintains reproductive capabilities.

HIV production occurs predominantly in macrophages. The predominant underlying mechanism in the pathogenesis of HAD is neuro-inflammation secondary to the activation of macrophages. Indirect neuronal injury occurs as a

result of cytokine release from microglia, macrophages and astrocytes, with neurodegeneration and cognitive impairment secondary to inflammation. Proliferation of microglia, macrophages and astrocytes associated with inflammation is a pathognomonic feature of HIV-associated cognitive impairment and inflammatory markers may possibly have value as markers of neurodegeneration in HAD.

Tissue macrophages and macrophages derived from monocytes (MDM), the blood derivatives of monocytes, are infected by HIV. Perivascular macrophages are one of the major lineages affected by HIV (Williams, 2002; Anderson, 2002) and meningeal macrophages which are rapidly replenished by bone marrow derived monocyte/macrophages provide a route for early HIV invasion (Williams, 2002). Once monocytes enter the brain they differentiate into macrophages and form multinucleated giant cells (MNGC). Multinucleated giant cells (MNGC) are rare in the CNS. In the presence of a foreign body or an intra-cellular pathogen which cannot be removed, as seen in retroviral infections, macrophages fuse and form MNGC. MNGC are the histopathological correlate of HAD and are pathognomonic of HIV-associated encephalitis (Williams, 2002). Further histopathological features of HIV-associated encephalitis include astrogliosis, microglia nodules, dentritic and axonal damage, and neuronal loss (Anderson, 2002; Kedzierska, 2004). The degree of monocyte infiltration and macrophage activation correlates with the degree of cognitive impairment (Boisse, 2008).

Macrophages can produce HIV for months without cytopathic effects (Kedzierska, 2002). Macrophages utilize the endosome and exosome system for production of HIV rather than vesicles budding from the plasma membrane. The latter process would result in cell death. This longevity of macrophages creates a viral sanctuary with viral persistence in the CNS. The sustained migratory

potential of brain macrophages poses an additional infection threat (Anderson, 2002).

Multinucleated giant cells and microglial nodules release pro-inflammatory mediators. Microglia are activated by pro-inflammatory cytokines with expression of additional pro-inflammatory cytokines, including IL-1β and TNF-α, chemokines, viral proteins and ROS (including NO, produced by iNOS) and superoxide, produced by NAPDH oxidase (Kedzierska, 2002; Saha, 2003; Dheen, 2007). Microglia may have cytotoxic effects through direct contact with neurons or indirectly via these neurotoxic mediators.

Neurodegeneration is promoted by inflammatory mediators including tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β). Cytokines mediate additional cytokine release through paracrine stimulation of macrophages and astrocytes with amplification of the inflammatory response. Cytokines augment the accumulation of glutamate in the extracellular space. The excess in inflammatory mediators impairs reuptake of glutamate due to suppression of the excitatory amino acid transporters with a resultant increase in extracellular glutamate, excitotoxicity and cell death (Alison, 2007).

Astrocytes provide support to both neurons and glia and are involved in the maintenance of blood brain barrier integrity. Astocytes regulate glutamate metabolism (Gorry, 2003) and modulate the immune response by presenting antigens to T-cells with stimulation of T-cell responses (Minagar, 2002).

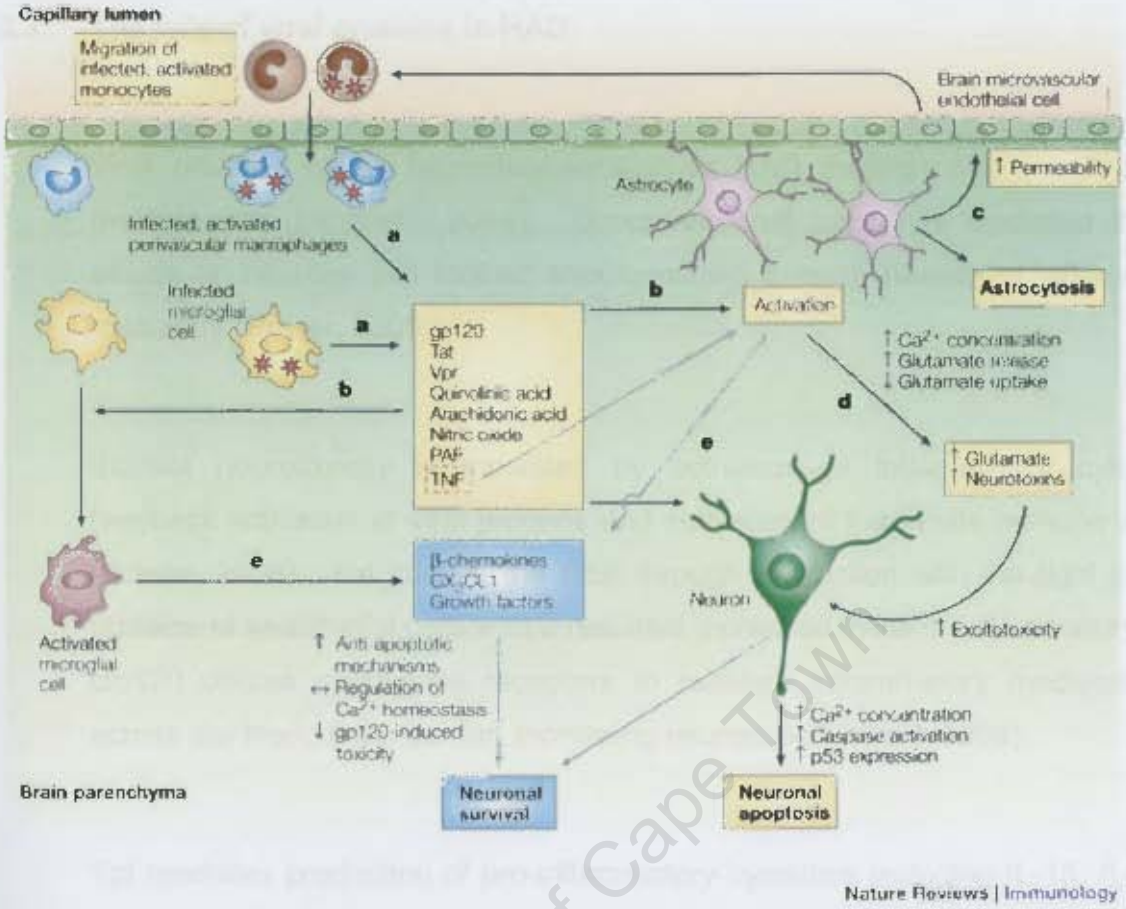
Astrocytosis is a defining feature of HIV encephalitis, occurring early after the initial infection with proliferation of astrocytes and astrocyte apoptosis mediated by cytokines. IL-1β increases the number and density of astrocytes and TGF-β1

inhibits astrocyte proliferation, but with an increase in size of astrocytes, which is a feature of astrocytosis. Astrocytes promote neurotoxicity through release of viral particles, production of cytokines and impairment of glutamate metabolism (Anthony, 2008). Astrocytes play an important role in the inflammatory process through the creation of a self-propagating cycle of cytokine production.

Paracrine immune activation by macrophages induces astrocyte apoptosis with secretion of cytokines. Astrocyte apoptosis cause a positive feedback with increased activation of macrophages and cytokine release.

Astrocytes play an important role in increased excitotoxicity. Glutamine released from astrocytes is taken up by neurons at the nerve terminals and converted to glutamate by glutaminase. Cytokines impair glutamate metabolism in astrocytes with an increased release and decreased uptake of glutamate (Hult, 2008) (Anderson, 2002). Excessive extracellular glutamate acts on post-synaptic glutamatergic receptors, including NMDA and non-NMDA receptors, leading to excitotoxicity and neuronal apoptosis (Alison, 2007).

Mechanisms involved in neuronal death include both apoptosis and necrosis (Boisse, 2008; Hult, 2008). Apoptotic cell death is initiated via caspases in milder insults (Brabers, 2006). Apoptosis, also called programmed cell death, involves cell shrinkage, membrane blebbing, nuclear pyknosis and early chromatin condensation. Necrosis takes place in severe insults with loss of ionic homeostasis, swelling and lysis (Chaparro-Huerta, 2002; Saha, 2003)



(Gonzales-Scarano F, 2005)

FIGURE 3: Mechanisms of Neurodegeneration and Neuroprotection in AIDS

3.3 The role of viral proteins in HAD

Viral proteins cause neurodegeneration in HAD through direct and indirect mechanisms (Minghetti, 2004). Direct neuronal toxicity is mediated through effects on neurons and indirect toxicity occurs through release of inflammatory mediators (Power, 2009).

Indirect neurotoxicity is mediated by activation of inflammatory cytokines, feedback activation of viral proteins and activation of the innate immune system (Boisse, 2008). Tat impairs the BBB through interaction with the tight junction proteins of endothelial cells with a resultant increased influx of inflammatory cells. Gp120 utilizes chemokine receptors to facilitate inflammatory mediator entry across the blood brain barrier, increasing neurotoxicity (Kaul, 1999).

Tat mediates production of pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α (Buscemi, 2007) (Ghafouri, 2006; Hult, 2008). Tat also mediates cytokine release by down regulation of adenosine 3', 5'-cyclic monophosphate (cAMP) with impaired suppression of cytokine production (Williams, R. 2009). The consequent release of cytokines accelerates HIV infection.

Tat and gp120 induce excitotoxicity through stimulation of the NMDA receptor by glutamate and L-cysteine, with increased intracellular calcium and NO levels, excitotoxicity and caspase-induced apoptosis (Ghafouri, 2006). Excitotoxicity is produced synergistically with other pro-inflammatory mediators and viral proteins. (Buscemi, 2007; Mayne, 2000; Kaneko, 1997). TNF-α exposure to HIV antigens also contribute to activation of astrocytes with an increased rate of cytokine release and apoptosis (Gorry, 2003).

Tat promotes HIV entry into cells through increased calcium influx (Minghetti, 2004). TNF-α may induce HIV replication in macrophages through induction of a transcriptional factor (Chen, 1997). Gp120 impairs glutamate metabolism by inhibiting uptake of glutamate through release of NO and arachidonic acid from astrocytes (Hult, 2008) (Minagar, 2002). Gp120 also up-regulates release of L-cysteine by macrophages and microglia through TNF-α and IL-1β (Brabers, 2006).

3.4 Inflammatory markers

3.4.1 Tumour Necrosis Factor alpha (TNF-α)

TNF-α plays an integral part in the pathogenesis of HAD. TNF-α is produced as a 26 kilodalton (kDa) membrane-bound polypeptide precursor which is cleaved by TNF-α converting enzyme to a 17 kDa bioactive subunit.

HIV dementia is associated with an increase in plasma TNF-α and levels correlate with severity and progression of disease (Sevigny, 2004). TNF-α increase the permeability of the blood brain barrier and opens a paracellular pathway for HIV entry (Saha, 2003). Increased production of adhesion molecules and chemokines by TNF-α facilitates entry of HIV infected monocytes and macrophages through the impaired blood brain barrier (Brabers, 2006).

Activated macrophages secrete IL-1β and TNF-α, with markedly higher levels of TNF-α in HAD (Saha, 2003). TNF-α shows paracrine activity through increased release of pro-inflammatory mediators by activated macrophages, microglia and astrocytes (Brabers, 2006) and TNF-α mediates cell death synergistically with other neurotoxins and viral proteins (Buscemi, 2007; Saha, 2003).

TNF-α mediates neurotoxicity through excitotoxicity. Autocrine effects of TNF-α result in amplification of glutamate release by astrocytes in the presence of chemokines. TNF-α, in association with IL-1β, upregulates iNOS expression with increased NO production and glutamate release (Saha, 2003). TNF-α inhibits uptake of glutamate by astrocytes (Brabers, 2006). TNF-α increases production of L-cysteine, an excitatory amino acid and precursor of glutamate and extracellular glutamate further inhibits L-cysteine uptake (Anderson, 2002). These increases in glutamate mediate cell death and neurodegeneration associated with HAD.

TNF-α mediates neurotoxicity through several other neurotoxins. TNF-α and IL-1β are associated with an increase in sphingomyelin and ceramide production causing oxidative stress through ROS production. Platelet activating factor (PAF) is stimulated by TNF-α, leading to increased microglia activity with phagocytosis, chemotaxis, calcium influx, and arachidonic acid release and superoxide anion production. PAF reciprocally induces production of TNF-α (Brabers, 2006). Genetic factors may influence the TNF-α levels in HAD. The TNF-α-308-A allele has a much higher frequency in HAD and is associated with increased TNF-α expression and increased cytokine production (Saha, 2003). TNF-α causes direct neurotoxicity through caspase activation after binding with TNF-α receptor on neurons (Saha, 2003; Chaparro-Huerta, 2002). TNF-α is the predominant mediator of the inflammatory cascade associated with neurodegeneration and HIV associated cognitive impairment.

However, in addition to the neurotoxic effects described, TNF-α also has a role in neuroprotection and may display anti-inflammatory properties. Neuroprotection was suggested through studies showing an attenuated rise of calcium in neurons pre-treated with TNF-α. TNF-α stabilizes calcium homeostasis via NMDA-

receptors and increases outward potassium flow with attenuated excitotoxicity. Upregulation of protective chemokines by TNF-α results in decreased neurotoxicity (Saha, 2003) and TNF-α may induce anti-apoptotic features. (Minagar, 2002). TNF-α plays a neuroprotective role in beta-amyloid toxicity (Brabers, 2006) which may be of importance in HIV-associated cognitive impairment.

3.4.2 Interleukin-1 beta (IL-1β)

IL-1β is produced by macrophages and causes macrophage activation (Williams, 2002). IL-1β release is regulated by caspase-1, the IL-1β converting enzyme (Brabers, 2006).

HIV dementia is associated with increased plasma levels of IL-1β, iNOS, TNF-α and caspase-1 compared to non-demented controls (Brabers, 2006). IL-1β mediates viral entry through impairment of the BBB and enhanced expression of adhesion molecules leading to an increase in monocyte infiltration. IL-1β activates astrocytes and, in association with TNF-α, induces iNOS expression in astrocytes with NO production (Brabers, 2006). IL-1β induces excitotoxicity through calcium influx secondary to stimulation of the NMDA-receptor by glutamate and L-cysteine. IL-1β is involved in oxidative stress through ceramide production leading to ROS-mediated neurotoxicity and apoptosis (Brabers, 2006).

Viral proteins are involved in the cytotoxic expression of IL-1β. Tat mediates NO production through caspase-1 activation of IL1-beta (Brabers, 2006) and Gp120 leads to increased production of IL-1β and ROS. IL-1β is involved in

neurodegeneration by these mechanisms. An increase in IL-1β levels relate to neuronal loss associated with HAD.

As with TNF-α, IL-1β fulfills a dual role. The aforementioned mechanisms are involved in neurodegeneration, but also play a role in neuroprotection. IL-1β protects through nerve growth factor, and in the presence of IL-1-induced NO a decrease in viral replication is seen (Brabers, 2006).

3.4.3 Transforming Growth Factor beta (TGF-β)

TGF beta shows predominantly neuroprotective mechanisms, but is also associated with proinflammatory mechanisms involved in neurodegeneration (Hurwitz, 1995). The inflammatory response induced by microglia impairs neurogenesis. TGF-beta acts as an anti-inflammatory cytokine (Dheen, 2007) to offer neuroprotection. TGF beta is associated with suppressed macrophage and T- and B-cell function and may ameliorate HIV infection. Increased TGF beta levels in the CSF were found to be inversely related to CSF viral load and disease severity (Alfano, 2005; Perella, 2003).

TGF beta has been associated with detrimental effects in HAD. The following mechanisms are involved in TGF beta associated neurodegeneration: TGF beta is associated with suppression of HIV early on in infection, but stimulates HIV replication later in the disease. TGF beta influences chemo-attraction of inflammatory cells through upregulation of TNF-α-induced chemokine expression (Hurwitz, 1995). Paracrine effects of IL-1β result in increased levels of TGF beta (Chao, 1994; Wahl, 1991). TGF beta antagonizes or augments the action of IL-1β depending on the stage of infection (Vitcovic, 1997). TGF beta production is upregulated by tat and gp120.

Apoptotic cell death in the central nervous system is not associated with a typical inflammatory response. An anti-inflammatory cytokine profile with increased levels of TGF beta is present to counter act a detrimental pro-inflammatory response. This initial protective anti-inflammatory response may evolve into a detrimental pro-inflammatory response during excessive inflammatory stimuli, as seen in experiments of induced systemic infection (Perry, 2002; Perry, 2004).

