

THE ASSOCIATIONS BETWEEN PLASMA HOMOCYSTEINE, VITAMIN B₁₂, FOLATE, THE APOLIPOPROTEIN E GENOTYPE AND ALZHEIMER'S DISEASE

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Dr Ilhaam Mohamed

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Abstract

Background

Alzheimer's disease (AD), the commonest form of dementia, affects people in both industrialised and developing countries. Risk factors for the development of AD include age, the presence of the Apolipoprotein $\epsilon 4$ allele, low vitamin B₁₂ and folate levels, and elevated plasma homocysteine concentrations. Most research involving the associations between these risk factors and AD have been conducted in Europe and North America. We know little about AD and its risk factors in a low to middle income country like South Africa, where nutrition is poor and the background population ApoE $\epsilon 4$ allelic frequency is high.

Objective

In this prospective observational study, I wished to determine the relationships between plasma homocysteine, vitamin B₁₂, folate, ApoE $\epsilon 4$ status and cognition in a sample of older persons from the greater Cape Town metropolitan area of the Western Cape region of South Africa.

Methods

Cognitively healthy controls and AD participants, diagnosed using NINCDS-ADRDA criteria, were recruited from the community. The study had both cross-sectional and longitudinal components. Cross-sectionally, I related non-fasting plasma homocysteine concentrations, vitamin B₁₂ levels, folate concentrations and the ApoE $\epsilon 4$ genotype to scores from a battery of cognitive tests including the Mini Mental State Examination (MMSE), the Cambridge Cognitive Examination (CAMCOG) and the Learning Subscale score of the CAMCOG. In the longitudinal analysis, I tested whether baseline plasma homocysteine concentrations related to cognitive decline one year after the initial assessment.

Results

One hundred and thirteen participants were recruited: 60 controls and 53 AD participants. Plasma homocysteine levels increased with age ($r_s = 0.418$, $p < 0.001$) and were inversely related to cognitive scores in all participants. Homocysteine concentrations were inversely related to vitamin B₁₂ and folate in all study participants (vitamin B₁₂ $r_s = -0.47$, $p < 0.001$, folate $r_s = -0.33$, $p = 0.001$). Homocysteine was inversely related to cognition but, in a regression model, this relation was confounded by the effects of age and years of education. Another regression model showed that vitamin B₁₂ and age independently predicted cognitive scores. There were more ApoE $\epsilon 4$ carriers in the AD group compared with controls and $\epsilon 4$ carrier status was significantly associated with AD. The ApoE $\epsilon 4$ allele modified the relationship between homocysteine and cognition. The association between homocysteine and cognition was strong in ApoE $\epsilon 4$ carriers (e.g. MMSE, $r_s = 0.33$, $p = 0.003$), but absent in $\epsilon 4$ non-carriers. High baseline homocysteine concentrations did not predict cognitive decline 1 year later.

Conclusions

These findings, the first from an African low to middle income country, are consistent with those from studies in industrialised countries. Plasma homocysteine levels increased with age and were inversely related to vitamin B₁₂ and folate. The ApoE $\epsilon 4$ allele strengthened the association between homocysteine and cognition, probably through mechanisms that increase neuronal susceptibility to homocysteine toxicity. My study supports the idea that homocysteine-lowering therapy can reduce the risk of developing AD or slow the progression of the disease.

DECLARATION

I, Ilhaam Mohamed, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Date:17/02/2014.....

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List of Abbreviations

AD	Alzheimer's disease
ApoE	Apolipoprotein E
ADI	Alzheimer's disease International
ADNI	Alzheimer's disease Neuroimaging initiative
AIDS	Acquired Immunodeficiency Syndrome
ALT	alanine transaminase
APP	amyloid precursor protein
AST	aspartate transaminase
A β	amyloid beta peptide
BADLS	Bristol Activities of Daily Living Scale
BBB	blood- brain barrier
BP	blood pressure
Ca	calcium
CAA	cerebral amyloid angiography
CAMDEX-R	The Cambridge Examination for Mental Disorders of the Elderly - Revised
CAMCOG-R	Cambridge Cognitive Assessment - Revised
CBL	
CBS	cystathionine- β -synthase
CD-RISC	Connor- Davidson Resilience Scale
CERAD	The Consortium to Establish a Registry for Alzheimer's disease
CLOX	The Executive Clock Drawing Task
CNS	central nervous system
CRP	C- reactive protein
CSF	cerebrospinal fluid
CT	computerised tomography
DECO	Détérioration de Cognition Observée
df	degrees freedom
diff	differential white blood cell count (monocytes, neutrophils etc.)
DNA	deoxyribonucleic acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
DVT	deep vein thrombosis
EDTA	ethylenediaminetetraacetic acid
EOAD	early onset Alzheimer's disease
ESR	erythrocyte sedimentation rate
FBC	full blood count
FBP	folate binding protein
GABA	gamma aminobutyric acid
GDS	Geriatric Depression Scale
GGT	gamma glutamyl transferase
GSH	Groote Schuur Hospital
Hcy	homocysteine

HIV	human immunodeficiency virus
holoTC	holotranscobalamin
IHD	ischaemic heart disease
IQR	interquartile range
LEQ	Life Events Questionnaire
LDLR	low density lipoprotein receptor
LOAD	late onset Alzheimer's disease
LMIC	low to middle income countries
M	mean
Mdn	median
MCI	mild cognitive impairment
MMA	methylmalonic acid
MMSE	Mini Mental State Examination
MRI	magnetic resonance imaging
MS	methionine synthase
MTHFR	methyltetrahydrofolate reductase
NAD+	oxidized nicotinamide co-factor
NADH	reduced nicotinamide co-factor
NEO-FFI	Neo-Five Factor Inventory
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease Related Disorders Association
NH ₃	ammonia
NHLS	National Health Laboratory Service
NMDA	N-methyl D-aspartate
NO	nitrous oxide
PCR	polymerase chain reaction
PET	positron emission tomography
ROS	reactive oxygen species
RPR	rapid plasma reagin
SAH	s-adenosyl homocysteine
SAM	s-adenosyl methionine
SD	standard deviation
SES	socioeconomic status
TCBV	total cerebral brain volume
THF	tetrahydrofolate
TM	thrombomodulin
TMT	Trail Making Test
TPT	The Placing Test
PSEN1	presenilin 1
PSEN2	presenilin 2

PSS	Perceived Stress Scale
VaD	vascular dementia
WMHs	white matter hyperintensities

Chapter One

Introduction

Studies in Europe and North America have shown that Alzheimer's disease (AD) is a multi-factorial degenerative process. Many studies have provided evidence of several risk factors, and proposed multiple disease mechanisms associated with neurodegeneration. Randomised controlled trials, although few, have focused on the treatment of modifiable risk factors for disease: vascular risk factors, vitamin deficiencies and plasma homocysteine- lowering therapies.

Vascular risk factors- hypertension, dyslipidaemia, diabetes, smoking and atrial fibrillation- are accepted risk factors for AD (de la Torre 2004). Plasma homocysteine, a risk factor for cardiovascular disease and stroke (McCully 1969; Boushey *et al.* 1995; Bots *et al.* 1999; Bostom *et al.* 1999), is also a risk factor for cognitive impairment and AD (Seshadri *et al.* 2002; Ravaglia *et al.* 2003). Elevated homocysteine levels are markers of vitamin B₁₂ and folate deficiency (Jacobsen 1998) and deficiency of these nutrients (and resultant hyperhomocysteinaemia), has been associated with cognitive decline in the elderly (Goodwin *et al.* 1983). The Apolipoprotein E ε4 allele is associated with an increased risk of atherosclerosis and AD (Stritmatter *et al.* 1993; Mahley *et al.* 1995). The interrelationships and synergistic activity amongst the above risk factors are thought to either initiate or potentiate the dementing process in the majority of AD cases (van Norden *et al.* 2011).

In South Africa it is estimated that there are at least 3.9 million persons over the age of 60 (Statistics SA 2011) and approximately 250 000 persons living with dementia (www.alzheimers.org.za). Most clinicians and researchers in academic medical centres in South Africa believe that dementia and Alzheimer's disease are underdiagnosed and under-reported in South Africa. Our country focuses on primary health care needs, and often healthcare workers are usually not trained to look for symptoms of cognitive impairment and associated diseases. Furthermore, we live in a multi-cultural society where lack of awareness and/or social stigmata surrounding memory impairment are common. The older population in lower and middle income countries is growing rapidly, and older persons are living even longer. The challenge of studying the epidemiology of dementias and related neurodegenerative disorders is considerable. These studies are expensive, time consuming and require large numbers of trained staff and field workers. Increased awareness and education about AD would allow for improved health care services and treatment. In the meantime, primary prevention should focus upon targets suggested by current evidence: risk factors for vascular disease, including hypertension, smoking, type II diabetes and hyperlipidaemia and nutritional deficiencies (ADI World Report 2009).

Little is known about the extent of AD and its associated factors in a South African context. In the Western Cape, we do not know how many persons are affected with AD, but anecdotal evidence suggests that it is not an uncommon disease. Moreover, South Africa has a population with a large number of individuals of low socioeconomic status. Dietary deficiency, especially that of vitamin B and folate are probably more common compared with first world countries.

In my research project, I aimed to describe the relationships between plasma homocysteine, vitamin B₁₂, folate and Alzheimer's disease in a group of older persons in the Western Cape. I hypothesised that elevated levels of plasma homocysteine and decreased concentrations of vitamin B₁₂ and folate are associated with normal ageing and cognitive impairment. Furthermore, because our native South African population has the highest ApoE ε4 allelic frequency in the world, I wished to determine whether carriers of this allele had a greater burden of disease compared with non-carriers and whether the association between plasma homocysteine and cognitive function was modified by the ε4 allele.

This study is clinically relevant since the above factors are potentially modifiable risk factors for disease. Modifiable risk factors justifies the early identification of disease. Early identification may prevent or delay the onset of new cases of disease, and slow disease progression in those with established AD.

Chapter Two

Background and Literature review

2.1) Definition of Dementia and neurocognitive disorders

Dementia is a syndrome characterized by an acquired global impairment of memory and other cognitive functions sufficient to interfere with normal life. There are many causes of dementia including Alzheimer's disease (AD) and cerebrovascular disorders, and most are progressive.

2.2) Definition and history of Alzheimer's disease

Alois Alzheimer (Figure 2.1), a lecturer at the Munich University Hospital, and a co-worker Emil Kraepelin, reported on an unusual case study involving a "peculiar severe disease process of the cerebral cortex" (Alzheimer 1906). Alzheimer's disease (AD) may be defined as a chronic, progressive degenerative disease of the brain, with complex multi-factorial risk factors and associations.

Figure 2.1: Alois Alzheimer 1909



*Archive for History of Psychiatry, Department of Psychiatry, University of Munich
Hippius et al. 2003*

Progressive mental deterioration in old age has been recognized and described throughout history. However, it was not until 1906 that Alois Alzheimer identified a collection of brain cell abnormalities which formed the basis of the disease.

AD refers to the clinico-pathological entity of the histological changes of neurofibrillary tangles and senile plaques in the brain in a patient with the clinical syndrome of dementia. The disease affects

neurones in the brain resulting in cognitive abnormalities, most prominently, the loss of memory, altered thinking and a decline in language skills as well as behavioural changes (Carlesimo *et al.* 1992).

Over the last decade, there has been a considerable improvement in the understanding of potential risk factors for AD, the processes leading to the formation of plaques and tangles in the brain, and the brain regions that are affected. Research has shown AD to be a complex disorder, which appears to be influenced by genetic components, vascular risk factors, socio-economic conditions and environmental and lifestyle aspects.

2.3) Prevalence of AD

Alzheimer's disease International (ADI), an umbrella organisation for national Alzheimer's Associations, in their 2009 World Alzheimer Report, estimated that there were 35.6 million people living with dementia worldwide in 2010. This is set to increase to 65.7 million by 2030 and 115.4 million by 2050.

In the 2013 World Alzheimer report the prevalence of dementia in sub-Saharan Africa was greater than that reported in 2009 (4 vs 4.76%). More than two-thirds of dementia cases live in low and middle income countries (LMIC) (<http://www.alz.co.uk/research/G8-policy-brief>).

Understanding the global burden of AD is important for 2 reasons:

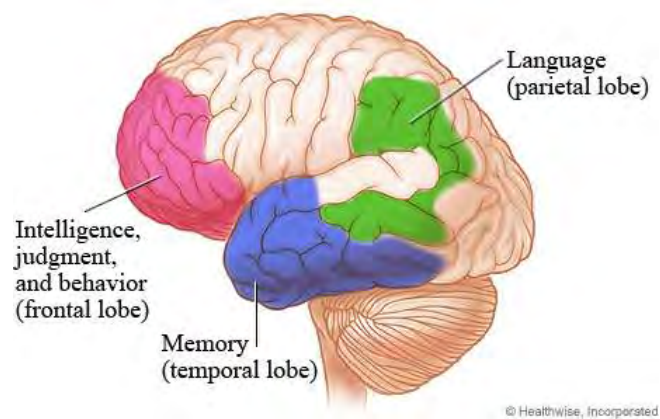
- I. It allows for increased awareness of AD in both developed and lower income countries. Increased awareness, in turn, will lead to better identification and management of the disease and also more effective support for patients and their families (Patel *et al.* 2001).
- II. Demographic ageing is proceeding more rapidly in LMIC compared with higher income countries (ADI 2009). As a result, chronic non-communicable diseases assume a greater significance in LMIC. We do not know how many older persons in South Africa are living with dementia and AD, and which sectors of the population are most severely affected by the disease.

2.4) Clinical presentation and diagnosis of Alzheimer's disease

2.4.1) Clinical features

The most prominent cognitive deficit in AD is memory. In AD, episodic memory is predominantly affected first and immediate recall or working memory spared. Language and visuo-spatial deficits appear as the disease progresses. The latter manifests as an impairment in topographical memory i.e. getting lost and confused in previously familiar places. Frontal lobe features and behavioural manifestations generally appear late in the disease process. At least 40% of persons have associated delusions and hallucinations (Rossor 1993). AD can affect all parts of the brain, but each person is affected differently as the disease progresses.

Figure 2.2: Alzheimer's disease and affected lobes of the brain



Anne C. Poinier
Healthwise incorporated
<https://myhealth.alberta.ca/health/Pages/conditions>

2.4.2) Neuropathology of Alzheimer's disease

Neurofibrillary tangles (NFTs) and amyloid or senile plaques are the neuropathological hallmarks of AD (Selkoe 1991, Tomlinson *et al.* 1970; Roth *et al.* 1996).

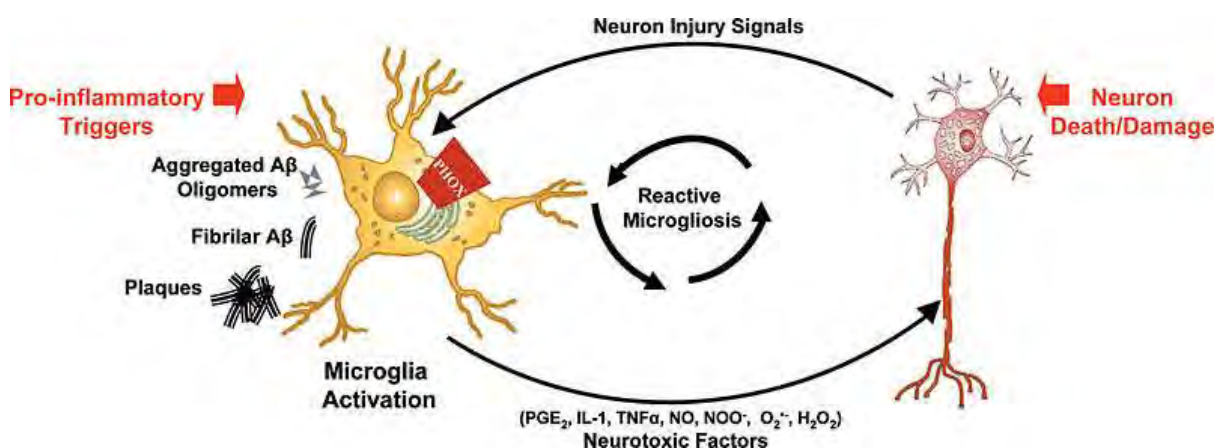
NFTs are intracellular nerve cell abnormalities. They are composed of twisted protein fibres found in the cytoplasm of neurones. In AD, these protein fibres are made up of abnormal hyperphosphorylated tau protein – a protein that functions in stabilising microtubules and forms an integral part of the neuronal cytoskeleton. Aggregation of tau protein leads to a disturbance of the

microtubule network. As a consequence of the latter, axoplasmic flow is disrupted and the neuronal cells collapse (Rossor 1993) (Figure 2.3).

Amyloid plaques are extracellular nerve abnormalities composed of dystrophic axons and dendrites, with a central amyloid core. The main component of these plaques is amyloid β ($A\beta$). $A\beta$ is processed from amyloid precursor protein (APP) by the action of β -secretase and γ -secretase enzymes. Rare genetic mutations in the gene coding for APP result in abnormal cleaving of APP and the production of amyloid β fragments, $A\beta_{1-40}$ and $A\beta_{1-42}$ (Chartier-Harlin *et al.* 1991). $A\beta_{1-40}$ is soluble and is found in cerebrospinal fluid (CSF) and brain interstitial fluid (Masters *et al.* 1985). Both forms of amyloid are found in plaques but $A\beta_{1-42}$ is less soluble and therefore has a greater propensity to aggregate and form plaques (Masters *et al.* 1985).

Despite many hypotheses on the causes of AD in recent times, the amyloid hypothesis remains the most widely accepted theory and the most investigated over the past 20 yrs. In short, this theory implies that deposition and accumulation of $A\beta$ in the brain is the primary factor driving AD pathogenesis (Selkoe 1991). The pathological process involving the formation of neurofibrillary tangles containing tau protein is thought to begin with an imbalance between the production and clearance of $A\beta$ (Masters *et al.* 1985). $A\beta$ is thought to be toxic to brain cells by generating inflammatory signals. If the accumulation of $A\beta$ peptides is excessive, this can activate microglia, the macrophages of the brain (Rogers *et al.* 1988). This results in a pro-inflammatory state that may eventually cause neuronal death.

Figure 2.3: Amyloid plaque toxicity in Alzheimer's disease



BMC Neuroscience (2008), vol. 9 issue. **Suppl 2**, S8-S8.

2.4.3) Diagnosis

Patient History, Examination and Neuropsychological Tests

The diagnosis of AD may be challenging for clinicians especially in the early stages of disease. A detailed history from the patient and their relatives is essential to obtain the overall picture of cognitive decline. Neuropsychological tests are often necessary in the earlier stages of the disease. The findings of the physical examination may suggest a cause for dementia. For example, dementia resulting from cerebrovascular disease may be accompanied by focal neurological signs.

Frequently used neuropsychological tests include the Mini-Mental State examination (MMSE) and the Cambridge Examination for Mental Disorders of the Elderly (CAMDEX). The MMSE was designed to detect patients with cognitive impairment amongst a psychiatric population (Folstein *et al.* 1975). It tests global cognitive function, with items assessing orientation, word recall, attention and calculation, language abilities and visuo-spatial ability. The CAMDEX is a standardized instrument for the diagnosis and grading of dementia (Roth *et al.* 1986). The Cambridge Cognitive Assessment - Revised (CAMCOG-R) questionnaire forms part of the cognitive section of the CAMDEX and has a maximum score of 105. All items of the MMSE are also incorporated into the CAMCOG. In most studies the CAMCOG cut-off point of 79 or 80 as suggested by Roth *et al.* seems satisfactory for discriminating between normal subjects and demented patients.

Clinical Diagnostic Criteria

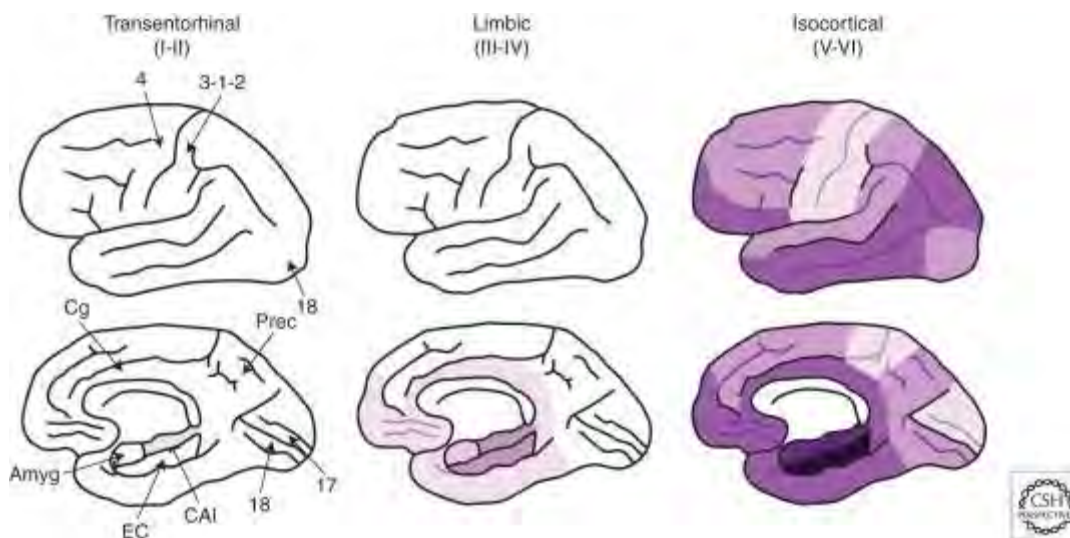
In clinical practice, the diagnosis of AD has been based on adherence to clinical criteria, such as the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease Related Disorders Association (NINCDS/ADRDA) and the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (McKhann *et al.* 1994). The NINCDS-ADRDA Alzheimer's Criteria (appendix A) were proposed in 1984 (McKhann *et al.* 1994). These criteria require that a clinical diagnosis of possible or probable AD be confirmed by neuropsychological testing whereas a definitive diagnosis of AD requires histopathologic confirmation (neurofibrillary tangles and amyloid plaques). These criteria have shown good reliability and validity (Blacker *et al.* 1994) (See appendices A and B).

Pathological classification

There are 2 commonly used systems to classify AD pathologically.

- 1) Heiko and Eva Braak published the Braak Staging of AD-related NFT and neurofibrillary threads (Braak *et al.* 1991). This staging system has been frequently used in histopathological studies (Figure 2.4). NFTs develop and spread in a predictable manner across the brain, providing the basis for distinguishing six stages of disease progression: the transentorhinal Braak stages I–II represent clinically silent cases; the limbic stages III–IV, incipient AD; and the neocortical stages V–VI, fully developed AD (Gotz *et al.* 2011).

Figure 2.4: Braak staging of Alzheimer's disease



Braak et al. 1991

Entorhinal stage (I-II) represents clinically silent periods of the disease with NFT involvement confined to trans-entorhinal layer. Limbic stages (III/IV) correspond with clinically incipient AD and neocortical stages (V/VI) represent fully developed AD, with NFT involvement of all areas of association cortex. Shading indicates the distribution of NFTs with darker colors representing increasing densities. Amyg = Amygdala; EC = Entorhinal cortex; CA1 = Cornus ammonis 1 hippocampal subfield; Cg = Cingulate cortex; Prec = Precuneus; 4 = Primary motor cortex; 3-1-2 = Primary sensory cortex; 17 = Primary visual cortex; 18 = Associative visual cortex.

- 2) The Consortium to Establish a Registry for Alzheimer's disease (CERAD), a multi-centre longitudinal study, has recognised standardized instruments for the evaluation of persons clinically diagnosed with AD. The most common aspect of the CERAD used relates to A β plaque score. Other parts of the CERAD include clinical neuropsychological, neuroimaging, and neuropathological tests.

The common consensus is to combine Braak and CERAD scores to determine whether there is a high, intermediate or low likelihood that post-mortem lesions are AD related (NIA-RI Consensus 1997) (Table 2.1).

