The Relationship between Alzheimer's Disease, Inflammation, the APOE Genotype and Neuronal Integrity

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DECLARATION

This work has not been previously submitted in whole, or in part, for the award of any degree. It is my own work. Each significant contribution to, and quotation in, this dissertation from the work, or works, of other people has been attributed, and has been cited and referenced.

Signature:_____ Date:_____

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder associated with progressive neuronal loss. Microglial cell activation in the central nervous system (CNS) may contribute to this process through the release of neurotoxic inflammatory mediators. Systemic inflammation, through signaling to the CNS, can further activate microglia and thus accelerate neurodegeneration. The apolipoprotein (APOE) ε4 allele has been associated with both an increased risk of developing AD and higher levels of inflammation. The aim of this study was to investigate the relationships between systemic inflammation, CNS inflammation, the APOE genotype, neuronal integrity and cognitive functioning in a cohort of elderly participants from the Western Cape region of South Africa. South Africa is a developing country where systemic infections are common and the ε4 allelic frequency is thought to be high. Sixty-eight cognitively healthy controls and 60 AD participants were recruited. Participants underwent a full clinical and cognitive assessment. APOE genotyping was performed and the following systemic inflammatory markers were measured: erythrocyte sedimentation rate (ESR), white cell count, monocyte count, tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), transforming growth factor- beta (TGF-β), IL-10 and osteopontin. Forty-eight control and 32 AD participants were re-assessed after one-year. Thirty-three participants underwent proton magnetic resonance spectroscopy for the detection of myo-inositol (MI), a marker of glial activation, and N-acetylaspartate and N- acetylaspartylglutamate (NAA+NAAG), a neuronal integrity marker, in the posterior cingulate gyrus. Results showed that mild AD participants had higher ESRs and IL-1β levels when compared with moderate AD, more severe AD and control participants. High baseline levels of TNF-α, low baseline levels of IL-10 and the presence of the ε4 allele were independently associated with a greater cognitive decline in AD. MI was increased in more severe AD participants and tended to be negatively correlated with episodic memory performance. NAA+NAAG levels were lower in AD participants compared with controls. In conclusion, the inflammatory response in AD changed with disease progression. Pro-inflammatory systemic changes were seen early in the disease but glial activation in the CNS was observed later. Thus, systemic inflammation may drive CNS inflammation and neurodegeneration. The ε4 allele had a detrimental effect in AD. The mechanism by which ε4 exerts its detrimental effects may relate to suppression of a protective anti-inflammatory response.

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LIST OF ABBREVIATIONS

[11]-(R)-PK1995	Carbon 11-labelled-(R)-PK1195
^{1}H-MRS	proton magnetic resonance spectroscopy
3T	3 Tesla
AD	Alzheimer's disease
ADAS-COG	Alzheimer's Disease Assessment Scale Cognitive Subscale
AICD	amyloid precursor protein intracellular domain
APOE	apolipoprotein E (gene)
APOE ε 2	apolipoprotein epsilon 2
APOE ε 3	apolipoprotein epsilon 3
APOE ε 4	apolipoprotein epsilon 4
ApoE	apolipoprotein E (protein)
ApoE2	apolipoprotein E2 (protein)
ApoE3	apolipoprotein E3 (protein)
ApoE4	apolipoprotein E4 (protein)
APP	amyloid precursor protein
Aβ	beta-amyloid
BADLS	Bristol Activities of Daily Living
BBB	blood-brain barrier
C1q	complement component 1 subcomponent Q
CAMCOG	Cambridge Examination for Mental Disorders in the Elderly
CAMCOG-R	Cambridge Examination for Mental Disorders in the Elderly Revised
CAMDEX-R	Cambridge Mental Disorders of the Elderly Examination - Revised
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
Cho	choline
CNS	central nervous system
COX	cyclooxygenase
Cr	creatine

CRP	C-reactive protein
CSF	cerebrospinal fluid
CT	computerised tomography
CUBIC	Cape Universities Brain Imaging Centre
DECO	Deterioration de Cognition Observee
df	degrees of freedom
EDTA	ethylenediaminetetraacetic acid
EPI	echo-planar imaging
ESE	effect size estimate
ESR	erythrocyte sedimentation rate
FDA	Food and Drug Administration
GDS	Geriatric Depression Scale
Gln	glutamine
Glu	glutamate
GPC	glyerophosphocholine
GPE	glycerophosphoethanolamine
IFN	interferon
IL	interleukin
IQR	interquartile range
kDa	kilodalton
LPS	lipopolysaccharide
MCI	mild cognitive impairment
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MI	myo-inositol
mM	millimolar
MMSE	Mini-Mental State Examination
MPRAGE	magnetization-prepared rapid acquisition with gradient echo
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy

NAA	N-acetylaspartate
NAA+NAAG	N-acetylaspartate and N-acetylaspartylglutamate
NHLS	The National Health Laboratory Service
NINCDS / ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association
NSAIDs	non-steroidal anti-inflammatory drugs
OPN	osteopontin
PET	positron emission tomography
pg/mL	picograms per millilitre
PGE_2	prostaglandin E_2
PiB	Pittsburgh compound B
ppm	parts per million
PRESS	point-resolved spectroscopy
rpm	revolutions per minute
Std Error	standard error
TE	echo time
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
TR	repetition time
TREM2	triggering receptor expressed on myeloid cells 2

INTRODUCTION

Alzheimer's disease (AD) is a chronic age-related neurodegenerative disorder affecting cognition, mood, behaviour and daily functioning. AD is the most common cause of dementia and is the main cause of disability in older adults in the developed world (Burns & Iliffe, 2009).

Although the exact prevalence of AD in South Africa is unknown, AD prevalence is known to increase exponentially with age (Gao, Hendrie, Hall, & Hui, 1998). The 2011 South African census estimated that approximately 556 800 of the 5.8 million people living in the Western Cape were over the age of 65 (Statistics SA). The number of people over the age of 50 years in South Africa is also projected to rise from the estimated 8 million in 2010 to 13.5 million in 2050 (U.S. Census Bureau, 2012). The rise in older adults will be accompanied by a rise in the prevalence of AD. The 2005 Delphi consensus concluded that there is already a high prevalence of AD in developing countries (Ferri et al., 2005). This study suggested that although there was a lower prevalence of dementia in Sub-Saharan Africa compared to European and North American countries, it was estimated that of the 24.4 million people living with dementia in 2001, 60% lived in low- to middle-income countries (Ferri et al., 2005). Anecdotal evidence from clinics in the Western Cape, South Africa, also suggests that AD is not uncommon.

AD is characterised by a cluster of neuropsychological deficits, commonly starting with memory impairment and gradually progressing into global cognitive deficits that include aphasia, apraxia, agnosia and visuospatial impairments. Behavioural and psychological symptoms such as delusions, hallucinations, depression, anxiety and apathy also accompany the progression of the disease (Rossor, 1993; Burns, Lawlor & Craig, 2004).

Most patients who develop AD have a late, insidious onset. Familial forms of AD have an earlier onset but these cases account for approximately 1 to 6% of all AD cases (Bekris, Yu, Bird, & Tsuang, 2010). The latter include rare autosomal dominant forms of the disease caused by missense mutations in the amyloid precursor protein (APP) or the

presenilin-1 and presenilin-2 genes located on chromosomes 21, 14, and 1 respectively (Goate et al., 1991; Sherrington et al., 1995; Levy-Lahad et al., 1995).

Many individuals who go on to develop AD can be identified during the prodromal phase of amnestic mild cognitive impairment (MCI). MCI is widely regarded as a transitional state between cognitively normal ageing and AD. An individual is regarded as having MCI when he/she has age-inappropriate memory decline, preserved general cognitive function and intact activities of daily living (Petersen et al., 2001a). Individuals diagnosed with MCI are up to 15 times more likely to develop AD, with an annual rate of conversion to AD from MCI estimated at 10 to 15% (Petersen et al., 2001b; Burns & Iliffe, 2009). Decline in cognition, from cognitively healthy to MCI to AD, may be assessed longitudinally using standardised cognitive tests that are quantifiable, reproducible, and for which normative data are available. Examples of these are the widely used Mini-mental State Examination (MMSE; Folstein, Folstein & McHugh, 1975) and the Cambridge Examination for Mental Disorders in the Elderly (CAMCOG; Huppert, Brayne, Gill, Paykel, & Beardsall, 1995).

Although these cognitive and behavioural manifestations of AD and MCI are fairly easily recognized by clinicians, the actual diagnosis of AD or MCI can be a lengthy process as the diagnostic decision has to follow a logical sequence and has to include multiple lines of evidence. These sources include a history of change in cognitive function from a spouse, partner or other family member, cognitive testing, a physical examination, blood tests and brain imaging. There are no specific diagnostic tests for AD. A diagnosis of possible or probable AD depends on an evaluation of the overall clinical picture and exclusion of other possible causes of cognitive impairment (Dubois et al., 2007; Burns & Iliffe, 2009). A definitive diagnosis of AD is only possible following histopathological examination of brain tissue. Beta-amyloid (Aβ) plaques and neurofibrillary tangles are characteristic pathological features of the condition; these will be discussed later in the Introduction (McKhann et al., 1984). In most cases the clinical diagnosis has to be made in the absence of definitive pathology. Therefore, the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association (NINCDS / ADRDA) developed criteria for the diagnosis of possible or probable AD (see Table 1).

Table 1
NINCDS / ADRDA Criteria for Possible and Probable Alzheimer's Disease

Possible Alzheimer's Disease	**Probable Alzheimer's Disease**
Presence of dementia symptoms, in the absence of any other neurological, psychiatric or systemic disorders.	Dementia – established by a clinical examination, history, and documented with neuropsychological tests (e.g., MMSE < 23, CAMCOG < 80).
Single, gradually progressive, severe cognitive deficit e.g. worsening amnestic syndrome.	Deficits in two or more areas of cognition with progressive worsening of memory and other cognitive functions.
Presence of a second systemic disease or brain disorder sufficient to produce dementia, but not considered to be *the* cause of dementia.	Absence of systemic disorders or other brain diseases that could account for the memory and cognitive deficits. No disturbances in consciousness. Age of onset > 40; usually after the age of 65.

These criteria were published in 1984 and new criteria have since been proposed for the diagnosis of AD. Dubois et al. (2007) proposed new diagnostic criteria for research purposes. These criteria involve the use of biomarkers on neuroimaging and cerebrospinal fluid (CSF) analysis (Dubois et al., 2007). In 2011, the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease published new criteria that incorporated the expanding field of biomarkers (McKhann et al., 2011). They proposed the use of biomarker tests including positron emission tomography (PET) amyloid imaging; low CSF $A\beta_{1-42}$ (a peptide fragment of Aβ) or elevated phosphorylated tau (McKhann et al., 2011). The use of the Pittsburgh compound B (PiB) in PET scanning was first published in 2004 by Klunk and colleagues (Klunk et al., 2004). The PiB compound is retained in areas of the cortex that contain significant Aβ deposits. PET imaging of the PiB compound has made it possible to measure or quantify the Aβ load in living patients. More recently, The Food and Drug Administration (FDA) in the United States approved the use of Amyvid (florbetapir F18 injection) as another PET tracer for Aβ plaques (Clark et al., 2011).

Other AD pathology biomarkers are found in the CSF. Andreasen and colleagues (2001) found increased tau protein concentrations and decreased $A\beta_{42}$ peptide levels in CSF of participants with probable AD. These authors noted that increased tau and decreased $A\beta_{42}$ have a predictive value for AD of greater than 90%. Although these markers are useful in a research setting, the National Institute on Aging-Alzheimer's Association workgroup do not advocate the use of AD biomarker tests in clinical practice as the tests currently lack standardization. The tests are also expensive and currently not widely available. The core NINCDS /ADRDA clinical criteria have good diagnostic accuracy and utility in most patients and in everyday practice these criteria are still widely used (McKhann et al., 2011). The accuracy of using the NINCDS / ADRDA diagnostic criteria has been established by Hogervorst and colleagues (2000). They investigated the inter-rater reliability and accuracy of independent medical doctors who used NINCDS/ADRDA criteria for the diagnosis of AD in patients. When compared with histopathological criteria, namely the Consortium to Establish a Registry for Alzheimer's Disease (CERAD), the diagnostic accuracy was 90% when the clinician diagnosed NINCDS/ADRDA probable AD. This study therefore indicated that the clinical diagnosis of probable AD using the NINCDS/ADRDA criteria correlated very well with a positive histopathological diagnosis.

There are numerous established risk factors for developing AD. The strongest of these risk factors remains increasing *age.*

Age: The prevalence of AD rises incrementally from 0.6% in individuals aged 65 to 69 years to 8.4% in individuals aged 85 years (Hebert et al., 1995). The prevalence of AD is approximately 45% in individuals over 90 years of age (Corrada, Brookmeyer, Berlau, Paganini-Hill, & Kawas, 2008).

Family history: Individuals with a first-degree relative who suffered from AD have a 10 to 30% increased risk of developing AD (Van Duijn et al., 1991).

Genetic factors: Familial forms of AD are related to mutations in APP gene or the presenilin-1 and presenilin-2 genes (Goate et al., 1991; Levy-Lahad et al., 1995; Sherrington et al., 1995). Individuals with Down syndrome also have AD pathology earlier in life (Wisniewski, Wisniewski, & Wen, 1985; Lamere, Blusztajn, Yamaguchi, Wisniewski, Saido, & Selkoe, 1996). The trisomy of chromosome 21, where the APP gene is located, in Down syndrome results in the increased production of Aβ (Rumble et al.,

1989). Aside from these inherited forms of AD, the Apolipoprotein E (APOE) gene has been strongly associated with the risk of developing AD in North America and Europe. The APOE gene has three common alleles (ε2, ε3 and ε4), and codes for a plasma protein involved with lipid transport. The presence of one or two ε4 alleles (heterozygous or homozygous) significantly increases the risk of AD. The odds ratio of developing AD with the ε2/ε2 or ε2/ε3 genotype is 0.6 whereas the odds ratio is 2.6 for the ε2/ε4 genotype, 3.2 for the ε3/ε4 genotype and 14.9 for the ε4/ε4 (Farrer et al., 1997). Other genes implicated in the development of AD include variants of the gene encoding for clusterin (Schrijvers, Koudstaal, Hofman, & Breteler, 2011). More recently a rare missense mutation in the triggering receptor expressed on myeloid cells 2 (*TREM2*) gene has been identified in Icelanders. The *TREM2* gene encodes for the triggering receptor expressed on myeloid cells 2, a glycoprotein thought to be involved in inflammation. The risk of developing AD in people with the *TREM2* mutation has been shown to be similar to the risk of developing AD in people with the ε4 allele (Jonsson et al., 2013).

Vascular risk factors: Vascular risk factors not only increase the risk of vascular dementia but also of AD. Numerous studies have shown an increased risk of AD with hypercholesterolemia, diabetes mellitus and hypertension (Whitmer, Sidney, Selby, Claiborne, Johnston, & Yaffe, 2005; Whitmer, Karter, Yaffe, Quesenberry, & Selby, 2009). Smoking has also been associated with an increased risk in some studies; however others have not found an association (Almeida, Hulse, Lawrence, & Flicker, 2002).

Lifestyle and Activity: An inverse relationship exists between physical activity and risk of AD. A large social network and having a partner also decreases the risk of AD (Fratiglioni, Paillard-Borg, & Winblad, 2004). Stress and high levels of the stress hormone cortisol increase an individual's risk of developing AD (Lupien et al., 1998; Wilson et al., 2005).

Education and Cognitive Reserve: Lower levels of education have been shown to increase the risk of developing AD (Kukull et al., 2002). Cognitive reserve is a term used to describe the brain's resilience to pathological damage. Numerous studies suggest that a large cognitive reserve is associated with higher socio-economic status, higher educational attainment and having an occupation, higher levels of literacy, and more participation in leisure activities (Stern, 2009). A larger cognitive reserve allows for better coping when brain damage occurs, probably due to the increased complexity of pre-morbid neuronal networks and cognitive processes (Stern, 2006).

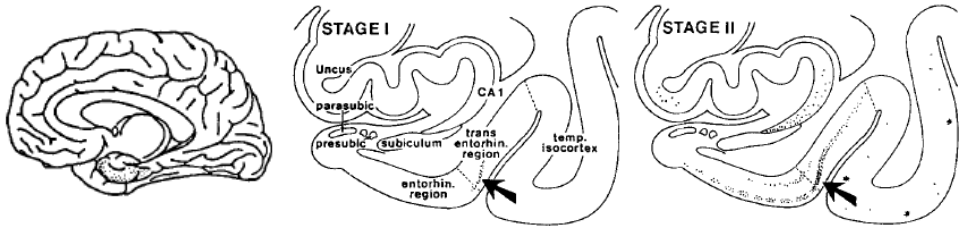
The neuropathology of AD includes extensive cerebral atrophy and the accumulation of neurofibrillary tangles, Aβ plaques and atrophy (Braak & Braak, 1995). *Neurofibrillary tangles* consist of abnormally phosphorylated tau proteins that cause the collapse of the neuronal cytoskeleton (Rossor, 1993; Minghetti, 2005). Braak and Braak (1995) developed the Braak staging for AD based on the presence of neurofibrillary tangles. Neurofibrillary tangles are thought to follow a predictable sequence during disease progression. Braak and Braak (1995) divided the development of neurofibrillary tangles into 6 stages:

Transentorhinal Stages I and II: Neurofibrillary tangles are confined to the transentorhinal cortex region (see *Figure 1a*). Clinically, stage I and II do not present with cognitive impairment because pathological changes are below the threshold at which clinical symptoms are noted.

Limbic Stages III and IV: In stages III and IV there is little destruction of the cerebral cortex. Severe changes are found only in few allocortical regions (the hippocampal formation, entorhinal and presubicular regions) and the surroundings. Mild changes are noted in the entorhinal and transentorhinal regions as well as the hippocampus (see *Figure 1b*). Stage III and IV do not involve changes to the neocortex and therefore patients in these stages do not meet the neuropathological criteria for AD (CERAD). Impairment of cognitive functioning, particularly episodic memory, is noted in these stages.

Neocortical Stages V and VI: Stage V and VI mark the later or end stages of AD. Neurofibrillary tangles are found in the cerebral cortex and severe destruction of the neocortical region is noted (see *Figure 1c*). Stage V and VI correspond with the CERAD criteria for the clinical diagnosis of AD.

a)



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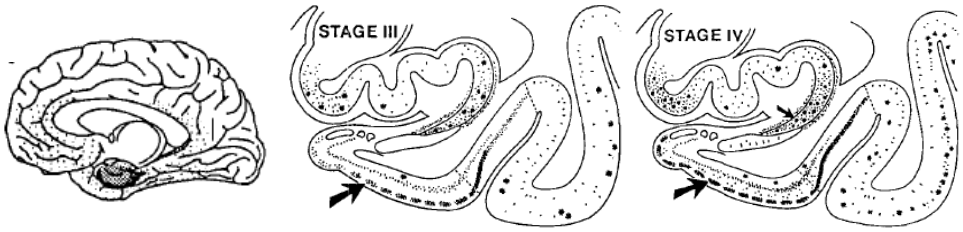

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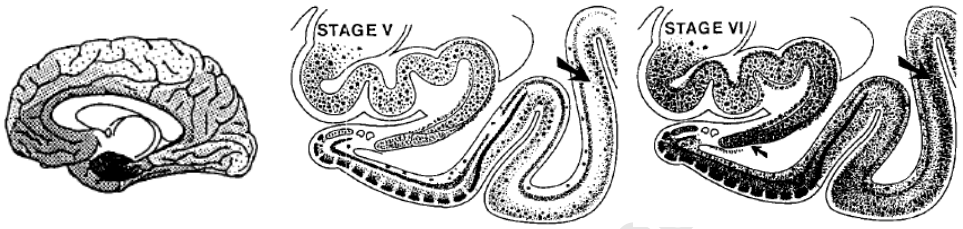


Figure 1: The Braak stages of AD. a) The transentorhinal stages I and II show neurofibrillary tangles (indicated by black dots) in the transentorhinal cortex (indicated by the arrow); b) stage III and IV represent the limbic stages. The limbic stages show destruction of the entorhinal (indicated by the arrow) and transentorhinal cortex as well as the hippocampus. c) the neocortical stages V and VI show destruction of the cortex and neocortical region. The first column of Figures a-c depicts a sagittal section through the brain. The second and third columns show a coronal view of the temporal region. The arrows point to the leading characteristics of each stage.
From: H. Braak & E. Braak, 1995, Neurobiology of Aging, 16, p. 272 & p. 275.

Aβ peptides are derived from the APP (see *Figure 2*). The APP is cleaved by enzymes called secretases. When β-secretase and γ-secretase cleave the APP, $A\beta_{1-40}$ and $A\beta_{1-42}$ are formed. The latter tend to aggregate into β-sheet fibrils that form extracellular Aβ plaques. These plaques are believed to be toxic to neurones. On the other hand, cleavage of the APP by α-secretase and γ-secretase, produce P3 fragments that are soluble, non-amyloidogenic and do not aggregate. Both pathways of APP cleavage occur in normal metabolism (Esch et al., 1990; Haass, Hung, Schlossmacher, Teplow, & Selkoe, 1993; Vassar et al., 1999).

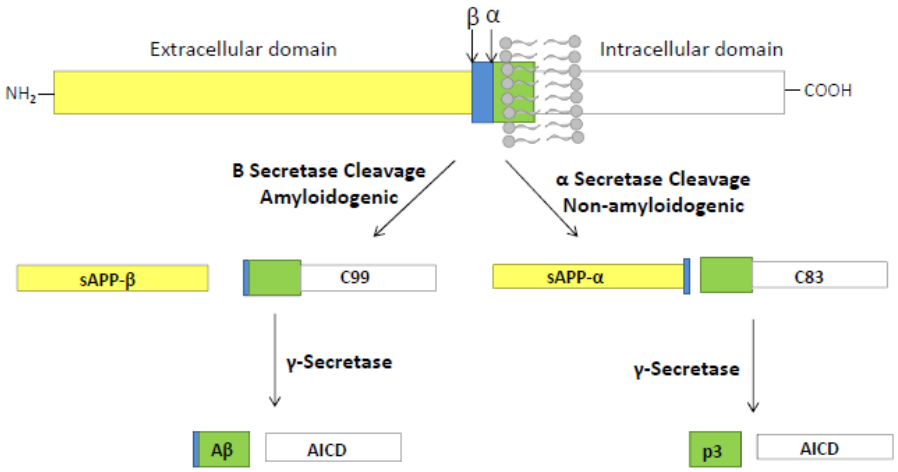


Figure 2: The pathways of amyloid precursor protein (APP) degradation. In the amyloidogenic pathway, cleavage of APP by β-secretase forms an extracellular soluble sAPP-β fragment and a membrane bound C99 fragment. Further degradation of C99 by γ-secretase forms extracellular Aβ and an APP intracellular domain (AICD; a soluble cytosolic fragment). In the non-amyloidogenic pathway, APP is cleaved by α secretase to yield a membrane bound C83 and extracellular sAPP-α. C83 is cleaved by γ-secretase to produce two short peptide fragments, p3 and AIDC.

The amyloid hypothesis proposes that excessive Aβ accumulation is the key pathological feature of AD and responsible for the clinical presentation of patients. Familial forms of AD in which single mutations in the APP gene, located on chromosome 21, causes an increased accumulation of Aβ, support the amyloid hypothesis. The three copies of the APP gene also cause trisomy 21 Down syndrome patients to develop AD usually by the age of 40 years (Wisniewski et al., 1985; Lamere et al., 1996). Evidence to support the amyloid hypothesis also comes from studies showing that in AD there is an imbalance between the production and clearance of Aβ plaques. The imbalance leads to a higher quantity of Aβ in brain tissue, leading to more neuronal damage and progression of the disease process. Evidence for this hypothesis is provided by Mawuenyena et al. (2010). They found that the rate of Aβ production in late onset AD patients and controls was not significantly different. However, in AD patients the rate of clearance of Aβ was 30% slower than controls. A deficit in clearance is probably due to decreased transport of Aβ across the blood-brain barrier (BBB) or decreased cellular uptake by macrophages (Minati, Edginton, Bruzzone, & Giaccone, 2009).

Newer evidence supporting the amyloid hypothesis comes from a genetic discovery. A missense mutation of the APP gene (A673T) has been found to be protective against AD.

Individuals with the A673T mutation produced less Aβ than individuals without the mutation. The mutation results in impaired β-secretase 1 cleavage of APP in carriers. This leads to a 40% reduction in the formation of amyloidogenic peptides. The mutation also seems to trump the APOE ε4 allele effect. In the study, 25 individuals with the mutation were homozygous for the ε4 allele. These individuals did not develop dementia despite their increased risk (Jonsson et al., 2012).

In 2002, Thal and colleagues described the progression of Aβ deposition, classifying it into 5 phases. The first phase includes Aβ deposition in the neocortex. This is followed by Aβ deposition in the allocortical regions in phase two. In the third phase, the putamen; caudate nucleus; substantia innominata; magnocellular cholinergic nuclei of the basal forebrain and the diencephalic nuclei contain Aβ plaques. Some brainstem nuclei exhibit Aβ deposition in the fourth phase. In the fifth phase deposition occurs in additional brainstem nuclei as well as the cerebellum (Thal, Rüb, Orantes, & Braak, 2002).

Aβ and neurofibrillary tanlges accumulate in specific parts of the brain, such as the neocortex and hippocampus, as well as in certain cells of subcortical cholinergic nuclei (e.g. in the nucleus basalis of Meynert) that project to the cerebral cortex. The brunt of neurofibrillary tangles accumulation occurs in the hippocampus and entorhinal cortex. This may account for the early episodic memory difficulties seen in AD patients (Rossor, 1993; Dickson, Crystal, Bevona, Honer, Vincent, & Davies, 1995).

Neurochemical and lesion studies have provided compelling evidence for the selective degeneration of cholinergic basal forebrain neurones in ageing and AD. The basal forebrain consists of a large group of cholinergic neurones. Neuronal extensions from the nucleus basalis of Meynert innervate the neocortex and amygdala and the neurones extending from the medial septal nucleus innervate mainly the hippocampus and the cingulate cortex (see *Figure 3*). The basal forebrain cholinergic system regulates limbic and cortical functioning. These cholinergic neurones degenerate and die in AD. This has led to the "cholinergic hypothesis", which states that a deficit of the neurotransmitter, acetylcholine, underlies the primary and earliest symptoms of memory loss in AD (Bartus, Dean Beer, & Lippa, 1982; Coyle, Price, & DeLong, 1983).

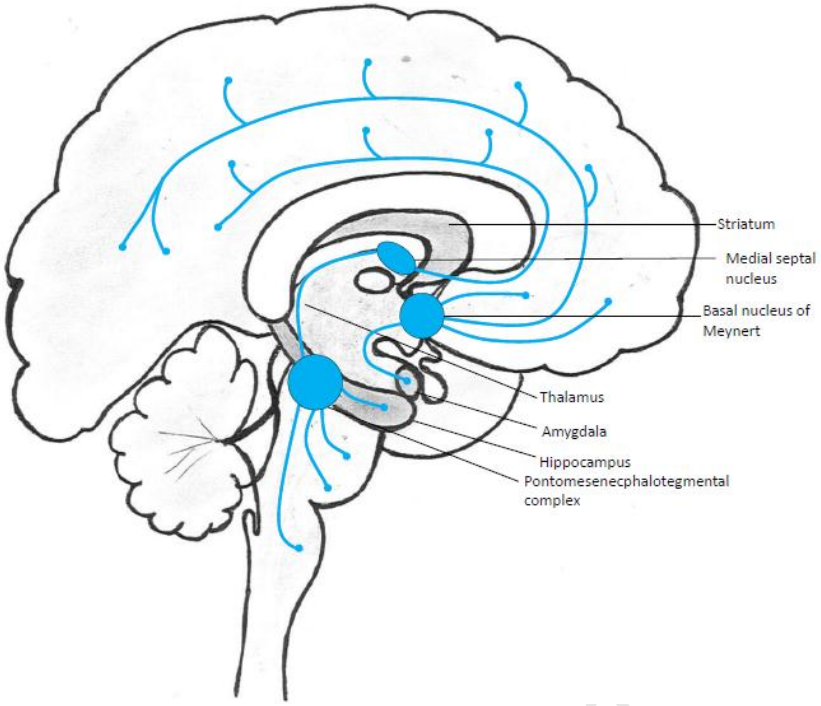


Figure 3: Projections of the cholinergic system. Neuronal extensions from the nucleus basalis of Meynert innervate the neocortex and amygdala and the neurones extending from the medial septal nucleus innervate mainly the hippocampus and the cingulate cortex.

There is no specific treatment for AD. Centrally acting acetylcholinesterase inhibitors, aimed at improving the acetylcholine deficit by inhibiting the enzyme that breaks it down, are often prescribed for symptomatic improvement in the early stages. Other drugs are in the clinical phases of development, with many of these being disease-modifying medications that are aimed at reducing the Aβ deposition (Mangialasche, Solomon, Winblad, Mecocci & Kivipelto, 2010).

NEUROINFLAMATION AND ALZHEIMER'S DISEASE

Another important and now widely accepted neuropathological feature of AD is neuroinflammation. *Neuroinflammation* may be defined as the presence of activated microglia and reactive astrocytes, as well as inflammatory mediators, in the central nervous system (CNS) (Minghetti, 2005).

Celsus (30 BC – 38 AD) was the first to describe the 4 classical signs of inflammation, namely dolor (pain), tumor (swelling), rubor (redness) and calor (heat). Later functio laesa

(loss of function) was added to form the 5 cardinal signs of inflammation. This classic innate response to infection in the periphery is caused by blood borne elements (e.g. cytokines, prostaglandins, histamines and nitric oxide) that are released at the site of injury. In 1892 Metchnikoff was the first to investigate the localised nature of inflammation by impaling starfish larvae with rose thorns. He observed mesenchymal cells around the site of injury and named these cells phagocytes (see McGeer & McGeer, 2003). In 1919 Del Rio Hortega identified the brains equivalent of phagocytes, microglia. In the 1980s there was a significant increase in research into neuroinflammation and neurodegenerative disorders. This research lead to the hypothesis that neuroinflammation may drive or contribute in part to neurodegenerative disease processes (Rozenmuller, Eikelenboom, & Stam, 1986; McGeer, Itagaki, & McGeer, 1988).

AD does not demonstrate the cardinal signs of calor, rubor, dolor and tumor and therefore the inflammatory response in the brain is sometimes termed "clinically silent" (Kandel, 2006). The inflammatory response in AD includes complement activation, chemokine alterations, elevated pentraxins, microglial and astrocyte activation, as well as biochemical features of oxidative stress and the release of neurotoxins (Rosenberg, 2005). Although neuroinflammation is largely considered a downstream consequence of Aβ and tau pathology, it may be an exacerbating factor in the pathological and clinical progression of the disease (Vehmas, Kawas, Stewart, & Troncoso, 2003). The rest of this section will describe some of the evidence implicating neuroinflammation in the pathogenesis of AD.

The presence of activated microglial and astrocytes in AD

Microglial cells and astrocytes form part of the CNS's innate immune system. In the sense of inflammation, these inflammatory cells are down-regulated and inactive in normal healthy brains. In AD, microglia become activated in areas of the brain associated with Aβ plaques and tau pathology. Activation is probably in response to the pathology and may represent an attempt to clear Aβ plaques by phagocytosis (Braak & Braak, 1995; see *Figure 4*). When microglia are activated, they change morphologically, developing into highly branched cells that are then capable of phagocytosis (Perry, Cunningham, Holmes, 2007).

Activated microglial cells have been found in mouse models of AD and post-mortem brain samples. In a transgenic mouse model using the mutant human APP gene, Aβ deposition was associated with immune responses. Matsuoka et al. (2001) found that these mice had activated microglia and astrocytes that increased synchronously with the increase in amyloid burden. In post-mortem studies, activated microglia surround plaques and neurofibrillary tangles in AD brains (Akiyama et al., 2000), see *Figure 4*. More recently, Gorlovoy, Larionov, Pham, & Neumann (2009) demonstrated that activated microglia may also lead to the aggregation of tau *in vivo*, further driving AD pathology. Gorlovoy et al. (2009) did this experiment by co-culturing activated microglial cells from mouse hippocampal tissue with neurones that had been exposed to the human tau gene. Neuronal cultures exposed to activated microglial cells had an increase in the life-time fluorescence resonance energy transfer signal, indicating that activated microglia producing inflammatory mediators stimulated the aggregation of tau in neurites.

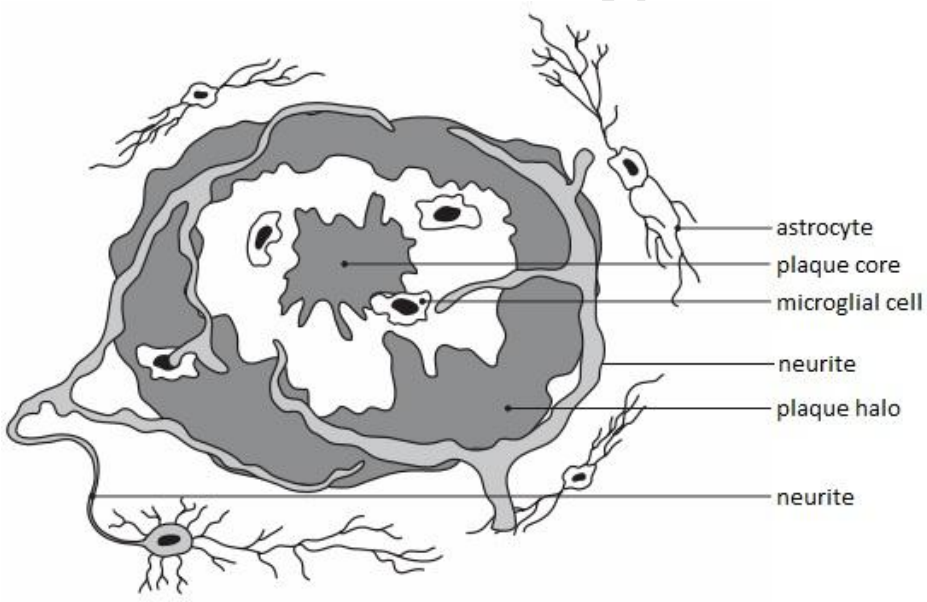


Figure 4: A schematic representation of activated microglial cells engulfing a β amyloid plaque.
From: M. Combrinck & J. Joska, 2008, Current Allergy & Clinical Immunology, 21, p.133.

Non-steroidal anti-inflammatory drugs (NSAIDs)

Indirect evidence for the detrimental effect of inflammation in AD is derived from population-based longitudinal studies, which found that the long-term use of some NSAIDs was associated with a reduced risk of AD (In'T Veld et al., 2001; Vlad, Miller,

Kowall & Felson, 2008; Côte et al., 2012). The risk of developing AD is further reduced with a longer duration of NSAIDs use (In'T Veld et al., 2001). NSAIDs inhibit cyclooxygenase (COX), the enzyme which catalyses the first step in the synthesis of prostaglandins and thromboxanes. The latter two chemicals are important mediators of inflammation (Minghetti, 2005). There are two major isoforms of COX, COX-1 and COX-2. COX-1 is constitutively expressed while COX-2 is inducible. COX-2 has been shown to be increased in the brain in early stage AD (Hoozemans et al., 2005). Chronic NSAID use may therefore protect against AD by suppressing neuroinflammation.

However, Weggen et al. (2001) demonstrated that the mechanisms by which NSAIDs protected against AD might be unrelated to their anti-inflammatory effects. Some NSAIDs, for example ibuprofen, have been found to alter the site of cleavage of the APP, thereby altering the Aβ fragment produced from the insoluble, amyloidogenic $A\beta_{1-42}$ to the soluble non-amyloidogenic $A\beta_{1-38}$ protein (Weggen et al., 2001). Therefore, NSAIDs may, in fact, reduce the Aβ burden in AD brains. Most clinical trials of NSAIDs in established AD have failed to show any benefit or slowing in the progression of AD (McGeer & McGeer, 2007). Some trials have shown that the administration of NSAIDs in the early stages of AD may actually be detrimental. The latter may be due to the inhibition of an early protective inflammatory response (Gasparini, Ongini, & Wenk, 2004).

Cytokine toxicity in AD

The activation of microglia has been shown to cause a disturbance in neuronal homeostasis and alterations to synapses (McGeer & McGeer, 2003). Neurotoxicity occurs as a result of the sustained secretion of high levels of cytokines and chemokines that damage neurones and perturb neuronal functions. Neurones are especially sensitive to cytokine signalling (Hoozemans, Veerhuis, Rozemuller, & Eikelenboom, 2006). One argument is that the propagation of microglial and astrocytes around the plaques drives a toxic cycle. Microglial and astrocytes cause neuronal damage, further APP cleavage and therefore increased Aβ deposition, neuronal injury, apoptosis and increased microglia activation (Gasparini et al., 2004). Schwartz and Schechter (2010) proposed that if microglia are intensely activated beyond a certain threshold and remain activated past a certain time period, they become a source of neurotoxicity.

Low levels of inflammation and high Aβ load

Cognitively healthy individuals with a high Aβ load, significant neurofibrillary tangle pathology but low levels of inflammation provide evidence that neuroinflammation may play a role in the clinical manifestation of AD (Parachikova et al., 2007). Parachikova and colleagues (2007) compared mild/moderate AD patients with cognitively healthy controls. Of the 14 cognitively healthy controls in this study (MMSE scores ranging from 25-30), 10 had AD pathology (high plaque and/or tangle pathology), 4 of whom had a significant Aβ load (6.15 – 13.5%). They found that mild/moderate AD patients had significantly higher levels of the inflammatory markers, major histocompatibility complex (MHC) class II mRNA and MHC II protein load, in the hippocampus compared with controls. Furthermore, MHC II protein levels correlated negatively with the MMSE. These findings show that despite the presence of significant plaque pathology, controls had lower inflammatory markers. The results support the notion that inflammation may correlate with the clinical dementia component of the disease. Thus, the presence of neuroinflammation may determine whether clinical signs result from the pathology of AD.

Systemic inflammation and cognition

The role of inflammation in cognitive decline is further validated by studies examining the effects of systemic inflammation on cognition.

High levels of systemic inflammatory markers and cognition:

Studies investigating the relationship between levels of systemic inflammation and decline in cognition contribute indirectly to evidence that neuroinflammation has a role in the pathogenesis of AD. Higher levels of inflammatory markers in the blood have been shown to predict cognitive decline. Inflammation may therefore be one reason for the cognitive decline seen in normal ageing (Schmidt, Schmidt, Curb, Masaki, White, & Launer, 2002a; Eikelenboom, van Exel, Hoozemans, Veerhuis, Rozemuller, & van Gool, 2010). Ageing has been shown to be accompanied by a mild, controlled, chronic increase in certain systemic pro-inflammatory cytokines. Franceschi, Bonafè, Valensin, & Benedictis (2000) coined the term "inflammaging" to describe this phenomenon. Some elderly individuals may shift from this normal, but slightly increased inflammatory state to an age-associated

diseased state. Inflammaging may serve as a prodrome or exacerbating factor for the development of AD, and may even be a risk factor (Giunta et al., 2008). The Honolulu-Asia Aging Study showed that cognitively healthy men with high levels of highly sensitive C-reactive protein (CRP) had a 3-fold increased risk for dementia compared to men with lower levels (Schmidt et al., 2002a). The shift from age-appropriate cognitive decline to a disease state falls on a continuum, suggesting a gradual shift from normal ageing to preclinical dementia (Burns & Iliffe, 2009).

The relationship between inflammation and cognition is also seen in cognitively impaired patients where high levels of peripheral or systemic inflammatory markers are significantly correlated with cognitive impairment (Holmes & Lovestone, 2003; Guerreiro, Santa, Brás, Santiago, Paiva, & Oliveira, 2007; Holmes et al., 2009). Guerreiro et al. (2007) found that levels of pro-inflammatory cytokines such as IL-1β and tumor necrosis factor alpha (TNF-α) in the blood were highest in AD patients when compared with cognitively healthy controls and participants with MCI. Holmes and colleagues (2009) found that increased baseline levels of serum TNF-α were associated with a fourfold increase in the rate of cognitive decline in AD patients over a 6 month period compared to AD patients with low baseline levels. A high baseline level of TNF-α was also associated with the degree of baseline cognitive impairment. High levels of systemic inflammatory markers in older adults may be due to multiple recurrent or chronic systemic infections throughout life. The mechanisms by which systemic infection may affect cognition are discussed below.

Peripheral infection and cognitive decline in AD:

Systemic infection and other inflammatory events in the periphery are a common cause of delirium in the elderly. Delirium is an acute, fluctuating condition affecting multiple cortical functions (American Psychiatric Association DSM-IV, 2000). Delirium has been associated with a significant increased risk of developing dementia (McCucker, Cole, Dendukuri, Belzile, & Primeau, 2001). Peripheral infection has been shown to accelerate cognitive decline and neurodegeneration in AD patients. In a 6-month follow-up study, AD patients who suffered a systemic inflammatory event had a two-fold increase in the rate of cognitive decline when compared with patients who had not suffered a systemic inflammatory event (Holmes et al., 2009).

The effect of systemic inflammation depends on the state of activation of the microglia. AD pathology partially activates microglia. These microglia become "primed". The systemic inflammatory response is thought to further activate primed microglia in the brain via neuro-immune signalling from the periphery to the CNS, leading to an exaggerated neuro-immune response (Perry, 2004). The increase in CNS inflammation is thought to increase neuronal damage, apoptosis and Aβ deposition, all of which drive the decline in cognitive functioning. The hypothesis that peripheral infection leads to increased CNS inflammation is well documented in animal studies where a systemic infection, mimicked by lipopolysaccharide (LPS), leads to activation of microglia in the CNS (Combrinck, Perry, & Cunningham, 2002). The mechanisms by which this occurs are further discussed in this chapter (The Link between Peripheral and Central Immune Systems, page 36).

The evidence summarised so far suggests that systemic inflammation can accelerate degeneration of neurones which, in-turn, leads to a decline in cognition. However, there has been much debate as to whether or not enhanced neuroinflammation is beneficial or detrimental to the process of neuronal stability and survival (Weninger & Yanker, 2001; Crutcher et al., 2006). One could argue that in the initial stages of AD, neuroinflammation is beneficial and that the primary mode of microglia during these stages is protective. Microglia and macrophages may attempt to reduce plaque formation by engulfing Aβ. Thus, the immune response improves neuronal survival. An early immune response may also repair damaged neurones (Crutcher et al., 2006). Combrinck et al. (2006) showed that high initial levels of prostaglandin E_2 (PGE_2), an anti-inflammatory-associated chemical mediator of inflammation, were associated with a longer survival of AD patients compared with AD patients with initial low levels of PGE_2.

Inflammation, an Early Event in AD

Inflammation in AD has been studied at length. However, few studies have investigated inflammation at the different stages of AD. Motta, Imbesi, Di Rosa, Stivala, & Malaguarnera (2007) investigated cytokines in serum in mild AD patients (patients with MMSE scores ranging from 18 – 20.2), and moderate AD patients (patients with MMSE scores ranging from 14.6 – 16.3). They showed that interleukin 12 (IL-12), IL-16, IL-18 and transforming growth factor beta (TGF-β) were higher in patients with mild AD,

progressively declining in moderate AD patients (Motta et al., 2007). Some CNS studies have shown similar results. As previously described, Braak and Braak (1995) devised a staging system of AD, which rates progression of the disease on a scale ranging from 1 to 6 according to the neurofibrillary tangle pathology. A lower Braak stage indicates lower quantities of neurofibrillary deposits. A post-mortem study showed an increase in the activation of COX-2 in response to an increase in neurofibrillary tangles from Braak stages 1 to 4 (Hoozemans et al., 2005). However, they also found that COX-2 expression subsides after Braak stage 4 (see *Figure 5*).

Parachikova et al. (2007) also demonstrated that microglia activation was an early event in the pathogenesis of AD. They showed that the gene expression of MHC II, a marker of activated microglia, was increased in prefrontal cortex tissue and hippocampal tissue from participants with mild to moderate AD when compared with post-mortem brain samples from non-demented participants. Combrinck et al. (2006) confirmed that inflammation subsides in moderate and advanced AD patients. In this study, CSF PGE_2 levels were higher in mild-moderate AD participants when compared with advanced AD participants. Therefore, the inflammatory response in MCI/early AD seems to change in nature, with high levels of the inflammatory markers COX-2, PGE_2 and MHC II found in the early stages of the disease process and lower levels at later stages (see *Figure 5*).

Many studies have not shown that inflammation subsides with the progression of AD. Bermejo et al. (2008) showed that IL-6, TNF-α and IFN-α were significantly raised in AD participants when compared with MCI participants and controls. Another study showed increased levels of TNF-α in sera of severe stage AD patients compared with controls; whereas mild/moderate AD patients did not have elevated TNF-α levels compared with controls (Bonotis, Krikki, Holeva, Aggouridaki, Costa, & Baloyannis, 2008).

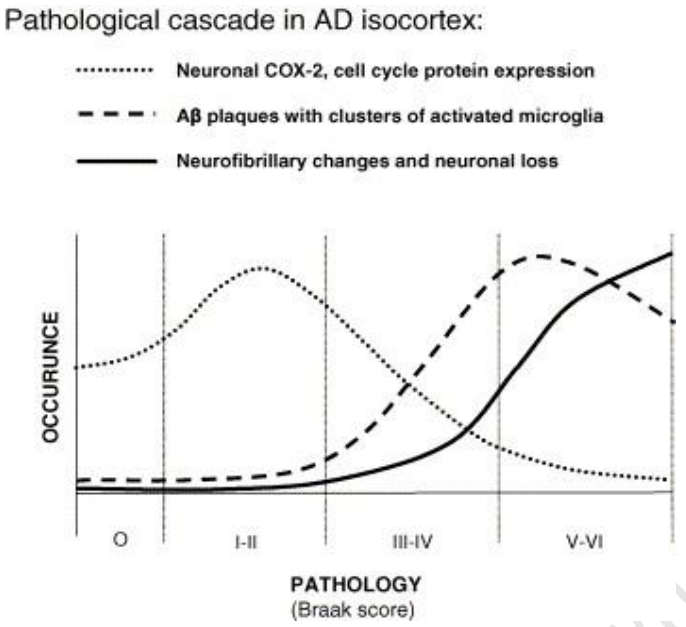


Figure 5: β-Amyloid and neurofibrillary tau pathology in the different stages, defined by Braak pathology, of Alzheimer's disease and their relationship to an inflammatory marker, cyclooxygenase 2 (COX-2).
From: J.J.M. Hoozemans, R.Veerhuis, J.M. Rozemuller, P. Eikelenboom, 2006, International Journal of Developmental Neuroscience, 24, p.158.

Interim Summary

The pathological features of AD include Aβ accumulation, neurofibrillary tangles, cerebral atrophy and neuroinflammation. Although neuroinflammation is largely considered a downstream consequence of Aβ and tau pathology, it may be an important factor in the pathogenesis and clinical progression of the disease (Vehmas et al., 2003).

Quiescent microglia are activated in response to AD pathology (Braak & Braak, 1995). Activated microglial surround plaques in an attempt to engulf them (Akiyama et al., 2000; Matsuoka et al., 2001). Activated microglial also secrete cytokines and other inflammatory molecules. These inflammatory molecules may drive the neurotoxic cycle, leading to neurodegeneration. Higher levels of inflammation have also been associated with an increase in the rate of cognitive decline in both cognitively healthy and AD participants (Schmidt et al., 2002a; Guerreiro et al., 2007; Holmes et al., 2009).

The inflammatory response in AD has been shown to occur early in the disease process (Parachikova et al., 2007). Some studies have found that this initial inflammatory event

may subside with the progression of the disease (Combrinck et al., 2006; Hoozemans et al., 2006).

Markers of Inflammation

In both sporadic and familial forms of AD, inflammatory cells are present (McGeer et al., 1988; McGeer & McGeer, 2003; Wyss-Coray & Rogers, 2012). Researchers have used different inflammatory markers to quantity inflammation. The rest of this section will outline some of the inflammatory markers studied in AD.

Cellular Markers

Activated microglial cells and astrocytes appear early in AD and microglia appear to surround plaques. Microglia are observed by immunohistochemical staining using antibodies against proteins displayed by the monocyte-phagocytic system. One of these proteins includes the MHC class II glycoproteins which are up-regulated in activated microglial cells (McGeer et al., 1988). Astrocytes, which provide supportive functions for neurones, have been found to be concentrated around Aβ plaques. Astrocytes migrate in response to monocyte chemoattractant protein-1 (MCP-1), a chemokine, and other chemo-attractants present in brain lesions. Astrocytes then adhere to $A\beta_{1-42}$, a process mediated by integrin. Adherence allows astrocytes to bind to Aβ enabling them to take up and degrade these plaques (Wyss-Coray et al., 2003).

Complement Proteins and Pentraxins

Pentraxins are important protein activators of the complement system. C- reactive protein (CRP), a pentraxin, is a protein in the blood. Originally it was believed that CRP was only produced in the liver and then secreted into the blood. However, genetic techniques have demonstrated that it is also produced locally in the brain. CRP is thought to assist in the process of phagocytosis by macrophages (Guyton & Hall, 2006). CRP has been shown to be up-regulated in AD (McGeer & McGeer, 2003).

The complement system is activated by pentraxins. The function of the complement system is “recognition, opsonization, inflammatory stimulation through anaphylatoxins and

direct killing through the membrane attack complement" (McGeer & McGeer, 2003). Recognition occurs through Complement Component 1, Q Subcomponent (C1q). Briefly, amplification then takes place through a cascade of 30 proteins in the complement pathway. This attracts and activates immune cells. Tangle and plaque aggregates have been shown to bind to C1q and to activate the classical complement cascade. C4d and C3d are some of the complement fragments that have been shown to be activated in AD and not cognitively healthy controls (Akiyama et al., 2000).

Other peripheral blood markers

The *erythrocyte sedimentation rate (ESR)* is the rate at which red blood cells precipitate over a period of 1 hour. It is a common haematological test that is performed by placing anti-coagulated blood in an upright capillary tube and measuring the rate (mm/hour) at which the red blood cells precipitate with gravity. When fibrinogen is present in high concentration it causes red blood cells to stick together, thus increasing the sedimentation rate. High levels of fibrinogen are present in the blood in inflammatory conditions (Guyton & Hall, 2006).

White blood cells form part of the innate immune system. They are produced particularly in bone marrow and partly in lymph tissue. After formation, white blood cells are transported to parts of the body where they are needed. An increase in the white cell count in peripheral blood usually indicates infection and/or inflammation (Guyton & Hall, 2006).

There are six types of white blood cells present in the blood, each with a distinct form and function. These include monocytes, neutrophils, eosinophils, basophils and lymphocytes. *Monocytes* make up 5-10% of the total white blood cell count. Monocytes have many roles in the immune system; one such role is to engulf, divide and differentiate into macrophages therefore replenishing the macrophage population in tissues. They also migrate from the bloodstream to other tissues where they engulf foreign matter by phagocytosis (Guyton & Hall, 2006). Microglial cells (the brain's immune defence) are most similar to monocytes. Microglia originate from monocytes present in the CNS. Peripheral monocytes produce the same cytokines that activated CNS microglial cells do (Davis, Foster, & Thomas, 1994).

Osteopontin

Osteopontin (OPN) is an extracellular phosphoprotein expressed by macrophages. OPN is involved in differentiation and immune cell activation as well as cell attachment and migration. OPN is thought to have a pro-inflammatory role as it acts as a cytokine in regulating macrophage function (Scatena, Liaw, & Giachelli, 2007). OPN in the blood has been found to be increased in AD patients and is associated with Aβ deposition (Wung et al., 2007). Comi et al. (2010) showed that OPN was increased in the early phases of AD. OPN levels in CSF were positively correlated with MMSE scores from AD patients and negatively correlated with disease duration. The increase in OPN was specific to AD and not found in age matched controls or patients with fronto-temporal dementia (Comi et al., 2010).

Cytokines – a double edged sword

Neuroinflammatory cytokines appear to have a dual role, with both beneficial and detrimental effects. Cytokines have therefore often been referred to as the double-edged sword of inflammation. When an immune response is triggered and microglia are activated, they release pro- and anti-inflammatory cytokines and chemokines. Cytokines are soluble communicating factors between cells. Cytokines encompass several subfamilies that include interleukins (ILs), interferons (IFNs), tumor necrosis factor (TNF), growth factors (GF) and chemokines (McGeer & McGeer, 2003; Wyss-Coray & Rogers, 2012).

Anti-inflammatory cytokines, such as transforming growth factor beta (TGF-β) and PGE_2, play a role in homeostasis and suppression of the inflammatory response. They down-regulate the pro-inflammatory cytokines and are involved in the resolution of inflammation (Boche, Cunningham, Docagne, Scott, & Perry, 2006). Higher TGF-β levels in transgenic mice expressing the human APP gene resulted in a 50% overall reduction in the Aβ load on the hippocampus and neocortex (Wyss-Coray et al., 2001). Parachikova et al. (2007) showed that the anti-inflammatory gene sequence for the TGF-β and IL-10 receptor were increased in mild/moderate AD post-mortem brain samples when compared with healthy controls.

In contrast, pro-inflammatory cytokines such as TNF-α and IL-1β may induce inflammation, phagocytosis, and cell death. Elevated blood levels of TNF-α and IL-1β have been found in MCI and AD patients compared with controls. Animal models have shown similar results. Mice expressing the mutant human APP gene had significantly higher levels of IL-1β and TNF-α in the cortex than control mice (Sly et al., 2001). Activation of these pro-inflammatory cytokines may be a common mechanism that contributes to neurodegenerative disease processes (González-Scaran & Baltuch, 1999).

Some cytokines have both anti and pro-inflammatory properties at different stages of the inflammatory process. Therefore, separating cytokines into anti- and pro-categories is problematic.

In summary, activated microglia release both pro-and anti-inflammatory cytokines. Microglia are in a quiescent state in a normal healthy brain and are activated in AD pathology. The pro-inflammatory cytokines may be neurotoxic and therefore cause neuronal damage. Anti-inflammatory cytokines counteract the pro-inflammatory effect and dampen the immune response.

The Link between Peripheral and Central Immune Systems

The CNS was previously believed to be an immunologically privileged site that was protected and isolated by the BBB from the peripheral immune system. However, new evidence suggests that there is communication between the peripheral and central immune systems (Guerreiro et al., 2007; Sonnen, Montine, Quinn, Kaye, Breitner, & Montine, 2008).

The BBB generally prevents cytokines moving from the CNS to the periphery and vice versa. However, it does allow for signalling between the two (Guerreiro et al., 2007; Sonnen et al., 2008; see *Figure 6*). There are 3 major routes of communication between the periphery and the CNS. The following section describes their respective roles.