The role of TGF beta, whether neuroprotective or neurodegenerative, needs to be ascertained by further research.

3.5 Systemic Infection

The release of pro-inflammatory cytokines during systemic infection is suggested as a mechanism of increased neurodegeneration. Systemic infection is associated with an increase in BBB permeability, increased entry of peripheral immune cells and neuronal toxicity (Solerte, 2000). Studies suggested that pro-inflammatory cytokines measured in the serum are increased in dementia. Systemic markers of inflammation may relate to inflammation in the brain. Thus the measurement of these systemic markers in the peripheral blood may be useful in the monitoring cognitive impairment in the peripheral blood.

In previous studies systemic inflammation relates to markers of inflammation in the brain. Lipopolysaccharide (LPS) induced a systemic inflammatory response with an increase in the serum levels of IL-1β, TNF-α and IFN-γ. Elevations of cytokines in HIV infection resembled the findings in systemic infection, suggesting a shared inflammatory mechanism (Munno, 1992). Further studies found increased IL-1β levels in the brain after induction of systemic inflammation with LPS (Combrinck, 2002; Cunningham, 2005; Lemstra, 2007). Systemic

infection is believed to gain entry to the central nervous system through the impaired blood brain barrier. Systemic cytokines induce an increase in permeability of the BBB through altered tight junctions, creating a paracellular entrance. The vagus nerve serves as another pathway of entry to the brain during systemic inflammation via neuro-humeral signaling from the periphery.

Alzheimer's disease and HIV dementia share a common pathogenic mechanism through the presence of beta-amyloid. Based on the shared pathogenesis of beta amyloidosis, systemic infection in HIV may contribute to the development of HIV dementia in a similar way to the increased cognitive impairment during systemic infection in Alzheimer's disease.

Neuroinflammation with excess cytokine production, including IL-1, TNF-α and interferon-gamma, leads to amyloid deposition. TNF-α stimulates amyloid production directly through the beta site of the amyloid precursor protein (APP) cleaving enzyme 1 (BACE1). Oxidative stress mediates amyloid beta deposition and an increased amyloid beta load in the brain. The presence of amyloid beta increases HIV infection rate through increased HIV cell fusion synergistically with APOE E4 (Mahley, 2009).

Chronic infections in the elderly lead to increased inflammatory and oxidative stress responses which could predispose subjects to Alzheimer's disease (AD). Systemic infection leads to increased inflammatory responses and associated neurodegeneration in the brain. Prior priming of glial cells in chronic neurodegenerative disease results in the transformation of the anti-inflammatory state of microglia and macrophages in the brain to a pro-inflammatory cytokine profile in the presence of systemic infection (Teeling, 2009). Thus, systemic inflammation and oxidative stress responses could initiate or exacerbate

inflammation and associated neurodegeneration in the brain with cognitive decline (Honjo, 2009).

During systemic infection in the elderly cognitive impairment worsened and remained impaired for a prolonged period after the systemic infection resolved (Holmes, 2003). Previous studies found a significant rise in systemic TNF-α (Sevigny, 2004; Paganelli, 2002; Xu, 2009) and IL-1β in AD patients, suggesting increased cytokine representation in the periphery (Crabb Breen, 2002). Systemic levels of TNF-α were associated with a prolonged detrimental effect on cognitive function (Holmes, 2009). A relationship was found between increased IL-1β in the serum and cognitive decline in patients with Alzheimer's dementia. Elevated levels of IL-1β in the brain after systemic inflammation and elevated levels of IL-1β in the peripheral blood, associated with cognitive decline, could imply a shared pathogenic mechanism. The increase in IL-1β levels were associated with memory impairment due to impaired synaptic functioning (Malek-Ahmadi, 1998; Teunissen, 2002; Solfrizzi, 2006).

Although we focus on a causal relationship between systemic inflammation and brain inflammation in HAD, studies have found changes in systemic cytokines reliant on central immune responses. Mice injected with amyloid into the brain showed not only an increase in IL-6 in the brain but also in the plasma levels of IL-6. This supports a complicated interrelationship between systemic and central immune responses of which the mechanism is still incompletely comprehended (Song, 2001).

3.6 Hypotheses: Inflammatory Markers

Abovementioned literature supports inflammation as an integral part of the pathogenesis of HIV-associated cognitive impairment. We hypothesized that

markers of inflammation in the peripheral blood and markers of oxidative stress in the peripheral blood will relate in patients with HIV-associated cognitive impairment. We further hypothesized that both the inflammatory markers in the peripheral blood, and the markers of oxidative stress in the peripheral blood will relate to inflammation in the brain or MRS in HIV-infected patients with cognitive impairment. We also hypothesized that the APOE E4 allele will correlate with markers of inflammation in the peripheral blood and the brain, and with HAD.

3.7 Methods: Inflammatory Markers

3.7.1 TNF-α Method

For quantitative measurement of TNF, we utilized the Assay Designs' human TNF TiterZyme® Enzyme Immunometric Assay (Catalog No. 900-099). The assay was performed according to the manufacturer's instruction. A monoclonal antibody against human TNF alpha is immobilized on a microtiter plate and used to bind human TNF alpha in the specimen. Streptavidin conjugated to Horseradish peroxidase subsequently binds to the biotinylated human TNF antibody. The colour generated after short incubation is read at 450nm. This optical density is directly proportional to the concentration of TNF present in the sample.

We used the seven standards provided to calculate a standard curve. The standard curve obtained from these results is presented in figure 4. Previously collected blood samples were spun down immediately after collection and supernatant serum pipetted and frozen at minus 70 degrees Celsius. 100µL of the serum was used to perform the assay.

3.7.2 Interleukin-1β Method

We utilized the TiterZyme® Enzyme Immunometric Assay (EIA) Human IL-1β kit from Assay Design Inc. (Catalog No. 900-130) with a sensitivity of less than 1pg/ml. The assay was performed according to the manufacturer's instruction. Samples were collected from participants as described (2.7, 3.7.1) and stored at minus 70 degrees Celsius until serum were thawed at room temperature to perform the assay.

The human IL-1β standards were prepared. The five standard samples, numbered 1 through 5, were 400, 160, 64, 25, 6 and 10,24 pg/ml respectively, as provided by the manufacturer. The standard curve of our assay is presented in figure 4.

The assay utilizes an antibody against human IL-1β which is immobilized on a microtiter plate and binds with human IL-1β. A biotinylated antibody binds with the captured human IL-1β on incubation. Streptavidin-horseradish peroxidase (HRP) solution is added and incubated to bind with the biotinylated human IL-1β antibody. The colour generated is read at an optical density of 450nm. The measured optical density is directly proportional to the concentration of human IL-1β in the sample.

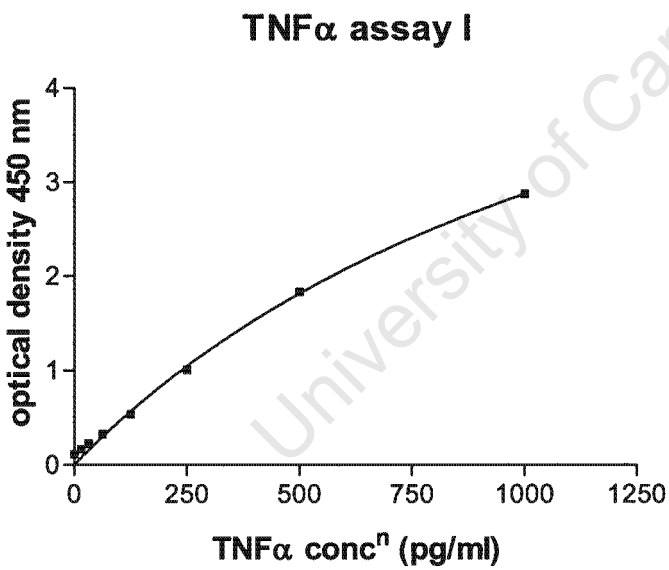
3.7.3 TGF-β1 Method

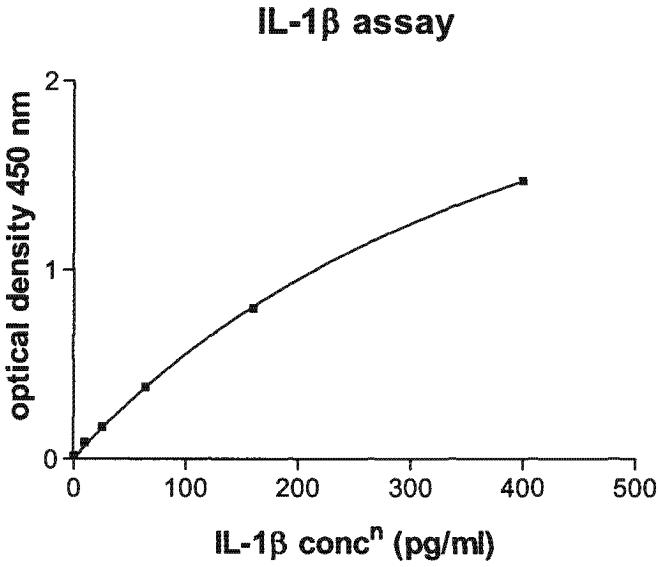
We utilized TiterZyme® EIA Human TGFβ-1 from Assay Design Inc. (Catalog # 900-155). The assay was performed according to the manufacturer's instruction. Serum was used to perform the assay and six standards were prepared with the samples according to the assay specifications. The standard curve of TGFβ-1 is depicted in figure 4.

The assay incorporates wells coated with monoclonal antibodies in binding TGFβ-1 after which a polyclonal antibody binds to bounded TGFβ-1. Addition of Streptavidin-horseradish peroxidase (HRP) conjugate and 3,3',5,5' tetramethylbenzidine (TMB) substrate elicit a colour response. The colour is read at an optical density of 450nm. The optical density provides a directly proportional measure of the level of TGFβ-1 in the sample.

FIGURE 4: The Standard Curves of TNF-α, IL-1 β and TGF-β

The following graphs represent the standard curves of the TNF-α, IL-1β and TGF-β1 assays. They represent a plot of the standards provided by the manufacturer against the optical density readings at 450nm measured using an ELISA plate reader.





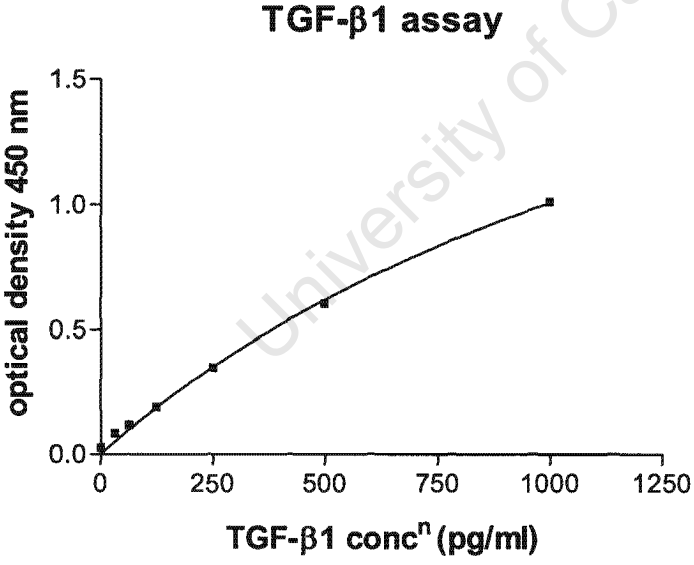


TABLE 5: Demographic data of participants

Variable	Distribution (N = normal)	HIV-negative controls (n = 10)	HIV -infected patients (n = 33)
		Mean ± Standard Deviation	Mean ± Standard Deviation
Haemoglobin	N	N/A	11.5 ± 1.8
Platelets	N	N/A	278.4 ± 75.5
		Median (Interquartile Range)	Median (Interquartile Range)
Age	Non N	23.5 (20.0 – 33.0)	30.0 (27.0 – 32.0)
White Cell Count	Non N	N/A	5.3 (4.3 - 6.7)
CD4 count	Non N	N/A	186.5 (91.5 - 251.0)

TABLE 5: Continued

Variable	Distribution (N = normal)	HIV-infected NP 0 (n = 9)	HIV-infected NP 1 (n = 13)	HIV-infected NP 2 (n = 12)
		Mean ± Standard Deviation	Mean ± Standard Deviation	Mean ± Standard Deviation
Hb	N	12.8 ± 2.1	11.9 ± 1.4	11.0 ± 1.7
		Median (Interquartile Range)	Median (Interquartile Range)	Median (Interquartile Range)
WCC	Non N	7.0 (3.5 - 9.7)	4.9 (3.9 - 6.6)	5.0 (4.5 - 6.1)
CD4 count	Non N	184.0 (92.0 - 314.5)	168.0 (71.3 - 271.3)	145.5 (86.3 - 204.8)
Age	Non N	29.0 (25.0 - 31.0)	31.0 (25.0 - 34.3)	31.0 (29.5 - 32.5)

TABLE 6: Descriptive data: Inflammatory Markers

Variable	Distribution (N = normal)	HIV negative controls (n = 10)	HIV -infected patients (n = 33)
		Median (Interquartile Range)	Median (Interquartile Range)
IL-1β	Non N	2.3 (2.0 - 4.8)	2.7 (2.1 - 3.6)
TNFα	Non N	23.7 (20.9 - 25.8)	23.8 (23.1 - 26.1)
TGFβ	Non N	511.2 (476.7 - 601.5)	481.8 (384.3 - 557.8)

Variable	Distribution (N = normal)	HIV-infected NP 0	HIV-infected NP 1	HIV-infected NP 2
		Median (Interquartile Range)	Median (Interquartile Range)	Median (Interquartile Range)
IL-1ß	Non N	1.7 (1.3 - 2.0)	2.8 (2.1 - 3.6)	3.6 (3.1 - 5.2)
TNFα	Non N	23.2 (23.0 - 25.7)	23.4 (23.0 - 26.6)	25.2 (22.8 - 30.9)
TGFβ	Non N	504.8 (485.0 - 545.8)	446.4 (373.5 - 730.0)	428.6 (323.0 - 551.1)

3.8 Results: Inflammatory Markers

We found a significant correlation between IL-1β levels in the peripheral blood and the various degrees of cognitive impairment.

Levels of IL-1β in the peripheral blood were highest in HIV-infected participants with severe cognitive impairment, compared to HIV-infected participants without cognitive impairment (Wilcoxon rank-signed test, Z-score = -2.816, p = 0.005). Refer to table 7.

IL-1β levels in the peripheral blood were significantly increased in HIV-infected participants with severe cognitive impairment compared to HIV-infected participants with mild cognitive impairment (Wilcoxon signed-rank test, Z-score = -2.342 p = 0.019). See table 7.

The correlation of IL-1β levels among groups was significant in a dose-dependent manner, with highest levels of IL-1β present in HIV-participants with greatest cognitive impairment (Chi-square, 10.182, p = 0.006). Refer to table 7. These findings support the hypothesis which relates systemic inflammation to cognitive impairment in HIV-infected individuals (hypothesis 1.6.1.).

A significant correlation between elevated levels of IL-1β, a systemic marker of inflammation, and decreased levels of N-acetyl aspartate, a marker of neuronal viability, was found when measured at an echo time of 135 milliseconds, in participants with HIV-associated cognitive impairment (Spearman's rank correlation coefficient = -0.373, p-value 0.019, n = 39). See table 7. The correlation between IL-1β and N-acetyl aspartate, as marker of neuronal loss on

MRS, supports the hypothesis relating systemic inflammation to brain inflammation in HAD (hypothesis 1.6.3). Previous studies found an association between elevated levels of IL-1β in the peripheral blood and cognitive impairment in AD (Holmes, 2003). Our study relates IL-1β, a systemic marker of inflammation, to N-acetyl aspartate, a marker of neuronal integrity on MRS used as a marker of inflammation in the brain, in patients with HAD. This supports the role of systemic inflammation in the pathogenesis of HIV-associated dementia. Whether IL-1β can be used as a systemic marker of cognitive impairment in HAD remains to be validated in a larger study involving greater numbers of participants.