Table 2.1: Staging in AD

Likelihood that AD pathological changes underlie dementia	Postmortem brain	
	CERAD plaque score	Braak neurofibrillary stage
High likelihood	frequent	stage V–VI
Intermediate likelihood	moderate	stage III–IV
Low likelihood	sparse	stage I–II

The National Institute on Aging and Reagan Institute (NIA-RI)

2.5.) Mild Cognitive Impairment (MCI)

MCI is a syndrome defined as cognitive decline greater than expected for an individual's age and education level, but not severe enough to affect activities of daily living (Petersen *et al.* 1999). It represents a prodromal form of incipient dementia conferring a 10-15% annual risk of converting to probable AD (Petersen *et al.* 1999). Identification of subtle brain changes in persons with cognitive impairment prior to disease conversion is important in understanding AD progression. Studies evaluating the importance of both clinical and imaging biomarkers early in the disease process are becoming more common. Genetic markers, cell-cycle biomarkers, oxidative stress parameters and pathological markers (serum A β) may be of value in detecting early disease (Gustaw-Rothenberg *et al.* 2010) (See Appendix C).

The Alzheimer's Disease Neuroimaging Initiative (ADNI) is a five-year public-private partnership to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of amnesic MCI and early probable AD (Risacher *et al.* 2009).

2.6) Risk factors for the development of Alzheimer's disease

2.6.1) Age

Many prevalence studies relating advanced age to AD have been conducted in the 1980s and 1990s. The majority of these studies have demonstrated that the incidence of dementia and AD is higher with advancing age within different population groups (Fratiglioni *et al.* 1991, Bachman *et al.* 1993, Lindsay *et al.* 2002). Population studies have shown that less than 1% of 60–64 year olds are affected compared with 40% in the elderly over 85 years (Rademakers *et al.* 2003). Other studies confirm that even in the oldest old (>85yrs), the risk of AD is increased (Aronsen *et al.* 1991, Fratiglioni *et al.* 1997).

Some researchers believe that cognitive decline is a normal consequence of the ageing process i.e. if all people live long enough they will eventually develop dementia (Levy 1994). Hippocampal brain volume has been shown to decrease with advancing age in cognitively healthy and impaired older persons (Jack *et al.* 1997). Another view is that AD and age are related due to the effect(s) of various age-related risk factors which either affect cognition directly, or work together to potentiate cognitive decline in older persons (Gao *et al.* 1998).

2.6.2) Gender

The 2009 World Alzheimer's report estimated that there are almost twice the number of women compared with men living with AD in America (3.4 million vs 1.8 million) (Herbert *et al.* 2003). This is not surprising as a number of studies have showed that AD is more prevalent in females compared with males in different population groups (Corso *et al.* 1992, Bachman *et al.* 1992, Fratiglioni *et al.* 1997, Friedland *et al.* 1998). Incident studies have generally found no sex differences (Nilsson *et al.* 1984, Bachman *et al.* 1993, Herbert *et al.* 2001). Studies reviewing sex differences in the pathologic indices of AD have also had mixed results, with most indicating no sex differences (Sandberg *et al.* 2001).

Despite years of research, it is still unclear whether AD is more common in females because they live longer, or because they are at a greater risk of AD compared with males. In support of the latter, it is thought that oestrogen loss associated with menopause may contribute to the development of AD. Study findings have highlighted the protective function of oestrogen on cognition (Paganini-Hill *et al.* 1994; Kawas *et al.* 1997).

2.6.3) Level of education

A low level of education has been linked to AD and dementia (Stern *et al.* 1994, Letenneur *et al.* 1999, Qui *et al.* 2001 Ngandu *et al.* 2007). Additionally, low levels of education and other markers of low socio-economic status (SES) such as low income, have also been associated with cognitive impairment (Evans *et al.* 1997).

Karp *et al.* showed that low education and a poor occupational history early on in life, may play a role in the development of dementia later on (Karp *et al.* 2004). In an earlier study, Evans *et al.* looked at 3 measures of SES namely education, occupational prestige and income, to the risk of AD. They found that all 3 measures separately predicted an increased risk of AD (Evans *et al.* 1997).

Different hypotheses have been suggested to explain such an association. One explanation is that education may increase brain reserve as well as mental activity throughout life which postpones the clinical manifestations of AD. Education may also be an indicator of intelligence, socio-economic status or other factors related to the first decade of life (Ngandu *et al.* 2007). These early life factors could potentially contribute to AD and therefore could modify disease outcome if prevented early enough. This is very relevant in South Africa where the majority of our population is poor, of low socio-economic status, poorly educated and of low occupational prestige.

2.6.4) Genetic Factors

Three genes responsible for early onset AD (EOAD) have been identified: the APP gene located on chromosome 21 and the two homologous presenilin genes, presenilin 1 (PSEN1) and presenilin 2 (PSEN2), located on chromosomes 14 and 1 respectively (Van Broekhoven *et al.* 1992). The $\epsilon 4$ allele of the apolipoprotein gene (ApoE) on chromosome 19 is the only known and well-established risk factor for late onset AD (LOAD) (Cedazo-Minguez *et al.* 2001).

2.6.5 Apolipoprotein E (ApoE)

ApoE was first described by Shore and Shore (Shore *et al.* 1973). It is a polypeptide composed of 229 amino acids whose main function is to modulate plasma lipoprotein and cholesterol levels. Most of ApoE is manufactured in the liver but a small amount is produced locally in the central nervous system (Elshourbagy *et al.* 1985). In the brain, cholesterol released from ApoE particles supports synaptogenesis and maintains synaptic connections after brain injury. Human ApoE has three common isoforms ($\epsilon 2$, $\epsilon 3$ and $\epsilon 4$) which have different effects on lipid and neuronal homeostasis (Farrer *et al.* 1997).

The ApoE $\epsilon 4$ allele has been associated with elevated plasma cholesterol and low density lipoprotein (LDL) levels and increased risk of cardiovascular disease (Davignon *et al.* 1988). ApoE $\epsilon 4$ allele is also a known risk factor for late onset AD (Corder *et al.* 1993, Strittmatter *et al.* 1993, Saunderson *et al.* 1993, Frisoni *et al.* 1995, Mahley *et al.* 2006). Different studies generally agree that ApoE $\epsilon 4$ is the strongest genetic risk factor for AD whereas ApoE $\epsilon 2$ is protective against AD (Corder *et al.* 1993, Saunders *et al.* 1993, Roses 1996). It has been demonstrated that, compared to persons with no $\epsilon 4$ alleles, individuals with one $\epsilon 4$ allele have a three-fold increased risk of AD while the risk is increased twelve-fold in those with two $\epsilon 4$ alleles (www.alzgene.org).

The ApoE distribution within different populations is variable. The frequency of ApoE $\epsilon 4$ carriers was shown to be the highest in the indigenous Khoi-San population group (“Bushmen”) in South Africa compared with other world populations (Sandholzer *et al.* 1995). The frequency of $\epsilon 4$ carriers is also greater in persons of African descent compared with other population groups (Gureje *et al.* 2006, Murray *et al.* 2006, Joska *et al.* 2010).

Magnetic resonance imaging and PET scans have shown that cognitively intact carriers of the $\epsilon 4$ allele have altered brain structure, decreased brain perfusion and differences in white matter compared with non-carriers (Bendlin *et al.* 2010). Recently Pievani *et al.* showed that hippocampal atrophy was greater in $\epsilon 4$ carriers compared with non-carriers (Pievani *et al.* 2011). Previous studies have reported that both hippocampal atrophy and the ApoE $\epsilon 4$ allele were significantly and independently associated with AD (Jack *et al.* 1998).

ApoE and neurobiological mechanisms of injury in Alzheimer’s disease

Physiologically, ApoE is responsible for regulating lipid metabolism in cells by directing the transport, delivery and the distribution of lipids from one cell to another (Mahley *et al.* 2000). In the brain, ApoE is mainly produced by astrocytes, and transports cholesterol to neurones via ApoE receptors. These ApoE receptors are members of the low-density lipoprotein receptor (LDLR) family (Fryer *et al.* 2005).

The ApoE $\epsilon 4$ allele could cause cognitive impairment via A β dependent mechanisms and A β independent mechanisms.

A β -dependent mechanisms of injury

In the early 1990s ApoE ϵ 4 was found to co-exist with amyloid plaques (Namba *et al.* 1991). The ApoE ϵ 4 dosage, heterozygous or homozygous state, has been associated with increased neuritic plaques in AD patients at autopsy i.e. patients with AD have higher plaque loads if they are ϵ 4 carriers compared with ϵ 4 non-carriers and even higher brain amyloid if they are homozygous i.e. carry two ϵ 4 alleles (Schmetchel *et al.* 1993, Tiraboschi *et al.* 2004). Studies have also demonstrated that cognitively healthy middle aged and elderly ApoE ϵ 4 carriers have higher brain amyloid levels (Morris *et al.* 2010).

The mechanism by which ApoE influences the onset and progression of AD is unclear. ApoE may be involved in the clearance of A β from the brain parenchyma, perhaps by acting as a molecular chaperone molecule for transporting A β peptides from the brain into the perivascular/cerebrospinal fluid spaces (Holtzman *et al.* 2000). Biologically, A β plaque load and accumulation could therefore be related to abnormal A β clearance mechanisms rather than excessive A β production (Masters *et al.* 1985). The ApoE isoforms can affect amyloid clearance in different ways. ApoE ϵ 3 has a higher binding affinity for A β compared with ApoE ϵ 4, leading to a better clearance of A β in ApoE ϵ 3 carriers but an accumulation of A β peptides in ApoE ϵ 4 carriers (Strittmatter *et al.* 1993; Ma *et al.* 1994). Structural analyses have provided insight into the mechanisms of the involvement of ApoE in atherogenesis and neurological disease. ApoE has two structural domains: the N-terminal domain and the C-terminal domain. The N-terminal domain contains the receptor-binding region and the C-terminal domain contains the lipid-binding region of ApoE. Impaired function of the N-terminal domain of ApoE ϵ 3 results in decreased clearance of lipoproteins and cholesterol and resultant hyperlipoproteinaemia (Mahley *et al.* 2000). In ApoE ϵ 4 carriers, the 2 domains interact, and this may account for the neurobiological effects observed in persons with AD. Domain interaction in ϵ 4 carriers stimulates A β production and potentiates A β -induced lysosomal leakage and apoptosis (Ye *et al.* 2005).

As discussed earlier on, the ApoE ϵ 4 allele is associated with an increased risk of atherosclerosis (Davygnon 1988). Cerebral amyloid angiopathy (CAA) is common in older persons and is associated with amyloid deposition in leptomeningeal and cortical arteries. CAA is also a pathological feature of AD (Selkoe 2001). The ApoE ϵ 4 allele together with other vascular risk factors for cerebrovascular disease, work synergistically to exacerbate AD pathology (Kalmijn *et al.* 1996). More recently it has been proposed that amyloid deposits in arteries trigger a secondary cascade of events including the release of inflammatory components, oxidative stress, alteration of the blood-brain barrier (BBB) permeability and leaking of toxic proteins into the brain parenchyma (Rostagno *et al.* 2007, Bell *et al.* 2012).

A β independent mechanisms

Plausible biological explanations that do not include A β peptides, called A β -independent mechanisms, are less well understood. ApoE ϵ 4 has been shown to be less effective in promoting neuronal repair and proper brain functioning compared with the ϵ 3 allele (Sorbi *et al.* 1995). Additionally, ApoE ϵ 3 stimulates neurite outgrowth, whereas ApoE ϵ 4 inhibits it and causes cytoskeletal instability (Nathan *et al.* 1994 and 1995). Mitochondrial dysfunction has been reported

in AD, which is modulated by the ApoE genotype, with a greater effect in $\epsilon 4$ than in $\epsilon 3$ carriers (Gibson *et al.* 2000). ApoE also causes greater membrane disruption and impaired synaptogenesis compared with ApoE $\epsilon 3$ (Mahley *et al.* 1997). Lastly, the ApoE $\epsilon 4$ allele has been linked to increased neuroinflammation associated with amyloid plaque formation in patients with AD (Guo *et al.* 2004).

It is possible that, in humans, both A β -dependent and independent effects of the ApoE $\epsilon 4$ allele mutually interact, thus potentiating the pathological and clinical expression of AD. Animal studies have showed that ApoE $\epsilon 4$ macrophages are associated with increased pro-inflammatory cytokines (tumor necrosis factor alpha, interleukin 1beta, macrophage inflammatory protein 1-alpha) compared with the $\epsilon 3$ allele (Huebbe *et al.* 2010).

2.6.6) Vascular risk factors

The conventional assumption is that there are two distinct forms of dementia: vascular dementia (VaD) and AD (Roth 1955). VaD has usually been associated with vascular risk factors and AD without. Epidemiological evidence however, indicates that vascular risk factors may be involved in the aetiology not only of dementia in general, but also specifically of AD (Kivipelto *et al.* 2001).

Although the amyloid hypothesis formed the basis for many investigations, the actual evidence available to substantiate the claim that amyloid deposition in the brain is the sole cause of AD, is limited. Growing evidence from epidemiological, pharmacological, neuroimaging and animal studies have suggested that AD is a vascular disorder caused by impaired cerebral perfusion (de la Torre *et al.* 1997).

Stroke and AD

Stroke and AD are no longer regarded as separate disease entities. It has been clear for some time that stroke and dementia are related (Snowdon *et al.* 1997, Pohjasvaara *et al.* 1998, Kalaria 2000). Persons who have had a stroke are twice as likely to develop dementia compared with those who are stroke-free (Leys *et al.* 2005).

The traditional view is that infarcted brain tissue is associated with dementia (Tomlinson *et al.* 1970). It is not clear however, whether stroke is able to initiate a dementing process with progressive deterioration. It is possible that vascular risk factors which are common to both stroke and AD, work synergistically with the effects of an infarct to further impair cerebral perfusion and hasten cognitive decline (Tatemichi *et al.* 1990, Gottesman *et al.* 2010).

White Matter Hyper-intensities

Studies relating to the involvement of "silent" brain injury and its association with AD have added to the evidence in support of a vascular cause for AD. Neuroimaging has allowed for the identification of cerebral infarcts that are silent but share the same risk factors as stroke (DeCarli *et al.* 2004).

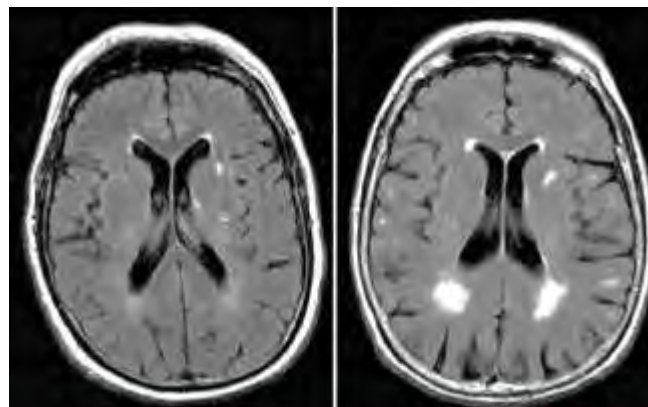
White matter hyperintensities (WMHs) is a radiological term for non-specific changes in the cerebral white matter which may be detected with high frequency magnetic resonance imaging in older persons. Leukoaraiosis (Greek: white rarefaction), are white matter changes seen using computed

tomography scanning. Despite having no clinically associated symptoms, it is believed that underlying vascular risk factors such as hypertension, diabetes, dyslipidaemia, smoking and atrial fibrillation are the primary cause of WMHs (Hachinski *et al.* 1987). Since these lesions are found in cognitively healthy older persons as well as impaired individuals, WMHs may be a useful marker of normal cognitive ageing and of clinically relevant cognitive decline (Steingart *et al.* 1987, Bronge *et al.* 2002).

The presence of WMHs would normally influence a clinician to favour a diagnosis of Vascular Dementia. However, in recent neuropathological studies of older persons with AD, it is thought that neurodegenerative processes such as gliosis, microglial infiltration, inflammation, and amyloid angiopathy may all contribute to WMHs. Some researchers believe that WMHs are more strongly associated with neurodegenerative diseases such as AD than with vascular disease (Appel *et al.* 2009).

In summary, it is possible that WMHs precede, coincide with, or result from, vascular and neurodegenerative processes. The degree of silent brain injury and WHM accrual over time may be directly related to the rate of cognitive decline.

Figure 2.5: White matter hyperintensities on MRI scan



Debette et al. 2010

Mechanisms of neuronal injury

The vascular hypothesis of injury arose when the Neuropathology Group of the Medical Research Council Cognitive Function and Ageing Study found that 80% of their sample population group, at autopsy, demonstrated vascular abnormalities in the brain and that these were worse in those with AD (The Medical Research Council Cognitive Function and Ageing Study (CFAS) 2001).

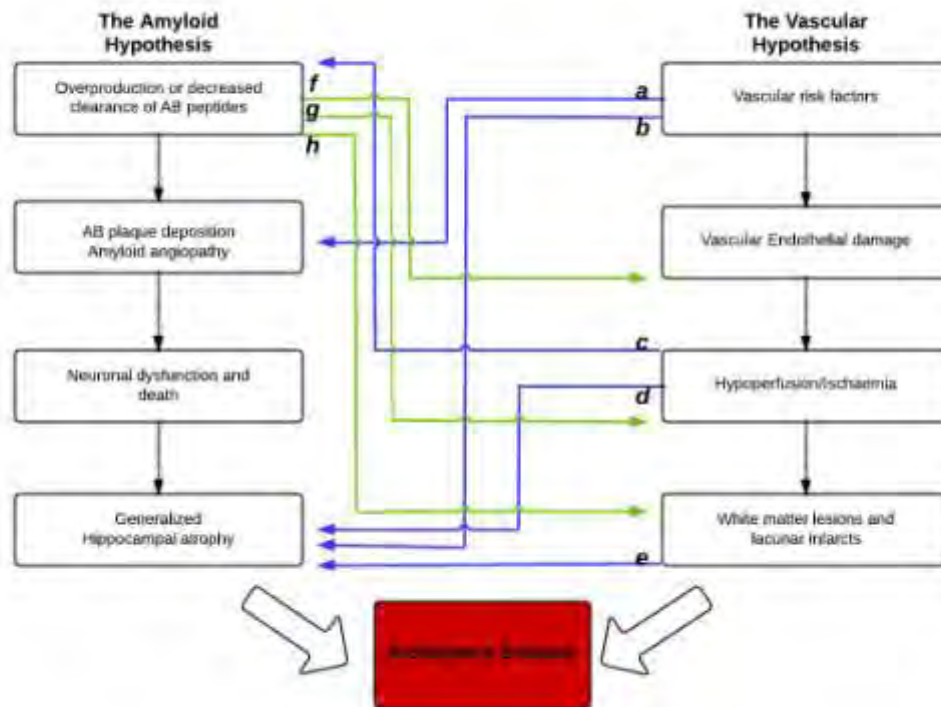
This theory implies that vascular risk factors induce neurovascular dysfunction, causing endothelial leakage by oxidative stress and inflammation. The latter leads to a hypoperfusion state or ischaemia, which is represented radiologically by infarcts and WMHs (de la Torre *et al.* 2004). White matter changes and infarcts are associated with cognitive decline (Steingart *et al.* 1987, Vermeer *et al.* 2003).

Thus, in patients with AD, the pathology is often mixed and the amyloid hypothesis alone is not a sufficient biological explanation for AD pathology. We can assume that the amyloid hypothesis and vascular hypothesis overlap in some way, or act synergistically to produce the cognitive impairment in AD. The Honolulu Asia Ageing Study, a community based study in old men, suggested that the amyloid burden of AD and vascular pathology both independently contributed to the diagnosis of AD. Thus, the co-existence of these two mechanisms is likely to result in a worse cognitive state than might be expected if they acted separately (Laurer *et al.* 2008).

Figure 2.6 explains possible biological mechanisms of interaction and overlap between the amyloid and vascular hypotheses of injury in AD proposed by several studies (Iadecola 2010, van Norden *et al.* 2011). The associations are as follows:

- a) Amyloid depositions occur in the brain, the pathological hallmark of AD (Selkoe 1991, Tomlinson *et al.* 1970, Roth *et al.* 1996). These deposits may also be found in cerebral blood vessels (cerebral amyloid angiopathy). Vascular risk factors in older adults are associated with amyloid angiopathy, which is characterised by amyloid deposition in the walls of leptomeningeal and cortical arteries and arterioles. Amyloid angiopathy is a frequent finding in persons with AD (Jellinger *et al.* 2002).
- b) Vascular risk factors such as hypertension and diabetes are associated with an increased risk of AD via the vascular pathway discussed previously (Sutton-Tyrell *et al.* 1997, Budge *et al.* 2002, Helzner *et al.* 2009). They have also been associated with medial temporal lobe atrophy (de Leeuw *et al.* 2006, Dhikav *et al.* 2011), which is strongly associated with AD (Seshadri *et al.* 2004).
- c) Hypoxia and ischaemia, resulting from vascular insufficiency, could induce AD changes via the vascular pathway or alternatively join the amyloid pathway by increasing the transcription of APP and its cleavage. This would result in an accumulation of AB peptides (Li *et al.* 2009).
- d) Decreased cerebral blood flow is also associated with silent brain injury which may be represented radiologically by WMHs (Hachinski *et al.* 1987). A small study has shown that WMHs are associated with hippocampal atrophy (e) (Korf *et al.* 2005).
- e) A β peptides are toxic to vascular endothelial cells (Rostagno *et al.* 2007). A β is associated with increased tau phosphorylation and increased NFT formation, mitochondrial dysfunction, oxidative stress, apoptosis and direct damage to vascular endothelial cell membranes (Gotz *et al.* 2011; van Norden *et al.* 2011).
- f) Cytotoxic effects of A β peptide on vascular smooth muscle cells results in vasoconstriction and hypoperfusion (Thomas *et al.* 1996). Recently evidence from animal studies has also shown that soluble forms of A β also induce arteriolar constriction (Dietrich *et al.* 2010).
- g) Plasma A β ₁₋₄₀ concentrations were also found to be independently associated with the extent of white matter hyperintensities in subjects with AD, mild cognitive impairment and CAA (Gurol *et al.* 2006).

Figure 2.6: Amyloid and vascular hypotheses in Alzheimer's disease



Adapted from van Norden et al. 2011

2.6.7) Nutritional Factors

Vitamin B₁₂ and cognition

Vitamin B₁₂ (cobalamin) is a water-soluble vitamin with a key role in the normal functioning of the brain and nervous system. Vitamin B₁₂ deficiency occurs in approximately 10% of the general population (Allan 2009) and 17% of the demented elderly population (Basun *et al.* 1994).

In the late 1990s and early 2000s, researchers highlighted the possibility of two types of vitamin B₁₂ deficiency. On the one hand there is clinical vitamin B₁₂ deficiency which is associated with haematological, neurological, and psychiatric manifestations. Neurological manifestations include paraesthesiae, peripheral neuropathy and demyelination of the corticospinal tract and dorsal columns of the spinal cord (subacute combined degenerative disease). Psychiatric symptoms include impaired memory, irritability, depression, dementia and, rarely, psychosis (Robert *et al.* 2003). On the other hand there is subclinical vitamin B₁₂ deficiency which consists of mild biochemical changes

that are consistent with vitamin B₁₂ deficient findings without the clinical manifestations as listed above (Lindenbaum *et al.* 1994, Carmel *et al.* 1999, Carmel *et al.* 2006).

Vitamin B₁₂ deficiency is common in older persons (Pennypacker *et al.* 1992, Lindenbaum *et al.* 1994, Gale *et al.* 1996, Clarke *et al.* 2003, Flood *et al.* 2006) and is believed to be subclinical rather than overt (Rosenberg *et al.* 1992). The cause of this deficiency is largely unknown, but it is hypothesised that age related-aclhorydia leads to decreased ability to extract vitamin B₁₂ from food protein (Carmel 2008, Allen 2009). Therefore, malabsorption or chronic dietary insufficiency may be a common cause of subtle vitamin B₁₂ deficiency in the elderly and is largely undiagnosed (Morris *et al.* 2012).

Epidemiological evidence linking low vitamin status with decline in neurocognitive function in the elderly has been described in earlier years (Goodwin *et al.* 1983). Since then, studies examining the relationship between vitamin B₁₂ and cognition have provided evidence of a link between low vitamin B₁₂ and poorer cognition (Riggs *et al.* 1996, La Rue *et al.* 1997, Clarke *et al.* 1998, Selhub *et al.* 2000).