Firstly, communication between the periphery and the CNS occurs via the BBB. Evidence for the BBB as a route of communication comes from studies showing that systemic infection may activate neuroinflammation (see *Figure 6*). Secretions of pro-inflammatory mediators or microbial products in the periphery are able to directly signal to the CNS. Peripheral cytokines and inflammatory mediators from the periphery signal to the endothelium of the BBB, which in turn signals to the perivascular macrophages located adjacent to the endothelial cells. The perivascular macrophages then signal to the resident microglia within the brain, which are then activated (Perry, 2004). The activated microglial cells are then capable of secreting cytokines and other toxic substances that damage neurones. In AD, primed microglia are further activated, enhancing the inflammatory response (Perry, 2004). In a demonstration of such interaction between the peripheral and central immune systems, Combrinck et al. (2002) showed that peripheral infection causes activation of microglia primed by prion disease, increasing pro-inflammatory cytokines in the diseased brains of prion-infected animals.

The central immune system may also influence the peripheral circulating system (see *Figure 6*). De Simoni, De Luigi, and colleagues (1995; 1998) showed that intra-cerebral administration of a LPS, an endotoxin from Gram-negative bacteria, induces peripheral systemic inflammation. Rats that were administered LPS centrally showed an increase in IL-1β, IL-6 and TNF-α cytokines in the serum. These authors have also suggested that the sympathetic nervous system exerts a tonic inhibitory control over the peripheral synthesis and secretion of inflammatory cytokines. The latter were enhanced by sympathetic nervous system denervation.

The second route of communication is by blood cytokines communicating directly with macrophages and other cells in the circumventricular organs of the brain that lack a BBB (Banks, Kastin, & Broadwell, 1995). Schwartz and Schechter (2010) also proposed that during neurodegeneration, monocytes might also infiltrate a leaky brain parenchyma from the circulatory system. These monocytes differentiate to form monocyte-derived macrophages. These cells are thought to regulate microglia by terminating their inflammatory response (Schwartz and Schechter, 2010).

The third route of communication between the peripheral and the central immune systems is via the vagal nerve sensory afferents from the thoracic abdominal cavity (see *Figure 6*). The vagus nerve is the 10th cranial nerve that conveys sensory information from the viscera to the CNS. The vagus nerve is activated by endotoxins and cytokines in the periphery, relaying the signal to the CNS. In-turn, vagus efferent fibres might have an anti-inflammatory effect through acetylcholine secretion. It is through this pathway that the vagus nerve is thought to modulate the systemic inflammatory response to pathogenic invasion (Tracey, 2002). Other evidence demonstrating the vagus nerve's function in peripheral and CNS communication comes from rat vagotomy studies. Vagotomised rats failed to display the behavioural depression in social exploration that the sham operated rats showed after a peripheral injection of IL-1β (Bluthé, Michaud, Kelley, & Dantzer, 1996).

Further evidence for the communication between the systemic immune system and the CNS is provided by the effect of an acute systemic illness on the CNS. A systemic infection causes an altered temperature, increased lethargy, decreased body weight, apathy, social withdrawal and depression. Collectively these behavioural changes are referred to as "sickness behaviour" and are mediated by the hypothalamus. These conditions are adaptive protective mechanisms aimed at creating suboptimal conditions for microbial replication and conservation of energy (Hart, 1988; Combrinck et al., 2002; Dantzer & Kelley, 2007; Holmes, Cunningham, Zotova, Culliford, & Perry, 2011).

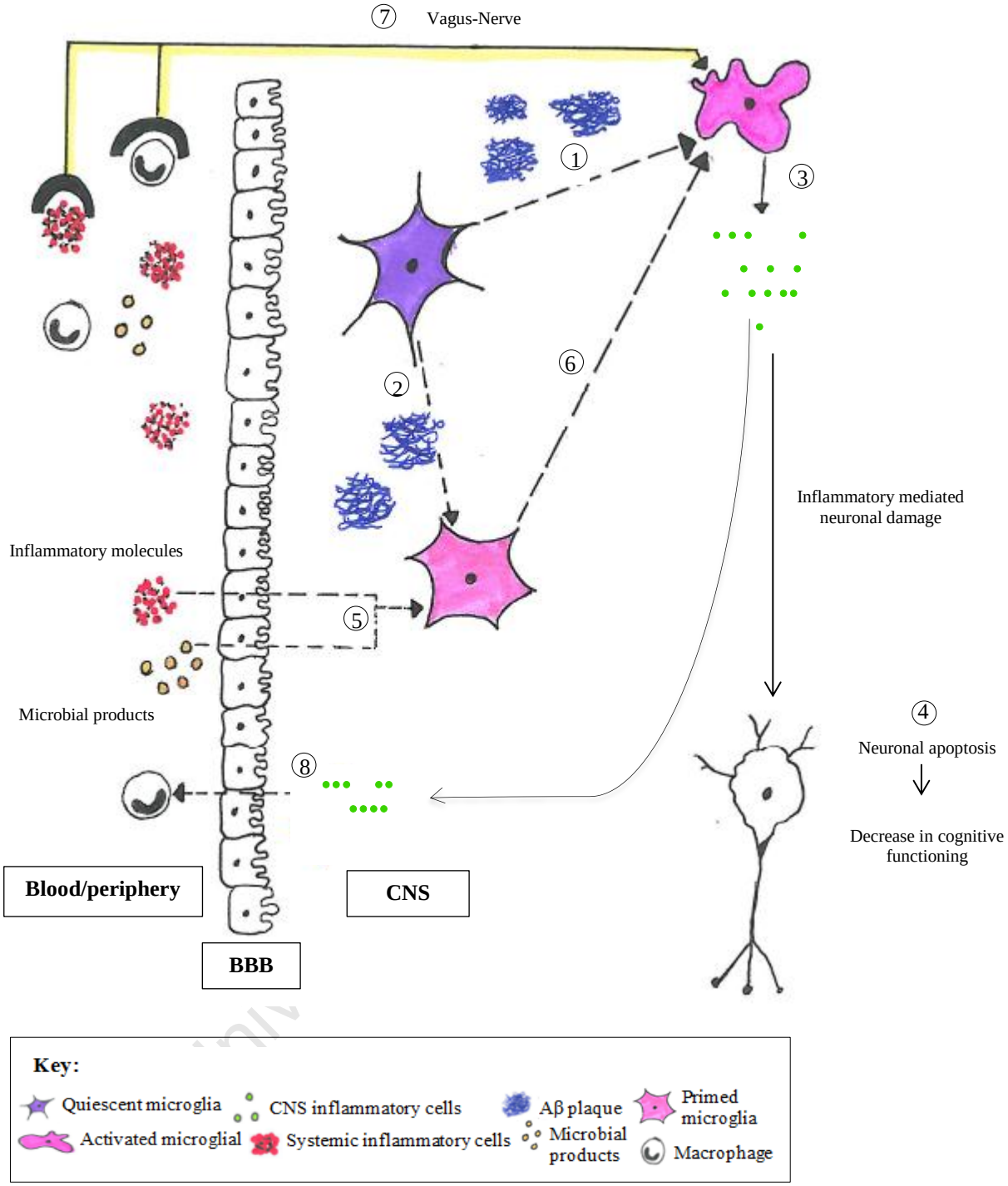


Figure 6: The link between the periphery and the central nervous system (CNS)
1: Resting microglia are activated by β-amyloid deposits. 2: Resting microglia are also "primed" or partially activated by β-amyloid deposits. 3: Activated microglial secrete cytokines. 4: Cytokines and other inflammatory molecules cause neuronal damage, increased apoptosis, neuronal cell loss and cognitive decline. 5: Inflammatory molecules or microbial products in the blood signal to the brain endothelium across the blood-brain barrier (BBB); the endothelial cells, in turn, signal to the perivascular macrophages leading to 6. 6: Primed microglia are further activated, exacerbating inflammation. 7: The vagus nerve senses inflammatory molecules in the viscera and signals to the CNS, activating CNS microglia. 8: There is some evidence that CNS inflammatory molecules are able to signal through the endothelium and activate monocytes in the blood.

Interim Summary

The BBB is generally thought of as a mechanism that protects the microenvironment of the brain/CNS from that of the periphery or systemic circulation. It does, however, allow for signalling between the two. There are 3 routes of communication. First, systemic cytokines or microbial products in the periphery signal to the brain endothelium across the BBB; the latter cells, in turn, signal to the perivascular macrophages and microglia of the CNS. The second route of communication is via circumventricular organs that lack a BBB (Banks, Kastin, & Broadwell, 1995). Lastly, inflammatory events in the periphery may be sensed in the abdominal cavity by the vagus nerve. The vagus nerve transmits these signals to the CNS. The vagus nerve might then modulate the systemic inflammatory response through its cholinergic innervation and acetylcholine secretion (Tracey, 2002).

During acute illness, inflammatory markers and microbial products in the periphery signal to the CNS. The hypothalamus causes an altered body temperature, apathy, social withdrawal, depression, lethargy and decreased body weight. This pathophysiological response is referred to as "sickness behaviour".

MAGNETIC RESONANCE IMAGING

Magnetic resonance spectroscopy (MRS) is a rapidly developing field of neuroimaging that provides a non-invasive, *in vivo* analysis of neurochemicals (Hajek & Dezortova, 2008). MRS permits identification of and changes in neurometabolites that reflect neuronal dysfunction, glial reaction, and energy metabolism. Identifying and being able to detect changes in neurometabolites has been used clinically in patients with a range of neurological and psychiatric disorders, which may contribute to the understanding of a variety of neurological diseases (Ross & Sachdev, 2004). PET scanning may also be used to detect neurometabolites. MRS has advantages over PET in that MRS is an easily repeatable and harmless examination whereas PET scans expose the person to radio-active isotopes. PET scans are also very expensive and require special facilities.

How MRS works

Electromagnetic energy of a certain wavelength is emitted from the scanner and directed at the tissue under examination. This tissue either absorbs or re-emits the energy, which can then be measured. From the distribution and the various intensities of the measured energy, called a spectrum, information about the chemical properties of the tissue sample can be obtained (Cousins, 1995). Each neurochemical is sensitive to the pulse sequence (the series of radio frequency pulses applied to the tissue) and echo time (TE; the time between the radio frequency pulses) and therefore these parameters need to be carefully selected depending on which neurochemical one wishes to examine (Valenzuela & Sachdev, 2001).

Proton spectroscopy (^{1}H-MRS) utilizes the nuclear magnetic resonance of protons. The protons spin in response to electromagnetic energy. Spinning of the neurometabolites protons, that are present in the tissue, produces a spectrum that is characteristic of that neurometabolite. The spectrum emitted from neurometabolites is detected from a 3 dimensional volume of tissue called a voxel. Metabolites are present in low concentrations in the brain, therefore one needs to examine a homogenous voxel of brain tissue ranging from 1 to 27cm^{3} in order to detect different signals. Water is present in high concentrations in the brain; therefore the water resonance within the voxel needs to be suppressed to detect the millimolar concentrations of neurometabolites (Cousins, 1995).

Besides water, the volume of interest contains other types of compounds: macromolecules such as lipids, metabolites greater than 1millimolar (mM) and metabolites less than 1mM in concentration. In human tissue the most important of these are the metabolites greater than 1mM. These metabolites include: lipids, lactate, N-acetylaspartate, glutamate-glutamine, creatine, choline and myo-inositol (Hajek & Dezortova, 2008). They are identified by their peaks at specific frequencies at which they resonate; this is usually depicted in a spectrum (see *Figure 7*). The peaks of the spectrum measure the intensity of the emitted energy from the metabolite in the voxel during ^{1}H-MRS: a higher peak indicates a higher concentration of the metabolite. Interpretation of these metabolites is discussed below (see *Figure 7* for the graphic presentation of metabolites).

Interpretation of Metabolites

Lipids

The broad peak of lipids occur at 0.9 and 1.2 parts per million (ppm). An increased lipid peak usually indicates necrosis in brain tumours (Lin, Ross, Harris, & Wong, 2005).

Lactate

Lactate generally has a double peak occurring close together at a frequency of 1.33 ppm. The presence of lactate is indicative of anaerobic glycolysis, which is detected in areas of the brain associated with hypoxia, mitochondrial dysfunction, encephalopathy, lactacidosis, or brain injury (Lin et al., 2005). An increase in lactate levels has also been linked to inflammation. Increased lactate levels were shown to decline with the resolution of inflammation and decreased presence and activity of macrophages (Lewiston, Theodore, Robin, 1976).

Glutamate-glutamine

The peak produced by glutamate-glutamine (Glu/Gln) is a mixture of closely related amino acids, amines and derivatives that are involved in the excitation and inhibition of neurotransmission. The glutamate-glutamine peak lies between 2.1 and 2.4 ppm, and has been used as a marker of stroke, lymphoma, hypoxia and other metabolic brain disorders (Lin et al., 2005). Moats, Ernst, Shonk, & Ross (1994) found a small but significant decrease in the glutamate-glutamine peak in AD compared with controls. Antuono and colleagues (2001) also found a reduction in the glutamate-glutamine peak in AD patients. In this study glutamate-glutamine positively correlated with the MMSE and the Instrumental Activities of Daily Living in AD participants.

Creatine (Cr)

The Cr peak refers to the sum of creatine and phosphocreatine, with the Cr peak lying at 3.0 ppm. Cr acts as a central energy marker of neurones and astrocytes. Cr is considered relatively "constant" in most of the areas of the brain and is therefore used as an internal reference, with the metabolite/Cr ratio commonly used (Lin et al., 2005). The Cr concentration is greater in grey matter compared with white matter. This needs to be kept

in mind when placing voxels anatomically and when comparing metabolite/Cr ratio between different brain regions (Ross & Sachdev, 2004).

Choline (Cho)

Cho or trimethylamine is an umbrella term used for several soluble components of the brain myelin and fluid-cell membranes that resonate at 3.2 ppm in the spectrum (Lin et al., 2005). Cho is used as a marker for membranes, with an alteration in the signal indicating rapid membrane turnover (e.g. presence of a tumour) or demyelination in inflammatory disease processes such as multiple sclerosis (Lin et al., 2005; Mader, Rauer, Gall, & Klose, 2008). An increase in Cho and the Cho/Cr has been reported in AD patients compared with controls (Lazeyras et al., 1998; Wang et al., 2012). Wang et al. (2012) also reported an inverse correlation between the Cho/Cr ratio and the global Pittsburgh compound B (PiB) retention in cognitively healthy adults. As discussed previously, PiB is used to quantify Aβ load. In this study, Cho/Cr was the only metabolic ratio that was correlated with measures of cognition. It is hypothesised that there is a cholinergic deficiency in AD patients. One explanation for the increase in the Cho peak in AD is thought to relate to the membrane phosphatidylcholine catabolism. Catabolism of the membrane phosphatidylcholine may be an attempt to provide free choline for this deficient cholinergic system. Another explanation is that there is an increase in membrane turnover due to neurodegeneration (Wang et al., 2012). However, Dixon and colleagues (2002) reported a decrease in Cho with cognitive impairment. Studies investigating Cho in AD are limited and further research needs to be done to clarify the conflicting results from the above studies.

N-acetylaspartate (NAA)

NAA is considered a neuronal marker, resonating at 2.00 ppm, and is the predominant signal produced in the human brain on ^{1}H-MRS (Hajek & Dezortova, 2008). NAA is located almost exclusively in neurones and altered (usually decreased) NAA levels indicate either temporary neuronal dysfunction or permanent neuronal loss (Mader et al., 2008). NAA has been correlated with brain maturity and is thought to be more concentrated in grey matter than in white matter. Thus, depletion of NAA has been found to be greater in the grey matter (Valenzuela & Sachdev, 2001; Hajek & Dezortova, 2008). This is important when comparing different brain regions.

Due to NAA's excellent signal characteristics on ^{1}H-MRS, many studies have found reproducible changes in the NAA/Cr ratio in AD (Kantarci et al., 2000; Dixon et al., 2002). Wang et al. (2012) found that in the posterior cingulate gyrus and the hippocampus, AD participants had lower NAA/Cr ratios compared with MCI and control participants. MCI participants also had a lower NAA/Cr ratio compared with controls.

Dixon et al. (2002) reported a relationship between NAA and cognitive functioning: the NAA concentration in the hippocampus was positively correlated with MMSE scores and CAMCOG scores of controls and AD patients. They suggested that NAA might be useful in assessing the functional status of the hippocampus as it may reflect the viability of neurones in this region.

Myo-inositol (MI)

MI is a simple sugar alcohol that acts as an organic osmolyte in the brain and is involved in the functioning of the second messenger system. The MI peak resonates at 3.6 ppm on MRS and is composed primarily of myo-inositol and, to a lesser proportion, inositol-1phosphate and glycine (Ross & Sachdev, 2004). An elevation of the MI is thought to be related to microglial cell and astrocyte activation. MI has been shown to be increased in inflammatory conditions such as multiple sclerosis (Lin et al., 2005; Mader et al., 2008).

The MI/Cr ratio has repeatedly been found to be increased in AD patients when compared with MCI participants and controls (Kantarci et al., 2000; Wang et al., 2009). Kantarci et al. (2000) reported a significant increase in the MI/Cr ratio in MCI patients compared with healthy controls. A further increase in the MI/Cr ratio was found in probable AD participants compared to MCI participants. Similar results were found by Wang et al. (2009) who reported an increase in the MI/Cr ratio in AD patients when compared with controls and MCI participants. A pathological study by Kantarci et al. (2011) used the retention of PiB to show the extent of amyloid burden in cognitively healthy individuals. They found that global retention of PiB and PiB retention in the posterior cingulate gyrus were positively correlated with the MI/Cr ratio.

Due to the decrease in NAA and the increase in MI commonly found in AD, many studies use the NAA/MI or MI/NAA ratios when analysing and interpreting data. These ratios are robust in discriminating AD from controls, with a positive predictive value of 98%, thus potentially making it a good diagnostic discriminant (Shonk et al., 1995). Regional differences in the MI/NAA have also been shown in AD patients. Wang et al. (2009) found an increase in MI/NAA ratio in the hippocampus compared with the posterior cingulate area (Wang et al., 2009). Parietal MI/NAA ratios were also inversely correlated with numerous subtests on the neuropsychology CERAD battery in a study by Ackl et al. (2005).

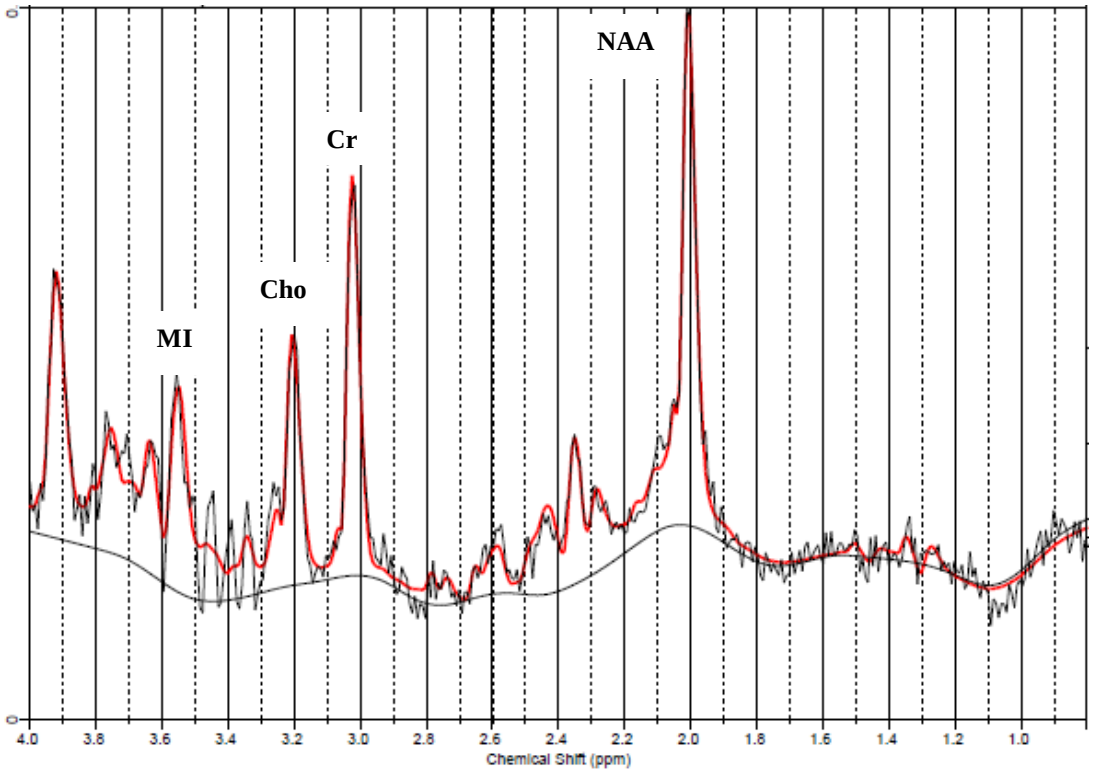


Figure 7: Spectrum acquired using proton spectroscopy (^{1}H-MRS) at 3 Tesla (3T) from the posterior cingulate gyrus in a healthy adult. The image depicts the peaks of: creatine and phosphocreatine (Cr) at 3 parts per million (ppm), glutamate (Glu) and glutamine (Gln) lies between 2.1 and 2.4 ppm (not labelled), myo-inositol (MI) at 3.6ppm, choline (Cho) at 3.2ppm, N-acetylaspartate (NAA) at 2ppm.
Note: This imagine was acquired during the pilot phase of the current study.

Brain Regions in Imaging

Imaging studies have mostly concentrated on volumetric analysis of the medial temporal structures such as the hippocampus and the entorhinal cortex. The hippocampus forms part of the limbic system and is buried in the depths of the temporal lobes. The entorhinal cortex region is located between the hippocampus and the transhippocampus spreading over the anterior borders of the parahippocampal gyrus (see *Figure 8a*). It is not surprising that research has focussed on these areas as this is where the neuronal loss is thought to typically start in AD (Rosenberg, 2005). Newer evidence suggests that the metabolism, perfusion and neuronal integrity of the posterior cingulate cortex are also affected in MCI and the early stages of AD (see *Figure 8b*; Nestor et al., 2003; Kantarci et al., 2011; Wang et al., 2012).

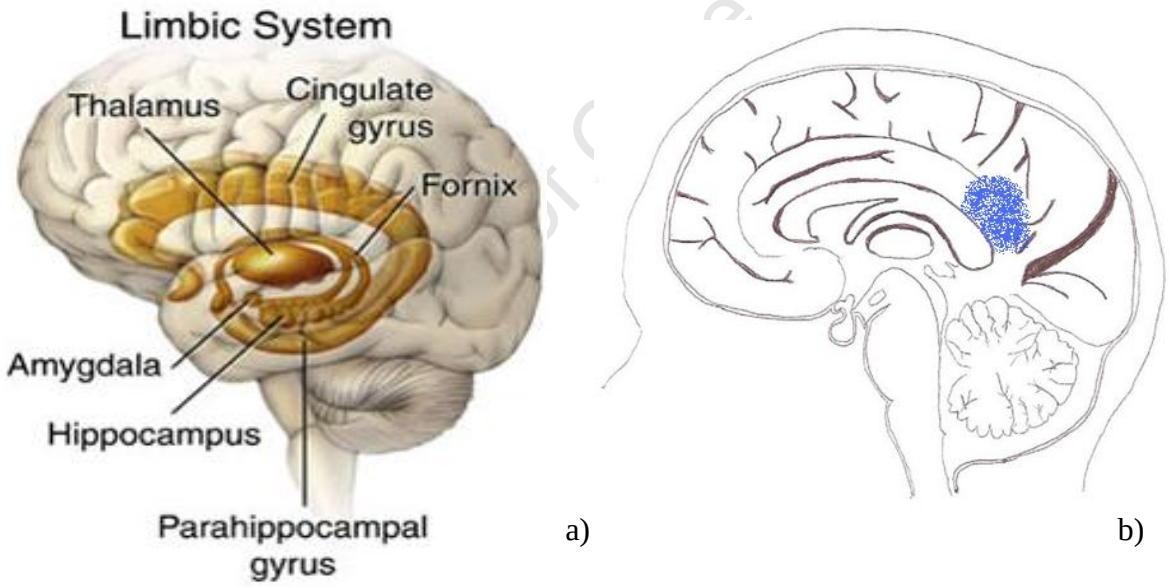


Figure 8: The anatomical location of the limbic system. a) The hippocampus forms part of the limbic system and is buried deep within the temporal lobes. b) A sagittal section of the brain depicting the anatomical location of the posterior cingulate (highlighted in blue). Figure 8a from: http://www.ahaf.org/alzheimers/about/understanding/anatomy-of-the-brain.html.

Pengas, Hodges, Watson, & Nestor (2010) investigated the role of the posterior cingulate region in MCI using volumetric magnetic resonance imaging (MRI). Their results show that when compared with controls, MCI participants had significant atrophy of the hippocampus and two posterior cingulate regions (Brodmann areas 29/30 and 23). Both the hippocampal and posterior cingulate regions had similar degrees of atrophy. The atrophy in

the posterior cingulate was not just a consequence of generalised atrophy as no significant difference in atrophy was found in the anterior cingulate between MCI and matched control participants. This study therefore supports the hypothesis for early pathological in the posterior cingulate in AD.

In a study by Choo et al. (2010), MCI and AD patients showed significantly reduced bilateral posterior cingulate cortex volumes in comparison to controls. AD patients had the lowest posterior cingulate cortex volumes. Anatomically, this study also showed that cingulum fibre disruption begins near the medial temporal structures, progressing to the posterior cingulate cortex. The progression of the disruption in cingulum fibres to the posterior cingulate cortex disruption coincides with the clinical progression of AD, from the preclinical to clinical stages. These results show that the posterior cingulate cortex is involved both directly and indirectly in AD: the direct involvement is the atrophy of the posterior cingulate cortex and the indirect involvement is the effect of the cingulum fibre degeneration due to the hippocampal atrophy (Choo et al., 2010).

Involvement of the posterior cingulate gyrus in early AD is further supported by a metabolic study. Nestor et al. (2003) calculated the metabolic rate of glucose using PET scanning. Metabolism was reduced in patients with mild AD throughout the limbic network and the posterior cingulate gyrus.

The hippocampus is relatively small and is anatomically in close proximity to areas of air and bone within the cranium, thus making it technically challenging to study using ^{1}H-MRS. Both bone and air distort the magnetic field homogeneity (Dixon et al., 2002). In AD research, distortion due to bone and air is exaggerated by the substantial quantity of CSF within the ^{1}H-MRS voxel. The presence of CSF is thought to be due to the large degree of atrophy of the medial temporal lobes causing an increase in the volume of the temporal horn of the lateral ventricles. All these factors lead to limiting spectra resolution, low signal-to-noise ratios and difficulty suppressing water signals (Dixon et al. 2002). The posterior cingulate is more homogenous than the hippocampus and therefore ideal in AD research as it also affected by AD pathology (Kantarci et al., 2000).

Interim Summary

^{1}H-MRS uses electromagnetic energy to identify changes in neurometabolites that reflect neuronal dysfunction, glial reaction, and energy metabolism therefore making it possible to identify these metabolites in a non-invasive way (Hajek & Dezortova, 2008). Two important metabolites in AD research are MI and NAA. MI is considered to be an astrocyte and glial marker and has been shown to be increased in inflammatory conditions (Lin et al., 2005; Mader et al., 2008). Many studies report an increase in MI in AD and MCI (Kantarci et al., 2000; Wang et al., 2009). NAA is a prominent marker of neuronal dysfunction and viability and has been shown to be significantly decreased in AD. The ratio between NAA and MI is robust in discriminating AD from controls and it is therefore frequently used in the literature (Shonk et al., 1995; Wang et al., 2009).

The hippocampus is technically a difficult area to examine with ^{1}H-MRS. The posterior cingulate is a more homogenous and has been shown to have early metabolic, perfusion and structural changes in MCI and AD. Thus, the posterior cingulate is an ideal region to examine using ^{1}H-MRS (Kantarci et al., 2000; Nestor et al., 2003; Kantarci et al., 2011; Wang et al., 2012).

APOLIPOPROTEIN E

The apolipoprotein E (APOE) gene codes for the 34 kilodalton (kDa) apolipoprotein E (ApoE) protein. The ApoE protein is produced in the CNS mainly by astrocytes, to a lesser extent by microglia and under certain conditions by neurones (Kim, Basak, & Holtzman, 2009). ApoE is mainly involved cholesterol transport and metabolism (Mahley, 1988).

As previously discussed, the APOE gene is encoded by a gene on chromosome 19 and has three main alleles: ε2, ε3 and ε4 (Utermann, Langenback, Beisiegel, & Weber, 1980). The ε3 allele has been shown to be the most common allele in Caucasian populations with an average frequency of 75%; the ε2 and ε4 allele frequencies are 8% and 15% respectively (Zannis, Kardassis, & Zannis, 1993). The ε4 allele has also been shown to be highest in sub-Saharan Africa countries (Corbo & Scacchi, 1999). Sandholzer and colleagues (1995)

showed that in the South African Khoi San population the presence of the homozygous ε4 allele (ε4/ ε4) was 3-5 fold more frequent when compared with a European population.

The presence of the APOE ε4 allele has been shown to increase the risk of developing AD. In a meta-analysis by Farrer et al. (1997), the odds ratio for developing AD was 2.6 for the ε2/ε4 genotype, 3.2 for the ε3/ε4 genotype and 14.9 for the ε4/ε4 genotype. Thus the risk is also dose-dependent. Homozygous (ε4/ε4) participants also have an earlier age of onset of AD than heterozygous participants (Farrer et al, 1997). However, the relationship between the risk of AD and the ε4 allele is thought to weaken with age, with some studies showing that the ε4 exerts it maximal effect before the age of 70 (Corder et al., 1994; Farrer et al., 1997; see *Figure 9*). Unlike the ε4 allele, the ε2 allele seems to have a protective effect, with the presence of the ε2 allele predicting a later age of onset of AD (Corder et al., 1994; Farrer et al., 1997). The odds ratio of developing AD with the ε2/ε2 or ε2/ε3 genotype is 0.6 (Farrer et al., 1997).

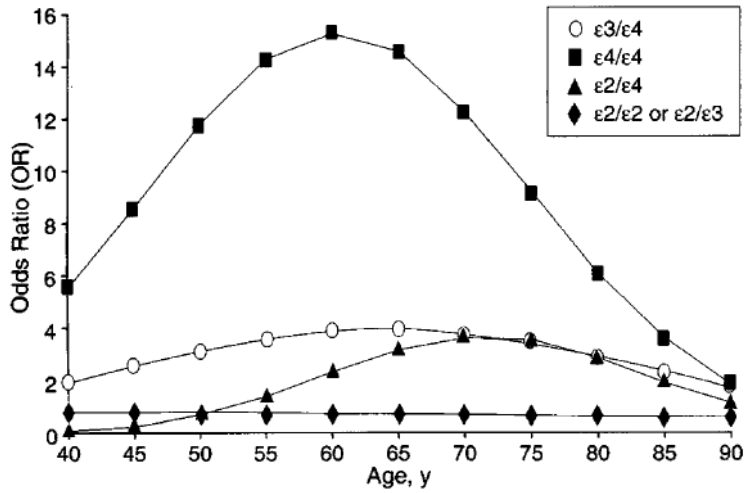


Figure 9: Relative odds of developing Alzheimer's disease according to apolipoprotein E (APOE) genotype and age among Caucasians. The odds ratio for the allelic variants ε2/ε4, ε3/ε4, ε4/ε4, and ε2/ε2 or ε2/ε3 are depicted according to the symbols in the box.
From: Farrer et al., 1997, Journal of the American Medical Association, 278, p.1353.

The relationship between the ε4 allelic variant and the risk of developing AD in Africa is still unclear. A study comparing African-Americans residing in Indianapolis versus people in Nigeria found that the APOE ε4 allele was not as strongly related to AD in the Nigerian cohort as in the African-American cohort (Osuntokun et al., 1995; Gureje et al., 2006).

This was also shown in a study in East Africa (Kenya and Tanzania) where non-demented elderly participants over the age of 65 had the same ε4 allele frequency as age-matched demented patients. In both groups, 25% of individuals had at least one ε4 allele (Sayi et al., 1997). These results suggest that, in Africa, the APOE ε4 genotype may not be as strongly related to AD in comparison to Western countries where there is a strong association. The lack of association suggests that both genetic and environmental factors may be responsible for increased risk of AD. Environmental factors, such as a high lipid and cholesterol intake typically seen in Western industrialized countries, *together* with the presence of the ε4 allele may strengthen the genetic association of ε4 and the risk of developing AD (Osuntokun et al., 1995; Gureje et al., 2006).

As well as its association with AD, the APOE ε4 allele has also been associated with poorer prognosis in multiple sclerosis (Schmidt et al., 2002b); poor recovery in traumatic brain injuries (Sorbi et al., 1995) and an increase in inflammation in patients following a cardiopulmonary bypass (Grocott et al., 2001). The increase in inflammation after bypass probably relates to, aside from APOE's role in cholesterol metabolism and transport, APOEs role in mediating the systemic and CNS's inflammatory responses (this is discussed later in this section). This response may be isoform specific (Lynch et al., 2003).

In summary, the APOE gene codes for the ApoE protein that is involved in lipid transport. In the CNS ApoE is secreted mainly by astrocytes. The gene has 3 allelic variants: ε2, ε3 and ε4. The ε4 allele has been shown to increase the risk of developing AD. Despite the apparent high frequency of the ε4 allele in Africa, little known about the risk of AD and the ε4 allele in this environment.

APOE and the Pathology of AD

The role of ApoE in the pathological processes of AD is not yet fully understood. It is thought that the APOE allele might differentially modulate the *clearance* and *accumulation* of Aβ plaques. The release of ApoE is increased in neurodegenerative diseases such as AD (Diedrich et al., 1991). ApoE binds to extracellular Aβ deposits, neurofibrillary tangles and cerebral vessel amyloid deposits (Namba, Tomonaga, Kwasaki,

Otomo, & Ikeda, 1991; Strittmatter et al., 1993). LaDu et al. (1994) found that ApoE3-Aβ binding was 20 fold greater than that of ApoE4-Aβ binding. Authors argue that the increase ApoE3 binding increases the clearance of Aβ plaques compared to the low ApoE4-Aβ binding. ApoE also affects the vascular drainage of Aβ through peripheral channels resulting in the accumulation of Aβ in the brain (Thal et al., 2007). Post-mortem studies provide evidence for the hypothesis that ε4 modulates the accumulation and clearance. AD homozygous ε4 brain tissue was shown to have greater Aβ plaques and neurofibrillary tangles when compared with AD patients with the APOE ε3 /ε3 genotype or the ε3/ε4 genotype (Tiraboschi et al., 2004). The ε4 isoform has been shown to increase the ratio of $A\beta_{1-40}$ to $A\beta_{1-42}$ in cerebral vessels, resulting in an accumulation of Aβ deposits and amyloid angiopathy (Fryer et al., 2005). The ε2 allele however, has been shown to provide some protection against the accumulation of plaques and tangles. The protective effect of the ε2 allele was also dominant in the case where there was an ε4/ε2 genotype (Nagy et al., 1995).

More recently the use of the PiB allowed researchers to investigate the accumulation of Aβ *in vivo*. Kantarci et al. (2012) showed that global retention of the PiB compound in cognitively healthy individuals progressively increased from ε2 to ε3 to ε4 carriers.

APOE and Cognition

The relationship between cognition and the ε4 allele is controversial. Henderson and colleagues (1995) showed that non-demented ε4 positive participants had a faster cognitive decline compared with non-demented participants with no ε4 allele. However, Small and colleagues (2000) found little evidence for an alteration in cognitive performance when comparing ε4 non-demented carriers with non-carriers (ε3 and ε2 allelic variants). All participants in the study performed similarly across all domains of cognition (Small et al., 2000). The relationship between APOE genotype and cognitive decline after the onset of AD is also controversial. Most studies, however, show that AD ε4 carriers have a greater rate of decline compared with AD participants with other alleles (Petersen et al., 1995). Martins and colleagues (2005) showed that the rate of cognitive decline in AD participants had a dose-dependent relationship with the ε4 allele, with homozygous patients declining faster than heterozygous patients.

Kantarci et al. (2012) were the first to examine the relationship between amyloid burden (using PiB retention), cognition and the APOE genotype in a cognitively healthy population. They found that the association between lower cognitive scores and higher retention of PiB was strongest in ε4 carriers. They suggested that APOE ε4, together with other factors such as cognitive reserve, mediates the relationship between Aβ load and cognitive function.

In summary, the ε4 allele is also thought to modulate the accumulation and clearance of Aβ plaques (Tiraboschi et al., 2004; Fryer et al., 2005; Thal et al., 2007). Studies have shown that ε4 carriers had a greater Aβ load (Tiraboschi et al., 2004; Kantarci et al., 2012). The ε4 allele mediates the relationship between Aβ load and decline in cognition. AD ε4 carriers have been shown to have a faster rate of cognitive decline (Kantarci et al., 2012).

Mechanism of APOE

The mechanisms by which the ε4 allele exerts its harmful effects over the ε2 and ε3 alleles are unclear. One hypothesis is that APOE may affect synaptic *plasticity* during *regeneration* and *repair.* ApoE is thought to be released in response to neuronal injury and may be needed for repair of the nervous system (Slezak & Pfrieger, 2003). Klunk, Panchalingam, Clure, Stanley, & Pettegrew (1998) showed that AD post-mortem brain samples with the ε4 allele had significantly higher phospholipid metabolites, glycerophosphocholine (GPC) and glycerophosphoethanolamine (GPE), compared with post-mortem brain samples from controls as well as AD ε4 negative samples. Both GPC and GPE are products of the breakdown of membrane phospholipids. The authors therefore argue that if the APOE alleles have different efficiencies for membrane repair then the higher levels of GCP and GPE may be a reflection of inefficient membrane repair mechanisms in ε4 carriers.

Evidence supporting this hypothesis is found in a study showing that cultured cortical neurones from an adult mouse that were incubated with human ApoE3 had greater neuronal outgrowths in comparison to neurones incubated with ApoE4. This relationship had a dose-dependent effect (Nathan et al., 2002). APOE ε4 has also been shown to be

more effective in blocking the endogenous protective ApoE production by activated microglia. This results in a decreased release of the ApoE which may impair neuronal repair processes (Guo et al., 2004).

APOE and Inflammation

ApoE is thought to have an immune modulating function (Mahley & Rall, 2000). ApoE has been shown by van den Elzen et al. (2005) to bind to antigens and then transport the antigens to antigen presenting cells. ApoE is also thought to be released from antigen presenting cells to survey the local environment for serum-borne lipid antigens (van den Elzen et al., 2005). ApoE has been shown to decrease T cell proliferation. The mechanism by which ApoE reduces T cell proliferation is thought to be due to ApoEs effect on IL-2. Kelly, Clay, Mistry, Hsieh-Li, & Harmony (1994) demonstrated that the addition of ApoE to mitogen-stimulated T-cells decreased the production of IL-2 by 50-65% when compared with non-ApoE exposed cells. The binding site or cell surface receptor that functions as the ApoE immunosuppressive receptor is, however, unknown (Mahley & Rall, 2000).

The immune modulatory role of ApoE is also thought to be isoform specific. The APOE ε4 allele has been shown to contribute to, or to exacerbate the inflammatory process and increase activation of microglia in AD. The ε4 allele is thought to be less effective at suppressing the activation of microglia than the other allelic variants. Support for this argument comes from post-mortem examination of the brains of individuals diagnosed with AD. Egensperger and colleagues (1998) showed an increase in the activation of microglia as well as the tissue area occupied by these cells, in the presence of the ε4 allele in post-mortem AD brains. The increase is dependent on the ε4 gene dose, with ε4/ε4 brains showing the greatest increase in microglia.

The ε4 allele may have a more robust pro-inflammatory effect than ε3 allele therefore exacerbating the detrimental effects of these cytokines (Guo et al., 2004). Lynch et al. (2003) found that transgenic mice carrying the human ε4 allele had higher levels of pro-inflammatory cytokines (TNF-α and IL-6) than those carrying the human ε3 allele. An *in vitro* study showed that glial cultures, in the absence of Aβ, released more IL-1β (a pro-

inflammatory cytokine) when stimulated with ApoE4 compared to stimulation with ApoE3. The release of IL-1β increased in a concentration-dependent manner (Guo et al., 2004).

Evidence for the pro-inflammatory effect of ε4 is also seen in other medical conditions. In patients who underwent a cardiopulmonary bypass, those with the APOE ε4 genotype had higher pro-inflammatory molecules IL-8 and TNF-α within 48 hours after surgery (Drabe et al., 2001).

In summary, the ε4 allele is thought to exacerbate the inflammatory process in AD. It may do so by increasing pro-inflammatory cytokines or by decreasing anti-inflammatory cytokines thereby leading to an unopposed pro-inflammatory effect.

In conclusion, the APOE ε4 allele may have properties that increase inflammatory responses that lead to a further increase in cognitive decline. Understanding the role of APOE in the progression of AD and APOE's relationship with neuroinflammation is important as it adds insight into the pathological process of AD. Drugs may be designed to interact with the APOE genotype and therefore mechanisms in which the isoforms interact with AD pathology need to be understood.

SUMMARY

AD is an age-related neurodegenerative disorder (Burns & Iliffe, 2009). Little is known about the prevalence of AD in South Africa. However, anecdotal evidence from our local clinics suggests that it is not uncommon. The strongest risk factor for AD is age and South Africa's population over the age of 50 years is projected to rise dramatically in the next few years (Hebert et al., 1995; US Census Bureau, 2012).

The neuropathological features of AD include accumulation of Aβ plaques, neurofibrillary tangles, neuronal loss and cerebral atrophy (Rossor, 1993; Braak & Braak, 1995; see *Figure 10*). The limbic areas (including the hippocampus) are most severely affected,

hence the early and pronounced memory impairments (Braak & Braak, 1995). Another important pathological feature of AD is neuroinflammation.

Evidence that inflammation is involved in the pathological process of AD comes from studies showing the presence of activated microglia deposition in the regions of Aβ and neurofibrillary tangles. Microglia are normally in a down-regulated, inactive state (see *Figure 10*). In AD the microglia become activated and release neurotoxic cytokines and other inflammatory molecules (Braak & Braak, 1995). The AD pathology itself also partially activates or "primes" microglial cells. Primed microglia are further activated during systemic infection resulting in an enhanced inflammatory condition leading to further cognitive decline (Perry, 2004). Other evidence that inflammation is involved in the pathological process of AD comes from large population-based longitudinal studies showing that long-term use of NSAIDs was associated with a reduced risk of developing AD. Parachikova et al. (2007) also showed that some cognitively healthy individuals with high Aβ loads had low levels of inflammation. This suggests that inflammation may be important in the clinical manifestation of AD.

Activation of microglia and subsequent cytokine release has been shown to occur early in the disease process in some studies. This initial inflammatory response may however subside with the progression of the disease (Hoozemans et al., 2005; Combrinck et al., 2006).

The BBB generally prevents cytokines and other inflammatory molecules from moving between the periphery and the CNS. It does, however, allow for signalling between the two. There are 3 routes of communication. First, systemic cytokines or microbial products in the periphery are able to signal to the brain endothelium across the BBB. The endothelium, in turn, signals to the perivascular macrophages and can further activate primed microglia in the brain parenchyma. The second route of communication is via circumventricular organs that lack a BBB (Banks, Kastin & Broadwell, 1995). Lastly, inflammatory events in the periphery are sensed in the abdominal cavity by the vagus nerve. The vagus nerve relays these signals to the CNS. The vagus nerve might then even modulate the systemic inflammatory response through its cholinergic activity (Tracey, 2002).

High levels of systemic inflammatory markers have been shown to be associated with a faster rate of cognitive decline in both cognitively healthy controls and AD patients (Schmidt et al., 2002a; Guerreiro et al., 2007; Holmes et al., 2009). Systemic inflammatory or infective events are also a major cause of delirium or acute transient cerebral dysfunction in the elderly (McCusker et al., 2001). Systemic infection may further activate primed microglia in the brain and so drive neurotoxic inflammatory damage. The latter could lead to accelerated neuronal loss and worsening cognitive functioning (Holmes et al., 2009).

Proton magnetic resonance spectroscopy (^{1}H-MRS) permits identification of, and changes in, metabolites that reflect neuronal dysfunction, glial reaction and energy metabolism (Ross & Sachdev, 2004). NAA is commonly investigated chemical in AD research using ^{1}H-MRS. NAA is a neuronal marker. A decrease in the NAA signal on ^{1}H-MRS indicates temporary neuronal dysfunction or permanent neuronal loss (Mader et al., 2008). MI is another metabolite that is often been studied. An increase in the MI peak on ^{1}H-MRS is related to microglial and astrocyte activation (Mader et al., 2008). The NAA/MI ratio is potentially a good diagnostic discriminate in AD (Shonk et al., 2005).

The APOE gene has 3 main alleles, ε2, ε3 and ε4. The presence of the ε4 allele has been shown to increase the risk of developing AD (Farrer et al., 1997). The ε4 allele has been shown to differentially modulate the accumulation and clearance of Aβ plaques leading to increased plaque density in AD (Fryer et al., 2005; Thal et al., 2007; Kantarci et al., 2012). AD ε4 carriers have also been shown to have a greater rate of cognitive decline compared with AD participants with other alleles (Petersen et al., 1995; Martins et al., 2005).

ApoE is also thought to have an immune modulating function (Mahley & Rall, 2000). Egensperger et al. (1998) showed increased activation of microglia in ε4 post-mortem AD brains compared with post-mortem brains of patients with other alleles.

In South Africa we have a rapidly increasing older population, high rates of systemic infection and high allelic frequency of ε4 in the indigenous population (Sandholzer,

Delport, Vermaak, & Utermann, 1995; Lawn, Bekker, Middelkoop, Myer, & Wood, 2006). For these reasons the current study aimed at investigating the relationships between systemic inflammation, CNS inflammation, APOE genotype, neuronal integrity and cognition in a population of older individuals in the Western Cape region of the country.

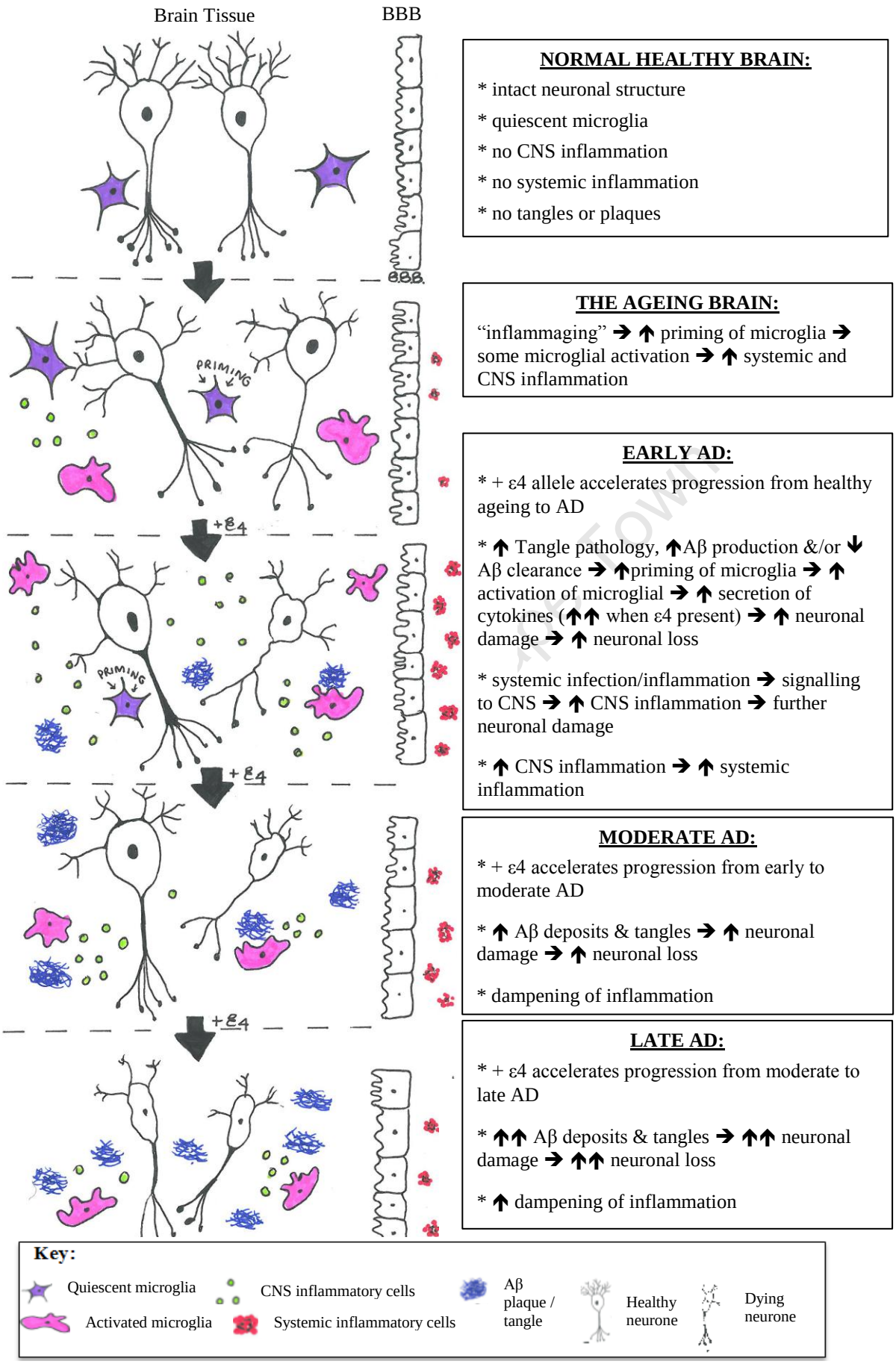


Figure 10: Summary of the Introduction. The figure depicts the pathological changes occurring in the CNS and inflammation in the systemic circulation, from a healthy individual to advanced or late stage Alzheimer's disease (AD). The presence of the ε4 allele (+ε4) accelerates the progression.

RATIONALE FOR THE PROPOSED RESEARCH

WHY IS RESEARCH ON OLDER ADULTS IMPORTANT?

Ageing is regarded as a triumph of development and is one of humanity's greatest achievements. As countries provide better nutrition, sanitation, health care systems and medical advances, their population ages (Osotimehin, 2012). Therefore, an ageing population has been widely perceived as being a problem only of high-income countries. Kinsella & He (2008) predict that the speed at which low- to middle-income countries age will however, outpace that of high-income countries. This is largely due to already high numbers of older adults and decreased fertility rates in the developed world. In South Africa the number of people over the age of 50 is expected to rise from 8 million in 2010 to 13.5 million in 2050 (U.S. Census Bureau, 2012).

As our population ages, individuals, families, communities and governments are faced with social, economic, demographic and cultural challenges. How we deal with these challenges will determine whether we reap the rewards of an ageing population. Understanding the health and disease status of the ageing population will allow governments to prepare their health care services, social support systems and design health care policies that cater for the specific needs of their population (He, Muenchrath, & Kowal, 2012).

The risk of non-communicable diseases increases with advancing age. As the number of people over the age of 50 increases, the rate of dementia prevalence can be expected to rise. A Delphi consensus study (Ferri et al., 2005) estimated that in 2001, 60% of the 24.3 million people with dementia came from developing countries. These numbers are expected to rise dramatically with an estimated 300% rate of increase from 2001 to 2040. This is compared to a 100% rate of increase in dementia cases in the developed world (Ferri et al., 2005). The prevalence of AD in South Africa is unknown. However, anecdotal evidence from local clinics suggests that it is not uncommon.

WHY STUDY AD IN SOUTH AFRICA?

It is important that AD be studied in an African context as the rate of dementia prevalence is expected to rise. There is a dearth of knowledge and research in this area and most risk factors for AD have been identified in Europe and North America. As mentioned in the Introduction, a study found that risk factors associated with AD, such as the APOE ε4 allele, were not as strongly related to AD in a Nigerian cohort as in an African- American cohort. These results suggest that additional environmental factors such as high cholesterol and lipid intake may, together with the ε4 allele, contribute to AD prevalence (Osuntokun et al., 1995; Gureje et al., 2006). The homozygous ε4 allelic frequency in indigenous populations of southern Africa has been shown to be 3-5 times higher when compared to European populations (Sandholzer et al., 1995). Therefore, research on the effects of the ε4 allele in our population will be very informative.

High levels of poverty, overcrowding and inadequate sanitation and nutrition have contributed to high levels of systemic infection in the Western Cape (de Swardt, Puoane, Chopra, & du Toit, 2005; Lawn et al., 2006). South Africa has one of the highest rates of tuberculosis and HIV/AIDS infections globally (Lawn et al., 2006). As discussed in the Introduction, Aβ and tau pathology prime microglia. During acute infection, microglia are further activated leading to an exaggerated inflammatory response. Holmes and colleagues (2003; 2009; 2011) have also showed that AD patients who acquire a systemic infection have a faster rate of cognitive decline than AD patients who remain healthy (Holmes et al., 2003; 2009; 2011). These studies were conducted in an industrialized country with a low prevalence of systemic infection; rates of systemic infection are much higher in South Africa.

The cost of dementia care is expected to increase more rapidly than the prevalence (WHO, 2012). Of the US$315 billion worldwide dementia care cost in 2005, it was estimated that one third of that was informal care. Therefore, informal care constitutes a major cost component, particularly in less developed regions (Wimo, Winblad, & Jönsson, 2007). It is essential that South Africa prepares for the financial implications of the rising cost of dementia care. With research, government will be able to allocate resources to assist in this regard.

THE USE OF BIOMARKERS

The use of biomarkers is helpful in understanding the disease process. A biomarker, as defined by the Biomarker Definition Working Group (Atkinson et al., 2001), is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological response to a therapeutic intervention". Biomarkers help identify the disease process and may be used in the predicting or monitoring the course of the illness. Inflammation is a pathological hallmark of AD. The use of inflammatory markers as biomarkers may be helpful in understanding the pathological process of the disease. Few biomarker studies have been performed in a developing world context where there is a high prevalence of systemic inflammatory conditions together with a high ε4 allelic frequency.

The Western Cape offers a unique setting for biomarker research. Although South Africa is largely regarded as a developing country, the University of Cape Town does have access to some sophisticated neuro-imaging equipment. There are only few places in Africa that have a 3 Tesla (3T) MRI scanner and experienced radiographers to perform the scans. The Cape Universities Brain Imaging Centre (CUBIC) is a research unit that allows us to perform MRS scans on our participants using a 3T scanner. Lumbar punctures were not permitted by the ethics committee in this study as there were no clinical/ diagnostic reason or beneficial therapeutic intervention for performing one. MRS is useful as it allows one to quantify metabolic changes in AD brains *in vivo*.

In summary, the identification of risk factors for AD such as APOE genotype is important in a population and environment-specific context. Identifying different patterns or markers of inflammation may also be important in developing reliable biomarkers, with special reference to our context. Understanding the relationships between these different factors is important as it may lead to a better understanding of the mechanisms underlying disease prevalence in this country. Research on the different stages of disease process and identifying biomarkers for these different stages may also be important in drug development and monitoring the response to treatment.

In conclusion, dementia is a devastating condition that imposes a huge burden of care on families, caregivers, as well as the health care system. There are few dementia services available to South African communities to provide support and relieve some of the burden of disease on caregivers. Medical conditions of the elderly also tend to be ignored due to a lack of resources in the South African health care settings. Cholinesterase inhibitors are currently not available in the state / public health service.