An increase in TGF-β levels measured in the peripheral blood related to an increase in myo-inositol levels, measured with MRS at an echo time of 30 milliseconds, in the brain (Spearman's rank correlation coefficient = 0.320, p-value 0.050, n = 38), presented in table 7. Myo-inositol is a marker of glial proliferation on MRS. A dual role is recognized for TGF-β, involving protective anti-inflammatory properties and pro-inflammatory neurotoxicity. Previous research in patients with Alzheimer's disease related an atypical immune response with increased levels of TGF-β in the brain to a cytokine profile associated with macrophages that have phagocytosed apoptotic cells (Perry, 2002; Perry, 2004). The correlation of an increased level of TGF-β in the systemic circulation with an increased level of myo-inositol, a marker of glial activation in the brain, implicates TGF-β as a possible systemic marker of intra-cerebral glial activation. This supports the relationship between systemic markers of inflammation and inflammation in the brain on MRS and HAD (hypothesis 1.6.3). Results on TGF-β bordered significance and the use of TGF-β as systemic marker of inflammation in the brain in HAD needs to be validated through larger numbers.

We found a significant correlation between TNF-α levels in the peripheral blood and conjugated diene levels in the peripheral blood (Spearman's rank correlation coefficient = 0.363, p-value 0.018, n = 42), tabulated in table 7. These markers related inflammation and oxidative stress in the peripheral blood but not to inflammation in the brain.

Within the group of HIV-infected participants with severe cognitive decline, levels of TNF-α in the peripheral blood and levels of IL-1β in the peripheral blood correlated (Spearman's rank correlation coefficient = 0.656, p = 0.020, n = 12), see table 7. This support increased systemic inflammation present in the peripheral blood in HIV-infected patients with severe cognitive impairment (hypothesis 1.6.1). Systemic inflammation has been related to cognitive impairment in several studies, as discussed in section 3.5 (Combrinck, 2002; Cunningham, 2005; Lemstra, 2007; Holmes, 2009; Honjo, 2009). Elevated systemic cytokines levels may be causally related to cognitive impairment in HAD. The latter could only be assessed in longitudinal studies as the cause must precede the effect.

IL-1β levels in the peripheral blood showed an inverse correlation with lactate, a measure of anaerobic respiration, measured at 135 milliseconds (Spearman's rank correlation coefficient = -0.812, p = 0.050, n = 6), result in table 7. This, however, is a tentative finding because of the small numbers and borderline significance.

We did not find a significant association between the APOE E4 allele and systemic inflammatory markers in HAD (hypothesis 1.6.11).

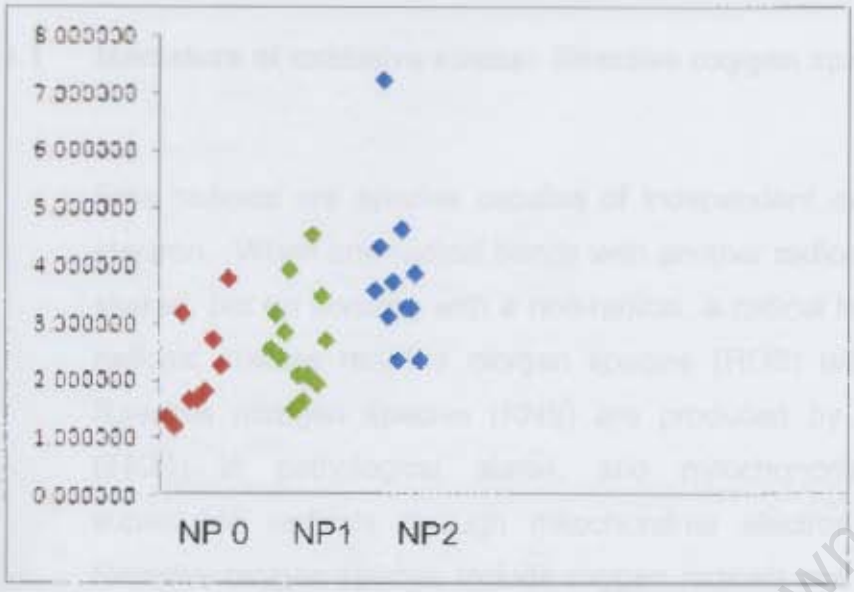
In summary, markers of inflammation in the peripheral blood correlated significantly with HIV-associated cognitive impairment. Markers of inflammation

in the peripheral blood also correlated with markers of oxidative stress in the peripheral blood and markers of inflammation in the brain measured on MRS. Our findings support the role of inflammation in the pathogenesis of HAD.

TABLE 7: Results: Inflammatory Markers

	NP 2 vs NP0	NP2 vs NP1	Among NP groups	N-acetyl aspartate - 135 milliseconds
IL1-beta	Wilcoxon rank-signed test Z-score = -2.816 p = 0.005	Wilcoxon signed-rank test Z-score = -2.342 p = 0.019	Chi-square, 10.182 p = 0.006	Spearman's rank correlation coefficient = -0.373 p-value 0.019 n = 39

	myo-inositol 30 milliseconds	conjugated diene	IL-1β NP2 group	IL-1β
TGF-beta	Spearman's rank correlation coefficient = 0.320 p-value 0.050 n = 38			
TNF-α		Spearman's rank correlation coefficient = 0.363 p-value 0.018 n = 42	Spearman's rank correlation coefficient = 0.656 p = 0.020 n = 12	
Lactate 135 milli-seconds				Spearman's rank correlation coefficient = -0.812 p = 0.050 n = 6



X – axis: Participants grouped according to degree of cognitive impairment

NP0: No cognitive impairment

NP1: Mild cognitive impairment

NP2: Severe cognitive impairment/dementia

Y- axis: IL-1β conc^{n} (pg/ml)

FIGURE 5: IL-1β Values of HIV-infected patients

4. OXIDATIVE STRESS

4.1 Mediators of oxidative stress: Reactive oxygen species

Free radicals are species capable of independent existence with an unpaired electron. When one radical bonds with another radical, the unpaired electron is shared, but on bonding with a non-radical, a radical is produced. Production of radicals creates reactive oxygen species (ROS) leading to oxidative stress. Reactive nitrogen species (RNS) are produced by inducible NO synthetase (iNOS) in pathological states, and mitochondrial metabolism produces superoxide radicals through mitochondrial electron flow (Reynolds, 2007). Reactive oxygen species include oxygen radicals and non-radical derivatives of oxygen, namely superoxide (O_2^-), hydroxyl radicals (OH^-), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2) and peroxyl radicals. RNS free radicals include nitric oxide (NO), peroxynitrite ($ONOO^-$) and peroxynitrous acid (ONOOH). Reactive oxygen species (ROS) are involved in the peroxidation of lipids and oxidation of proteins resulting in excitotoxicity and apoptotic cell death (Halliwell, 1993; Emerit, 2004).

Oxidation-related mitochondrial damage preferentially affects areas of high dopamine content. Therefore subcortical areas, including the putamen, caudate and substantia nigra show increased vulnerability to oxidative stress.

Oxidative stress plays an important role in neurodegeneration (Sacktor, 2004). In HAD the critical balance between endogenous reactive oxygen species and anti-oxidant defenses is impaired, resulting in oxidative stress. HIV infected patients are especially vulnerable to oxidative stress by age, opportunistic infections and illicit drug use (Valcour, 2004). Host responses against abundant

pathogens lead to activation of glial cells in response to inflammation with formation of an excess of endogenous reactive oxidative species (ROS).

4.2 Excitotoxicity

Excitotoxicity is a common pathway leading to apoptosis as a result of oxidative stress induced by inflammation, protein oxidation and impaired mitochondrial function (Emerit, 2004). Oxidative stress develops due to activation of glutamate receptors with an increase in intracellular calcium levels. The increase in calcium levels lead to excitotoxicity and apoptotic cell death. Excess extracellular glutamate induces excitotoxicity through overstimulation of the excitatory glutamate receptors. The modulatory site of the N-methyl-D-aspartate (NMDA) receptor is stimulated by glutamate with subsequent influx of calcium.

Extracellular glutamate concentrations are finely regulated to prevent excitotoxicity. Astrocytes mediate glutamate homeostasis through the glutamate-glutamine cycle. After uptake, glutamate is converted to glutamine through glutamine synthetase, requiring ATP. Subsequently, glutamine is released for uptake by neurons and conversion back to glutamate by glutaminase. Disruption of the glutamate-glutamine cycle results in an increase in extracellular glutamate concentrations, excessive stimulation of excitatory receptors and an increase in calcium influx (Emerit, 2004). The increase in intracellular calcium levels induces several enzymes, including iNOS, with production of ROS and RNS via the arachidonic acid cascade, resulting in excitotoxicity.

4.3 HAD and oxidative stress

In HAD several mechanisms are associated with increased excitotoxicity. HIV dementia is associated with dysregulation of astrocyte function due to up regulated cytokine release (Annunziato, 2003). Cytokines secreted by activated macrophages, including TNF-α and IL-1β, induce oxidative stress through induction of inducible nitric oxide synthetase (iNOS). iNOS initiates the production of nitric oxide (NO) in macrophages and astrocytes (Valcour, 2004). NO releases glutamate and glutamate activates the NMDA receptor with associated calcium influx, excitotoxicity and apoptosis (Pocernich, 2005).

Neurons are not directly affected by HIV in HIV-associated neurodegeneration. Macrophages and microglia are predominantly infected with subsequent release of viral and non-viral products (Sacktor, 2004). Activated mononuclear phagocytes, including macrophages, microglia and multinucleated giant cells, induce oxidative stress through the release of cytokines and chemokines and through an increase in neurotoxins, including free radicals, glutamate and arachidonic acid metabolites (Emerit, 2004; Reynolds, 2007). The increase in cytokine release, including IL1-β and TNF-α, leads to neuronal death through excitotoxicity (Pocernich; Viviani, 2001)

HIV-infected astrocytes, macrophages and microglia release tat and gp120. HIV proteins induce neuronal death through indirect oxidative damage. Gp120 and tat mediate oxidative stress through the release of free radicals. Tat-associated activation of astrocytes induces iNOS with overproduction of NO. NO in association with TNF-α facilitates an increase in glutamate with stimulation of the NMDA receptor and excitotoxicity. The increase in intracellular calcium in addition leads to ROS formation, including superoxide, hydrogen peroxide, nitric

oxide and peryoxynitrite, which are neurotoxic (Pocernich, 2005; Steiner, 2006; Turchan, 2003; Kruman, 1998).

4.4 Lipid peroxidation

Lipid peroxidation is mediated by free radical induced oxidative stress. Polyunsaturated fatty acids (PUFA) are prevalent in the brain and are especially vulnerable to lipid peroxidation compared to monounsaturated and saturated fatty acids.

Lipid peroxidation occurs through formation of peroxyl radicals. A self-propagating cycle of damage to lipid membranes, due to abstraction of hydrogen atoms from polyunsaturated fatty acids in the presence of oxygen, produces peroxyl radicals. Lipid peroxidation causes membrane damage with impaired membrane functioning, increased permeability of the membrane to calcium and other ions, and inactivation of receptors (Halliwell, 1993).

4.4.1 HAD and lipid peroxidation

Increased levels of oxidative stress correlate with disease severity in HIV-associated cognitive impairment. Oxidative stress in the brain of HIV dementia patients is demonstrated by increased levels of sphingomyelin, ceramide and 4-HNE, a lipid peroxidation product, produced through increased breakdown of lipid membranes.

In HIV dementia an increase in sphingolipid levels is found secondary to impaired lipid metabolism. The rise in sphingomyelin and ceramide, which is followed by

an increase in 4-hydoxynonenal (4-HNE), is associated with disease progression (Steiner, 2006).

Sphingomyelin, ceramide and 4-HNE levels in the CSF show significant increases in patients with dementia (Sacktor, 2004; Pocernich, 2005). These increases of 4-HNE and ceramide in the CSF of active HAD and the increase of sphingomyelin, but not 4-HNE and ceramide, in inactive HAD may aid in the classification of HAD subjects according to disease severity (Sacktor, 2004; Bandaru, 2007).

4.5 Protein Oxidation

Oxidative damage includes damage to proteins and breakage of the deoxyribonucleic acid (DNA) strand. Damage result from direct insults to thiol groups, or indirectly through lipid peroxidation (Steiner, 2006).

Reactive oxygen species cause oxidative damage to the amino acid side-chains of proteins with production of protein carbonyls. Protein oxidation is mediated through peroxynitrite ($ONOO^-$), a powerful oxidant formed by a reaction between nitric oxide and superoxide, leading to formation of protein carbonyls. The structural damage to proteins results in breakdown of enzymes, impaired receptor-mediated cellular signaling, defective transport mechanisms, and damage to DNA with miscoding, mutations, and neuronal death (Valcour, 2004).

4.5.1 HAD and protein oxidation

Protein carbonyl levels are used markers of oxidative damage to proteins. HAD is associated with elevated levels of peroxynitrite, 4-HNE and protein carbonyls due to protein oxidation (Sacktor, 2004; Valcour, 2004; Pocernich, 2005). HIV proteins induce the formation of protein carbonyls independently and increased levels of protein carbonyls are associated with severe HIV-associated dementia (Turchan, 2003).

HIV proteins mediate direct neurotoxicity through oxidative stress. Tat stimulates expression of iNOS, with production of nitric oxide and neurotoxic peroxynitrite (ONOO), after reacting with superoxide anions (O_2^-). Tat protein increases intracellular calcium through mitochondrial dysfunction. Tat is also associated with elevated levels of protein carbonyls as a result of oxidative damage (Turchan, 2003).

4.6 Oxidative stress induced by mitochondria

Mitochondria produce oxidative stress through oxidative phosphorylation. Adenosine triphosphate (ATP) is synthesized by the respiratory chain during oxidative phosphorylation with utilization of oxygen (Emerit, 2004). Reactive oxygen species (ROS) are formed during the normal process of oxidative phosphorylation.

The lipid peroxidation product, 4-HNE, impairs function of mitochondria. Mitochondrial dysfunction leads to overproduction of ROS during oxidative phosphorylation and apoptotic cell death (Steiner, 2006).

Oxidative stress related to impaired energy systems induced by mitochondrial failure lead to accumulation of extracellular glutamate.

4.7 The role of APOE in oxidative stress

The APOE E4 allele is associated with dysregulation of lipid metabolism. Sphingomyelin, ceramide and cholesterol levels are significantly increased in the presence of the APOE E4 allele (Steiner, 2006). The apolipoprotein E4 isoform is associated with accumulation of cholesterol during sphingomyelin breakdown due to impaired metabolism of cholesterol esters and triglycerides (Bandaru, 2007). The increase in lipid oxidation is associated with increased oxidative stress.

Individuals who have an APOE E4 allele show increased markers of oxidative stress, an increase in mithochondrial dysfunction and decreased glutathione levels in HAD (Steiner, 2006).

The APOE E4 allele is associated with an increased in lipid peroxidation and a decrease in anti-oxidant potential in the presence of HIV proteins, including tat (Pocernich, 2005).

4.8 Anti-Oxidants and HAD

Mitochondrial glutathione (GSH) is an endogenous electron donor which demonstrates anti-oxidant properties. GSH reduces ROS and nitrogen free radicals, and binds lipid peroxidation products to decrease oxidative stress.

Decreased levels of GSH are associated with increased oxidative stress. Decreased manganese superoxide dismutase levels are another indicator of increased oxidative stress (Pocernich, 2005).

Glutathione forms an important part of the anti-oxidative stress response. Cystine is the rate limiting factor in glutathione production. After importation into the cell, cystine is reduced to cysteine. Cysteine is then transformed to gamma-glutamyl-cysteine which reacts with glycine to form glutathione (GSH) (Alison, 2007).

Increased stimulation of protective pathways may idiosyncratically result in upregulation of glutathione production with increased cystine import and excessive export of glutamate. An excess in extracellular levels of glutamate during oxidative stress impairs anti-oxidant mechanisms through suppression of cystine-release required for production of glutathione.

Anti-oxidant defense systems provide further protection through manganese superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and NADPH regenerating enzymes (Dheen, 2007). Manganese superoxide dismutase fulfills the role of an anti-oxidant and decomposes O_2^- into O_2 and H_2O_2. HIV proteins directly impair anti-oxidant defense mechanisms through decreased levels of glutathione and manganese superoxide dismutase with an increase in oxidative stress (Emerit, 2004). Tat induces feedback inhibition of gluthathione synthetase with a further reduction of glutathione levels. The resultant glutathione deficiency impairs antioxidant mechanisms (Reynolds, 2007). Decreased levels of glutathione in HIV infected patients were associated with increased mortality (Steiner, 2006).