The mechanisms by which vitamin B₁₂ deficiency affects cognition are however, unclear. Researchers are not sure if the deficiency affects cognition directly, or if it is a consequence of ageing which contributes to the neurodegenerative disease process. Biologically, low vitamin B₁₂ and its effect on cognition may be mediated by high plasma levels of the compounds homocysteine and methylmalonic acid (MMA) (Carmel 1996). More recently researchers have provided evidence that vitamin B₁₂ deficiency is also linked to brain atrophy and white matter changes (Vogiatzoglou *et al.* 2008, Smith *et al.* 2009).

Classical vitamin B₁₂ deficiency is associated with damage to the white matter in the spinal cord and in the brain. This has been attributed to damage to myelin as a result of deficient methylation of myelin basic protein (Weir *et al.* 1999). These white matter changes in the brain have been linked to low levels of vitamin B₁₂, MMA and holotranscobalamin (holoTC) (de Lau *et al.* 2009). In a longitudinal study, low-normal baseline vitamin B₁₂ status also predicts whole brain atrophy in community-dwelling elderly individuals (Vogiatzoglou *et al.* 2008).

Folate and cognition

Folates are essential components of the human diet and are synthesized by micro-organisms and plants. The human body needs folate to synthesize deoxyribonucleic acid (DNA), repair DNA, and methylate DNA as well as to act as a cofactor in biological reactions (Mattson *et al.* 2003).

Opinions relating to folate deficiency and its effects on the nervous system have differed over the last 50 years. At present, the benefits and risks of folic acid supplementation in nervous system disorders have received both positive and negative reviews.

Folate is involved in methylation processes which are essential for brain development during the early stages of brain growth (Georgieff *et al.* 1999). It is also important in maintaining proper brain functioning later in life (Selhub *et al.* 2000). Folate deficiency generally occurs as a result of inadequate intake, decreased absorption or impaired metabolic utilization. It is associated with congenital neural tube defects, megaloblastic anaemia and psychiatric diseases such as

schizophrenia and psychosis (Reynolds 1976). In many cases, patients present with neurological symptoms in the absence of haematological signs (Reynolds *et al.* 1979).

Several studies have provided evidence of an association between red cell folate, cerebrospinal fluid (CSF) folate and more recently plasma homocysteine on the one hand, and cognitive decline and AD on the other (Reynolds *et al.* 1976, Clarke *et al.* 1998, Bottiglieri *et al.* 2000, Mattson and Shea 2003, Ramos *et al.* 2005). Folate and vitamin B₁₂ deficiency have shown independent associations with neurological abnormalities, but are also linked to elevated homocysteine levels (Ravaglia *et al.* 2005, Kim *et al.* 2008). Interactions between these nutritional factors and high homocysteine levels have been a focus of many studies in Europe and North America and form the basis for much of the discussion later on in this chapter.

In elderly populations, the incidence of folate deficiency is higher and is often related to depression and cognitive impairment (Sneath *et al.* 1973, Clarke *et al.* 1998). Snowdon *et al.* showed a correlation between low folate and cerebral atrophy which was greater in persons with AD compared to those without AD (Snowdon *et al.* 2000). Other studies have demonstrated that folate and vitamin B₁₂ deficiency doubled the risk of AD (Wang *et al.* 2001).

The mechanisms by which folate deficiency cause cognitive impairment are not clear. Folate deficiency could cause cognitive decline via vascular mechanisms mediated by the consequences of high homocysteine concentrations in low folate conditions (pg. 43). Alternatively, folate deficiency could lead to changes in the numerous methylation processes in the brain. Folate provides the methyl group for the conversion of homocysteine to methionine. Methionine is then activated to S-adenosyl methionine (SAM), a major donor for several methyltransferase reactions. In low folate conditions, SAM levels are low and homocysteine levels are elevated. Low folate levels results in damage to DNA and impaired DNA repair. The latter could result in genetic mutations or lead to cell apoptosis (Kruman *et al.* 2000).

Vitamin B₁₂ and folate treatment in cognitive impairment

Folic acid supplementation in pregnancy and folate fortification of foods has reduced the incidence of neural tube defects especially in developing countries such as South Africa (Sayed *et al.* 2008). Controlled trials examining vitamin B₁₂ and folate supplementation to reduce plasma homocysteine concentrations and cognitive decline have resulted in mostly negative findings (Sun *et al.* 2007, Aisen *et al.* 2008, Malouf *et al.* 2008). I shall discuss this later on.

Plasma homocysteine and its interactions and interrelationships are the focal points in this study. Robert Clarke has added the 'homocysteine hypothesis' to vascular and amyloid theories surrounding the aetiology of AD. Clarke, along with other researchers, has identified mild to moderately elevated plasma homocysteine levels as a potential modifiable risk factor for AD. It is difficult to elaborate on the association between homocysteine and AD. Their interaction is complicated by the added effects of ApoE ε4 status, vitamin B₁₂ and folate deficiency as well as vascular disease. In the discussion that follows, I shall highlight the main findings in the literature pertaining to plasma homocysteine and its relationship to cognitive impairment.

2.7) Plasma Homocysteine

Background

In 1932, Butz and Du Vigneaud, biochemists at the University of Illinois, discovered a new amino acid resulting from removal of the methyl group of methionine – a compound regularly consumed in our diet (Du Vigneaud *et al.* 1999). Homocysteine, as it was named, is a non-protein sulphur containing amino acid which occurs naturally in the human body.

The biomedical significance of plasma homocysteine only became known in the 1960s. Kilmer McCully *et al.* discovered an association between elevated plasma homocysteine levels and arteriosclerosis (McCully 1969). These elevated plasma homocysteine levels were the result of underlying vitamin B₁₂ and folate deficiencies. The role of nutritional deficiencies and elevated plasma homocysteine levels in vascular disease challenged conventional theories regarding saturated fats and cholesterol dietary risk factors, and resulted in an explosion of research pertaining to plasma homocysteine and its determinants in the years following McCully's discovery. Plasma homocysteine has been implicated in other important disease processes such as rheumatoid arthritis, hormonal imbalances, renal failure, cancer and neurodegenerative diseases like AD (Kuhn *et al.* 1998, Wollensen *et al.* 1999, Wu *et al.* 2002).

2.8) Homocysteine and AD

The interaction between the determinants of plasma homocysteine and homocysteine is complex and often confusing. It is not clear whether these factors act independently or if, when combined, act synergistically to potentiate cognitive impairment.

The determinants of plasma homocysteine concentrations

Plasma homocysteine refers to the sum of protein-bound, free oxidised and reduced species of homocysteine in plasma. It is usually 5-15µmol/L in healthy subjects (Ueland *et al.* 1993). Hyperhomocysteinaemia may be classified as moderate (15–30mmol/L), intermediate (31–100mmol/L), or severe (>100mmol/L) (Selhub *et al.* 2000). Moderate to severe cases of hyperhomocysteinaemia are rare and often related to genetic mutations (Selhub *et al.* 2000). Elevated plasma homocysteine levels (>15mmol) are linked to multiple clinical conditions such as vascular disease, whereas persons with lower homocysteine levels have overall better physical and mental health (Refsum *et al.* 2006).

Table 2.2 summarises the factors that are thought to be causes of elevated plasma homocysteine concentrations. For the purposes of this study, I have elaborated on 3 relevant determinants of hyperhomocysteinaemia, viz. age, vascular disease and the nutritional factors, vitamin B₁₂ and folate.

Table 2.2
Causes of elevated plasma homocysteine levels

Genetic	Methylenetetrahydrofolate reductase (MTHFR) deficiency MTHFR defect (rare) Cystathionine beta synthase deficiency (CBS) deficiency (heterozygotes) CBS defect (homocystinuria – homozygotes) (rare) Functional methionine synthase deficiency (rare)
Nutritional Causes	Folic acid deficiency Vitamin B12 deficiency Vitamin B6 deficiency
Systemic factors	Renal disease Cancer Hypothyroidism Psoriasis Diabetes mellitus Acute phase of stroke or myocardial infarction
Physiological factors	Increased age Male sex Menopause Race
Drugs	Anticonvulsants (phenytoin, carbamazepine) Oral contraceptives Methotrexate Nitrous oxide Trimethoprim Sulfasalazine

Homocysteine Metabolism (Selhub 1999)

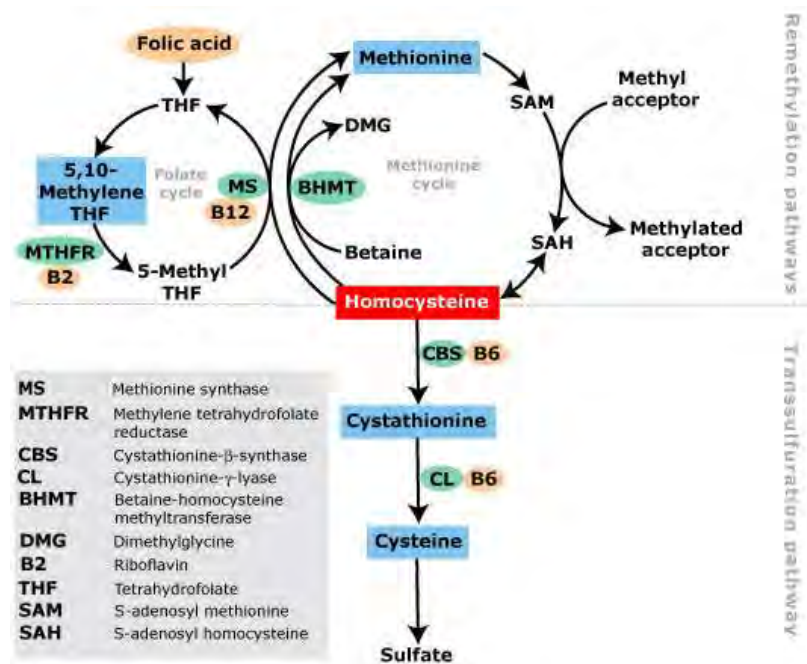
Homocysteine metabolism is at the intersection of two metabolic pathways: remethylation and transsulfuration. The remethylation pathway allows for the elimination of the body's free homocysteine. In this pathway, homocysteine receives a methyl group from N-5-methyltetrahydrofolate (N-5 MTHF) or from betain to form methionine. The reaction with N-5MTHF occurs in all tissues and is vitamin B₁₂ dependent and requires the enzyme methionine synthase (MS), whereas the reaction with betain occurs in the liver, is vitamin B₁₂ independent and is dependent on the enzyme betain-homocysteine-methyltransferase. A considerable proportion of

methionine is then activated by ATP to form S-adenosylmethionine (SAM). SAM serves primarily as a universal methyl donor to a variety of acceptors. S-adenosylhomocysteine (SAH), the by-product of these methylation reactions, is subsequently hydrolysed, thus regenerating homocysteine, which then becomes available to start a new cycle of methyl-group transfer.

In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine catalysed by cystathionine β -synthase (CBS). Cystathionine is hydrolysed γ -cystathionase (CYS), to form cysteine and α -ketobutyrate. This process is called transsulfuration because it results in the creation of sulphate by-products, which are then excreted in the urine. In addition to the synthesis of cysteine, the transsulfuration pathway effectively catabolizes excess homocysteine.

Homocysteine metabolism in the brain is different from systemic homocysteine metabolism. In the liver and kidneys, there is an alternate pathway for remethylation of homocysteine to methionine via the action of MS and that is through the action of betain-homocysteine-methyltransferase which has not been detected in brain cells. CNS cells and neurones do not seem to express the complete transsulfuration pathway, therefore the metabolism of homocysteine in the brain is dependent on vitamin B₁₂ and folate. If vitamin B₁₂ and folate are deficient homocysteine is elevated and may cause damage via several pathways.

Figure 2.7: Homocysteine metabolism



Filip Cristiana and Zamosteanu Nina

<http://www.homocysteine2011.com/homocysteine-metabolism#sthash.VE2jZyl0.dpuf>

Age, plasma homocysteine concentrations and cognition

Plasma homocysteine concentrations increase with advancing age and many studies have shown a positive correlation between these two variables (Selhub *et al.* 1993, Nygard *et al.* 1998). There are however, age-related diseases which may account for the elevated plasma homocysteine concentrations observed in the elderly. These diseases include renal dysfunction and vascular disease (Wollensen *et al.* 1999, Steingart *et al.* 1987). Researchers have often questioned whether these factors together with age cause elevated homocysteine levels and in this way potentiate cognitive impairment or whether they act independently. Brattstrom *et al.* showed that plasma homocysteine concentrations increased with age, but that this relationship, in turn, was largely influenced by serum creatinine concentrations (Brattstrom *et al.* 1994). Other studies confirm that renal function, as measured by serum creatinine concentrations, is a major determinant of plasma homocysteine levels (Norlund *et al.* 1998, Bostom *et al.* 1999, Wollensen *et al.* 1999). This relationship could be the result of poor kidney function arising in patients with a long history of vascular disease (Moustapha *et al.* 1994).

Vascular disease, elevated homocysteine concentrations and cognition

In the 1980s, and with improvements in quantifying plasma homocysteine concentrations (Refsum *et al.* 1985), it became generally accepted that hyperhomocysteinaemia was a potent independent risk factor for atherosclerotic vascular disease (Clarke *et al.* 1991; den Heijer *et al.* 1996; Selhub *et al.* 1995; Ueland *et al.* 2000).

Clarke established that elevated homocysteine levels were independently associated with vascular disease (Clarke *et al.* 1991). Boushey *et al.* concluded that homocysteine was an independent graded risk factor for arteriosclerotic vascular disease. The odds ratio of cardiovascular disease with a 5µmol increment in homocysteine concentration was 1.5, and the odds ratio for cerebrovascular disease with the same increment in homocysteine concentration was 1.6 (Boushey *et al.* 1995). Eikelboom *et al.* demonstrated that moderate elevations in plasma homocysteine concentrations were also independently associated with vascular disease (Eikelboom *et al.* 1999). Other studies have also shown a link between mild to moderate levels of homocysteine with cardiovascular disease, cerebrovascular disease and stroke (Clarke *et al.* 2002; de Koning *et al.* 2003).

Elevated plasma homocysteine levels have also been linked to stroke and myocardial infarction (MI) (Bots *et al.* 1999). As part of the Rotterdam Study, Bots *et al.* looked at homocysteine levels in a cohort of 144 MI participants and 120 stroke participants. They showed that the risk of MI and stroke were directly related to plasma homocysteine concentrations in older persons independent of age, sex and other vascular risk factors (Bots *et al.* 1999). These results were supported by other researchers (Verhoef *et al.* 1996, McIlroy *et al.* 2002). In a meta-analysis, Wald *et al.* showed a graded independent association between plasma homocysteine concentrations and ischaemic heart disease (IHD), deep vein thrombosis (DVT) and stroke (Wald *et al.* 2002). Nygard showed that plasma homocysteine was a strong predictor of mortality in patients with confirmed coronary artery

disease. Other studies, such as the homocysteine studies collaboration, found that homocysteine levels were, at most, a modest predictor of IHD and stroke in healthy populations (Nygard *et al.* 1997, Homocysteine Studies Collaboration 2002).

Previously I discussed WMHs as possible biomarkers for underlying small vessel disease and silent brain injury (Hachinski *et al.* 1987, DeCarli *et al.* 1999). WMHs are associated with an increased risk of stroke (Vermeer *et al.* 2003) and elevated homocysteine levels are linked to stroke and atherosclerotic outcomes (Perry *et al.* 1995, Bots *et al.* 1997 and 1999). Studies examining the relationship between white matter lesions and plasma homocysteine are few, but the evidence is that hyperhomocysteinaemia may be a risk factor for white matter lesions (Wright *et al.* 2005, Fuh 2010). A recent study revealed that high plasma homocysteine levels are associated with the presence of silent brain infarcts (Seshadri *et al.* 2008).

In summary, there is clear evidence to suggest that vascular injury to the brain, both overt and silent, is associated with AD and with elevated plasma homocysteine concentrations. By association, therefore, elevated plasma homocysteine could causally relate to AD as well.

Many of the studies which have determined an association between AD and plasma homocysteine concentrations have also included vitamin B₁₂ and folate measurements. Due to their closely linked metabolic associations, they are frequently studied together to determine the effects on cognition.

Homocysteine, vitamin B₁₂, folate and cognition

The first study reporting the association between plasma homocysteine and AD was published in 1990 (Regland *et al.* 1990). Our current knowledge regarding plasma homocysteine and its association with cognitive impairment is based on retrospective, prospective and interventional study findings.

Retrospective studies have shown that plasma homocysteine levels are higher in AD patients compared with cognitively healthy controls (Clarke *et al.* 1998, McCaddon *et al.* 1998, Mizrahi *et al.* 2004, Gallucci *et al.* 2004). Prospective studies following participants over a period of time have generally demonstrated that elevated homocysteine concentrations at baseline predicted cognitive decline and dementia at a later stage (Seshadri *et al.* 2002, Ravaglia *et al.* 2005, Haan *et al.* 2007, Oulhaj *et al.* 2010). The majority of randomised controlled trials in patients with AD using folate, vitamin B₁₂ or a combination of both, have demonstrated that lowering plasma homocysteine levels did not prevent cognitive decline (Sun *et al.* 2007, Aisen *et al.* 2008).

In 1998 Clarke *et al.* hypothesised that elevated homocysteine levels, by then an accepted vascular risk factor, may also be relevant to AD. They demonstrated that elevated homocysteine as well as low vitamin B₁₂ and folate levels, were present in patients with AD (Clarke *et al.* 1998). Several other studies since then have supported these findings (McCaddon *et al.* 1998, Selley 2003, Linnebank *et al.* 2010). Although these researchers were not clear as to which factors determined the elevated plasma homocysteine concentrations in the participants with AD, the majority described an inverse association between homocysteine and vitamin B₁₂ and folate levels i.e. plasma homocysteine concentrations increased with lower concentrations of vitamin B₁₂ and folate.

Several longitudinal studies, with greater participant numbers, subsequently showed that persons with high homocysteine levels had a greater risk of Alzheimers' disease compared to those with lower homocysteine levels (Seshadri *et al.* 2002, Ravaglia *et al.* 2005). Seshadri *et al.* demonstrated

that elevated homocysteine concentrations in cognitively healthy persons was associated with an increased risk of AD after a median of 8 years follow-up. They showed that baseline plasma homocysteine concentrations greater than 14mmol/l nearly doubled the risk of AD at a later stage, and that each 5 mmol/l increment in homocysteine concentration increased the risk of AD by 40%. More importantly, the association between homocysteine with cognitive decline was independent of age, sex, education, renal function and other vascular risk factors. Other studies supported these findings and showed that elevated homocysteine concentrations and low folate levels were independent predictors of dementia and AD. Ravaglia *et al.* reported a 2-fold increase in the risk of dementia associated with hyperhomocysteinaemia (>15mmol/l) in an elderly Italian population group (Ravaglia *et al.* 2005). More recently, researchers showed that elevated homocysteine concentrations in midlife was an independent risk factor for dementia in women later on in life, and that elevated homocysteine levels predicted conversion of MCI to AD (Annerbo *et al.* 2006, Oulhaj *et al.* 2010, Zylberstein *et al.* 2011).

Whilst most studies have shown an association between plasma homocysteine and cognitive impairment, few studies by comparison have demonstrated no association between these two variables. Some researchers have found that high plasma homocysteine levels were not related to a decline in memory scores over time and that age was a significant confounder in all the analyses (Luchsinger *et al.* 2004). Similarly, Kalmijn *et al.* showed that elevated homocysteine levels were associated with advancing age but not with cognitive decline (Kalmijn *et al.* 1996).

In several of the studies mentioned above, authors have linked associations between low vitamin B₁₂ and folate levels with high homocysteine levels and poorer cognition. Cross-sectional studies over the past decade have demonstrated a positive relationship between serum vitamin B₁₂ levels and cognition i.e. better vitamin status is associated with better cognition. The inverse relationship between vitamin B₁₂ and plasma homocysteine concentrations has been well documented in literature from European and North American studies (Stabler *et al.* 1988, Selhub *et al.* 1993, Ubbink *et al.* 1993, McCaddon *et al.* 1998).

A few studies have demonstrated that plasma homocysteine concentrations correlated negatively with MMSE scores, and that serum vitamin B₁₂ and serum folate levels were positively correlated with MMSE scores (Bernard *et al.* 1998, Stewart *et al.* 2002; Duthie *et al.* 2002, Hin *et al.* 2006). Seshadri *et al.* found that homocysteine and vitamin B₁₂ and folate were negatively correlated, and that vitamin B₁₂ and folate were positively correlated with cognition (Seshadri *et al.* 2002).

Important evidence of these associations was identified by Robert Clarke who reported an association between raised plasma homocysteine levels, low serum vitamin B₁₂ and low serum folate with confirmed histological changes in participants with AD (Clarke *et al.* 1998). The vast majority of studies subsequently have produced equivocal findings (Quadri *et al.* 2004, Kim *et al.* 2008). Most have indicated that both vitamin B₁₂ and folate together are associated with cognitive impairment but failed to show that low vitamin B₁₂ concentrations in isolation was a risk for AD and dementia (Wang *et al.* 2001). Evidence for an association between folate alone with AD or dementia has been more consistent. The majority of these authors agreed that lower folate levels are associated with cognitive decline (Ramos *et al.* 2005, Ravaglia *et al.* 2005, Kim *et al.* 2008).

There are several theories that might explain the difficulty in establishing a clear link between low vitamin B₁₂ concentrations and poor cognition. Firstly, it is more useful to identify cognitive impairment in persons with low vitamin B₁₂ levels than to look for low vitamin B₁₂ concentrations in

cognitively impaired persons (Smith *et al* 2009). This is because many other factors, in addition to low levels of vitamin B₁₂ levels, could account for the cognitive impairment in AD.

Secondly, total plasma vitamin B₁₂ concentrations are thought to be poor markers of vitamin status in tissues (Refsum *et al.* 2006). The vitamin B₁₂ carrier protein holotranscobalamin (holoTC), plasma homocysteine concentrations or methylmalonic acid (MMA) could more accurately represent the tissue availability of vitamin B₁₂ compared with total plasma B₁₂ (McCracken *et al.* 2006). Vitamin B₁₂ is carried on one of two carrier proteins, haptocorrin and transcobalamin. Transcobalamin, when saturated, becomes holoTC. HoloTC binds less than 25% of cobalamin but is considered to be more biologically active because it transports cobalamin to various cells in the body whereas haptocorrin does not. Therefore, measurements of holoTC provide a more accurate representation of the tissue availability of vitamin B₁₂ than the serum B₁₂ (Refsum *et al.* 2006).

Thirdly, the association between vitamin B₁₂ status and cognition is influenced by the folate status of the population studied. In the 1940s and 1950s high levels of folic acid were used to treat anaemia (Reynolds 1976). Despite the improvement haematologically, low doses of folate could aggravate neurological symptoms associated with vitamin B₁₂ deficiency and potentiate cognitive impairment (Reynolds 1976, Savage *et al.* 1995). Selhub *et al.* showed that in a folic acid fortified population group, the high folic acid/low vitamin B₁₂ combination (<148pmol/l) worsened cognition whereas the normal folate/low vitamin B₁₂ combination improved cognition (Selhub *et al.* 2009). A more recent study showed that the combination of high folate levels and low-normal vitamin B₁₂ concentrations (187-256pmol/l) predicted cognitive decline (Morris *et al.* 2012). In another study however, authors showed that the high folate/low B₁₂ combination in a non-folate fortified population, reduced the risk of cognitive impairment (Doets *et al.* 2013). Therefore, the safety of folic acid fortification especially in older persons, who are at a higher risk of vitamin B₁₂ deficiency, is uncertain.

Finally, some studies have made no distinction between clinical and subclinical vitamin B₁₂ deficiency as it is understood today (Carmel 2012). Additionally, there are no clear biological vitamin B₁₂ cut-off values that has been standardised amongst studies. Some use traditional low/high values, where others use groupings of vitamin B₁₂ (low, subclinical, and normal). Although there are very few studies which have tried to overcome the failure of initial studies, a longitudinal study in 2007 by Clarke *et al* tried to account for all the discrepancies listed previously. They found that when controlling for possible confounding variables, MMA levels, holoTC concentrations and plasma homocysteine, biological markers of low vitamin B₁₂ status, predicted cognitive decline over a 10 year period (Clarke *et al.* 2007).