Understanding the association between systemic inflammation, CNS inflammation, neuronal loss, APOE genotype and cognitive functioning may lead to better preventative public health interventions and education on AD in a context specific to a South African population. Research provides knowledge that will lead to increased public awareness. Research also creates a foundation with which to lobby government.

AIMS AND HYPOTHESES

The overall aim of this study is to investigate the relationship between systemic inflammation, CNS inflammation, neuronal loss and the APOE genotype in a population of elderly South Africans in the Cape Town region. Furthermore, the aim is to identify how these inflammatory and genetic factors influenced cognition over a period of one year. A diagrammatic framework is represented in *Figure 11*.

To assess systemic inflammation, pro-inflammatory (TNF-α, IL-1β) and anti-inflammatory (TGF-β_1, TGF-β_2, TGF-β_3, IL-10) cytokines, the total white cell count, monocyte count and the ESR in peripheral blood will be measured. ^{1}H-MRS imaging for the MI and N-acetylaspartate and N- acetylaspartylglutamate (NAA+NAAG) peaks will be examined as an index of CNS inflammation and neuronal dysfunction respectively.

HYPOTHESES

A number of hypotheses will be tested in a cross-section study, longitudinal study and a MRS study:

Cross-Sectional and Longitudinal Studies:

Hypothesis 1: Systemic inflammatory markers will be highest in the early stages of AD, declining with the progression of the disease. Participants with moderate AD and cognitively healthy controls will have comparable levels of inflammatory markers, whereas participants with severe AD will have lower levels of inflammatory markers when compared with the controls.

Hypothesis 2: AD and control participants, either homozygous or heterozygous for the APOE ε4 allele, will have lower cognitive scores compared with participants with no ε4 allele.

Hypothesis 3: Participants, either homozygous or heterozygous for the APOE ε4 allele, will have higher baseline levels of systemic inflammatory markers compared with participants with no ε4 allele.

Hypothesis 4: High levels of baseline systemic inflammatory markers will predict a faster rate of cognitive decline over a one-year period.

Hypothesis 5: The presence of the ε4 allele (either homozygous or heterozygous) in participants will exacerbate their rate of cognitive decline when compared with participants with no ε4 allele.

MRS Study:

Hypothesis 6: MI, a central nervous system inflammatory marker, will be highest in the early stages of AD, declining with the progression of the disease.

Hypothesis 7: There will be a direct relationship between the NAA+NAAG peak on MRS in the posterior cingulate gyrus and the hippocampus, and cognitive functioning; i.e. the better the cognitive functioning, the higher the NAA+NAAG peak.

Hypothesis 8: The ratio between MI and NAA+NAAG will be negatively correlated with cognition.

Hypothesis 9: Participants, either homozygous or heterozygous for the APOE ε4 allele, will have higher baseline levels of MI and lower levels of NAA+NAAG compared with participants with no ε4 allele.

Hypothesis 10: There is a direct association between systemic inflammation and CNS inflammation and neuronal loss. MI, a marker of CNS inflammation in the posterior cingulate and hippocampus, will be positively correlated with systemic measures of inflammation (cytokines, OPN, white cell count, monocyte count and ESR) and negatively

correlated with NAA+NAAG. NAA+NAAG will also be negatively correlated with systemic inflammatory markers.

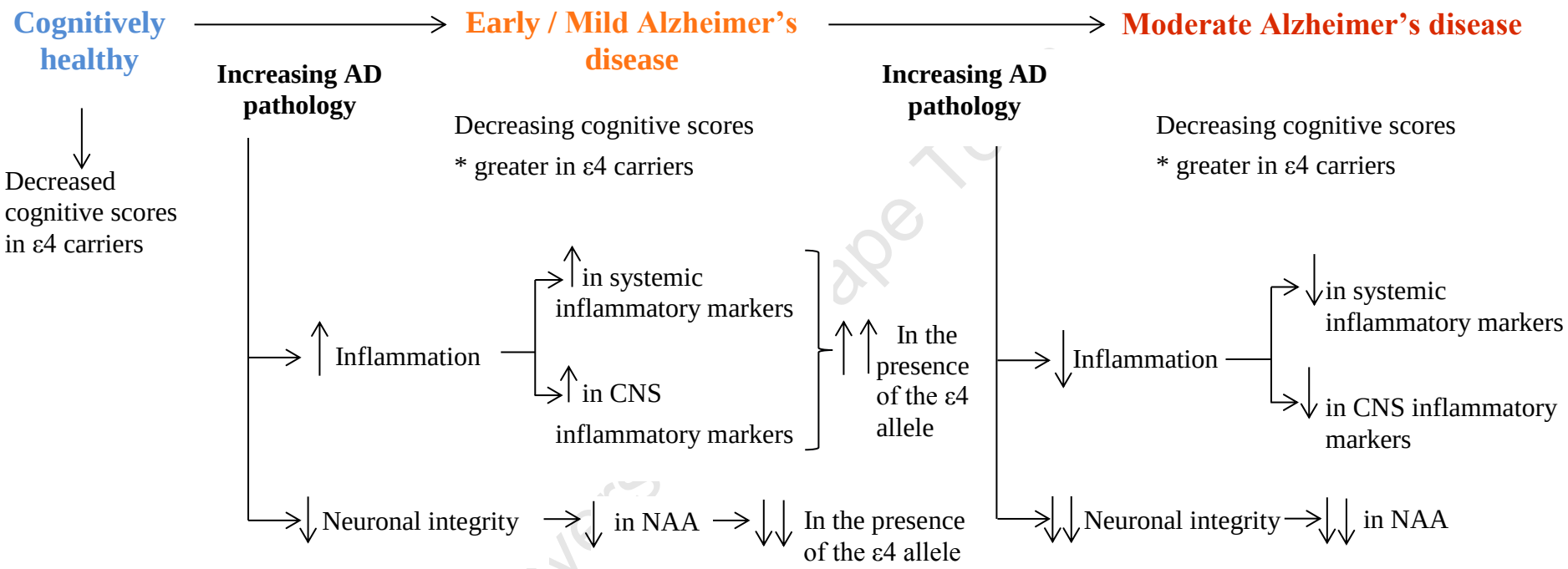


Figure 11: A diagrammatic representation of the study's underlying framework. The diagram shows the progression from cognitively healthy (left) to moderate Alzheimer's disease (AD) (right). It is hypothesised that the progression from cognitively healthy to early or mild AD is accompanied by an increase in systemic and CNS inflammation and a decrease in neuronal integrity. The progression from early AD to moderate AD is then accompanied by a further decrease in neuronal integrity and a dampening of inflammation. The hypotheses predict that the presence of the ε4 allele a) increases the rate of cognitive decline b) increases systemic and CNS inflammatory markers c) decreases neuronal integrity.

METHODS

RESEARCH DESIGN AND SETTING

The study consisted of three sections:

(1) A cross-sectional component that investigated the relationship between peripheral inflammatory markers, APOE genotype and cognition.

(2) Another cross-sectional component that investigated the relationship between neurometabolites on MRS, the APOE genotype, peripheral inflammatory markers and cognition.

(3) A longitudinal component that investigated the relationship between peripheral inflammatory markers at baseline, APOE genotype and cognitive decline over a 1-year period.

This study formed part of a larger observational study investigating risk factors for cognitive impairment in a sample of older adults from the Cape Town area. All study procedures were conducted at the Research Unit in the Neurology Ward at Groote Schuur Hospital or at the participants' places of residence. The MRS component was conducted at the Cape Universities Brain Imaging Centre (CUBIC) at the Medical Campus of Stellenbosch University, Tygerberg Hospital.

PARTICIPANTS

The Cross-sectional and Longitudinal Studies

Participants were recruited from the Cape Town metropolitan region between the year 2009 and 2012. Older adults with mild to moderate AD were recruited from community Old Age Homes in Cape Town and the Geriatric Medicine and Memory Clinics of the Groote Schuur Hospital, Cape Town. Participants were also recruited via referrals from local general practitioners and neurologists. Pamphlets outlining the objective of the study were placed in general practitioners consulting rooms and interested participants were

encouraged to speak to their doctor about participation. Cognitively healthy, community-dwelling older adults were also recruited from community Old Age Homes and via word of mouth advertising. In some cases, spouses or relatives of enrolled AD participants were interested in participating in the study and if suitable, these participants were enrolled.

The MRS Study

Participants enrolled in the study were contacted and screened for contra-indications to MRI. Due to the large cost of MRI scans, a limited number of controls were invited to participate in this section of the study. Controls were selected based on age; older controls were invited to take part. This was done to try match the AD and control groups as far as possible. AD and control participants with no contra-indications to MRI scanning were invited to undergo MRI and MRS scanning after signing additional consent.

Inclusion and Exclusion Criteria

Individuals who were 55 years of age or older, who had basic literacy (i.e., the ability to speak, read and write in English or Afrikaans), and who had an MMSE score of more than 12 were eligible for the study. The inclusion and exclusion criteria for the cross-sectional and longitudinal studies are summarized in Table 2.

Participants with clinically overt systemic inflammatory conditions or infections were excluded. Participants who had an ESR above 40mm per hour were also excluded.

For the MRS study, participants were excluded if they had any of the following: pacemaker, aneurism clips, artificial heart value, vena cava filters, prosthesis, shrapnel in their eye or body, neurostimulator, cochlear implant (ear) or hearing aid (Appendix A). Participants who had an ESR above 40mm per hour at the time of their MRS scan were excluded from analyses that involved peripheral blood inflammatory markers.

Table 2
Inclusion and Exclusion Criteria for the Current Study

Inclusion criteria	Exclusion criteria
Age 55 years old and older Basic literacy • Basic ability to speak, read, and write in English or Afrikaans Cognition (controls) • Reported normal cognition Cognition (patients) • Possible or probable AD by NINCDS/ADRDA criteria • MMSE score > 12 Informant • Availability of close relative/ friend or caregiver who could provide information about cognitive change and functional abilities	Medical history Diagnosis of: • HIV/AIDS • Uncontrolled hypertension • Uncontrolled diabetes mellitus • Any other medical condition that, in the opinion of the investigator, precluded the patient from participation Presence of infection • Clinical features of infection at the time of assessment and blood-taking • ESR > 40mm per hour Psychiatric history • Presence of any major disorder (e.g. depression: GDS>8) Neurological history • Presence of any other neurological disorder e.g. Parkinson's disease, Huntington's disease • Stroke within the last 6 months • Significant head injury within the last year

Sample Size Estimation

A sample size calculation (power = 0.8) was done. ESRs for some participants that had been recruited in the larger study were available. Therefore the use of the ESR for *a priori* power calculations was a practical choice. A sample size of 15 participants in each group (mild AD, moderate AD, severe AD and controls) in the cross-sectional analysis was

needed. A significant difference in the frequency of the APOE ε4 allele was seen in a case-control study of 33 AD patients and 35 controls (Combrinck et al., 2006).

Post-hoc power calculations for differences in inflammatory markers at the different stages of AD were done. Ideal power (1 - β) was set at .80 and α = .05, two-tailed:

IL-10: when the mild AD and moderate AD groups were compared, an actual power of 0.06 was achieved with an effect size of 0.08. When the mild AD and severe AD groups were compared, an actual power of 0.34 was achieved with an effect size of 0.58. When the mild AD and control groups were compared, an actual power of 0.20 was achieved with an effect size of 0.35.

TNF-α: when the mild AD and moderate AD groups were compared, an actual power of 0.28 was achieved with an effect size of 0.53. When the mild AD and severe AD groups were compared, an actual power of 0.16 was achieved with an effect size of 0.36. When the mild AD and control groups were compared, an actual power of 0.32 was achieved with an effect size of 0.49.

TGFβ_1: when the mild AD and moderate AD groups were compared, an actual power of 0.05 was achieved with an effect size of 0.002. When the mild AD and severe AD groups were compared, an actual power of 0.05 was achieved with an effect size of 0.008. When the mild AD and control groups were compared, an actual power of 0.06 was achieved with an effect size of 0.109.

TGFβ_2: when the mild AD and moderate AD groups were compared, an actual power of 0.05 was achieved with an effect size of 0.07. When the mild AD and severe AD groups were compared, an actual power of 0.05 was achieved with an effect size of 0.03. When the mild AD and control groups were compared, an actual power of 0.05 was achieved with an effect size of 0.03.

MI: when the AD and control groups were compared, an actual power of 0.06 was achieved with an effect size of 0.09.

Ethical Considerations

Ethical approval for all study procedures was obtained from the Research Ethics Committee of the University of Cape Town, Faculty of Health Sciences and Groote Schuur Hospital (Approval #: 346/2008). All procedures were conducted according to the ethical guidelines and principles of the International Declaration of Helsinki (2002) and the South African Guidelines for Good Clinical Practice (Department of Health, 2006).

All participants were required to sign informed consent (Appendix B). Informed consent was obtained before the participant was enrolled in the study and before the participant had their scan. In the case of those with cognitive impairment, a close relative, friend or caregiver was also required to sign the consent forms. The consent form emphasized that participants were free to withdraw from the study at any point without this affecting their general medical care.

Anonymity with regards to laboratory tests, data storage and publications was ensured. On enrolment all participants were assigned a study number. The first participant in the study was assigned the number 001 and each subsequent participant was assigned a study number in numerical order. All samples, including those sent to the laboratory for tests, were labelled with the participants' study number and not their name. Files with the participants' study observations were stored in a locked room. Only researchers involved in the study had access to this room.

Participants were reimbursed for transport costs to and from either Groote Schuur Hospital or the imaging centre and their homes.

PROCEDURE FOR STUDY

The Cross-Sectional and Longitudinal Study

Participants that were interested in taking part in the study were contacted. Participants were briefly screened to see if they were suitable for the study. Arrangements were then made for the participants' first *baseline visit*. See *Figure 12* for a summary of the study procedures.

The majority of participants were assessed at the Research Clinic in the Neurology Unit, Groote Schuur Hospital. Exceptions were made in some instances where the participant was unable to attend. In these cases they were assessed in their place of residence. The study was explained to the participant and, if he/she was interested in participating, informed consent was obtained (see Appendix B). All participants included in the study had an informant – usually a spouse or another close relative - who was required to be in regular contact with the participant. The informant completed the Deterioration de Cognition Observee (DECO) and the Bristol Activities of Daily Living (BADLS questionnaires - see Information Gathering and Measures for details). Socio-demographic information, including data about age, handedness, gender, and level of education achieved was collected from the participant and informant. The participant also completed the Geriatric Depression Scale (GDS - see Information Gathering and Measures for details).

All participants underwent cognitive testing (see Information Gathering and Measures for details). Tests were conducted in English. English was the second language of 33 participants. However, all participants were all able to speak English fluently. Cognitive testing took approximately 90 minutes to complete.

A medical doctor performed a comprehensive clinical assessment that included a full medical history, a general physical examination and a detailed examination of the nervous system. Blood tests for electrolytes, glucose, cholesterol, syphilis serology, liver function, renal function, serum vitamin B12, folate levels and thyroid function (thyroid stimulating hormone) were performed to exclude participants with systemic, metabolic or endocrine

disorders that might contribute to cognitive impairment. Additional blood was taken for inflammatory markers and apolipoprotein (APOE) genotyping. Blood samples were sent to The National Health Laboratory Service (NHLS) for the white cell count, monocyte count and ESR. Blood for cytokine analysis and APOE genotyping was stored until analysis (see Information Gathering and Measures for details).

Seven participants underwent computerised tomography (CT) scanning for additional diagnostic clarification. In addition to these 7 participants, 14 participants were sent for CT or MRI scans by their medical doctors before they were enrolled in our study. Researchers excluded all participants that were not eligible for the study (see Inclusion and Exclusion Criteria). The remaining participants were then assigned to groups by a neurologist and neuropsychologist (including myself) using the NINCDS/ADRDA criteria (see Table 1) for the diagnosis of possible and probable AD (McKhann et al., 1984). The diagnostic process included a multi-step procedure. A neurologist and neuropsychologist used the DECO and BADLS scores in conjunction with information gleaned during the history taking to determine the degree of cognitive decline and level of activities of daily living for each participant. The neurologist rechecked the medical history notes, blood tests and clinical examination notes to make sure that these investigations did not reveal any other systemic or brain disease that could account for cognitive impairment. The neurologist also examined the MRI or CT scans of the 21 participants who had undergone scanning. The neuropsychologists calculated the participants' cognitive scores to determine the level of functioning on the different cognitive domains. Once all the information was bought together a diagnosis was made.

All participants were invited to participate in a follow-up assessment 12 months after their baseline visit. All participants were contacted and arrangements were made for their follow-up assessment. At this follow-up visit the CAMCOG cognitive test battery (which included the MMSE and learning subscale) was repeated (see *Figure 12*).

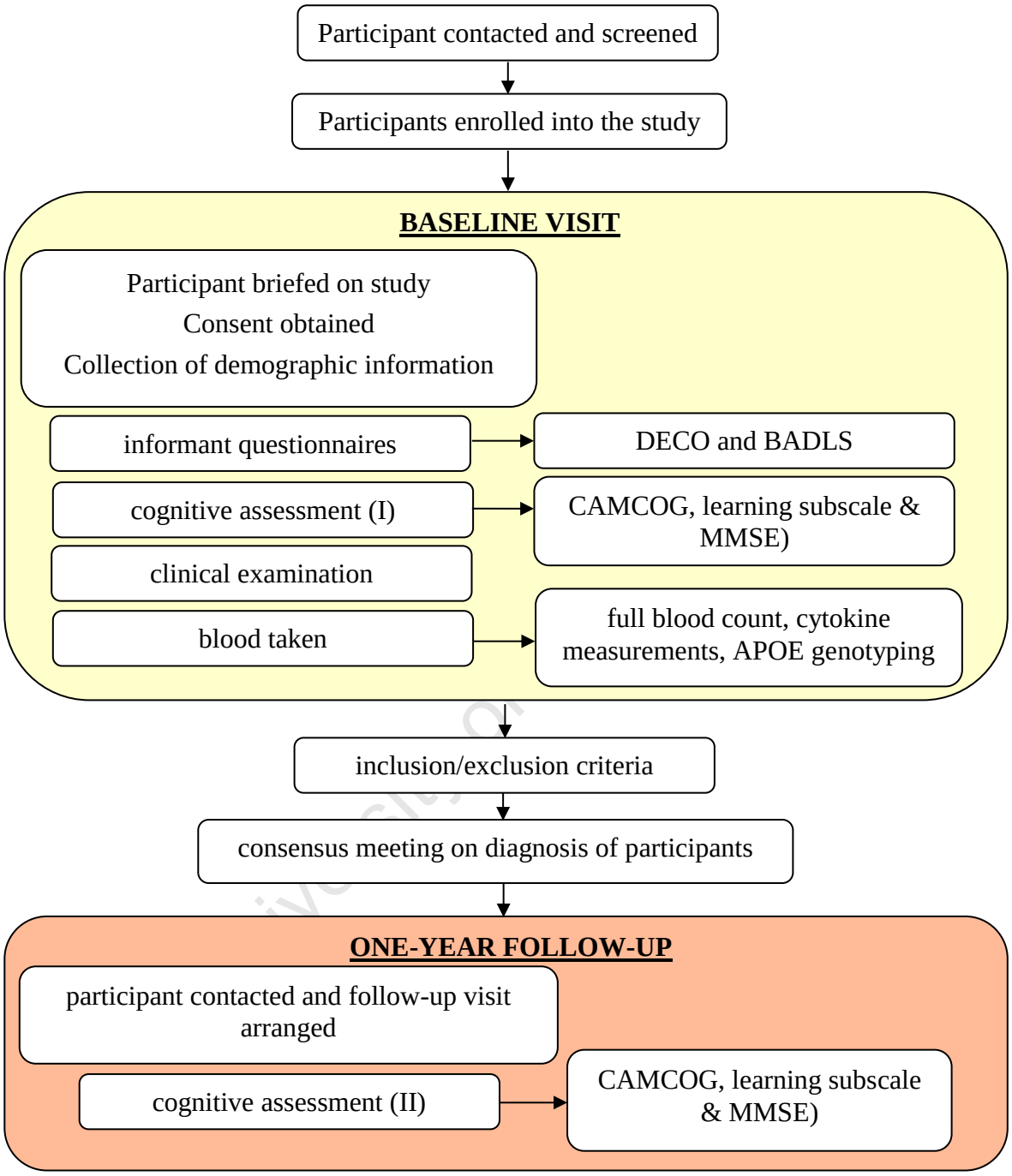


Figure 12: Procedures for the cross sectional (■) and longitudinal (■) studies.

THE MRS STUDY

The MRS imaging study was performed as a separate cross-sectional study within the larger study. It involved participants for whom there were no contra-indications to performing MRI scans (see Appendix A for a list of contra-indications). Participants were contacted and screened and if suitable, arrangements were made for them to go to CUBIC.

AD participants were accompanied to CUBIC by a caregiver. All participants were first administered the MMSE and learning subscale from the CAMOCG test battery on the morning (or day) of their scan. Cognitive testing took approximately 30 minutes. The radiographer then prepared the participant for the scan by making sure all metal objects had been removed. The scanning procedure lasted 20 minutes.

To reduce the amount of stress placed on participants before scanning, blood was taken after the scan was complete. The NHLS of Groote Schuur Hospital analysed blood samples for the total white cell count, differential white cell (including monocyte) count and ESR. Whole blood was kept on ice prior to centrifugation at 4000 revolutions per minute (rpm) for 10 minutes in a standard laboratory bench top centrifuge machine. Serum for cytokine analysis was then stored at -80^0 C until further analysis (see cytokine measurements, under Peripheral Inflammatory Measures).

My role in this study consisted of the following categories:

1) Recruitment of participants.
2) Contacting and pre-screening participants referred to the study by general practitioners, neurologists as well as participants that had heard about the study from their community.
3) Scheduling participant's baseline and follow-up visits.
4) Briefing the participant on the study and documentation of consent procedures.
5) Collection of demographic information.
6) Neuropsychological test battery administration and scoring at baseline and follow-up.
7) Specimen collection and laboratory processing of samples.

8) Diagnosis consensus of participants.

9) Contacting, screening and orchestrating the participants visit to CUBIC.

10) Briefing participants on procedures at CUBIC as well as neuropsychological test administration and sample collection at CUBIC.

11) Processing and analysis of all MRS data.

INFORMATION GATHERING AND MEASURES

Sociodemographic and Lifestyle Measures

A) The Deterioration de Cognition Observee (DECO)

The DECO (Richie & Fuhrer, 1996; Appendix C) is a 19-item questionnaire in which an informant rates the degree of cognitive change in the participant over the past year. The DECO items include questions about daily activity levels, memory (semantic and visual memory for places and procedures), visuospatial performance and the learning of new skills. For example, the informant was asked: "How well does he/she manage his/her money?" The informant was asked to rate each item as, compared to 1 year ago, *Better or About the Same, Not as Well*, or *Much Worse*. Responses were scored as 2, 1 or 0 respectively, with a maximum score of 38 indicating no change in functioning over the past year. A score less than 25 is generally taken to indicate the presence of dementia. Studies using the DECO have shown the questionnaire to have high test-retest reliability and good face validity (Ritchie & Fuhrer, 1996). The DECO has previously been used in South African research studies (Lenger, de Villiers, & Louw, 1996; Heckmann et al., 2004).

B) The Bristol Activities of Daily Living Scale (BADLS)

The BADLS (Bucks, Ashworth, Wilcock, & Siegfried, 1996; Appendix D) is a 20-item scale that was specifically designed to obtain information from caregivers about daily living abilities of patients with dementia. The BADLS includes items related to hygiene, dressing, eating and handling of finances. The BADLS has good test-retest reliability and good construct validity (Burns, Lawlor, & Craig, 2004). This test has previously been used in a South African research study (Jelsma, Mkoka, Amosun, & Nieuwveldt, 2004).

C) The Geriatric Depression Scale (GDS)

The GDS (Yesavage et al., 1983; Appendix E) is a 15-item self-reported questionnaire that requires participants to respond by answering yes or no to items enquiring about how they felt over the past week. The GDS is often used in geriatric clinical assessments and was developed as a measure for depression in the elderly. Both the original and shortened versions of the GDS display high internal consistency, test-retest reliability and validity in American studies (Yesavage et al. 1983). A cut-off score of 8 was used to indicate the presence of depression in the current study. The GDS has previously been used in a South African study examining depression and social support in elderly people (Rodriguez, Brathwaite, & Dorsey, 2002).

Neuropsychological Measures

A) The Cambridge Cognitive Examination for Mental Disorders of the Elderly – Revised (CAMCOG-R; Huppert et al., 1995) is a section of a larger instrument, the Cambridge Mental Disorders of the Elderly Examination - Revised (CAMDEX- R; Roth, Huppert, Mountjoy, & Tym, 1988). The CAMCOG-R investigates eight aspects of cognition: orientation, language, memory, calculation, concentration and attention, praxis, abstract thinking and visuospatial perception. Sub-scores for each of these cognitive domains were calculated. The CAMCOG-R consists of 67 items. If the participant gets the item right, one point is awarded. Some items require the participant to give more than one answer, for these items one point for each answer is given. For example, one of the 67 items requires the participant to recall three items. For this item, a maximum of 3 points is awarded. The participant can achieve a maximum possible score of 105. Lower scores indicate cognitive impairment. The CAMCOG-R (which will now be referred to as the CAMCOG) can be used to rate the severity of cognitive impairment/dementia.

A few of the CAMCOG items were altered in order to suit the South African population. These items included questions relating to historical events and recognition of famous people. For example, questions like “Who was the Prime Minister of South Africa during the Second World War?” and “Which Island off the coast of South Africa was used as a

leper colony, military base and prison?" were asked. The CAMCOG booklet, which contains pictures relating to some of the items that were changed, was reprinted with images that were more appropriate for administration to a South African population. The CAMCOG has high test-retest and inter-rater reliability (O'Connor, Pollitt, Brook, & Reiss, 1989), and has been used in several longitudinal and cross-sectional studies of the neuropsychology of dementia (Walker, Allen, Shergill, & Katona, 1997; Nielsen, Lolk, Andersen, Andersen, & Kragh-Sørensen, 1999; Martins, Oulhaj, de Jager, & Williams, 2005).

B) The Learning Subscale

The CAMCOG *learning subscale* (scored out of 17) tests both visual and verbal memory recall. The learning subscale score is therefore a marker of episodic memory impairment, the first cognitive domain to decline in most AD patients (Mickes et al., 2007). The learning subscale score in this study was used as a proxy of disease severity and progression (Combrinck et al., 2006).

C) The Mini-Mental State Examination (MMSE)

The MMSE (Folstein et al., 1975), which is incorporated within the CAMCOG, is a common tool used in the assessment of dementia. The test consists of 19 questions that cover various cognitive functions including orientation, language, memory, attention, naming of objects, following of verbal and written commands, sentence production and the ability to copy a complex figure. A maximum score of 30 can be obtained on the MMSE, with lower scores indicating cognitive impairment. The MMSE has been used in many studies to examine the relationship between cognitive function and neuroinflammation (see, e.g., Guerreiro et al., 2007). This measure has previously been used in an AD study by Heckmann et al. (2004) in South Africa, and is commonly used in South African clinical practice.

Peripheral Inflammatory Measures

A) White cell count, ESR, and monocyte count

Systemic markers of inflammation *viz*. the ESR, total white cell count and differential white cell (including monocyte) count were measured at the participants first visit (baseline visit) and at the time of the MRS study. At baseline, blood was taken from each participant. Samples were collected between 12h00 and 13h00 on the day of the participant's clinical and cognitive assessment. For the MRS study, blood was taken after the participant had their scan. All blood samples for the total white cell count, monocyte count and ESR were analysed at the Groote Schuur Hospital NHLS.

B) Cytokine Measurements

Blood samples from participants were collected at baseline and after neuroimaging for cytokine analysis. Blood was taken from each participant, placed on ice and centrifuged within one hour at 4000 rpm for 10 minutes in a standard bench top laboratory centrifuge machine. Serum supernatants were pipetted into cryotubes and stored in a -80°C freezer until they were analysed.

Due to the number of wells per plate, some controls were excluded from the cytokine analysis. The youngest controls were excluded in an attempt to match the groups on age. Cytokines included in the analysis were anti-inflammatory cytokines (TGF-β_1, TGF-β_2, TGF-β_3 and IL-10) and pro-inflammatory cytokines (TNF-α and IL-1β). The serum samples were transported on ice to the Centre for Proteomic & Genomic Research (CPGR) at the University of Cape Town for analysis. All procedures were conducted according to manufacturers' instructions that were included in the kit (Appendix F - H). All buffers, diluents, standards and controls were supplied with the kits.

Cytokines (TNF-α, IL-1β and IL-10) were measured using a Bio-Plex Pro Assay (Bio-Rad Laboratories; Appendix F) according to the manufacturer's instructions. Bio-Plex assays use xMAP technology. xMAP technology employs tiny colour-coded beads called microspheres or microbeads to detect molecules of interest. These colour-coded microspheres are coated with a capture antibody that allows coupling with the molecule of

interest. In the current study's assay, the beads were coated with antibodies for the cytokines under investigation. The microbeads were first diluted in buffer –following which 50µl of the microbead solution was added into each well of a 96 well plate. 50µl of diluted serum from each participant (diluted with standard diluent in a ratio of 1:4, i.e. 10ml neat serum from each participant), 50µl of each standard[1] (provided by the manufacturer) and 50µl of each of the two controls (samples of known concentration provided by the manufacturer) were then pipetted in duplicate into the wells of the plate and incubated according to the manufacturer's instructions. This process allows for the immobilized antibodies to bind to each cytokine.

The wells were then washed to remove any unbound protein. 50µl of biotinylated antibodies were then added to each well. Biotinylated antibodies detect the cytokine of interest (see *Figure 13*). Following incubation at room temperature and washing, 50µl of the streptavidin-phycoerythrin conjugate was added. Streptavidin-phycoerythrin binds to the biotinylated detection antibodies. Phycoerythrin serves as a fluorescent indicator. After 30 minutes of incubation the plates were washed and the micro-particles re-suspended with wash buffer.

Bio-Plex sandwich immunoassay

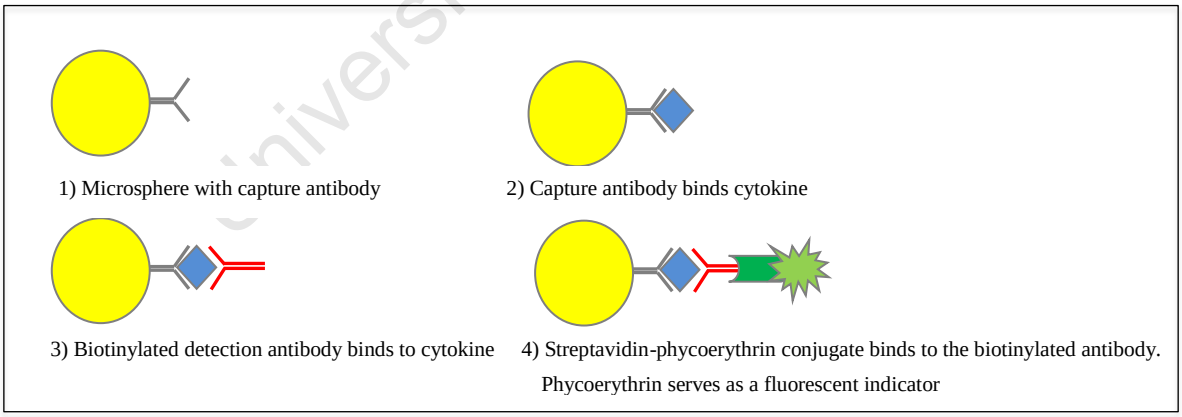


Figure 13: xMAP assay principle.
Modified from: Bio-Plex Pro-Assays manufacturer's booklet, page 3.

[1] Standards (samples with standard concentrations) were used to generate a standard curve for each assay. A best-fit curve was then be plotted and the cytokine concentration calculated from the best-fit curves equation.

The 96 well plates were then placed into the Bio-Plex machine for analysis. In the Bio-Plex machine, each micro particle was passed through 2 lasers. The first laser excites the internal dye of the microsphere and the label on the detection antibody compound. This allows for the Bio-Plex Manager software to detect which particular microsphere is passing through, i.e. the microsphere, with the known capture antibody, is identified therefore allowing for the identification of the cytokine. The second laser determines the magnitude of the detection antibody signal. This signal is directly proportional to the amount of cytokine bound to the microsphere-capture antibody complex. Multiple readings were made on each bead set and an average of the multiple readings is calculated by the computer software and reported. Multiple readings on the microbeads validate the results. The classification and quantification of each microbead readings were made on each individual particle using the Bio-Plex Manager software. The software calculates the standard curve for the assay, the type of microsphere detected and the concentration of the cytokine of interest in each of the wells.

TGF-β_1, TGF-β_2 and TGF-β_3 were analysed using the Fluorokine MAP TGF-β Multiplex Kit according to manufactures instructions (R&D Systems, cat# LKT001; Appendix G). The Multiplex Kit simultaneously assessed the levels of all 3 TGF-β molecules in a single sample. Again, the Multiplex kit uses xMAP technology as described above. Latent TGF-β first needs to be activated to become immuno-reactive. To activate TGF-β, 100µl of serum was added to 20µl of 1N hydrochloric acid. The mixture was incubated for 10 minutes at room temperature and neutralized by adding 20µl of NaOH/0.5M HEPES (prepared according to manufacturer's instructions). The serum was then diluted with Calibrator Diluent RD5-49 in a ratio of 1:10.71 thus making a final dilution factor of 1:15. 50µl of the diluted serum samples, standards (provided by the manufacturer) and controls (provided by the manufacturer) were added to the 96 well plate in duplicate. The xMAP technology–based procedures described above were then performed for quantification of each TGF-β cytokine.

C) Osteopontin (OPN)

The MILLIPLEX MAP Human Bone Panel 1 (Millipore, cat # HBN1A-51K), a multiplex assay kit, was used to analyse OPN. Procedures were carried out according to the manufacturer's instructions (Appendix H). Serum was diluted in a ratio of 1:4 in Assay

Buffer provided in the kit. 25µl of diluted serum, standards and controls were added to each well in duplicate as described above. Quantification of OPN was then carried out using xMAP technology, as described above.

Proton Magnetic Resonance Spectroscopy (^{1}H-MRS) and Neurometabolite Analyses

A small pilot project took place before the commencement of participant scanning. Three young, healthy adults volunteered to undergo ^{1}H-MRS scanning. Consent from these volunteers was obtained. During this pilot phase a radiologist, neurologist, physicist and myself made sure that all parameters outlined below were programmed and correct on the scanner. Voxel sizes from previous literature were used and the neuroradiologist ensured that the radiographer at CUBIC knew the exact anatomical location of the voxel placement.

In order to gain some insight to the mechanism of measuring neurometabolites, the explanation in this section is of necessity highly technical.

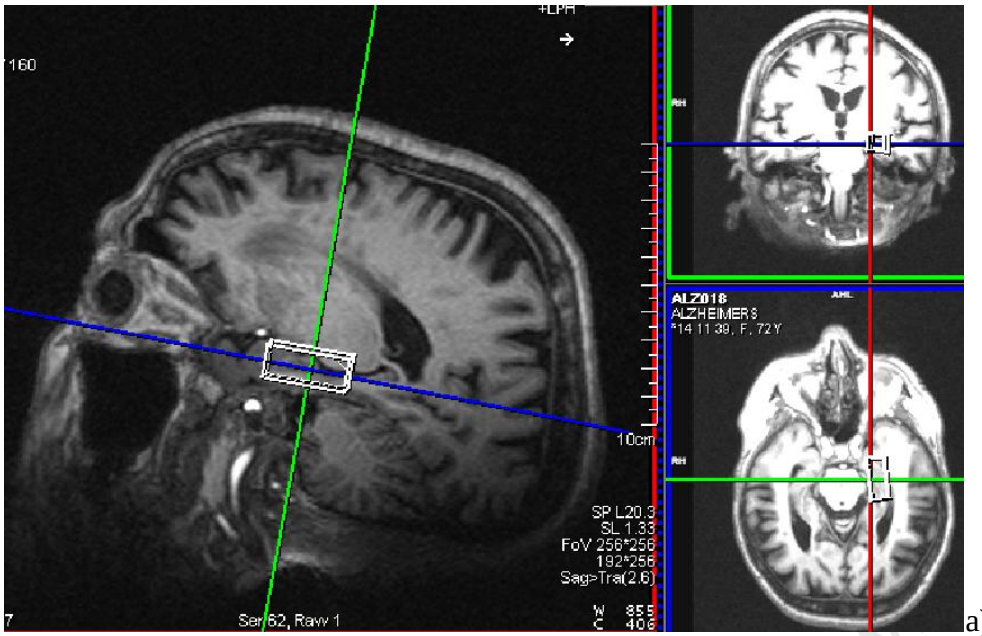
All scans were performed using a 3T Siemens Allegra (Siemens Medical Systems, Erlangen, Germany) scanner. Scans were performed by an experienced radiographer at CUBIC. Sagittal multi-echo magnetization-prepared rapid acquisition with gradient echo (MPRAGE) images were acquired with the following parameters: resolution = 1x1x1mm^3, slices = 160, repetition time (TR) = 2530ms, four echo times (TE): TE 1 = 1.53ms, TE 2 = 3.21ms, TE 3 = 4.89ms, TE 4 = 6.57ms, T1 = 1100ms, flip angle = 7 degrees, base resolution = 256, phase resolution = 75%, bandwidth = 651 Hz/pixel. Structural images were acquired for the placement of the single voxels over the region of interest. ^{1}H-MRS was performed using voxels placed on the hippocampus and posterior cingulate. ^{1}H-MRS data were obtained using an echo-planar imaging (EPI) volumetric navigated point-resolved spectroscopy (PRESS) sequence (Hess, Tindall, Andronesi, Meintjes, & van der Kouwe, 2011a), with real-time shim and motion correction. The following parameters were used: TR= 2000ms, TE= 30ms. Water signal were suppressed. In addition, water unsuppressed spectra were acquired in the voxel using 7 different TE's: TE =30ms, 50ms, 75ms, 100ms, 144ms, 500ms, 1000ms; TR=4000 and two averages were acquired for each TE. This was done to estimate the fraction composition of grey matter, white matter and

CSF in the voxels of interest. This allows for more reliable metabolite levels (Ernst, Kreis, & Ross, 1993)

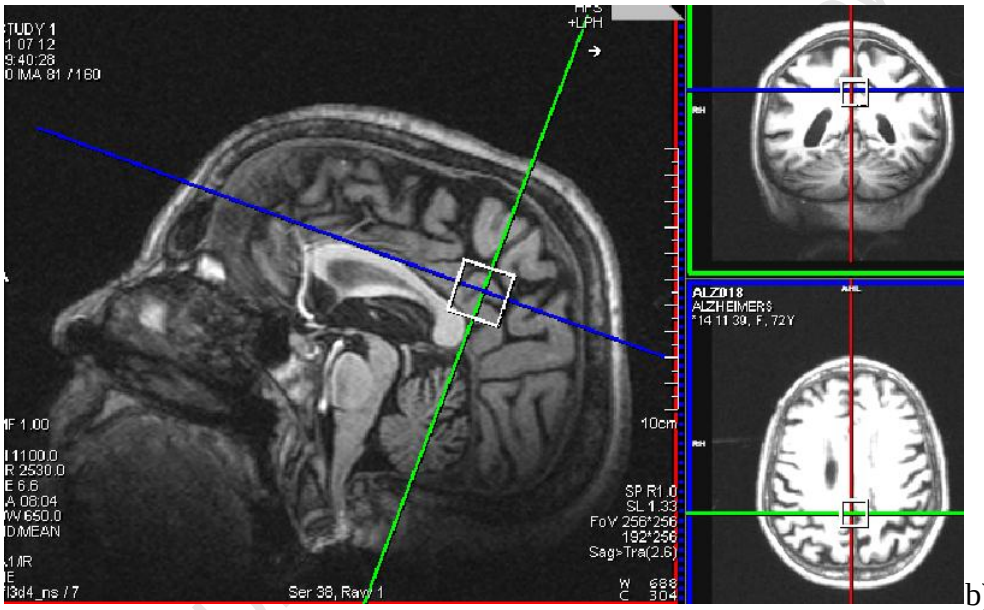
An 8000mm^3 (20 × 20 × 20mm) voxel, prescribed on a mid-sagittal T1 weighted image, over the midline of the right and left posterior cingulate gyri was placed. The anterior border of the splenium, the superior border of corpus callosum and the cingulate sulcus were the anatomical landmarks used to define the anterior inferior and the anterior superior border of the 8000mm^3 voxel (see *Figure 14a*). The voxel placement and size were used according to Kantarci et al. (2000).

A 4320mm^3 (30 x 12 x 12mm) voxel was placed over the left temporal region and included the entorhinal cortex, subiculum, hippocampal proper and dentate gyrus (see *Figure 14b*).

Pre-processing of the water-suppressed data was done according to the method of Hess, van der Kouwe, & Meintjes (2011b). Pre-processing of the data using the technique outlined by Hess et al. (2011b) ensures narrower line widths and higher signal to noise ratios. This makes the results more reliable. The corrected water-suppressed spectra were quantified using the software package LC model (Provencher, 2003). The water signal from the water-unsuppressed spectra of each TE measurement were also quantified using the LC Model and then modelled as a function of TE using a tri-exponential function (SigmaPlot; version 11). This estimates the fraction of grey matter, white matter and CSF in the voxel of interest. Because metabolites are found in different concentrations in white matter, grey matter and CSF, these fractions are then used to obtain absolute quantification of metabolite (Hess et al., 2011b).



a)



b)

Figure 14: Location of the a) 4320mm^3 left hippocampal voxel b) 8000mm^3 posterior cingulate voxel. Both were acquired on a T1 weighted localizing image.
Note: This image is taken from a pilot participant within this study.

Absolute metabolite concentrations are reported as unsuppressed data were acquired. Reporting the absolute concentrations is considered the gold standard as it takes into account the different tissue fractions and is therefore more reliable (Valenzuela & Sachdev, 2001). In the results section the N-acetylaspartate and N- acetylaspartylglutamate (NAA+NAAG) concentration is reported.

APOE Genotyping

At the participants first, baseline visit peripheral blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes. These were kept on ice and centrifuged at 4000 rpm for 10 minutes. The buffy coat layer containing the white blood cells was pipetted into cryotubes and stored in a -80°C freezer until analysis.

The buffy coat layer was thawed on ice. DNA was then extracted from the buffy coat. QIAamp DNA Blood Mini kits were used for purification of total DNA. The QIAamp DNA purification procedure was carried according to manufacturer's instructions. All buffers and washers were supplied with the kits. A QIAamp spin columns were provided in the kits. The spin columns have silica membranes that DNA binds to. The spin columns are removed and placed in new microcentrifuge tubes throughout the process.

20µl QIAGEN Protease was pipetted into a microcentrifuge tube together with 200µl of the buffy coat sample from the participant. 200µl of Buffer AL was added. Microcentrifuge tube was mixed by pulse vortex for 15 seconds.

The sample was incubated for one hour at 56 °C. 200µl of 96% - 100% ethanol was added to the sample and mixed for 15 seconds. The QIAamp spin column was removed and placed into a QIAamp spin column tube. 200µl of Buffer AL was added to the microcentrifuge tube and centrifuged again at 8000 rpm for 1 minute. 500µl of Buffer AW1 was added and centrifuged at 8000 rpm for one minute. 500µl of Buffer AW2 was added and centrifuged at 14000 rpm for 3 minutes. The QIAamp spin column was placed in a clean microcentrifuge tube and 200µl of Buffer AE was added. The microcentrifuge tube was incubated at room temperature for 1 minute and then centrifuged at 8000 rpm for 1 minute.

DNA restriction isotyping for rapid genotyping of the three common APOE alleles was then performed. Procedures were carried out according to Hixson and Vernier (1990). In the first step, restriction digestion involved amplification, using oligonucleotides, of a 228 base pair sequence of the APOE gene that encompasses the regions coding for amino acid

112 and 158. The 228 base pair sequence was amplified to a 237 base pair sequence. The amplification products were then digested using the *HhaI* restriction enzyme. *HhaI* cleaves the GCGC encoding 112arg and 158arg but does not cleave the GTGC encoding 112cys and 158cys. Thus it distinguishes the ε2, ε3 and ε4 alleles. The fragments were then separated by electrophoresis on a 10% acrylamide gel. The ε2, ε3 and ε4 fragments resulted in several bands. Fragment sizes of a DNA standard were used to identify the isoforms present.

STATISTICAL ANALYSES

Data were collected and checked for missing information. Statistical tests were performed at the 0.05 level. All statistical analyses were completed using a computer software package, Statistica 11 (Borovikov & Borovikov, 1998) and the Statistical Package for the Social Science (SPSS) version 21.0 (Kirkpatrick & Feeney, 2008). Graphs were plotted using Graph Pad Prism 5, SPSS 21.00 and Statistica 11.

Unless otherwise stated, all assumptions for the specific statistical analyses were upheld. The level for statistical significance was set at $\alpha = 0.05$. Where data were not normally distributed, log-transformations were done. In all cases where data were still skewed after such transformations, non-parametric statistical measures were used. For between-group comparisons Mann-Whitney *U* tests were performed. Non-parametric correlations were performed using Spearman's ranked correlations. In the case where data was normally distributed, ANOVA analysis and Student's *t*-test were used for between-group comparisons. Pearson's correlation analysis was used for correlations of normally distributed data. Regression models were used in cases where there were small sample sizes within the groups. Chi-squared analysis was used for between-group categorical comparisons. Details about specific analyses are provided in the Results section.

RESULTS

THE CROSS-SECTIONAL STUDY

Final Sample Composition

One hundred and fifty-one participants were initially enrolled in the study. Four participants withdrew before their first visit. Nineteen participants were excluded from the study according to the exclusion criteria. Details of these exclusions are summarised in the figure below (see *Figure 15*).

Exclusion after visit 1: At the end of the cognitive testing and completion of the self-rated questionnaires, 4 participants were excluded due to the presence of moderate to severe depressive symptoms (a GDS score greater than 8). A further 8 participants were excluded after their medical examination: 2 because they had parkinsonian signs, 2 were excluded due to the presence of significant psychiatric symptoms other than depression, 3 were thought to have dementia not due to Alzheimer's disease and 1 participant had epilepsy.

Exclusion on blood results: Seven participants were excluded because their ESRs were above 40 mm per hour.

At a diagnostic consensus meeting (consisting of a neurologist and neuropsychologist) each of the 128 participants were assigned to either the control group (n = 68) or the possible/probable AD group (n = 60) according to the NINCDS/ ADRDA criteria (see Procedure for Study, page 71 and Table 1).

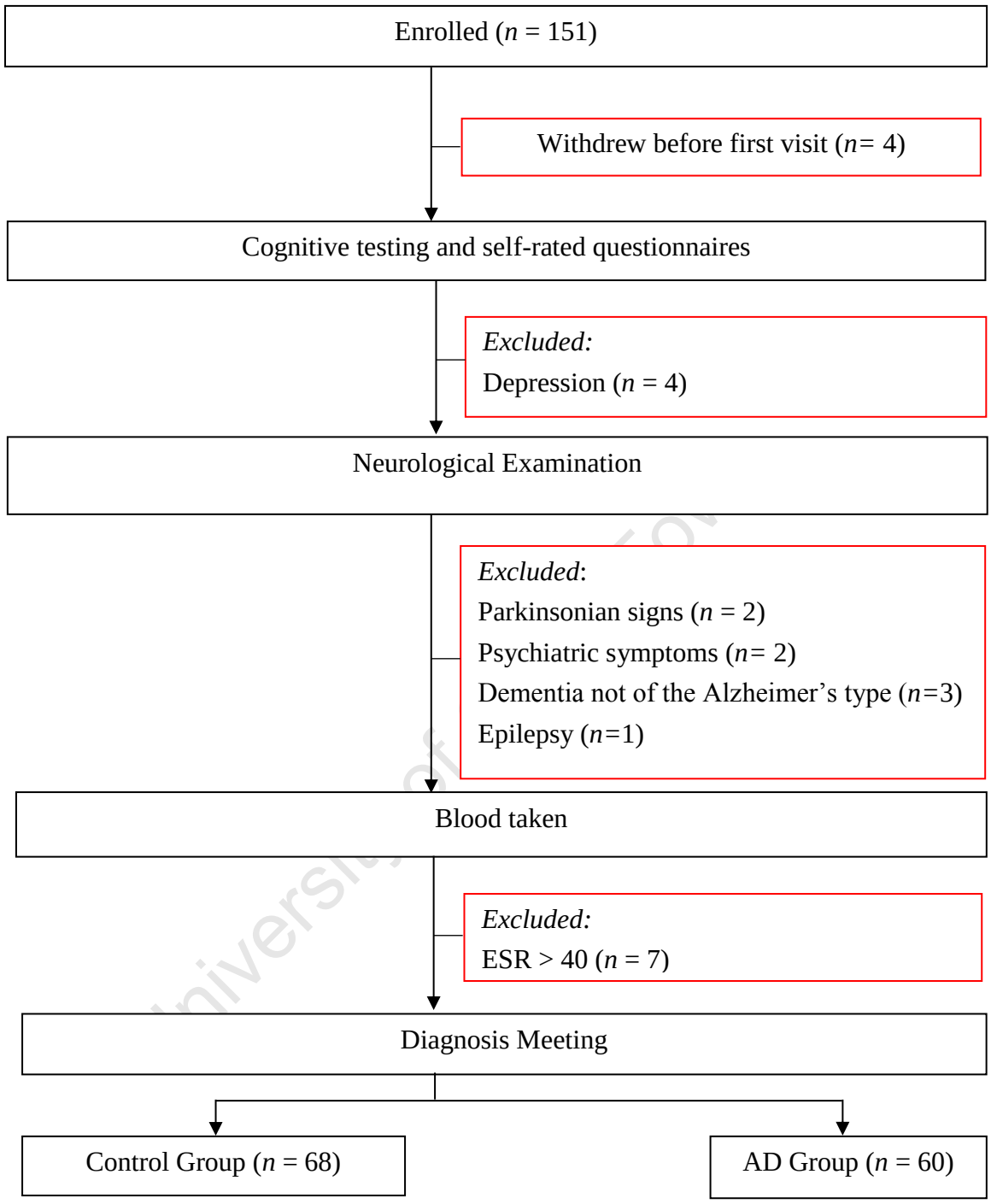


Figure 15: Flow diagram of participant enrolment and attrition.

Final Sample: Demographic Characteristics and Cognitive Scores

Demographic data were collected from all participants at their baseline visit. Table 3 describes the demographic characteristics of the study population (n = 128).

Age: The participants' ages ranged from 58 to 100 years (M = 73.9, SD = 9). The age data were normally distributed and therefore a Student's t-test was used to compare groups. Control participants (M = 71.3) were significantly younger than AD participants (M = 76.8; $p < 0.001$).

Sex: There were 37 males and 91 females in the final sample. Pearson's Chi-Squared analysis of these data showed that there were no significant between-group differences with regard to sex distribution.

Level of education: This variable was defined as the total number of years of successfully completed education. If the person repeated a year at school, the two years were counted as one. The number of years of education ranged from 3 to 25 in the sample population (M = 11.8, SD = 4.6). Control participants (M = 13.8) had significantly more years of education when compared with AD participants (M = 9.5; $p < 0.001$).

Table 3
Demographic Data for the Cross-Sectional Study's Participants

	Group					
	Control (n = 68)	AD (n = 60)	t / χ^2	df	p	ESE
age	71.3 (8.8)	76.8 (8.3)	3.64	126	< .001*	0.61
sex (M:F)	16:52	21:39	2.04	1	.153	-0.13
level of education	13.8 (4.7)	9.5 (3.2)	-5.78	126	< .001*	-0.94

Note: For age and level of education, means are presented with standard deviations in parentheses. The mean ± 1 standard deviation was used. Level of education was measured as the number of years of successfully completed education. ESE = effect size estimate; for continuous variables (age, level of education) the ESE is Cohen's d; for sex the ESE is Phi. See Appendix I: Table A - E for raw data.
*Control and AD groups differed significantly, $p < .001$.

Cholinesterase inhibitor use: None of the AD participants in this study were on cholinesterase inhibitors at baseline or at follow-up.

Cognitive Testing

All participants underwent cognitive testing at their first, baseline visit. Cognitive tests included the CAMCOG, which incorporated the MMSE and learning subscale. Details of the cognitive tests scores are given in Table 4. Cognitive scores were not normally distributed, therefore Mann-Whitney *U* tests were used for between-group comparisons. Cognitive scores, together with the DECO, BADLS and clinical history, formed part of the diagnostic criteria. Therefore cognitive scores were, as expected, significantly different between the controls and AD participants.

CAMCOG: Total CAMCOG scores for both groups ranged from 38 to 101 out of a total of 105 (for the entire sample, *Median* = 85, *IQR* = 68 – 94.5). Control participants (*Median* = 94) predictably performed better than the AD participants (*Median* = 67.5) on the total CAMCOG score ($p < .001$).

MMSE: The participants' MMSE scores ranged from to 12 to 30 out of a total of 30 (for the entire sample, *Median* = 27, *IQR* = 22 – 29). Control participants (*Median* = 29) had higher scores on the MMSE compared with AD participants (*Median* = 21.5; $p < .001$).

Learning subscale: Learning subscale scores for all participants ranged from 1 to 17 out of a total of 17 (*Median* = 12.5, *IQR* = 7 – 14). Control participants (*Median* = 14) performed better than the AD participants (*Median* = 7) on the learning subscale score ($p < .001$).

Table 4
Between-Group Comparisons of Neuropsychological Test Scores at Baseline

Cognitive test	Group					
	Control (n = 68)	AD (n = 60)	*U*	*df*	*p*	*r*
CAMCOG total	94 (89 – 96)	67.5 (52 – 76)	25	126	<.001*	-0.85
MMSE	29 (28 – 30)	21.5 (16 – 24)	105	126	<.001*	-0.82
Learning subscale	14 (13 – 15)	7 (4 – 10)	208	126	<.001*	-0.77

Note: For the total CAMCOG, the Mini-Mental State Examination (MMSE) and the learning subscale the median is presented with the interquartile range in parentheses. Mann-Whitney *U*, the degrees of freedom (*df)* and the effect size *r,* are presented. See Appendix I: Table A - E for raw data.
*Control and AD groups differed significantly, $p < .001$.

The Relationship Between Systemic Inflammatory Markers and Cognition

Blood samples from all participants (68 controls, 60 AD participants) were compared with respect to ESR, white cell count and monocyte count. Two AD participant did not have a serum sample. Cytokine and OPN analysis was done on a smaller sample because of restrictions on the number of wells per plate. The youngest 9 control participants were excluded. Table 5 displays the demographic data for the participants that were included in the cytokine and OPN analysis. For the cytokine and OPN analysis the final groups consisted of 58 AD and 59 control participants.

The AD participants were still older ($p = .009$) and had fewer years of education ($p < .001$) than the controls.