4.9 Hypotheses: Markers of Oxidative Stress

In the light of this literature review we hypothesized that markers of oxidative stress in the peripheral blood will relate to markers of inflammation in the peripheral blood, as well as with markers in inflammation in the brain as measured by MRS in HIV-infected patients with cognitive impairment. We hypothesized that ORAC, as measure of total anti-oxidant capacity, will inversely relate to systemic markers of oxidative stress and markers of inflammation, both in the brain and peripheral blood. In addition we investigated the association of APOE E4 with oxidative stress in HAD.

4.10 Method: Markers of Oxidative Stress and Anti-oxidant Capacity

4.10.1 Thiobarbituric Acid Reactive Substances (TBARS) Method

We mixed 50µl of serum with 6.25 µl of butylated hydroxyl toluene (BHT) in ethanol and added 50 µl of 0.2mol/l orthophosphoric acid in 2ml Eppendorf micro test tubes. It was vortexed for 10 seconds.

We added 6.25 µl thiobarbituric acid (TBA) reagent (0.11mol/l: TBA dissolved in 50ml 0.1 mol/l sodium hydroxide (NaOH) and it was vortexed again.

The mixture was then incubated at 90 degrees Celsius for 45 minutes in a water bath.

The tubes were put on ice for two minutes to stop the reaction and then cooled to room temperature for a further 5 minutes.

TBARS were extracted once with 500 µl butanol.

50 µl saturated sodium chloride (NaCl) was added to facilitate phase separation and then centrifuged at 12000rpm for 1 minute.

The upper phase was pipetted into a multititer plate reader.

Absorption was read at 535nm and 572nm and the calculations made using the two wavelengths and previously prepared calibration curves (Jentzsch, 1996).

4.10.2 Conjugated Dienes Method

Lipid extraction was performed by the Folch method (Folch, 1957).

Chloroform and methanol was mixed in a 2:1 ratio by volume.

The serum sample was diluted with the chloroform methanol mixture to a 20 fold dilution of the original serum sample and mixed with water of 0.2 of the sample volume.

The sample was then split into two phases by centrifugation at 2400rpm. The upper and lower phase was calculated in a 40% to 60% volume ratio. The upper phase was removed by pipette.

The lower phase was then diluted in a 2:1 ratio with the chloroform-methanol mixture (Vansankari, 1995).

A double extraction was performed to ensure the purity of the lipids.

The extract was then dried under nitrogen atmosphere.

The dried lipid extract was diluted with chloroform and aliquotted into vials.

The lipid sample was dried finally to ensure concentration of pure lipids (Folch, 1957).

The dried lipid was dissolved in cyclohexane to a lipid concentration of 100µg/ml.

The serum lipid per oxidation was read spectrophotometrically between 300 and 220nm (232) (Corongui, 1983).

4.10.3 Lipid hydroperoxides (LOOH) Method

The FOX assay was used to assess the amount of lipid peroxides in the samples. Reduction of peroxides by Fe^{2+} generates Fe^{3+} in acidified samples. The generated Fe^{3+} forms a complex with xylenol orange. The absorbance of the 1:1 XO/ Fe^{3+} complex can be read at 560nm by spectrometry in which the concentration of lipid hydro peroxides is represented by the colour intensity.

The Ferric-Xylenol Orange (FOX) reagent consisted of 90% of methanol, 10% 250mM sulphuric acid (H_2SO_4), 880mg of BHT, 98mg of ferrous ammonium sulfate hexahydrate (250µM) and 76mg of xylenol orange (100 µM). Two individual samples were prepared. The first with equal amounts of sample and 10mM triphenylphosphine (TPP), a LOOH reducing agent which was combined in methanol by vortex and incubated for 30 minutes to allow complete reduction of ^{-}OOH. The second sample was prepared without the reducing agent, substituting the TPP with methanol. The FOX agent was added to both samples and the difference in absorbance read at 560nm exactly 10 minutes after the reagent was added. It was then read against a standard curve (DeLong, 2002).

4.10.4 Oxygen Radical Absorbance Capacity (ORAC) Method

The ORAC assay is an inhibition method. Inhibition of free radical action is measured after a free radical generator has been added to the sample. The fluorescence of R-phycoerythrin is measured as indicator of damage through free radical action. A decrease in fluorescence of R-phycoerythrin is an indicator of the anti-oxidant capacity of the sample. 2, 2' azobiz (2-amidinopropane) dihydrochloride is used as the free radical generator. The free radical action is followed until completion and the area under the curve is calculated.

We added 10µl of 2, 2' azobiz (2-amidinopropane) dihydrochloride to the reaction mixture.

The reaction mixture consisted of 300µl R-phycoerythrin and 20 µl of Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) for acquisition of the standard curve. The sample mixture consisted of 300µl R-phycoerythrin and serum (1:200 dilutions). The serum was prepared by precipitation of proteins through ethanol and the sample was diluted with a phosphate buffer (Wang, 2004).

TABLE 8: Descriptive data: Oxidative stress

Variable	Distribution (N = normal)	HIV negative controls (n = 10)	HIV -infected patients (n = 33)
		Median (Interquartile Range)	Median (Interquartile Range)
CD	Non N	86.2 (74.2 - 98.7)	109.8 (70.8 - 138.2)
TBARS	Non N	1.7 (1.4 - 2.4)	1.2 (1.0 - 1.8)
LOOH	Non N	213.5 (180.2 - 268.7)	173.3 (143.0 - 215.7)
		Mean ± Standard Deviation	Mean ± Standard Deviation
ORAC	N	1.4 ± 0.8	1.5 ± 0.5

TABLE 8: Descriptive data: Oxidative stress (Continued)

Variable	Distribution (N = normal)	HIV-infected NP 0	HIV-infected NP 1	HIV-infected NP 2
		Median (Interquartile Range)	Median (Interquartile Range)	Median (Interquartile Range)
LOOH	Non N	193.5 (144.4 - 227.6)	164.5 (132.8 - 188.4)	164.2 (151.8 - 222.3)
CD	Non N	127.6 (76.5 - 148.0)	79.3 (64.8 - 126.5)	127.3 (82.1 -154.0)
TBARS	Non N	1.2 (1.0 - 2.4)	1.2 (1.0 - 1.6)	1.5 (0.8 - 2.3)
		Mean ± Standard Deviation	Mean ± Standard Deviation	Mean ± Standard Deviation
ORAC	N	1.8 ± 0.7	1.5 ± 0.5	1.7 ± 0.4

4.11 Results: Markers of Oxidative stress

A significant inverse correlation between the marker of anti-oxidant capacity in the peripheral blood and a marker of brain inflammation, specifically glial proliferation, namely myoinositol, was found in HIV-infected participants with severe cognitive impairment. Oxygen Radical Absorbance Capacity (ORAC) correlated inversely with myo-inositol, measured at 30 milliseconds on MRS (Spearman's rank correlation coefficient = -0.587, p = 0.045, n = 12), results in figure 9. The correlation between decreased anti-oxidant capacity in the peripheral blood and increased glial proliferation may signify increased inflammation in the brain in a setting of impaired anti-oxidant defenses. Impaired anti-oxidative defenses relate to increased oxidative stress, which could mediate cognitive impairment in HIV-infected individuals (hypothesis 1.6.6). This finding supports our hypothesis (hypothesis 1.6.8) which relates impaired anti-oxidative defenses to an increase in inflammation in the brain of participants with HAD.

Another systemic marker of oxidative stress correlated with inflammation in the brain (hypothesis 1.6.2). Lipid hydroperoxides, measured in the peripheral blood, showed a significant inverse correlation to N-acetyl aspartate, a marker of neuronal viability on MRS, in HIV-infected participants with severe cognitive impairment (Spearman's rank correlation coefficient = -0.697, p = 0.025, n = 10) results presented in figure 9. This supports the role of systemic oxidative stress in neurodegeneration associated with increased inflammation in the brain of patients with HAD (hypothesis 1.6.5). Increased systemic oxidative stress may relate to neuronal toxicity and loss. However, the numbers are small in this correlation and further studies with greater numbers are needed to validate the role of oxidative stress in cognitive impairment associated with HAD.

We found a significant correlation between conjugated dienes and TNF-α (Spearman's rank correlation coefficient = 0.363, p-value 0.018, n = 42), results presented in figure 9. This relation between conjugated dienes and TNF-α relates to inflammation in the systemic circulation and cannot be extrapolated to the central nervous system. We screened for systemic causes of inflammation, but a systemic cause unrelated to inflammation in the brain might still explain the result.

No further correlations were found between oxidative stress, anti-oxidant defenses and inflammatory markers systemically or in the brain measured with MRS. Neither levels of oxidative stress nor the anti-oxidant capacity differed significantly among the various HAD groups (hypotheses 1.6.4 and 1.6.7).

We did not find correlations between the presence of the APOE E4 allele and either oxidative stress or anti-oxidant capacity in the peripheral blood in patients with HAD (hypothesis 1.6.12).

In conclusion, we found significant relationships between systemic oxidative stress and inflammation in HAD. A marker of oxidative stress and a marker of inflammation in the brain, measured with MRS, correlated significantly in HIV-infected patients with cognitive impairment. We also found a significant inverse relationship between the total anti-oxidant capacity in the blood and a marker of inflammation in the brain on MRS, in support of our hypothesis on the protective role of total anti-oxidant capacity in HAD. These findings support the role of oxidative stress in the development of cognitive impairment in HAD.

TABLE 9: Results: Markers of Oxidative Stress

	myo-inositol **30 milliseconds**	**N-acetyl aspartate**	**TNF-α**
Oxygen Radical Absorbance Capacity (ORAC)	Spearman's rank correlation coefficient = -0.587 p = 0.045 n = 12		
Lipid hydro-peroxides		Spearman's rank correlation coefficient = -0.697 p = 0.025 n = 10	
conjugated dienes			Spearman's rank correlation coefficient = 0.363 p-value 0.018 n = 42

5. MAGNETIC RESONANCE SPECTROSCOPY

5.1 The role of imaging in HAD

Several radiological techniques has been used to evaluate changes in HAD including magnetic resonance imaging (MRI), diffusion tensor imaging (DTI), diffusion weighted imaging (DWI), positron emission tomography (PET) and magnetic resonance spectroscopy (MRS). Various abnormalities on these radiological investigations are associated with HAD, of which the metabolites on MRS were used as surrogate markers of neuroinflammation in HIV-associated cognitive impairment in this study. Reduced levels of N-acetyl aspartate (NAA) and increased levels of myo-inositol represent neuronal loss and glial activation respectively and occur early in the disease. MRS markers relate to cognitive impairment, CD4 count, CSF and plasma viral load and severity of disease (Price, 2007) and MRS has been suggested for monitoring disease progression, with early initiation of treatment in patients at risk of cognitive impairment (Nath, 2008).

5.2 MRS Physics

Magnetic Resonance Spectroscopy utilizes the nuclear magnetic resonance of individual molecules to obtain a magnetic spectrum. Proton spectroscopy (^{1}H-MRS) relies on the spinning properties of protons to produce this magnetic resonance spectrum. When placed in a strong magnetic field, atoms with spin align themselves in relation to the magnetic field which is calculated in units of tesla. Magnetic resonance spectroscopy employs spinning of atoms and the magnetic properties related to it to achieve the spectrum of MRS. Molecules of different chemical groups acquire different magnetic field strengths and behave

differently in an applied magnetic field and these differences distinguish them on spectroscopy (Cady, 1990).

5.3 Magnetic resonances

The concentration of the metabolite is calculated by measuring the area under the metabolite peak of the resonances. Most of the relevant proton resonances are represented in a small bandwidth of 8 parts per million (ppm). One of the challenges faced in spectroscopy of the brain is the strong resonance due to tissue water. Water suppression pulses are used to diminish the resonance of the water molecules to permit interpretation of the various metabolite resonances of interest. Tissues with high magnetic susceptibility, including bone and sinuses, may produce artifact if the voxel is chosen too close to the structure. The artifact may broaden the spectrum to such an extent that the spectrum of the metabolites in question becomes indistinguishable (Cady, 1990).

5.4 Chemical Shift Imaging (CSI)

Spectroscopy is performed on either a single voxel, single voxel spectroscopy (SVS), or by involving multiple voxels, known as chemical shift imaging (CSI). In single voxel spectroscopy a single voxel, the size ranging from one to eight cubic centimeters, is used to evaluate a small area with high signal to noise ratio but partial volume effects may decrease specificity for focal metabolite abnormalities (Roc, 2007). Chemical shift imaging is more time consuming and produces a metabolite map of a larger area, which is subdivided in voxels of one cubic centimeter or larger, permitting separate analyses.

5.5 MRS of the brain

The metabolites of note in spectroscopy of the brain include N-acetyl aspartate, myo-inositol, creatine, choline, lactate, lipids, and glutamine and glutamate. The resonances of different metabolites peak at different times in the spectrum of MRS. Depending on the metabolite in question, relevant parameters including repetition time and echo time needs to be selected. An echo time of 20-35 ms is applicable in metabolites whose relaxation time is relatively short, including myo-inositol, glutamine and glutamate. Metabolites with long relaxation times, such as N-acetyl aspartate (NAA), creatine (Cr), choline (Cho) and lactate and lipids, require long echo times in the order of 135-270 ms.

5.6 MRS in HIV

HAD pathology studied with MRS shows a predilection for the subcortical gray matter (Chang, 1999). Structural abnormalities, with a decrease in volume, functional impairment of basal ganglia, and a decrease in metabolites, especially the lentiform nucleus (Meyerhoff, 1999; Roc, 2007), support involvement of the subcortical structures in HIV-associated cognitive impairment (Paul, 2007).

Previously several studies reported improvement of metabolites on HAART including reversal of N-acetyl aspartate abnormalities (Roc, 2007). Correction of N-acetyl aspartate levels in patients receiving HAART implies a reversible pathological process underlying cognitive impairment and not necessarily irreversible damage due to apoptosis (Salvan, 1997). MRS detects abnormalities earlier than conventional magnetic resonance studies in the asymptomatic group of HIV infected patients. Early detection of a reversible cause in a patient with HAD with prompt initiation of HAART raises the possibility of reversal of the

underlying pathology, rearborization of dendrites, and an improvement of cognitive impairment (Meyerhoff, 1993).

In future, metabolites levels on MRS may be used in conjunction with other clinical measures to classify severity of cognitive impairment in HIV. MRS could play an important role in the evaluation and early detection of changes in HIV infected patients (Salvan, 1997).

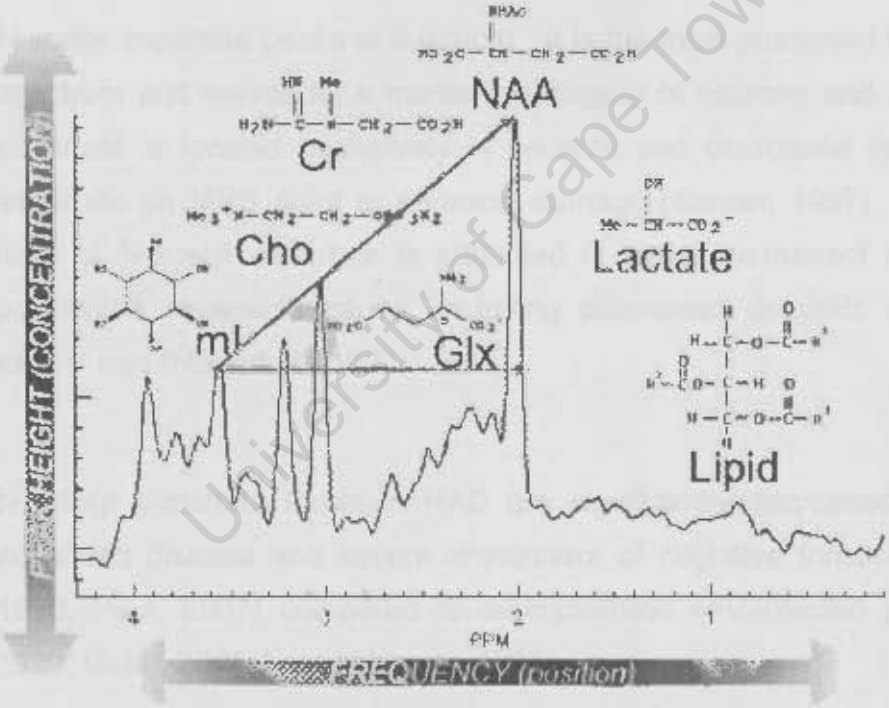


FIGURE 6: Metabolite resonances on MRS

This figure demonstrates the standard peaks observed in the magnetic resonance spectra and the frequencies at which they occur. Please refer to the text (section 5.7) for detailed information regarding the relevant peaks.