Plasma homocysteine may be causal in AD or a biomarker of underlying nutritional deficiency i.e. high levels of plasma homocysteine levels could be toxic to the brain vasculature and neurones, or, these high levels could reflect subclinical vitamin B₁₂ deficiency which we know to be prevalent in older persons (Flood *et al.* 2006, Clarke *et al.* 2003). The interest in homocysteine is because plasma homocysteine levels may be lowered using folate, vitamin B₁₂ or a combination. Plasma homocysteine may be a modifiable risk factor (Haan *et al.* 2007). Controlling homocysteine concentrations could also have an impact on disease progression (Seshadri *et al.* 2002). Clarke and Smith in their studies both suggested the need to perform randomised controlled trials to determine

the relevance of vitamin B₁₂ supplementation for the prevention of dementia (Clarke *et al.* 2007, Smith 2008).

Homocysteine lowering therapy

Most trials have shown that vitamin B₁₂ and/or folate supplementation does not slow cognitive decline (Sun *et al.* 2007, Aisen *et al.* 2008). Malouf *et al.*, in their review, found that folate and vitamin B₁₂ supplementation reduced homocysteine levels, but did not alter the cognition in both cognitively healthy and impaired participants (Malouf *et al.* 2008). More recently another meta-analysis showed that vitamin B₁₂ supplementation alone did not improve cognition in those with or without cognitive impairment (Ford *et al.* 2012).

Despite these findings, there has been an abundance of evidence from observational studies which consistently demonstrate the interrelationships between plasma homocysteine, folate and cognition and, to a lesser extent, vitamin B₁₂ and cognition. Plasma homocysteine could still be a modifiable risk factor, but standardised criteria for treatment in preventing its adverse effects on cognitive function have not yet been established (Morris 2012). Older persons should therefore be encouraged to maintain a good dietary intake of vitamin B₁₂ until such standardised criteria become available. Smith *et al.* recently proposed that people who are cognitively impaired with normal biochemical vitamin B₁₂ concentrations in the low-normal range (up to 300p mol/l), should be treated with vitamin B₁₂ to improve symptoms. The treatment in elderly persons in the same biochemical B₁₂ range, who do not show symptoms, is still unclear (Smith *et al.* 2012).

2.9) Homocysteine and brain atrophy

Brain atrophy in older persons is common. It occurs in both healthy and demented individuals but is much more accelerated in persons with AD. Total cerebral brain volume (TCBV) and hippocampal volumes are accepted as measures of neuronal loss and have been associated with impaired cognitive function and the risk of AD (Seshadri *et al.* 2004).

Deposition of tau protein, formation of neurofibrillary tangles and accumulation of A β contributes to hippocampal atrophy together with damage caused by several other factors. Vascular risk factors, hypertension, diabetes, dyslipidaemia and homocysteine may contribute to hippocampal atrophy (de Leeuw *et al.* 2006, Dhikav *et al.* 2011). Carriers of the ApoE ϵ 4 genotype have been shown to have greater temporal lobe atrophy and poorer memory functions than non-carriers (Dhikav *et al.* 2011).

Several studies have highlighted the association between homocysteine and brain pathology. Williams *et al.* found that homocysteine damaged the hippocampus in normal healthy elderly persons (Williams *et al.* 2002). In the Sydney Stroke Study (131 stroke patients, 81 healthy controls), higher homocysteine levels were related to increased number of strokes and greater cognitive impairment, in particular, frontal-executive function and attention. In the control group, homocysteine was related to increased subcortical atrophy (Sachdev 2004). Most researchers have shown an inverse relationship between plasma homocysteine levels and grey matter volume. A

recent study concluded that this relationship was due to age and cardiovascular risk factors rather than homocysteine itself (Ford *et al.* 2012).

More positive associations have been discovered in studies investigating the effects of B vitamin and folate supplementation (with subsequent homocysteine lowering) on brain volume. In the VITACOG trial, researchers showed that vitamin B treatment decreased plasma homocysteine levels. There was a 31.7% reduction in the final homocysteine concentration compared with placebo. They also found that the accelerated rate of brain atrophy in elderly patients with MCI was slowed by the administration B vitamins (Smith *et al.* 2008). More recently the same group reported the outcome of vitamin B supplementation on cognition in the same study. They concluded that B vitamins appeared to slow cognitive and clinical decline in people with MCI, particularly in those with elevated homocysteine (de Jager *et al.* 2012).

Despite the association between homocysteine and AD, the mechanisms by which they occur remain controversial. How homocysteine may predispose, cause and potentiate AD has been the subject of many human and animal studies. Potential mechanisms of association between hyperhomocysteinaemia and AD may be explained by factors which are common to both entities, and by the effects of homocysteine itself on the nervous system.

2.10) Proposed biological mechanisms of homocysteine-induced cognitive impairment

Vascular Mechanisms

Studies of cellular, animal and human models have suggested several mechanisms by which homocysteine may be involved in cerebrovascular neuropathology. These biological mechanisms, in the context of high plasma homocysteine, ultimately result in cerebral hypoperfusion. This has been well described in the vascular hypothesis of injury in AD (pg. 27 figure 2.6). Plasma homocysteine could reduce cerebral blood flow by enhancing atherogenesis (Steingart *et al.* 1987, Vermeer *et al.* 2003). This could occur via mechanisms causing endothelial injury or as a result of increased thrombosis within vascular cells.

Homocysteine could also stimulate collagen synthesis causing vascular smooth muscle proliferation potentiating atherosclerosis and vascular damage (Tsai *et al.* 1994 and 1996).

Thrombosis activation

Normal haemostasis depends, in some measure, on the balance between pro-aggregatory and pro-thrombotic platelet thromboxane A₂, and vascular prostacyclin, which inhibits platelet aggregation and is antithrombotic. Homocysteine is believed to cause an increase in platelet thromboxane production with enhanced platelet adhesion, without an increase vascular prostacyclin, thereby causing vascular thrombosis ischaemia and cell death (Wang *et al.* 2006).

Structural damage

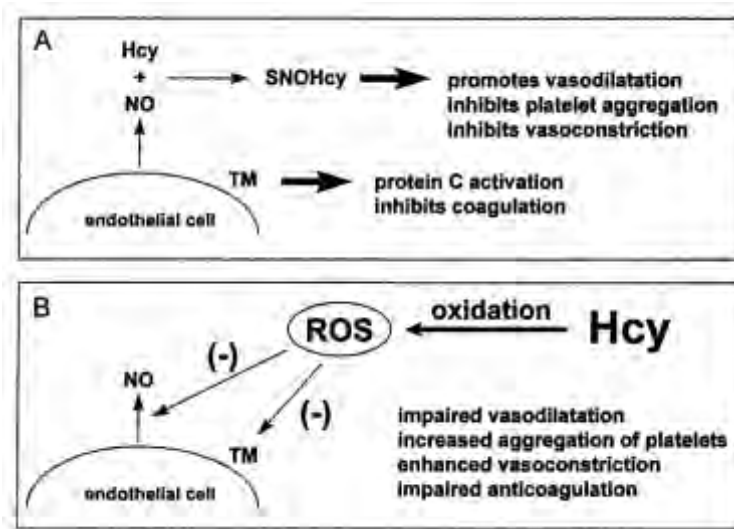
Normal functioning of the blood- brain barrier (BBB) is critical for proper neuronal function including synaptic transmission, remodelling and neurogenesis (Marlatt *et al.* 2008). Homocysteine alters the integrity of the BBB in both AD patients and transgenic mice, facilitating the release of toxic molecules into the central nervous system. These molecules potentiate cell damage and death (Zhou *et al.* 2011).

Oxidative stress

Reduced clearance of reactive oxygen species (ROS) and oxidative deactivation of nitric oxide (NO), produce an imbalance in the oxygen homeostasis in the brain (Weiss *et al.* 2003, Marlatt *et al.* 2008). The brain has a higher oxygen consumption relative to other parts of the body and it is essential that it should be capable of compensating for acute changes in oxygen metabolism and blood flow. If the mechanisms controlling the ROS balance in the brain are impaired for any reason, abnormal cellular and neural processes could occur. Elevated plasma homocysteine levels could increase the production of ROS, increasing calcium influx and enhancing excitotoxicity causing apoptosis (Jacobsen 2000).

Oxidation of homocysteine could also lead to the production of hydrogen peroxide and other ROS which may inactivate NO and thrombomodulin (TM). NO is a potent vasodilator and inhibitor of platelet aggregation. It is also an important mediator of endothelial, platelet and smooth muscle function. At normal plasma concentrations, homocysteine could react with endothelium-derived nitrous oxide to form s-nitroso-homocysteine (SNOHcy) which enhances the vasoprotective effects of NO. TM expressed on the surface of endothelial cells inhibits coagulation by activation of protein C (Figure 2.8). High levels of homocysteine-produced ROS therefore cause impaired vasodilation, increased platelet aggregation, enhanced vasoconstriction and impaired TM anticoagulant activity (Lentz 1997).

Figure 2.8: Oxidative stress: inactivation of nitric oxide



Lentz 1997.

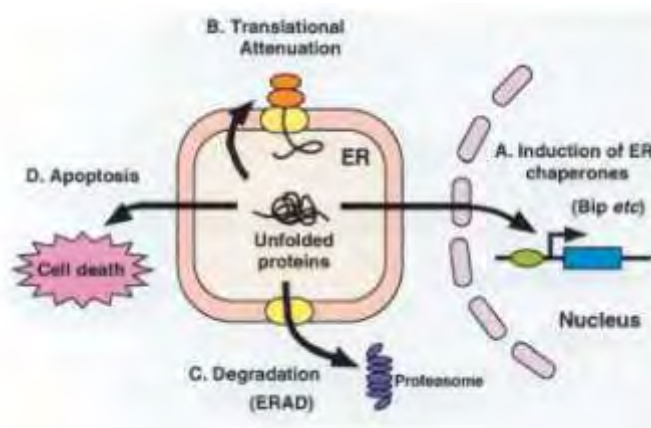
Inflammatory response

Increased plasma homocysteine concentrations result in the increased production of pro-inflammatory cytokines such as monocyte chemoattractant protein (MCP-1) and Interleukin-8 (IL-8) from microglia, the macrophages in the brain. These cytokines cause vascular inflammation which promotes atherogenesis (Poddar *et al.* 2001, Collins *et al.* 2001).

Endoplasmic Reticulum Stress (ER stress)

The endoplasmic reticulum (ER) is the principal site for protein synthesis and maturation in the cell. Physiologically, it regulates protein production, folding and modification. ER stress refers to a state where proteins become unfolded or misfolded and tend to accumulate (Lee *et al.* 2001, Ron *et al.* 2001). Oxidative stress, ischaemia and disturbances of calcium homeostasis may induce ER stress (Minamino *et al.* 2010). Overload of misfolded proteins triggers the unfolded protein response (UPR) in the ER. The UPR has 2 main functions: it aims to restore normal cell functioning by decreasing protein translation (Figure 2.9 B) and secondly it aims to provide the necessary molecular chaperones involved in normal protein folding (Figure 2.17 A). If this does not occur within a specific time frame, the UPR initiates apoptosis and cell death (Ji and Kaplowitz 2004) (Figure 2.17 D). Plasma homocysteine is believed to cause ER stress by disrupting disulfide bond formation resulting in misfolding of proteins, triggering the UPR and ER-initiated apoptosis (Ji *et al.* 2004).

Figure 2.9: ER stress and the UPR response



Araki *et al.* 2003

Mitochondrial dysfunction

Mitochondria are dynamic ATP-generating organelles that contribute to many cellular functions including intracellular calcium regulation, and free radical scavenging. In neurons, mitochondria are numerous in synapses and are responsible for energy production. They are major producers of ROS and at the same time are targets of ROS toxicity. Elevated homocysteine levels producing ROS damage mitochondria causing dysfunctional energy homeostasis and eventual cell death (Pagani *et al.* 2011).

Homocysteine hypomethylation

It has been shown that elevated homocysteine levels as well as folate and vitamin B₁₂ deficiency cause decreased levels of s-adenosyl methionine (SAM), a major methyl group donor, and increased concentrations of s-adenosyl homocysteine (SAH), an inhibitor of methyl-transferases (Gharib *et al.* 1983). A combination of these changes results in decreased overall cellular methylation (Caudill *et al.* 2001). Synthesis and catabolism of several neurotransmitters, as well as the maintenance of DNA methylation are all important biological reactions where the methyl groups are required (Mattson *et al.* 2003).

The lower ratio of SAM/SAH due to elevated homocysteine levels induces DNA damage and apoptosis in neuronal cells (Kruman *et al.* 2000). DNA hypomethylation caused by low vitamin B₁₂ and folate levels could also be responsible for the activation and overexpression of genes involved in AD pathology (Richardson 2003).

Other markers of vitamin B₁₂ deficiency, such as MMA, could also contribute to the pathogenesis of Alzheimer's disease through similar mechanisms such as hypomethylation, A β and tau aggregation, oxidative stress and apoptosis (Obeid *et al.* 2006).

A β elevation and tau phosphorylation

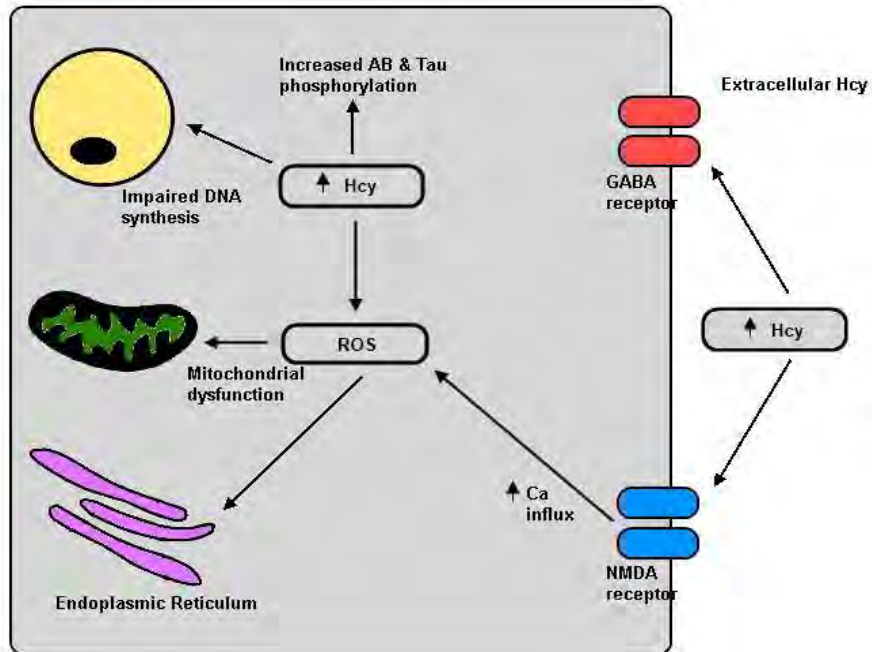
Studies have demonstrated a positive association between plasma homocysteine levels and AB₁₋₄₀ (Irizarry *et al.* 2005). Recent studies provide evidence that elevated homocysteine levels could directly affect A β and tau metabolism and cause an increase in amyloid deposition in the brain (Obeid *et al.* 2006; Stanger *et al.* 2009). Alternatively, homocysteine toxicity could make neurones more vulnerable to damage by A β peptides (Ho *et al.* 2001). Homocysteine also increases tau phosphorylation through an imbalance between SAM and SAH in experimental animal models (Vafai *et al.* 2002).

Excitatory damage

N-methyl-D-aspartate (NMDA), homocysteate, and N-methyl glutamate were found to be the most potent excitatory neurotransmitters causing neuronal necrosis (Olney *et al.* 1971). Glutamate is an important neurotransmitter that plays a key role in long-term potentiation and is important for memory and learning. NMDA is an excitotoxin which may bind to the NMDA receptor mimicking the action of glutamate. In a study of cultured neuronal and glial cells, homocysteine acted as an agonist at the glutamate binding site of the NMDA receptor, causing an increase in cytoplasmic calcium ion and ROS influx within cells (McCully 2009). Activation of the NMDA receptor on endothelial cells may cause intimal hyperplasia (Chen *et al.* 1971) and in smooth muscle cells, cellular proliferation (Qureshi *et al.* 2005).

Gamma (γ)-Aminobutyric (GABA) acid is the chief inhibitory neurotransmitter in the central nervous system. It plays a role in regulating neuronal excitability throughout the nervous system. Homocysteine may compete with GABA at the GABA receptor and may affect its inhibitory function (McCully 2009). The overall effect is increased brain excitotoxicity and neuronal damage.

Figure 2.10: Summary of the homocysteine neurotoxicity in neurones

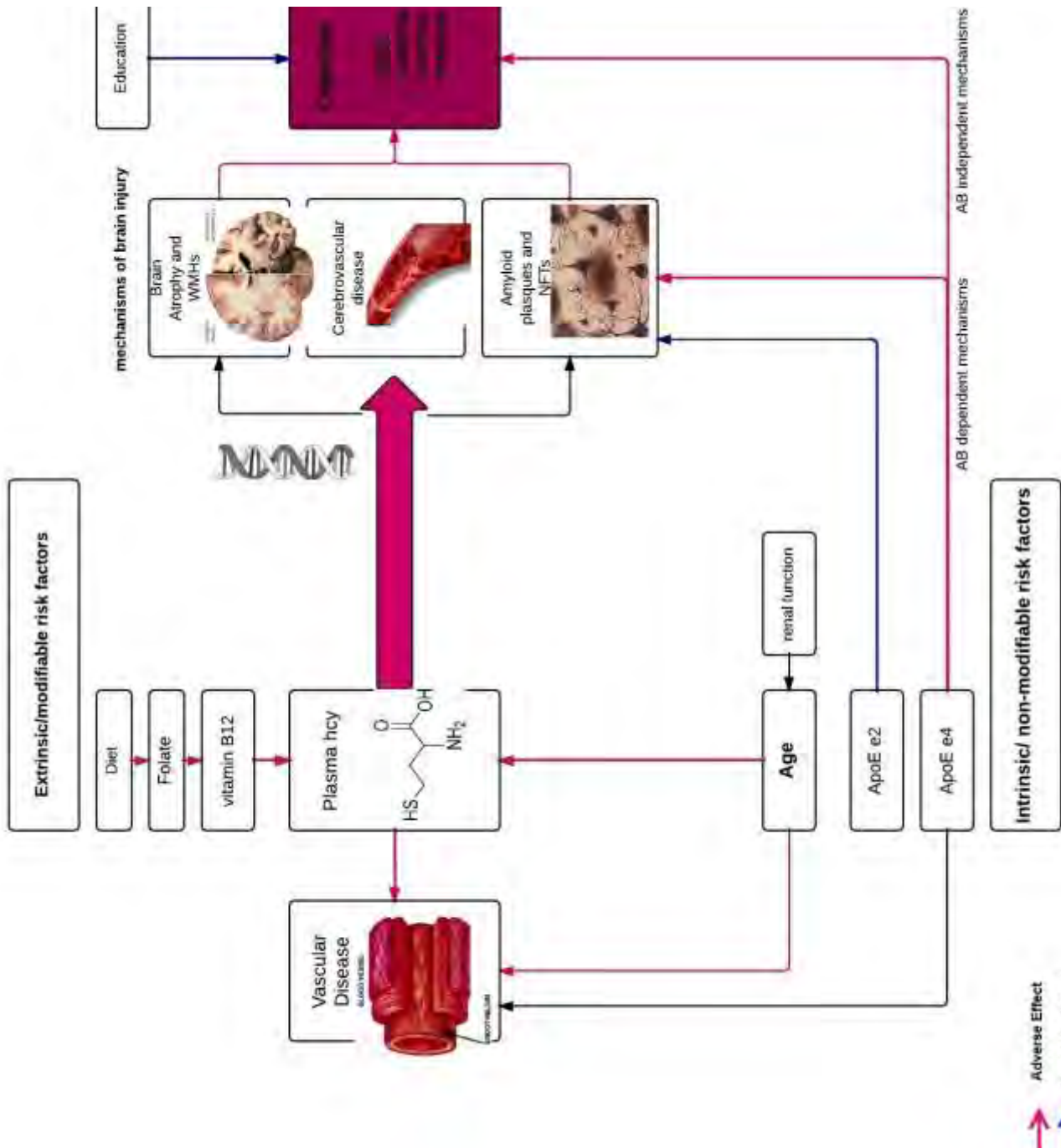


*Neurotoxic effects of homocysteine (Hcy). Hcy may enter the cell from the extracellular space. It may activate the NMDA receptor causing calcium influx and increased ROS within the neuron. ROS causes mitochondrial dysfunction and ER-stress. Hcy can also induce neuronal injury through oxidative stress and production of ROS. Elevated Hcy levels are associated with increased A β deposits, enhanced tau phosphorylation and DNA damage and subsequent neuronal death
Adapted from: Stanger et al. 2009*

Figure 2.11 is a summary of what is currently understood about the associations between plasma homocysteine, vitamin B₁₂, folate, ApoE ε4 and cognition.

- Vitamin B₁₂, folate, vascular disease, the ApoE ε4 allele and plasma homocysteine have all been associated with cognitive impairment and AD.
- Improved SES and greater years of education are associated with better cognition.
- Poor diet, common in older people, could cause deficiencies of folate and vitamin B₁₂ levels. Vitamin B₁₂ contributes to cognitive impairment via mechanisms inducing white matter changes and atrophy in the brain. Folate is associated with poor cognition through biological mechanisms such as homocysteine hypomethylation or via the neurotoxic effects of plasma homocysteine.
- The ApoE ε4 allele is a risk factor for late onset AD whereas the ApoE ε2 allele is protective against AD. The ε4 allele is associated with atherosclerosis and vascular disease. It could also induce cognitive impairment via vascular pathways or through abnormal Aβ metabolism. Genetic risk factors are non-modifiable but there is evidence to suggest that the ApoE ε4 renders an individual more susceptible to environmental (modifiable) factors.
- The amyloid and vascular hypotheses have been formulated to explain the pathogenesis of AD. This theory implies that vascular factors work synergistically with the effects of impaired Aβ metabolism to worsen cognition.
- Plasma homocysteine (homocysteine hypothesis) has also been causally linked to AD. Homocysteine concentrations are inversely related to vitamin B₁₂ and folate levels. Homocysteine levels also increase with age. Other age related risk factors such as declining renal function and vascular could contribute to cognitive decline include.
- Plasma homocysteine is causally linked to vascular diseases in the brain, both overt and silent. Homocysteine may induce cognitive impairment via mechanisms which cause cerebrovascular disease, white matter changes and hippocampal atrophy. Homocysteine has been linked to increased amyloid deposition and other neurotoxic mechanisms in the brain.

Figure 2.11: Risk factors associated with Alzheimer's disease



Summary

Plasma homocysteine and its association with cognitive impairment is the main focus of this research study. I have discussed the main studies from North America and Europe which have demonstrated that elevated plasma homocysteine levels are associated with vascular disease as well as neurodegenerative diseases such as AD (Clarke *et al.* 1991, Seshadri *et al.* 2002). Plasma homocysteine concentrations are also determined by the effects of age and renal function (Nygard *et al.* 1998, Brattstrom *et al.* 1994).

Homocysteine concentrations are also largely influenced by vitamin B₁₂ and folate status as a result of their closely linked metabolism (Stabler *et al.* 1988, Selhub *et al.* 1993). Plasma homocysteine is linked to AD via its toxicity to the endothelial blood vessels in the brain. However, other processes involving elevated plasma homocysteine levels could also cause damage to neurones. These include oxidative stress, inflammation, mitochondrial dysfunction and excitatory damage.

A large body of evidence suggests that low concentrations of vitamin B₁₂ (which may be better represented by high plasma homocysteine levels) are common in older persons. This deficiency is associated with impaired cognition. Therefore, it is possible that B vitamin supplementation could be used to reduce plasma homocysteine levels which could, in turn, reduce the risk of developing AD. RCTs have been largely inconclusive thus far and there are no standardised vitamin B₁₂ and folate treatment guidelines available to reduce or slow the rate of cognitive decline.