Table 5
Demographic Data for the Cross-Sectional Sample Included in the Cytokine and OPN Analysis

	Group					
	Control (n = 59)	AD (n = 58)	t / χ^2	df	p	ESE
age	72.9 (8.3)	77.0 (8.4)	2.65	115	.009*	0.49
sex (M:F)	15:44	20:38	1.14	1	.284	-0.10
level of education	14.1 (4.6)	9.5 (3.2)	-6.28	115	< .001**	-1.17

Note: For age and level of education, means are presented with standard deviations in parentheses. The mean ± 1 standard deviation was used. Level of education was measured as the number of years of successfully completed education. ESE = effect size estimate; for continuous variables (age, level of education) the ESE is Cohen's *d*; for sex the ESE is Phi. See Appendix I: Table A - E for raw data.
Control and AD groups differed significantly, *p < .01, **p < .001.

Mann-Whitney *U* tests were used to compare the inflammatory markers in all AD participants and the controls. No significant differences in any of the inflammatory markers were found (see Table 7).

Hypothesis 1 predicted that peripheral inflammatory markers in the early stages of AD would be highest. Systemic inflammatory markers would then decline, below that of control participants, as participants reached more advanced stages of AD. In other words, there would be a biphasic relationship between systemic inflammatory markers and the stage of AD.

In order to test hypothesis one, AD participants were divided into 3 subgroups based on their learning subscale scores. The learning subscale is a test of episodic memory, which is usually the first cognitive domain to decline in AD. It is also the most severely affected aspect of cognitive function in AD. The first group formed the *early/mild AD* (n = 13) subgroup. Participants with learning subscale scores greater and equal to 11/17 were assigned to this group. The second group, the *moderate AD* group (n = 22), consisted of AD participants with learning subscale scores of 7/17 to 10/17. Participants who fell into the *more severe* AD group (n = 25) had learning subscale scores equal to or below 6/17.

Note that participants who fell into the severe AD group were severe in terms of their learning subscale score. As previously mentioned, all participants in the study were mild/moderate stage AD and were able to provide consent at the start of the study. They were also able to complete cognitive testing.

One-way ANOVA analysis and Tukey HSD post-hoc test showed that controls (M = 71.3, SD = 8.8) were younger than mild AD participants [(M = 78.9, SD = 9.2), $F(3,124)$ = 5.68, p =.0017, partial ηp^2 = 0.12], and severe AD participants [(M = 78.2, SD = 7.8), $F(3,124)$ = 5.68, p =.003, partial η_p^2 = 0.12].

There were no detectable levels of $TGF\beta_3$ in any of the participants, therefore $TGF\beta_1$ and $TGF\beta_2$ levels are only reported.

In all participants, Spearman's ranked correlation analysis showed no correlation between age and ESR, white cell count, monocyte count, OPN or any of the cytokines, see Table 6. Therefore, age was not controlled for when the group's inflammatory markers were compared.

Table 6
The Correlation between Systemic Inflammatory Markers and Age in all Participants

Inflammatory Marker	*r*	*p*
ESR	0.17	.062
White cell count	0.07	.429
Monocyte count	0.1	.288
IL-1β	0.06	.556
IL-10	0.09	.357
TNF-α	-0.12	.185
TGF-β_1	-0.05	.618
TGF-β_2	-0.12	.186
OPN	0.15	.098

Note: The test statistic is Spearman's rho. See Appendix I: Table A - E for raw data.

The systemic inflammatory markers were not normally distributed. Non-parametric Mann-Whitney *U* tests were therefore used for between-group comparisons. Table 7 details the median and interquartile range for each group.

The mild AD group (*Median* = 20) had significantly higher ESRs compared with controls (*Median* = 8), *U* = 254, *p* = .018, *r* = -0.26, and moderate AD participants (*Median* = 6), *U* = 75, *p* = .031, *r* = 0.37 (Table 7, *Figure 16*). The moderate AD, severe AD and control groups did not significantly differ with respect to their ESRs. The mild AD group (*Median* = 2.5) also had a significantly higher IL-1β level compared with controls (*Median* = 2), *U* = 253, *p* = .05, *r* = -0.21, moderate AD participants (*Median* = 2), *U* = 75, *p* = .031, *r* = 0.37, and severe AD participants (*Median* = 2), *U* = 94, *p* = .047, *r* = 0.32 (Table 7; *Figure 17*). Again, the moderate AD, severe AD and control groups did not significantly differ from one other with respect to their IL-1β levels. OPN was highest in mild AD group compared with the controls, moderate and severe AD groups. This however did not reach significance.

The severe AD group had significantly higher total white cell count (*Median* = 7.41) compared with the moderate AD group (*Median* = 6.09), *U* = 165, *p* = .047, *r* = -0.29. There were no between-group differences with respect to the monocyte count, TGF-β_1, TGF-β_2 and IL-10 measurements.

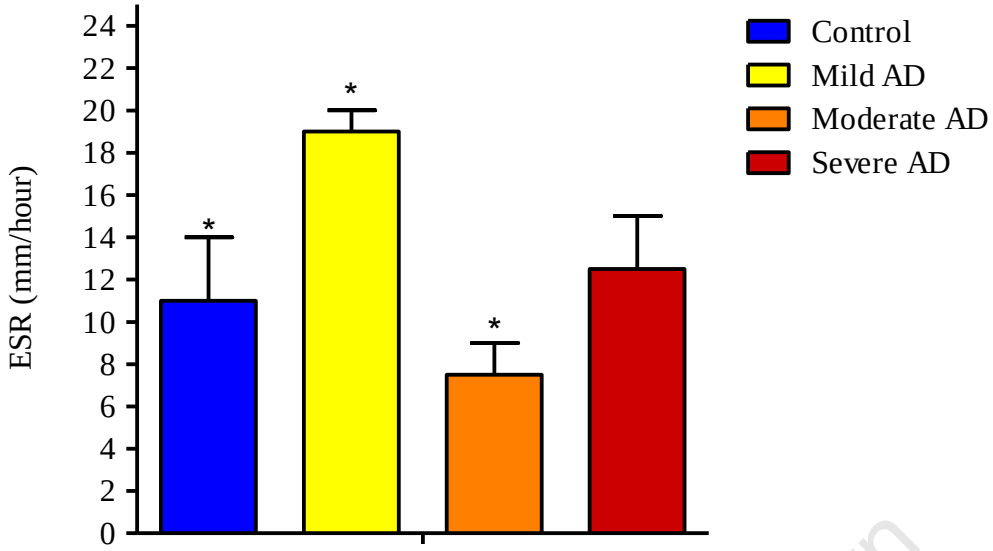


Figure 16: Median erythrocyte sedimentation rates (ESRs) for the control (*n* = 68), mild AD (*n* = 13), moderate AD (*n* = 22) and severe AD groups (*n* = 25). Error bars represent the upper interquartile range.
* The mild AD group differed significantly from the moderate AD group and the controls, $p < .05$.

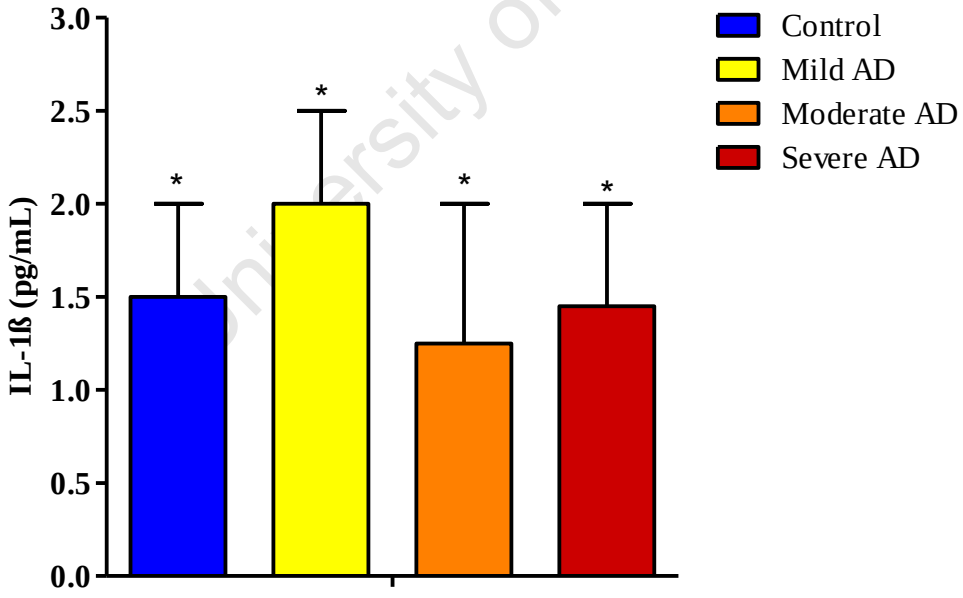


Figure 17: Median interleukin 1 beta (IL-1β) for the control (*n* = 59), mild AD (*n* = 13), moderate AD (*n* = 21) and severe AD (*n* = 24) groups. Error bars represent the upper interquartile range.
* The mild AD group differed significantly from the control, moderate and severe AD groups, $p < .05$.

Table 7

Peripheral Inflammatory Marker Concentrations for the AD Subgroups and the Control Participants

		Group			
Outcome variable	Control (n = 68)	AD (n = 60)	Mild AD (n = 13)	Moderate AD (n = 22)	Severe AD (n = 25)
ESR	8 (3 – 17)*	11 (5 – 22)	20 (12 – 30)	6 (4 – 13)*	10 (7 – 22)
White cell count	6.64 (5.55 – 7.68)	6.98 (5.46 – 7.78)	5.66 (4.94 – 9.67)	6.09 (5.41 – 7.54)	7.41 (6.18 – 8.48)†
Monocyte count	0.39 (0.32 – 0.48)	0.40 (0.3 – 0.53)	0.37 (0.29 – 0.43)	0.36 (0.29 – 0.51)	0.42 (0.34 – 0.53)
	Control (n = 59)	AD (n = 58)	Mild AD (n = 13)	Moderate AD (n = 21)	Severe AD (n = 24)
IL-1β	2 (1.5 – 2.5)*	2 (1.5 – 2.5)	2.5 (2 – 3.5)	2 (1.5 – 2)*	2 (1.5 – 2.4)*
IL-10	4.5 (3.5 – 5.5)	4.5 (3.5 – 5.5)	5 (3.8 – 7)	4.5 (4 – 5.5)	4 (3.3 – 5.4)
TNF-α	3.5 (3 – 4)	3.5 (3 – 4)	3.5 (3.3 – 5)	3.5 (3 – 4)	3.5 (3 – 3.8)
TGF-β_1	999 (178.3 – 1476.5)	899.9 (186.5 – 1533.5)	914 (138.3 – 1430.8)	933 (218.3 – 1612.5)	816.9 (189.4 – 1411.4)
TGF-β_2	5.5 (3.5 – 8)	5.4 (3.5 – 7)	5 (3.3 – 7)	5.5 (4.5 – 6.8)	6.55 (3.25 – 7.75)
OPN	594 (367.30 – 1005.3)	7.7.75 (248.8 – 1312.5)	853 (416.8 – 1293.8)	701 (319.8 – 1533.8)	610.4 (207.5 – 1215.15)

Note. Inflammatory markers medians are presented with the interquartile range in parentheses. The erythrocyte sedimentation rate (ESR) was expressed as mm in 1 hour; the total white cell and monocyte counts are represented as cells x10^9/l. Interleukin 1 beta (IL-1β), interleukin 10 (IL-10), tumor necrosis factor alpha (TNF-α), transforming growth factor beta 1 and 2 (TGF-β_1 and TGF-β_2) and osteopontin (OPN) concentrations are expressed in picograms per millilitre (pg/mL). See Appendix I: Table A - E for raw data.

* Significantly different from the mild AD group, $p < .05$. † Significantly different from the moderate AD group, $p < .05$.

The Relationship Between APOE Genotype and Cognition

APOE demographics

APOE genotyping was available for 123 participants (64 controls, 59 AD participants). Table 8 shows the allelic frequency (ε2, ε3 and ε4) in all participants as well as the control and AD groups. The allelic frequency was calculated by dividing the number of each allele by the total number of alleles in all participants, in controls or in AD participants. The most common allele in both the control and AD groups was the APOE ε3 allele, with allelic frequencies of 0.703 and 0.551 respectively. Pearson's Chi-Squared analysis showed that the control group (0.703) tended to have a higher ε3 allelic frequency when compared with the AD group (0.551; $p = .074$). The allelic frequency of ε4 was significantly higher in the AD group (0.356) compared with the control group (0.203; $p = .025$. In both the control and AD group, the ε2 allelic frequency was the lowest.

Table 8
Allelic Frequency of the APOE ε2, ε3 and ε4 Alleles

APOE Allele	Group						
	All participants ($n = 123$)	Control ($n = 64$)	AD ($n = 59$)	χ^2	*df*	*p*	*r*
ε2	0.093	0.094	0.093	0.68	1	.795	-0.23
ε3	0.630	0.703	0.551	3.18	1	.074	-0.16
ε4	0.276	0.203	0.356	5.00	1	.025*	0.20

Note: The test statistic is Pearson's Chi-Squared (χ^2). The degrees of freedom *(df)* and the effect size estimator *r* are given. See Appendix I: Table A - E for raw data.
* Control and AD groups differed significantly, $p < .05$.

Table 9 and *Figure 18* to *20* show the distribution of the different APOE genotypes among the control and AD participants. The most frequent APOE genotype in all the participants was ε3/ε3, 39% of participants had this APOE genotype. When the groups were examined separately, the most frequent genotype in the controls remained the ε3/ε3 combination. Pearson's Chi-squared analysis showed that significantly more controls had the ε3/ε3 combination when compared with the AD group ($p = .026$). Forty-eight percent of controls had the ε3/ε3 genotype compared with 28% of AD participants.

The ε2/ε2 genotype was the least common genotype frequency in all participants (1%). Only one AD participant had the ε2/ε2 genotype (2%). There were no controls with this genotype. In the control group, the ε2/ε4 genotype was the 3rd least common genotype after ε2/ε2 (0%) and ε4/ε4 (3%). Five percent of controls had the ε2/ε4 genotype. Three percent of AD participants in the AD group had the ε2/ε4 genotype.

Forty-seven percent of all participants had at least one ε4 allele, 8% were homozygous. Thirty-eight percent of controls had at least one ε4 allele; 3% were homozygous. In the AD group, the most common APOE genotype was ε3/ε4. Forty-one percent of AD participants had the ε3/ε4 allele and 14% had the ε4/ε4 genotype. Chi-Squared analysis showed that the AD group had a significantly higher number of participants who were homozygous for the ε4 (ε4/ ε4) genotype compared with the control group (p = .034). In total, 58% of AD participants had at least one ε4 allele.

When adjusted for age, the odds ratio (95% CI = 0.965 – 4.954) for AD was 2.2 fold higher in participants with one ε4 allele compared with participants with no ε4 allele. Homozygous ε4 participants had a 9 times higher odds (95% CI = 1.619 – 48.721) of having AD compared with participants with no ε4 allele.

Table 9
The Distribution of the APOE Genotype

Allele	Group			χ^2	*df*	*p*	ESE
	All participants (*n* = 123)	Control (*n* = 64)	AD (*n* = 59)				
ε2 / ε2	1	0	1	1.09	1	.296	0.09
ε2 / ε3	16	9	7	0.13	1	.717	-0.03
ε3 / ε3	48	31	17	4.97	1	.026*	-0.20
ε2 / ε4	5	3	2	0.13	1	.716	-0.03
ε3 / ε4	43	19	24	1.63	1	.202	0.12
ε4 / ε4	10	2	8	4.47	1	.034*	0.19

Note: The test statistic is Pearson Chi-Squared (χ^2) for between-group differences with respect to the control and AD group. The degrees of freedom *(df)* and the effect size estimator (ESE) *r* are given. See Appendix I: Table A - E for raw data.
* Control and AD groups differed significantly, $p < .05$.

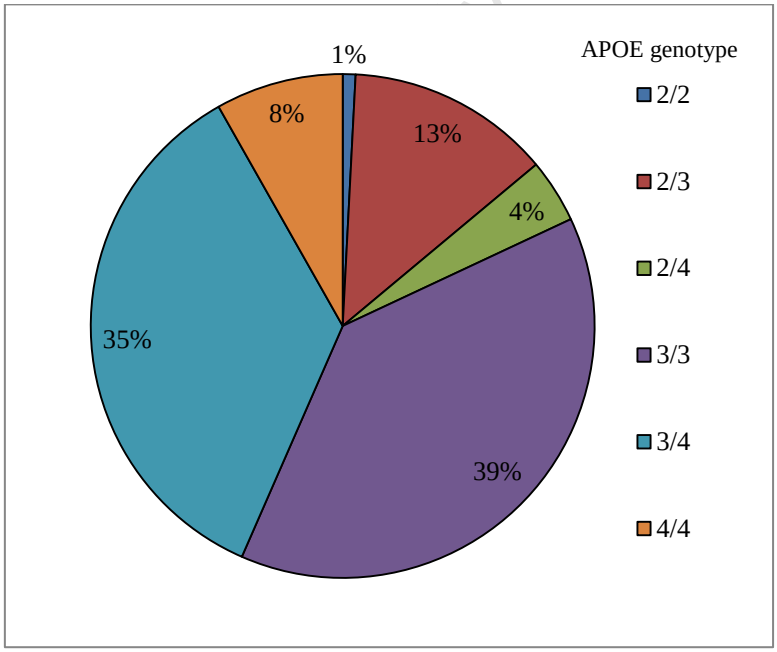


Figure 18: The percentage distribution of the APOE genotypes in all participants.

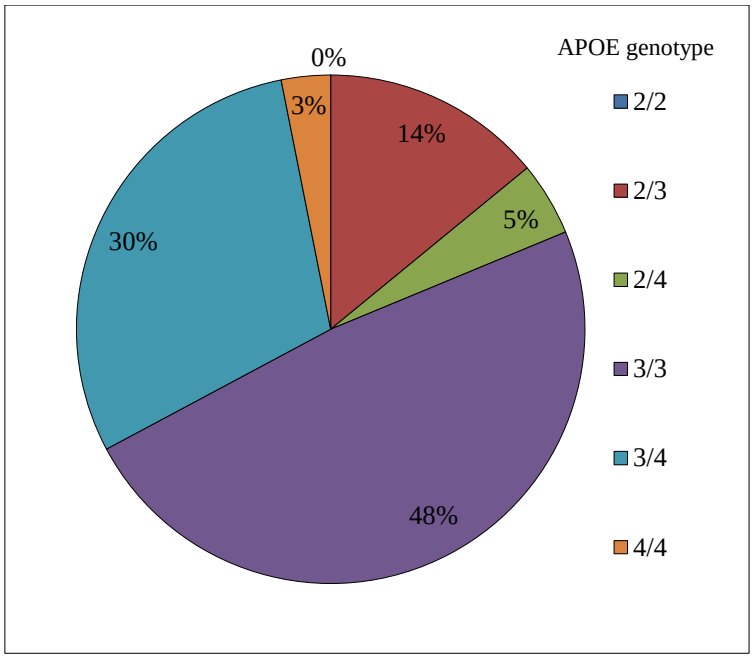


Figure 19: The percentage distribution of the APOE genotypes in the control participants.

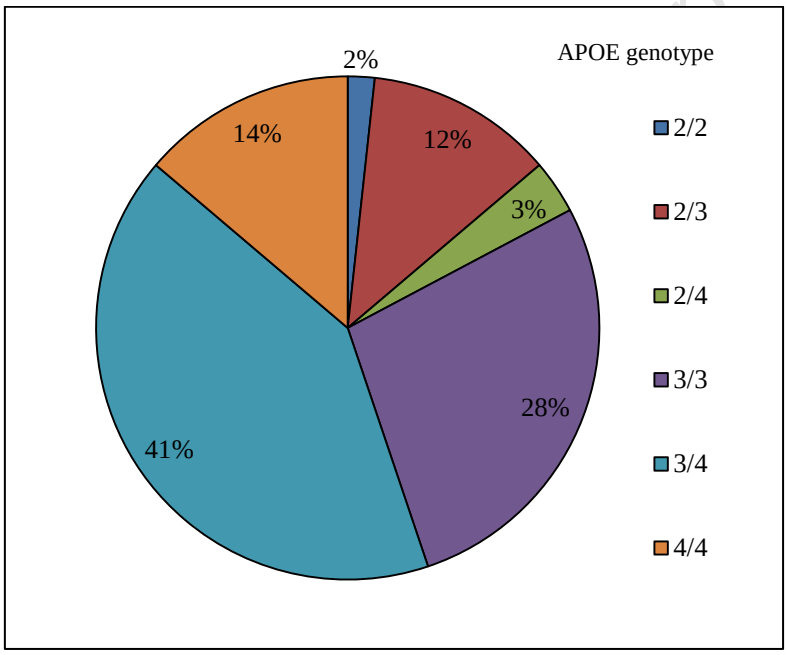


Figure 20: The percentage distribution of the APOE genotypes in AD participants.

APOE and cognitive functioning

The control and AD groups were divided into subgroups based on their genotype. APOE ε4 carriers formed the *ε4 carrier* subgroup and participants who had the APOE ε2 and/or APOE ε3 alleles formed the *ε4 non-carrier* subgroup. The ε4 carrier subgroup included ε4 homozygous (ε4/ε4) and heterozygous participants (ε2/ε4 and ε3/ε4). Table 10 outlines the details of the between group comparison.

AD participants with at least one ε4 allele tended to have lower MMSE scores (*Median* = 21) when compared with ε4 non-carrier participants (*Median* = 24; $p = .053$). However, there was no significant difference on CAMCOG scores between AD ε4 carriers and non-carriers. Similarly, there was no significant difference on the learning subscale between AD ε4 carriers and non-carriers.

When the control group was divided according to presence/absence of the ε4 allele, there were no significant differences in scores on the CAMCOG, MMSE and learning subscale between the ε4 carrier and non-carrier subgroups.

Table 10
Cognitive Scores for the APOE ε4 Carrier and APOE ε4 Non-Carrier Subgroups

Cognitive Measure	Control: APOE ε4 Non-Carrier (*n* = 40)	Control: APOE ε4 Carrier (*n* = 24)	*U*	*df*	*p*	*r*
CAMCOG	95 (89.5 – 97)	93.5 (88.5 – 95.5)	381.5	62	.174	-0.17
MMSE	29 (28 – 29)	29 (28 – 30)	437.5	62	.560	0.07
Learning subscale	14 (13 – 15)	14 (13 – 15)	415.0	62	.371	0.11
	AD: APOE ε4 Non-Carrier (*n* = 34)	AD: APOE ε4 Carrier (*n* = 25)	*U*	*df*	*p*	*r*
CAMCOG	68 (56 – 76)	66.5 (52 – 76)	419.5	57	.939	-0.01
MMSE	24 (17 – 26)	21 (16 – 23)	298.5	57	.053	-0.25
Learning subscale	8 (5 – 11)	7 (4 – 10)	367.5	57	.371	-0.11

Note: For the total CAMCOG, the Mini-Mental State Examination (MMSE) and the learning subscale the median is presented with the interquartile range in parentheses. Mann-Whitney *U*, the degrees of freedom (*df*) and the effect size *r,* are presented. See Appendix I: Table A - E for raw data.

The Relationship Between APOE Genotype and Systemic Inflammation

Hypothesis 3 stated that participants, either homozygous or heterozygous for the APOE ε4 allele, would have higher baseline levels of systemic inflammatory markers compared with the non-ε4 carriers. In order to investigate the relationship between the ε4 allele and inflammation, between-group comparisons were done using the Mann-Whitney *U* test as well as linear regression analysis.

The control and AD groups were divided into an APOE ε4 carrier subgroup and APOE ε4 non-carrier subgroup. The APOE ε4 carrier subgroup included ε4 homozygous and heterozygous participants whereas the APOE ε4 non-carrier subgroup included participants that did not have any ε4 alleles. Forty control participants had ESR, total white cell count and monocyte count data. Thirty-five control participants had cytokine and OPN data. Twenty-four AD participants had ESR, total white cell count and monocyte count data. Twenty-two AD participants had cytokine and OPN data. Table 11 and Table 12 outline the details of the Mann-Whitney *U* tests, for between-group comparison, for the control and AD group respectively.

There were no significant differences in any of the inflammatory markers when the control APOE ε4 carrier and non-carrier groups were compared.

Likewise, there were no significant differences in any of the inflammatory markers when the AD APOE ε4 carriers and non-carriers were compared.

Table 11

Inflammatory Marker Differences in the Control APOE ε4 Non-Carriers and APOE ε4 Carriers

Inflammatory marker	Control					
	APOE ε4 Non-carrier (*n* = 40)	APOE ε4 Carriers (*n* = 24)	*U*	*df*	*p*	*r*
ESR	9 (3 – 16)	8.00 (5.5 – 18)	441.5	62	.598	0.07
White cell count	6.58 (5.66 – 7.80)	6.64 (5.33 – 7.4)	474.5	62	.945	-0.01
Monocyte count	0.38 (0.33 – 0.49)	0.41 (0.29 – 0.44)	442.0	62	.603	-0.07
	APOE ε4 Non-carrier (*n* = 35)	APOE ε4 Carriers (*n* = 22)				
IL-1β	2 (1.5 – 2.5)	2 (1.5 – 2)	384.0	55	.993	< -0.01
IL-10	4.5 (3.5 – 5.5)	4.9 (3.3 – 5.5)	360.5	55	.694	0.05
TNF-α	3.5 (3 – 4.3)	3.5 (2.8 – 4)	319.5	55	.287	-0.14
TGF-β_1	999 (244 – 1436.5)	1003.75 (108.8 – 1738.5)	384.5	55	.486	0
TGF-β_2	5.5 (3.5 – 8)	5.5 (4 – 8.5)	362.0	55	.999	0.05
OPN	719.8 (409.8 – 999)	528.4 (311.8 – 948)	342.0	55	.712	-0.09

Note. Inflammatory markers medians are presented with the interquartile range in parentheses. The test statistic is the Mann-Whitney *U*. The degrees of freedom (df) and estimated effect size, r, are displayed. The erythrocyte sedimentation rate (ESR) was expressed as mm in 1 hour; the total white cell and monocyte counts are represented as cells $x10^9$/l. Interleukin 1 beta (IL-1β), interleukin 10 (IL-10), tumor necrosis factor alpha (TNF-α), transforming growth factor beta 1 and 2 (TGF-β_1 and TGF-β_2) and osteopontin (OPN) concentrations are expressed in picograms per millilitre (pg/mL). See Appendix I: Table A - E for raw data.

Table 12
Inflammatory Marker Differences in the AD APOE ε4 Non-Carriers and APOE ε4 Carriers

Inflammatory marker	AD					
	APOE ε4 Non-carrier (*n* = 24)	APOE ε4 Carriers (*n* = 34)	*U*	*df*	*p*	*r*
ESR	18.5 (5 – 25)	10 (5 – 16)	342.5	56	.392	-0.11
White cell count	7.31 (4.98 –9.08)	6.35 (5.66 – 7.59)	398.0	56	.881	-0.02
Monocyte count	0.45 (0.30 – 0.54)	0.36 (0.3 – 0.47)	324.0	56	.325	-0.31
	APOE ε4 Non-carrier (*n* = 25)	APOE ε4 Carriers (*n* = 33)				
IL-1β	2 (1.5 – 2.5)	2 (1.5 – 2.5)	409.5	56	.969	0.01
IL-10	4.5 (3.5 – 6.3)	4.5 (3.8 – 5.5)	411.0	56	.987	<0.01
TNF-α	3.5 (3 – 4)	3.5 (3 – 4)	402.5	56	.881	-0.02
TGF-β_1	577.8 (160 – 1533.5)	1069.0 (192.3 – 1458.8)	366.0	56	.470	0.09
TGF-β_2	5.25 (3 – 7)	5.5 (3.5 – 8)	379.0	56	.604	0.06
OPN	593.5 (172.3 – 1092.8))	850 (325 – 1321.5)	338.0	56	.245	0.15

Note. Inflammatory markers medians are presented with the interquartile range in parentheses. The test statistic is the Mann-Whitney *U*. The degrees of freedom (df) and estimated effect size, r, are displayed. The erythrocyte sedimentation rate (ESR) was expressed as mm in 1 hour; the total white cell and monocyte counts are represented as cells x10^9/l. Interleukin 1 beta (IL-1β), interleukin 10 (IL-10), tumor necrosis factor alpha (TNF-α), transforming growth factor beta 1 and 2 (TGF-β_1 and TGF-β_2) and osteopontin (OPN) concentrations are expressed in pictograms per millilitre (pg/mL). See Appendix I: Table A - E for raw data.

For linear regression analysis, the presence or absence of the ε4 allele was the independent variable. Dummy variables were created for the presence (1) and absence (0) of the ε4 allele. The individual systemic inflammatory markers were the dependent variables. Table 13 details the results from the linear regression analysis for each inflammatory marker. The control and AD group were analysed together and as individual groups.

Linear regression analysis showed that across both groups, the presence of one ε4 allele was not significantly associated with any of the systemic inflammatory markers. In the control group, the ε4 allele tended to be positively associated with IL-10 (p = .099). In other words, controls with the ε4 tended to have higher baseline levels of IL-10. In the AD group, ε4 tended to be negatively associated with IL-10 (p = .078). But neither reached significance (p = .05).

Table 13

The Association between Inflammation and APOE ε4 in all Participants and in the Control and AD Groups

APOE ε4												
Inflammatory marker	Group											
	All Participants (*n* = 123)				Control (*n* = 64)				AD (*n* = 59)			
	β	Std Error	R^2	*p*	β	Std Error	R^2	*p*	β	Std Error	R^2	*p*
ESR	-.008	1.820	<.001	.933	.067	2.356	.004	.600	-.139	2.875	.019	.304
White cell count	-0.17	.341	<.001	.849	-.032	.449	.001	.803	-.032	.546	.001	.814
Monocyte count	-.121	.021	.015	.189	-.080	.028	.006	.529	-.179	.034	.032	.186
IL-1β	-.071	.218	.005	.454	-.039	.216	.002	.771	-.110	.385	.012	.410
IL-10	-.045	3.807	.002	.635	.220	4.256	.049	.099	-.233	6.243	.054	.078
TNF-α	-.150	.355	.022	.110	-.142	.221	.020	.292	-.210	.673	.044	.113
TGF-β_1	.038	135.072	.001	.690	.021	196.541	<.001	.876	.064	192.129	.004	.634
TGF-β_2	.057	.588	.003	.544	.035	.836	.001	.798	.073	.869	.005	.584
OPN	.134	163.023	.018	.153	.119	253.375	.014	.378	.146	219.782	.021	.275

Note. β = standardized coefficients for beta. Std Error = standard error. The erythrocyte sedimentation rate (ESR) was expressed as mm in 1 hour; the total white cell and monocyte counts are represented as cells $x10^9$/l. Interleukin 1 beta (IL-1β), interleukin 10 (IL-10), tumor necrosis factor alpha (TNF-α), transforming growth factor beta 1 and 2 (TGF-β_1 and TGF-β_2) and osteopontin (OPN) are represented as picograms per millilitre (pg/mL). See Appendix I: Table A - E for raw data.

Summary of the Cross-Sectional Results

The final sample number for the cross-sectional analysis was 128, and consisted of 68 controls and 60 AD participants. Control participants had significantly more years of education and were younger when compared with the AD group. Age was not correlated with any of the systemic inflammatory markers; therefore age was not controlled for in the analyses.

Systemic Inflammation and Cognition

Hypothesis one stated that in AD, systemic inflammatory markers would be highest in the early stages. The AD group was divided into mild, moderate and more severe subgroups based on their learning subscale scores. The mild AD group had a significantly higher ESR compared with controls (p = .018) and moderate AD participants (p = .031). There were no significant differences between the moderate AD, severe AD and control groups.

Mild AD participants also had a significantly higher IL-1β levels compared with the control group (p = .05), moderate AD group (p = .031) and severe AD group (p = .047). Again, there were no significant differences between the moderate AD, severe AD and control groups.

APOE genotype and Cognition

APOE genotyping was available for 123 participants, which included 64 controls and 59 AD participants. The most frequent allele in both groups was ε3. The allelic frequency for the ε3 allele in the control group was 0.703 and 0.551 in the AD group. The controls tended to have a higher ε3 frequency compared with the AD group (p =. 074). AD participants had a significantly higher frequency of the ε4 allele when compared with controls (p = .025). The most common genotype in the controls was the ε3/ε3 genotype whereas the ε3/ε4 genotype was the most common in the AD group. The AD group had significantly more ε4 homozygous participants than the control group (p = .034). The ε2 allele was the least common allele. The ε2 allelic frequency in controls was 0.094 and 0.093 in AD participants. Only one participant was homozygous for the ε2 allele.

The AD and control groups were divided up into APOE ε4 carriers and non-carriers. Mann-Whitney *U* test was used to compare between-group differences with respect to cognitive measures (CAMCOG, MMSE and learning subscale). In the controls, there was no significant difference between the APOE ε4 carriers and non-carriers on any of the cognitive measures. In the AD group, the APOE ε4 carriers tended to have poorer scores on the MMSE when compared with the APOE ε4 non-carriers (p = .053). There were no between-group differences on the learning subscale and the CAMCOG when AD APOE ε4 carriers and non-carriers were compared.

APOE genotype and Inflammation

When the subgroups were compared using Mann-Whitney *U* tests, the control APOE ε4 carrier and non-carrier subgroups did not differ on any of the inflammatory measures. This was true for the AD APOE ε4 carrier and non-carrier subgroups too. Linear regression showed a tendency for APOE ε4 to be positively associated with IL-10 in the control group and negatively associated with IL-10 in the AD group.

THE LONGITUDINAL STUDY

Final Sample Composition

Figure 21 depicts the participant attrition at follow-up. One hundred and six participants (59 controls, 47 AD participants) were contacted after one year for follow-up. The remaining 22 participants (9 Controls, 13 AD participants) from the cross-sectional study were not contacted as they had been recruited in the final year of the study. Twenty-six participants were not followed-up for the following reasons:

Withdrew from the study: Five Control and 8 AD participants withdrew from the study after the first year. Reasons for withdrawal were increased frailty or simply no further interest in participating in the study.

Deceased: One Control and 3 AD participants died after their first visit.

Relocated: Three Control participants and 1 AD participant relocated to different parts of South Africa.

Advanced disease: Two AD participants had progressed to advanced dementia and therefore were unable to consent to further testing.

Loss to follow-up: 1 AD participant was no longer contactable.

Conversions: 2 Control participants converted to MCI after their baseline visit.

Eighty participants were followed-up after one year; this included 48 Controls and 32 AD participants.

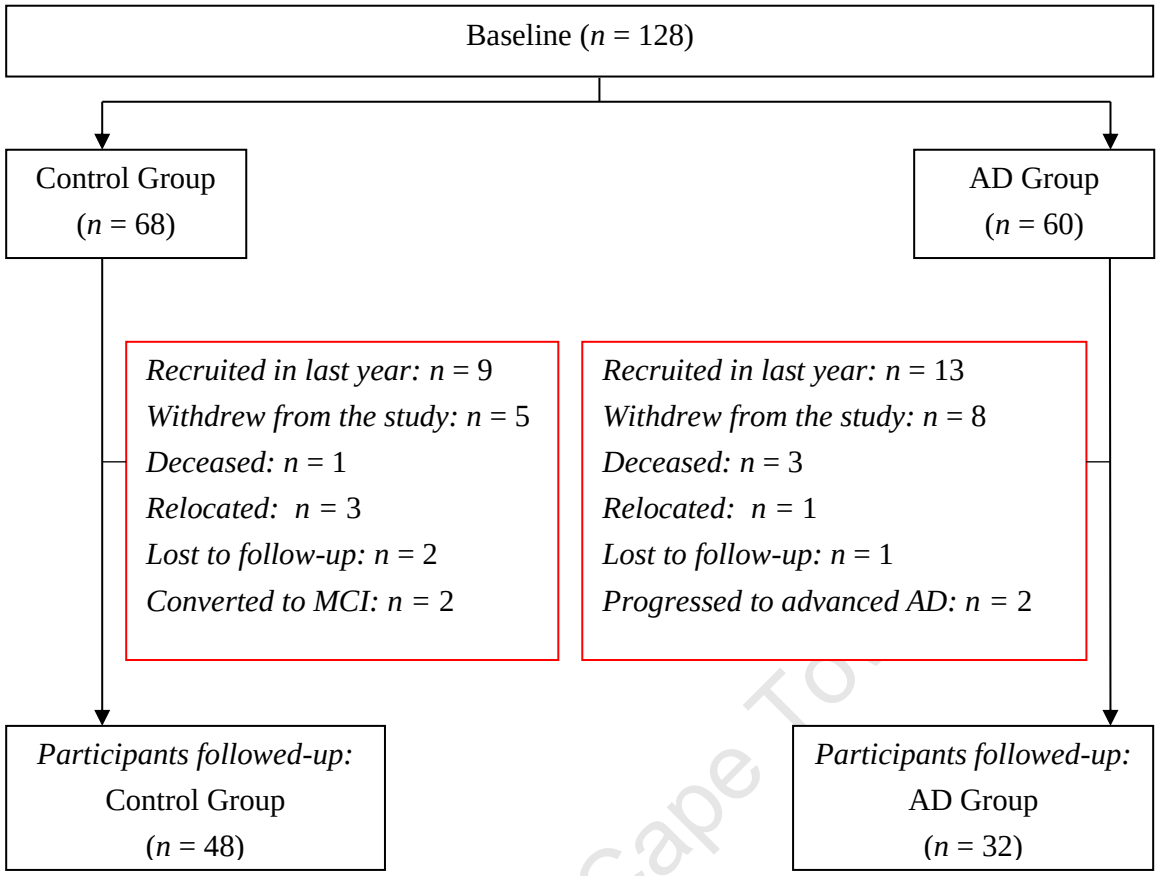


Figure 21: Participant attrition at one-year follow-up.

Final Sample: Demographic Characteristics and Cognitive Scores

Demographic data were obtained from all participants at their baseline visit. Table 14 describes the demographic characteristics of the participants that were followed-up (n = 80).

Age: At baseline, follow-up participants' ages ranged from 58 to 91 years (M = 72.5, SD = 8.6). The age data were normally distributed and therefore a Student's t-test was used to compare groups. There was no significant difference between the control participants and the AD participants that were followed-up (p = .115).

Sex: There were 23 males and 57 females in the final follow-up sample. Pearson's Chi-Squared analysis of these data showed that there were no significant between-group differences with regard to sex distribution.

Level of education: Level of education was defined as the total number of years of successfully completed education. The number of years of education ranged from 3 to 25 in the sample population (M = 12.1, SD = 4.6). Control participants (M = 13.9) had significantly more years of education when compared with AD participants (M = 9.4; p < 0.001).

Table 14
Demographic Data for the Longitudinal Study's Participants

	Group					
	Control (n = 48)	AD (n = 32)	t / χ^2	df	p	ESE
age	71.2 (8.2)	74.3 (9.1)	1.59	78	.115	0.36
sex (M:F)	11:37	12:20	1.99	1	.158	-0.16
level of education	13.9 (4.6)	9.4 (3.1)	-4.90	78	< .001*	-1.11

Note: For age and level of education, means are presented with ±1 standard deviations in parentheses. Level of education was measured as the number of years of successfully completed education. ESE = effect size estimate; for continuous variables (age, level of education) the ESE is Cohen's d; for sex the ESE is Phi. See Appendix I: Table A - E for raw data.
*Control and AD groups differed significantly, p < .001.

All participants had cognitive testing at their 12-month follow-up. The cognitive test battery included the CAMCOG, the learning subscale of the CAMCOG, and the MMSE. Table 15 and *Figure 22* to *24* depict the baseline scores of the participants who were followed-up and the follow-up scores of the two groups. Cognitive decline was measured by subtracting the participant's raw baseline score from their follow-up score. Therefore, a negative number indicates a decline in cognitive functioning; whereas a positive number indicates improvement in cognition. The median and interquartile range for cognitive decline was calculated from each participant's raw scores.

CAMCOG: The median change in CAMCOG scores are presented in Table 15. AD participants had lower CAMCOG scores at baseline and at the one-year follow-up compared with controls. The AD group declined, on average, by 7 points on the CAMOCG over 12 months whereas the control group improved by 1 point. The change in CAMCOG scores was significantly different between the AD and control groups ($p < .001$).

MMSE: At baseline and at follow-up the AD group had significantly lower MMSE scores when compared with controls. The AD group declined by 2 points on the MMSE over 12 months. There was no change in the median scores on the MMSE in the control group. The AD groups MMSE score declined more over 12 months than the control group ($p = .049$).

Learning Subscale: AD participants had significantly lower learning subscale scores at baseline and at their follow-up. The median change in the learning subscale over 12 months was -1.5 in the AD group and 0 in the control group. The change in the learning subscale score in one year was greater in the AD group compared with the control group ($p < .001$).

Table 15
Cognitive Test Scores at Baseline and One-Year Follow-up

Cognitive test	Group					
	Control (n = 48)	AD (n = 32)	*U*	*df*	*p*	*r*
CAMCOG 1	94 (89 – 96)	64.5 (52 – 75.5)	7.0	78	< .001*	-0.84
CAMCOG 2	94 (31.5 – 97)	57 (46.5 – 70)	8.5	78	< .001*	-0.83
CAMCOG 2 –1	1 (-1 – 2)	-7 (-11 – -2.5)	272.5	78	< .001*	-0.54
MMSE 1	29 (28 – 29)	21.5 (17 – 24)	21.0	78	< .001*	-0.82
MMSE 2	29 (27 – 30)	19 (16.5 – 22.5)	22.5	78	< .001*	-0.82
MMSE 2 – 1	0 (-1 – 1)	-2 (-4 – 1.5)	566.5	78	.048**	-0.22
LS 1	14 (13 – 15)	7 (4 – 10)	57.5	78	< .001*	-0.78
LS 2	14.5 (14 – 15.5)	5.5 (2.5 – 7.5)	18.0	78	< .001*	-0.83
LS 2 – 1	0 (-0.5 – 2)	-1.5 (-3 – 0)	360.5	78	< .001*	-0.45

Note. Medians are presented with interquartile ranges in parentheses. The CAMCOG 1, Mini Mental State Examination (MMSE) 1 and learning subscale (LS) 1 represent baseline cognitive scores. The CAMCOG 2, MMSE 2 and LS 2 represent 1 year follow-up scores. Cognitive decline was measured by subtracting the baseline score (1) from the follow-up score (2). See Appendix I: Table A - E for raw data.
Control and AD groups differed significantly, * $p < .001$, ** $p < .05$.

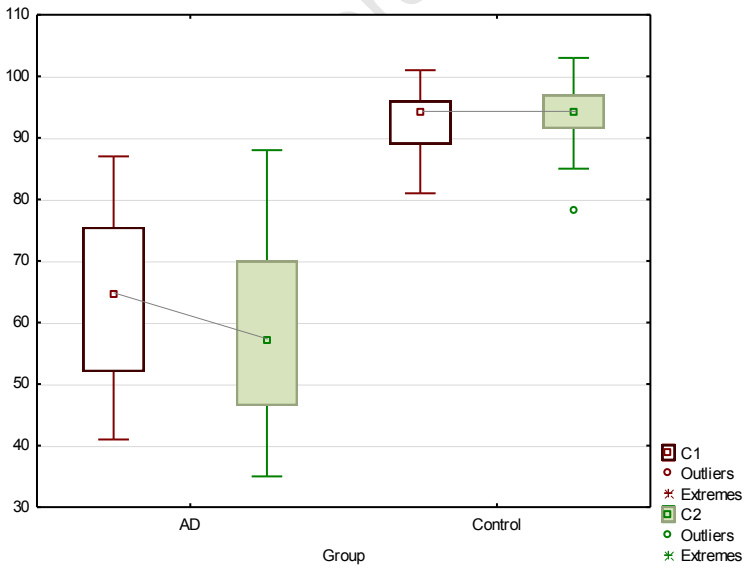


Figure 22: Baseline and follow-up CAMCOG scores for AD (n = 32) and control (n = 48) groups. C1 represents the baseline score. C2 represents the follow-up score.

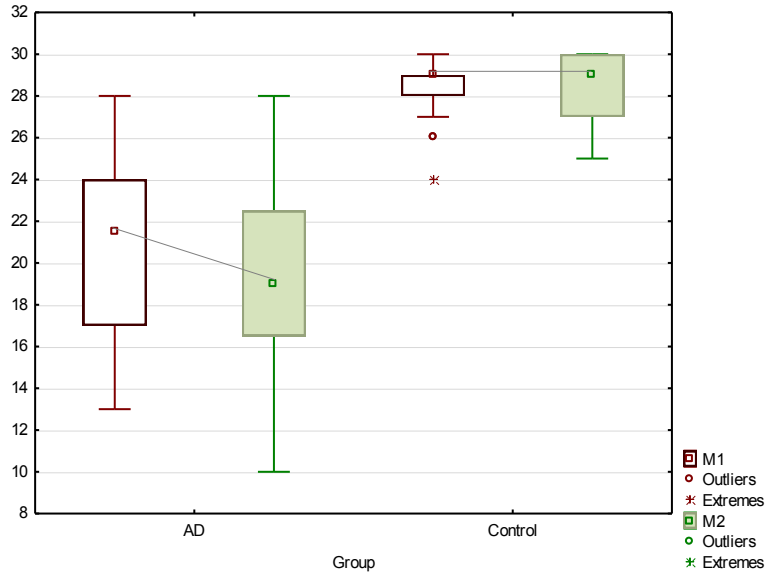


Figure 23: Baseline and follow-up Mini-Mental State Examination (MMSE) scores for AD (*n* = 32) and control (*n* = 48) groups. M1 represents the baseline MMSE score. M2 represents the follow-up score.

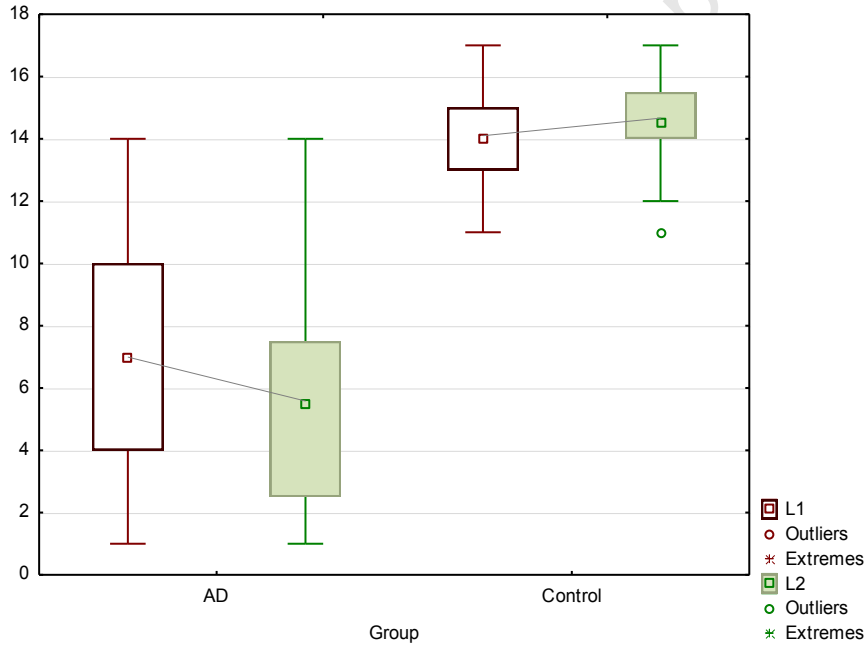


Figure 24: Baseline and follow-up learning subscale scores for AD (*n* = 32) and control (*n* = 48) groups. L1 represents the baseline score. L2 represents the follow-up score.

When both groups were analysed together, Spearman's ranked correlation showed that age was not correlated with cognitive decline on the CAMCOG (r = -0.139, p = .650), MMSE (r = -0.104, p = .235), or learning subscale (r = -0.08, p = .515). Similarly, when the control and AD groups were analysed separately, age was not correlated with cognitive decline on the CAMCOG (r = -0.190, p = .364), MMSE (r = 0.112, p = .476), or learning subscale (r = -0.019, p = .937) in the control group or in the AD group; CAMCOG (r = 0.014, p = .483), MMSE (r = -0.213, p = .246), or learning subscale (r = 0.011, p = .824). Therefore the following analyses did not control for age.

Education was also not significantly related to cognitive decline on the CAMCOG (r = 0.149, p = .197), MMSE (r = -0.022, p = .843) or the learning subscale (r = 0.054, p = .632).

The Effect of Systemic Inflammation on Cognitive Decline

Hypothesis 4 stated that high levels of baseline systemic inflammation would predict a faster rate of cognitive decline over a one-year period.

Linear regression analysis was used to examine the independent effect of inflammation on cognitive decline. APOE ε4 was added to the model as an independent variable to account for the APOE ε4 related decline in cognition. The dependent variable in the regression analysis was cognitive decline on the CAMCOG (CAMCOG 2 – CAMCOG 1), MMSE (MMSE 2 – MMSE 1) and learning subscale (learning subscale 2 – learning subscale 1). The independent variables were the presence or absence of the APOE ε4 allele and the systemic inflammatory markers (ESR, white cell count, monocyte count, IL-10, TGF-β_1, TGF-β_2, TNF-α, IL-1β and OPN). Linear regression analyses were performed by adding the APOE ε4 status (present or absent) as an independent variable together with each individual inflammatory marker as a new model. Groups were analysed together and separately.

CAMCOG

Table 16 depicts the results from the linear regression analysis for the CAMCOG. The *p* value is the independent effect of the inflammatory marker. As previously mentioned, ε4 was added to the model to account for the effect of the ε4 allele on cognitive decline. A *positive association* indicates that a high level of the baseline systemic inflammatory marker was associated with a smaller decline on the cognitive score. A *negative association* indicates that a high level of the baseline systemic inflammatory marker was associated with a greater or faster decline on the cognitive score.

When the two groups were analysed together, the total white cell count ($p = .050$) and TNF-α ($p = .019$) were independently negatively associated with cognitive decline on the CAMCOG (*Figure 25* and *26* respectively). In other words, high levels of baseline TNF-α and total white cell count were associated with greater decline on the CAMCOG. IL-10 was positively associated with a decline on the CAMCOG ($p = .032$), i.e. the higher the baseline level of IL-10 the greater decline on the CAMCOG, see *Figure 27*.

Similarly in the AD group, high baseline levels of white cell count and TNF-α predicted greater cognitive decline on the CAMCOG, $p = .023$ and $p = .049$ respectively (*Figure 25* & *26*). The monocyte count also tended to be negatively associated with cognitive decline on the CAMCOG ($p = .082$). High baseline levels of IL-10 tended to predict a slower rate of cognitive decline on the CAMCOG ($p = .068$; *Figure 27*), but this did not reach significance.

In the control group, there were no significant associations between decline on the CAMCOG and any of the systemic inflammatory markers.

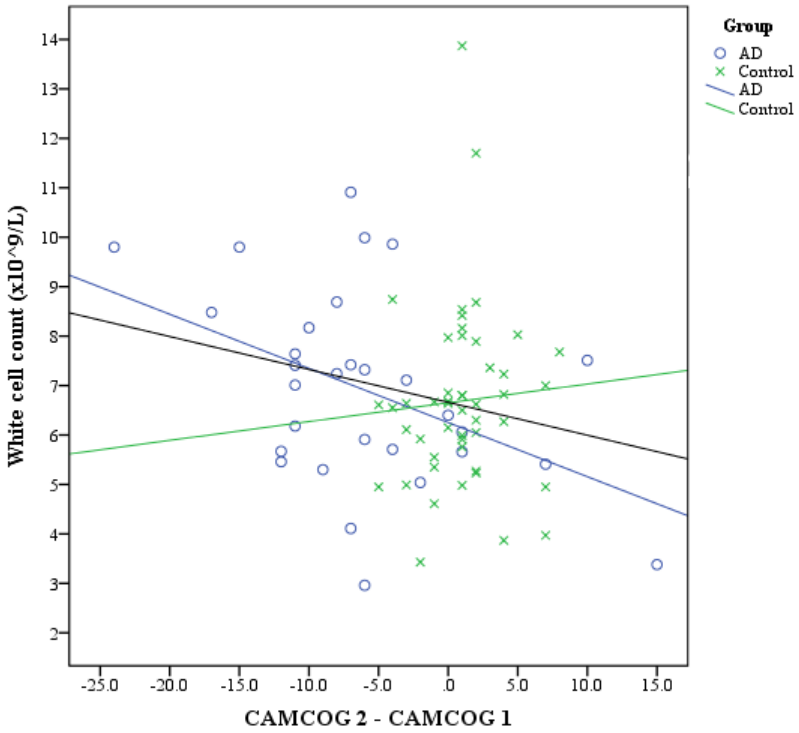


Figure 25: The relationship between the white cell count and decline on the CAMCOG (CAMCOG 2 – CAMCOG 1). The black line represents the association when all participants were examined together.

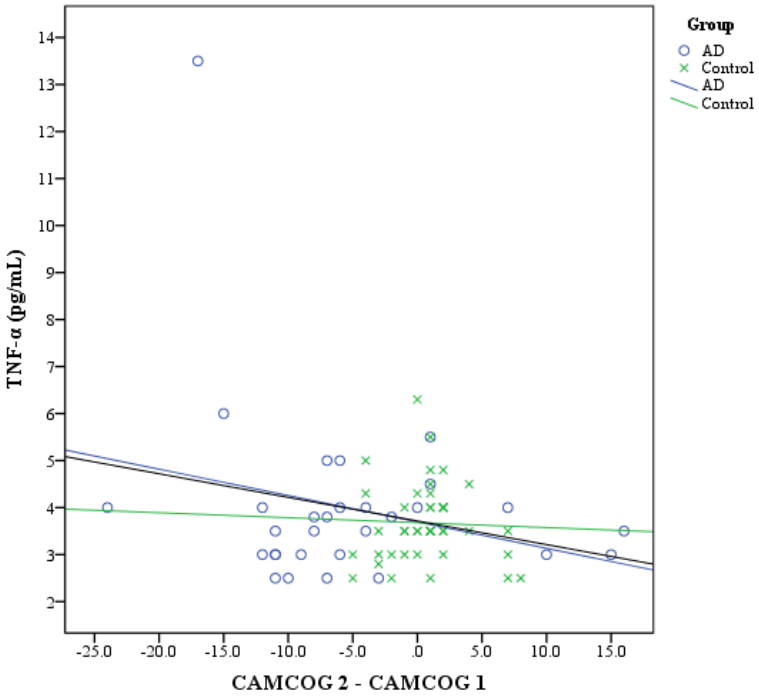


Figure 26: The relationship between tumor necrosis alpha (TNF-α) and decline on the CAMCOG (CAMCOG 2 – CAMCOG 1). The black line represents the association when all participants were examined together.