5.7 Metabolites on MRS in HAD

5.7.1 N-Acetyl Aspartate

Altered metabolites on MRS in HIV-associated cognitive impairment were first reported by Meyerhoff *et al.* (Meyerhoff, 1993). N-acetyl aspartate levels in HIV-infected patients with cognitive impairment were significantly reduced compared to HIV-negative controls. The decrease in N-acetyl aspartate levels reflects neuronal damage and correlates with disease severity.

N-acetyl aspartate peaks at 2,02ppm. It is the most prominent resonance in the spectrum and serves as a marker of integrity of neurons and axons. N-acetyl aspartate is located exclusively in neurons and decreased levels of N-acetyl aspartate on MRS point to neuronal damage (Salvan, 1997). The decreased level of N-acetyl aspartate is attributed to either permanent neuronal loss or possibly a reversible cause, including decreased dendritic arborization and axonal loss (Meyerhoff, 1993).

N-actetyl aspartate levels in HAD are significantly decreased in groups with advanced disease and severe impairment of cognitive functioning (Meyerhoff, 1999; Paul, 2007) compared to asymptomatic HIV-infected patients (Chang, 1999; Gujar, 2005; Laubenberger, 1996).

High levels of N-acetyl aspartate correlated with better scoring on neuropsychological tests. Decreased N-acetyl aspartate levels from the sub-cortical areas, especially the lentiform nucleus and thalamus, correlated with impaired Z-scores on the Grooved Pegboard Test for fine motor and speed ability, as well as immediate and delayed memory and abstraction testing

(Meyerhoff, 1999). Functional impairment correlated with testing of specific domains related to basal ganglia function (Paul, 2007).

Confounding factors include age and CD4 count. N-acetyl aspartate levels are significantly affected by HIV independent of age (Chang, 2004), while CD4+ count did not correlate with N –acetyl aspartate levels in earlier studies (Salvan, 1997).

5.7.2 Myo-inositol

Myo-inositol levels are increased in HIV-associated dementia. Myo-inositol is a marker of glial proliferation and levels increase during astrocyte proliferation. The myoinositol peak is seen at 3,56ppm in studies using a short echo time.

Myo-inositol levels show significant changes in HIV infected individuals with cognitive impairment (Chang, 2004). Simultaneous raises in myo-inositol, choline and creatine on MRS are indicative of glial proliferation as shared underlying pathology in HIV dementia (Chang, 1999). Increased levels of myo-inositol in the basal ganglia as measured by MRS are associated with cognitive impairment in HIV infected patients (Paul, 2007; Gujar, 2005). Elevated myo-inositol levels in the basal ganglia of patients with cognitive impairment correlate with decreased scoring on neuropsycological testing (Paul, 2007). A study by Chang *et al.* showed a significant increase in myo-inositol levels in a dose dependent manner according to the degree of cognitive impairment.

The myo-inositol levels in HIV-infected subjects with cognitive impairment were higher compared to HIV-infected subjects who were asymptomatic or the control group (Chang, 1999; Chang, 2004).

Myo-inositol levels are a more sensitive marker than choline levels in patients with moderate cognitive impairment and are elevated earlier than N-acetyl aspartate levels in HAND (Paul, 2007). Myo-inositol also serves as surrogate marker of an impaired blood brain barrier because of an increase in glial activation associated with indirect damage due to inflammation (Avison, 2004).

Changes in myo-inositol levels correlate with a decrease in CD4+ count to below 100 (Salvan, 1997). Myo-inositol levels are significantly affected by HIV irrespective of the changes in myo-inositol levels due to age (Chang, 2004).

5.7.3 Choline

Choline peaks at 3,2ppm. The peak represents all choline containing compounds including phosphocholine, glycerophosphocholine and phosphatidylcholine. They form part of cell membranes and increased choline levels are associated with increased membrane synthesis, an increased number of cells, increased membrane breakdown secondary to demyelination or tumour activity.

Increased choline levels are present in most patients with HIV, including asymptomatic individuals (Gujar, 2005). Choline levels are higher in all groups of HIV infected patients with cognitive impairment compared to HIV-infected patients without cognitive impairment. High levels of choline are secondary to increased cell membrane turnover associated with apoptosis and glial proliferation (Meyerhoff, 1996; Meyerhoff, 1999). Increases in choline levels correlate with the degree of severity of cognitive impairment in HIV infected subjects (Chang, 1999). On MRS an increase in choline levels in the basal

ganglia was associated with cognitive impairment in HIV-infected patients (Paul, 2007; Chang, 2004; Meyerhoff, 1999).

CD4+ count related to choline levels in a concentration-dependent manner (Salvan, 1997). Choline levels in the basal ganglia are significantly affected by HIV independent of age (Chang, 2004). Reversal of choline abnormalities on HAART supports a reversible pathological process underlying cognitive impairment, thus advocating early detection of cognitive impairment in order to initiate HAART (Roc, 2007).

5.7.4 Creatine

In this study creatine was used as reference value to calculate the N-acetyl aspartate and myo-inositol ratios. The creatine peak represents phosphocreatine and occurs at 3,0ppm. It is a marker of brain metabolism and due to the stability of creatine it is used as a reference value to calculate metabolite levels. Creatine levels on MRS do not differ among HIV-infected subjects, HIV-negative controls (Meyerhoff, 1993) or HIV-infected subjects grouped according to degree of cognitive impairment (Meyerhoff, 1999; Chang, 1999). The stability of total creatine renders it the ideal standard reference (Laubenberger, 1996; Chang, 1999; Chang, 2004).

5.7.5 Lactate and Lipid

Other metabolites which have significance in MRS studies of HIV related cognitive impairment are lactate and lipid.

Lactate forms a doublet peak at 1,33ppm. At low echo times (TE) of 20 to 35ms and very high echo times of 270 to 288ms lactate forms a peak above the baseline. At intermediate echo times (TE) of 135-144ms the lactate peak inverts to form a peak below the baseline which distinguish lactate from lipids and other macromolecules in the same location. Lactate is at or below detection levels in the normal brain. The lactate peak represents hypoxia, ischaemia, tumours or mitochondrial disorders and increases during anaerobic glycolysis and with impaired oxidative phosphorylation.

Elevated lactate levels are a marker of anaerobic oxidation and inflammation in subjects with severe cognitive impairment. In the final irreversible stages of dementia leakage of lactate hydrogenase during apoptosis may contribute to the elevation of lactate (Roc, 2007). Lipid and lactate show promise as sensitive markers of cognitive impairment in patients on treatment, because they remain unaltered regardless of HAART (Roc, 2007).

The lipid resonance is found at 0,9ppm to 1.5ppm. Elevated lipid levels are secondary to an increase in membrane turnover. Oxidative stress, with associated lipid peroxidation, and necrosis of brain tissue, seen in tumours, cause an elevation of the lipid peak. Subcutaneous fat is a possible source of contamination of the resonance.

Increased lipid levels were found in HIV infected subjects with no or mild cognitive impairment and are attributed to increased lipid peroxidation associated with membrane turnover in oxidative stress. Elevated lipid and lactate levels in all subgroups of patients suggest the presence of oxidative stress early in the disease process which persists as the disease progresses. Continued lipid elevation after initiation of HAART may point to an irreversible component of lipid peroxidation related to HIV reservoirs resistant to HAART. The persistence of

oxidative stress and inflammation in the presence of HAART, implies impaired penetrance of HAART with continued viral replication and progression of neurological disease despite therapy (Roc, 2007).

5.7.6 Glutamate and glutamine

Glutamate and glutamine produce multiple resonances between 2,2ppm and 2,4ppm when using short echo times. Glutamate increases in conditions causing hyperammonemia, for example in hepatic encephalopathy, but no significant changes in glutamate or glutamine are found in HIV-associated cognitive impairment (Chang, 1999).

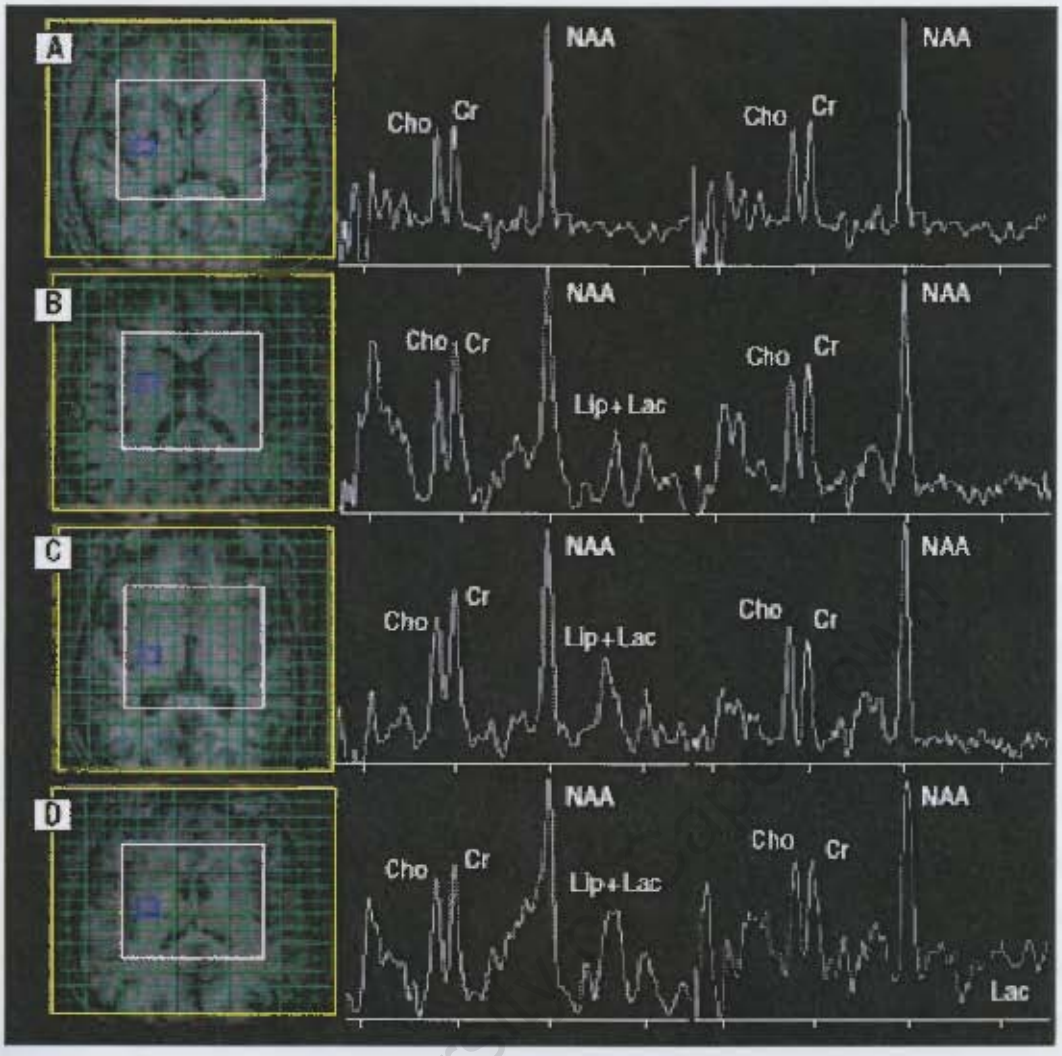


FIGURE 7: MRS in HAD (Roc AC et al. Arch Neurol. 2007; 64(9): 1249-1257)

The study by Roc *et al.* compared MRS of the lentiform nuclei in participants with various degrees of cognitive impairment.

Spectrum A: MRS performed on a HIV-negative control. The NAA peak represents viable neurons. Note the absence of a lipid and lactate peak.

Spectrum B: A HIV-infected participant with mild cognitive impairment. There is a lipid and lactate (Lip+Lac) peak visible, indicating the presence of anaerobic respiration.

Spectrum C: A HIV-infected participant with moderate cognitive impairment. The lipid and lactate and choline (Cho) peaks represent cell damage associated with HAD.

Spectrum D: MRS in a HIV-infected participant with severe cognitive impairment. The lipid and lactate and choline peaks are visible, representative of neuronal damage.

5.8 Hypotheses: Magnetic Resonance Spectroscopy

In the light of the existing data on MRS in HAD presented, we employed MRS to measure metabolites in the brain as markers of inflammation in the brain in HAD. Our hypothesis was that markers of inflammation on MRS in the brain will relate to systemic markers of inflammation and oxidative stress in HIV-infected patients with cognitive impairment. We also looked for a relationship between brain inflammation on MRS and the APOE E4 allele.

5.9 Method: Magnetic Resonance Spectroscopy

Magnetic Resonance Spectroscopy was performed on a Siemens 3 Tesla Magnetom Allegra Scanner. Two CSI MRS sequences were performed; one with an echo time (TE) of 30 milliseconds and another with an echo time of 135 milliseconds. The short echo time measured metabolites with a short relaxation time, including myo-inositol, and the long echo time measured metabolites with long relaxation times such as N-acetyl aspartate. The repetition time was 2000 milliseconds. The voxel size in the grid was 1.3cm X 1,3cm X 2.00cm with a matrix size of 16 voxels X 16 voxels. MRS slices included deep gray matter of the lentiform nucleus. Voxels were chosen to avoid close proximity to the ventricles, limiting the possibility of interference by water resonance. Water suppression was achieved by pre-scanning the patients to obtain the water resonance value. Acquisition time amounted to 8 minutes.

The LC Model Version 6.2-1 computer software package was used to analyze data.

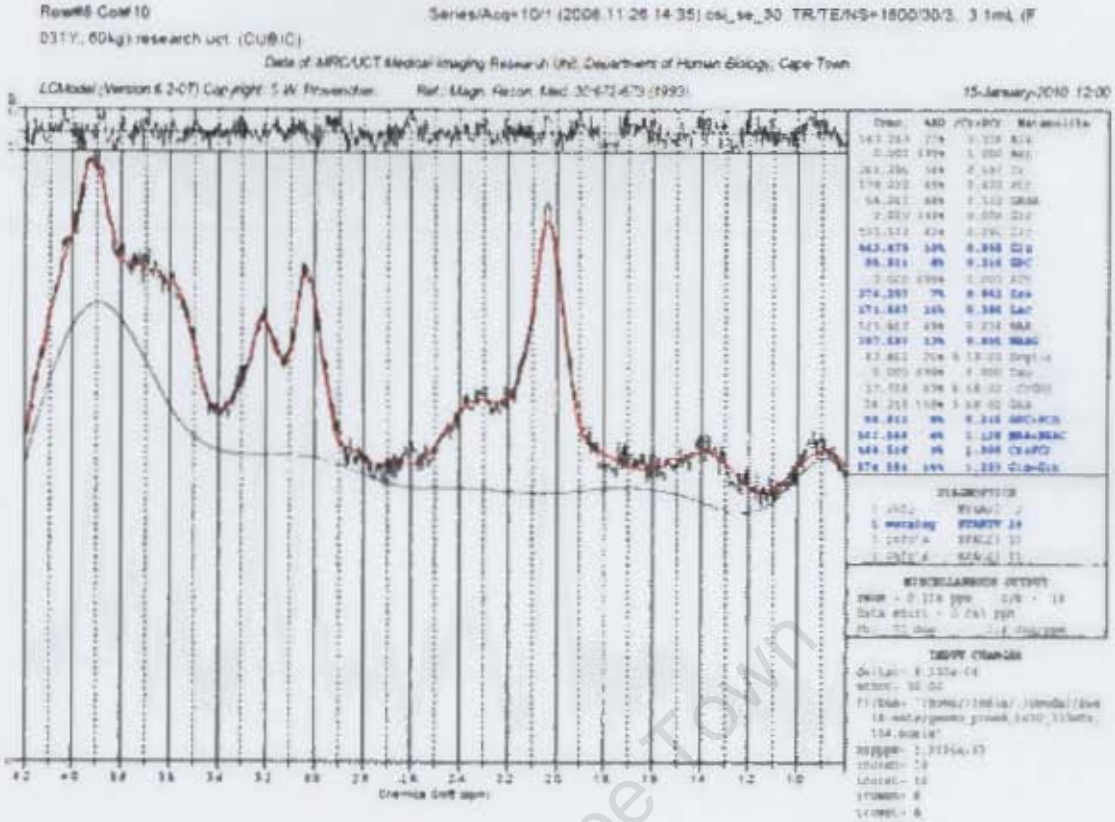


FIGURE 8: MRS Spectrum from this study: HIV-infected patient at echo time (TE) 30 milliseconds

The N-acetyl aspartate (2,02ppm), myo-inositol (3,56ppm), choline (3,2ppm), creatine (3,0ppm), lipid (0,9ppm) and lactate (1,33ppm) peaks are demonstrated. Slight drift of the baseline demonstrate some of the technical difficulties encountered with MRS.