The ApoE ε4 allele is a well-known risk factor for late onset AD but its relationship with plasma homocysteine has, to my knowledge, not been well researched. Few studies have shown that the ε4 allele may modify the relationship between plasma homocysteine and cognition (Elias *et al.* 2008) and vitamin B₁₂ and cognition (Vogiatzoglou *et al.* 2013).

In South Africa we have no reliable data concerning homocysteine levels and their role in dementia and AD. What we do know is that AD is not uncommon in South Africa and the Western Cape, and that nutritional deficiency is probably more common in our lower income and older population groups. In my research, I hope to test the hypothesis that plasma homocysteine is related to cognition. I should also like to test its associations with vitamin B₁₂ and folate in a sample of older adults with and without cognitive impairment in the Western Cape. Furthermore, I should also like to test the influence of the ApoE ε4 allele on the relationship between plasma homocysteine and cognitive function. Research from Europe and North America hints at the promise of homocysteine-lowering therapy as a way of delaying or preventing cognitive impairment in older persons. My hope is that this will also apply to our part of the world where vitamin deficiencies are more common and the ApoE ε4 allele more frequent in the rapidly ageing local population.

Chapter Three

Aims and Hypotheses

3.1) Introduction

In Chapters One and Two, I discussed how AD relates to certain risk factors, in particular, plasma homocysteine and its determinants, vitamin B₁₂ and folate. To our knowledge, little is known about homocysteine and its associations with AD in an African and South African context.

I wanted to study these inter-relationships for the following reasons:

1. Our impression, and that of colleagues in the fields of cognitive neurology and psychiatry, is that cognitive impairment of varying degrees is under-reported and under-diagnosed in older persons in South Africa. This project allowed us to determine the association between various risk factors and AD in a small group of community dwelling older persons recruited from the greater Cape Town metropole.
2. The South African population consists of a large number of people of poor socio-economic status. Dietary deficiencies, especially the B group of vitamins, are probably more common compared with first world countries.
3. The indigenous population of South Africa is known to have the highest frequency of the $\epsilon 4$ allele of the ApoE gene in the world (Sandholdzer *et al.* 1995; Masemola *et al.* 2007).
4. Homocysteine, vitamin B₁₂ and folate are potentially modifiable risk factors. Early identification may prevent or delay the onset of new cases of disease, and slow disease progression in those with established AD.

Based on these observations I set out to determine more specifically how plasma homocysteine and vitamin deficiencies related to one another and to the degree of cognitive impairment.

3.2) Specific Aims

This study contained both cross sectional and longitudinal components:

- In a cross sectional analysis, I wished to determine:
 - a) The relationship between homocysteine and cognition in all participants.
 - b) The relationship between vitamin B₁₂ and folate on the one hand, and cognition on the other, in all participants.
 - c) The relationship between homocysteine and vitamin B₁₂ and folate in all participants.

For a)-c), I also wished to establish whether these relationships were true for both AD and control participants analysed separately.

- d) To determine the association between homocysteine and cognition in carriers of the ApoE ε4 allele compared with non-carriers.
- In the longitudinal analysis I wished to determine whether baseline homocysteine levels predict subsequent rates of cognitive decline in patients with established AD over a 1 year period. My hypothesis is that participants with high baseline plasma homocysteine levels will decline faster when compared to those with lower baseline homocysteine levels.

3.3) Hypotheses

Hypothesis 1 (H1): Homocysteine levels increase with age.

My hypothesis is that plasma homocysteine levels increased with age in all participants, AD participants and cognitively healthy control participants. As discussed in section 2.7 pg. 35, this finding has been a common one in many studies in industrialized countries.

Hypothesis 2 (H2): Plasma homocysteine concentrations are inversely related to vitamin B₁₂ and folate.

I set out to test the hypothesis that baseline plasma homocysteine concentrations would be inversely associated with vitamin B₁₂ and folate status in patients with AD, as well as in the cognitively healthy participant group. That is, the lower the vitamin B₁₂ and folate concentrations, the higher the plasma homocysteine levels.

Furthermore, I hypothesised that this inverse association between homocysteine and vitamin B₁₂/folate would be greater in AD participants compared with cognitively healthy controls.

Hypothesis 3 (H3): Plasma homocysteine concentrations are directly associated with vascular risk factors.

The link between homocysteine concentrations and vascular risk factors have been well documented (pg. 35). I set out to test the hypothesis that plasma homocysteine concentrations would be higher in persons with vascular risk factors. I also wished to test the hypothesis that persons with increasing numbers of vascular risk factors also showed higher homocysteine levels.

Hypothesis 4 (H4): Serum Vitamin B₁₂ and folate are directly related to cognitive function.

For the most part, studies have shown that lower levels of vitamin B₁₂ are associated with poorer cognition (Goodwin *et al.* 1983, section 2.7 pg. 36). My hypothesis was that when controlling for age,

vitamin B₁₂ and cognition would be directly related i.e. the higher the vitamin B₁₂ levels, the better the cognitive score; the lower the vitamin B₁₂ levels, the poorer the cognition.

Similarly, low folate levels have been associated with poor cognition (pg. 36). I wished to determine the relationship between cognition and serum folate in all participants, AD participants and cognitively healthy controls.

Hypothesis 5 (H5): Plasma homocysteine concentrations are inversely related to cognitive function in all participants.

I wished to test the hypothesis that plasma homocysteine levels are inversely related to cognitive function scores in both healthy controls and AD participants i.e. the higher the homocysteine levels the poorer the cognitive function. As cognition itself might be related to age, I also wished to determine whether age confounded the relationship between plasma homocysteine concentrations and cognition.

Hypothesis 6 (H6): The association between plasma homocysteine and cognition is greater in ApoE ε4 carriers compared with non-carriers.

Elias *et al* showed that homocysteine levels were inversely related to cognitive function, and that the association was greater in ApoE ε4 carriers compared with non-carriers (Elias *et al.* 2007). I therefore wished to test the hypothesis that the association between homocysteine and cognition is greater in ApoE ε4 carriers compared with non-carriers i.e. the inverse relationship between hcy and cognition would have a stronger magnitude of association in participants with 1 or more ε4 alleles compared with those with no ε4 alleles.

Hypothesis 7 (H7): AD participants with high baseline serum homocysteine concentrations will decline faster when compared to those with lower baseline homocysteine levels.

I wished to test the hypothesis that participants with AD and a high baseline homocysteine level, had a faster rate of cognitive decline over a 1 year period compared to those with lower baseline homocysteine levels. Previous studies have demonstrated that individuals with MCI and high-normal homocysteine levels declined more rapidly than those with low-normal homocysteine concentrations (Oulhaj *et al.* 2010).

Chapter Four

Methods and Study Design

Introduction

This project formed part of a larger study which aimed to determine, for the first time, whether a number of established and putative risk factors for dementia in general and AD more specifically, derived from studies in Europe and North America, also applied to our South African population. The study sample included elderly volunteers from the greater Cape Town metropole area and elderly persons referred from the Memory Clinic at Groote Schuur Hospital.

The study contained both cross-sectional and longitudinal components. Recruitment of participants took place between 2008 and 2012.

I joined the research team in 2009, and developed an interest in homocysteine concentrations and its relevance in AD. Several overseas studies have highlighted the close association between vitamin deficiencies and high homocysteine levels. As a result of these, I formulated specific aims and hypotheses for study in a sample population from the Western Cape.

4.1) Study Design

This study was a prospective observational study with both cross-sectional and longitudinal components. In the cross-sectional analysis, total plasma homocysteine, vitamin B₁₂ and folate were studied across the spectrum of cognition, from normal to mild to moderate AD. In the longitudinal study, those participants with normal or mild memory impairment had annual repeat cognitive assessments after initial testing to determine whether these putative risk factors measured at baseline predicted subsequent cognitive decline.

4.2) Sample size

I aimed for a sample size of n=150 for cross-sectional analysis, as previous European and North American Studies have shown this number to be sufficient to produce significant differences in plasma homocysteine concentrations. Reasons for the “attrition” included participants declining cognitively to the point of no longer being able to give informed consent, increased frailty and death.

In total, 113 participants were involved in the study. Statistical significance was set at the α level of 0.05.

4.3) Recruitment of Participants: patients and controls

Participants were recruited from the greater Cape Town metropole area. About one third of the study sample of elderly participants, both patients and controls, were recruited through the Rehoboth Age Exchange, a community organisation and Old Age Home in Hanover Park, a working class district in the greater Cape Town metropole. Other potential volunteers for the study responded to advertisements, placed in various family practices in Cape Town's southern suburbs. More than half of the demented elderly study population, patients with MCI as well as patients with mild or moderate AD, were referred from the Memory Clinic of the Department of Geriatric Medicine at Groote Schuur Hospital (GSH) and the Institute of Ageing of the University of Cape Town.

Inclusion Criteria

- participants >55 years of age
- cognitively intact (normal) participants or participants with mild to moderate Alzheimer's disease diagnosed according to the NINCDS-ADRDA as possible or probable AD (McKhann 1994).
- basic literacy i.e. ability to speak, read and write in English, Afrikaans or Xhosa
- participants must have had a close relative or someone who knew them well who was available to provide information about their cognitive abilities as required in the DECO questionnaire.

Exclusion Criteria

- a major psychiatric disorder e.g. depression. Participants were screened for depression using the Geriatric Depression Scale (GDS).
- another major neurological disorder e.g. Parkinson's or Huntington's disease
- a recent major stroke (within the last 6 months)
- a significant recent head injury (within the last year) associated with loss of consciousness
- known HIV/AIDS
- uncontrolled hypertension
- uncontrolled diabetes mellitus
- any medical condition that, in the opinion of the investigator, precluded the patient from participating in the study
- alcoholism & drug abuse
- heavy smoking (>20 cigarettes per day)
- oral steroid treatment (precluded only the cortisol study)
- no access to a freezer at home (for storage of the salivary samples)
- illiteracy
- severe dementia i.e. Mini-mental State Examination (MMSE) score < 12

4.4) Ethics

This observational study was approved by the Research Ethics Committee of the Faculty of Health Sciences of the University of Cape Town (REC Ref: 346/2008), and has adhered to the principles laid down in the Helsinki Declaration of 2008 (World Medical Association 2008) and South African Medical Research Council guidelines for research on human subjects (South African Medical Research Council 2002).

4.5) Study Procedure

Baseline visit

Interested participants from the community and referred elderly persons from the Memory Clinic at GSH were screened telephonically to assess whether they met the inclusion criteria for the study. In the case of the AD patients, a spouse, son, daughter or relative was contacted. If the participant was deemed eligible, an initial appointment was arranged. Participants were interviewed and assessed in the Department of Neurology in our research clinic facility at Groote Schuur Hospital (GSH). In the early stages of the project, initial meetings took place at the Rehoboth Age Exchange Centre in Hanover Park.

Participants were booked for their cognitive evaluation and clinical assessment on the same day. Each participant evaluation took approximately 3 hours in total. Elderly persons who became easily fatigued and could not complete the entire evaluation in one session were given a break before we completed our assessment.

Pre-interview and Consent

At an initial interview, the Détérioration de Cognition Observée (DECO) questionnaire, was administered to a relative or someone who had at least monthly contact with the participant over a period of three years. Participants were screened for depression, using the Geriatric Depression Scale (GDS). Participants with a GDS >7 were excluded from the study. We obtained written consent from all suitable participants and their relatives. These assessments are discussed in detail later on (see section 4.6 pg. 56).

Lifestyle and Neuropsychological Evaluation

Demographic and lifestyle questionnaires were administered in English by the patient and/or relative, before the battery of neuropsychological tests were performed. The relevant tests are explained in more detail later (see section 4.6 pgs. 56 and 57).

Baseline Clinical Assessment

The clinical assessment of participants included a detailed medical history and a general and neurological examination.

A detailed description of the participant's past history and current complaints was recorded. This included relevant medical and family history e.g. hypertension, diabetes and a family history of AD. Cognitive symptoms and other relevant medical complaints were noted. We obtained a detailed medication/drug history, including vitamin supplementation, from all participants. A general examination included measurements of blood pressure, pulse rate, height and weight. Cardiovascular, respiratory, abdominal and a thorough neurological examination were all included in the clinical evaluation.

A history of co-morbid vascular risk factors was obtained. The presence of vascular risk factors were presented using the Co-morbid score, a scale based on the number of vascular risk factors obtained from the medical history of each participant. The Co-morbid Score ranged from 0-4 and included previously diagnosed vascular risk factors viz. hypertension, diabetes mellitus and dyslipidaemia as well as a history of current or past smoking. If any one of these individual risk factors were present, a score of '1' was ascribed and the final co-morbid score was an addition of the number of risk factors present.

Special Investigations

Blood Investigations

Blood tests as part of a dementia screen and special research bloods were taken on the same visit as the clinical evaluation. These included:

- Full blood count (FBC),
- Erythrocyte sedimentation rate (ESR)
- Electrolytes (sodium and potassium)
- renal function tests (urea and creatinine)
- calcium
- liver function tests- albumin, aspartate transaminase (AST), alanine transaminase (ALT), gamma glutamyl transferase (GGT)
- C-reactive protein (CRP)
- fasting blood glucose
- cholesterol
- thyroid function tests
- syphilis serology
- serum vitamin B₁₂
- serum folate

Specific research blood samples included:

- plasma homocysteine
- whole blood (containing white blood cell DNA) for ApoE genotyping

Blood storage and processing are discussed further in section 4.7 pg. 58.

Neuroimaging

Participants underwent computerised tomography (CT) scanning at GSH if clinically indicated or for diagnostic clarification. Participants referred from the specialist clinics already had neuroimaging studies to exclude space occupying lesions as a cause for their cognitive impairment.

4.6) Instruments and Measures

Cognitive evaluation included demographic and lifestyle questionnaires and specific neuropsychological tests. See Table 4.1 for a list of all the instruments and questionnaires that were used in this study.

Table 4.1: Summary of Instruments and Measures

Instruments and Measures	Brief Explanation	Score and Interpretation
<i>Demographic and lifestyle Measures</i>		
Geriatric Depression Scale (GDS)	Self-Rating scale of 15 yes/no questions related to how participant felt over the past week.	Scores >7 are indicative of depression
BADLS (Bristol Activities of Daily Living)	Carer-rating scale assessing 20 activities of daily living over past 2 weeks.	Score 0 indicates total independence Score 60 indicates total dependence
The Détérioration de Cognition Observée (DECO)	19 item questionnaire by a relative/informant	Scores range from 0 to 38, a low score indicating poor performance.
<i>Neuropsychological Measure</i>		
The Cambridge Examination for Mental Disorders of the Elderly Revised (CAMCOG-R)	67 items including 19 item MMSE questionnaire	Scored 0-105. Scores < 80 are indicative of dementia.
The Mini Mental State Examination (MMSE)	Measures orientation to place and time, immediate recall, short term verbal memory, language, construct ability and calculation.	Scored out of 30 >25 normal >10 severe dementia 10-19: mild dementia 19-24: Moderate dementia

The Détérioration de Cognition Observée (DECO)

In 1992 Ritchie and Fuhrer developed the DECO from retrospective data based on interviews with the relatives of dementia patients. The DECO is a questionnaire containing a series of 19 items covering cognitive and behavioural changes which are presented to a relative of a patient likely to have cognitive impairment (see Appendix D). Questions focus on memory changes for people, places and events. It also includes questions about changes in the participant's activity level and learning of new skills. The respondent indicates whether, compared to last year, the behaviour of the participant in these situations is more or less the same (2), less (1) or much less (0) than 1 year ago. Scores range from 0 to 38, a low score indicating poor performance. The DECO has a demonstrated high internal consistency and test-retest reliability (Ritchie *et al.* 1996), and has been shown to be useful in predicting dementia in South African studies (Lenger *et al.* 1996).

The Geriatric Depression Scale (GDS)

In the assessment of depression in old age, the Geriatric Depression Scale (GDS) (Brink *et al.* 1982; Yesavage *et al.* 1983) is currently one of the most widely used depression self-reports. Dementia in the elderly is often mistaken for depression and the syndrome of 'pseudodementia', characterised by psychomotor retardation and memory impairment, may be mistaken for dementia. Brink *et al.* in their study selected 100 items with yes/no answers that had been shown to be useful in distinguishing elderly depressed subjects from elderly normal subjects. These questions were administered in a self-rating form to 47 subjects. The 30 questions which showed the highest correlation with the total score were chosen for inclusion in the GDS. The GDS was found to have 92% sensitivity and an 89% specificity when evaluated against diagnostic criteria.

The GDS Long Form is a 30-item self-report questionnaire in which participants are asked to respond by answering yes or no in reference to how they felt over the past week. A Short Form GDS consisting of 15 questions was developed in 1986 (Sheikh *et al.* 1986). Questions from the Long Form GDS which had the highest correlation with depressive symptoms in validation studies were selected for the short version. Scores of 0-4 are considered normal, depending on age, education, and complaints; 5-8 indicate mild depression; 9-11 indicate moderate depression; and 12-15 indicate severe depression. The GDS-Short Form and GDS-Long Form were found to be highly correlated ($r = .89$) and to have similar high sensitivity rates (Leshner *et al.* 1994).

The GDS-Short Form was used in this study. This version is more easily used by physically ill and mildly to moderately demented patients who have short attention spans and/or feel easily fatigued. It takes about 5 to 7 minutes to complete (see Appendix E).

The Bristol Activities of Daily Living Scale (BADLS)

Activities of daily living refers to everyday tasks that people undertake that do not normally require any assistance. In the assessment of persons with suspected dementia, it is important to identify whether normal activities of daily life have been compromised and if so, how severely they affect everyday life.

The BADLS is an assessment of the functional capacity of participants. It is administered by a relative or carer, and was designed specifically for use in patients with dementia (Bucks *et al.* 1996). It is an assessment of 20 daily living activities e.g. eating, drinking, showering, use of the toilet etc. (see Appendix F). Researchers have shown that it has good test-retest reliability and that it correlates well with the Mini Mental State examination. The BADLS has a minimum possible score of 0 (totally independent) and a maximum score of 60 (totally dependent). South African researchers have made use of the BADLS in their studies in elderly persons (Tipping *et al.* 2006).

The Cambridge Cognitive Assessment - Revised (CAMCOG-R)

The CAMCOG (Roth *et al.* 1986) is a neuropsychological test that forms part of the Cambridge Examination for Mental Disorders of the Elderly -Revised (CAMDEX). The revised version was published in 1995 (Huppert *et al.* 1995).

The CAMCOG-R consists of 67 items, including the 19 items from the Mini Mental State Examination (MMSE) (Folstein *et al.* 1975). It was used as a test of cognitive functioning in all participants and was slightly modified to suit our South African population e.g. we used a picture of Nelson Mandela instead of the Queen.

The test is divided into 8 subscales: Orientation, Language, Memory and Learning, Attention, Praxis, Calculation, Abstract thinking, and Perception.

- The orientation subscale is comprised of 10 items taken from the MMSE.
- In the language subscale, comprehension is assessed through nonverbal and verbal responses to spoken and written questions, and expression is assessed through tests of naming, repetition, fluency and definitions.
- The memory subscale assesses remote memory (famous events and people), recent memory (news items, prime minister, etc.), and learning (the recall and recognition of non-verbal and pictorial information learned incidentally as well as intentionally). Attention is assessed by serial sevens and counting backwards from 20.
- Praxis is assessed by copying, drawing, and writing as well as carrying out instructions.
- In the calculation subscale, the participant is asked to perform an addition and a subtraction question involving money.
- For the abstraction subscale, the participant is asked about similarities between an apple and a banana, a shirt and a dress, a chair and a table, and a plant and an animal.
- In the perception subscale, the participant is asked to identify photographs of famous people and familiar objects from unusual angles, in addition to the tactile recognition of coins (Huppert *et al.* 1996).

The CAMCOG takes approximately 30 min to complete and is scored out of 105. Generally scores less than 80 are indicative of dementia.

The MMSE is a widely used and well validated screening tool for cognitive impairment. It measures orientation to place and time, immediate recall, short term verbal memory, language, constructional ability and calculation. It is scored out of 30 with scores greater than 25 generally indicating normal cognition. A score of 19 -24 indicates mild cognitive impairment; scores between 10-19 are usually associated with moderately advanced cases of AD and scores less than 10 indicate severe impairment.

The total CAMCOG, MMSE and Learning Subscale scores were most frequently used in this study for diagnosis and for testing hypotheses related to cognitive function. Dementia-free participants were defined as NINCDS-ADRDA criteria negative, CAMCOG scores > 80/105, MMSE \geq 24/30 and a Learning Subscale score \geq 13/17.

4.7) Blood Investigations

Blood sample collection and storage

Routine Blood Samples

These were part of the standard dementia screen and were sent to the routine hospital laboratory, the National Health Services Laboratory (NHLS). A summary of blood collection tubes, storage and special instructions is given in Table 4.2.

Research Blood Samples

Blood samples for plasma homocysteine were collected in a 4ml ethylene-diamine-tetra-acetic acid (EDTA) tube and stored on ice. These samples were centrifuged at 4000rpm for 10 minutes in a bench top laboratory centrifugation machine within an hour of collection. The supernatants were aspirated, aliquotted and stored in a laboratory freezer at -80°C .

Two 4 ml EDTA-containing blood tubes were collected for ApoE genotyping. These samples were centrifuged within the hour and the buffy coat layer containing the white blood cells (and DNA) was aspirated. The latter was also stored at -80°C until analysed. The total volume of all blood collected did not exceed 40mls.

Table 4.2: Summary of blood investigations

Blood Tube	Blood Investigation	Volume Collected	Storage
<u>Tubes with gel to assist serum RBC separation</u> 4 tubes in total Routine chemistry Blood tests	urea	3 x 5mls	room temperature
	creatinine and electrolytes (Na, K)	1 x 2mls	
	Calcium		
	AST		
	ALT		
	GGT		
	TSH		
	CRP		
	total Cholesterol		
	vitamin B ₁₂ and folate		
RPR screen			
<u>EDTA tube</u>	FBC diff & WCC	4mls	room temperature
2 tube	Serum homocysteine	4mls	on ice. Supernatants store at -80°C
<u>Fluoride tube</u>	glucose	1-2 mls	room temperature
1 tube			
<u>Citrated tube</u>	ESR	2mls	room temperature
1 tube			

Analysis of blood tests

Blood samples forming part of the routine dementia screen were sent to the National Health Laboratory Service (NHLS) Groote Schuur Hospital, and the frozen plasma samples for homocysteine analysis were sent to a private laboratory (Path Care) for analysis.

ApoE genotyping was performed by Mrs Felicity Leisegang in the Department of Chemical Pathology, University of Cape Town.

4.8) Research Blood Tests: assay methods

Plasma Homocysteine

The samples were thawed at room temperature prior to analysis. The Path Care private laboratory performed these analyses according to the manufacturer's instructions.

Homocysteine was measured using an enzymatic cycling assay. The Beckman DXC Chemistry Analyser (Beckman Coulter Inc.) and liquid-stable reagents (Catch Homogenous Enzymic Homocysteine reagent) were used by the Path Care Laboratories. The Catch Homocysteine enzymatic assay has been formulated into a 3-reagent, homogeneous liquid system. Reagents were made up of the following active ingredients:

- lactate dehydrogenase
- serine
- reduced nicotinamide co-factor (NADH)
- cystathionine β -synthase (CBS)
- cystathionine β -lyase (CBL)

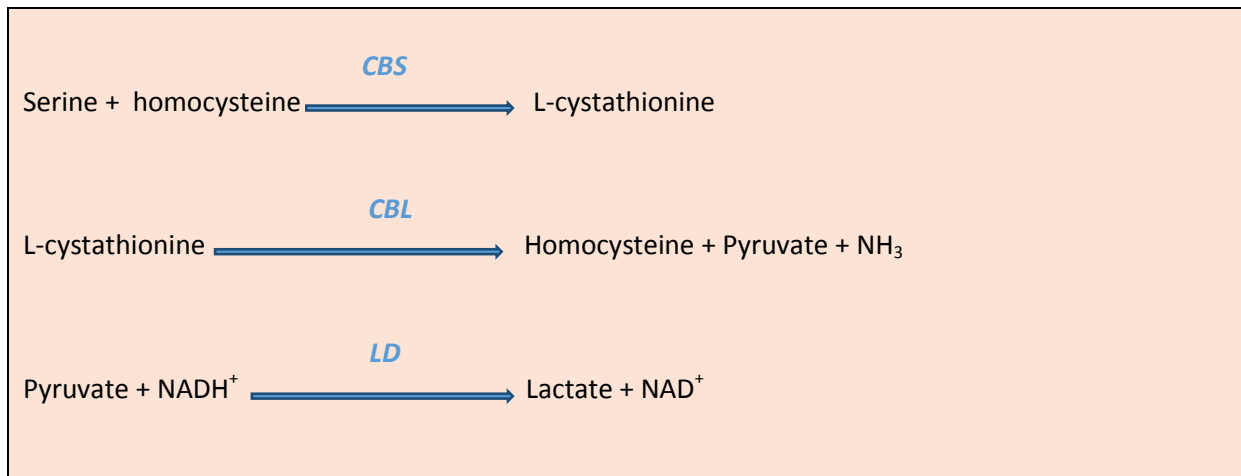
Homocysteine Quantification Method

The steps used in the enzyme cycling assay are summarised in figure 4.1.