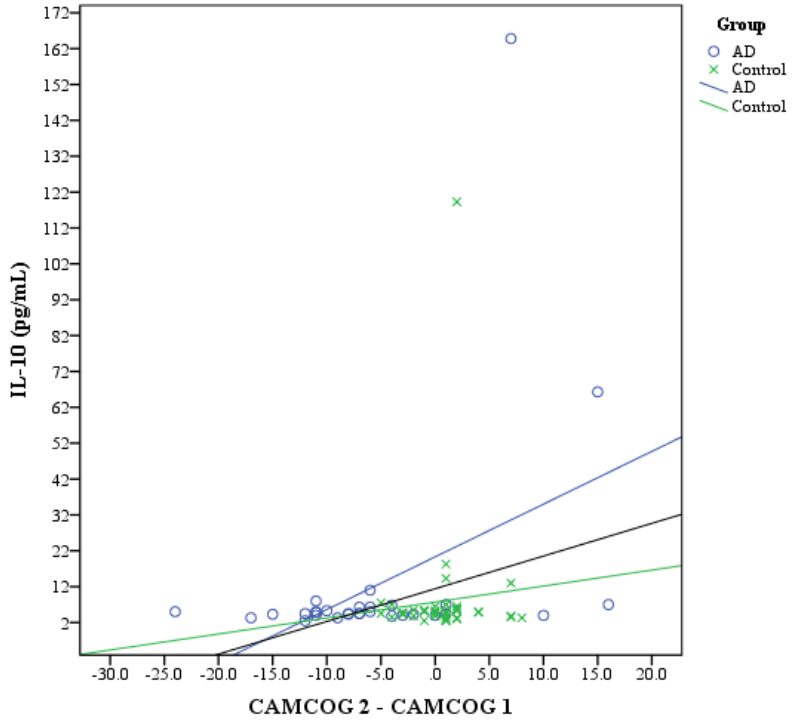


Figure 27: The relationship between interleukin 10 (IL-10) and decline on the CAMCOG (CAMCOG 2 – CAMCOG 1). The black line represents the association when all participants were examined together.

Table 16
The Association Between Cognitive Decline on the CAMCOG and Systemic Inflammatory Markers

CAMCOG												
Inflammatory marker	All participants				Control				AD			
	β	Std Error	R^2	*p*	β	Std Error	R^2	*p*	β	Std Error	R^2	*p*
ESR	-.045	.078	.025	.697	-.042	.048	.133	.768	-.078	.147	.086	.687
White cell count	-.223	.379	.076	**.050***	.093	.233	.140	.511	-.407	.664	.245	**.023***
Monocyte count	-.125	6.435	.038	.281	.119	3.811	.145	.401	-.325	11.798	.183	.082
IL-1β	.110	1.026	.048	.352	-.022	.565	.145	.885	.288	2.010	.213	.104
IL-10	.249	.032	.098	**.032***	.003	.026	.144	.986	.377	.051	.232	.068
TNF-α	-.273	.538	.109	**.019***	.006	.538	.144	.968	-.354	.756	.248	**.049***
TGF-β_1	.037	.001	.038	.752	.214	.001	.190	.142	.030	.002	.131	.871
TGF-β_2	.002	.268	.036	.989	.085	.146	.152	.563	-.035	.544	.131	.847
OPN	.052	.001	.039	.660	-.034	<.001	.146	.819	-.065	.003	.134	.723

Note. β = standardized coefficients for beta, Std Error presents the standard error. Abbreviations for the inflammatory markers are as follows: erythrocyte sedimentation rate (ESR), interleukin 1 beta (IL-1β), interleukin 10 (IL-10), tumor necrosis factor alpha (TNF-α), transforming growth factor beta 1 and 2 (TGF-β_1 and TGF-β_2), osteopontin (OPN). See Appendix I: Table A - E for raw data.
* Significant effect on CAMCOG, $p < .05$.

MMSE

Table 17 depicts the results from the linear regression analysis for the MMSE. When the groups were analysed together, IL-10 tended to be positively associated with decline on the MMSE (p = .077), i.e. the higher the baseline level of IL-10, the smaller the decline on the MMSE in all participants (see *Figure 28*). TNF-α was negatively associated with decline on the MMSE (p = .027), i.e. the higher the baseline level of TNF- α, the greater the decline on the MMSE (see *Figure 29*). There were no significant associations between decline on the MMSE and the systemic inflammatory markers in the control group (Table 17).

In the AD group, IL-10 tended to be positively associated with decline on the MMSE (p = .082), the higher the baseline level of IL-10 the smaller the decline on the MMSE (see *Figure 28*). No other associations were found in the AD group.

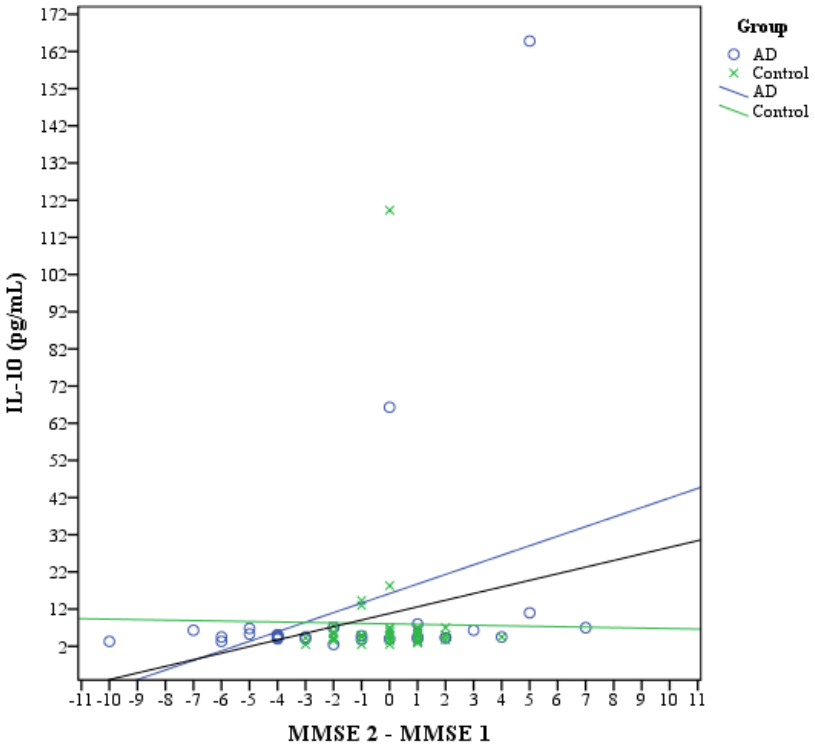


Figure 28: The relationship between interleukin 10 (IL-10) and decline on the Mini Mental State Examination (MMSE) (MMSE 2 – MMSE 1). The black line represents the association when all participants were examined together.

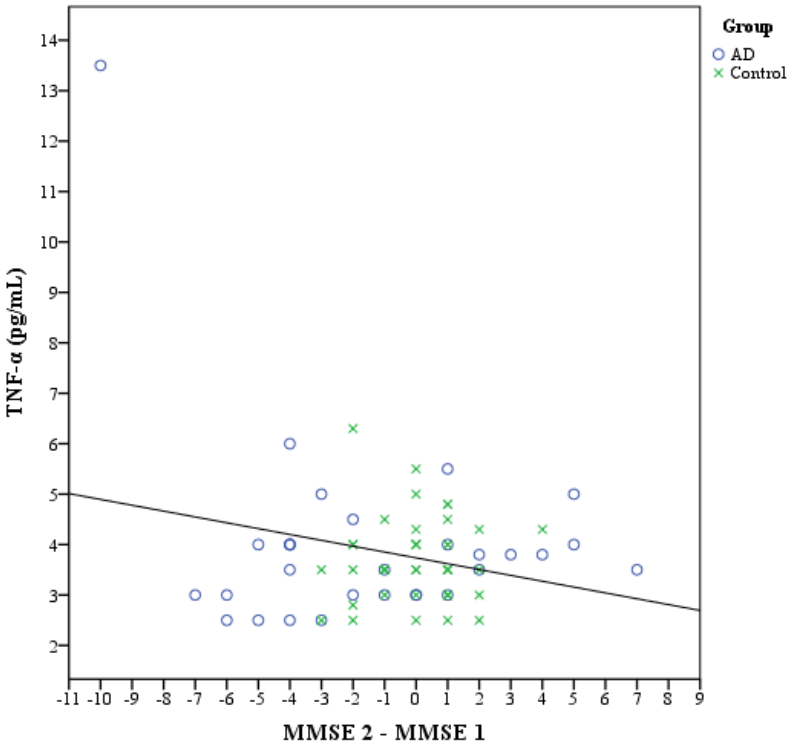


Figure 29: The relationship between tumor necrosis alpha (TNF-α) and decline on the Mini Mental State Examination (MMSE) (MMSE 2 – MMSE 1) in all the participants.

Table 17

The Association Between Cognitive Decline on the MMSE and Systemic Inflammatory Markers

MMSE												
Inflammatory marker	All participants				Control				AD			
	β	Std Error	R^2	*p*	β	Std Error	R^2	*p*	β	Std Error	R^2	*p*
ESR	-.063	.034	.013	.593	-.028	.025	.019	.852	-.063	.074	.005	.756
White cell count	.082	.168	.014	.475	.048	.121	.021	.751	.136	.370	.022	.483
Monocyte count	.082	2.76	.016	.485	.166	1.953	.046	.266	.070	6.274	.006	.725
IL-1β	.083	.445	.027	.486	.113	.298	.032	.477	.110	1.024	.017	.569
IL-10	.208	.014	.063	.077	.018	.014	.020	.912	.345	.025	.112	.082
TNF-α	-.261	.233	.087	**.027***	.011	.286	.020	.942	-.304	.379	.092	.119
TGF-β_1	.088	<.001	.028	.461	.032	<.001	.021	.839	.173	.001	.035	.370
TGF-β_2	.113	.115	.033	.340	-.075	.078	.025	.634	.246	.258	.065	.200
OPN	.121	<.001	.034	.311	-.118	<.001	.033	.458	.307	.001	.097	.110

Note. β = standardized coefficients for beta, Std Error presents the standard error. Abbreviations for the inflammatory markers are as follows: erythrocyte sedimentation rate (ESR), interleukin 1 beta (IL-1β), interleukin 10 (IL-10), tumor necrosis factor alpha (TNF-α), transforming growth factor beta 1 and 2 (TGF-β_1 and TGF-β_2), osteopontin (OPN). See Appendix I: Table A - E for raw data.

* Significant effect on MMSE, $p < .05$.

Learning Subscale

Table 18 depicts the results from the linear regression analysis for the learning subscale. When the AD and control groups were analysed together the ESR (p = .025) and TNF-α (p = .029) measures were negatively associated with decline on the learning subscale (see *Figure 30* and *31* respectively). I.e. the higher the level of ESR and TNF-α at baseline, the greater the decline on the learning subscale. When the control group was analysed separately, high levels of ESR at baseline predicted greater decline on the learning subscale score, p = .028 (see *Figure 30*). There were no other significant associations between systemic inflammatory markers and decline on the learning subscale in the control group. In the AD group, the monocyte count and TGF-β_2 levels were negatively associated with decline on the learning subscale, p = .032 (*Figure 32*) and p = .034 (*Figure 33*) respectively. High baseline levels of OPN also tended to predict greater decline on the learning subscale over one-year, p = .061. No other significant associations were found in the AD group.

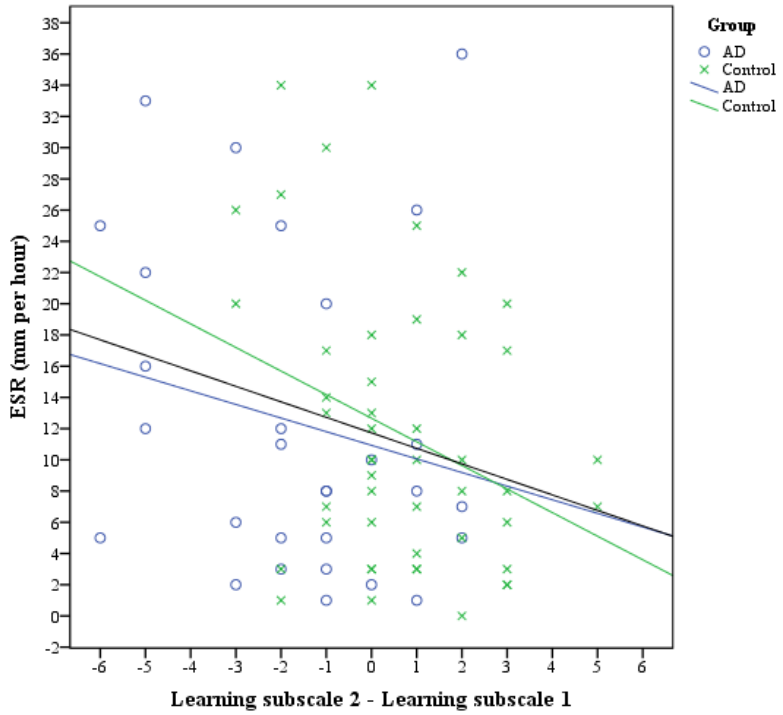


Figure 30: The relationship between the erythrocyte sedimentation rate (ESR) and decline on the learning subscale in all the participants. The black line represents the effect of ESR when both groups were examined together.

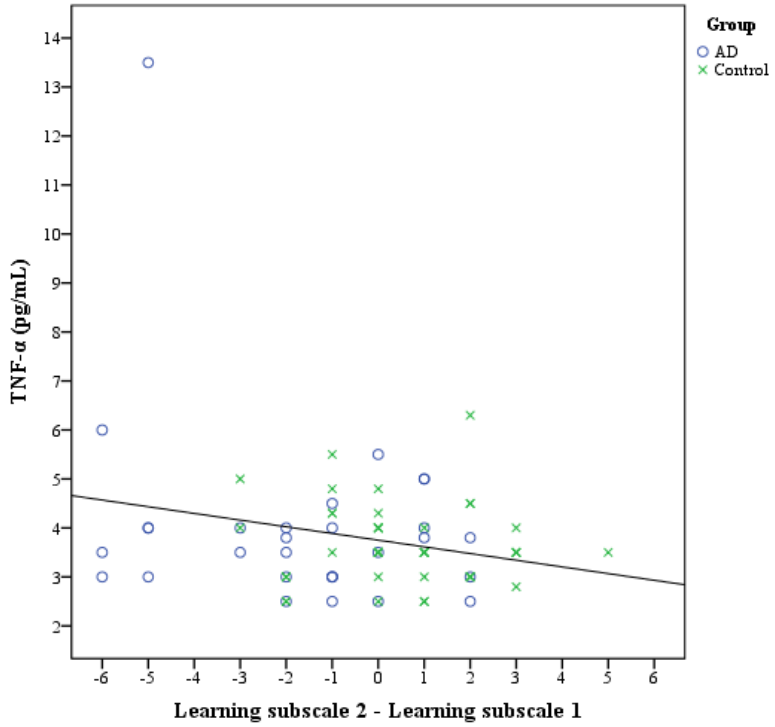


Figure 31: The relationship between tumor necrosis alpha (TNF-α) and decline on the learning subscale in all the participants. The black line represents the effect of TNF-α when both groups were examined together.

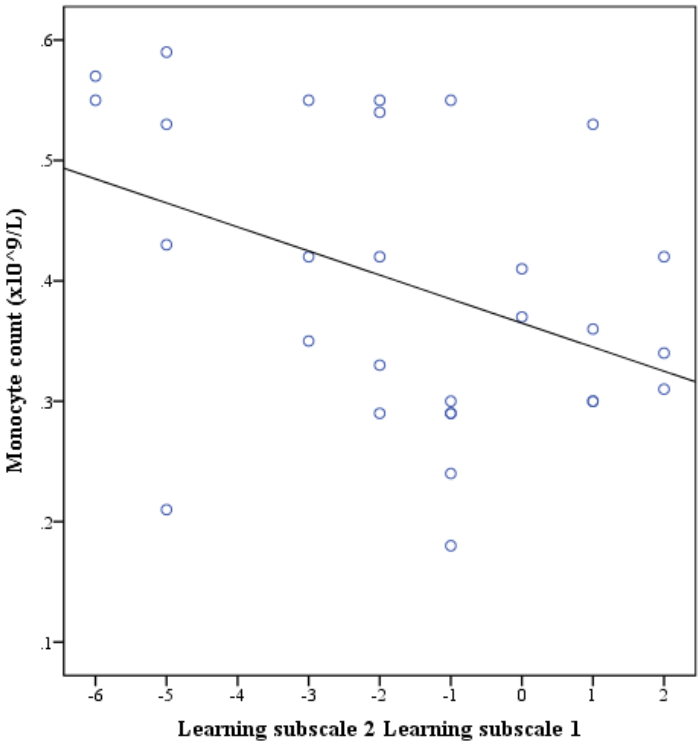


Figure 32: The relationship between the monocyte count and decline on the learning subscale in AD participants.

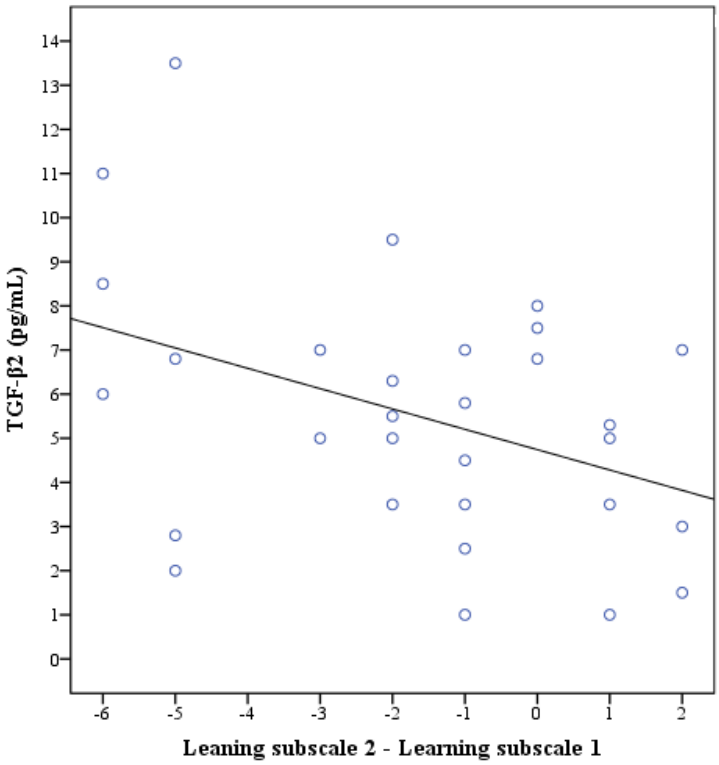


Figure 33: The relationship between transforming growth factor beta 2 (TGF-β_2) and decline on the learning subscale in AD participants.

Table 18
The Association Between Cognitive Decline on the Learning Subscale and Systemic Inflammatory Markers

Learning Subscale												
Inflammatory marker	All participants				Control				AD			
	β	Std Error	R^2	*p*	β	Std Error	R^2	*p*	β	Std Error	R^2	*p*
ESR	-.257	.028	.078	**.025***	-.324	.030	.105	**.028***	-.215	.046	.044	.282
White cell count	-.162	.148	.045	.158	-.031	.156	.001	.841	-.293	.235	.087	.125
Monocyte count	-.140	2.398	.032	.232	.075	2.546	.006	.622	-.412	3.644	.166	**.032***
IL-1β	-.012	.361	.030	.921	-.137	.351	.027	.388	.169	.640	.033	.381
IL-10	.142	.011	.050	.227	.087	.016	.015	.591	.317	.016	.095	.112
TNF-α	-.256	.189	.094	**.029***	-.084	.336	.015	.599	-.288	.240	.082	.141
TGF-β_1	-.039	<.001	.031	.741	.238	<.001	.065	.128	-.237	.001	.060	.216
TGF-β_2	-.095	.093	.039	.422	.152	.091	.031	.334	-.397	.154	.160	**.034***
OPN	.014	<.001	.030	.907	.088	<0.001	.016	.584	-.357	.001	.128	.061

Note. β = standardized coefficients for beta, Std Error presents the standard error. Abbreviations for the inflammatory markers are as follows: erythrocyte sedimentation rate (ESR), interleukin 1 beta (IL-1β), interleukin 10 (IL-10), tumor necrosis factor alpha (TNF-α), transforming growth factor beta 1 and 2 (TGF-β_1 and TGF-β_2), osteopontin (OPN). See Appendix I: Table A - E for raw data.
* Significant effect on learning subscale, $p < .05$.

The Effect of the APOE ε4 on Cognitive Decline

Table 19 displays the decline in cognition over 12 months in APOE ε4 carriers and ε4 non-carriers. Again, a negative number indicates a decline in cognitive functioning; whereas a positive number indicates improvement in cognition.

Table 19
Changes in Cognitive Test Scores Over One-Year in APOE ε4 Carriers and Non-Carriers

	Control Group	
Cognitive test	APOE ε4 Non-carriers (n = 28)	APOE ε4 Carriers (n = 19)
CAMCOG 2 – CAMCOG 1	0.5 (-2 – 1)	2 (1 – 4)
MMSE 2 – MMSE 1	0 (-1 – 1)	0 (-1 – 1)
LS 2 – LS 1	0 (-0.5 – 2)	1 (-1 – 2)
	AD Group	
	APOE ε4 Non-carriers (n = 11)	APOE ε4 Carriers (n = 20)
CAMCOG 2 – CAMCOG 1	-6 (-11 – 10)	-7.5 (-11 – -4)
MMSE 2 – MMSE 1	0 (-6 – 3)	-2.5 (-4 – 1.5)
LS 2 – LS 1	-1 (05 – 1)	-2 (-3 – -0.5)

Note. Medians are presented with interquartile ranges in parentheses. Cognitive decline is measured by subtracting the baseline score (1) from the follow-up score (2) for the CAMCOG, Mini Mental State Examination (MMSE) and learning subscale (LS).
See Appendix I: Table A - E for raw data.

Hypothesis 5 stated that the presence of the ε4 allele (either homozygous or heterozygous) in participants would exacerbate their rate of cognitive decline when compared to participants with the ε3 or ε2 allele.

To test the above hypothesis, linear regression analysis was used. The presence or absence of the APOE ε4 allele was the independent variable. Each inflammatory marker was also added to the model as an independent variable to examined if the APOE ε4 allele was *independently* associated with cognitive decline, and that the decline was not associated

with inflammation. The dependent variable was decline in cognition on the CAMCOG, MMSE and learning subscale.

Across both groups, the APOE ε4 allele (either homozygous or heterozygous) was not associated with cognitive decline on the MMSE, β = -0.113, R^2 = 0.013, *p* = .343, or the learning subscale, β = -0.138, R^2 = .019, *p* = .229. On the CAMCOG, the presence of the ε4 allele tended to be associated with greater cognitive decline, β = -0.188, R^2 = 0.035, *p* = .099; i.e. the ε4 allele predicted greater decline on the CAMCOG in all participants in one-year.

When the groups were analysed separately, the presence of the ε4 allele was positively associated with decline on the CAMCOG, β = 0.362, R^2 = 0.131, *p* = .012 in the control group (see *Figure 34*). Controls with the ε4 allele tended to decline less than the controls without the ε4 allele. The presence of ε4 was not associated with decline on the MMSE, β = -0.136, R^2 = 0.018, *p* = .362, or the learning subscale score, β = 0.011, R^2 <0.001, *p* = .942, in the controls.

In the AD group, the ε4 allele was negatively associated with decline on the CAMCOG, β = -0.359, R^2 = 0.187, *p* = .047 (see *Figure 35*). AD participants with the ε4 allele had a greater decline on the CAMCOG compared with AD participants with no ε4 allele. There was no significant association between the presence or absence of the ε4 allele and the MMSE, β = -0.037, R^2 = 0.001, *p* = .843 or the learning subscale score, β = -0.077, R^2 = 0.006, *p* = .680.

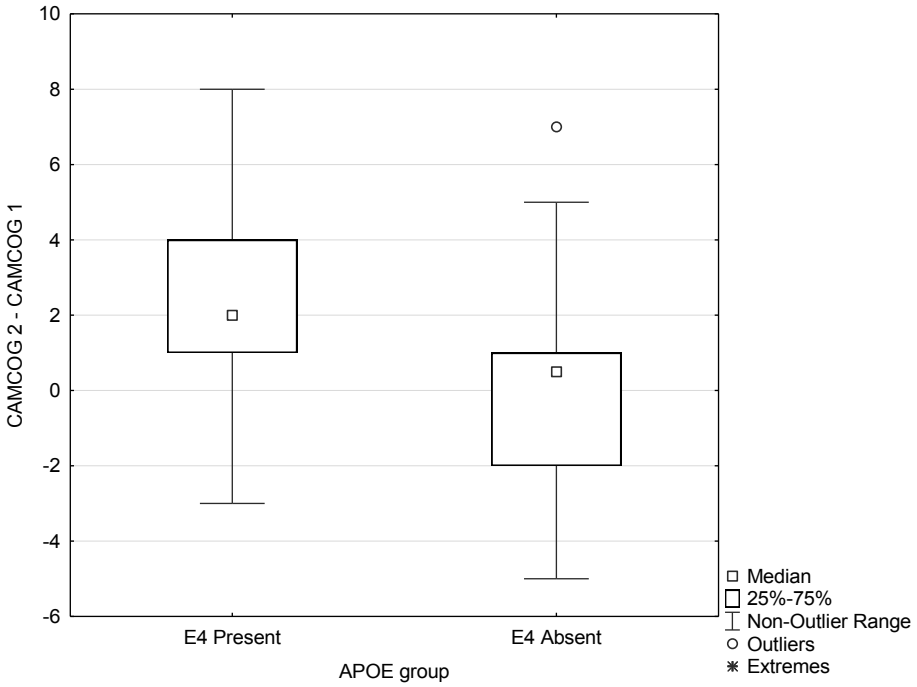


Figure 34: The relationship between the presence and absence of the APOE ε4 allele and cognitive decline on the CAMCOG in control participants (ε4 Present *n* = 19; ε4 Absent *n* = 28). Cognitive decline was measured by subtracting the baseline score from the follow-up score.

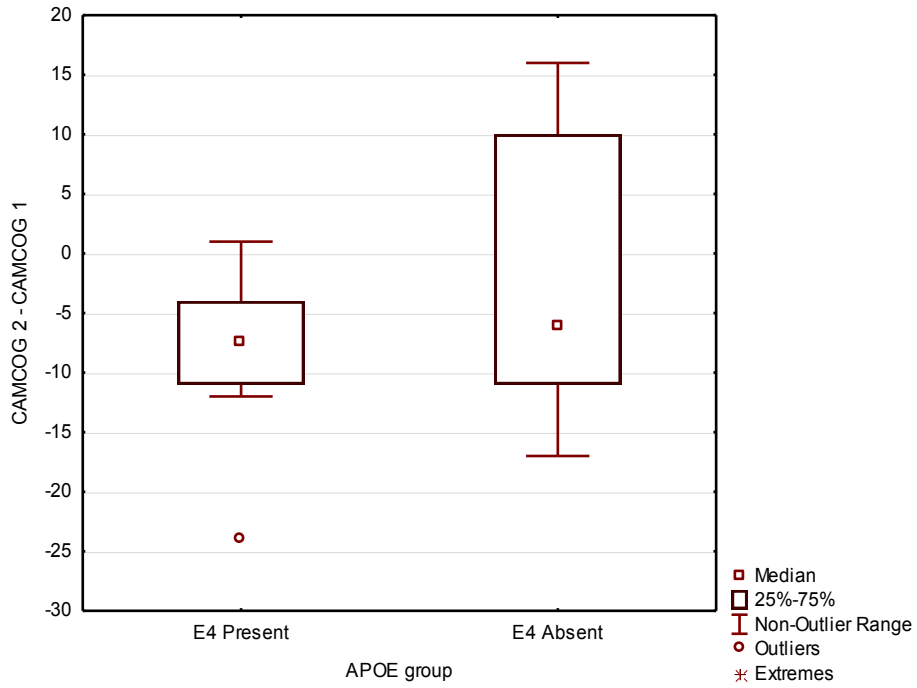


Figure 35: The relationship between the presence and absence of the APOE ε4 allele and cognitive decline on the CAMCOG in AD participants (ε4 Present *n* = 20; ε4 Absent *n* = 11). Cognitive decline was measured by subtracting the baseline score from the follow-up score.

Longitudinal Study Summary

Eighty participants were followed-up after 12 months. As expected, AD participants declined faster than controls on the CAMCOG, MMSE and learning subscale. The AD participants had a median change of -7 on the CAMCOG, -2 on the MMSE and -1.5 on the learning subscale. Decline in cognition was measured as the follow-up score minus the baseline score.

The effect of inflammation on cognition

Linear regression analysis was used to take into account the effect of the APOE ε4 allele on cognitive decline. This was done to examine the independent effect of inflammation on cognitive decline.

Across all participants, high baseline levels of TNF-α were associated with greater decline on the CAMCOG, MMSE and learning subscale. A high total white cell count at baseline was associated with a greater decline on the CAMCOG. High baseline levels of ESR were associated with greater decline on the learning subscale.

In all participants, IL-10 was positively associated with decline on the CAMCOG. I.e. high levels of IL-10 were associated with smaller decline on the CAMCOG across all participants. Similarly, high levels of IL-10 tended to be associated with smaller decline on the MMSE in all participants.

In the AD group, high baseline levels of TNF-α and the total white cell count were associated with a greater decline on the CAMCOG. The monocyte count also tended to be negatively associated with decline on this measure. A high monocyte count and high levels of TGF-β_2 were associated with greater decline on the learning subscale in AD participants. OPN tended to be negatively associated with decline on the learning subscale, i.e. high levels of OPN at baseline tended to be associated with a greater decline on the learning subscale.

High levels of IL-10 in the AD group tended to be associated with smaller decline on the CAMCOG and MMSE.

In the control group, a high baseline ESR was associated with greater decline on the learning subscale.

The effect of APOE ε4 on cognition

Linear regression analysis was used to determine the effect of APOE ε4 on cognitive decline. In controls, the absence of the ε4 allele was associated with greater decline on the CAMCOG over one year. However, in the AD group, the presence of the ε4 allele was associated with a greater decline on the CAMCOG over one year. There were no significant associations between the ε4 allele and decline on the MMSE or learning subscale.

THE MRS STUDY

Final Sample Composition

AD and control participants already enrolled in the study were contacted and requested to participate in the MRS study. As far as possible, the control and AD group were matched on age and education. AD participants that had been enrolled within the last two years of the study were asked to take part. Controls that were older and had fewer years of education were contacted.

Fifty participants were approached to participate in the MRS study; this included 21 controls and 29 AD participants. Four controls and 4 AD participants declined to participant. The mains reasons for refusal were increased frailty and claustrophobia. One control was excluded from the MRS study because of metal plates in her neck. One AD participant was excluded as he/she had a prosthetic heart value. Sixteen controls and 24 AD participants were eligible for, and agreeable to, MRI scanning. *Figure 36* summarises the participant attrition for the MRS study. One Control and 4 AD participants were unable to tolerate the 20-minute duration of the scan and their scans had to be terminated. Full MRS data were not obtainable from these participants. One control and 1 AD participant had ESRs over 40 mm in one hour. These participants MRS data were used but their systemic inflammatory markers were excluded. During the data analysis stage, neurometabolite readings from 2 AD participants could not be obtained for technical reasons. The final sample consisted of 33 participants: 15 Controls and 18 AD participants. Due to exclusion of participants with ESRs above 40 mm per hour, the final sample numbers for analysing the relationship between systemic inflammatory markers and neurometabolites consisted of 14 Controls and 17 AD participants.

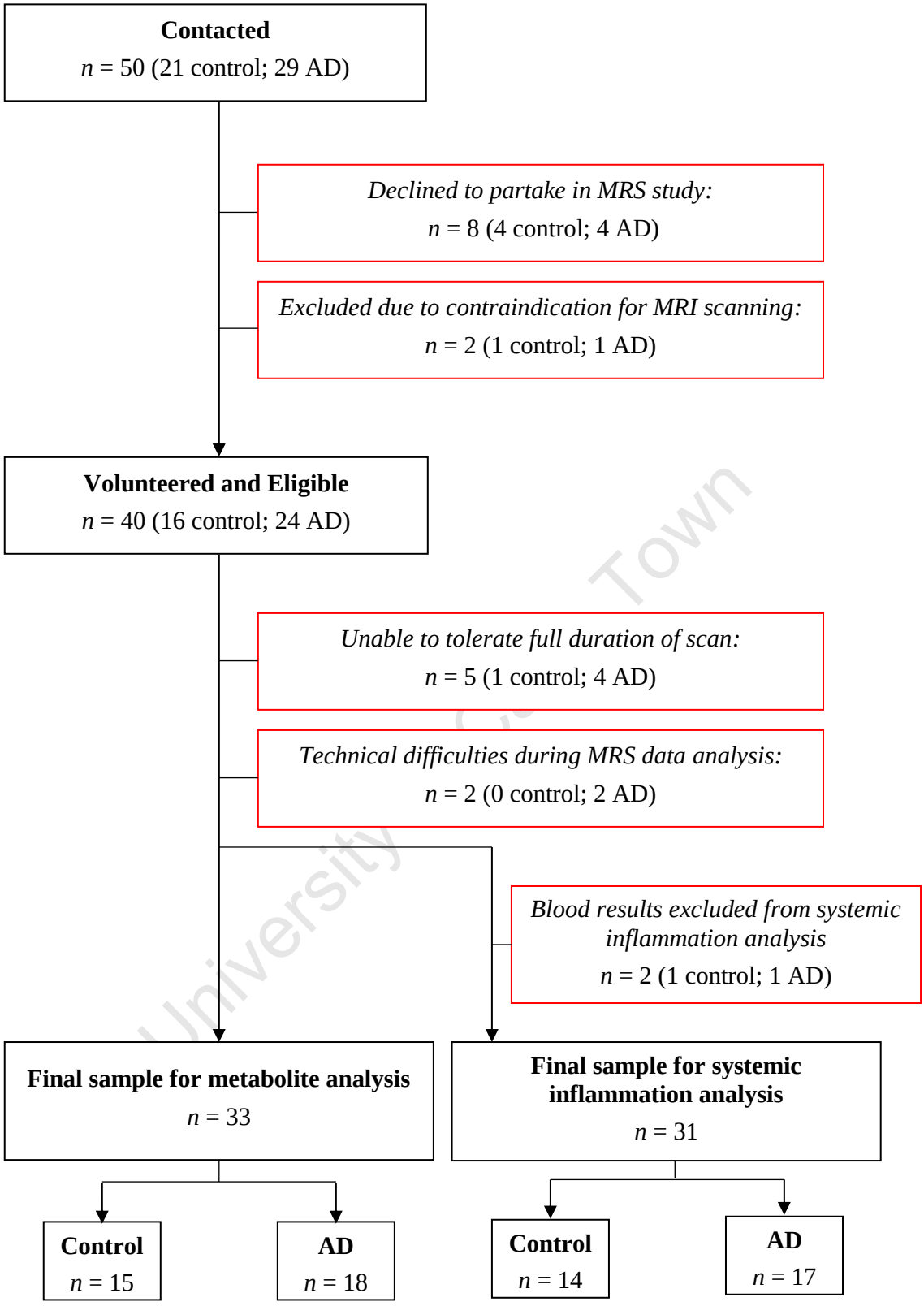


Figure 36: Participant attrition for MRS study.

Final Sample: Demographic Characteristics and Cognitive Scores

Table 20 summarises the demographic characteristics of participants in the MRS study.

Age: The participants' (AD and controls) ages ranged from 61 to 87 years (M = 74.35, SD = 7.46). The age data were normally distributed and therefore Student's t-test was used to do between-group comparisons. There was no significant age difference between the control and AD group (p = .451).

Sex: There were 16 males and 17 females in the final sample. Pearson's Chi-Squared analysis of these data showed that there were no significant between-group differences with regard to sex distribution (p = .373).

Level of education: Again, this variable was defined as the total number of years of successfully completed education. The number of years of education in all participants ranged from 5 to 18 years (M = 11.32, SD = 3.14). Education was normally distributed and therefore the Student's t-test was used to compare groups. Control participants (M = 13.2) had significantly more years of education when compared with AD participants (M = 10.1; p = .002).

APOE ε4: Sixty-four percent of all participants who were included in this MRS study did not carry an APOE ε4 allele. Thirty-six percent of all participants were either homozygous or heterozygous for the ε4 allele, i.e. carried at least one APOE ε4 allele.

Table 20
Demographic Data for the MRS Study's Participants

	Group					
	Control (n = 15)	AD (n = 18)	t / χ^2	df	p	ESE
age	72.9 (6.3)	74.9 (8.1)	0.76	31	.451	0.27
sex (M:F)	6:9	10:8	0.79	1	.373	-0.16
level of education	13.2 (3.00)	10.1 (2.3)	-3.41	31	.002*	-1.22
APOE ε 4	27%	44%	1.12	1	.290	0.18

Note: For age and level of education, means are presented with standard deviations in parentheses. Level of education was measured as the number of years of successfully completed education. Apolipoprotein ε4 (APOE ε4) was given as a percentage of controls and AD participants with at least one ε4 allele (either homozygous or heterozygous). ESE = effect size estimate: for continuous variables (age and level of education) the ESE is Cohen's *d*; for categorical variables (sex and APOE ε4) the ESE is Phi. See Appendix J: Table A &B for raw data.
* Control and AD groups differed significantly, $p < .005$.

All participants were administered the MMSE and learning subscale tests from the CAMCOG on the morning of, and prior to, the scan itself. Table 21 shows results of the two groups scores' for the learning subscale and the MMSE. For both cognitive measures, scores were not normally distributed.

MMSE: In all participants, the MMSE scores ranged from 12 to 30 (*Median* = 27, *IQR* =19 – 29). The MMSE is scored out of a total of 30. Controls (*Median* = 29) had significantly higher MMSE scores than AD participants (*Median* = 19; $p < .001$).

Learning subscale: The participants' learning subscale scores ranged from 0 to 17 (*Median* = 13, *IQR* = 6 – 15). The maximum score on the learning subscale is 17. Controls (*Median* = 15) had significantly higher learning subscale scores than AD participants (*Median* = 6.5; $p < .001$).

Table 21
Between-Group Comparisons of Neuropsychological Test Scores for the MRS Study

Cognitive test	Group					
	Control (n = 15)	AD (n = 18)	U	df	p	r
MMSE	29 (27 – 29)	19 (17 – 24)	19	31	< .001*	-0.73
Learning subscale	15 (14 – 16)	6.5 (3 – 9)	10	31	< .001*	-0.78

Note. Medians are presented with interquartile ranges in parentheses. Raw scores for the Mini-Mental State Examination (MMSE) and learning subscale are presented. See Appendix J: Table A &B for raw data.
* Control and AD groups differed significantly, $p < .001$.

As previously indicated, a total of 18 AD participants had complete MRS data. The 18 AD participants were divided up into two groups. AD participants with a learning subscale score equal to and greater than 7/17 were considered *mild/moderate AD* (n = 9) and those who scored below 7/17 were considered *more advanced AD* participants (n = 9). The AD group was not divided into three separate sub-groups: mild, moderate and severe, as was done previously done in the cross-sectional study because of the smaller numbers in the imaging study.

Technical difficulties were experienced while acquiring MRS data from the hippocampal areas. MRS data from the hippocampal areas were therefore not obtainable. The rest of this section will only report results from the posterior cingulate gyrus.

Inflammatory data and neurometabolites were normally distributed. Therefore, statistics for normally distributed data were used in this section. When the control and mild/moderate AD group were compared using the Student's *t*-test, there was no significant difference in their age (t (23) = 0.60, p = .558, d = 0.25) or education (t (23) = -1.82, p = .082, d = -0.76). When the control group was compared with the advanced AD group, there was no significant difference in the age of the two groups (t (23) = 0.71, p = .488, d = 0.30). However, the control group did have a significantly greater number of years of education (t (23) = -3.77, p = .001, d = -1.57). There were no significant between-group differences with respect to age (t (17) = 0.17, p = .867, d = 0.08) or education (t (17) = -1.88, p = .079, d = 0.91) when the mild/moderate and advanced AD groups were

compared. Education was not correlated with any of the neurometabolites and therefore education was not adjusted for in the analyses.

The Relationship Between CNS Inflammation in the Posterior Cingulate Gyrus and Cognitive Scores

Hypothesis 6 predicted that myo-inositol (MI), a marker of glial cell activation in the CNS, would be highest in the early stages of AD, declining with the progression of the disease. Two different statistical tests were used to test this hypothesis: one-way ANOVA to compare between-group differences and Pearson's correlation analysis. For Pearson's correlation analyses all AD participants were examined together and not as separate sub-groups. Table 22 and Table 23 detail the results from the one-way ANOVA and Pearson's correlation analyses respectively.

Mild/moderate AD participants (learning subscale score ≥ 7/17) did not have a significantly higher MI level when compared with controls or advanced AD participants. However, one-way ANOVA and a post-hoc Tukey test showed that more advanced AD (M = 5.03) participants (learning subscale score < 7/17) had a significantly higher MI level compared with mild/moderate AD participants (M = 4.24; p = .011; see *Figure 37*). Pearson's correlation between MI and cognition showed that when controls were analysed, there was no correlation between MI and the learning subscale or the MMSE. However, when the AD group was analysed, the learning subscale tended to be negatively correlated with MI (p = .093; see *Figure 38*). There was no significant correlation between MI and the MMSE in the AD group.

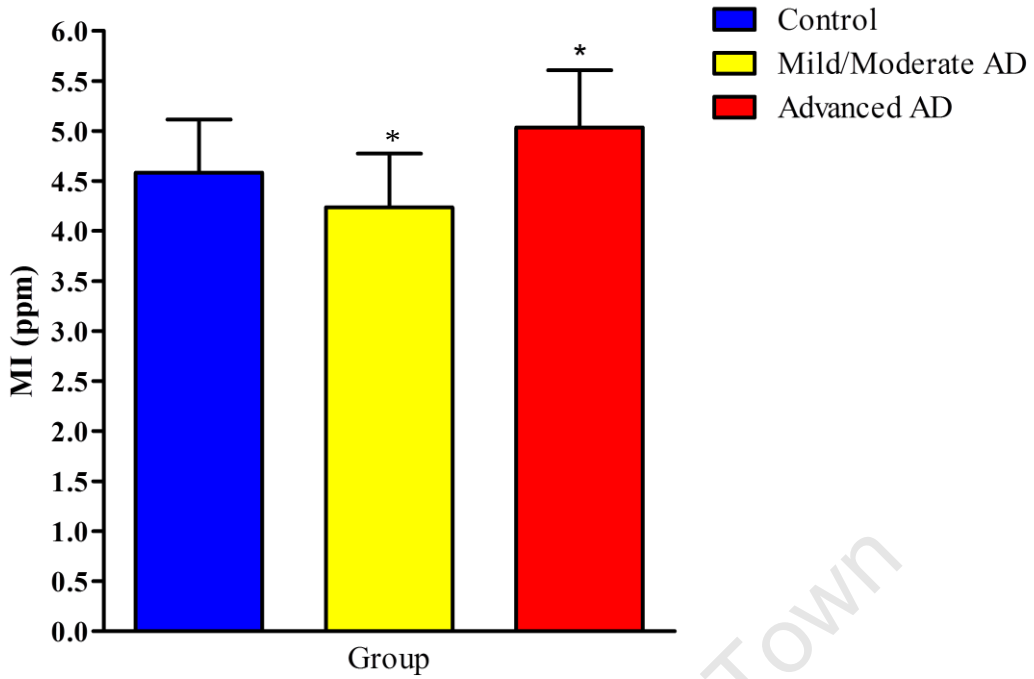


Figure 37: Bar graph showing myo-inositol (MI) in the control (n = 15), mild/moderate (n = 9) and advanced AD (n = 9) groups. MI is measured in parts per million (ppm).
* The mild/moderate and advanced AD groups differed significantly, $p < .05$.

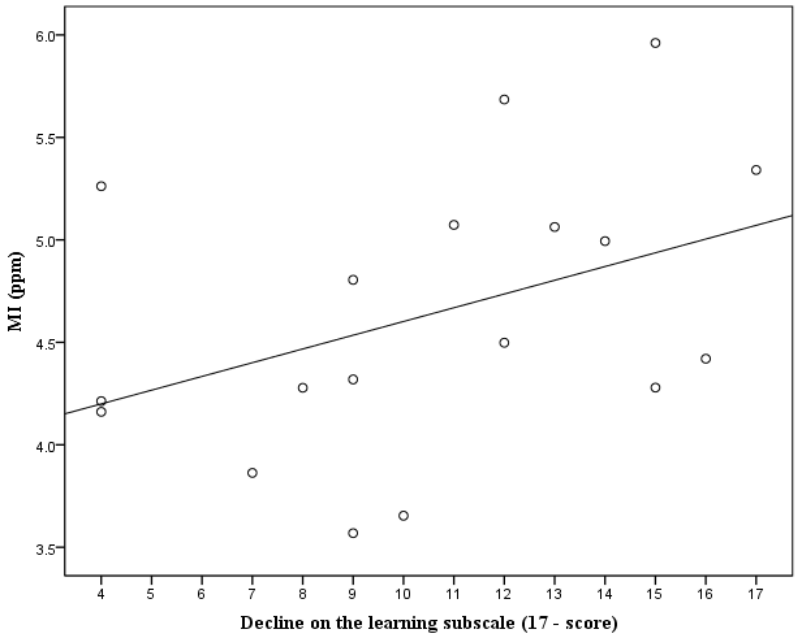


Figure 38: The correlation between myo-inositol (MI) and decline on the learning subscale score in the AD group.

The Relationship Between Neuronal Integrity in the Posterior Cingulate Gyrus and Cognitive Scores

Hypothesis 7 stated that there would be a direct relationship between the NAA+NAAG peak on MRS, i.e. the better the cognitive functioning, the higher the NAA+NAAG peak. Again, this hypothesis was tested using one-way ANOVA and Pearson's correlation analysis (see Table 22 and 23 respectively).

One-way ANOVA analysis showed that the NAA+NAAG neurometabolites were significantly higher in the controls (M = 6.03) compared with the mild/moderate AD group (M = 5.64; p = .003) and the advanced AD group (M = 5.78; p = .02), see *Figure 39*. There was no significant difference between the mild/moderate AD group and the advanced AD group (p = .783). Pearson's correlation analysis showed no correlations between NAA+NAAG and the learning subscale score or the MMSE in the control and AD groups.

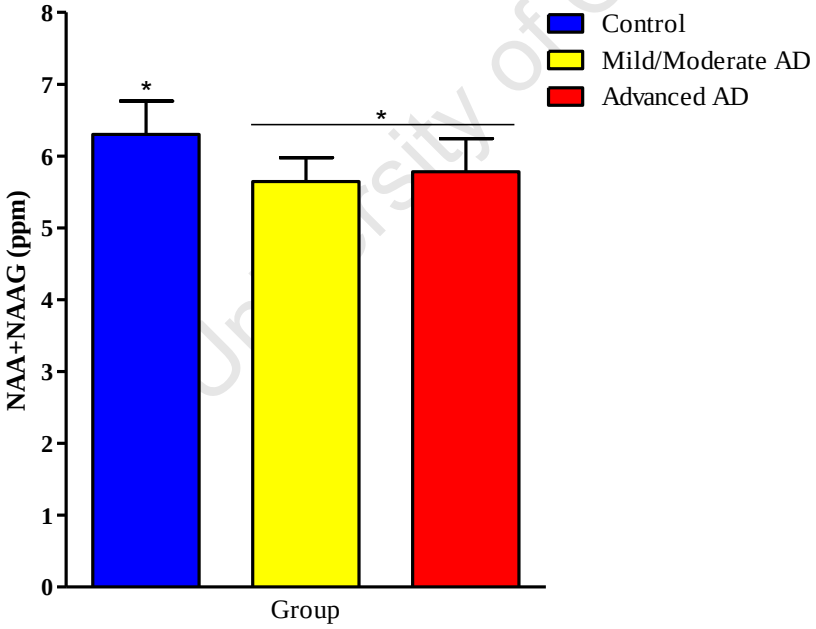


Figure 39: Bar graph showing N-acetylaspartate and N-acetylaspartylglutamate (NAA+NAAG) in the control (n = 15), mild/moderate (n = 9) and advanced AD (n = 9) groups. NAA+NAAG is measured in parts per million (ppm).
* The mild/moderate and advanced AD groups differed significantly from the control group, $p < .05$.

The Relationship Between Neuronal Integrity, CNS Inflammation and Cognitive Scores

Hypothesis 8 stated that the ratio between MI and NAA+NAAG would be negatively associated with cognition measured on the learning subscale and the MMSE. One-way ANOVA analysis was used to compare the three groups: mild/moderate AD, advanced AD and control groups (Table 22; *Figure 40*). One-way ANOVA analysis and a post-hoc Tukey test showed that advanced AD participants (M = 0.88) had a higher MI/(NAA+NAAG) ratio compared with controls (M = 0.73; p = .005) and the mild/moderate AD group (M = 0.75; p = .044). There was no significant difference between the control and mild/moderate AD groups for the MI/(NAA+NAAG) ratio (p = .806). Pearson's correlation analysis was also used (Table 23). Pearson's correlation analysis showed that the MI/(NAA+NAAG) ratio in the posterior cingulate gyrus was not correlated with the learning subscale score or MMSE in the AD and control groups.

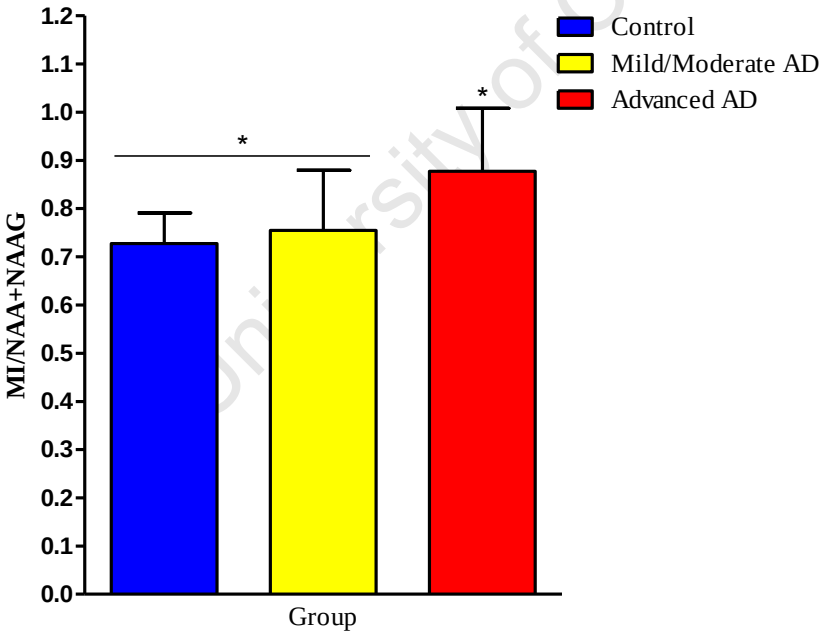


Figure 40: Bar graph showing the ratio of MI to N-acetylaspartate and N-acetylaspartylglutamate (NAA+NAAG) in the control (n = 15), mild/moderate (n = 9) and advanced AD (n = 9) groups. MI and NAA+NAAG are measured in parts per million (ppm).
* The control and mild/moderate AD groups differed significantly from the advanced AD group, p < .05.

Table 22
Between-Group Differences in Neurometabolites in the Posterior Cingulate Gyrus

Neurometabolite	Group			*F*	*p*	Partial η^2
	Control (n=15)	Mild/Moderate AD (n = 9)	More Advanced AD (n= 9)			
MI	4.58 (0.53)	4.24 (0.54)	5.03 (0.57)	4.89	.015*	0.25
NAA+NAAG	6.30 (4.46)	5.64 (0.33)	5.78 (0.46)	7.81	.001†	0.34
MI/(NAA + NAAG)	0.73 (0.06)	0.75 (0.12)	0.88 (0.13)	6.22	.006**	0.29

Note: Means are presented with standard deviations in parenthesis. Absolute metabolite concentrations for myo-inositol (MI) and N-acetylaspartate and N-acetylaspartylglutamate (NAA+NAAG) are given in parts per million (ppm). The mean for ratio between MI and NAA+NAAG is presented (MI/NAA+NAAG). See Appendix J: Table A &B for raw data.
* The more advanced AD group and the mild/moderate AD group differed significantly, $p < .05$. ** Controls and mild/moderate AD participants differed from the more advanced AD group, $p < .01$. † Controls differed from the mild/moderate AD participants and the more advanced AD group, $p < .001$.

Table 23
Correlation between Cognitive Scores and Neurometabolites in the Posterior Cingulate Gyrus

	Controls (*n*= 15)				AD (*n* =18)			
	LS		MMSE		LS		MMSE	
Neurometabolite	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
MI	.130	.644	-.201	.473	-.408	.093	-.233	.353
NAA+NAAG	.010	.973	-.349	.202	-.210	.402	.285	.252
MI/(NAA+NAAG)	.203	.467	.001	.998	-.264	.289	-.315	.203

Note: The Pearson correlation statistic is *r.* Abbreviations are as follows: myo-inositol (MI), N-acetylaspartate and N-acetylaspartylglutamate (NAA+NAAG), the ratio between MI and NAA+NAAG, MI/(NAA+NAAG). The cognitive measures are the learning subscale (LS) and the Mini-Mental State Examination (MMSE). See Appendix J: Table A &B for raw data.

The Relationship Between CNS Inflammation, Systemic Inflammatory Markers and Neuronal Integrity

Hypothesis 10 predicted that there would be a direct association between systemic inflammation, CNS inflammation and NAA+NAAG. Firstly, MI, a marker of CNS inflammation, in the posterior cingulate and hippocampus would be positively correlated with the systemic measures of inflammation (cytokines, OPN, white cell count, monocyte count, and ESR). Secondly, MI would be negatively correlated with NAA+NAAG. Thirdly, NAA+NAAG would also be negatively correlated with systemic inflammatory markers.

The inflammatory markers were normally distributed therefore Pearson's correlation analyses were used to analyse the data. Table 24 shows the correlation statistics for the control and AD group.

In the control group, the monocyte count was positively correlated with NAA+NAAG, p = .004. NAA+NAAG correlated positively with the ESR and white cell count, but this did not reach significance. TNF-α and OPN were both negatively correlated with NAA+NAAG. MI had a positive correlation with the white cell count and a negative correlation with TNF-α. These correlations however, were not significant.

In the AD group, NAA+NAAG was negatively correlated with TGF-β_1 and TGF-β_2, p = .025 and p = .023 respectively (see *Figure 41* and *Figure 42*).

When the relationship between MI and NAA+NAAG was investigated, MI and NAA+NAAG were not correlated in the AD group (r = -0.928, p = .714). However, in the control group, NAA+NAAG was positively correlated with MI (r = 0.654, p = .008).