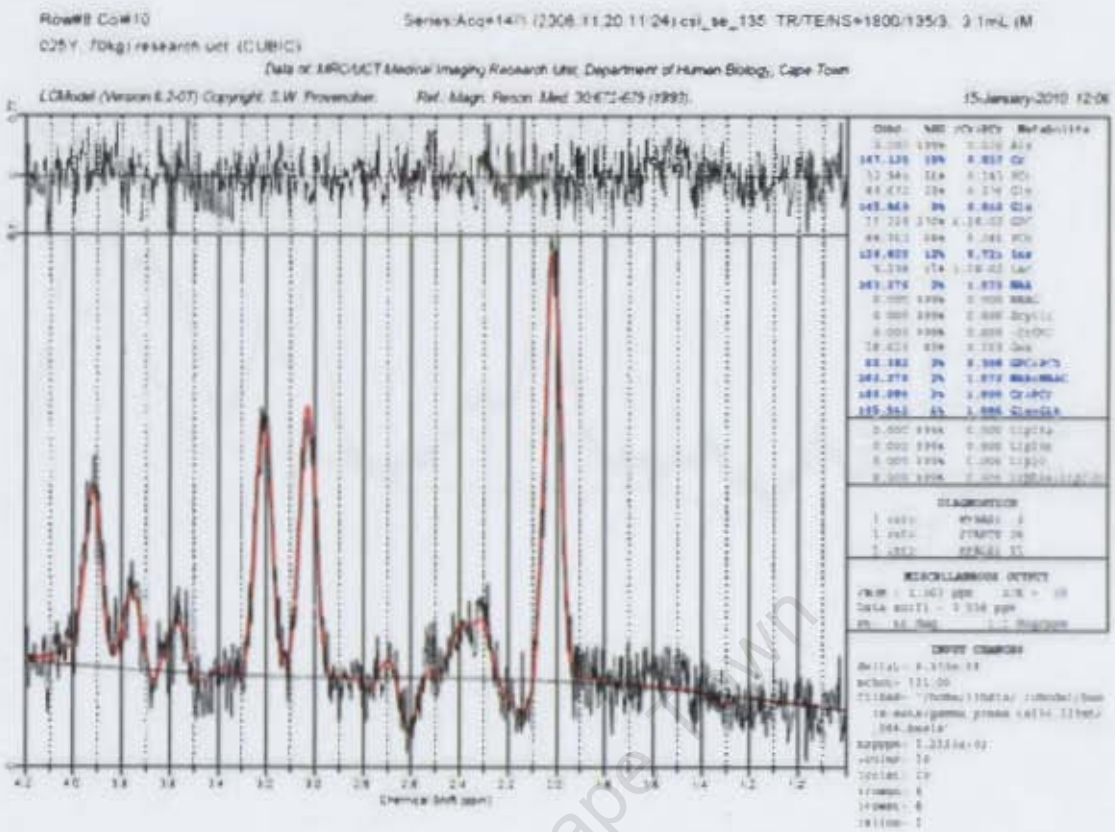


FIGURE 9: MRS Spectrum from this study: HIV-infected patient at echo time (TE) 135 milliseconds

Note the prominent N-acetyl aspartate peak (2,02ppm) evident at 135 milliseconds. The creatine peak can be seen at 3,0ppm. Choline (3,2ppm) and myo-inositol (3,56ppm) peaks are present. The latter may relate to increased inflammation in the brain.

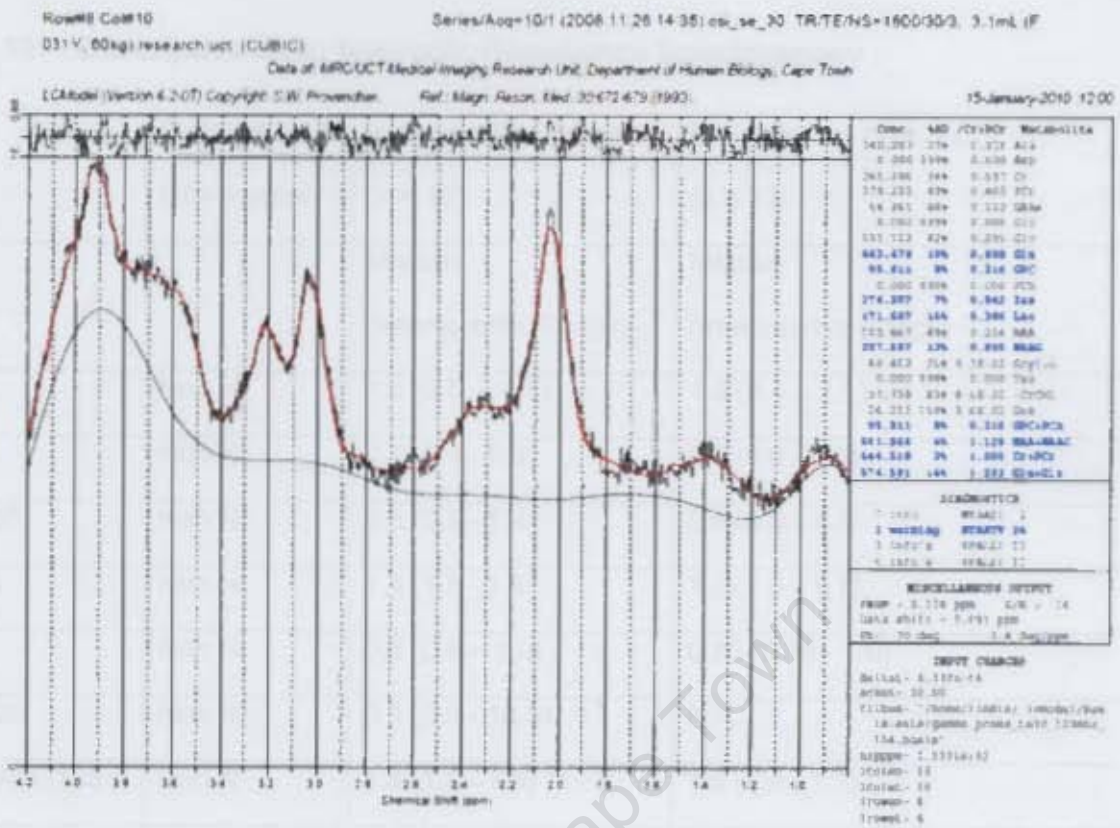


FIGURE 10: MRS Spectrum from this study: HIV-infected patient at echo time (TE) 30 milliseconds

The N-acetyl aspartate peak (2,02ppm) is present. Lactate (1,33ppm), choline (3,2ppm) and creatine (3,0ppm) peaks are evident.

TABLE 10: Descriptive data: Magnetic Resonance Spectroscopy

Variable	Distribution (N = normal)	HIV negative controls (n = 10)	HIV -infected patients (n = 33)
		Median (Interquartile Range)	Median (Interquartile Range)
NAA 30	Non N	1.1 (0.5 – 1.5)	1.2 (0.4 – 2.1)
MI 30	Non N	0.7 (0.4 - 0.9)	0.7 (0.6 - 0.9)
Lactate 30	Non N	0.6 (0.5 - 1.4)	0.5 (0.4 - 1.3)
NAA 135	Non N	1.4 (1.5 - 1.6)	1.3 (1.5 - 1.7)
MI 135	Non N	0.9 (0.8 – 1.0)	0.82 (0.75 - 1.0)
lactate 135	Non N	0.6 (0.4 - 10.5)	N/A
NAAN/NAAG135	Non N	1.4 (1.4 - 1.5)	1.5 (1.4 - 1.7)

Variable	Distribution (N = normal)	HIV-infected NP 0	HIV-infected NP 1	HIV-infected NP 2
		Median (Interquartile Range)	Median (Interquartile Range)	Median (Interquartile Range)
NAA 30	Non N	1.2 (0.9 – 1.5)	0.9 (0.1 – 1.4)	1.0 (0.3 – 1.5)
MI 30	Non N	1.5 (0.3 – 2.7)	0.9 (0.5 – 1.3)	0.8 (0.4 – 1.2)
Lactate 30	Non N	0.4 (0.4 - 1.1)	1.1 (0.6 - 1.7)	0.45 (0.4 - 0.53)
NAA 135	Non N	1.5 (1.4 - 1.6)	1.5 (1.3 -1.7)	1.4 (1.2 - 1.5)
MI 135	Non N	N/A	N/A	N/A
lactate 135	Non N	N/A	0.8 (0.3 - 13.5)	N/A

5.10 Results: Magnetic Resonance Spectroscopy

We found significant correlations between markers of systemic inflammation and markers of inflammation in the brain on MRS. Results have been discussed in the chapter on inflammation (see section 3.8). We also found significant associations between markers of inflammation in the brain on MRS and systemic oxidative stress and anti-oxidant capacity (see section 4.11). Results on inflammation in the brain on MRS which has not been presented in the chapters on systemic markers of inflammation and oxidative stress will now be discussed.

Increased levels of lactate, measured at an echo time of 135 milliseconds, correlated with an increased white cell count in the peripheral blood (Spearman's rank correlation coefficient = 0.829, p-value 0.042, n = 6), table 11. The increase in white cell count, a marker of systemic inflammation, relates systemic infection to brain inflammation, as measured by lactate, marker of anaerobic respiration. The correlation coefficient is quite strong but the result should be interpreted in the light of the small sample size and the p-value which is not strongly significant.

Inflammation in the brain measured with MRS did not differ significantly among the various HAD groups (hypothesis 1.6.9). The APOE E4 allele did not show significant correlations with inflammation in the brain as measured on MRS and HAD (hypothesis 1.6.13).

These findings prove our hypotheses that markers of inflammation in the brain on MRS correlate with systemic markers of inflammation and oxidative stress, including anti-oxidant capacity, in HIV-infected patients with cognitive

impairment. Our findings support the presence of a relationship between systemic inflammation and inflammation in the brain in HAD.

TABLE 11: Results: Magnetic Resonance Spectroscopy

	White cell count
Lactate – 135 milliseconds	Spearman's rank correlation coefficient = 0.829 p-value 0.042 n = 6

6. The Apoliprotein E Genotype

The apoliprotein E4 (APOE E4) allelic variant of the apolipoprotein E (APOE) gene is associated with an increased risk of cognitive impairment in neurodegenerative diseases and in HIV-infected patients (Deary, 2002). The APOE E4 allele has several associations with HIV. The presence of the APOE E4 allele is associated with a greater risk for cognitive impairment in patients with reduced $CD4^+$ counts and increased viral loads (Corder, 2007; Burt, 2008). Amyloid beta deposits are increased in HIV dementia and this may, in the presence of the APOE E4 allele, predispose patients to cognitive impairment. Allelic frequencies of APOE E4 are especially high in African populations, possibly placing them at risk for increased cognitive impairment (Sandholzer, 1995; Gerdes, 2003). The presence of the APOE E4 allele has been associated with acceleration of disease and faster progression to death (Burt, 2008). The association between APOE E4 and HAD may be age-dependent, with an increased risk of cognitive decline in older HIV infected patients (Pomara, 2008; Valcour, 2004).

The apoliprotein E4 (APOE E4) allele has been associated with an increased risk for neurodegenerative diseases including, Alzheimer's disease, earlier onset of Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis (Deary, 2002). The APOE E4 allele is associated with accelerated cognitive decline in the elderly and in HIV infected patients. Increased glial activity, over-expression of cytokines and augmentation of direct effects of viral proteins, lead to accelerated cognitive decline in the presence of the APOE E4 allele.

Neurodegeneration is accelerated in the presence of the APOE E4 allele due to impaired neuronal repair mechanisms in the presence of increased inflammatory mediators secondary to chronic activation of microglia by HIV. Impaired neuronal repair leads to increased cognitive decline and neuropathy and the APOE

genotype is associated with an increased risk of cognitive impairment in HIV-infected patients. HIV-infected subjects with an APOE E4 allele show an increased prevalence of mild suspected dementia (30% versus 15%). Neuropathy is also increased in frequency and severity (70% versus 39%) in HIV infected patients with an APOE E4 allele (Corder, 1998).

6.1 APOE Genetics

The apolipoprotein E (APOE) gene is situated on chromosome 19, location 19q13.2, and is formed by 3597 base pairs. The APOE gene shows polymorphism and has common three alleles APOE E2, APOE E3 and APOE E4. The polymorphisms are situated on exon 4 in positions 3937 and 4075.

The APOE gene codes for the apolipoprotein E (ApoE) protein. ApoE is a 34 000 molecular weight glycoprotein of 299 amino acids which consists of two structural domains separated by a hinge region. The N-terminal domain forms a four helix anti-parallel bundle which contains the receptor binding region. The N-terminal domain is formed by amino acids 1-191 and the receptor binding region by amino acids 134-150 and Arg-172. The C-terminal domain contains the major lipid binding region formed by amino acids 244-272 (Mahley, 2009). The amino acid sequences differ between the three isoforms due to isoform-specific inclusion of cystein and arginine in positions 112 and 158. ApoE2 has two cystein amino acids in position 112 and 158 while ApoE3 has a cystein and an arginine and APOE4 has an arginine amino acid in both positions. The differences in the primary structure of the isoforms lead to differences in secondary and tertiary structures and function (Gerdes, 2003). Arginine-112 alters the structure of apolipoprotein E4, resulting in increased binding to large triglyceride-rich very low density lipoproteins (VLDL) receptors compared to the

preferred binding of the E2 and E3 isoforms to small phospholipid-rich receptors (Mahley, 2009).

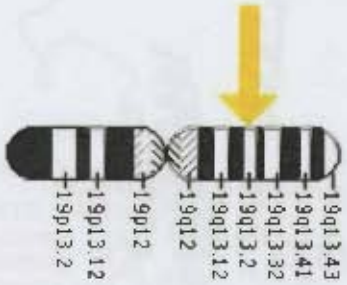


FIGURE 11: The APOE Gene

Cytogenetic Location: 19q13.2

Molecular Location on chromosome 19: base pairs 50,100,878 to 50,104,489

The APOE gene is located on the long (q) arm of chromosome 19 at position 13.2

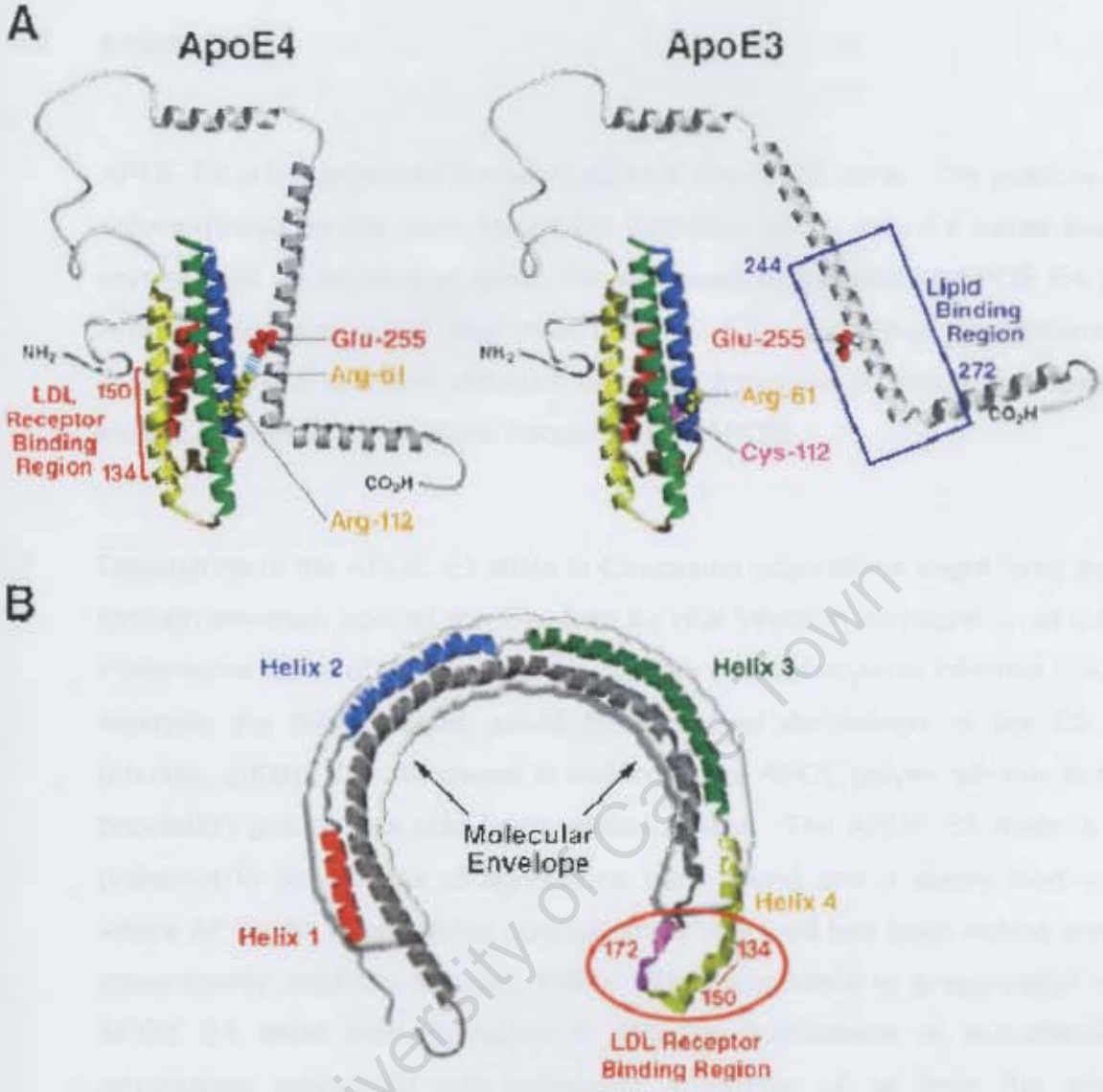


FIGURE 12: Apolipoprotein E

The Apolipoprotein E4 isoform demonstrates the ability of Arg-112 to bind to Arg-61, thus mediating the conformational change of ApoE E4.