Plasma samples containing homocysteine, when mixed with reagent, react with serine to form L-cystathionine in the presence of the enzyme CBS. L-cystathionine is then converted to homocysteine, pyruvate, and ammonia (NH₃) with a second enzyme, CBL. The result of using these anabolic and catabolic enzymes operating simultaneously is a cycle in which (1) cystathionine and homocysteine were continuously interconverted, (2) serine is degraded, and (3) pyruvate and ammonia are created.

Pyruvate is measured by the enzymatic conversion using lactate dehydrogenase to produce lactate. This enzymatic conversion reaction requires the cofactor NADH, which is converted to NAD⁺ (oxidized nicotinamide co-factor). The concentration of homocysteine is directly proportional to the amount of NADH⁺ converted to NAD⁺. The oxidation of NADH to NAD⁺ is measured by monitoring the absorbance at 340 nm.

Figure 4.1: Homocysteine Quantification
Catch Inc.



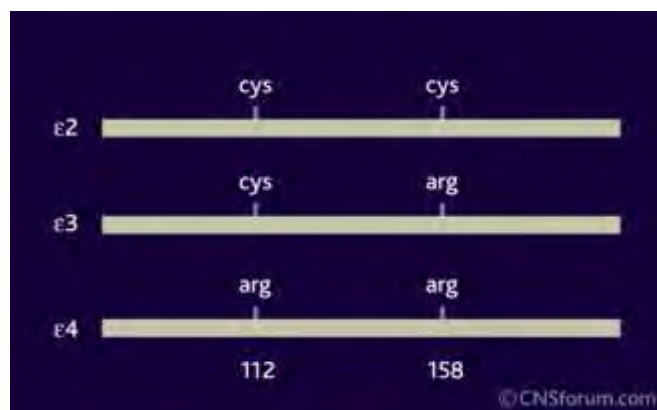
4.9) ApoE Genotyping

Blood samples for ApoE genotyping were collected in 5ml EDTA-containing blood tubes. 109 buffy coat samples were prepared and stored at -80°C. Specimens were sent to the Department of Chemical Pathology, University of Cape Town, for ApoE genotyping.

Restriction Isotyping Method

The 3 isoforms of the ApoE gene differ in the amino acid sequence at 2 sites: amino acid 112 and 158 (Figure 4.2).

Figure 4.2 ApoE gene isoforms



J Cell Mol Med 2001; 5:254–266. *Physiological Reviews* 2001; 81: 741–766. <http://www.cnsforum.com>

Restriction Enzyme Isoform Genotyping (restriction isotyping) was used for rapid typing of the ApoE isoforms, ε2, ε3 and ε4 (Hixson *et al.* 1990). This method involved the following processes:

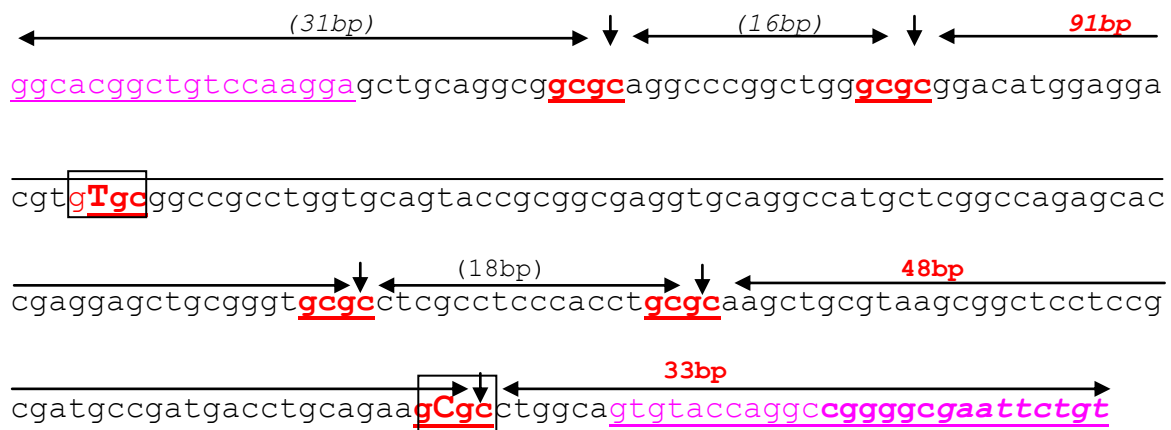
Step 1: polymerase chain reaction (PCR) Amplification

ApoE gene sequences that incorporate the region coding for amino acid 112 and 158 were amplified using PCR amplification. This method uses cycles of heating and cooling to unravel the double helix DNA structure and as a result, allows for DNA synthesis in the presence of DNA polymerases. PCR amplification facilitates the construction of thousands of copies of a specific genetic sequence for analysis. A primer is added at the end of the amplified sequence to increase its length to 237bp, allowing for better strand separation.

Step 2: Restriction Enzyme Digestion

A restriction enzyme is one that is able to cut DNA at a specific nucleotide sequence known as a restriction site. Restriction enzymes are used to distinguish gene alleles by specifically recognising single base changes in the DNA. Our samples were digested with Hha1 restriction enzyme (gcgc) to generate specific DNA fragments (Figure 4.3).

Figure 4.3: Restriction Enzyme Digestion



Allele ε3 is shown below. Primers are given in purple and cut sites in red. The two bases of interest are in capital letters with the diagnostic restriction enzyme cut site shown in the box.

Step 3: Gel Electrophoresis

Gel electrophoresis is used to separate these DNA fragments according to length. The lab used 10% acrylamide gel to separate strands. The patterns formed by the fragments are interpreted to identify the different isoforms. The restriction enzyme cut the PCR fragment in several places resulting in several bands some of which were common between all isoforms while others were diagnostic.

Step 4: Diagnosis (Table 4.3 and Figure 4.4)

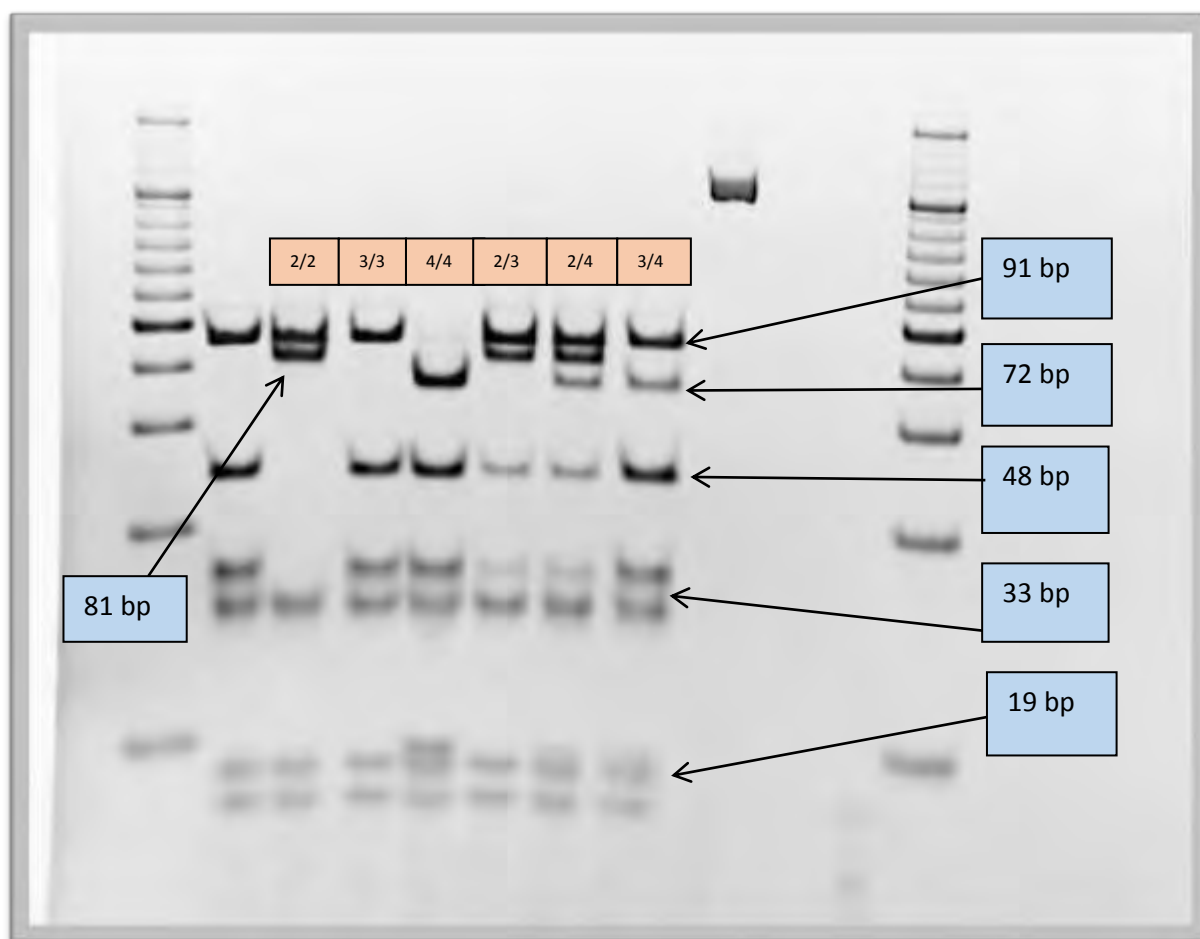
1. Genotype $\epsilon 2/\epsilon 2$ has one 81 base pairs (bp) band.
2. Genotype $\epsilon 3/\epsilon 3$ is diagnosed by the absence of the 81 and 72 bp bands.
3. Genotype $\epsilon 4/\epsilon 4$ has 1 72bp band.
4. Genotype $\epsilon 2/\epsilon 3$ is diagnosed by the presence of the 81 bp band (allele2), no 72 bp band (excludes $\epsilon 4$) and the presence of the 48 and 33 bp bands. The 48 and 33 bp band come from allele $\epsilon 3$ as allele $\epsilon 2$ does not have these bands. In addition a 19 bp band is also present.
5. Genotype $\epsilon 2/\epsilon 4$ has both 81 and 72 bp bands.
6. Genotype $\epsilon 3/\epsilon 4$ is diagnosed by the presence of a 72 bp band (allele $\epsilon 4$), no 81 bp band (excludes allele $\epsilon 2$) and the presence of the 91bp band. The 91 bp band comes from allele $\epsilon 3$ as allele $\epsilon 4$ does not have these bands. A 19 bp band is also present.

Table 4.3: Diagnostic Interpretation of ApoE genotyping: Chemical Pathology Lab GSH

Isoform type	Diagnostic band sizes present on 10% acrylamide gel	Additional bands
$\epsilon 2/\epsilon 2$	91 81	31 18 16
$\epsilon 3/\epsilon 3$	91 48 33	31 18 16
$\epsilon 4/\epsilon 4$	72 48 33 19	31 18 16
$\epsilon 2/\epsilon 3$	91 81 48 33	31 18 16
$\epsilon 2/\epsilon 4$	91 81 72 48 33 19	31 18 16
$\epsilon 3/\epsilon 4$	91 72 48 33 19	31 18 16

Hixson et al. 1990

Figure 4.4: Hha1 digested PCR products run on a 10% Acrylamide gel



Dept Chem Pathology, UCT

4.10) Vitamin B₁₂ and Folate

Serum folate and vitamin B₁₂ concentrations in our samples were determined using the Elecsys Folate III assay and Elecsys Vitamin B₁₂ assays respectively. These were routine automated diagnostic tests done at the NHLS at GSH and were carried out according to manufacturer's instructions.

These assays are binding radioassays which allowed for in vitro quantitative determination of serum folate and vitamin B₁₂ concentrations. Both assays use the competitive test principle.

Intrinsic factor specific for vitamin B₁₂ and natural Folate Binding Protein (FBP) specific for folate are used to determine the concentration of vitamin B₁₂ and folate in the sample. Sample folate competes with added folate (labelled with biotin) for binding sites on FBP (labelled with ruthenium complex). Vitamin B₁₂ in the sample competes with B₁₂ (labelled with biotin) for binding sites on ruthenium-labelled intrinsic factor complex. Voltage applied to the electrode induces a chemiluminescent emission measured with a photomultiplier. Results are then determined using an instrument-specific calibration curve provided via the reagent barcode.

Clinical diagnostic criteria and classification of participants

Based on neuropsychological, clinical and biochemical analyses, we were able to classify patients into two main groups – those with normal cognition and those with impaired cognition.

Weekly meetings involved group discussions of each case and an overall diagnosis based on cognitive scores, clinical impression and collateral information from the participant's relative/informant. The NINCDS-ADRDA (McKhann *et al.* 1984) criteria were used to classify participants into NINCDS-ADRDA negative (controls). Participants classified as possible or probable were included in the AD group. Diagnostic criteria proposed by Petersen *et al.* were used to identify patients with MCI) (See Appendix C).

Follow-up

All participants received feedback in the form of a summary letter with baseline blood results and cognitive scores. Recommendations for follow-up investigations/treatment were included in the letter for participants with abnormal blood results. Results were posted to participants, their relatives and/or their general practitioner. One year after the initial assessment, participants were invited to undergo repeat cognitive evaluations. The physical examination and blood investigations were not repeated at this visit.

4.11) Collation of Data

All data from the larger study were captured on an Excel spreadsheet. These included cognitive test scores at baseline and follow-up scores 1 year later. I added data that had relevance to my study: demographic data for patients and controls, homocysteine, vitamin B₁₂ and folate concentrations ApoE data and Co-morbid scores. In total I compiled a data spreadsheet for 113 participants.

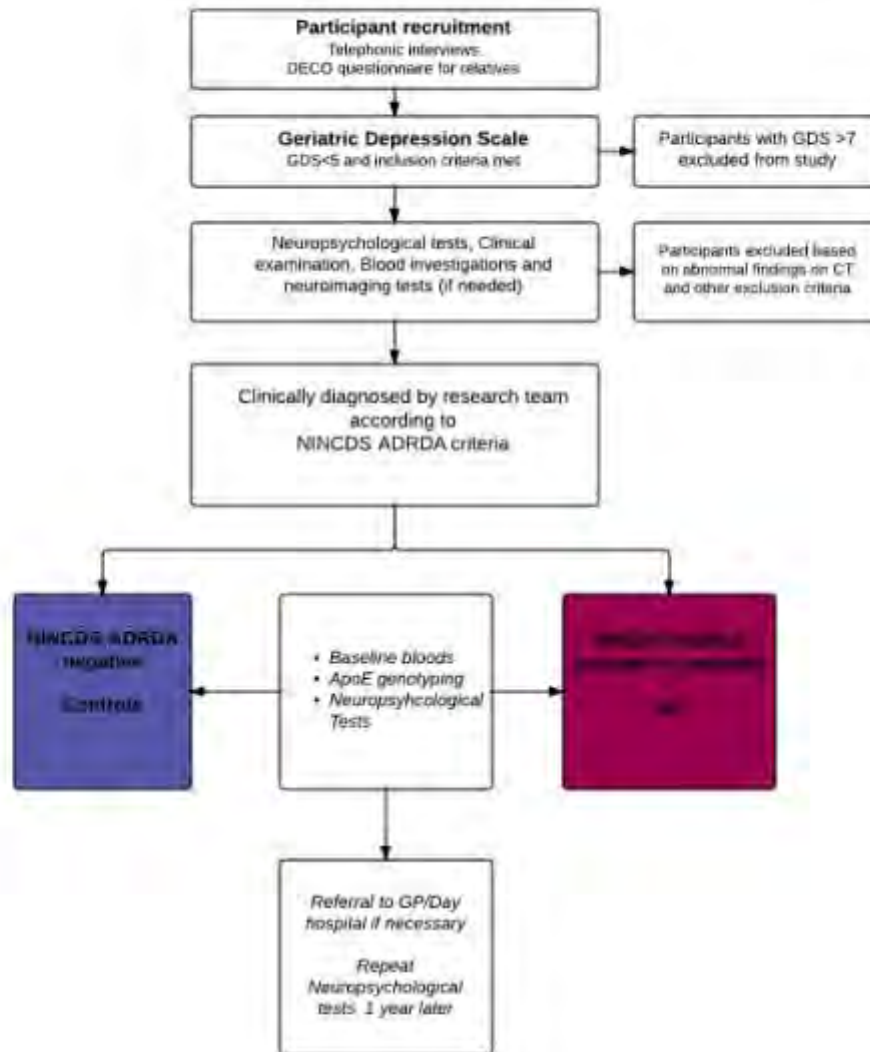
4.12) Data Analysis

To analyse data statistically, I transferred all data onto another spreadsheet in Statistica (version 21), the software programme used for data analysis. All data had to be reorganised into groups for both continuous and categorical statistical analysis. The level of significance was set at $\alpha=0.05$. Further details regarding statistical analyses are outlined in Chapter 5.

4.13) Study Process summary

Figure 4.5 is a summary of the methods used in this study. Participant numbers and the attrition rate are outlined in figure 5.1.

Figure: 4.5: Summary of Methods



Chapter Five

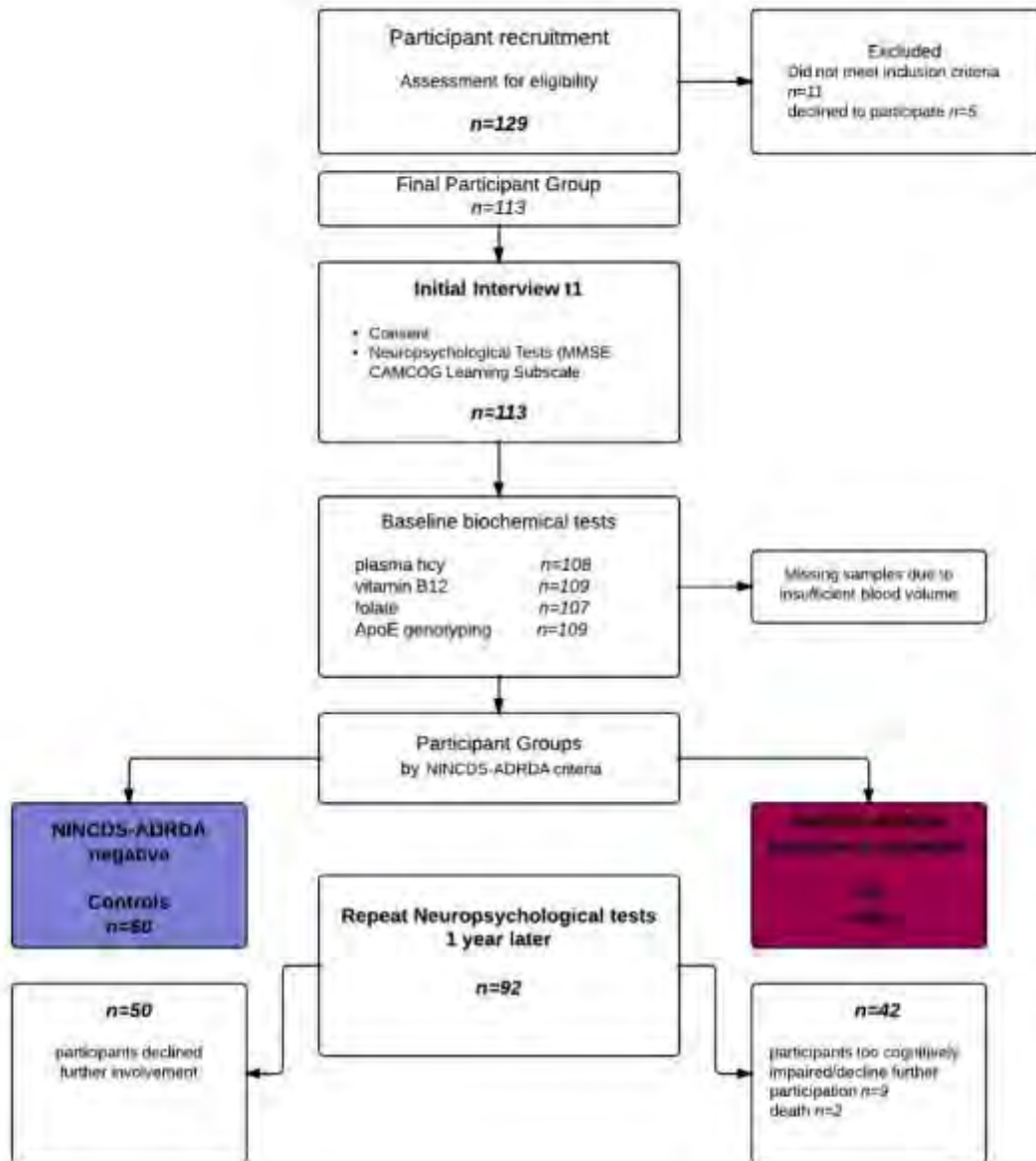
Results

5.1) Study summary

The final sample comprised 113 study participants in total, of whom 60 were controls and 53 AD participants. Demographic characteristics, cognitive test scores, blood test results and ApoE genotyping were collected from all participants at baseline (t1). Follow-up cognitive assessments were conducted 1 year after the baseline assessment (t2). In both AD and control participants there were losses due to either participant death or to participants declining to be involved in further evaluations. Further data losses occurred as a result of missing laboratory data or insufficient sample volumes for the appropriate assay (see figure 5.1).

I used the Shapiro Wilk test to assess whether the continuous variables were normally distributed or not. Age was the only variable that was normally distributed ($p=0.107$). Other variables MMSE₁ and MMSE₂ ($p<0.001$), CAMCOG₁ and CAMCOG₂ ($p<0.001$), Learning Subscale₁ and Learning Subscale₂ ($p<0.001$), serum folate ($p<0.001$), serum vitamin B₁₂ ($p<0.001$), years of education ($p=0.001$) and plasma homocysteine ($p<0.001$) were all found to have non-normal distributions.

Figure 5.1: Flow diagram of study process



5.2) Demographic characteristics

Table 5.1 shows baseline age, sex and education characteristics of all study participants.

Table 5.1: Participant demographic characteristics

	All participants n=113	Control Group n=60	AD group n=53	Statistical comparison AD vs. Controls		
				χ^2	df	p
Age	74.8 (8.9)	73.2 (8.9)	76.6 (8.6)	-2.02	1	0.043**
Sex (M:F)	30:83	12:48	18:35	2.8	1	0.093
Level of Education	10.5 (8-15)	14 (10-17)	8 (7-11)	27.8	1	<0.001*
<p><i>Age is expressed in years as mean values with the standard deviations in parentheses</i> <i>The level of education is expressed as median number of years with the interquartile ranges (IQR) in parentheses.</i> <i>*significant at the 0.01 level</i> <i>**significant at the 0.05 level</i></p>						

Age

Age in the study group ranged from 59 to 101 years ($M=74.8$, $SD=8.9$). The independent samples t-test showed that the AD participant group were significantly older ($M=76.6$ vs 73.2 , $p=0.045$) than the control participant group.

Sex

Overall, there were more females than males in the study population (see table 5.1). These differences however, were not statistically significant between the AD and control participant groups ($\chi^2=2.8$, $p=0.093$).

Level of education

The number of years of education ranged from 3 to 33 years in all participants ($Mdn=10.5$, $IQR=8-15$). AD participants had significantly lower levels of education compared with controls ($Mdn=8$, $p<0.001$).