Table 24
Correlation Analyses between Peripheral Inflammatory Markers, MI and NAA+NAAG

Peripheral inflammatory marker	Control (n =14)				AD (n =17)			
	MI		NAA+NAAG		MI		NAA+NAAG	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
ESR	.213	.583	.300	.434	-.335	.204	.054	.843
White cell count	.303	.428	.331	.385	-.386	.140	.049	.858
Monocyte count	.297	.437	.842	**.004***	-.084	.757	.178	.509
IL-1β	-.053	.893	-.110	.779	-.437	.090	-.201	.456
IL-10	.032	.935	.146	.708	-.231	.389	-.037	.890
TNF-α	-.331	.384	-.460	.213	-.323	.222	.370	.158
TGF-β_1	.088	.822	.100	.798	.155	.566	-.558	**.025***
TGF-β_2	.125	.750	.071	.856	-.033	.903	-.562	**.023***
OPN	-.057	.884	-.473	.198	-.006	.982	.062	.819

Note: The test statistic is Pearson's *r*. MI = myo-inositol, NAA+NAAG = N-acetylaspartate and N-acetylaspartylglutamate. Abbreviations for the inflammatory markers are as follows; erythrocyte sedimentation rate (ESR), interleukin 1 beta (IL-1β), interleukin 10 (IL-10), tumor necrosis factor alpha (TNF-α), transforming growth factor beta 1 and 2 (TGF-β_1 and TGF-β_2), osteopontin (OPN).). See Appendix J, Table A &B for raw data. See Appendix J: Table A &B for raw data.
* Significant correlation, $p < .05$.

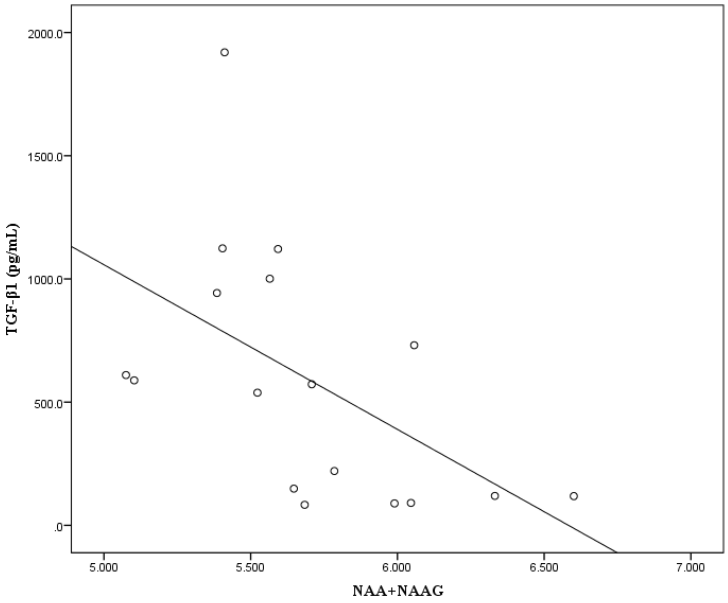


Figure 41: Inverse correlation between N-acetylaspartate and N-acetylaspartylglutamate (NAA+NAAG) and transforming growth factor beta 1 (TGF-β_1) in the AD group.

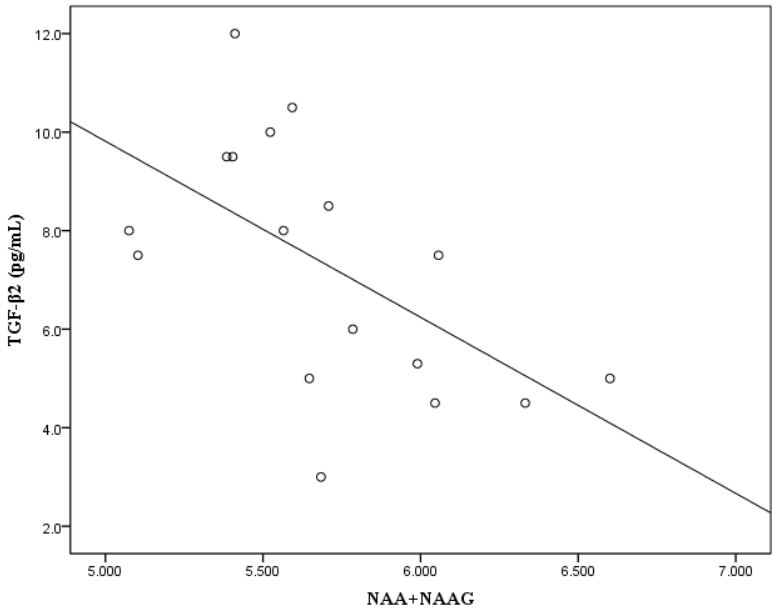
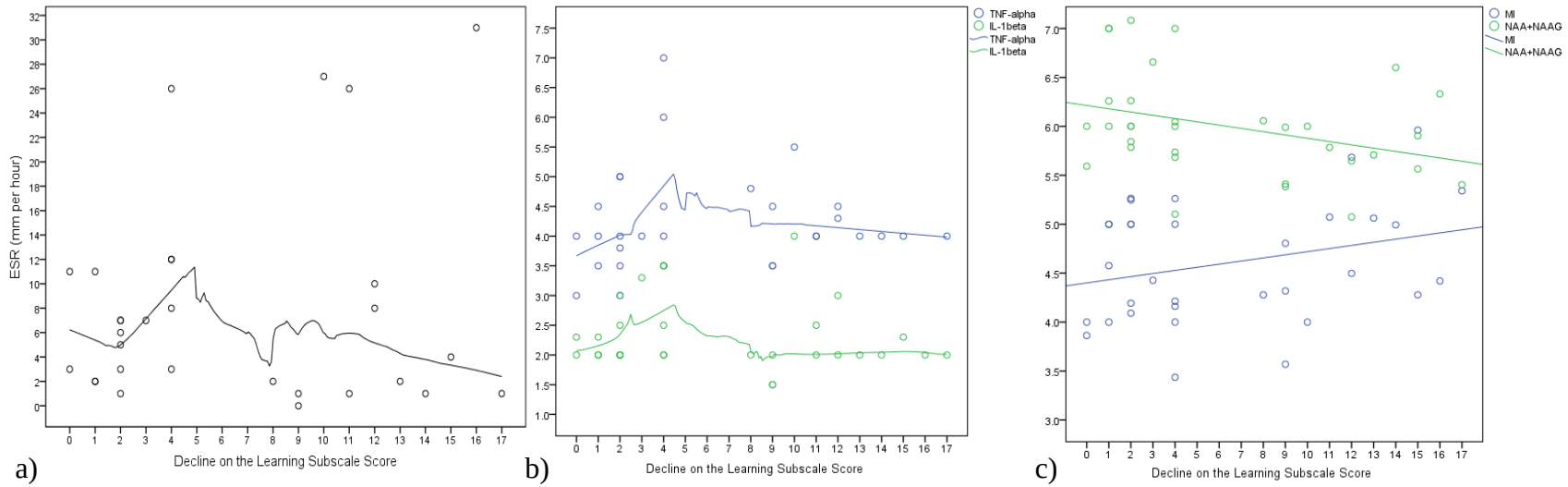


Figure 42: Inverse correlation between N-acetylaspartate and N-acetylaspartylglutamate (NAA+NAAG) and transforming growth factor beta 2 (TGF-β_2) in the AD group.

Figure 43 a, b and *c* depict the relationship between the decline on the learning subscale (indicative of disease progression) and the ESR; IL-1β and TNF-α; as well as MI and NAA+NAAG in all participants. From the graphs it can be seen that in the periphery, inflammatory markers: ESR, IL-1β and TNF-α rise when there is a 1 to 8 point loss on the learning subscale. Inflammatory markers then decline with a further decrease in learning subscale scores. In the CNS, MI rises when there is a decline on the learning subscale and continues to rise with further decline on the learning subscale. NAA+NAAG declines with a decline on the learning subscale. This decline continues with disease progression.



a) b) c)

Figure 43: The relationship between a) erythrocyte sedimentation rate (ESR), b) tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) c) myo-inositol (MI) and N-acetylaspartate and N-acetylaspartylglutamate (NAA+NAAG) and decline on the learning subscale in all participants. The y-axis represents a) the ESR (mm/hr), b) TNF-α and IL-1β in picograms per millilitre (pg/mL), c) MI and NAA+NAAG levels in parts per million (ppm).

The Relationship Between the APOE ε4 Allele, CNS Inflammation and Neuronal Integrity in the Posterior Cingulate Gyrus

Hypothesis 9 predicted that participants, either homozygous or heterozygous for the APOE ε4 allele, would have higher baseline levels of MI and lower levels of NAA+NAAG compared with participants that did not carry an ε4 allele. Linear regression analysis was used to test this hypothesis. Dummy variables for the presence (1) or absence (0) of the APOE ε4 allele were created. The APOE ε4 allele was the independent variable. MI and NAA+NAAG and the MI/(NAA+NAAG) ratio were the dependent variables.

Table 25 details the results from the linear regression analyses. In the control and AD group, MI and the MI/NAA+NAAG ratio were positively associated with the ε4 allele. NAA+NAAG was negatively associated with the ε4 allele. In other words participants with at least one ε4 allele had a lower NAA+NAAG and higher MI peak on MRS. However, the regression results for MI, MI/(NAA+NAAG) and NAA+NAAG did not reach significance.

Table 25
The Association between the Presence of the APOE ε4 Allele, MI and NAA+NAAG

	Control				AD			
Neurometabolite	β	Std Error	R^2	*p*	β	Std Error	R^2	*p*
MI	.246	.334	.060	.377	.175	.314	.031	.488
NAA+NAAG	-.123	.299	.015	.663	-.005	.195	<.001	.986
MI:NAA+NAAG	.372	.036	.138	.173	.125	.067	.016	.622

Note. β = standardized coefficients for beta, Std Error represents the standard error. Myo-inositol (MI), N-acetyl aspartate and N-acetylaspartylglutamate (NAA+NAAG) and the ratio between the two are presented in the table. See Appendix J: Table A & B for raw data

The MRS Study Summary

A total of 33 participants were included in the MRS study, 15 controls and 18 AD participants. To examine the relationship between neurometabolites and cognition the AD group was divided into a mild/moderate group and an advanced AD group based on their learning subscale scores.

The relationship between CNS inflammation in the posterior cingulate gyrus and cognition.

One-way ANOVA showed that the advanced AD group had higher MI levels compared with the moderate AD group. There were no differences between the control group and the moderate or advanced AD groups. There was no correlation between MI and the MMSE or the learning subscale in the control group. In the AD group, MI tended to be negatively correlated with cognition on the learning subscale but not the MMSE.

The relationship between neuronal integrity in the posterior cingulate gyrus and cognition.

One-way ANOVA showed the NAA+NAAG was higher in the control group compared with the mild/moderate AD group and the advanced AD group. There was no significant difference in NAA+NAAG when the moderate AD and advanced AD groups were compared. Pearson's correlation analysis showed no significant correlations between NAA+NAAG and the MMSE or the learning subscale in the control and AD group.

The relationship between the ratio of CNS inflammation to neuronal integrity and cognitive functioning.

More advanced AD participants had a higher MI/(NAA+NAAG) ratio compared with controls and mild/moderate AD participants. There was no significant difference in the ratio when the control and mild/moderate groups were compared. There was also no significant correlation between MI/(NAA+NAAG) and the MMSE or the learning subscale score in the control or AD groups.

The relationship between systemic inflammation, CNS inflammation and neuronal integrity

In the control group, the monocyte count was positively correlated with NAA+NAAG. There was a moderate positive correlation between NAA+NAAG and the ESR as well as the total white cell count. NAA+NAAG also had a strong negative correlation with TNF-α and OPN.

In the AD group TGF-β_1 and TGF-β_2 were negatively correlated with NAA+NAAG. MI had a strong negative correlation with IL-1β and a moderately negative correlation with the ESR, white cell count and TNF-α.

The relationship between APOE ε4, neuronal integrity and CNS inflammation

Linear regression analysis showed that although the presence of APOE ε4 allele was associated with a higher MI and lower NAA+NAAG in the posterior cingulate gyrus in control and AD participants, however these associations were not significant.

SUMMARY

The key finding from the cross-sectional, longitudinal and MRS analyses are summarised in Table 26.

Table 26
Summary of Key Findings

	Systemic Inflammation	CNS Inflammation and Neuronal Integrity	APOE ε4 and Cognition	APOE ε4 Inflammation and Neuronal Integrity
Cross-Sectional Study	* ESR and IL-1β raised in mild AD participants compared with the control, moderate AD and severe AD groups. * Severe AD participants had a higher white cell count compared with moderate AD participants.	-	* AD participants had a significantly higher ε4 allelic frequency compared with controls. * The age-adjusted odds ratio for AD was 2.2 in heterozygous ε4 carriers and 9 in homozygous ε4 carriers compared with non-ε4 carriers. *AD ε4 carriers tended to have lower MMSE scores compared with non-carriers	* Controls: ε4 carriers tended to have higher IL-10 levels compared with non-carriers. * AD: ε4 carriers tended to have lower IL-10 levels compared with non-carriers.
Longitudinal Study	* AD: high baseline levels of TNF-α and the white cell count were associated with greater decline on the CAMCOG. * AD: high baseline levels of the monocyte count and TGF-β_2 were associated with a greater decline on the learning subscale; high levels of OPN also tended to be associated with the learning subscale. * AD: high levels of IL-10 at baseline were associated with less of a decline on the CAMCOG and MMSE. * Controls: High baseline levels of ESR were associated with a greater decline on the learning subscale.	-	* Controls: non-ε4 carriers had a greater decline on the CAMCOG in one year compared with ε4 carriers. * AD: ε4 carriers had a greater decline on the CAMCOG in one year compared with non-carriers.	-
MRS Study		* Advanced AD participants had higher MI levels compared with moderate AD participants. * AD: MI tended to be negatively correlated with the learning subscale. * NAA+NAAG was higher in controls compared with all AD participants.	-	* The presence of ε4 allele did not have a significant effect on the MI or NAA+NAAG peaks.
	* Controls: The monocyte count was positively correlated with NAA+NAAG; TNF-α and OPN had a strong negative correlation with NAA+NAAG. * AD: TGF-β_1 and TGF-β_2 were negatively correlated with NAA+NAAG. * AD: MI tended to be negatively correlated with IL-1β.			

DISCUSSION

AD is a devastating neurodegenerative disease that affects millions of patients, families and caregivers worldwide. South Africa is currently on the verge of an epidemic that the developed world is already experiencing as the population over the age of 55 continues to increase at a rapid rate, despite the HIV/AIDS epidemic. High rates of infection, inadequate access to primary health care, poverty and low levels of education increase the vulnerability of our ageing population to age-related cognitive disorders. Without local knowledge of the disease processes, risk factors and prevalence of these conditions in South Africa, we shall be ill equipped to deal with this impending epidemic. Public health facilities currently do not provide for geriatric care in general, and dementia in particular. Greater public and government awareness will be required to formulate and implement health policies around dementia.

This study investigated the relationships between cognitive functioning, the APOE genotype, CNS inflammation, systemic inflammation and neuronal integrity, in a sample population of older adults from the greater Cape Town metropolitan area. The study consisted of 3 sub-studies: a cross-sectional, a longitudinal and a MRS study.

Firstly, participant demographics will be discussed. The findings from each hypothesis will then be integrated, setting each into context alongside relevant previously published literature. The limitations of the study and some directions for future research will also be discussed.

THE PARTICIPANT POPULATION

Age and Education

One hundred and twenty-eight participants, 68 controls and 60 AD participants, were included in the cross-sectional analyses. The control group was younger and had more years of education than the AD group. Age is known to be the strongest risk factor for AD with the incidence rising from 0.6% in individuals aged 65 to 69 years to 8.4% in individuals aged 85 years (Hebert et al., 1995). Therefore it is not surprising that controls

were younger than the AD participants. Similarly, lower levels of education are a risk factor for AD. Controls with lower levels of education are more likely to develop AD compared to those with a higher educational attainment (Kukull et al., 2002; Mickes et al., 2007).

The difference in education levels between the control and AD group raises the question of cognitive reserve. Cognitive reserve is a term used to describe the resilience of neurones to Aβ and tau pathology. Several studies have shown that some cognitively healthy individuals have significant pathological features of AD at autopsy. However, the AD pathology did not result in dementia (for a review see Stern, 2009). Cognitive reserve is thought to account for the discrepancy between the pathological features of AD and its clinical manifestations (Albert, 1995). A larger cognitive reserve has been shown to be associated with higher educational attainment, higher socio-economic status, exposure to occupations and participation in leisure activities (Stern, 2006). A larger cognitive reserve allows the person to cope better when Aβ and tau pathology begin accumulating. This is probably due to increased complexity of neuronal networks and higher cognitive processes (Stern, 2006).

Cognitive reserve has several implications for the current research. Some control participants, although performing within the normal range on cognitive tests, may have had AD pathology. This may have impacted on other factors investigated, such as inflammation. The use of PiB to measure Aβ load might have been helpful in addressing Aβ accumulation in control participants.

In the current study, age was not correlated with the inflammatory markers. This was unexpected as ageing has been shown to be accompanied by a low-grade inflammatory state. For a review see Vasto et al. (2007) and Franceschi et al. (2000). Sadeghi, Schnelle, Thomas, Nishanian, & Fahey (1999) showed that when compared with younger participants (age range: 20 - 47 years), older adults (age range: 72 – 100 years) had increased blood IL-1β and IL-6 levels. Activated microglia are thought to be the primary source of the age-related increase in CNS IL-6 (Ye & Johnson, 1999). One reason why there was not a positive correlation in the current study might be because our participants were all older adults within a narrower age range. Therefore, the variations in

inflammatory markers were less. This study also investigated a disease that is associated with inflammation.

Cholinesterase inhibitor use

One of the strengths of the current study was that there were no participants on cholinesterase inhibitors at baseline or at follow-up. Although modest in their effects, cholinesterase inhibitors have been shown to slow or stabilize decline in cognition (Lanctôt et al., 2003; Hansen, Gartlehner, Webb, Morgan, & Jonas, 2008). The use of cholinesterase inhibitors was, therefore, not a factor when measuring cognitive decline in the current study. Similarly, the use of cholinesterase inhibitors did not influence inflammatory markers. The 'cholinergic anti-inflammatory pathway' provides a physiological mechanism that links the neurotransmitter acetylcholine with inhibition of inflammatory processes (Nizri, Hamra-Armitay, Sicsic, Lavon, & Brenner, 2006). Therefore, AD participants taking cholinesterase inhibitors might have had lower inflammatory markers than AD participants not using these drugs.

APOE Genotype

Of the 123 participants that were genotyped, the most common allele was APOE ε3. Sixty-three percent of all participants carried this allele. Corbo & Scacchi (1999) showed in their study examining the world distribution of APOE alleles, that 75.2% of participants carried the ε3 allele. As expected, ε2 was the least common allele in all participants. The allelic frequency of ε2 was 9.3 % in AD participants and 9.4% in controls. This was similar to frequencies found in a Nigerian study, where the allelic frequency was 10.2% in AD participants and 11.1% in controls (Gureje et al., 2006). The frequency of ε2 in the current study was also similar to that found in an indigenous Southern African population, the Khoi San (7.7%; Sandholzer et al., 1999). The ε2 allele is thought to be protective and its presence predicts a later age of AD onset (Corder et al., 1994; Farrer et al., 1997). However, this protective effect of the ε2 allele has not been studied in a South African context.

The allelic frequency of ε4 was higher in AD participants when compared with controls (35.6% vs. 20.3% respectively). The allelic frequency of the controls was similar to that

reported in a Nigerian study (21.7%). However, the AD participants in the current study had a higher ε4 allelic frequency compared with AD participants from Nigeria, 35% vs. 26% respectively (Gureje et al., 2006). A study on East Africans in Kenya and Tanzania found no difference in the ε4 allelic frequency between AD patients and controls, 25% in both groups (Sayi et al., 1997). When all participants were taken together, the allelic frequency of ε4 in the current study was 27.6%.

The frequency of the homozygous ε4 genotype was higher than that reported in a meta-analysis that included African Americans, Hispanics and Japanese AD patients (Farrer et al., 1997). In this review the frequency of the homozygous ε4 genotype was highest in Caucasian AD participants (14.8%), which is similar to the 14% found in the current study (Farrer et al., 1997). The frequency of the homozygous ε4 genotype in AD participants in the current study was also higher than that of a Nigerian AD population (Gureje et al., 2006).

The ε4 allelic frequency in the current study was lower than the 37% frequency found in the indigenous Khoi San population (Sandholzer et al., 1999). A possible explanation for this may be the varied genetic profile of the population in the Western Cape region of South Africa. Cape Town is historically a colonial port city servicing trade between Europe, Africa and Asia. Its population is therefore genetically diverse, with European, African and Asian influences.

The age-adjusted odds of having AD with one ε4 allele was 2.2 compared with non- ε4 carriers. The homozygous ε4 state was associated with an odds ratio of 9 compared with non- ε4 carriers. These ratios were higher than those found in a Nigerian population (Gureje et al., 2006) but were similar to those calculated by Farrer et al. (1997) for a European/ North American ("Caucasian") population. Unlike other African countries, the population of the Cape Metropole region live in urban (Western), industrialised environments. The reason there was a relationship between the ε4 allele and AD, which was not seen in other African studies, may be because of this lifestyle difference. Our participants may have had higher cholesterol levels, higher body mass index, more hypertension, more diabetes and their diet may contain more processed carbohydrates and a lower amount of fibre. In addition they were probably less physically active than other

rural African populations. All these environmental factors might have contributed to the association between AD and the ε4 allele being seen in this urban environment (Hendrie et al., 2011).

The sample size in the current study was small and this was not primarily a genetic or prevalence study. Therefore, the figures have to be interpreted cautiously. However, they do indicate a high population prevalence of the ε4 allele. Furthermore, ε4 was a risk for the development of AD in the study population. Further larger, prevalence studies would be needed to verify these findings.

In summary, findings from this study showed that the population sample investigated had similar ε2 and ε3 allelic frequencies when compared with other African studies (Sayi et al., 1997; Gureje et al., 2006). The ε4 allelic frequency was higher compared with AD participants in other African studies but similar to those found in European and North American studies (Farrer et al., 1997; Sayi et al., 1997; Gureje et al., 2006). The ε4 allelic frequency was lower than that found in an indigenous South African population (Sandholzer et al., 1999). The most likely explanation for this is the mixed genetic population in the Western Cape. Further dementia prevalence studies are needed to determine whether ε4 is a strong risk factor for developing AD in South Africa.

THE RELATIONSHIP BETWEEN SYSTEMIC INFLAMMATION, CNS INFLAMMATION AND COGNITION

Both CNS and systemic inflammation have been shown to be associated with neurodegeneration and cognitive decline. High levels of poverty, overcrowding and inadequate access to primary health care have led to high rates of systemic infection in South Africa. The Western Cape has the highest reported infection rate for tuberculosis in the world (Lawn et al., 2006). High levels of systemic infection may predispose our population to faster rates of cognitive decline. Therefore, one of the aims of this study was to investigate the role of systemic inflammation and CNS inflammation in cognitive functioning and cognitive decline.

Systemic Inflammation and Cognitive Functioning

Hypothesis 1 stated that systemic inflammatory markers would be highest in the early stages of AD, declining with the progression of the disease. To test this hypothesis, the AD group was divided into mild, moderate and more severe AD subgroups based on their learning subscale scores. The learning subscale is a proxy for episodic memory impairment and has been used by Combrinck et al. (2006) as a proxy of disease status and progression. Participants with early or mild AD had significantly higher ESRs when compared with controls and moderate AD participants. The ESR is a non-specific marker of inflammation. A raised ESR indicates an increase in blood fibrinogen, a clotting factor associated with inflammatory conditions.

To my knowledge, this is the first study that has shown a rise in ESR in early AD. Other studies investigating non-specific inflammatory markers have focused on CRP as a peripheral immune marker. Although CRP was measured to exclude participants with infections in this study, the CRP values were not reported. The CRP measure reported by the NHLS varied significantly in range and we did not feel that the CRP was a reliable measure of peripheral inflammation. Holmes and colleagues (2011) also showed that there was no relationship between raised CRP and behavioural symptoms during acute infection. These authors therefore argued that the CRP was not a good indicator of systemic

inflammation in the elderly and that CRP was not involved in the systemic to CNS communication (Holmes et al., 2011).

All participants with overt inflammatory conditions and participants with an ESR over 40mm per hour were excluded. However, participants with conditions such as ischaemic heart disease, osteoarthritis and diabetes were not excluded. These conditions may be associated with low-grade systemic inflammation. Of the 13 participants in the mild AD group, one participant had ischaemic heart disease and arthritis, one participant had arthritis and another had asthma. The control, moderate AD and severe AD groups also consisted of participants with these conditions. Therefore, it is unlikely that these three participants accounted for the increase in ESR in this group.

Blood IL-1β was also significantly increased in mild AD participants compared with controls. In moderate and severe AD participants IL-1β was lower than in mild AD participants and similar to control levels. Although these are cross-sectional results, it may be assumed that these changes also occur longitudinally as participants progress from normal to MCI to moderate and then severe AD. IL-1β is a member of the interleukin 1 family and is a pro-inflammatory cytokine. IL-1β is produced by activated macrophages and is an important mediator in the inflammatory response (Griffin, Sheng, Roberts, & Mrak, 1995). IL-1 initiates activation of T cells, up-regulates the expression of adhesion molecules and induces the expression of other pro-inflammatory cytokines. The up-regulation of adhesion molecules, pro-inflammatory cytokines and T-cell activation amplify the immune response (Griffin et al., 1995).

Griffin and colleagues first proposed that microglial and astrocyte activation was accompanied by an elevated IL-1 expression in the CNS of AD patients in 1989 (Griffin et al., 1989). IL-1β levels in serum have also been shown to be associated with decreased cognitive functioning (Guerreiro et al., 2007). Licastro et al. (2000) showed that AD participants had increased levels of IL-1β and IL-6 in the peripheral blood when compared with cognitively healthy controls. These studies, together with the increase in IL-1β in mild AD in the current study, are in keeping with a meta-analysis that showed IL-1β was significantly raised in peripheral blood when AD participants were compared with controls (Swardfager, Lanctôt, Rothenburg, Wong, Cappell, & Herrmann, 2010).

Most studies however, have not investigated IL-1β across the stages of AD progression. Although the study by Guerreiro et al. (2007) included an MCI group in their analysis, the study did not investigate systemic inflammatory markers at different stages of AD. This was also true for the Licastro et al. (2000) study. An increase in IL-1β in early AD might account for the overall increase in IL-1β found in the AD group in their studies. Future work should compare IL-1β levels at different stages of AD in a larger population, involving a longitudinal follow-up of participants from cognitively healthy to advanced AD.

The increase in blood ESR and IL-1β in mild AD parallels the early inflammatory changes found in the CNS by others (Yermakova & O'Banion, 2001; Hoozemans et al., 2005; Combrinck et al., 2006). A post-mortem study of the mid-temporal cortex showed that COX-2 expression was increased during the Braak neurofibrillary tangle stages 0 – II. It then declined with an increase in AD neurofibrillary tangle pathology (Hoozemans et al., 2005). Yermakova & O'Banion (2001) report similar findings. They showed that COX-2 expression was slightly increased in post-mortem human hippocampal samples of mild AD patients. COX-2 expression declined and was significantly lower in AD patients with Braak staging V – VI and a clinical dementia rating of 5 compared with mild patients and controls. An increase in CSF levels of PGE_2 was found in early AD participants in a study by Combrinck et al. (2006). PGE_2 levels declined with disease progression to levels below that of controls in more advanced AD (Combrinck et al., 2006). IL-1β has been shown to induce COX-2 and PGE_2 expression in human neuroblasma cell lines (Hoozemans, Veerhuis, Janssen, Rozemuller, & Eikelenboom, 2001).

IL-1 frequently manifests synergistically with the pro-inflammatory marker TNF-α (Dinarello, 2000). However, there were no significant between-group differences with regards to TNF-α in the current study. This was unexpected, as previous literature has shown that AD is accompanied by an increase in TNF-α levels. A study by Guerreiro et al. (2000) found that TNF-α levels were increased in MCI participants compared with controls and were highest in AD participants. A meta-analysis that pooled 14 studies found that TNF-α was significantly elevated in the peripheral blood of AD patients compared with controls (Swardfager et al., 2010). TNF-α levels have also been associated with disease

severity. High levels of TNF-α were associated with a higher Alzheimer's Disease Assessment Scale Cognitive Subscale (ADAS-COG) score, where a higher score on the ADAS-COG indicates more severe disease (Holmes et al., 2011). The lack of significance in the current study might be due to a small sample size or technical factors related to assay technique: the Luminex technology may be less sensitive than the traditionally used ELISA assays at detecting TNF-α.

Another inflammatory marker that was raised in the between-group analysis was the white cell count. More severe AD participants had significantly higher total white cell counts when compared with moderate AD participants. This may indicate that although there is a decline in the pro-inflammatory marker IL-1β, there may still be an inflammatory response of a different nature in the systemic circulation. There was however, no significant difference between groups when comparing the monocyte count. There was also no significant difference between the subgroups with regard to cytokines traditionally regarded as anti-inflammatory *viz.* TGF-β_1, TGF-β_2 and IL-10. The TGF-β result was unexpected. Previous reports had shown an increase in TGF-β in early AD patients. TGF-β is an anti-inflammatory cytokine that plays a pivotal role in injury and repair (Finch, Laping, Morgan, Nichols, & Pasinetti, 1993). Motta et al. (2007) found an increase in TGF-β_1 in early AD participants, which then declined with disease progression. The MMSE scores of the mild AD participants in the current study were comparable to the early AD group in the study by Motta et al. (2007). Swardfager et al. (2010), in a meta-analysis, also reported an increase in TGF-β in peripheral blood and CSF in AD participants compared with controls.

The lack of significant between-group difference with regards to anti-inflammatory cytokine IL-10 is in line with the meta-analysis of Swardfager et al. (2010). The latter did not find a difference in serum IL-10 in controls and AD patients when 4 studies were pooled. Kim et al. (2011) also found no differences in IL-10 between cognitively healthy controls, MCI and AD participants.

In summary, the current study showed that the systemic inflammatory profile in AD changes with the progression of the disease. Perhaps the decline in cognition from normal ageing to MCI is accompanied by an increase in anti-inflammatory cytokines (see *Figure*

44). One reason that there was no increase in anti-inflammatory cytokines in the current study might be because it did not include a MCI group. During this anti-inflammatory stage, macrophage activation may be defined as an alternative activation or an M2 state (Boche, Perry, & Nicoll, 2013). Support for the idea that early AD is associated with an anti-inflammatory profile comes from Motta et al. (2007) who showed that mild AD was accompanied by an increase in TGF-β. Parachikova et al. (2007) also showed that TGF-β and IL-10 receptor mRNA was increased in post-mortem prefrontal cortex samples of mild/moderate AD participants (MMSE scores ranging from 17 – 22) compared with non-demented controls. Similarly, CSF samples from early AD participants showed an increase in the anti-inflammatory lipid compound, PGE_2 (Combrinck et al., 2006). During this early phase, inflammation may be beneficial as inflammatory mediators are involved in the processes that attempt to clear or phagocytose Aβ plaques (Wyss-Coray et al., 2001).

The early proposed anti-inflammatory response may then followed by a predominately pro-inflammatory response as the disease progresses to mild/moderate AD (see *Figure 44*). Macrophage activation might change from M2 to M1 or the classic activation state (Boche et al., 2013). In the current study there was a rise in the pro-inflammatory marker IL-1β in early/mild AD (defined by a learning subscale of greater than and equal to 11/17). Motta et al. (2007) also show an increase in pro-inflammatory cytokines IL-18, IL-16 and IL-12 in mild AD (AD participants with MMSE scores ranging from 18 to 20.2).

The reason for the rise in pro-inflammatory cytokine and ESR in early AD in the current study is unclear. It is known that a gradual rise in systemic inflammation is a common consequence of ageing (Franceschi et al., 2000). Additionally, a lifetime of exposure to multiple infections may contribute to this increase. A heightened immune activation in early AD may also be the result of the increase in Aβ in the blood. In a study on participants with known autosomal dominant mutations for familial AD, Aβ was shown to increase in the peripheral circulation years before the onset of disease (Bateman et al., 2012). Late onset AD is thought to follow a similar pattern to familial AD. The increase in systemic inflammation may be an attempt to clear systemic Aβ. The rise in systemic inflammatory cytokines may also be due to the pathological changes occurring in the CNS. De Simoni et al. (1995) and De Luigi et al. (1998) showed that central inflammation may activate the peripheral immune response. Therefore, the immune response to Aβ and tau in

the CNS might activate systemic inflammation through signalling from the CNS to the periphery.

The increase in peripheral pro-inflammatory cytokines may also be due to decreased anti-inflammatory cholinergic innervation by the vagus nerve. The cholinergic hypothesis states that in AD there is a decrease in the neurotransmitter, acetylcholine (Bartus et al., 1982). The use of cholinesterase inhibitors in AD is thought to improve acetylcholine-mediated neurone-to-neurone transmission. However, recent evidence suggests that the benefit of cholinesterase inhibitor use may not be restricted to improved neuronal transmission. It may, in part, be due to its anti-inflammatory effect. In an animal model, acetylcholinesterase inhibitors suppressed TNF-α and IL-1β production in mice with experimental autoimmune encephalomyelitis. The vagus nerve may suppress systemic pro-inflammatory cytokines via acetylcholine. This anti-inflammatory activity of the vagus nerve was shown to be mediated by the α7 nicotinic acetylcholine receptor (Nizri et al., 2006). Decreased vagal-cholinergic activity may lead to a reduced inhibition of inflammation in the systemic circulation. This, in-turn, may lead to an unopposed pro-inflammatory state (van Gool, van de Beek, & Eikelenboom, 2010).

The current study showed that the pro-inflammatory systemic state declined with disease progression. Motta et al. (2007) also showed a decline in IL-12, IL-16 and IL-18 from mild AD to moderate and severe AD. The decline in pro-inflammatory cytokines may either be due to progressive immune dysfunction or to an adaptive immune response. Evidence for progressive immune dysfunction comes from Guerreiro et al. (2007). They showed that when cells from AD and MCI patients were stimulated with LPS, there was a smaller increase in the amount of TNF-α, IL-6, IL-12 and IL-1β secreted *in vitro* from patients' cells compared with healthy age-matched controls. They suggested that a compromised capacity to respond to extra inflammatory challenges occurs in AD (Guerreiro et al., 2007).

The decrease in pro-inflammatory cytokines does not, however, necessarily mean that an inflammatory response ceases. It is possible that other inflammatory markers, not measured in the current study, are increased in more severe AD. One indication that this may be the case is that the total white cell count in the current study was raised in severe AD participants when compared with moderate AD participants. The ESR in more severe

AD participants was also not significantly different from mild AD participants, who had a raised ESR when compared with controls and moderate AD groups. Advanced AD participants were not studied and only participants with an MMSE score above 12 were included. Inclusion of a more severe AD group and a wider range of inflammatory markers should be investigated in future work. However, this may be problematic from an ethical and consent point of view.

Systemic Inflammation and Cognitive Decline

In the longitudinal study, Hypothesis 4 predicted that higher levels of baseline systemic inflammatory markers would predict a faster rate of cognitive decline. Linear regression analysis, controlling for the presence of the APOE ε4 allele, showed that high baseline levels of IL-10 were associated with slower decline on the CAMCOG and the MMSE when all participants were analysed together and when the AD group was analysed separately.

IL-10 is an anti-inflammatory cytokine that inhibits the secretion of pro-inflammatory cytokines, thereby suppressing inflammation and modulating disease progression. Rat astroglial-microglial cell co-cultures that were incubated with LPS and rat IL-10 showed decreased LPS-induced pro-inflammatory cytokines TNF-α, IL-6 and IL-1β, when compared with astrogial-microglial cultures that were incubated with LPS alone (Ledeboer, Brevé, Poole, Tinders, & van Dam, 2000). Two polymorphisms in the regulatory region of the IL-10 gene were found to be associated with both low plasma levels of IL-10 and an increased risk of AD. The polymorphisms were not associated with APOE genotype. Therefore, the risk of developing AD was independent of APOE (Lio et al., 2003; Ma, Tang, Lam, & Chiu, 2005). The finding in the current study that low IL-10 was associated with greater cognitive decline, together with the genetic evidence (Lio et al., 2003) and *in vitro* studies (Ledeboer et al., 2000), provide evidence that IL-10 is protective in AD. This is in keeping with a study showing that high levels of the PGE_2 in the CSF at baseline were related to longer survival time in AD participants (Combrinck et al., 2006). PGE_2 acts as an inflammatory mediator as it limits and controls the inflammatory response.

High levels of the pro-inflammatory cytokine TNF-α at baseline were associated with greater decline on the CAMCOG, MMSE and learning subscale when all participants (control and AD) were analysed together. Similarly, high levels of TNF-α at baseline were associated with greater decline on the CAMCOG in the AD group. Holmes et al. (2009) found that the positive change in the ADAS-COG over 6 months was greater in AD participants with high baseline blood TNF-α levels when compared with AD participants with low baseline TNF-α levels. A greater positive change on the ADAS-COG indicates greater decline in cognitive functioning. Lower cognitive scores at follow-up are consistent with the increase in neuropsychiatric symptoms at follow-up. A pilot study showed that participants with lower TNF-α levels at baseline had lower Neuropsychiatric Inventory scores at a 6-month follow-up (Holmes et al., 2011).

In general, the current study showed that high levels of non-specific inflammatory markers (white cell count, monocyte count and OPN) at baseline predicted faster decline in one year on at least one of the cognitive measures in AD participants. These results add to the current literature showing that higher levels of inflammatory markers predict a faster rate of cognitive decline. A large, 25-year follow-up study showed that men with high levels of CRP, a non-specific marker of inflammation, had a 3-fold increase in the risk of dementia (Schmidt et al., 2002a). High baseline levels of alpha 1-antichymotrypsin, a protein induced during inflammation, were associated with increased decline on the MMSE over a 3-year period (Dik, Jonker, Hack, Smit, Comijs, & Eikelenboom, 2005).

In the control group, high baseline levels of ESR predicted a greater decline on the learning subscale. This is in line with the above literature linking high levels of systemic inflammatory markers with cognitive decline. Controls with high ESRs could also be 'preclinical' AD participants. The current study showed that early AD participants had elevated ESRs. The rise in ESR and decline in the learning subscale could indicate that these participants have early MCI. Future work following control participants with high ESRs to see if they convert to MCI / AD would be interesting.

IL-1β in the current study was not associated with decline on any of the cognitive measures when participants were followed-up after 12 months. Holmes and colleagues (2003) found that AD patients with detectable levels of IL-1β at baseline declined faster over 2 months

compared with AD patients with no detectable levels of IL-1β. None of the patients fulfilled the Confusion Assessment Method diagnostic criteria for delirium at baseline or at follow-up (Holmes et al., 2003). One explanation for the lack of association between IL-1β and cognitive decline in the current study could be that AD participants were grouped together, irrespective of the stage of their disease. As discussed previously, IL-1β was significantly raised in the early stages of AD, declining with disease progression. During the early stages of AD (when IL-1β was highest), cognitive decline over one-year is relatively slow compared with decline during moderate disease. This is because cognitive decline in AD is not linear and generally follows a decreasing logistic function curve (Martins et al., 2005). Ideally, when comparing the rate of decline over one year, studies should compare participants at similar clinical stages of the disease process. This will control for the differences in the inflammatory markers noted at the different stages of AD. Due to small numbers in the AD group at baseline and at follow-up this was not possible in the current study. However, clinical disease stage also does not necessarily correlate with pathological changes.

Other evidence that increased inflammation leads to an increase in cognitive decline comes from studies examining the effect of infection on cognition. Holmes et al. (2009) showed that AD patients who experienced an acute inflammatory event in a 6-month period had a faster rate of cognitive decline when compared with AD patients who did not experience an inflammatory event (Holmes et al., 2009). The mechanism by which high levels of cytokines lead to a faster cognitive decline is discussed further in the section: The Relationship Between Systemic Inflammation and CNS Inflammation (page 168).

CNS Inflammation and Cognitive Functioning

In the MRS study, Hypothesis 6 predicted that MI, a marker of glial activation, would be highest in early AD, declining with disease progression. In order to investigate this hypothesis the AD group was divided into a mild/moderate subgroup and advanced subgroup based on their learning subscale scores. Results from the one-way ANOVA statistical analysis showed that mild/moderate AD participants did not have higher MI levels in the posterior cingulate gyrus when compared with controls and more advanced AD participants respectively. However, the advanced AD participants had significantly

higher MI level compared with the mild/moderate AD group, indicating that MI increases with progression to later, moderate AD.

An increase in MI levels in both MCI and AD patients has been reported in many studies (Miller et al., 1993; Moats et al., 1994; Shonk et al., 1995). Kantarci et al. (2000) found that, compared with controls, MCI participants had increased MI levels in the posterior cingulate. Martinez-Bishal, Arana, Marti-Bonmati, Mollá, & Celda (2004) found that MCI participants had lower MI/Cr ratios in the posterior cingulate gyrus compared with AD participants. Wang and colleagues (2009; 2012) also found that MI/Cr was increased from controls to MCI to AD participants in the left hippocampus and the posterior cingulate respectively (Wang et al., 2009; Wang et al., 2012).

MI has also been shown to be correlated with cognitive scores. Parnetti et al. (1997) found that MI correlated inversely with MMSE scores and duration of disease in frontal white matter of AD patients. The MI/Cr ratio was also negatively correlated with MMSE scores across the cognitive spectrum, from cognitively healthy controls to AD patients (Rose et al., 1999). The current study supports these findings: MI tended to be negatively correlated with episodic memory scores in AD participants, although not significantly. However, MI was not correlated with the MMSE in the control or AD group. The lack of correlation between MI and the MMSE may relate to the sensitivity of the MMSE. The MMSE is probably less sensitive than the learning subscale to pathological changes occurring in individuals within a mild to moderate AD group. Huang et al. (2001) found that although MI was increased from controls to MCI participants in the occipital lobe and the left and right parietal areas, there was no correlation between MMSE scores and MI across the cognitive spectrum from cognitively healthy to AD participants (Huang et al., 2001).

An increase in microglial cell activation in AD has been shown in both post-mortem and PET imaging studies. A post-mortem study by Hoozemans et al. (2005) showed that microglia activation increased with increasing AD pathology. In this study investigators measured glial fibrillary acid protein (a protein expressed by astrocytes), KP1 (a marker of phagocytotic microglia) and CR3/43 (a marker of activated microglia) in the mid-temporal cortex of post-mortem brain tissue. The density of immune-reactivity of the two microglial cell markers and the astrocyte marker were positively correlated with the Braak scores for

neurofibrillary tangles and the Aβ deposits. These authors hypothesised that microglial cell activation occurs later in the disease process and is highest in Braak stage V – VI (Hoozemans et al., 2005), this is in keeping with the increase in MI in later, moderate stage AD found in the current study.

Microglial cell activation has also been studied *in vivo* using Carbon 11-labelled-(R)-PK1195. [11C]-(R)-PK1195 is a PET ligand that is used as a marker of microglial cell activation. MMSE scores correlated inversely with [11C]-(R)-PK1195 binding in the posterior cingulate gyrus in AD participants (Edison et al., 2008).

There was no difference between the mild/moderate AD and the control group in the current study. One reason for this may be that MI was measured in the posterior cingulate gyrus and not the hippocampus. As discussed in the Introduction (page 46), the posterior cingulate gyrus has been shown to undergo early metabolic and structural changes in MCI and early AD, which is why this area was chosen to be investigated (Nestor et al., 2003; Kantarci et al., 2011; Wang et al., 2012). It is also a more homogenous tissue, making it ideal for imaging. Unfortunately, for technical reasons, neurometabolites from the hippocampal areas could not be measured in the current study. Dixon and colleagues (2002) were one of the few groups to examine neurometabolites in the hippocampus. They note that the hippocampus was a challenging area to investigate due to its close proximity to air and bone. Kantarci et al. (2000) also noted that obtaining spectra from a small voxel without partial voluming was difficult. Perhaps results from the hippocampus may have been more sensitive to early MI changes. Wang et al. (2009) did not find between-group differences with regard to MI/Cr in the posterior cingulate gyrus between control, MCI and AD participants. However, they did find between-group differences in MI/Cr in the hippocampus. MI/Cr in the hippocampus may therefore be more sensitive in early AD than in the posterior cingulate.

The increase in MI may be specific to AD. Shonk et al. (1995) showed that the increase in MI/Cr ratio in the occipital lobe was specific to AD and was not found in other dementias. Therefore, MI might be a distinguishable marker for AD. Rose et al. (1999) also showed that MI levels were relatively stable and did not fluctuate over the course of the day. This is useful when imaging patients, as one does not need to worry about diurnal fluctuations.

^{1}H-MRS measurements of MI may therefore be useful in monitoring disease progression in AD.

In summary, the current study has shown that microglia activation increased in the more advanced stages of moderate AD. Research examining activated microglial cells in post-mortem samples as well as *in vivo* studies using ^{1}H-MRS and PET, taken together with the current study's findings, indicate that microglial cell activation continues to increase with progression of the disease.

The Relationship between Systemic Inflammation and CNS Inflammation

Studies have shown that high levels of peripheral inflammation and infection lead to a faster rate of cognitive decline in AD participants (Holmes et al., 2009; 2011). However, the clinical implications of raised systemic inflammatory markers, in the absence of any obvious acute infection, remain unclear. Hypothesis 10 therefore investigated the relationship between systemic inflammation and CNS inflammation.

The current data showed that IL-1β tended to be negatively correlated with MI in AD participants. The negative correlation between IL-1β and MI suggests that these two inflammatory processes are somehow linked. As discussed previously, IL-1β was raised in early AD and declined in moderate and severe AD. However, MI was increased in more advanced AD participants compared with mild/moderate AD participants. This suggests that the rise in IL-1β in the systemic circulation preceded microglial activation in the CNS. Therefore, it may well be that the rise in IL-1β in the systemic circulation is the precursor to microglia activation in the CNS.

In the normal healthy brain, microglia are in a down-regulated, quiescent state. In the presence of AD pathology, microglia are partially activated or "primed" (Perry, 2004). This inflammatory response is not the same as that which occurs in acute infection where there is a robust increase in inflammatory markers. Systemic inflammatory changes can be relayed to the brain via three main routes of systemic-CNS communication. Firstly, systemic cytokines are able to signal to the CNS via the BBB (Perry, 2004). The second

route of communication is directly to the CNS via the circumventricular organs that lack a BBB (Banks et al., 1995). The vagus nerve also transmits signals to the CNS from sensory afferent fibres in the thoracic abdominal cavity (Bluthe et al., 1996). For a discussion of these mechanisms see the Introduction (page 36). The increased levels of IL-1β and ESR may have signalled to the CNS via one or more of these routes. CNS microglia are reactive to the systemic inflammatory stimuli and primed microglia are further activated. Further activation results in an increase in MI, observed in the later stages of moderate AD in the current study. The further activation of microglia leads to an increase in cytokines, chemokines, proteases and nitric oxide. These toxic inflammatory molecules, together with reactive oxygen species and an increase in glutamate, lead to further neuronal damage and more Aβ aggregation (Akiyama et al., 2000). It is most likely via this mechanism that systemic infection leads to a decline in cognitive functioning (Combrinck, Perry, & Cunningham, 2002; Perry et al., 2007, Holmes et al., 2009).

In conclusion, the rise in systemic inflammatory markers in the early AD participants may have triggered or driven the activation of microglia noted in the more moderately-advanced stages of AD.

Summary

The nature of the inflammatory response in AD appears to change during the course of the disease and may be divided into three phases (see *Figure 44*). On the basis of previous literature, I should like to propose that the initial response in MCI / early AD is "anti-inflammatory" in nature and may be beneficial as inflammatory cells assist in the attempt to remove Aβ plaques (Wyss-Coray et al., 2001; Combrinck et al., 2006; Motta et al., 2007; Parachikova et al., 2007). Although this study did not delineate the precise timing of the anti-inflammatory cytokine increase, the findings do support the hypothesis that anti-inflammatory cytokines are protective. AD participants with higher levels of the IL-10 at baseline declined less on cognitive measures. This is in line with literature showing that higher levels of the PGE_2 were associated with longer survival in AD patients (Combrinck et al., 2006). This predominately anti-inflammatory response is then followed by a more pro-inflammatory response in early AD (see *Figure 44*). In the current study, higher levels

of TNF-α, a pro-inflammatory cytokine, and non-specific inflammatory markers, measured systemically during early or mild AD were associated with a more rapid cognitive decline. The increase in pro-inflammatory molecules might arise from a decrease in the anti-inflammatory cholinergic vagal response (Nizri et al., 2006). Pro-inflammatory cytokines may also be released in response to elevated Aβ in the peripheral blood and/or brain (Bateman et al., 2012). A combination of the above mechanisms may also be operating.

There is evidence to support the idea that peripheral pro-inflammatory cytokines are able to signal to the CNS. Microglia are sensitive to peripheral immune signalling and primed microglia are further activated in response to this signalling (Perry, 2004). Systemic pro-inflammatory cytokines that are elevated in the early stages of AD might signal to the CNS. This may account for the further consequential activation of microglial cells in the CNS.

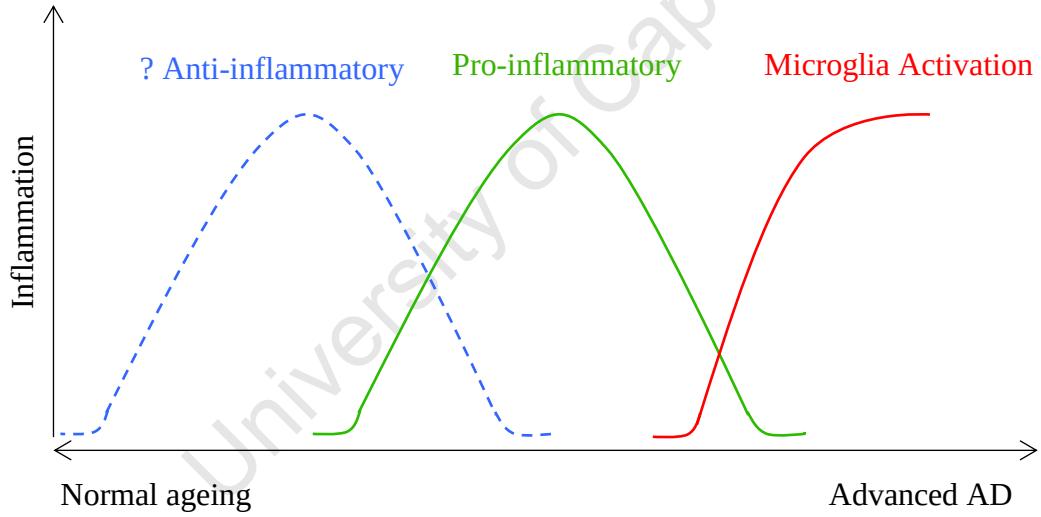


Figure 44: The proposed tri-phasic inflammatory response in the progression from normal ageing to advanced Alzheimer's disease (AD). In the MCI / early AD phase, the inflammatory profile is predominantly "anti-inflammatory". This is then followed by a predominantly pro-inflammatory response in early / mild AD. In the later stages of moderate AD, there is significant brain microglial activation.

Longitudinal studies, with repeated cytokine measurements together with repeated cognitive assessments would better characterise the changes in inflammatory markers and lead to a better temporal definition of the aforementioned scheme (*Figure 44*). The long-term use of NSAIDs reduces the risk of dementia (Breitner et al., 2011; In'T Veld et al.,

2001; Vlad, Miller, Kowall & Felson, 2008; Côte et al., 2012), yet current drug trials targeting inflammation in AD patients have failed (Gasparini, Ongini, & Wenk. 2004; McGeer & McGeer, 2007). Breitner et al. (2011) showed that NSAIDs taken before the onset of dementia reduced the risk of AD. However, they had harmful or no benefit once in patients with the disease. Understanding and characterising the different stages of inflammatory response, both at a peripheral and CNS level, are crucial to the design of targeted treatment options.

NEURONAL INTEGRITY AND ITS RELATIONSHIP WITH SYSTEMIC INFLAMMATION, CNS INFLAMMATION AND COGNITION

NAA is thought to be a marker of neuronal integrity. A decrease in NAA in the CNS may indicate different processes occurring: either neuronal loss or a decreased level of functioning or reduced metabolic integrity of neurones that are still viable (Valenzuela & Sachdev, 2001). As discussed, inflammation is thought to drive a neurotoxic cycle leading to neurodegeneration and subsequent cognitive decline (Akiyama et al., 2000). The aim of Hypothesis 7 was to investigate the relationship between neuronal integrity and cognition and the aim of Hypothesis 10 was to investigate the relationship between neuronal integrity and both CNS and systemic inflammatory markers.

The Relationship between Neuronal Integrity in the Posterior Cingulate Gyrus and Cognitive Functioning

In order to investigate the relationship between NAA+NAAG and cognition, the AD group was divided into mild/moderate and more advanced AD groups based on their learning subscale. In the current study, NAA+NAAG in the posterior cingulate gyrus was decreased in the mild/moderate AD group and the advanced AD group when compared with controls. There was no difference between the mild/moderate and more advanced AD group. Kantarci et al. (2000) also showed a reduction in NAA/Cr in the posterior cingulate when AD patients were compared with controls. Medial temporal lobe reduction in NAA in AD patients compared with cognitively healthy controls was also found by Jessen et al. (2000). The reduction in medial temporal lobe NAA was not due to generalised atrophy as there was no difference in NAA concentrations between control and AD groups in the primary motor and sensory cortices (Jessen et al., 2000). Dixon et al. (2002) also found decreased NAA levels in the left hippocampal area when AD participants were compared with controls.

Although the current study found that NAA+NAAG was decreased in early AD, NAA+NAAG levels in the mild/moderate AD and more advanced AD groups were not significantly different from each other in the posterior cingulate. This suggests that

although NAA+NAAG distinguished control participants from AD participants, it was not a sensitive marker for disease progression in the posterior cingulate. Another explanation may be that AD participants that underwent ^{1}H-MRS scanning all had moderate disease and therefore NAA+NAAG levels between the mild/moderate and more "advanced" group were not distinguishable. I.e. the advanced group (defined by a learning subscale score below 7) were still not that advanced. A larger number and wider range of AD patients are needed to investigate whether NAA is a sensitive marker for disease stage. However, there are ethical considerations and practical difficulties related to scanning more advanced disease patients; these would need to be factored into future studies.

The reduction in NAA+NAAG in the mild/moderate AD group is interesting. Some studies have suggested that NAA is *not* a sensitive marker of pathology in MCI or early AD (Kantarci et al. 2000; Martinez-Bishal et al., 2004). Huang et al. (2001) divided AD patients into severely demented (MMSE scores ≤ 9), moderately demented (MMSE score of 10 – 19) and mildly demented (MMSE ≥ 20) groups. The moderately demented and severely demented groups had lower NAA levels compared with the control group in all three regions of interest (occipital, right and left parietal lobes), whereas the mildly demented group didn't differ from the control group in any of the regions of interest (Huang et al., 2001). This study suggested that parietal and occipital reductions in NAA occur slightly later in the disease process and continues to decline with disease progression. The current data do not support this hypothesis.