6.2 Epidemiology

APOE E4 is the proposed ancestral allele of the APOE gene. The position of the polymorphism on the gene favors the formation of E3 from E4 rather than the reverse and social studies relate the increased prevalence of APOE E4 in the African population to the later origin of APOE E3 in the era of the modern man. A hypothesis of selective pressure driven by infectious diseases is proposed to explain the difference in allelic frequencies of APOE.

Dominance of the APOE E3 allele in Caucasian populations might have evolved through selection against the E4 allele by viral infection on migration to Europe. Preferential death of APOE E4 allele carriers in a cataclysmic infective insult, for example the great plague, could have caused dominance of the E3 allele (Mahley, 2009). The difference in distribution of APOE polymorphisms between population groups has also been related to diet. The APOE E3 allele is more prevalent in populations of agricultural background and a stable food supply, where APOE E4 is present in populations where food has been scarce and only intermittently available (Corbo, 1999). The hypothesis of preservation of the APOE E4 allele from extinction in foraging populations is associated with advantages associated with increased absorption of fat from the intestine providing effective survival during famine. The tendency of an increased frequency of APOE E4 allele in the north of Europe supports this hypothesis with persistence of the APOE E4 allele in the hunter/gatherer population of the north despite later inward migration of agricultural farmers.

The APOE E2 allele is least frequent and the E3 allele most frequent. The allelic frequencies in the majority of studies from North America and Europe are quoted as 7%, 77% and 16% for E2, E3 and E4 respectively. The genotypic frequencies

quoted are 0.5% for E2/E2, 11% for E2/E3, 2% for E2/E4, 59% for E3/E3, 25% for E3/E4 and 3% for E4/E4 (Corder E.H., 1998; Farrer, 1997).

APOE E4 frequency is relatively high in African population. In the Asian populations the E4 allele is less frequent and the homozygous E4 state is associated with a significant increase in risk of AD in the Japanese population (Farrer, 1997). The higher frequencies of APOE E4 in the foraging population include the Khoi-San (0.370), Pygmies (0.407), Australians (0.260), Aborigines of Malaysia (0.240), Papuans (0.368), Native Americans (0.280) and Lapps (0.310). Studies based on Nigerian populations also showed higher APOE E4 allelic frequencies of 0.217 and 0.310 (Sahota, 1997; Eichner, 2002; Gureje, 2006).

The homozygous APOE E4 genotype is 3-5 fold more common in the Khoi-San population compared to Caucasians (10% versus 2-3%) and a study in the Shona of Zimbabwe revealed equally high allelic frequency of E4 (22.3%) and a homozygous E4 genotype frequency of 5.9% (Tanayanyiwa). Populations with a foraging decent, like the Khoi-San, may have a genetic predisposition to these disorders when exposed to the Western lifestyle (Sandholzer, 1995; Gerdes, 2003). Atherosclerosis and Alzheimer's disease have been associated with APOE E4 allele, Western diet and longevity.

6.3 Lipid Metabolism and APOE

ApoE is produced within the brain and does not need to cross the brain barrier (Guo, 2004). In the central nervous system ApoE is secreted mainly by astrocytes, but neurons and other activated microglial cells may secrete ApoE in the presence of stressors including advanced age, amyloid beta deposition and

oxidative stress (Mahley, 2009). ApoE is involved in neuroprotection through redistribution of lipids involved in neuronal repair (Mahley, 2009).

Apolipoprotein E regulates cholesterol metabolism utilizing low density lipoprotein (LDL) receptors for transport and metabolizing lipoproteins containing cholesterol and triglycerides. Apolipoprotein E4 binds to triglyceride rich, very low density lipoproteins receptors in contrast to preferred binding of the E2 and E3 alleles to small phospholipid-rich receptors (Mahley, 2009). Binding to very low density lipoproteins receptors predisposes apolipoprotein E4 to increased clearance. The accelerated clearance of apolipoprotein E4 leads to decreased levels of apolipoprotein E4 in the serum and CSF, and an increased rate of neurodegeneration secondary to impaired protective mechanisms. Decreased levels of apolipoprotein E4 secondary to preferential degradation could lead to impaired neuronal regeneration precipitated by a decrease in cholesterol delivery to neurons. The decreased availability of cholesterol is associated with impaired protective mechanisms, including a decrease in synaptogenesis, dentrite branching and axonal repair. Neurite branching is less extensive in the presence of the APOE E4 allele compared to APOE E3 (Turchan-Cholewo, 2006) and inadequate regeneration of neurons following injury may accelerate neurodegeration in the presence of the APOE E4 allele compared to the other polymorphisms. These impaired protective mechanisms are associated with increased membrane damage and apoptosis compared to the APOE E3 allele (Riddel, 2008; Mahley, 2009).

The E4 isoform of apolipoprotein differs structurally from the other polymorphisms and is inherently more unstable due to its molten globule state.

The structural differences increase the proteolytic tendency of ApoE4 and astrocytes preferentially degrade apolipoprotein E4 causing ApoE4 to undergo

proteolytic cleavage to a much greater extent than ApoE3 (Riddel, 2008). The increased breakdown of ApoE is associated with accumulation of cytotoxic proteolytic fragments in the cytosol which cause neurotoxicity and neurodegradation (Riddel, 2008; Mahley, 2009).

6.4 APOE and inflammation

ApoE is involved in pro-inflammatory and anti-inflammatory responses in the central nervous system. ApoE mediates an anti-inflammatory response by decreasing glial cell activation, with a concomitant reduction in the production of ApoE. These anti-inflammatory changes are genotype-specific and the APOE E4 allele displays impaired anti-inflammatory modalities, with persistence of the amyloid beta-associated inflammatory stimulus. This results in decreased suppression of amyloid beta secretion with an increase in amyloid beta load (Guo, 2004).

ApoE4 demonstrates independent pro-inflammatory properties, seen as an increase in production of IL-1β. ApoE4 is associated with a significantly greater increase in IL-1β compared to Apolipoprotein E3 (Guo, 2004). A selective increase in IL-1β levels is present despite inhibition of the amyloid beta-related inflammatory response by ApoE. Apolipoprotein E4 stimulated interleukin-1beta independent of amyloid beta in an isoform dependent manner. Some studies reported an increased inflammatory response with increased cytokine production of TNF-α in individuals with an APOE E4 allele compared to subjects without the E4 allele (Colton, 2004), but later studies failed to replicate the findings (Turchan-Cholewo, 2006).

6.5 Impaired protection against oxidative stress associated with APOE

ApoE is involved in anti-oxidant homeostasis and the APOE E4 allele may be less protective against oxidative insults than the E3 allele. Increased microglial responsiveness may be responsible for early neurodegeneration in subjects with an APOE E4 allele secondary to excessive oxidation and nitration of neuronal cells.

Mitochondria are involved in plasticity, synaptogenesis and neuronal dendrite growth and ApoE E4 induced mitochondrial toxicity manifests through impaired dendrite formation and neurotoxicity (Mahley, 2009). Decreased suppression of amyloid beta by APOE E4 leads to activation of macrophages and astrocytes with increased production of nitric oxide synthetase (Guo, 2004). Reactive nitrous oxygen species (RNOS) are involved in neurodegenerative processes in the central system and the APOE E4 allele is associated with augmentation of the inducible nitric oxide synthetase (iNOS) pathway in macrophages resulting in higher levels of NO. The increased cumulative redox imbalance is cytotoxic, causes neurotoxicity and predisposes an individual with an APOE E4 allele to accelerated neurodegeneration (Colton, 2004). The allele specific anti-oxidant capacity predisposes a subject who has an E4 allele to microglial-associated inflammatory damage due to impaired anti-oxidant defenses (Valcour, 2004).

6.6 APOE and Alzheimer's Disease

The APOE E4 allele is associated with an increased risk for development of Alzheimer's disease (AD). The amyloid related mechanism involved in AD associated cognitive decline may share a pathological mechanism with cognitive decline in HIV. The risk for AD is associated with the APOE E4 allele in a dose

dependent way. An eightfold increase in risk is associated with the homozygous state of APOE E4 compared to heterozygous individuals (Corder, E.H., 1998). The homozygous state was associated with an increased risk in all races, but African-Americans had a weaker association than other races (OR 5.7 compared to an OR of 12.5 and 33.1 in Caucasians and Japanese respectively) (Farrer, 1997). The E2 allele is associated with a protective effect, but due to the relative infrequency of this allele difficulty in achieving numbers impairs acquisition of data (Corder, E.H., 1998).

6.7 APOE and amyloid deposition in Alzheimer's Disease

The pathophysiology of Alzheimer's disease relates to amyloid beta levels with associated glial activation. The decreased levels of apolipoprotein E4 lead to decreased clearance of amyloid beta with an increased burden of amyloid beta. The excess amyloid beta results in amyloid beta-associated glial activation with increased neuroinflammation (Riddel, 2008). Glial activation is modified in the presence of the APOE E4 allele and may influence disease progression in several ways. AD has been associated with increased inflammation due to excess glial activation in the presence of beta-amyloid plaques. Increased activation of glial cells in the presence of amyloid beta may lead to neurotoxicity due to excess secretion of cytokines and inflammatory mediators. Activated glial cells secrete ApoE to attenuate the inflammatory response associated with amyloid deposition. ApoE associates with amyloid beta, forms complexes and facilitate clearance of beta-amyloid via lipoprotein receptors.

Diffuse plaques found in HIV infected patients is a precursor of plaques associated with AD and cognitive decline. CSF amyloid beta 1-42 levels are raised in HIV infected patients (Valcour, 2004).

6.8 APOE and Inflammation in Alzheimer's Disease

AD is associated with increased levels of pro-inflammatory cytokines, including IL-1 and IL-6. In the absence of the APOE gene there is an increase in the levels of IL-1β, IL-6, TNF-α and other inflammatory cytokines, demonstrating the anti-inflammatory properties of apolipoproteins (LaDu, 2001). The increase in neuroinflammation in subjects with AD who have an APOE E4 allele is secondary to a relative decrease in APOE E4 levels compared to other APOE isoforms (Guo, 2004). Neurological symptoms in HIV infection share common underlying pathological mechanisms with Alzheimer's disease. An increased level of TGF-1β was present in subjects with AD compared to controls (Finch, 2007).

6.9 APOE and Infection in Alzheimer's Disease

Infectious agents have been implicated in AD in association with the APOE genotype. The APOE E4 allele may render the brain vulnerable to infection-related damage due to ineffective repair with resultant cognitive impairment. The APOE E4 allele is more prevalent in AD patients infected with Herpes simplex virus type-1 (HSV1) (Itzhaki, 2010; Honjo, 2010; Honjo, 2009; Dobson, 2003). HSV1 may play a role in Alzheimer's related cognitive decline, with HSV1 DNA present in beta-amyloid plaques.

6.10 Predisposing factors to HAD in the presence of the APOE E4 allele

Other risk factors for cognitive impairment in the presence of the APOE genotype have been identified, including age, gender and stress levels. A significant increased risk of cognitive impairment is present in subjects with an APOE E4 allele with an increase in age compared to subjects without an APOE E4 allele.

Studies found the risk of cognitive decline to be increased between the ages of 40 and 60 years with a subsequent decrease.

The association between APOE E4 allele and HIV-associated dementia (HAD) might be age-dependent (Pomara, 2008). Age has a modulatory effect on HIV-associated cognitive impairment in the presence of the APOE E4 allele (Valcour, 2004; Burt, 2008). The Hawaii Aging with HIV Cohort found a significantly increased incidence (40%) of HAD in an older age group above 50 years in the presence of one or more APOE E4 alleles compared to 17% in subjects without an APOE E4 allele (Valcour, 2004).

Female sex is significantly associated with an increased risk for AD and may be a dominant risk factor (Farrer, 1997). Individuals with an E4 allele who are exposed to stress have an increased susceptibility to cognitive decline compared to individuals without an E4 allele (Peavy, 2007).

Impaired immune function secondary to decreased ApoE4 levels may predispose an individual to impaired neuronal repair and increased neurodegeneration. Neuronal repair is dependent on normal immune function (Colton, 2004). Antigen-presenting cells secrete apolipoproteins to bind serum-borne lipid antigens. Bound antigens are delivered to the endosomal compartments of antigen presenting cells by apolipoproteins. Apolipoproteins mediate T-cell activation by the presentation of the antigens to the antigen presenting cells.

6.11 APOE in HIV

Apolipoprotein E is involved in cholesterol metabolism. In the brain astrocytes and microglia are primarily involved in the secretion of cholesterol. Cholesterol forms part of the HIV envelope and variance in lipid metabolism between APOE isoforms may alter cholesterol metabolism and influence the HIV disease progression (Burt, 2008; Mahley, 2009).

The homozygous state of the APOE E4 polymorphism showed a significant acceleration of HIV disease progression due to an increased rate of HIV infection. Apolipoproteins act as viral fusion inhibitors due to their homology to the fusion domains of viral fusion proteins and interact with HIV 41 through amphipatic helices, with inhibition of viral fusion. APOE E4 inhibits fusion less efficiently than the E3 allele, predisposing cells to a higher frequency of HIV-target cell fusion and an increased rate of viral entry. An accelerated mortality associated with the homozygous state of APOE E4 allele is attributed to increased entry of HIV in the presence of the APOE 4 allele (Burt, 2008). The APOE E4 allele is possibly associated with a shortened survival in subjects with the APOE E4 allele (Valcour, 2008). The altered fusion properties of APOE E4 additionally predispose subjects to viral infection including HSV and susceptibility to opportunistic infections (Mahley, 2009) which may relate to the increased susceptibility to viral infections seen in individuals with AD. The presence of the APOE E4 allele is not associated with an increased risk of acquiring HIV infection (Burt, 2008).

6.11.1 Oxidative stress and APOE in HAD

Products of lipid metabolism are increased in patients with HAD secondary to oxidative stress and the APOE E4 allele is associated with increased levels of 4-HNE in neuronal cells, indicating a relative increase in oxidative stress and neuronal loss (Turchan-Cholewo, 2006). Polyunsaturated fatty acids are broken down by free radicals leading to the production of aldehydes including 4-HNE. 4-HNE may impair mitochondrial function leading to further oxidative stress (Turchan-Cholewo, 2006).

6.11.2 APOE and HIV proteins

Neurotoxicity associated with HIV proteins is amplified by the presence of the APOE E4 allele. Amplified neurodegeneration in the presence of the APOE E4 allele may be related to increased neurotoxicity caused by higher levels of oxidative stress mediated by the HIV tat protein (Turchan-Cholewo, 2006). The presence of HIV proteins, gp120 and tat, is associated with increased levels of oxidative stress and neurotoxicity in subjects with an APOE E4 allele compared to subjects without an APOE E4 allele. It presents in a dose dependent manner with maximal toxicity in homozygous individuals. Tat protein increases oxidative stress and impairs mitochondrial function.