In summary, AD participants were older and had fewer years of education compared with controls.

5.3) Cognitive test scores

Table 5.2 shows baseline cognitive test scores in all participants.

Table 5.2: Baseline Cognitive Scores in all participants, AD and Control participants

	Group			Statistical comparisons AD vs. Controls	
	All participants n=113	Control n=60	AD n=53	<i>U</i>	<i>p</i> value
MMSE₁ (30)	27 (22-29)	29 (28-29)	22 (18-24)	126	<0.001*
CAMCOG₁ (105)	84 (68-93)	92 (88-96)	67 (56-76)	34	<0.001*
Learning Subscale₁ (17)	12 (8-14)	14 (13-15)	7 (4-10.5)	104.5	<0.001*
<i>Scores are given as median values with the IQRs in parentheses.</i>					
<i>* significant at the 0.01 level</i>					

Baseline cognitive scores included the MMSE₁, CAMCOG₁ and the Learning Subscale₁. As expected, scores of the AD group were poorer compared with control participants (see table 5.2). The Mann Whitney U test was used to compare the cognitive scores between the 2 groups. AD participants' scores on all 3 measures of cognition were significantly lower than those of the controls (see table 5.2: MMSE₁: $U=126, p<0.001$; CAMCOG₁: $U=34, p<0.001$; Learning Subscale₁: $U=104.5, p<0.001$).

5.4) Biochemical Tests

Table 5.3 shows blood measurements for plasma homocysteine, serum vitamin B₁₂ and folate taken at baseline.

Table 5.3: Baseline biochemical data for all participants, AD and Control participants

	Group			Statistical comparisons AD vs. Controls	
	All participants	Control	AD	<i>U</i>	<i>p</i>
Plasma hcy (mmol/l)	n=108 10.2 (7.3- 13)	n=59 9.3 (6.7-11.2)	n=49 10.6 (8.3-14.95)	1017	0.016**
Serum Vitamin B₁₂ (pmol/l)	n=109 350 (300-475)	n=58 414 (268- 521)	n=51 317 (199-446)	1219	0.053
Serum Folate (nmol/l)	n=107 30 (21-45)	n=58 36 (23-45)	n=49 25 (19-41)	997	0.007*
<i>All values are given as medians with IQR in parentheses.</i>					
<i>* significant at the 0.01 level</i>					
<i>**significant at the 0.05 level</i>					

Plasma homocysteine (hcy)

Hcy concentrations ranged from 5-28 mmol/l (*Mdn*= 10.2) and were significantly higher in AD participants (*Mdn*=10.6) compared with controls (*Mdn*=9.3). The Mann Whitney U test was used to evaluate the difference between the groups ($U=1016, p=0.016$).

Vitamin B₁₂

Serum Vitamin B₁₂ levels ranged from 115- 1468 pmol/l. Participants with AD tended to have lower vitamin B₁₂ levels (*Mdn*=317 pmol/l) compared with controls (*Mdn*=414pmol/l). This difference almost reached statistical significance ($U=1219, p=0.053$).

Serum Folate

Folate levels ranged from 12 to >45 nmol/l. AD participants had lower folate levels (*Mdn*=25nmol/l) compared with cognitively healthy controls (*Mdn*=36nmol/l). Differences in folate levels between AD and control participant groups were statistically significant ($U=997, p=0.007$).

In summary, AD participants had lower scores on all cognitive tests compared with controls. Participants in this group had higher plasma homocysteine levels and lower vitamin B₁₂ and folate levels compared with cognitively healthy controls.

5.5) Baseline ApoE data

The ApoE data were expressed as allelic frequencies (table 5.4), and also described in groups based on the presence or absence of the $\epsilon 4$ allele in all participants as well as in the AD and control groups.

Table 5.4 shows the breakdown of the allelic frequencies amongst all participants, AD and control groups. This is represented graphically in figure 5.2. Table 5.5 is a 2x2 table depicting AD and control participants with respect to their $\epsilon 4$ carrier status ($\epsilon 4+$ = $\epsilon 4$ carrier, $\epsilon 4-$ = $\epsilon 4$ non-carrier). Figure 5.3 is a graphic representation.

Table 5.4: ApoE allelic frequencies in all participants, AD and control groups

	Group			Statistical comparisons AD vs. Controls		
	All participants n=109	Control n=58	AD n=51	χ^2	df	p
ε4 non carriers						
ε2/ ε2	1		1			
ε3/ ε2	17	9	7	0.25	1	0.617
ε3/ ε3	38	26	13	4.3	1	0.037**
ε4 carriers						
ε2/ ε4	5	3	2	0.2	1	0.655
ε3/ ε4	41	18	23	0.61	1	0.435
ε4/ ε4	7	2	5	1.29	1	0.257
. Values are expressed as numbers with percentages in parentheses.						
** significant at the 0.05 level						

Figure 5.2: Bar graph showing distribution of ApoE allelic frequencies

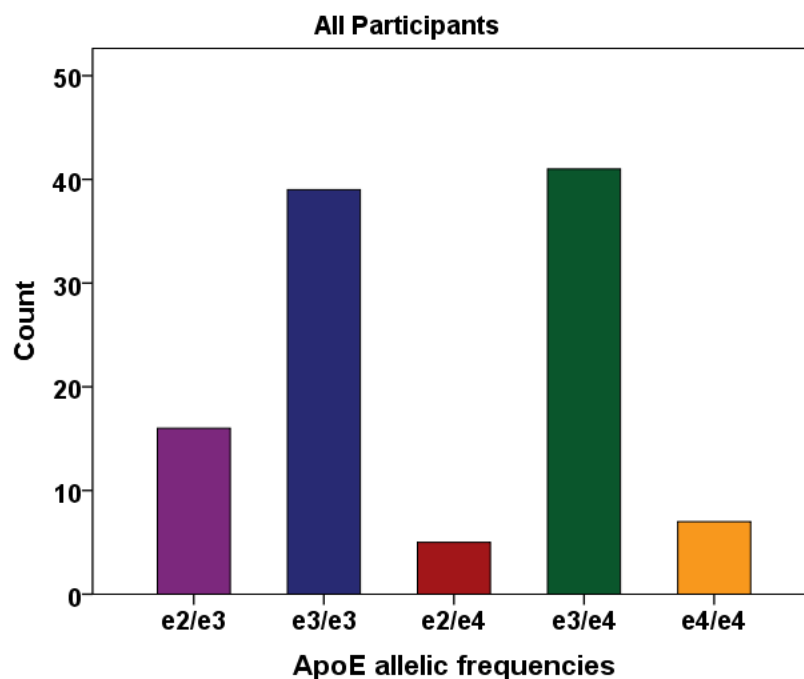
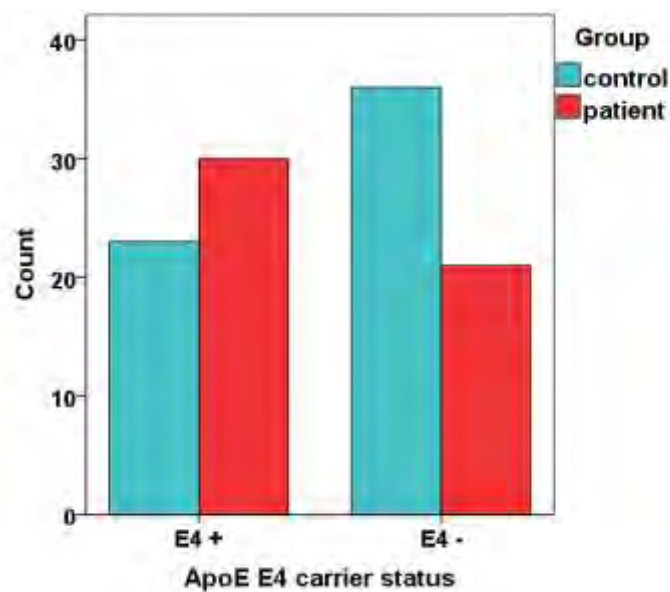


Table 5.5: A 2x2 table comparing ApoE ε4 carrier status in AD and control participant groups

		ε4 carrier/ε4 non-carrier and AD/control group cross-tabulation			
		Group		Total n and (%) of ε4 carriers in all participants	
		AD n (%)	Control n (%)		
ApoE ε4 status	ε4+	30 (27)	23 (21)	53 (48)	$\chi^2=4.3$ $p=0.038^{**}$ OR=2.2 $p=0.039^{**}$ CI [0.2,5.97]
	ε4-	21 (19)	35 (33)	56 (52)	
	Total				

****significant at the 0.05 level**
 () indicate % in within group as a whole

Figure 5.3: The distribution of participants in ApoE ε4 carrier and ε4 non-carrier groups



From the data it was noted that:

- In all participants, $\epsilon 3/\epsilon 4$ was the most common genotype (36%) followed by $\epsilon 3/\epsilon 3$ (34%), $\epsilon 2/\epsilon 3$ (16%), $\epsilon 4/\epsilon 4$ (6%) $\epsilon 2/\epsilon 4$ (4%) and lastly $\epsilon 2/\epsilon 2$ (1%).
- In the AD group, the majority of participants carried at least 1 $\epsilon 4$ allele. In this group, 30 participants were $\epsilon 4$ carriers compared with 23 in the control group (Table 5.5). The $\epsilon 3/\epsilon 4$ genotype was most common in the AD group (43%), and 9% were homozygous $\epsilon 4/\epsilon 4$.
- In the control group the majority of participants were $\epsilon 4$ non-carriers (64%) compared with the AD group (36%). The most common genotype was homozygous $\epsilon 3/\epsilon 3$ (44%).

Chi-square analysis was used to compare the AD and control groups with respect to their ApoE genotype. With the exception of a significant difference between AD and controls in the $\epsilon 3/\epsilon 3$ genotype ($\chi^2 = 4.3, p = 0.037$), no other comparisons were statistically relevant with respect to the genotype (see table 5.4).

In a 2x2 contingency table analysis (Table 5.5), the difference in $\epsilon 4$ allelic status between participant groups was significant ($\chi^2 = 4.3(1), p = 0.038$). The odds of having AD were 2.2x greater in $\epsilon 4$ carriers compared with non-carriers (OR=2.2, $p = 0.039$, CI [0.2, 5.97]).

In summary, more AD participants carried the $\epsilon 4$ allele compared with controls and $\epsilon 4$ carrier status was significantly associated with the presence of AD.

5.6) Co-morbid score

Table 5.6: Baseline Co-morbid Scores in all participants, AD and control participants

Co-morbid score	Group			Statistical comparisons AD vs. Controls		
	All participants n=109	Control n=58	AD n=51	χ^2	df	P
0	31	19	12	1.6	1	0.21
1	50	26	24	0.08	1	0.78
2	22	9	13	0.73	1	0.394
3	5	3	2	0.2	1	0.655
4	1	1	0			
<i>Co-morbid score breakdown in all participants, AD and Control groups</i>						

Figure 5.4: Co-morbid Score breakdown in all participants

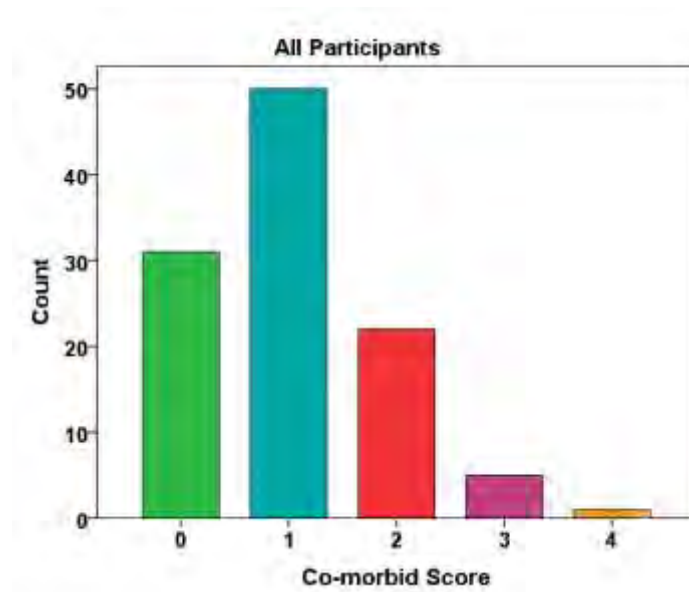


Table 5.6 shows the distribution of these vascular risk factors within the study population and separately amongst control and AD groups. Figure 5.4 shows the distribution graphically. Group comparisons were made using Pearson's chi-square analysis. The results showed that there were no significant differences between AD and control participants in terms of their co-morbid scores.

AD participants and controls did not differ significantly in terms of the presence of vascular risk factors.

5.7) Results by hypothesis

5.7.1) H1: Plasma homocysteine (hcy) increases with age

Hcy and age were analysed as continuous variables. I used Spearman's Rank Order Correlation to determine the relationship between hcy and age in all participants and also separately in the AD and control participant groups.

Hcy and age were strongly correlated in all study participants, AD and control group ($r_s = 0.418$, $p < 0.001$). The magnitude of the association was stronger in the AD group ($r_s = 0.457$, $p = 0.001$) compared with the control participant group ($r_s = 0.315$, $p < 0.015$).

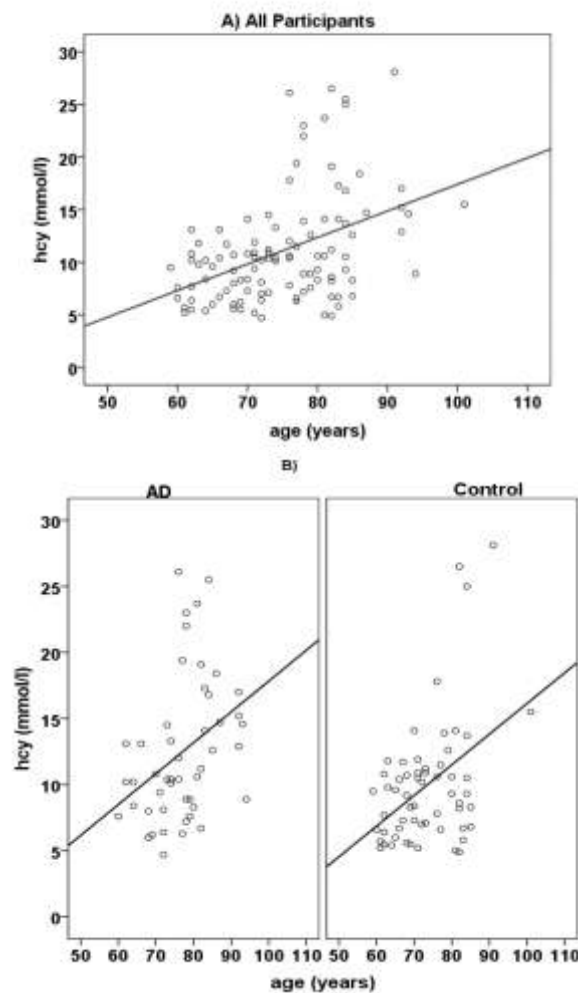
Table 5.7 summarises these findings and figure 5.5 demonstrates the relationship graphically.

Table 5.7: Correlation between hcy and age in all study participants, AD and Control groups.

Plasma homocysteine				
Age	Number (n)		Spearman's rho(r_s)	p value
	All Participants	108	0.418	0.001*
	AD group	49	0.457	0.001*
	Control group	59	0.315	0.015**

*correlation is significant at the 0.01 level
 **correlation is significant at the 0.05 level

Figure 5.5: Association between hcy level and age in A) all participants and B) AD and control groups.



Hypothesis one is therefore confirmed i.e. as the participants' ages increased, so did their hcy concentrations. Hcy levels were significantly related to age in all participants taken together, as well as in AD and controls participant groups analysed separately.

5.7.2) H2: Hcy concentrations are inversely related to vitamin B₁₂ and folate

I analysed the relationship between hcy and vitamin B₁₂ and folate both continuously and categorically.

Continuous variable Analysis

Folate

Spearman's rank order correlation showed a significant inverse relationship between hcy and folate in all study participants ($r_s=-0.33$, $p=0.001$). This relationship held true for the control group ($r_s=-0.29$, $p=0.027$) and tended towards significance in the AD group ($r_s=-0.28$, $p=0.059$). Table 5.9 summarises these results.

Vitamin B₁₂

Spearman's rank order correlation showed a significant inverse relationship between hcy and vitamin B₁₂ in all study participants ($r_s=-0.47$, $p<0.001$). This relationship held true for both control ($r_s=-0.32$, $p=0.014$) and AD groups ($r_s=-0.49$, $p=0.001$), with a greater magnitude of association in the AD population group. Table 5.8 summarises the correlation results and figure 5.6 is a graphic representation of the relationship.

Table 5.8: Correlation between hcy and serum vitamin B₁₂ levels

Plasma homocysteine				
		n	Spearman's rho	p value
Vitamin B ₁₂	All Participants	109	-0.47	<0.001*
	AD	51	-0.49	0.001*
	Controls	58	-0.32	0.014**
*significant at the 0.01 level				
**significant at the 0.05 level				

Figure 5.6: The relationship between vitamin B₁₂ and hcy concentrations in A) all participants and B) control and AD groups.

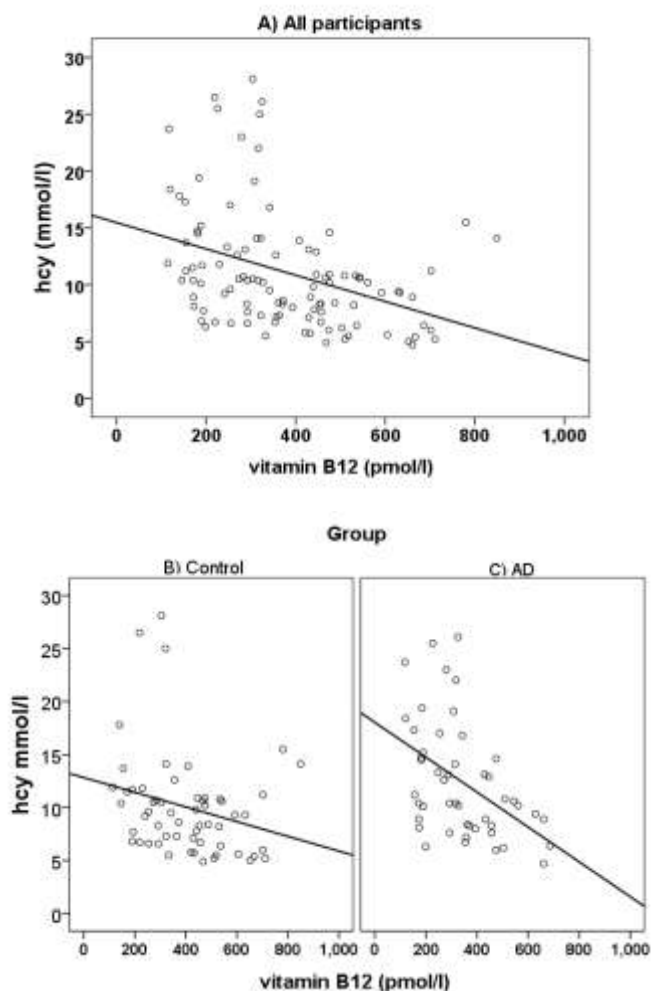


Table 5.9: Correlation between hcy and folate levels

Plasma homocysteine				
Folate		n	Spearman's rho	p value
	All Participants	107	-0.33	0.001*
	AD	49	-0.28	0.059
	Controls	58	-0.29	0.027**
*significant at the 0.01 level **significant at the 0.05 level				

In summary, these analyses revealed that hcy levels were inversely related to vitamin B₁₂ levels in all participants, as well in AD and control participants. H2 is therefore proven: hcy levels are inversely related to serum vitamin B₁₂ and serum folate.

5.7.3) H3: Hcy concentrations are directly associated with vascular risk factors.

The Krustal-Wallis statistical test was used to establish whether hcy concentrations increased as the co-morbid scores of the participants increased. This analysis showed no significant association between vascular risk factors and hcy concentration in all participants ($\chi^2(4) = 2.8, p=0.587$), the AD group ($\chi^2(3) = 1.7, p=0.621$) and the control group ($\chi^2(4) = 2.8, p=0.593$).

In this study, hcy concentrations did not differ significantly according to the number of vascular risk factors.

Addendum to H1-3: Causes of elevated plasma homocysteine in this study.

H1-H3 evaluated the relationships between hcy and its various determinants: age, vitamin B₁₂, folate and vascular risk factors.

I used a multiple linear regression analysis with hcy as the dependent variable and age, vitamin B₁₂, folate and vascular risk factors as independent predictor variables, to determine which of these factors independently predicted hcy concentrations in this study population.

The results showed that age ($\beta=0.481, p<0.001$) and vitamin B₁₂ ($\beta=-0.29, p=0.017$) independently predicted hcy levels. Serum folate and the presence of vascular risk factors did not predict changes in hcy concentrations. (see table 5.10).

Table 5.10: Regression analysis: hcy and independent variables age, vitamin B₁₂, folate and vascular risk factors.

Dependent Variable	Independent Variables	β	p value	95% CI
plasma hcy (mmol/l)	• age	0.48	<0.001*	[0.14,0.4]
	• vitamin B ₁₂	-0.29	0.017*	[-0.1,-0.002]
	• folate	-0.12	0.198	[-0.05,0.1]
	• Vascular risk factors	-0.006	0.468	[-1.143,0.67]
<i>Dependent variable hcy, and independent variables age, vitamin B₁₂, folate and vascular risk factors.</i>				
<i>*significant at the 0.01 level</i>				

5.7.4) H4: Serum vitamin B₁₂ and folate are directly related to cognitive function.

Folate

Spearman's rho revealed no association between folate and MMSE₁ scores ($r_s=0.15$, $p=0.118$). However, the relationship between folate and CAMCOG₁ and Learning Subscale₁ scores was significant i.e. the higher the folate levels, the higher the CAMCOG₁ and Learning Subscale₁ scores. (CAMCOG $r_s=0.19$, $p=0.046$, Learning Subscale $r_s=0.26$, $p=0.008$) (See table 5.11 and figure 5.7).

Table 5.11: Correlation between folate and cognitive score

Serum folate in all participants		
	Spearman rho (r_s)	p value
MMSE ₁	0.15	0.118
CAMCOG ₁	0.19	0.046**
Learning Score ₁	0.26	0.008*
<i>*significant at the 0.01 level</i>		
<i>**significant at the 0.05 level</i>		

Vitamin B₁₂

To define the relationship between serum vitamin B₁₂ and cognition I analysed the data both continuously and categorically. Regression analysis was used to control for age as a possible confounding variable.

Continuous variable analysis

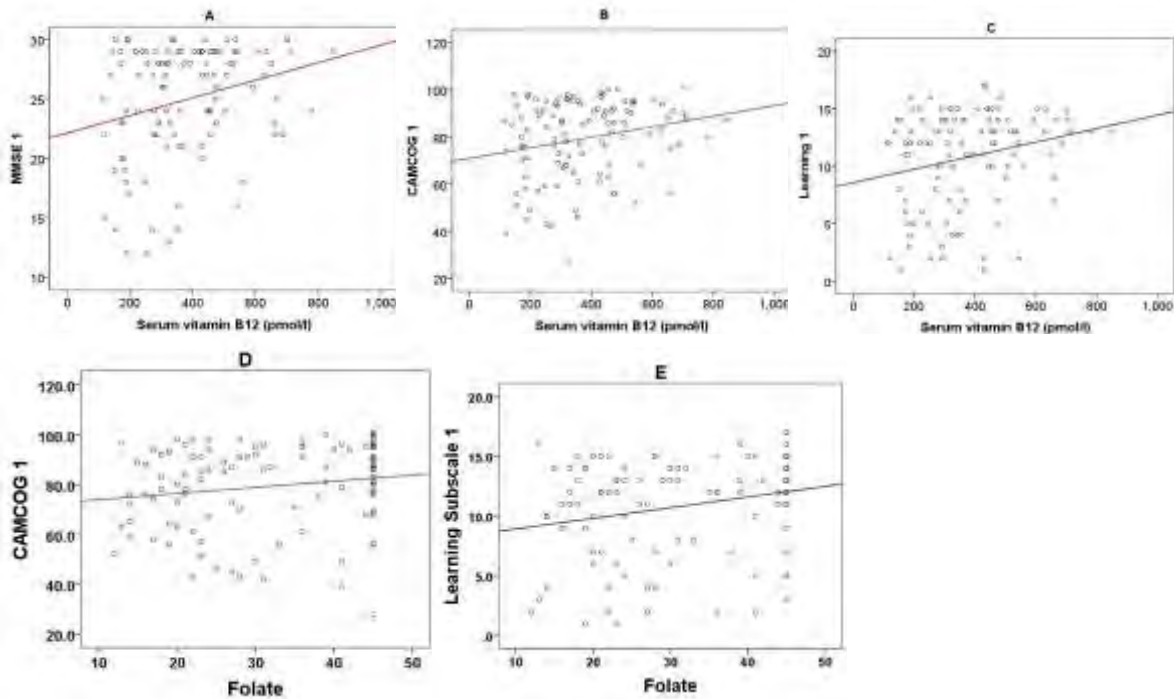
Cognition and serum vitamin B₁₂ were positively correlated in all study participants i.e. as vitamin B₁₂ levels increased, cognition improved.