NAA was not correlated with the MMSE or learning subscale in the current study. This is in keeping with results found by Jessen et al. (2000). In their study, cognitive scores on the ADAS-COG also did not correlate with the NAA/Cr ratio. In a follow-up study by the same group, they did find a relationship between the MMSE and NAA/Cr. Cognitive decline on the MMSE was positively associated with the NAA/Cr decline in individuals followed-up after 23 months (Jessen et al., 2001). This suggests NAA may be a useful longitudinal marker.

The lack of association between the cognitive scores and NAA+NAAG might be because NAA+NAAG was measured in the posterior cingulate cortex in the current study. Hippocampal NAA measurements may be more sensitive to early pathological changes.

Dixon et al. (2002) found that the total left hippocampal NAA was positively correlated with the CAMCOG scores in AD participants. Rose et al. (1999) also found a positive correlation between NAA in the hippocampus and MMSE scores in cognitively healthy and AD participants. A volumetric study also showed that the NAA/Cho ratio was positively correlated with volume reductions in the left hippocampus (Schuff, Amend, Knowlton, Norman, Fein, & Weiner, 1999). Therefore, hippocampal NAA measurements may be better correlated with cognitive functioning in mild to moderate AD participants than the posterior cingulate gyrus because of the area's early episodic memory involvement in AD.

Neuronal Integrity and its Relationship with CNS Inflammation, Systemic Inflammation

An increase in inflammatory molecules and cytokines has been associated with an increase in neurodegeneration. Hypothesis 10 investigated whether inflammation and neuronal integrity were correlated in the population examined.

NAA+NAAG and MI were not correlated in the AD group. This was not surprising given that NAA+NAAG was decreased in all AD participants (mild/moderate and more advanced AD) and MI was only significantly raised in more advanced AD participants. These results suggest that a decrease in NAA+NAAG occurs before a significant increase in MI and therefore these processes are asynchronous. Other studies have also found that the decrease in NAA and the rise in MI are asynchronous. However, these studies showed that the rise in MI precedes the decrease in NAA (Kantarci et al., 2000; Wang et al., 2012). The rise in MI preceding the decrease in NAA is supported by a study showing that non-demented Down syndrome adults, who are predisposed to developing AD, had increased MI concentrations and no decline in NAA concentrations (Huang et al., 1999). Wang and colleagues (2012) showed a difference in MI levels in control, MCI and AD participants in the temporal lobes but no difference in NAA levels. These authors suggested that the early increase in MI occurred before significant loss of neuronal integrity occurs (Wang et al., 2012). Huang et al. (2001) also showed that MI increased in the parietal lobes of mild AD participants compared with controls, whereas a decrease in NAA was only evident in moderate AD participants. Failure to demonstrate an MI increase preceding NAA+NAAG

decline in the current study might be due to small numbers in the mild/moderate group. The early decrease in NAA+NAAG might also be because the signal strength of NAA+NAAG on ^{1}H-MRS is much stronger than that of MI, making it easier to detect small differences.

Studies using PET imaging with [11C]-(R)-PK1195, a marker of activated microglia, and PiB, a marker of Aβ load, support the idea that microglial activation is not correlated with Aβ pathology and therefore potentially neurodegeneration. [11C]-(R)-PK1195 did not correlate with PiB retention in the posterior cingulate gyrus in AD participants, i.e. microglia activation did not correlate with Aβ load (Edison et al., 2008). Wiley et al. (2009) also found no correlation between [11C]-(R)-PK1195 and PiB retention in the posterior cingulate cortex and the frontal cortex. The lack of correlation between Aβ load and microglial cell activation suggests that these pathologies may occur independently (Okello et al., 2009).

The current study supports the idea that MI may be a more robust and sensitive indicator of AD progression than NAA+NAAG in AD patients as it was correlated with episodic memory scores (Siger, Schuff, Zhu, Miller, & Weiner, 2009). However, MI was not shown to be sensitive marker in early disease, whereas NAA+NAAG was significantly decreased in early AD.

The lack of correlation between MI and NAA+NAAG probably accounted for the decreased sensitivity of the MI/NAA+NAAG ratio to disease stage in the current results. Others have reported that the MI/NAA+NAAG ratio was sensitive to disease stage that MI/NAA was correlated with cognitive scores (Shonk et al., 1995; Ackl et al., 2005). This was not found in the current study. However, these studies examined different brain regions. Shonk et al (1995) examined the occipital and frontal lobes and Ackl et al. (2005) examined the hippocampal area. This could be one reason for the conflicting results.

Studies showing that high levels of systemic inflammation are associated with an increase in neurodegeneration (Holmes et al., 2009; Perry et al., 2007) lead to the hypothesis that NAA+NAA would be negatively correlated with systemic markers of inflammation.

Results from the current study showed that TGF-β_1 and TGF-β_2 were inversely correlated with NAA+NAAG in AD participants. I.e. the higher the systemic levels of TGF-β the lower the NAA+NAAG peak in the CNS. As mentioned, TGF- β is an anti-inflammatory cytokine. This result suggests that systemic TGF- β increases with neurodegeneration, contrary to the proposed hypothesis that anti-inflammatory markers are raised in the beginning stages of AD, declining with disease progression (see page 170). This result could indicate that the anti-inflammatory profile predominates in the later stages of moderate AD. However, in the cross-sectional study, which incorporated a larger number of AD participants, there was no rise in TGF-β_1 and TGF-β_2 with disease progression. The finding that TGF-β_1 and TGF-β_2 correlated with NAA+NAAG might just be due to a Type I error. Further studies are needed to clarify this result.

In summary, if NAA+NAAG is assumed to be present exclusively in neurones, the current study suggest that neuronal loss and/or dysfunction occurs in mild to moderate AD participants in the posterior cingulate gyrus. The early reduction in NAA+NAAG also suggests that NAA+NAAG is sensitive to early neuronal changes in the posterior cingulate gyrus of AD participants. Neuronal integrity and microglia activation seemed to occur at different times or independently of each other.

NAA+NAAG is a useful marker as it measures the viability of neurones. Therefore, NAA sizes may be more sensitive to cognitive changes than regional volumes.

THE ROLE OF THE APOE GENOTYPE IN COGNITIVE FUNCTIONING AND ITS RELATIONSHIP WITH INFLAMMATION AND NEURONAL INTEGRITY

Until recently, with the discovery of *TREM* 2, the APOE ε4 allele was the only confirmed genetic marker that was strongly associated with late-onset AD. The APOE ε4 allele has also been shown to be associated with cognitive functioning (Petersen et al., 1995; Bretsky, Guralnik, Launer, Albert, & Seeman, 2003) and to be related to inflammatory processes in AD and other diseases (Egensperger et al., 1998; Grocott et al., 2001). These associations are particularly important in the South African population given the high prevalence of the APOE ε4 allele found in the current study. The allelic frequency of the ε4 allele in all participants in the current study was 27.6%, with a frequency of 35.6% in AD participants and 20.3% in controls. The associations between the APOE ε4 allele, cognition (Hypothesis 2 and 5), systemic inflammation (Hypothesis 3) and CNS inflammation (Hypothesis 9) were explored.

The Relationship Between the APOE Genotype and Cognition

While the association between the ε4 allelic variant and Aβ load has been clearly established in studies from the industrialised world (Tiraboschi et al., 2004; Thal et al., 2007), the relationship between the APOE genotype and cognition is controversial with many studies showing conflicting reports. This study therefore aimed at investigating the effect of the APOE ε4 allele on cognition in AD and control participants in a cross-sectional and longitudinal study.

Hypothesis 2 investigated the effect of the ε4 allele cross-sectionally. It was hypothesised that participants in the AD and control groups with at least one APOE ε4 allele would have poorer cognitive scores when compared with ε4 non-carriers. AD ε4 carriers tended to have poorer scores on the MMSE when compared to ε4 non-carriers. There was no difference in the CAMCOG or learning subscale scores of AD ε4 carriers and non-carriers. Likewise, in the control group, there was no difference in ε4 carriers versus non-carriers on any of the cognitive measures. Other studies have also shown no effect of the ε4 allele on cognitive functioning. A cross-sectional study by Growdon, Locascio, Corkin, Gomez-Isla,

& Hyman (1996) found that AD APOE ε4 carriers did not perform more poorly than non-carriers on cognitive tests. This study was, however, small and may have lacked sufficient power. Small et al. (2000) also showed in a cross-sectional analysis that there was no significant effect of the APOE ε4 genotype on general cognition in cognitively healthy older adults.

Many longitudinal studies in developed countries have shown that control participants carrying the ε4 allele were at a higher risk of developing AD compared with controls carrying other APOE alleles (Farrer et al., 2007). Therefore, some investigators have argued that the poorer cognitive performance found in ε4 positive controls in some studies may be due to the over-representation of preclinical AD participants in the control group. If participants with preclinical dementia were retrospectively excluded from the analyses in the cognitively healthy group, the difference in rate of cognitive decline between ε4 carriers and non-carriers disappeared (Bondi, Salmon, Galasko, Thomas, & Thal, 1999; Bunce Fratiglioni, Small, Winblad, & Backman, 2004). In the current study's cross-sectional analysis, similar cognitive scores in controls with or without the ε4 allele support the findings that ε4 is not related to cognition in cognitively healthy participants.

In the longitudinal section of this study, Hypothesis 5 predicted that participants with at least one ε4 allele would decline faster over one year when compared with ε4 non-carriers. When control participants were followed-up for 12 months, the presence of the ε4 allele was associated with a *less* of a decline on the CAMCOG. However, in AD participants the presence of the ε4 allele was associated with a *greater* decline on the CAMCOG over 12 months.

The finding that the APOE ε4 allele was associated with increased decline on the CAMCOG in AD participants is in agreement with a study by Martins et al. (2005). Martins and colleagues (2005) also used the CAMCOG as a measure of cognitive functioning in AD participants. The presence of the APOE ε4 allele predicted a faster rate of cognitive decline on the CAMCOG when AD ε4 carriers were compared with ε2 carriers (Martins et al., 2005). The same study also found that APOE ε4 had a dose-dependent effect on decline on the CAMCOG, with ε4/ε4 patients declining faster than ε3/ε4 patients. Unfortunately, due to the small numbers in the current study, the dose-

dependent effect of ε4 could not be examined. Cosentino et al. (2008) also showed that ε4 carriers in the early stages of AD had a faster rate of cognitive decline over a 4-year period when compared with ε4 non-carriers. Cognitive decline was measured using a composite score comprising of various tests that assessed memory, language, visuospatial functioning, abstract reasoning and executive speed. The study suggested that the effect of ε4 was restricted to participants in early AD and was not evident in more severe participants. However, the latter finding may have been due to floor effects on neuropsychological tests seen in advanced AD participants.

The mechanisms by which APOE ε4 exerts its detrimental effects after the onset of AD pathology may also relate to the role of the ApoE protein in repair and regeneration. ApoE is released by microglia in response to neuronal injury (Slezak & Pfrieger, 2003). Soares et al. (2012) showed that ApoE protein levels were lower in AD patients with the ε3/ε4 and ε4/ε4 genotype compared with patients with the ε2/ε3, ε3/ε3 and ε2/ε4 genotypes. Cultured neurones incubated with ApoE4 had fewer neuronal outgrowths when compared with neurones cultured with ApoE3 (Nathan et al., 2002). Reduced neuronal outgrowths in ε4 carriers could lead to impaired neuronal repair due to damage caused by Aβ and tau pathology. Clinical evidence from traumatic brain injury patients has also shown that patients carrying the APOE ε4 allele were twice as likely to have an unfavourable outcome 6 months after a traumatic brain injury than ε4 non-carriers (Teasdale, Nicoll, Murray, & Fiddes, 1997). This is consistent with the notion that ε4 only influences cognitive functioning after the event of neuronal injury (Small et al., 2000).

The ε4 allele has not been associated with cognitive decline in AD patients in other studies. Growdon et al. (1996) showed that over an average of 5.5 years, AD ε4 carrier and non-carrier participants did not differ in their rates of cognitive decline. The authors of this study estimated that the APOE genotype of participants accounted for less than 10% of the variance in the rate of cognitive decline (Growdon et al., 1996). In the current study, the ε4 allele was not associated with decline on the MMSE or learning subscale in AD group. However, the small scoring range of these two tests may explain this result. On average, AD participants declined by 2 points on the MMSE and 1.5 points on the learning subscale. Although AD participants did decline, the change in scores on the MMSE and learning subscale were small. AD participants declined 7 points on the CAMCOG in one

year, showing a greater change in functioning. It is therefore important to consider the sensitivity of the cognitive test scales when assessing cognitive decline.

It is also important to note that decline on the CAMCOG, as shown by Martins et al. (2005), is not linear. Therefore, comparing the rate of decline between two groups of AD patients at different stages of AD using linear models needs to be interpreted with caution. Ideally one should adjust for the number of years of disease duration when comparing rates of cognitive decline so that participants with equivalent disease stages are compared. However, this also poses problems as disease duration/symptoms and pathological stages of disease may also be asynchronous.

In the current study, control ε4 carriers showed a smaller decline on the CAMCOG than ε4 non-carriers over one year. Bunce et al. (2004) showed that the ε4 allele did not affect cognitive decline in cognitively healthy individuals carrying the ε4 allele. The strength of their study was the long duration of participant follow-up, *viz* 3 and 6 years, respectively. One limitation, however, was that their only measure of cognition was the MMSE. Although the MMSE is a good screening tool for established dementia, it may not be sensitive enough to detect early cognitive changes (especially episodic memory) in cognitively healthy and MCI individuals. Smith et al. (1998) also showed that cognitively healthy ε4 carriers did not differ on any of the 5 Mayo Cognitive Factor Scores when compared with ε4 non-carriers. This study, like the current study, also found that AD ε4 carriers and AD non-ε4 carriers differed on cognitive measures (Smith et al., 1998).

One reason for the lack of association between ε4 and cognitive *decline* in controls may be that the ε4 allele is a thrifty allele. In the absence of AD pathology, the APOE ε4 allele may be protective in our South African participants. APOE is involved in cholesterol transport and metabolism (Mahley, 1988). In populations where food supply is low or has until recently been scarce and food quality is poor, carrying the ε4 might still be beneficial. In these populations, the ε4 allele might help in rebalancing the cholesterol levels that might otherwise be too low. However, when the intake of cholesterol is high, the ε4 allele becomes detrimental and predisposes one to increased risk of cognitive decline (Scacchi et al., 1997).

Another explanation for the lack of association between ε4 and decline in cognitive functioning in control participants may be that APOE ε4 does not directly influence cognitive performance. APOE ε4 may act only as a modulating effect on the processes that actually cause cognitive impairment. Therefore, the effects of ε4 on cognitive functioning may only be evident after significant onset of Aβ and tau pathology. Kantarci et al. (2012) showed that ε4 interacts with Aβ to produce its effect on cognition. In cognitively healthy participants, the presence of the ε4 allele modified the relationship between retention of PiB (a measure of Aβ load) and cognition. In their study cognitively healthy ε4 carriers had the highest retention of PiB, indicating a higher Aβ load. Linear regression analysis also showed that the relationship between PiB retention and cognitive functioning was strongest in ε4 carriers (Kantarci et al., 2012). The lack of association between ε4 and decline in cognitive functioning in controls may therefore be due to the fact that the control participants studied lacked sufficient Aβ and tau pathology for the detrimental effects of ε4 to be observed. In other words, AD pathology is required for ε4 to exert effects on cognition. Larger studies utilising sophisticated measures of Aβ quantification, such as PiB imaging, are needed to test these hypotheses.

Martins et al. (2005) argue that neuropathological changes, rather than neuropsychological changes, may be more sensitive at detecting the effect of ε4. A neuropathological study has shown that ε4 carriers had a greater accumulation of Aβ and neurofibrillary tangles in brain tissue when compared with participants who were homozygous for the ε3 allele (Tiraboschi et al., 2004). Measuring pathological Aβ changes was not within the scope of the current study. Ideally one would measure Aβ in the CSF (for which ethics approval was not available in the current study) or using the PET tracer PiB.

Another factor to consider is the type of neuropsychological tests used in the current study. These tests may be less sensitive to decline in cognition in cognitively healthy participants. Participants were also only followed-up for one-year. In a 7-year follow-up study, Bretsky et al. (2003) showed that the risk of cognitive decline in highly functioning ε4 carriers was greater than non-carriers. However, this effect was less significant at the 3-year time interval. The current study's follow-up time may be too short to examine the true effect of ε4 on cognition in control participants.

It is important to note that the ε4 allele is not the only factor to consider when investigating cognitive decline. A number of elderly people remain cognitively healthy despite being homozygous for the ε4 gene. Certainly age and education need to be considered. Both advancing age and fewer years of education have been associated with cognitive decline (Hebert et al., 1995; Kukull et al., 2002). However, environmental factors, such as stress, also need to be considered (Breitner & Welsh, 1995).

Summary

This study found an association between the APOE ε4 allele and cognitive functioning in AD participants. In the cross-sectional study AD ε4 carriers tended to have poorer scores on the MMSE compared with AD non-carriers. In the longitudinal study, ε4 carriers had a greater decline on the CAMCOG. This is in keeping with current literature showing that AD patients with the APOE ε4 allele have poorer cognitive functioning and a faster rate of cognitive decline compared with non-carriers (Martins et al., 2005; Cosentino et al., 2008).

There was no association between cognitive functioning at baseline and the APOE ε4 allele in control participants. APOE ε4 may not directly influence cognitive functioning and may only modulate the pathological features of AD that cause cognitive decline. The controls studied may have lacked sufficient Aβ and tau pathology for the effect of ε4 to be evident.

The Relationship Between the APOE Genotype and Inflammation

Many studies have investigated the effect of the APOE ε4 genotype on inflammatory processes (Egensperger et al., 1998; Lynch et al., 2003; Guo et al., 2004). The current study investigated the relationship between APOE genotype, systemic inflammation and the CNS inflammatory marker, MI in the posterior cingulate gyrus.

In the cross-sectional study, Hypothesis 3 predicted that participants, either homozygous or heterozygous for the APOE ε4 allele, would have higher baseline levels of systemic inflammatory markers compared with participants with the other allelic variants. Linear regression analysis showed that in the control group the presence of the ε4 allele was

associated with higher baseline IL-10 levels. However, in the AD group the ε4 allele was associated with lower IL-10 levels.

There are two possible explanations for the difference in the relationship between ε4 and IL-10 in controls and AD cases. As discussed, APOE ε4 is a thrifty allele and in the absence of AD pathology, APOE ε4 may be beneficial in the South African population. The beneficial response in controls might include higher levels of IL-10, an anti-inflammatory cytokine. The onset of AD pathology might change the "protective" ε4 effect to that seen in AD, which is harmful.

A second, and perhaps more plausible explanation for ε4 tending to predict higher IL-10 levels in controls may be that control ε4 carriers have higher levels of Aβ and tau pathology. As discussed, controls carrying the ε4 allele have a higher risk of developing AD compared with ε4 non-carriers. In a study by Soares et al. (2012), 25% of the 58 healthy controls that converted to MCI within one year had at least one ε4 allele. Similarly, of the 164 MCI participants that converted to AD, 67.7% were ε4 positive. The rise in IL-10 levels ε4 positive in control participants may be due to increased AD pathological features. As hypothesized before (see The Relationship between Inflammation and Cognition, page 157), the rise in IL-10 may be the early protective anti-inflammatory response to AD pathology. The use of PET imaging for a measurement of Aβ load will help in identifying controls that have increased AD pathology. Monitoring controls with high IL-10 for conversion to MCI to AD could be the interesting subject of future studies.

In AD participants, APOE ε4 tended to be associated with lower baseline IL-10 levels. The decrease in the anti-inflammatory cytokines may result in an unopposed or predominant pro-inflammatory state. This is in keeping with the literature showing that the APOE ε4 has a pro-inflammatory effect (Lynch et al., 2003; Guo et al., 2004). Guo et al. (2004) showed that rat glial cultures that were stimulated with exogenous ApoE4 released more IL-1β when compared with glial cell cultures stimulated with ApoE3. Another study also showed that mice carrying the human APOE ε4 gene had higher levels of serum pro-inflammatory markers, TNF-α and IL-6 compared with APOE ε3 counterparts (Lynch et al., 2003).

The current study also investigated the association between CNS inflammation and the APOE ε4 allele. MI was used as the CNS inflammatory marker. Hypothesis 9 predicted that participants, either homozygous or heterozygous for the APOE ε4 allele, would have higher levels of MI in the posterior cingulate gyrus compared with participants with other allelic variants. Linear regression analysis showed that the APOE ε4 allele was associated with increased levels of MI, but this was not significant. Kantarci et al. (2002) also found no significant effect of APOE ε4 on MI in the posterior cingulate gyrus in both control and AD participants. However, both this study and the current study were based on small sample sizes. Larger studies are needed to determine whether MI is a sensitive marker for detecting differences in glial activation between ε4 carriers and non-carriers. ^{1}H-MRS has the advantage of measuring *in vivo* glial activation in a non-invasive way.

Other studies investigating different inflammatory markers have found an association between the APOE genotype and inflammatory markers in the CNS (Egensperger et al., 1998; Lynch et al., 2003). In a post-mortem study by Egensperger et al. (1998), hippocampal and frontal cortical tissue derived from AD ε4 positive patients had an increased number of activated microglial cells compared with AD cortical tissue from ε3 carriers. This finding was independent of disease duration and age at death. Lynch and colleagues (2003) replicated the finding by Egensperger and colleagues (1998) in an animal model. Lynch et al. (2003) used an LPS model of induced inflammation in mice that expressed the human ε3 and ε4 genes. This study was performed to establish whether systemic and brain inflammatory responses were influenced by the APOE isoform. TNF-α levels in brain homogenates of APOE ε4 animals stimulated with LPS were higher compared with APOE ε3 animals. IL-6 was also significantly higher in APOE ε4 animals compared with APOE ε3 animals (Lynch et al., 2003).

As with cognition and ε4, one mechanism by which the APOE ε4 and inflammation may be related is via the decreased release of ApoE in ε4 carriers. ApoE4 production has been shown to be decreased compared with the ApoE3 and ApoE2 (Slooter et al., 1998; Sullivan et al., 2011). ApoE is thought to protect against bacterial infection. Rats injected subcutaneously with LPS in the absence of ApoE emulsion had higher pro-inflammatory cytokines TNF-α and IL-1 serum levels when compared with rats administered LPS with ApoE enriched emulsion. Rats administered LPS with no ApoE also had higher mortality

rates when compared with rats administered LPS with and ApoE. Therefore, ApoE decreases LPS induced production of pro-inflammatory cytokines by decreasing the release of these cytokines (van Oosten et al., 2001).

ApoE also increases plaque clearance (Thal et al., 2007). Increased release of ApoE in ε4 non-carriers may result in increased plaque clearance, decreasing plaque pathology. This may result in a decrease in plaque-induced inflammation. Similarly, the association between inflammation and ε4 may also be due to the increase in Aβ in AD ε4 positive patients (Tiraboschi et al., 2004; Fryer et al., 2005). Higher levels of Aβ may result in an increased inflammatory response. APOE ε4 has also been shown to decrease IL-1 receptor agonist, a cytokine that inhibits pro-inflammatory cytokines. This might lead to an unopposed pro-inflammatory effect (Egensperger et al., 1998).

The relationship between the APOE genotype and inflammation is important as it has been shown to affect treatment with NSAIDs. Szekely et al. (2008) examined NSAID use and the risk of dementia. They found that APOE ε4 carriers who took NSAIDs had a reduced risk of developing dementia compared with ε4 carriers who did not take NSAIDs. The reduction in risk was not evident in ε4 non-carriers taking NSAIDs. The risk not related to whether the person took non-$A\beta_{42}$ -lowering NSAIDs or $A\beta_{42}$ -lowering NSAIDs. $A\beta_{42}$ – lowering NSAIDS were classified according to evidence that the NSAID selectively lowered $A\beta_{42}$. These findings suggest that ε4 carriers might derive a greater benefit from taking anti-inflammatory drugs than non- ε4 carriers (Szekely et al., 2008).

In summary, the APOE ε4 was associated with lower plasma IL-10 levels at baseline in AD participants. This is in keeping with the current literature showing that ε4 carriers have a predominately pro-inflammatory profile (Lynch et al., 2003; Guo et al., 2004). However, in controls APOE ε4 was associated with higher IL-10 levels. One explanation for the current finding is that control ε4 carriers have higher Aβ and tau pathology compared with non-carriers. The increased pathology may activate the immune system leading to an early rise in anti-inflammatory molecules such as IL-10.

There was no significant effect of APOE genotype on the CNS inflammatory marker MI. This is contrary to other CNS evidence that CNS inflammatory markers are increased in ε4 carriers (Egensperger et al., 1998; Lynch et al., 2003). Larger studies are needed to investigate the effect of ε4 on MI. MI is a useful CNS inflammatory marker as it provides non-invasive measure of CNS inflammation, but it may not be sensitive enough to detect small differences.

APOE Genotype and Neuronal Integrity

NAA+NAAG is a marker of neuronal integrity and viability. In this study, NAA+NAAG was used to determine whether there is a difference in neuronal integrity between APOE ε4 carriers versus non-carriers. In the MRS study, Hypothesis 9 predicted that AD and control participants with the APOE ε4 allele would have lower levels of NAA+NAAG when compared with AD and control participants with no ε4 allele, respectively.

In the current study, linear regression analysis showed no significant association between the APOE ε4 allele and NAA+NAAG in the posterior cingulate gyrus in controls and AD participants. In other words, the presence of the APOE ε4 allele was not associated with decreased levels of NAA+NAAG. Kantarci et al. (2002) also found that control and AD ε4 carriers did not have lower levels of NAA in the posterior cingulate gyrus when compared with non-carriers. Similarly, a study investigating NAA in the hippocampus also found no difference in NAA levels between ε4 carriers and non-carriers in both the AD and control groups (Dixon et al., 2002).

These findings are in keeping with some anatomical and molecular studies. A study in community-dwelling mild AD participants found no association between APOE ε4 and hippocampal volumes (Walsh, Slater, Nair, & Attia, 2013). A post-mortem study used an ELISA technique to measure synaptic protein markers syntaxin 1, synaptophysin and PSD95 in the superior temporal cortex of AD patients. It found no association between ε4 and synaptic protein markers in AD patients (Love, Siew, Dawbarn, Wilcock, Ben-Shlomo, & Allen, 2006).

There are, however, some studies that have shown anatomical and metabolic changes in ε4 carriers versus non-carriers. Lehtovirta et al. (1995) showed that AD patients with the ε4/ε4 genotype had smaller right hippocampal volumes when compared with AD patients with other genotypes. Similarly, Tohgi et al. (1997) found reduced right hippocampal volumes in cognitively healthy controls who were carriers of the ε4 allele when compared with non-carriers. Although Love et al. (2006) did not find an association between APOE genotype and synaptic markers in AD patients, they did find an association between the synaptic density and APOE genotype in controls. APOE ε2 carriers had a higher synaptic density measure (an index that used the cumulative measure of syntaxin 1, synaptophysin & PSD95) compared with ε4 carriers whose synaptic density measure was low (Love et al., 2006). Kantarci et al. (2012) also showed that global cortical (which included the bilateral parietal cortex and posterior cingulate gyrus) retention of PiB increased from cognitively healthy APOE ε2 carriers to APOE ε3 carriers, with APOE ε4 carriers having the highest retention of PiB.

Metabolic changes in the brain have also been shown to be altered in the presence of the ε4 allele. In AD post-mortem brain tissue, there was an overall trend for increased metabolic abnormalities in APOE ε4 carriers compared with AD ε3 carriers. These metabolic changes included abnormalities in glycerophosphoethanolamine and phosphocholine (Klunk et al., 1998). A decrease in glucose activity in the parietal lobe, measured using PET imaging, was also found in ε4 carriers at risk of dementia compared to at-risk non-carriers. At-risk participants were defined as participants with at least two relatives with AD (Small et al., 1995). Decreased metabolic activity is suggestive of decreased neuronal activity. The evidence from these anatomical, synaptic density and metabolic studies suggests that there is increased neurodegeneration and decreased metabolic activity when cognitively healthy and AD ε4 carriers are compared with non-carriers.

The lack of association between ε4 and NAA+NAAG in the current study may be due to the small sample size that was used in the imaging section. Kantarci et al. (2002) also argued that the effect of APOE ε4 and neuronal integrity (measured by the NAA peak) might relate to different pathological features of the disease process and are therefore not directly related.

In summary, APOE ε4 was not associated with decreased NAA+NAAG levels in AD or control participants. There are conflicting reports on the association between APOE ε4 and anatomical and metabolic features.

Summary

In the current study, the APOE ε4 allele was associated with a detrimental effect on cognitive function in AD participants. The mechanisms by which ε4 might exert its detrimental effects may be through the decreased release of ApoE protein in AD ε4 patients (Soares et al., 2012). ApoE aids clearance of Aβ and plays a role in regeneration and repair (Slezak & Pfrieger, 2003). The ApoE4 isoform may also be less effective in the clearance of Aβ and in neuronal repair than the ε3 and ε2 isoform. The ε4 allele was also related to decreased levels of protective anti-inflammatory cytokine IL-10 in AD participants. This might explain the increased pro-inflammatory state found in AD ε4 carriers in other studies (Lynch et al., 2003; Guo et al., 2004).

In the control group, the ε4 allele was not associated with cognitive scores at baseline. At follow-up, the *absence* of the ε4 allele was associated with a greater decline on the CAMCOG. It could be argued that the reason the ε4 allele was not related to cognitive decline in the controls is that in the absence of Aβ and tau pathology, ε4 is beneficial in the South African population. The ε4 allele may also modify the relationship between Aβ and cognition (Kantarci et al., 2012). Therefore, in the absence of Aβ the detrimental effects of the APOE ε4 allele are not observed. An alternative reason may be that the study was underpowered and the neuropsychological tests used are not sensitive enough to detect small changes in a short follow-up period in cognitively healthy individuals. It is therefore proposed that a longer follow-up time is needed to explore the relationship between ε4 and cognition in cognitively healthy controls.

Systemic IL-10 was raised in control participants carrying the ε4 allele compared with non-ε4 carriers. A possible explanation for this is that the ε4 positive controls were at higher risk of developing MCI and AD (Soares et al., 2012). The increase in IL-10 may be an

inflammatory response to early Aβ and tau accumulation. Future work would be needed to further explore this relationship.

A clear understanding of the relationship between the ε4 allele, cognition and inflammation is important since it might explain the failure of current clinical trials using immuno-modulating therapies.

SUMMARY

An overall summary of the discussion is outlined in *Figure 45*. The figure depicts the inflammatory and neuronal changes that occur in the progression from cognitively healthy to the more advanced stages of AD. The effect of the APOE ε4 allele on cognitive functioning, inflammation and neuronal integrity is also included in the figure.

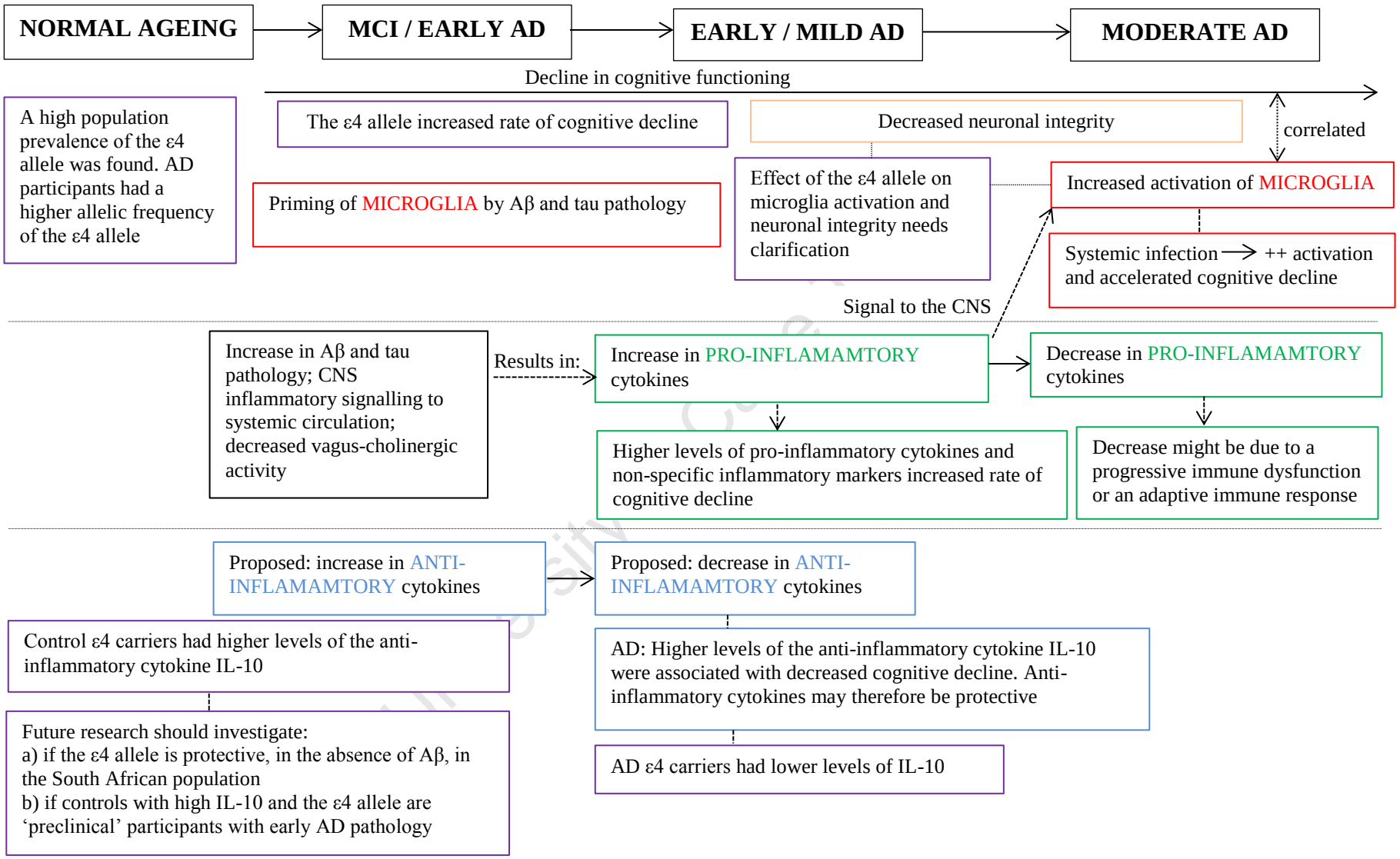


Figure 45: Summary of the Discussion. The figure describes the inflammatory, neuronal and cognitive changes that occur during the progression from normal ageing (left) to moderate AD (right). The role of the APOE ε4 genotype in these processes is also outlined.

LIMITATIONS AND RECOMMENDATIONS FOR FUTURE WORK

This study was conducted in a predominantly poor urban population from the greater Cape Town metropole. Various difficulties were encountered during its execution. Patients were often not easily contactable and transport difficulties meant that if patients arrived, they were usually late. The notion of participating in a research study, with no direct beneficial effect to the patient, was a difficult concept for many. The lack of benefit of treatment had to be emphasised and re-emphasised at the initial consent procedure and subsequent study visits. Therefore, there were many limitations in the current study. The following section will outline a number of these limitations. Recommendations for future research, that might address these limitations, will also be discussed.

A limitation in AD research in general, is the confidence with which the diagnosis of AD can be made clinically. A definitive diagnosis of AD is only possible following histopathological examination of brain tissue. The positive predictive value of a "probable" AD diagnosis is around 90 – 95% compared with CERAD histopathological evidence. The specificity for a "possible" AD diagnosis is around 50 – 60% (Hogervorst et al., 2000). This study included both possible and probable AD participants, many of whom had vascular risk factors. The composition of the AD group was therefore likely to include both definite AD participants and those with mixed AD and vascular dementia.

The second limitation in the current research is the small sample size at baseline and at follow-up. AD in the underprivileged communities is poorly understood in South Africa. AD, like other mental illnesses, also remains a stigmatised condition (Ineichen, 2000). Families and caregivers therefore rarely seek help. Alternatively, older adults with dementia are taken to see traditional healers. Traditional healers in South Africa incorporate psychological, spiritual and some medical elements into their practice (Stafforda, Pedersenb, van Stadena, & Jäger, 2008). Therefore, patients with early AD rarely enter the health care systems where the recruitment of participants for the study took place. Inadequate access to health care settings also means that if/when patients do visit clinics, they usually present with other illnesses and have advanced AD with behavioural symptoms.

The sample size for the MRS study was also small. This is accounted for by the fact that some AD participants were unable to tolerate the full duration of the 20-minute period in the scanner, as well as the cost involved in scanning participants. An alternative to direct brain imaging would be the analysis of inflammatory markers in the CSF. Ethical approval for the collection of CSF for research purposes was not granted for this study and consequently could not be used in aiding the characterization of the CNS inflammatory markers. A larger longitudinal study investigating the use of ^{1}H-MRS may be useful in our communities.

Increasing awareness of early symptoms of AD and educating communities about dementia will encourage families to seek help early in the disease process. Monitoring and longitudinal follow-up of MCI and AD patients may then be facilitated.

The third limitation in the current study is that MCI participants were not investigated. MCI is considered the transitional state between cognitively healthy ageing and AD. The larger study, in which this study is nestled, aimed to recruit MCI participants. However, over the 5-year study period, very few participants were identified as having MCI. Again, this highlights the lack of awareness when seeking medical attention for early cognitive complaints. Population screening may be a way of identifying MCI participants. Inclusion of an MCI group will help identify the factors that contribute to the conversion from MCI to AD in our population and enable us to study systemic and CNS inflammatory markers in the transitional state between healthy ageing and AD.

Another limitation in the current study relates to medication use and systemic inflammatory markers. A full list of medications taken by all participants was obtained. Participants were not excluded for taking NSAIDs. Unfortunately, due to the large number of older adults on NSAIDs (especially low dose aspirin) the exclusion of participants on NSAIDs was not possible. The use of NSAIDs may alter the levels of systemic inflammatory markers thereby skewing inflammatory data analysis relating to disease stage.

Participants with clinically overt systemic inflammatory conditions, infections and participants with an ESR greater than 40mm per hour were excluded. However, certainty that participants did not have some form of subclinical systemic infection or inflammatory condition at the time blood was taken was not possible. This again may have influenced the inflammatory marker analysis.

Multiple acute systemic infections, over a number of years, have been shown to increase the likelihood of dementia in older adults (Dunn, Mullee, Perry, & Holmes, 2005). Acute infection has also been associated with a decline in cognitive functioning in AD patients (Holmes et al., 2009). These factors are therefore especially important in the South African context where poverty and poor access to primary health care facilities result in high rates of infection. Therefore, participants with these conditions are *extremely* relevant in AD research and should be incorporated into research.

The fifth limitation is the exclusion of depressed participants from the study. Depressive symptoms have been shown to be associated with abnormal memory performance (Basso & Bornstein, 1999). Depression is generally regarded as an important differential diagnosis to consider in someone suspected of having dementia. Most research studies therefore exclude participants with depression. However, because one of the symptoms of AD might include depression, excluding participants with cognitive complaints and depression limits the sample size. The prevalence of depression in dementia has been shown to range from 11% to 86% (Burns, 1991). Lyketsos et al. (1997) showed that 27% of AD participants suffered from minor depressive episodes. Depressive symptoms have also been shown to increase with increasing cognitive impairment (Ross, Arnsberger, & Fox, 1998). Future studies need to address the problem of excluding participants with depression while making sure that lower scores on cognitive tests are not due to depressive symptoms. Perhaps closer monitoring and shorter follow-up intervals of participants with depressive symptoms will help identify those who are declining from their baseline cognitive scores; a decline in cognitive scores may aid the diagnosis of possible AD.

The sixth limitation addresses cognitive decline measurements in the study. The neuropsychological tests applied in the current study have floor and ceiling effects. Floor and ceiling effects might underestimate the rate of decline in some participants. Non-linear

modelling of cognitive decline accounts for the floor and ceiling effects better than linear models used in the current studies analysis (Martins et al., 2005). The length of time that participants were followed-up was also short, cognitive decline was measured over one year. As described, decline in AD does not follow a linear trend (Martins et al., 2005). Therefore, patients at the early stages of AD decline more slowly over one year than patients in the middle stages of the disease process. Interpretation of cognitive decline results, when comparing AD participants who are at different stages of the disease process, needs to be done with caution. Examining factors that contribute to decline when the AD group is made up of individuals at different stages of the disease may yield results that can be difficult to interpret. Individual variability in cognitive decline also poses substantial difficulties when comparing AD patients (Plassman & Breitner, 1996).

Ideally, longitudinal cohort studies of longer duration together with much larger numbers of participants ought to be conducted so that participants can be followed-up from cognitively healthy to advanced stages of AD. Longitudinal studies should also include multiple inflammatory measures together with yearly MI and NAA+NAAG measures. The current study on inflammatory profiles is cross-sectional and therefore the change in the inflammatory profile could only be inferred from differences in controls, mild and moderate stages of AD. Longitudinal cohort studies will enable investigators to examine the inflammatory profile, neurometabolite changes, effect of APOE genotype and factors contributing to cognitive decline at the different stages of AD within individuals. However, such studies are also expensive and require numbers of staff in a resource-poor setting, such as South Africa.

The current study shows that the research into the relationship between systemic inflammation, CNS inflammation, neuronal integrity, the APOE genotype and cognitive functioning is worth pursuing in South Africa. Longitudinal studies will help clarify the nature of the inflammatory profile through the different stages of the disease process. Also, paired comparisons with repeated measures in individuals would then be possible, increasing statistical significance of the results.

CONCLUSION

This is, to my knowledge, the first study to investigate the relationship between systemic inflammation, CNS inflammation, APOE genotype, neuronal integrity and cognitive functioning in a sample of AD and cognitively healthy older South Africans. It was undertaken in a predominately poor urban population under sub-optimal conditions compared with studies performed in industrialised countries. Nevertheless, the results from the study provide an interesting framework for further investigations.

This study showed that the inflammatory response in AD changes with disease progression. I proposed that in the MCI or early stages of AD, there might be a predominantly anti-inflammatory response. During this phase inflammation may be protective as higher levels of anti-inflammatory cytokines were associated with slower cognitive decline. This finding may be important as promotion of this early protective response might limit the progression of the disease. The anti-inflammatory phase is then followed by one which is predominantly pro-inflammatory. An increase in systemic IL-1β in participants with early AD was found in the current study. This study also showed a rise in peripheral blood ESR in early AD. Higher levels of pro-inflammatory markers at baseline were associated with greater decline in cognition. The increase in pro-inflammatory cytokines in the systemic circulation might drive the activation of primed microglia, leading to the increased microglial activation seen in my study in the moderately advanced stages of AD.

An association between the APOE ε4 allele and AD was also found in this study. The results confirmed the relationship found in developed countries between the presence of the ε4 allele and increased cognitive decline in AD (Henderson et al., 1995; Petersen et al., 1995; Martin et al., 2005). This is also, to my knowledge, the first study in South Africa to show an association between the ε4 allele and lower levels of the anti-inflammatory cytokine, IL-10, in AD. The relationship between the ε4 allele, systemic inflammation and CNS inflammation is especially important in a South African context where rates of systemic infections and the prevalence of the ε4 allele are both high. People infected with HIV are also living longer and there may soon be a collision between the HIV epidemic

and the age-associated neurodegenerative diseases. Thus a clearer understanding of the association between systemic inflammation, CNS inflammation and neurodegeneration is extremely important. My study has been the first to examine the interrelationships between various inflammatory and genetic factors in the pathogenesis of AD in a South African population. Data from this study may be extrapolated to other conditions of systemic infection and inflammation. In doing so it may contribute to the limited knowledge base concerning the complex interrelationships of AD with other diseases prevalent in South Africa.

Over the past few years the failure of many phase II and phase III trials of disease modifying drugs has offered little hope to AD sufferers and their families. The greatest benefit from treatment may come from preventing the chain of events that lead to neurodegeneration and cognitive decline. Amongst these interventions the prevention and prompt treatment of infections in older people may turn out to be very important. This may limit systemic inflammation and therefore reduce neuroinflammation and its consequent cognitive decline. Characterising inflammation at the different stages of disease, identifying reliable biomarkers and understanding how the APOE ε4 allele exerts its effects may also lead to better designed clinical trials that are appropriately timed.

Alois Alzheimer first described the clinico-pathological entity of AD over one hundred years ago. It continues to be one of the most debilitating health conditions especially of ageing populations. The continued lack of research in South Africa leads to poor public awareness and education. Poor public awareness increases fears and stigma, leading to further social isolation of patients suffering from AD, their carers and their families. Further research is also needed to determine the true prevalence of the disease in the country and to further elucidate the mechanisms of the disease process. Understanding AD in a South African context will help raise awareness and create a positive framework for carers, health care workers and the public health services to work together in order to alleviate some of the burden of disease.

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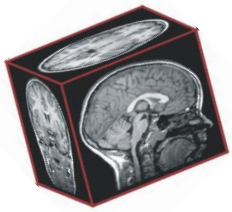
APPENDIX A



Cape Universities Brain Imaging Centre (CUBIC)

MRI Volunteer Screening Form

Volunteer Information:

Name	Contact number
Date of Birth	Project name
Weight	Principle investigator

The following information is very important to ensure your safety and to prevent any interference during the MR procedure.

Please answer the following questions (mark with an X):	**Yes**	**No**	**Don't know**
Pacemaker			
Aneurism clips			
Artificial heart valve			
Vena cava filter			
Prosthesis (e.g. eye, breast etc)			
Shrapnel in eye or body			
Neurostimulator			
Cochlear implant (ear) or hearing aid			
? Diabetic			
? Renal impairment			
? Asthma			
? Allergies			
? Any other implants (e.g. screws, plates, joint replacements)			
? Pregnant			
? Previous MRI investigation with intravenous contrast			
Is there any other device implanted or are there any other ailments that you think that we should be aware of?			

I hereby acknowledge that the potential risks of the examination have been explained to me and that during the course of the investigation it may be necessary for the intravenous injection of a contrast agent.

Attention: It is the policy of this institution not to discuss results of the MR investigation with the patients for ethical reasons. All enquiries in this regard should be directed to the referring physician.

Signature: **Date:**

APPENDIX B

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT: Risk factors for the development of cognitive impairment in the elderly

PROTOCOL NUMBER:

PRINCIPAL INVESTIGATOR: Dr Marc I Combrinck

ADDRESS: Divisions of Neurology and Geriatric Medicine, Department of Medicine, E7 Room 63, Groote Schuur Hospital, Private Bag X3, Observatory, Cape 7935

CONTACT NUMBER: +27 21 404 3198 or 404 3120

We are inviting you to participate in a research project. Please take some time to read the information presented here. It explains the details of the project. If there are any aspects of the project you do not understand, please do not hesitate to ask the study staff or doctor. It is important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Your participation in the study is entirely voluntary and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. It will not affect any future medical treatment you may need. You are also free to withdraw from the study at any point, even if you did initially agree to take part. You do not have to give a reason for withdrawing.

This study has been approved by the Research Ethics Committee of the Faculty of Health Sciences of the University of Cape Town. It will be conducted according to the ethical guidelines and principles of the International Declaration of Helsinki, the South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

This trial is being run by the Division of Neurology and the Institute of Ageing in Africa in the Department of Medicine at the University of Cape Town. We aim to recruit a total of 150 participants over a period of 3 years.

What is this research study all about?

Some people develop memory problems as they get older. Many elderly people have mild memory difficulties. However, in a few, the problem may be more severe. We are interested in finding out more about what causes the difficulties with memory and other higher brain functions. In order to do so, we should like to investigate these possible causes using a number of methods. These include blood tests, questions you would need to answer about yourself, tests of memory and other higher brain functions, a CT scan of the brain, and tests of your saliva. In some instances we may, with your special permission, perform tests on the fluid covering your brain and spine by doing a lumbar puncture. We shall also ask some of you to undergo a special scan known as an MRI at our research facility at Tygerberg Hospital.

We are interested in testing people both with memory difficulties and those without, so that we can compare the two groups. In this way we might be able to identify causes of memory impairment. Our research findings may help to prevent, or to better diagnose and treat, these conditions in the future.

Procedures

If you agree to take part in the study and you meet all the conditions required to enter the study, you will be invited to visit our clinic on two separate days. At these visits to our clinic we shall:

(1) take a detailed history.
(2) interview your relative/friend (someone who knows you well) to find out whether he/she thinks you have any memory difficulties.

(3) perform a full physical examination. This will include measuring your blood pressure, listening to your heart and chest, examining the nervous system and testing your urine.

(4) take approximately 36 ml (7 tablespoons) of blood to look for possible causes of memory impairment. These will include:

(i) a blood sugar (glucose) (for diabetes)
(ii) a blood cholesterol level
(iii) tests of liver, kidney and thyroid gland function
(iv) haemoglobin, blood cell counts
(v) tests for vitamins or vitamin deficiences (vitamin B-12 and homocysteine)
(vi) a test for syphilis. (This test is routinely performed in hospital clinics because syphilis used to be a common cause of impairment of higher brain function in the past)
(vii) a sample to measure inflammation in the blood (cytokines)

(viii) one sample will be used to prepare DNA from your blood. We shall test this for a gene called apolipoprotein E (APOE). Some forms of this gene may be associated with a higher risk of developing memory problems. However, this test does not diagnose that you have, or will necessarily develop, memory problems. We are doing it purely for research; it may help us to better understand some causes of memory difficulties.

(ix) A test for a chemical in the blood known as clusterin. This chemical may be increased in people with memory difficulties.

(5) We shall ask you to complete questionnaires about yourself and we shall perform tests of your memory and other higher mental functions. These will be conducted in a quiet, relaxed atmosphere. We expect that these tests will be of about two hours' duration. However, there will be opportunities to rest in-between tests.

(6) We shall perform a CT scan of the brain. This is a special X-ray photograph that will show us the structure of your brain. It can detect lumps or masses inside the head and it might also give us some idea about brain shrinkage. It will also show any obvious strokes that you might have had. If you like, we shall show you the pictures at the second visit.

(7) In some instances we may wish to perform a lumbar puncture to obtain a sample of the fluid (the cerebrospinal fluid or CSF) that surrounds the brain and the spinal cord. We shall only perform this test with your special permission and if we think the test will help diagnose and treat the cause of your memory problems. We shall send the sample to the laboratory to test for infection or inflammation. We shall also store a sample in the freezer to be analysed at a later stage for chemicals related to inflammation (cytokines).

If we think a lumbar puncture is necessary and if you agree, the test will be performed in our Neurology Ward at the Groote Schuur Hospital. You will need to rest in bed, lying flat, for a couple of hours afterwards. The test is usually well tolerated, especially in elderly people, and there are usually no side-effects. Occasionally some people develop headaches after the test, often a day or two later. We generally advise people to have a restful two days after the procedure and to drink plenty of fluids. Should headaches occur, they are usually relieved by lying down flat and taking simple pain-killers like paracetamol. Very rarely, some people (usually young adults) develop quite bad headaches and for this we may need to re-admit you to our hospital ward for pain relief. We shall telephone all participants a day or two after the lumbar puncture to make sure they are okay.

(8) We shall measure the levels of a stress hormone, cortisol, in your saliva ("spit"). You or your relative will be asked to swab a sample of your saliva on three consecutive mornings at 09h00 a.m. We shall provide the swabs and the containers as well as clear instructions about how to collect and store the samples. The samples need to be kept refrigerated and one of the staff of our study group will collect them from you.

If we find any abnormal results that we think will need further treatment, we shall contact you. With your permission, we shall refer you to to the appropriate health services. For example, we may diagnose hypertension or diabetes mellitus for the first time. If we find that you or your relative/friend has a significant

memory problem that is interfering with your daily living activities, we shall refer you to a Memory Clinic. Your permission will always be sought first.

(9) Some participants in the study will be asked to undergo a special type of brain scan which will be performed at the Cape Universities Brain Imaging Centre (CUBIC) at Tygerberg Hospital. This scan is called an MRI (magnetic resonance imaging) scan. It provides a detailed picture of the brain including the parts involved in memory. We are also able to measure chemicals in parts of the brain using what's known as magnetic resonance spectroscopy (MRS).

We shall provide transport for you to the facility at Tygerberg Hospital where necessary. On arrival we shall ask you and your relative questions regarding the following: whether you have had a cardiac pacemaker, a neuro-stimulator, previous eye injuries or a foreign body, previous neurosurgery, previous other surgery involving metal implants, a cochlear implant, whether you have been a welder or metal grinder and whether you are claustrophobic or not. The scan will require you to lie on your back on a table that will move into the scanning machine for the 30 to 40 minutes it will take for the scan to be completed.

During this time you will be able to close your eyes and rest. You will also be able to talk to the study doctor/assistant at all times during the scan should you experience any discomfort. The scan is a safe procedure if you have been screened correctly for the presence of any magnetic material on or inside your body. As the scan is done in a relatively confined space, occasionally some people become anxious. This does not happen often, and if you feel anxious beforehand, we will spend time allowing you to get used to the surroundings before we begin. When the magnet in the machine is switched on, it will make some loud banging noises, but you will be clearly warned when this will take place. At this time you will feel nothing and the noise is not harmful to you in any way. To minimise the possible discomfort associated with this, we will give you some soft earplugs and will also put earphones on so that you can listen to music if you so choose.

After the two baseline visits and the three collectins of saliva, we would like to re-assess your memory functions again at one, two and three years later, respectively, provided you continue to consent to participation in the study.

What will your responsibilities be?
You will be required to attend the study visit at the appropriate time and to participate as fully as you can with the tests and questionnaires. You should answer the questions as fully and honestly as you can. If there are any questions that you cannot, or do not wish to answer, you should tell us so.
Will you benefit from taking part in this study?
You will receive little direct benefit from the study.
However, you will undergo a thorough medical check-up as part of the research protocol. As previously indicated, we shall, with your permission, refer you to the appropriate medical services if any treatable abnormalities are found.
Are there any risks in your taking part in this research?

You may feel uncomfortable about answering some of the questions about yourself or your friend/relative. Some people don't like talking, or knowing about, problems related to memory or thinking. You should feel free to mention your feelings or concerns to any member of the study team.

If you do not agree to take part, what alternatives do you have?
You are free not to participate in the study or to refuse parts of the study.

Who will have access to your medical records?
The information collected about you will be treated as confidential and protected. If it is used in a publication or thesis, your identity will remain anonymous. Only the direct study team will have full access to the information. If we need to refer you to a clinic for treatment, we will provide them with the relevant information needed to treat your condition.
Will you be paid to take part in this study and are there any costs involved?

You will not be paid to take part in the study but your transport costs will be covered for the study visit. You will be re-imbursed for the sum of R150-00. There will be no costs involved for you, if you do take part.

Is there anything else that you should know or do?