6.11.3 APOE and CD4 count

A low CD4+ count is an independent risk factor for dementia. $CD4^+$ count correlates with HAD. A $CD4^+$ count of less than 100 is associated with disease progression. The lower the $CD4^+$ count, the higher the risk of developing HAD,

the nadir of the $CD4^+$ count being the most valuable predictor (Brew, 2008; Nath, 2008).

The rate of cognitive impairment is dependent on the APOE genotype and the CD4+ count in HAD. Patients with the APOE E4 allele has a faster rate of decline with decreased CD4+ counts compared to groups without the APOE E4 allele. The APOE E2 allele shows the slowest decline in CD4+ count in a dose dependent manner, with stable CD 4 counts and delayed cognitive decline in the homozygous APOE E2 genotype (Corder, 2007).

6.11.4 APOE and Viral load

A high viral load is associated with the APOE E4 allele in a dose dependent manner (Valcour, 2004).

6.12 The potential benefit of APOE genotyping

APOE genotyping may provide benefit in management of HIV infected individuals in future. Differences in host-viral interaction with accelerated progression of disease depending on the APOE genotype, suggest early genotyping and initiation of HAART to alter disease progression. Development of APOE E4 targeted therapeutic agents should be considered in HIV related pathology due to shared pathological disease processes (Burt, 2008).

6.13 Hypotheses: APOE E4 Allele

Literature reviewed in the previous sections suggests a role of the APOE E4 allele in the pathogenesis of HIV-associated cognitive impairment. We hypothesized that the APOE E4 allele relates to markers of systemic inflammation, systemic markers of oxidative stress and inflammation in the brain measured by MRS.

6.14 Method: APOE genotyping

*prep*GEM™ was used to prepare single DNA strands for DNA extraction. *prep*GEM™ lyse cells and strips DNA from nucleoproteins. The DNA was then used to determine the APOE genotype.

The method was performed in a DNA free environment to prevent contamination. Blood samples were collected from patients at their physical examination following informed consent. Blood samples were frozen within 1 hour of collection. Samples of whole blood, stored in citrated tubes in a minus 70 degrees Celsius freezer, were used for DNA extraction.

We used the method describe by Hixson (Hixson, 1990). ApoE restriction enzyme isoform genotyping (restriction genotyping) were used to amplify apolipoprotein E gene sequences containing the amino acid position 112 and 158. The amplification products were digested and subjected to electrophoresis. Subsequent typing of homozygotic and heterozygotic combinations of the individual apolipoprotein E alleles was performed. This enabled us to calculate the frequency of the homozygous and heterozygous state of the apolipoprotein E4 allele.

6.15 Results: APOE Genotype

6.15.1 Allelic frequencies of HIV-infected Participants

The allelic frequencies and genotype frequencies are tabulated in table 12.

TABLE 12: APOE frequencies (n = 35)

Allele	Allelic Frequency
E2	8.1%
E3	58,1%
E4	33, 7%

Genotype	Genotypic Frequency
E2/E2	0%
E2/E3	11.6%
E2/E4	4.7%
E3/E3	32.6%
E3/E4	39.5%
E4/E4	11.6%

6.15.2 Allelic frequencies of the Newborn Controls

300 stored cord blood samples, collected from black newborn babies, were genotyped to establish the population frequency of APOE. The allelic frequencies and frequencies of the various genotypes are tabulated in table 13.

TABLE 13: APOE Population frequencies (n = 300)

Allele	Allelic Frequency
E2	20%
E3	50%
E4	30%

Genotype	Genotypic Frequency
E2/E2	6%
E2/E3	17%
E2/E4	11%
E3/E3	26%
E3/E4	30%
E4/E4	9,6%

6.15.3 APOE Allelic frequencies compared

The genotyping of our study group and the 300 standard samples is in keeping with reported data with the dominant allelic frequency of E3 with 58.1% and 50% respectively and the dominant genotype of E3/E4 (39.5% and 30% respectively). The frequency of the E4 allele is much higher than the Caucasian, and even non-Caucasian standards, in literature with an E4 allelic frequency of 33.7% in the study group and 30% in the standard samples, compared to 16% reported in literature. The results on the homozygous E4 genotype (11.6% in the study population and 9.6% in the standard samples) are in keeping with the findings on the Khoi-San (10%) (Sandholzer, 1995) and the Shona (14, 8 %) (Tanayanyiwa), but much higher than the reported prevalence in the Western population (3%) (Corder, E.H., 1998; Farrer, L.A., 1997).

TABLE 14: The APOE E4 allelic frequency grouped according to cognitive impairment

Cognitive Function	E4 Allelic Frequency	Percentage
NP = 0 (n = 9)	9	26.5
NP = 1 (n = 13)	13	38.2
NP = 2 (n = 12)	12	35.3
Total	34	100

The allelic frequency across the different groups of cognitive impairment was not significant (hypothesis 1.6.10). HIV-infected participants without cognitive impairment, HIV-infected participants with mild cognitive impairment and HIV-infected participants with dementia showed similar APOE E4 allelic frequencies. The APOE genotype of HIV-infected participants without cognitive impairment compared to HIV-infected participants with severe cognitive impairment was also not significant (Chi-squared = 0.43, p = 0.56)

Further, the APOE genotype did not correlate with markers of systemic inflammation, markers of oxidative stress or markers of inflammation in the brain on MRS of patients with HAD (hypotheses 1.6.11, 1.6.12 and 1.6.13).

6.15.4 Results: The APOE E2 allele

We did not find a significant difference between the frequency of the APOE E4 allele in the HIV-infected population and the newborn population. However, the allelic frequency of the APOE E2 allele in the HIV-infected population was significantly lower compared to the APOE E2 allelic frequency in the newborn population (n = 33, Chi-squared, 2.5, p = 0.001). Protective mechanisms were previously associated with the APOE E2 allele in HIV (Corder, E., 2007; Farrer, 1997). This decrease in the allelic frequency of APOE E2 in the HIV-infected population in our study could support the protective role of the APOE E2 allele in

HIV infection. The decrease in the allelic frequency of the APOE E2 allele in the HIV-infected population has subsequently been replicated in a follow-up study with greater numbers performed by dr Joska on this study population (Joska, in press).

Although the APOE E4 allele did not prove significant in our study as hypothesized, the APOE genotype may play a role in the pathogenesis of HIV-associated cognitive impairment through protection by the APOE E2 allele.

7. DISCUSSION

Neuroinflammation is involved in the pathogenesis of HIV-associated dementia through indirect neuronal injury. Several previous studies found associations between systemic inflammation and markers of inflammation in the brain (Honjo, 2009). These studies originated predominantly from North America and Europe, leaving Africa, with its significant burden of HIV, unexplored. This study is, to our knowledge, the first in Africa to investigate the relation of systemic inflammation to brain inflammation in HAD with clinical, biochemical and neuroimaging techniques.

We found a significant correlation between systemic inflammatory markers and the degree of cognitive impairment in HAD in a dose dependent way. Participants with severe cognitive impairment had higher levels of IL-1 beta compared to participants with mild cognitive impairment and participants without cognitive impairment. The increase in levels of systemic markers of inflammation, which rise in conjunction with impaired cognitive function in patients with HAD, suggest a causal relationship between systemic infection and cognitive impairment in HAD, but will need to be validated in a longitudinal study.

Inflammatory markers in the peripheral blood correlated significantly with markers of inflammation on MRS. We found significant correlations between systemic oxidative stress and cognitive impairment associated with inflammation in the brain in HAD. The direct association between systemic markers of oxidative stress and brain inflammation, and the inverse relationship between anti-oxidant capacity and inflammation in the brain, support the involvement of systemic oxidative stress in the neuropathogenesis of HAD. These findings support our hypothesis on the association between systemic inflammation oxidative stress and inflammatory changes in the brain related to HAD.

Systemic inflammation and inflammation in the brain may interrelate, as suggested by the findings of our study. Systemic inflammation may induce inflammation in the brain in a complex bi-directional relationship, resulting in indirect injury and neurodegeneration due to the associated inflammatory response (Combrinck, 2002; Cunningham, 2005). Development of central inflammation secondary to systemic inflammation may predispose HIV-positive patients in particular to accelerated cognitive decline due to the increased prevalence of opportunistic infections in the immune-compromised HIV-infected host.

The influence of the association between inflammatory markers and cognitive decline in HAD will need to be assessed in the long term. The significance of the association between the markers of inflammation and the pathogenesis of HIV-associated cognitive impairment found in this study, and whether it predicts prognosis in HAD, remains to be confirmed. The relationship between systemic infection, systemic inflammation and cognitive impairment due to brain inflammation may prove to be an important underlying pathogenesis in HAD.

We are in need of surrogate markers to assess disease severity, progression of disease, response to management in HAD. Monitoring of the inflammatory process with systemic markers, similar to the markers in our study, will allow advancement in treatment options and prevention of cognitive impairment in HAD. Therapeutic options, including HAART, might address the underlying inflammatory cause, with modification of the disease process and reversal or even prevention of cognitive impairment.

The advent of HAART attenuated neurological complications of HIV, but the burden of HIV associated neurocognitive impairment persists despite therapy. HAART has the possibility to reverse cognitive impairment in HAD, making early diagnosis vital. An unanswered question is whether cognitive impairment

associated with increased peripheral cytokines is reversible or not. Systemic inflammation may lead to temporary neuronal dysfunction which could be reversed if systemic inflammation and immune activation is reduced. As many of the participants of this study would have subsequently gone on to HAART, re-evaluation of systemic inflammation, and the associated inflammatory changes in the brain in these participants while on HAART, will be of interest. The association between systemic inflammation and cognitive impairment in HAD may be reversible on HAART.

The frequency of the APOE E4 allele in our study resembled the high frequency of the APOE E4 allele found in other studies performed in Africa (Sandholzer, 1995). The frequency of the APOE E4 allele is much higher than numbers stated in Western populations (Corder, E.H., 1998; Farrer, L.A., 1997). The high prevalence of the APOE E4 allele may predispose HIV-infected African individuals to a disproportionate risk of cognitive impairment. Our study did not find an association between the APOE E4 allele and either markers of systemic inflammation, systemic oxidative stress and inflammation in the brain or cognitive impairment in HIV-positive participants. This might be due to a lack of sufficient numbers. The APOE E2 allele was significantly less frequent in the HIV-positive group compared to the newborn controls. This may imply a protective role of the E2 allele against cognitive impairment in HAD. Research conducted on larger numbers of the same population has subsequently supported this finding (Joska, 2010). Longitudinal studies need to clarify the relationship between the APOE E2 allele and HAD.

Limitations of the study include a small number of participants and the cross-sectional nature of the study. I initiated this as a pilot study, which has subsequently developed in a longitudinal study. Some of our results have already been validated in this longitudinal study with greater numbers (Joska, in press).

Due to the cross-sectional nature of the study, we could only investigate associations between inflammation and HAD. Longitudinal studies will need to assess the causal relationship between systemic inflammation and brain inflammation in HAD.

Although we attempted to exclude active systemic and opportunistic central nervous system infections on screening, we could not exclude all possible infections. We did not have permission to perform lumbar punctures to exclude the presence of an infectious cause of central nervous system inflammation. Systemic inflammatory markers correlated with the systemic oxidative stress markers. This supports the presence of systemic inflammation. Seeing that we relate systemic inflammation to inflammation in the brain, any systemic infection able to mount a systemic inflammatory response, regardless of etiology, may be involved in inflammation in the brain and neurodegeneration. This might rectify the unknown presence of opportunistic infections, because they add to systemic inflammation nonetheless, and we are measuring systemic inflammation as common pathogenesis, independent of cause.

In conclusion, we found significant relationships among markers of systemic inflammation, systemic oxidative stress and inflammation in the brain on MRS in patients with HAD. The complexity of the respective relationships among systemic inflammation, oxidative stress and neuroinflammation, as determined in our study, is depicted in figure 12; a summary of our findings on inflammation in HAD.

This study is, to our knowledge, the first in Africa to investigate the role of neuroinflammation in HAD with the use of systemic markers of inflammation and neuroimaging in relation to the APOE genotype. In this study systemic inflammation and oxidative stress showed significant associations with inflammation as underlying pathogenesis of cognitive impairment in HAD.

I initiated this study to investigate the role of inflammation in the neuropathogenesis of HAD through evaluation of systemic markers of inflammation and oxidative stress, markers of inflammation in the brain on MRS and their correlation with cognitive impairment. This study served as a pilot study for what has since developed into a longitudinal study conducted by the Department of Psychiatry (Dr John Joska), UCT, in collaboration with Assoc Prof Marc Combrinck of the Neurology Unit at Groote Schuur Hospital.

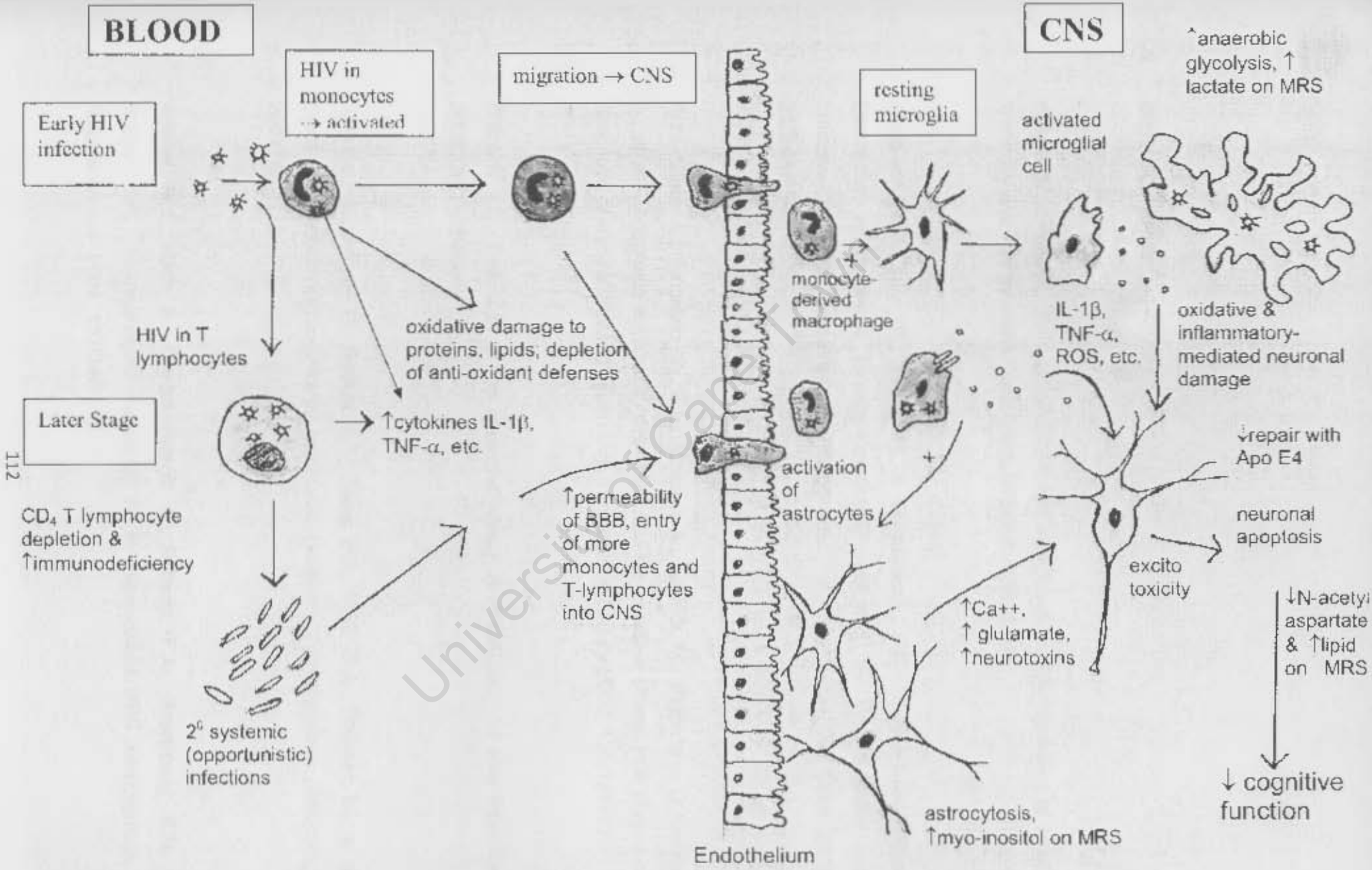


FIGURE 13: The relationship between inflammation and HAD: Our findings in summary

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