- **You should inform your family practitioner or usual doctor that you are taking part in a research study.**
- **You can contact Dr Marc Combrinck at tel 021-4043198 or 404 3120 if you have any further queries or encounter any problems.**
- **You can contact the Research Ethics Committee of the Health Sciences Faculty of the University of Cape Town 021-4066338 if you have any concerns or complaints that have not been adequately addressed by your study doctor.**
- **You will receive a copy of this information and consent form for your own records.**

Declaration by participant and/or friend/relative/guardian
By signing below, I ……..……., hereby agree to take part in the research study entitled: "Risk Factors for Cognitive Impairment in the Elderly"

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2012.

.....

Signature of participant Signature of witness

.....

Signature of relative/friend/guardian Signature of witnes

Declaration by investigator

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below).*

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2012.

Signature of investigator Signature of witness

Declaration by interpreter

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) ……..…….. using the language medium of Afrikaans / Xhosa *(delete whichever is not applicable).*
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....….....…….. on (*date*) ……...…….. 2012.

..... **.....**

Signature of interpreter Signature of witness

APPENDIX C

Deterioration Cognitive Observee (DECO)

We would like you to tell us how your relative was a year ago. The following questions ask about a number of everyday situations. We would like you to tell us whether in these situations he/she is doing about the same, not as well or much worse, than a year ago. Place a tick in the relevant column to show your response.

	Better or about the same	Not as well	Much worse
1. Does he/she remember as well as before which day of the week and which month it is?			
2. When he/she goes out of the house, does he/she know her way as well as before?			
3. Have there been changes in his/her ability to remember his/her own address or telephone number			
4. In the house, does he/she remember as well as before where things are usually kept?			
5. And when an object isn't in its usual place, is he/she capable of finding it again?			
6. In comparison with a year ago, how well is he/she able to use household appliances (washing machine, etc….)?			
7. Has his/her ability to dress or undress changed at all?			
8. How well does he/she manage his/her money, for example, doing the shopping?			
9. Apart from difficulties due to physical problems, has there been a reduction in his/her activity level?			
10. How well can he/she follow a story on television, in a book or told by someone?			
11. And writing letters for business or to friends; does he/she do this as well as a year ago?			
12. How well does he/she recall a conversation you had with him/her a few days ago? Has this changed over the past year?			
13. And if you remind him/her of this conversation, does he/she still have difficulty remembering it in comparison to a year ago?			
14. Does he/she forget what he/she wanted to say in the middle of a conversation? Has this changed over the past year?			
15. In a conversation, does he/she sometimes have difficulty finding the right word?			
16. In comparison with a year ago, how well does he/she recognize the faces of people he/she knows well?			
17. And how well does he/she remember the names of these people?			
18. In comparison with a year ago, how well does he/she remember other details concerning people he/she knows well; where they live, what they do?			
19. Over the past year, have there been changes in his/her ability to remember what has happened recently?			

APPENDIX D

The Bristol Activities of Daily Living Scale (BADLS)

This questionnaire is designed to reveal the everyday ability of people who have memory difficulties of one form or another. For each activity (Nos. 1-20), statements a-e refer to a different level of ability. Thinking of the **last 2 weeks**, tick the box that represents your relative's/friend's ability.

Only 1 box should be ticked for each activity.

(If in doubt about which box to tick, choose the level of ability which represents their average performance over the last 2 weeks).

1. FOOD

a. Selects and prepares food as required **[ ]**
b. Able to prepare food if ingredients set out **[ ]**
c. Can prepare food if prompted step by step **[ ]**
d. Unable to prepare food even with prompting and supervision **[ ]**
e. Not applicable **[ ]**

2. EATING

a. Eats appropriately using correct cutlery **[ ]**
b. Eats appropriately if food made manageable and/or uses spoon **[ ]**
c. Uses fingers to eat food **[ ]**
d. Needs to be fed **[ ]**
e. Not applicable **[ ]**

3. DRINK

a. Selects and prepares drinks as required **[ ]**
b. Can prepare drinks if ingredients left available **[ ]**
c. Can prepare drinks if prompted step by step **[ ]**
d. Unable to make a drink even with prompting and supervision **[ ]**
e. Not applicable **[ ]**

4. DRINKING

a. Drinks appropriately **[ ]**
b. Drinks appropriately with aids, beaker/straw etc. **[ ]**
c. Does not drink appropriately even with aids but attempts to **[ ]**
d. Has to have drinks administered (fed) **[ ]**
e. Not applicable **[ ]**

5. DRESSING

a. Selects appropriate clothing and dresses self **[ ]**
b. Puts clothes on in wrong order or back to front or dirty clothing **[ ]**
c. Unable to dress self but moves limbs to assist **[ ]**

d. Unable to assist and requires total dressing [ ]
e. Not applicable [ ]

6. HYGIENE

a. Washes regularly and independently [ ]
b. Can wash self if given soap, flannel, towel, [ ]
c. Can wash self if prompted and supervised [ ]
d. Unable to wash self and needs full assistance [ ]
e. Not applicable [ ]

7. TEETH

a. Cleans own teeth/dentures regularly and independently [ ]
b. Cleans teeth/dentures if given appropriate items [ ]
c. Requires some assistance, toothpaste on brush, brush to mouth, etc. [ ]
d. Full assistance given [ ]
e. Not applicable [ ]

8. BATH/SHOWER

a. Bathes regularly and independently [ ]
b. Needs bath to be drawn/shower turned on but washes independently [ ]
c. Needs supervision and prompting to wash [ ]
d. Totally dependent, needs full assistance [ ]
e. Not applicable [ ]

9. TOILET/COMMODE

a. Uses toilet appropriately when required [ ]
b. Needs to be taken to the toilet and given assistance [ ]
c. Incontinent of urine or faeces [ ]
d. Incontinent of urine and faeces [ ]
e. Not applicable [ ]

10. TRANSFERS

a. Can get in/out of chair unaided [ ]
b. Can get into a chair but needs help to get out [ ]
c. Needs help getting in and out of a chair [ ]
d. Totally dependent on being put into and lifted from chair [ ]
e. Not applicable [ ]

11. MOBILITY

a. Walks independently [ ]
b. Walks with assistance, i.e. furniture, arm for support [ ]
c. Uses aids to mobilize, i.e. frame, sticks etc. [ ]
d. Unable to walk [ ]
e. Not applicable [ ]

12. ORIENTATION—TIME

a. Fully orientated to time/day/date etc. [ ]
b. Unaware of time/day etc but seems unconcerned [ ]
c. Repeatedly asks the time/day/date [ ]
d. Mixes up night and day [ ]
e. Not applicable [ ]

13. ORIENTATION—SPACE

a. Fully orientated to surroundings [ ]
b. Orientated to familiar surroundings only [ ]
c. Gets lost in home, needs reminding where bathroom is, etc. [ ]
d. Does not recognize home as own and attempts to leave [ ]
e. Not applicable [ ]

14. COMMUNICATION

a. Able to hold appropriate conversation [ ]
b. Shows understanding and attempts to respond verbally with gestures [ ]
c. Can make self understood but difficulty understanding others [ ]
d. Does not respond to or communicate with others [ ]
e. Not applicable [ ]

15. TELEPHONE

a. Uses telephone appropriately, including obtaining correct number [ ]
b. Uses telephone if number given verbally/visually or predialled [ ]
c. Answers telephone but does not make calls [ ]
d. Unable/unwilling to use telephone at all [ ]
e. Not applicable [ ]

16. HOUSEWORK/GARDENING

a. Able to do housework/gardening to previous standard [ ]
b. Able to do housework/gardening but not to previous standard [ ]
c. Limited participation even with a lot of supervision [ ]
d. Unwilling/unable to participate in previous activities [ ]
e. Not applicable [ ]

17. SHOPPING

a. Shops to previous standard [ ]
b. Only able to shop for 1 or 2 items with or without a list [ ]
c. Unable to shop alone, but participates when accompanied [ ]
d. Unable to participate in shopping even when accompanied [ ]
e. Not applicable [ ]

18. FINANCES

a. Responsible for own finances at previous level [ ]
b. Unable to write cheque but can sign name and recognizes money values [ ]
c. Can sign name but unable to recognize money values [ ]
d. Unable to sign name or recognize money values [ ]
e. Not applicable [ ]

19. GAMES/HOBBIES

a. Participates in pastimes/activities to previous standard [ ]
b. Participates but needs instruction/supervision [ ]
c. Reluctant to join in, very slow, needs coaxing [ ]
d. No longer able or willing to join in [ ]
e. Not applicable [ ]

20. TRANSPORT

a. Able to drive, cycle or use public transport independently [ ]
b. Unable to drive but uses public transport or bike etc [ ]
c. Unable to use public transport alone [ ]
d. Unable/unwilling to use transport even when accompanied [ ]
e. Not applicable [ ]

Thank you for taking the time to complete this questionnaire.

APPENDIX E

Geriatric Depression Scale (GDS)

Choose the best answer for how you have felt over the past week:

1. Are you basically satisfied with your life? YES / NO

2. Have you dropped many of your activities and interests? YES / NO

3. Do you feel that your life is empty? YES / NO

4. Do you often get bored? YES / NO

5. Are you in good spirits most of the time? YES / NO

6. Are you afraid that something bad is going to happen to you? YES / NO

7. Do you feel happy most of the time? YES / NO

8. Do you often feel helpless? YES / NO

9. Do you prefer to stay at home, rather than going out and doing new things? YES / NO

10. Do you feel you have more problems with memory than most? YES / NO

11. Do you think it is wonderful to be alive now? YES / NO

12. Do you feel pretty worthless the way you are now? YES / NO

13. Do you feel full of energy? YES / NO

14. Do you feel that your situation is hopeless? YES / NO

15. Do you think that most people are better off than you are? YES / NO

APPENDIX F

Bio-Plex Pro Assay (Biorad), manufactures booklet: Pages 4 and 5.

NEW MAGNETIC Assays! Now includes instructions for:
Transforming Growth Factor Assays (TGF-β) – TGF-β1, TGF-β2, TGF-β3
Rat Cytokine Assays – Expanded and Improved
Mouse Cytokine Assays – Key Th17 pathway markers

Bio-Plex Pro™ Assays

Cytokine, Chemokine, and Growth Factors Instruction Manual

For technical support, call your local Bio-Rad office, or in the U.S., call 1-800-424-6723. For research use only. Not for diagnostic procedures.

BIO-RAD

Assay Quick Guide

For experienced users, the following guide can be used to prepare and run a single 96-well assay plate. For more information on a given step, refer to the detailed instructions in the corresponding section of the manual

Table 1. Assay quick guide.

Refer to Section	Initial Preparation
3	1. Plan the plate layout
1	2. Start up/warm up the Bio-Plex system (up to 30 min) • Meanwhile, equilibrate assay reagents to room temperature (RT) • Begin to thaw samples
2	3. Prime wash station or calibrate vacuum manifold
1	4. Calibrate the system (now, or later during an incubation)
4	5. Reconstitute a single vial of standards in 500 µl of the appropriate diluent, vortex and incubate on ice (30 min) • For serum and plasma samples, use Bio-Plex standard diluent* • For culture supernatant, use culture medium** • For lavage, sputum, or other fluid, use buffer similar to sample**
	6. Prepare the 8 point standard dilution series and blank. • Add 72 µl diluent to tube S1, and 150 µl diluent to tubes S2-8 and blank. • Transfer 128 µl reconstituted standard into S1 • Then serially dilute 4 fold from S1 thru S8 by transferring 50 µl between tubes. Vortex between transfers
5	7. Once thawed, prepare 1x samples • Activate TGF-β samples by adding 1 volume of 1 N HCl to 5 volumes of sample (eg, 5 µl to 25 µl of sample). Vortex, incubate at RT 10 min. Neutralize by adding the same volume (5 µl) • Dilute serum, plasma and lysates in Bio-Plex sample diluent • Dilute culture supernatants in culture medium** • Dilute lavage, sputum, or other fluid in buffer similar to sample**
6	8. Prepare 1x coupled beads in assay buffer, protect from light • From 10x stock: Add 575 µl beads to 5,175 µl buffer • From 20x stock: Add 288 µl beads to 5,472 µl buffer
	9. Make sure samples and standards are at RT before dispensing

* Refer to section 4 for instructions on preparing an appropriate standard diluent for TGF-β assays when running serum or plasma samples.

** Make sure to add protein carrier such as BSA to a final concentration of 0.5% to lavage, sputum, and serum free culture medium samples as described in section 5. Samples and standards should be reconstituted and/ or diluted in this same medium.

Table 1. Assay quick guide, con't.

Refer to Section	Running the Assay
7	1. Prewet filter plate with 100 µl assay buffer (skip for flat bottom)
	2. Add 50 µl of 1x beads to the assay plate
	3. Wash 2 times with 100 µl wash buffer
	4. Add 50 µl samples, standards, blank, controls
	5. Cover and incubate in the dark at RT with shaking at 300 RPM • 30 min - Human Group I,II and Mouse Group I,II • 1 hour - Mouse Group III and Rat Group I • 2 hours – TGF-β With 10 min remaining, prepare 1x Detection Ab in detection antibody diluent. • From 10x stock: Add 300 µl Ab to 2,700 µl diluent • From 20x stock: Add 150 µl Ab to 2,850 µl diluent
	6. Wash 3 times with 100 µl wash buffer
	7. Add 25 µl of detection antibody
	8. Cover and incubate in the dark at RT with shaking at 300 RPM • 30 min - Human Group I,II; Mouse Group I,II,III; and Rat Group I • 1 hour - TGF-β
8	Meanwhile, prepare software protocol; enter normalized standard S1 values
7	With 10 min remaining, prepare 1x SA-PE in assay buffer, From 100x stock: Add 60 µl SA-PE to 5,940 µl assay buffer. Protect from light
	9. Wash 3 times with 100 µl wash buffer
	10. Add 50 µl of strepavidin-PE
	11. Cover and incubate in the dark at RT with shaking at 300 RPM • 10 min - Human Group I,II; Mouse Group I,II,III; and Rat Group I • 30 min - TGF-β
	12. Wash 3 times with 100 µl wash buffer
8	13. Resuspend beads in 125 µl assay buffer, shake at 1100 RPM for 30 sec
	14. Read plate • Low PMT (Low RP1) – Human group I,II; Mouse group I,II,III and TGF-β • High PMT (High RP1) - Rat group I

APPENDIX G

Luminex, TGF-β Mulitplex (R&D), manufactures booklet: Pages: 6-9.

Luminex® Performance Assay

TGF-β Multiplex Kit

Catalog Number LKT001

For the simultaneous quantitative determination of transforming growth factor beta 1, 2, and 3 (TGF-β1, TGF-β2, and TGF-β3) concentrations in cell culture supernates, serum, plasma, urine, and human milk.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

ACTIVATION/NEUTRALIZATION REAGENT PREPARATION

The following solutions may be stored at room temperature for up to one month in polypropylene bottles.

Caution: *Wear protective clothing and safety glasses during preparation or use of these reagents. Refer to the appropriate MSDS prior to use.*

1N HCl (100 mL) - Slowly add 8.33 mL of 12N HCl to 91.67 mL of deionized water. Mix well.

1.2N NaOH/0.5M HEPES (100 mL) - Slowly add 12 mL of 10N NaOH to 75 mL of deionized water. Mix well, and add 11.9 g of HEPES. Mix well, and bring the final volume to 100 mL with deionized water.

SAMPLE ACTIVATION PROCEDURE

To activate latent TGF-β to immunoreactive TGF-β detectable in this assay, follow the activation procedure outlined below for all sample types. Assay samples immediately after neutralization (pH 7.2-7.6). **Use polypropylene test tubes. Do not activate the TGF-β Standard Cocktail.**

1. If samples contain particulates, centrifuge for 2 minutes at 5000 x g before proceeding.
2. Add 20 µL of 1N HCl to 100 µL of sample. Mix well, and incubate for 10 minutes at room temperature.
3. Add 20 µL of 1.2N NaOH/0.5M HEPES. Mix thoroughly.

SAMPLE PREPARATION

Activated cell culture supernates and activated urine samples require a 1:3.57 dilution in Calibrator Diluent RD5-49 after activation. This is a 1:5 final dilution factor. A suggested 1:3.57 dilution is 112 µL of activated/neutralized sample + 288 µL of Calibrator Diluent RD5-49. Mix thoroughly.

Activated serum, plasma, and human milk samples require a 1:10.70 dilution in Calibrator Diluent RD6-50 after activation. This is a 1:15 final dilution factor. A suggested 1:10.70 dilution is 50 µL of activated/neutralized sample + 485 µL of Calibrator Diluent RD6-50. Mix thoroughly.

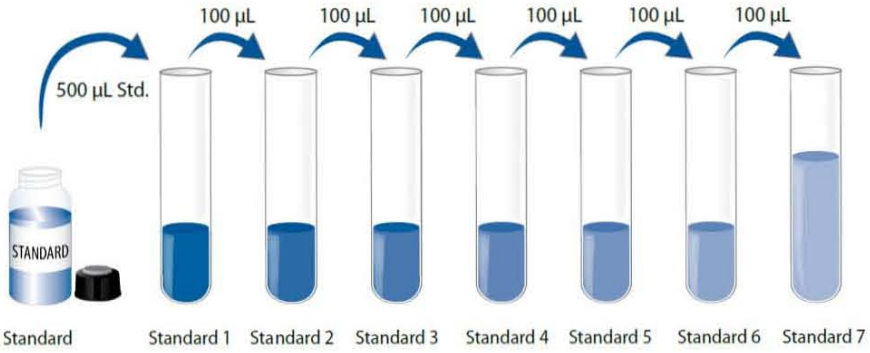
REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Standard - Reconstitute the Standard Cocktail with Calibrator Diluent RD5-49 (*for cell culture supernate and urine samples*) or Calibrator Diluent RD6-50 (*for serum, plasma, and human milk samples*). Refer to the Standard Card for the reconstitution volume and assigned values. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 500 µL of the reconstituted Standard into the Standard 1 tube. Pipette 200 µL of the appropriate Calibrator Diluent into the remaining tubes. Use Standard 1 to produce a 3-fold dilution series (below). Mix each tube thoroughly before the next transfer. Standard 1 serves as the high standard. The appropriate Calibrator Diluent serves as the blank.



DILUTED MICROPARTICLE COCKTAIL PREPARATION

1. Centrifuge the Microparticle Concentrate vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial to resuspend the microparticles, taking precautions not to invert the vial.
3. Dilute the Microparticle Concentrate in the mixing bottle provided.

Number of Wells Used	Microparticle Concentrate	+	Microparticle Diluent
96	500 µL	+	5.00 mL
72	375 µL	+	3.75 mL
48	250 µL	+	2.50 mL
24	125 µL	+	1.25 mL

Note: *Protect microparticles from light during handling. Diluted microparticles cannot be stored. Prepare microparticles within 30 minutes of use.*

DILUTED BIOTIN ANTIBODY COCKTAIL PREPARATION

1. Centrifuge the Biotin Antibody Concentrate vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial, taking precautions not to invert the vial.
3. Add 500 µL of Biotin Antibody Concentrate to the Biotin Antibody Diluent. Mix gently.

STREPTAVIDIN-PE PREPARATION

Use a polypropylene amber bottle or a polypropylene tube wrapped with aluminum foil. Protect Streptavidin-PE from light during handling and storage.

1. Centrifuge the Streptavidin-PE vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial, taking precautions not to invert the vial.
3. Dilute the 100X Streptavidin-PE to a 1X concentration by adding 55 µL of Streptavidin-PE to 5.5 mL of Wash Buffer.

INSTRUMENT SETTINGS

Adjust the probe height setting on the Luminex analyzer to avoid puncturing the membrane. Refer to the instrument manual.

a) Assign the bead region for each analyte being measured (see page 1)
b) 50 events/bead
c) Minimum events: 0
d) Flow rate: 60 µL/minute (fast)
e) Sample size: 50 µL
f) Doublet Discriminator gates at approximately 7500 and 15,500
g) Collect Median Fluorescence Intensity (MFI)

Note: *For the Bio-Rad Bio-Plex analyzer, set the gates at 4300 and 10,000. The CAL2 setting for the Bio-Rad Bio-Plex analyzer should be set at the low RP1 target value.*

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

Note: *Protect microparticles and Streptavidin-PE from light at all times.*

1. Prepare all reagents, working standards, and samples* as directed in the previous sections.
2. Pre-wet the filter-bottomed microplate by filling each well with 100 µL of Wash Buffer. Remove the liquid through the filter at the bottom of the plate using a vacuum manifold designed to accommodate a microplate.

   Note: *After each final wash cycle and subsequent reagent addition, blot the bottom of the plate with a paper towel to prevent wicking.*
3. Add 50 µL of Standard or activated sample per well.
4. Resuspend the diluted microparticle mixture by inversion or vortexing. Add 50 µL of the microparticle cocktail to each well of the pre-wet filter-bottomed microplate. Securely cover with a foil plate sealer. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Using a vacuum manifold device designed to accommodate a microplate, wash by removing the liquid, filling each well with Wash Buffer (100 µL) and removing the liquid again. All of the liquid must be removed through the filter at the bottom of the plate to avoid any loss of microparticles. Complete removal of liquid is essential for good performance. Perform the wash procedure three times.
6. Add 50 µL of diluted Biotin Antibody Cocktail to all wells. Securely cover with a foil plate sealer and incubate for 1 hour at room temperature on the shaker set at 500 ± 50 rpm.
7. Repeat the wash as in step 5.
8. Add 50 µL of diluted Streptavidin-PE to all wells. Securely cover with a foil plate sealer and incubate for 30 minutes at room temperature on the shaker set at 500 ± 50 rpm.
9. Repeat the wash as in step 5.
10. Resuspend the microparticles by adding 100 µL of Wash Buffer to each well. Incubate for 1 minute on the shaker set at 500 ± 50 rpm.
11. Read within 90 minutes using the Luminex or BioRad Analyzer.

*Samples require activation and dilution. See the Sample Activation and Sample Preparation sections.

CALCULATION OF RESULTS

Use the Standard concentrations on the Standard Value Card and calculate 3-fold dilutions for the remaining levels. Average the duplicate readings for each standard and sample and subtract the average blank Median Fluorescence Intensity (MFI).

Create a standard curve for each analyte by reducing the data using computer software capable of generating a five parameter logistic (5-PL) curve-fit.

Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

APPENDIX H

Milliplex Map (Millipore), manufactures booklet: Pages: 9-12

MILLIPLEX® MAP

HUMAN BONE PANEL 1A KIT PROTOCOL
96 Well Plate Assay

 # HBN1A-51K

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Antibody-Immobilized Beads

Sonicate each individual antibody-bead vial for 30 seconds; vortex for 1 minute. Add 150 µL from each antibody-bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Bead Diluent. Vortex the mixed beads well. Unused portion may be stored at 2-8°C for up to one month.

Example 1: When using 3 antibody-immobilized beads, add 150 µL from each of the 3 bead sets to the Mixing Bottle. Then add 2.55 mL Bead Diluent.

Example 2: When using 4 antibody-immobilized beads, add 150 µL from each of the 4 bead sets to the Mixing Bottle. Then add 2.40 mL Bead Diluent.

B. Preparation of Quality Controls

Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 µL deionized water. Invert the vial several times to mix and vortex. Allow the vial to sit for 5-10 minutes and then transfer the controls to appropriately labeled polypropylene microfuge tubes. Unused portion may be stored at ≤ -20°C for up to one month.

C. Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 30 mL of 10X Wash Buffer with 270 mL deionized water. Store unused portion at 2-8°C for up to one month.

D. Preparation of Serum Matrix

This step is required for serum or plasma samples only.

Before the assay, add 1.0 mL of Deionized Water and 4.0 mL of Assay Buffer to the bottle containing the lyophilized Serum Matrix, gently swirl the bottle and then place the bottle on bench for 10 minutes. Transfer the reconstituted Serum Matrix solution to an appropriately-labeled polypropylene tube. Unused portion may be stored at ≤ -20°C for up to one month.

E. Preparation of Human Bone Panel 1 Standard

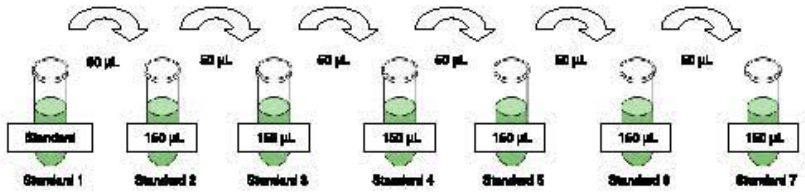
1.) Prior to use, reconstitute the lyophilized Human Bone Panel 1 Standard with 250 µL Deionized Water to give a concentration prescribed in the Quality Control Analysis Sheet. Invert the vial several times to mix. Allow the vial to set for 5-10 minutes to make sure that the standards are completely reconstituted, and then transfer the standard to an appropriately-labeled polypropylene microfuge tube. This stock solution will be used as the Standard 1. The unused portions of this stock may be stored at ≤ -20°C for up to one month.

2.) Preparation of Working Standards

Label six polypropylene microfuge tubes as Standard 2, Standard 3, Standard 4, Standard 5, Standard 6, and Standard 7. Add 150 µL of Assay Buffer to each of the six tubes. Prepare 1:4 serial dilutions by adding 50 µL of the reconstituted Standard 1 to the Standard 2 tube, mix well and transfer 50 µL of the Standard 2 to the Standard 3 tube, mix well and transfer 50 µL of the Standard 3 to the Standard 4 tube, mix well and transfer 50 µL of the Standard 4 to Standard 5 tube, mix well and transfer 50 µL of the Standard 5 to the Standard 6 tube, mix well and transfer 50 µL of the Standard 6 to the Standard 7 tube and mix well. The 0 pg/mL standard (Background) will be the Assay Buffer.

Tube Number	Standard Dilution	Volume of Deionized Water to Add	Volume of Standard to Add
1	Original (refer to analysis sheet for exact concentration)	250 µL	0

Tube Number	Standard Dilution	Volume of Assay Buffer to Add	Volume of Standard to Add
2	1:4	150 µL	50 µL of Standard Tube 1 (Original Standard)
3	1:16	150 µL	50 µL of Standard Tube 2 (1:4 Standard)
4	1:64	150 µL	50 µL of Standard Tube 3 (1:16 Standard)
5	1:256	150 µL	50 µL of Standard Tube 4 (1:64 Standard)
6	1:1024	150 µL	50 µL of Standard Tube 5 (1:256 Standard)
7	1:4096	150 µL	50 µL of Standard Tube 6 (1:1024 Standard)



IMMUNOASSAY PROCEDURE

- Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines.
- Allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Diagram the placement of Standards [0 (Background), Standard 7, 6, 5, 4, 3, 2, and 1], Controls 1 and 2, and Samples on Well Map Worksheet in a vertical configuration. (Note: Most instruments will only read the 96-well plate vertically by default.) It is recommended to run the assay in duplicate.
- Set the filter plate on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Prewet the filter plate by pipetting 200 µL of Assay Buffer into each well of the Microtiter Filter Plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Remove Assay Buffer by vacuum. **(NOTE: DO NOT INVERT PLATE.)** Blot excess Assay Buffer from the bottom of the plate with an absorbent pad or paper towels.
3. Add 25 µL Assay Buffer to all wells.
4. Add 25 µL of each Standard or Control into the appropriate wells. Assay Buffer should be used for the 0 pg/mL standard (Background).
5. Add 25 µL of Assay Buffer to the sample wells.
6. Add 25 µL of Serum Matrix to the background, standards, and control wells.
7. Add 25 µL of Sample into the appropriate wells. (Serum and plasma samples should be diluted 1:4 in Assay Buffer.)
8. Vortex Mixing Bottle and add 25 µL of the Mixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
9. Seal the plate with a plate sealer, cover it with the lid. Wrap a rubber band around the plate holder, plate and lid and incubate with agitation on a plate shaker overnight (16-20 hours) at 4°C.
   (The total volume should be 100 µL in each well for the overnight incubation.)

Add 200 µL Assay Buffer per well

↓ Shake 10 min, RT

↓ Vacuum

- Add 25 µL Assay Buffer to all wells
- Add 25 µL Standard or Control to appropriate wells
- Add 25 µL Assay Buffer to background and sample wells
- Add 25 µL Serum Matrix to background, standards and control wells
- Add 25 µL Samples to sample wells
- Add 25 µL Beads to each well

↓ Incubate overnight at 4°C with shaking

10. Gently remove fluid by vacuum. **(NOTE: DO NOT INVERT PLATE.)**
11. Wash plate 3 times with 200 µL/well of Wash Buffer, removing Wash Buffer by vacuum filtration between each wash. Blot excess Wash Buffer from the bottom of the plate with an absorbent pad or paper towels.
12. Add 50 µL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)
13. Seal, cover with lid, and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). **DO NOT VACUUM AFTER INCUBATION.**
14. Add 50 µL Streptavidin-Phycoerythrin to each well containing the 50 µL of Detection Antibodies.
15. Seal, cover with lid and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
16. Gently remove all contents by vacuum. **(NOTE: DO NOT INVERT PLATE.)**
17. Wash plate 3 times with 200 µL/well Wash Buffer, removing Wash Buffer by vacuum filtration between each wash. Wipe any excess buffer on the bottom of the plate with a tissue.
18. Add 100 µL of Sheath Fluid to all wells. Resuspend the beads on a plate shaker for 5 minutes.
19. Run plate on Luminex 100™ IS, 200™, or HTS.
20. Save and analyze the Median Fluorescent Intensity (MFI) data using a weighted 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples.

↓ Vacuum and wash 3X with 200 µL Wash Buffer

Add 50 µL Detection Antibodies per well

↓ Incubate 1 hour at RT

↓ Do Not Vacuum

Add 50 µL Streptavidin-Phycoerythrin per well

↓ Incubate for 30 minutes at RT

↓ Vacuum and wash 3X with 200 µL Wash Buffer

Add 100 µL Sheath Fluid per well

Read on Luminex (50 µL, 50 beads per bead set)

APPENDIX I

Table A – E contains the raw data from the cross-sectional and longitudinal studies.

Abbreviations and measurements are as follows:

Sex = M - Male; F - Female

CAMCOG 1 = CAMCOG at baseline

MMSE 1 = Mini-Mental State Examination at baseline

LS 1 = learning subscale at baseline

CAMCOG 2 = CAMCOG at follow-up

MMSE 2 = Mini-Mental State Examination at follow-up

LS 2 = learning subscale at follow-up

WCC = white cell count ($x10^9/l$)

MC = monocyte count ($x10^9/l$)

ESR = erythrocyte sedimentation rate (mm in 1 hour)

IL-1β = Interleukin 1 beta (pg/mL)

IL-10 = interleukin 10 (pg/mL)

TNF-α = tumor necrosis factor alpha (pg/mL)

TGF-β_1 and TGF-β_2 = transforming growth factor beta 1 and 2 (pg/mL)

OPN = osteopontin (pg/mL)

Note: Missing data are represented by blank cells. In the CAMCOG 2, MMSE, LS 2 columns, missing data from these columns means that the person was not followed-up. Names and study numbers for participants were removed for confidentially reasons.

Table A

	Group	Age	Sex	Year of Education	CAMCOG 1	MMMSE 1	LS 1	CAMCOG 2	MMSE 2	LS 2	No of E4 alleles	WCC	MC	ESR	IL-1β	IL-10	TNF-α	OPN	TGF-β1	TGF-β2
1	AD	78	F	6	66	21	14	62	20	11	1	9.86	0.55	30	2.50	3.80	3.50	714.50	1216.00	5.00
2	AD	74	F	6	49	14	7				1	5.3	0.49	16	2.30	6.30	2.50	2570.30	933.00	6.50
3	AD	62	F	14	77	24	14	62	20	8	0	9.8	0.57	25	4.00	4.30	6.00	584.50	2924.00	11.00
4	AD	91	M	9	57	17	8	46	16	2	0	7.64	0.55	5	1.50	4.80	3.00	2029.30	1612.50	6.00
5	AD	84	F	8	41	17	4	51	17	3	0	7.51	0.55	20	2.30	4.00	3.00	122.30	1912.50	7.00
6	AD	60	M	10	68	18	10	60	20	4	1	8.69			2.00	4.30	3.50	970.00	2096.30	8.50
7	AD	71	F	9	76	23	4	74	25	6	1	5.04	0.34	5	2.00	4.30	3.80	882.80	1738.50	7.00
8	AD	90	F	5	45	14	3				1	6.29	0.43	10	1.50	6.50	3.50	2308.30	1253.00	10.30
9	AD	72	M	8	50	16	3	47	13	3	1	7.11	0.37	10	2.00	4.00	2.50	368.80	1245.80	8.00
10	AD	85	F	13	75	23	5	64	19	3	1	7.41	0.42	3	2.30	5.00	3.50	248.80	1453.30	6.30
11	AD	76	F	16	78	24	7	66	20	4	1	5.67	0.35	6	2.00	4.50	4.00	169.00	1873.50	7.00
12	AD	67	M	13	87	28	11	88	26	10	1	5.66	0.29	1	4.50	7.00	4.50	240.50	1709.00	5.80
13	AD	78	F	10	54	21	7				1	5.66	0.27	13	1.00	4.00	3.00	1312.50	1723.00	7.00
14	AD	82	F	5	52	17	7	45	11	6	0	4.11	0.24	8	2.00	4.50	2.50	757.80	1533.50	4.50
15	AD	91	F	5	56	17	13				1	4.98	0.32	34	3.00	5.50	5.00	4375.50	1100.00	10.00
16	AD	93	F	8	68	22	11				0	9.67	0.4	30	3.50	7.00	5.80	1293.80	1829.50	15.50
17	AD	78	F	9	73	27	12				0	11.49	0.8	19	2.50	5.00	3.00	416.80	1430.50	7.00
18	AD	91	F	8	68	24	12	83	24	14	0	3.38	0.31	36	3.00	66.30	3.00	853.00	577.80	3.00
19	AD	81	F	8	38	13	5				0	5.01	0.29	18	1.50	3.00	3.50	2127.00	885.50	9.00
20	AD	63	M	5	72	23	10	79	28	11	0	5.41	0.36	1	3.00	164.80	4.00	319.80	877.50	5.00
21	AD	73	M	7	45	12	4				0	6.94	0.37	7	2.50	3.80	3.00	1066.50	1369.50	7.50
22	AD	59	M	12	73	23	2	74	24	2	0	6.06	0.41	2	1.50	4.50	5.50	151.00	1725.30	6.80
23	AD	72	F	6	45	13	7	37	17	5	1	7.24	0.54	11	2.50	4.50	3.80	2673.80	2222.50	9.50
24	AD	67	M	11	79	22	10	73	26	7	2	5.91	0.42	2						
25	AD	69	F	12	86	27	10				1	6.27	0.32	6	2.00	4.30	4.30	325.00	175.00	1.50
26	AD	72	M	17	70	20	7				1	5.31	0.45	4	2.00	6.50	2.50	300.30	997.00	4.00
27	AD	82	M	8	69	23	3				1	10.6	0.53	9	3.00	5.50	3.50	647.80	167.50	2.00

Table B

	Group	Age	Sex	Year of Education	CAMCOG 1	MMMSE 1	LS 1	CAMCOG 2	MMSE 2	LS 2	No of E4 alleles	WCC	MC	ESR	IL-1β	IL-10	TNF-α	OPN	TGF-β1	TGF-β2
28	AD	77	M	16	63	19	3	54	13	2	1	5.3	0.3	8	2.50	3.30	3.00	183.00	748.30	3.50
29	AD	83	F	4	81	27	12				0	4.91	0.23	23	2.50	5.00	3.50	138.00	914.30	4.00
30	AD	78	M	8	78	27	11	72	23	6	1	7.32	0.43	12	2.00	5.00	4.00	1060.00	470.00	2.80
31	AD	73	F	11	75	23	7	68	20	8	1	7.42	0.3	11	2.00	4.50	5.00	421.30	1671.30	5.30
32	AD	82	F	13	67	26	5				0	7.61	0.54	8	1.50	3.50	3.00	41.80	350.00	2.50
33	AD	63	M	10	59	22	10	48	18	8	1	7.01	0.55	12	2.00	4.00	2.50	109.00	1458.80	5.50
34	AD	61	F	8	61	20	2	54	23	3	0	10.91	0.53	26	2.00	6.30	3.80	172.30	109.80	1.00
35	AD	74	F	5	49	14	6	65	21	6	0				2.50	7.00	3.50	159.50	1551.00	7.50
36	AD	85	F	8	57	24	6	40	14	1	0	8.48	0.53	22	2.00	3.30	13.50	245.80	298.50	2.00
37	AD	79	F	8	44	14	4	35	10	2										
38	AD	66	F	9	73	21	7	49	17	2	2	9.8	0.59	33	1.50	5.00	4.00	449.30	378.00	6.80
39	AD	82	F	7	51	14	1	45	19	2	2	9.99	0.3	8	3.50	11.00	5.00	573.00	741.50	3.50
40	AD	69	F	14	80	24	9	68	22	4	2	5.46	0.21	16	1.50	2.50	3.00	1629.80	1262.30	13.50
41	AD	66	F	8	64	21	1				2	7.78	0.34	20	1.00	3.50	3.50	1153.80	2105.30	15.00
42	AD	78	M	8	61	21	4	51	16	6	1	8.17	0.42	7	2.00	5.30	2.50	232.00	186.50	1.50
43	AD	68	F	8	56	25	7	50	18	6	0	2.96	0.18	5	2.00	6.30	3.00	142.80	451.30	2.50
44	AD	71	M	15	83	27	13				0	5.71	0.39	20	11.30	73.00	19.50	331.30	81.00	1.00
45	AD	79	M	12	76	24	9	72	19	8	1	5.71	0.29	3	3.00	6.80	4.00	203.80	80.30	1.00
46	AD	85	F	6	42	14	8	42	15	6	1	6.4	0.33	25	2.00	4.00	4.00	1572.50	218.30	5.00
47	AD	81	F	9	52	16	4	41	17	2	1	6.18	0.29	5	1.00	8.00	3.00	1016.80	120.80	3.50
48	AD	78	M	9	84	25	14				1	5.51	0.37	4	1.00	3.00	2.50	1394.50	136.50	4.00
49	AD	87	F	8	51	16	2				1	6.16	0.31	38	1.50	3.50	3.50	1276.50	175.80	4.50
50	AD	79	M	10	84	28	14				0	4.94	0.27	17	1.00	3.00	3.30	870.80	138.30	5.00
51	AD	88	F	14	67	20	5				2	7.59	0.59	9	2.00	3.00	4.50	1801.50	192.30	4.00
52	AD	77	M	12	79	21	11				2	3.4	0.21	4	1.00	3.00	3.50	1321.50	97.00	3.30
53	AD	79	M	10	76	26	10				0	2.89	0.28	5	0.80	3.50	3.00	1092.80	160.00	4.00

Table C

	Group	Age	Sex	Year of Education	CAMCOG 1	MMMSE 1	LS 1	CAMCOG 2	MMSE 2	LS 2	No of E4 alleles	WCC	MC	ESR	IL-1β	IL-10	TNF-α	OPN	TGF-β1	TGF-β2
54	AD	84	F	8	79	26	10				0	9.74	0.51	31	1.50	5.50	4.00	1533.80	520.50	6.30
55	AD	80	F	8	49	15	3				0	9.84	0.49	26	1.50	3.00	3.50	1600.50	559.50	7.00
56	AD	79	F	16	74	25	1				0	7.11	0.53	25	1.00	2.00	3.00	2279.30	118.30	3.00
57	AD	80	F	8	44	12	5				1	9.18		14	1.00	2.00	3.00	552.50	1069.00	8.00
58	AD	83	F	12	77	26	8				0	7.59	0.51	1	1.00	2.50	4.00	701.00	119.50	5.50
59	AD	77	M	12	75	22	8				0	7.54	0.55	0	1.50	4.00	3.50	593.50	89.00	5.25
60	AD	78	F	7	57	16	2				2	7.17	0.48	35	2.75	5.00	4.00	850.00	1243.00	10.00
1	Control	66	M	11	94	29	13	91	27	16	1	6.64	0.35	8	2.00	4.30	2.80	311.80	1581.80	9.00
2	Control	59	F	14	100	29	17	103	30	16	0	7.36	0.52	14						
3	Control	62	F	9	99	30	16	98	30	17	1	5.35	0.56	3	2.00	5.00	3.50	5040.50	2372.50	13.50
4	Control	66	F	19	94	28	16	95	25	14	1	8.42	0.41	34	2.00	4.30	2.50	152.00	1982.00	8.50
5	Control	76	M	12	91	29	15	93	30	12	1	6.62	0.43	26	2.50	3.00	4.00	1833.30	1385.00	7.00
6	Control	69	F	8	91	24	15	92	28	14	0	8.54	0.5	13	2.00	4.50	4.30	1409.30	1814.80	6.50
7	Control	70	F	15	81	28	13	78	27	13	0	6.11	0.43	3	1.50	4.50	3.50	305.80	1436.50	6.50
8	Control	67	F	16	94	28	15	94	30	15	0	6.85	0.44	13	5.50	5.00	4.30	740.30	1823.30	8.00
9	Control	63	F	15	97	29	15	97	30	17	0	6.64	0.33	18	2.00	4.50	3.00	770.50	1369.50	5.50
10	Control	61	F	10	89	28	12	93	27	15	1	3.87	0.25	6						
11	Control	89	M	12	87	26	14	85	27	14	0	3.43	0.23	12	2.50	5.50	3.00	999.00	334.00	3.00
12	Control	69	F	16	87	28	14	94	29	16	0	4.95	0.33	10	2.00	3.50	3.00	809.30	1408.30	5.00
13	Control	84	F	9	85	26	13	85	27	13	0	7.97	0.5	34	2.00	6.50	3.50	594.00	1563.00	6.00
14	Control	83	F	3	87	28	14	89	28	15	0	7.89	0.42	4	3.00	5.80	4.00	359.00	1500.50	6.50
15	Control	75	F	8	85	28	15	93	29	16	1	7.68	0.56	10	2.00	3.30	2.50	504.80	1943.00	7.00
16	Control	100	F	8	84	24	13					8.32	0.37	30	2.80	5.00	3.00	1005.30	962.80	6.00
17	Control	58	F	9	91	30	13	96	28	16	0	8.03	0.51	17						
18	Control	75	F	8	87	28	13	87	26	15	0	6.68	0.36	22	3.50	3.80	6.30	3503.00	2395.30	4.50
19	Control	76	M	8	85	28	14				1	6.64	0.41	7	1.50	3.50	2.50	330.80	810.00	4.00

Table D

	Group	Age	Sex	Year of Education	CAMCOG 1	MMMSE 1	LS 1	CAMCOG 2	MMSE 2	LS 2	No of E4 alleles	WCC	MC	ESR	IL-1β	IL-10	TNF-α	OPN	TGF-β1	TGF-β2
20	Control	59	F	7	84	28	12	88	28	17	1	7.23	0.51	7						
21	Control	83	M	12	89	27	13	86	29	11	0	4.99	0.36	1	1.80	4.80	3.00	562.80	696.00	3.50
22	Control	81	F	12	85	28	11	86	27	14	0	8.01	0.58	20	2.50	14.30	3.50	367.30	1320.00	4.00
23	Control	83	F	18	93	30	14	97	29	14	1	6.82	0.38	10	2.00	5.00	3.50	854.00	719.30	4.00
24	Control	83	M	23	96	28	13	97	30	14	0	13.87	0.79	12	2.50	7.00	3.50	913.80	1092.00	4.50
25	Control	79	F	14	94	29	15	93	30	15	1	4.61	0.39	3	2.50	5.50	3.50	948.00	1452.50	7.00
26	Control	79	M	20	95	29	12	94	27	15	0	5.55	0.39	2	2.50	5.30	4.00	243.80	778.00	3.50
27	Control	69	F	18	96	28	15	97	29	15	0	4.98	0.32	15	3.30	5.00	4.80	899.30	1476.50	6.00
28	Control	80	F	18	88	28	11	90	29	16	0	8.68	0.53	10	2.50	5.30	3.50	498.00	2565.30	8.30
29	Control	72	F	11	91	27	14	93	25	14	1	5.27	0.41	10	3.00	5.80	4.00	154.30	1197.50	5.50
30	Control	81	M	18	90	28	11	94	27	13	0	6.27	0.35	0	2.00	4.80	4.50	466.50	1358.00	5.50
31	Control	78	M	12	95	29	12	96	27	15	0	6.8	0.44	2	1.00	3.50	3.50	923.50	1125.30	11.50
32	Control	81	F	9	87	29	13				0	7.45	0.31	36	2.00	5.50	4.00	1021.50	1503.50	13.50
33	Control	65	F	15	90	30	14	97	29	17	2	7	0.29	3	1.50	13.00	3.50	1804.50	1387.50	9.80
34	Control	66	F	18	100	29	15				0	6.01	0.53	1	1.50	4.00	3.50	782.80	1393.50	10.00
35	Control	67	F	22	97	30	16				1	5.31	0.29	6	1.00	2.50	3.00	1883.00	778.00	8.50
36	Control	66	F	3	96	30	15													
37	Control	62	F	13	95	29	13	96	30	13	0	5.76	0.32	18	1.30	3.00	4.00	1148.50	941.30	9.50
38	Control	85	F	10	86	24	13				1	7.84	0.44	15	2.00	37.00	2.50	154.50	1836.50	9.00
39	Control	82	F	11	96	29	15	97	28	15	0	5.91	0.38	8	2.50	4.50	3.50	1107.30	927.30	5.50
40	Control	62	F	14	98	30	15	94	30	12	0	8.74	0.48	20	2.00	7.00	5.00	1149.00	1027.25	10.50
41	Control	71	F	22	95	30	14	96	30	14	2	6.8	0.43	1	1.50	2.50	4.00	377.80	178.30	5.00
42	Control	70	M	23	100	29	16	99	28	14	0	6.67	0.37	3	2.80	2.50	3.00	98.30	530.00	5.50
43	Control	72	F	8	86	29	14				1	6.65	0.34	23	2.00	4.80	3.50	446.30	1770.80	6.00
44	Control	62	F	16	98	30	14	100	30	14		6.3	0.43	6	2.00	6.50	4.00	1354.00	1193.50	13.00
45	Control	70	F	15	98	29	15	93	29	16	0	4.95	0.36	7	2.00	4.50	3.00	126.30	198.50	1.50

Table E

	Group	Age	Sex	Year of Education	CAMCOG 1	MMMSE 1	LS 1	CAMCOG 2	MMSE 2	LS 2	No of E4 alleles	WCC	MC	ESR	IL-1β	IL-10	TNF-α	OPN	TGF-β1	TGF-β2
46	Control	73	F	12	99	29	17	97	29	15	0	5.92	0.17	27	1.50	3.80	2.50	445.30	72.50	0.50
47	Control	73	F	12	101	30	15	96	28	15	0	6.61	0.28	9	2.50	7.50	2.50	477.00	130.00	1.50
48	Control	69	F	25	91	28	13	98	30	14	1	3.97	0.23	3	2.00	3.80	2.50	236.80	81.30	0.50
49	Control	77	F	17	96	28	15	96	29	14	1	6.15	0.44	17	2.00	5.30	3.50	127.30	1738.50	5.50
50	Control	68	F	10	96	29	15	98	30	17	1	6.05	0.28	5	2.00	3.30	3.00	552.80	128.30	1.50
51	Control	61	F	14	88	29	13	90	30	12	1	11.7	0.43	30	3.50	5.50	4.80	317.00	106.00	1.00
52	Control	63	F	20	98	30	15				0	7.16	0.5	18	2.00	3.50	4.50	397.50	168.50	4.50
53	Control	63	M	20	95	30	14	96	30	13	1	6.5	0.43	7	3.00	18.30	5.50	940.00	106.00	3.50
54	Control	62	F	16	94	30	13	95	27	14	1	8.16	0.29	25	1.50	2.50	3.50	4184.30	99.30	3.50
55	Control	67	F	12	94	29	14	90	29	13	0	6.55	0.33	6	2.50	3.50	4.30	304.00	49.50	0.00
56	Control	76	M	10	91	29	12	92	30	14	0	5.99	0.31	8	1.30	7.00	4.50	583.50	280.00	6.30
57	Control	82	F	15	92	29	13				0	6.51	0.32	2	1.00	3.00	2.50	1105.80	115.00	3.50
58	Control	82	F	15	91	29	12				0	6.03	0.43	3	1.00	3.00	3.30	1955.50	142.00	2.80
59	Control	66	F	14	98	29	14	100	29	15	1	5.23	0.27	19	1.00	119.30	3.50	641.50	108.80	4.50
60	Control	64	M	12	96	30	14				0	7.7	0.52	13	1.00	3.50	3.30	409.80	742.30	8.00
61	Control	60	F	13	97	29	13				0	4.26	0.31	1						
62	Control	73	M	10	96	28	15				0	8.52	0.48	9	1.50	3.00	3.50	477.00	244.00	4.50
63	Control	65	F	16	96	30	11				0	9.13	0.38	7	1.00	3.00	3.50	993.80	133.50	3.50
64	Control	66	F	15	93	26	14				0	4.66	0.48	5	1.50	3.00	4.50	719.80	999.00	8.00
65	Control	61	F	22	100	30	15				0	5.17	0.23	2						
66	Control	60	F	13	96	30	13				0	4.66	0.36	2						
67	Control	76	M	16	96	29	14				1	7.57	0.47	8	2.00	5.25	5.00	552.00	71.50	5.00
68	Control	66	M	16	98	30	14					7.09	0.48	4						

APPENDIX J

Table A – B contains the raw data from the MRS Study.

Abbreviations and measurements are as follows:

Sex = M - Male; F - Female

MMSE = Mini-Mental State Examination

LS = learning subscale

WCC = white cell count ($x10^9/l$)

MC = monocyte count ($x10^9/l$)

ESR = erythrocyte sedimentation rate (mm in 1 hour)

IL-1β = Interleukin 1 beta (pg/mL)

IL-10 = interleukin 10 (pg/mL)

TNF-α = tumor necrosis factor alpha (pg/mL)

TGF-β_1 and TGF-β_2 = transforming growth factor beta 1 and 2 (pg/mL)

OPN = osteopontin (pg/mL)

MI = myo-inositol (parts per million)

NAA+NAAG = N-acetylaspartate and N-acetylaspartylglutamate

MI:NAA+NAAG = ratio of MI to NAA+NAAG

Note: Missing data are represented by blank cells. Names and study numbers for participants were removed for confidentially reasons.

Table A

	Group	Age	Sex	Year of Education	No of E4 alleles	LS	MMSE	WCC	MC	ESR	IL-1β	IL-10	TNF-α	OPN	TGF-β1	TGF-β2	MI	NAA+ NAAG	MI: NAA+NAAG
1	AD	62	F	14	0	7	18	8,57	0,58	27	4	5,5	5,5	721,25	538,3	10	3,654	5,523	0,7
2	AD	67	M	13	1	10	27	5,08	0,31	3	2	3,8	3	537,00	1121,5	10,5	3,863	5,593	0,7
3	AD	63	M	5	0	13	25	4,29	0,27	3	3,5	117	7	696,75	83,5	3	4,161	5,684	0,7
4	AD	59	M	12	0	0	19	5,37	0,51	1	2	3	4	863,25	1124	9,5	5,341	5,404	1,0
5	AD	67	M	11	2	3	27	5,49	0,45	1	2	3	4	857,75	118,5	5	4,994	6,601	0,8
6	AD	73	F	11	1	8	20				2	3,5	4,5	847,00	1919,5	12	3,569	5,411	0,7
7	AD	63	M	10	1	5	18	6,54	0,48	8	2	5	4,3	135,00	149	5	5,685	5,647	1,0
8	AD	85	F	8	0	1	14	6,22	0,48	31	2	3	9,5	2264,00	119,5	4,5	4,42	6,332	0,7
9	AD	78	M	8	1	6	16	6,22	0,42	1	2	4,5	4	2910,25	220,5	6	5,073	5,785	0,9
10	AD	68	F	8	0	4	18	3,1	0,23	2	2	4	4	587,75	572,5	8,5	5,063	5,708	0,9
11	AD	79	M	12	1	8	19	5,82	0,41	1	1,5	5,5	3,5	510,50	943	9,5	4,805	5,385	0,9
12	AD	81	F	9	1	2	17	4,68	0,3	4	2,3	5	4	656,75	1000,8	8	5,961	5,565	1,1
13	AD	87	F	8	1	2	12										4,279	5,904	0,7
14	AD	79	M	10	0	13	28	4,67	0,22	12	3,5	5,5	6	917,25	91	4,5	4,213	6,046	0,7
15	AD	79	M	10	0	13	21	3,34	0,3	12	2,5	4,5	4	1311,75	588,5	7,5	5,262	5,103	1,0
16	AD	80	F	8	0	5	14	8,15	0,35	10	3	5	4,5	1765,50	610	8	4,498	5,075	0,9
17	AD	83	F	12	0	9	24	7,24	0,4	2	2	4	4,8	633,00	731	7,5	4,278	6,057	0,7
18	AD	77	M	12	0	8	22	7,54	0,55	0	1,5	4	3,5	593,50	89	5,3	4,319	5,99	0,7
1	Control	66	M	11	1	16	29				2	4,5	4	1044,25	1237,5	12	4,842	6,121	0,8
2	Control	59	F	14	0	17	29	6,35	0,32	11	2,3	4,5	4	1872,50	1158	10,5	4,158	5,687	0,7
3	Control	62	F	9	1	15	29	5,13	0,31	6	2	4	3,5	633,50	1797	12,5	4,945	6,281	0,8
4	Control	76	M	12	1	13	30	6,04	0,45	26	2	4	4,5	870,75	83,5	4,5	5,33	6,806	0,8
5	Control	70	F	15	0	13	29	12,03	0,66	8	2	4,5	3,5	2600,75	473,3	7	4,235	6,021	0,7
6	Control	67	F	16	0	15	27	7,56	0,38	7	2	4	3	484,00	209,5	6	4,72	6,16	0,8
7	Control	63	F	15	0	16	28	7,42	0,42	2							4,978	6,893	0,7
8	Control	69	F	16	0	16	26	6,11	0,67	11	2	3,3	3,5	605,50	566,5	6,8	4,315	6,911	0,6

Table B

	Group	Age	Sex	Year of Education	No of E4 alleles	LS	MMSE	WCC	MC	ESR	IL-1β	IL-10	TNF-α	OPN	TGF-β1	TGF-β2	MI	NAA+ NAAG	MI: NAA+NAAG
9	Control	80	F	18	0	16	29	7,74	0,47	2	2,3	4	4,5	1119,75	466,3	7,5	4,577	6,26	0,7
10	Control	81	M	18	0	15	27	7,09	0,46	1	3	4	3,8	1662,00	529,5	7	5,249	6,263	0,8
11	Control	78	M	12	0	15	27	7,72	0,63	5	2	4,5	4	1084,00	1385,3	11,8	5,265	7,083	0,7
12	Control	73	F	12	0	15	30	5,19	0,27	7	2,5	4	5	835,75	729,3	8,3	4,193	5,786	0,7
13	Control	68	F	10	1	15	28	6,83	0,4	3	2	3,5	5	1219,75	72	4	4,091	5,844	0,7
14	Control	76	M	10	0	14	30	6,32	0,47	7	3,3	6	4	1004,00	1329,3	9,5	4,427	6,657	0,7
15	Control	73	M	10	0	13	29										3,436	5,736	0,6