Table 5.12 shows a summary of the results and figure 5.7 is a graphic representation of the relationship between cognitive scores and vitamin B₁₂ in all participants. This relationship was statistically significant between vitamin B₁₂ and MMSE₁ ($p=0.032$), CAMCOG₁ ($p=0.039$) and the Learning Subscale₁ scores ($p=0.027$). When the groups were analysed separately, there was only a significant association between vitamin B₁₂ concentrations and MMSE₁ in the AD group ($r_s=0.31$, $p=0.026$).

Table 5.12: Correlation between cognitive scores and serum vitamin B₁₂ levels

Serum vitamin B ₁₂ in all participants		
	Spearman rho (r_s)	p value
MMSE ₁	0.235	0.014**
CAMCOG ₁	0.198	0.039**
Learning Score ₁	0.211	0.027**
<i>**significant at the 0.05 level</i>		

Figure 5.7:
The relationships between serum vitamin B₁₂ levels and cognition as measures by A) MMSE₁, B) CAMCOG₁, and the C) Learning Subscale₁ scores.
The relationships between folate levels and cognition as measures by D) CAMCOG₁ and the E) Learning Subscale₁ scores.



Categorical analysis

Using guidelines for vitamin B₁₂ levels suggested by Carmel, I divided the vitamin B₁₂ data into low/subclinical (<258pmol/l) and normal-high (>258pmol/l) groups (Carmel *et al.* 2006). A 2x2 contingency table was used to relate high and low vitamin B₁₂ levels to AD and control groups.

A total of 109 vitamin B₁₂ data samples (51 AD, 58 control participants) were available for analysis. In all participants, 27% samples had a value <258pmol/l and 73% of samples a value >258pmol/l. The breakdown within each participant group is shown in table 5.13, and figure 5.8 shows the distribution of AD and control participants in low and high vitamin B₁₂ groups. There were more AD participants in the low vitamin B₁₂ group compared with controls (16 AD vs. 14 controls). There were more controls compared with AD participants in the high vitamin B₁₂ group (44 controls vs. 36 AD), but these differences were not statistically significant.

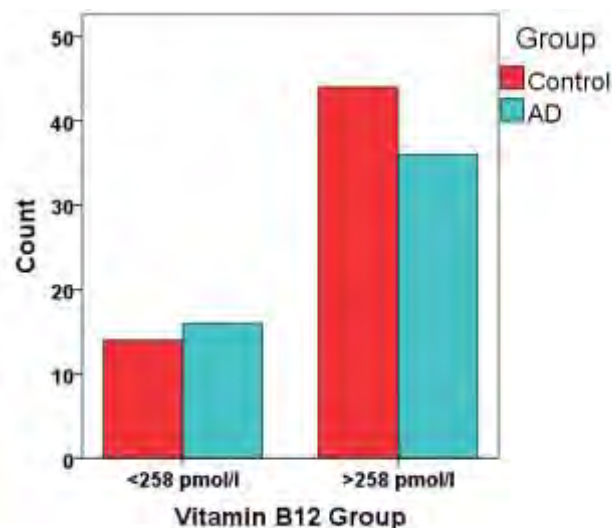
Despite the differences between groups, statistical analyses revealed that low vitamin B₁₂ levels and the presence of AD were not associated with each other ($\chi^2(1, N=110)=0.608, p=0.286$).

Furthermore, the odds of cognitive impairment (AD status) in the low vitamin B₁₂ group was not significantly increased compared with the high vitamin B₁₂ group ($OR=2.1, p=0.14, CI [0.79, 5.9]$).

Table 5.13: Serum vitamin B₁₂ high/low and AD/control group cross-tabulation

		Group		Total n (%)	$\chi^2=2.4$ $p=0.122$
		AD n	Control n		
Vitamin B ₁₂ group	low	16	14	30 (27)	OR=2.1 $p=0.14$ CI [0.79,5.9]
	high	35	44	79 (73)	
				109 (100)	

Figure 5.8: Clustered bar chart showing the distribution of AD and control participants in high and low vitamin B₁₂ groups



In another analysis, I showed that as the ages of participants' increased, the MMSE ($r_s = -0.34$, $p < 0.001$) CAMCOG ($r_s = -0.35$, $p < 0.001$) and Learning Subscale scores decreased ($r_s = -0.29$, $p = 0.003$) i.e. cognition was significantly associated with advancing age. Using regression analysis, I wished to analyse the relationship between vitamin B₁₂ and cognition when controlling for age as a possible confounding variable.

Regression analysis

A single model using a multiple regression method was used for all 3 cognitive measures. Table 5.14 summarises the indices used to describe the association between vitamin B₁₂ and the cognitive scores.

MMSE₁

Both independent variables, vitamin B₁₂ and age, accounted for 14% of the variance in MMSE₁ score ($r^2=0.14$, $F(2) = 8.8$, $p<0.001$). Both vitamin B₁₂ concentrations and age made significant predictions to the MMSE₁ score. For every unit increase in serum vitamin B₁₂ concentrations, MMSE₁ scores improved by 0.23 units ($\beta=0.23$, $p=0.012$).

CAMCOG₁ and the Learning Subscale₁

Similarly, both vitamin B₁₂ concentrations and age made significant predictions to the CAMCOG₁ and Learning Subscale₁ scores. For every unit increase in serum vitamin B₁₂ concentrations, CAMCOG₁ scores improved by 0.19 units ($\beta=0.19$, $p=0.036$), and the Learning Subscale₁ score by 0.21 units ($\beta=0.21$, $p=0.024$).

These variables accounted for 13% of the variance in CAMCOG₁ score ($r^2=0.13$, $F(2) = 8.4$, $p=0.001$) and 10% of the variance in the Learning Subscale₁ score ($r^2=0.1$, $F(2) = 5.5$, $p=0.005$).

Age had the opposite effect on all 3 measures of cognition. A unit increase in the age of the participants resulted in a 0.28, 0.29 and 0.21 unit decline in the MMSE₁ ($\beta=0.28$, $p=0.003$), CAMCOG₁ ($\beta=0.29$, $p=0.002$), and the Learning₁ ($\beta=0.21$, $p=0.024$) scores respectively.

Table 5.14: H4 Multiple linear regression

	Variable	β	p value	95% CI
MMSE ₁	<ul style="list-style-type: none">• vitamin B₁₂• age	0.23 -0.28	0.012** 0.003*	[0.001, 0.012] [-0.24, -0.005]
CAMCOG ₁	<ul style="list-style-type: none">• vitamin B₁₂• age	0.19 -0.29	0.036** 0.002*	[0.001, 0.004] [-0.88, -0.2]
Learning ₁	<ul style="list-style-type: none">• vitamin B₁₂• age	0.21 -0.2	0.024** 0.032**	[0.001, 0.01] [-0.18, 0.008]
<i>Dependent variables MMSE₁, CAMCOG₁ and Learning Subscale₁, and independent variables vitamin B₁₂ and age.</i> <i>*significant at the 0.01 level</i> <i>** significant at the 0.05 level</i>				

In summary, continuous analyses revealed significant positive relationships between folate and CAMCOG and the Learning Subscale scores. The relationship between vitamin B₁₂ and all cognitive measures (MMSE, CAMCOG and the Learning Subscale) in all participants was significant but did not hold true for AD and control groups when analysed separately. However, in a categorical analysis, there was no association between AD cognitive status and vitamin B₁₂ levels. Folate however, showed no significant association with MMSE₁ score. Regression analysis demonstrated that both vitamin B₁₂ and age independently predicted cognitive scores.

H4 is therefore proven with respect to vitamin B₁₂. Vitamin B₁₂ concentrations were independently and significantly associated with cognition: as the vitamin B₁₂ levels increased, cognitive scores increased. Folate did not show any relationship with MMSE₁ in this study.

5.7.5) H5: Hcy concentrations are inversely related to cognitive function in all participants

To determine the relationship between hcy levels and cognitive function as measured by the baseline MMSE₁, CAMCOG₁ and the Learning Subscale₁, I analysed the data both as continuous and as categorical variables.

Continuous variable analysis

Spearman's rank order correlation was used to determine the relationship between hcy and cognition in all study participants. The analysis revealed an inverse association between hcy and MMSE₁ ($r_s = -0.276$, $p = 0.004$), CAMCOG₁ ($r_s = -0.267$, $p = 0.005$) and the Learning Subscale₁ ($r_s = -0.259$, $p = 0.007$) i.e. as hcy concentrations increased, cognitive function deteriorated. This inverse association was not significant when analysed in control and AD groups separately.

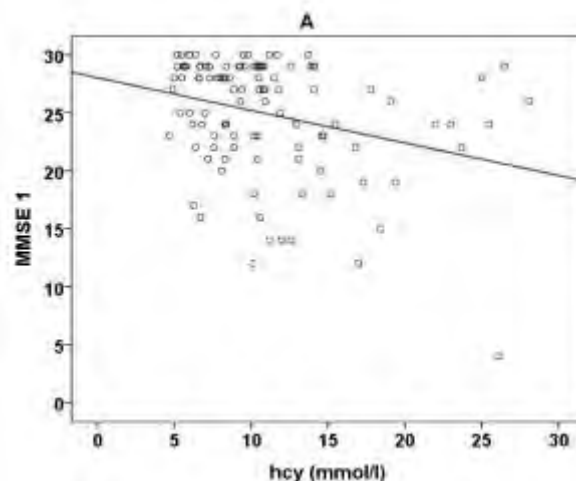
Table 5.15 summarises these findings and figure 5.9 is a graphic representation of the relationship.

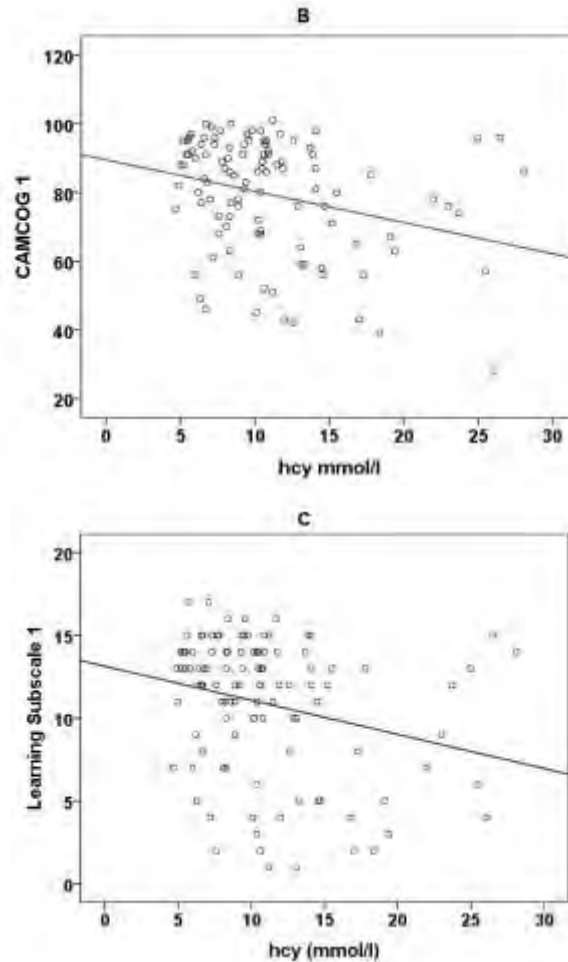
Table 5.15: Correlation between hcy and MMSE₁, CAMCOG₁ and the Learning Subscale₁ at baseline in all study participants.

Plasma homocysteine		
	Spearman rho(r_s)	p value
MMSE ₁	-0.276	0.004*
CAMCOG ₁	-0.267	0.005*
Learning Subscale ₁	-0.259	0.007*

**correlation is significant at the 0.01 level*

Figure 5.9: The relationship between hcy concentration and A) MMSE₁, B) CAMCOG₁ and C) Learning Subscale₁ scores in all participants.





Categorical analysis

Using guidelines set out by the Path Care Laboratory in South Africa (Fiekerstrand *et al.* 1993, Clarke *et al.* 1998), I divided the hcy data into low (<15mmol/l) and high (>15mmol/l) groups. I used a 2x2 contingency table to compare high and low hcy concentrations in AD and control participants.

A total of 108 samples were available for analysis, 91 samples with a value <15mmol/l and 17 with a hcy concentration >15mmol/l. Figure 5.10 represents the distribution of AD and control participants in high and low hcy groups. The graph shows a greater proportion of controls in the low hcy group (54 controls vs. 37 AD), and a greater number of AD participants in the high hcy group (12 AD vs. 5 controls).

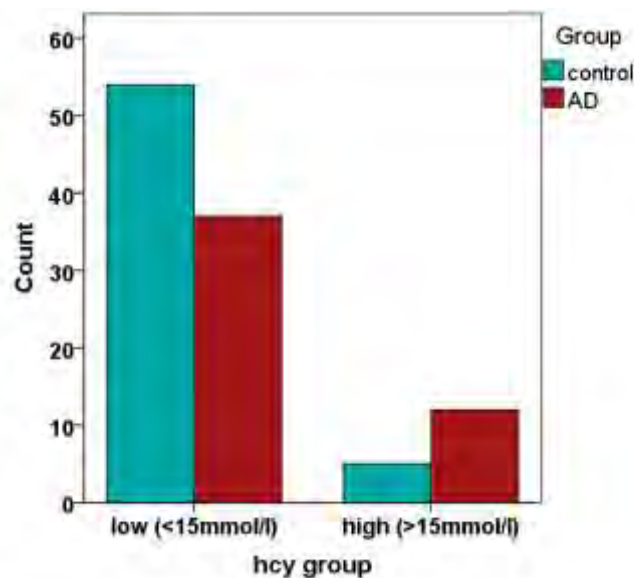
As can be seen by the frequencies cross tabulated in table 5.16, there was a significant relationship between high hcy levels and cognitive status (AD vs controls) ($\chi^2(1, N=108) = 5.18, p=0.023$). Furthermore, the odds of cognitive impairment in the high hcy group was 3.5x greater compared with the low hcy group ($OR=3.5, p=0.011, CI [1.14, 10.78]$).

Table 5.16 Hcy high/low and AD/control group cross-tabulation

		Group		Total	$\chi^2=5.18$ $p=0.023^{**}$ $OR=3.5$ $p=0.011^{**}$ <i>CI</i> [1.14,10.78]
		AD	Control		
Hcy group N=108	high	12	5	17	
	low	37	54	91	
	Total			108	

Figures are given in numbers
***significant at the 0.05 level*

Figure 5.10: The distribution of AD and control participants in high and low hcy groups.



In summary, the continuous analyses indicated that hcy concentrations were significantly negatively correlated with cognitive scores. The categorical analyses indicated that significantly more AD participants were in the high hcy group compared with the low hcy group.

From H1, I noted that hcy concentrations were significantly associated with age. Since AD patients were older and had fewer years of education than the controls, these 2 factors could confound the association between hcy and cognition. For this reason I decided to perform a regression analysis to determine whether the relationship between hcy and cognition was truly independent of other potential confounders.

Regression Analysis

Three linear regression models for each cognitive measure were constructed for the analysis. I used simple linear regression to make predictions in model 1, and multiple linear regression for predictions in models 2 and 3.

Model 1

A simple linear regression analysis was used to determine whether cognition as measured by MMSE₁, CAMCOG₁ and the Learning Subscale₁, could be predicted by hcy levels.

In table 5.17, indices of the strength of the variable hcy, in predicting cognitive scores, are presented. Results from the regression analysis suggest that 7% of the variance in the MMSE₁ ($r^2=0.07$, $F(1) = 7.67$, $p=0.007$), 8% in the CAMCOG₁ ($r^2=0.08$, $F(1) = 9.3$, $p=0.003$) and 7% in the Learning Subscale₁ ($r^2=0.07$, $F(1) = 7.7$, $p=0.007$), could be explained by hcy concentrations.

Hcy significantly predicted changes in MMSE₁ score ($\beta=-0.26$, $p=0.007$), CAMCOG₁ score ($\beta=-0.28$, $p=0.003$), and the Learning Subscale₁ ($\beta=-0.26$, $p=0.007$). A 1 unit increase in the hcy concentration resulted in a decrease in the MMSE score by 0.26 units. Similarly, for every unit increase in hcy concentrations, CAMCOG₁ and Learning Subscale₁ decreased by 0.28 and 0.26 units respectively (see table 5.14).

Table 5.17: H4 Model 1 Simple Linear Regression

	MMSE ₁	CAMCOG ₁	Learning Subscale ₁
r	0.26	0.28	0.26
r²	0.07	0.08	0.07

	Variable	β	<i>p</i> value	95% CI
MMSE₁	<ul style="list-style-type: none"> hcy 	-0.26	0.007*	[-0.4,-0.07]
CAMCOG₁	<ul style="list-style-type: none"> hcy 	-0.28	0.003*	[-1.5,-0.32]
Learning₁	<ul style="list-style-type: none"> hcy 	-0.26	0.007*	[-0.35,-0.06]

Dependent variables MMSE₁, CAMCOG₁ and Learning Subscale₁ scores, and independent variable hcy.
**significant at the 0.01 level*

Model 2

Multiple linear regression was used to determine whether hcy predicted changes in cognition as measured by MMSE₁, CAMCOG₁ and Learning Subscale₁ when controlling for age. In table 5.18, indices of the strength of 2 independent predictors hcy and age, are presented.

Results of the analysis revealed that 13% of the variance in MMSE₁ score ($r^2=0.13$, $F(2)=7.7$, $p=0.001$), 14% of the CAMCOG₁ score ($r^2=0.14$, $F(2)=8.2$, $p=0.001$) and 10% of the Learning Subscale₁ ($r^2=0.1$, $F(2)=5.8$, $p=0.004$), could be explained by hcy and age.

Additional findings included the following for each measure of cognition:

MMSE₁: When controlling for age, hcy concentrations were not significant in predicting changes in MMSE₁ score ($\beta=-0.16$, $p=0.143$). Age was the only significant variable within the model: a 1 unit increase in the age of a participant resulted in a 0.27 unit decline in the MMSE₁ score ($\beta=-0.27$, $p=0.009$).

CAMCOG₁: age made significant predictions to the CAMCOG₁ score. A 1 unit increase in the age of the participant resulted in a 0.26 unit decline in the CAMCOG₁ score ($\beta=-0.26$, $p=0.01$). Hcy concentrations did not quite reach significance: a 1 unit increase in hcy concentration resulted in a 0.17 unit decline in CAMCOG₁ score ($\beta=-0.17$, $p=0.088$).

Learning Subscale₁: hcy concentrations and age as predictor variables tended towards, but did not quite reach significance within this model: a 1 unit increase in hcy concentrations resulted in a Learning score decline by 0.18 units ($\beta=-0.18$, $p=0.077$). A 1 unit increase in the age of participants resulted in a 0.19 unit decline in the Learning Subscale₁ ($\beta=-0.19$, $p=0.065$).

In summary, hcy was not independently associated with changes in the MMSE₁ score. Hcy concentrations tended towards significance in explaining the changes in the CAMCOG₁ scores. Plasma hcy concentrations as well as age tended towards significance in explaining changes in the Learning Subscale₁ score.

Table 5.18: H4 Model 2 Multiple linear regression

	MMSE ₁	CAMGOG ₁	Learning Score ₁
r	0.36	0.37	0.32
r ²	0.13	0.14	0.1

	Variable	β	<i>p</i> value	95% CI
MMSE₁	• hcy	-0.15	0.143	[-0.32 -0.46]
	• age	-0.27	0.009*	[-0.25, -0.36]
CAMCOG₁	• hcy	-0.17	0.088	[-1.2, -0.85]
	• age	-0.26	0.01*	[-0.86,-0.12]
Learning₁	• hcy	-0.18	0.077	[-0.31,-0.16]
	• age	-0.19	0.065	[-0.18, 0.006]

Dependent variables MMSE₁, CAMCOG₁ and Learning Subscale₁, and independent variables hcy and age.
**** significant at the 0.01 level**

Model 3

Prior to analysing model 3 which included a third predictor variable, years of education, I used Spearman's rank order correlation to determine the relationship between 1) age and education, and 2) education and cognition.

Results showed that the age and education were negatively correlated and that the association was significant ($r_s=-0.276$, $p=0.003$) i.e. fewer years of education were associated with more advanced age. Education was positively correlated with all 3 measures of cognition (see table 5.19) i.e. more years of education were associated with better cognition.

Table 5.19: Correlation results between years of education and MMSE₁, CAMCOG₁ and the Learning Subscale₁ at baseline.

Years of Education		
	Spearman rho(r_s)	<i>p</i> value
MMSE₁	0.59	<0.001*
CAMCOG₁	0.65	<0.001*
Learning Subscale₁	0.47	<0.001*

**significant at the 0.01 level*

Table 5.20 summarises the indices used to describe the strengths of the 3 predictor variables in explaining the MMSE₁ score. The results show that 20% of the variance in MMSE₁ could be explained by hcy, age and years of education ($r^2=0.2$, $F(3)=12.6$, $p<0.001$). Similarly 40% of the variance in the CAMCOG₁ score ($r^2=0.4$, $F(3)=22.5$, $p<0.001$) and 22% of the variance in the Learning Subscale₁ ($r^2=0.22$, $F(3)=9.5$, $p<0.001$) could be explained by these 3 variables.

Hcy concentrations and age were not significantly associated with changes in MMSE₁. The number of years of formal education was the only significant predictor variable within this model. A unit increase in the years of education caused a 0.49 unit increase in the MMSE₁ score ($\beta=0.49$, $p<0.001$). Similarly for cognitive measures CAMCOG₁ and the Learning Subscale₁, hcy concentrations and age were not significant. A unit increase in the number of years of education resulted in a 0.54 unit increase in the CAMCOG₁ score ($\beta=0.54$, $p<0.001$) and a 0.36 unit increase in the Learning Subscale₁ score ($\beta=0.36$, $p<0.001$).

Table 5.20: H4 Model 3 Multiple linear regression

	Variable	β	p value	95% CI
MMSE₁	• hcy	-0.11	0.23	[-0.26, 0.6]
	• age	-0.14	0.124	[-0.17, 0.2]
	• education	0.49	<0.001**	[0.32, 0.67]
CAMCOG₁	• hcy	-0.13	0.122	[-0.98, 0.12]
	• age	-0.12	0.182	[-0.56, 0.11]
	• education	0.54	<0.001**	[1.35, 2.5]
Learning₁	• hcy	-0.15	0.127	[-0.27, 0.03]
	• age	-0.1	0.323	[-0.14, 0.005]
	• education	0.36	<0.001**	[0.16, 0.5]
<i>Dependent variables MMSE₁, CAMCOG₁, Learning Subscale₁ and independent variables hcy, age and years of education.</i> <i>*significant at the 0.01 level</i>				

Although continuous variable and categorical analyses suggested that hcy and cognition were inversely related to each other, this association did not hold true when controlling for the confounding effects of age and years of education.

H5 is therefore not proven, hcy concentrations were not associated with cognition when controlling for age or the years of education.

5.7.6) H6: The association between hcy and cognition is greater in ApoE ϵ 4 carriers compared with non-carriers.

I analysed the data for this hypothesis using correlation and regression analysis.

Continuous variable analysis

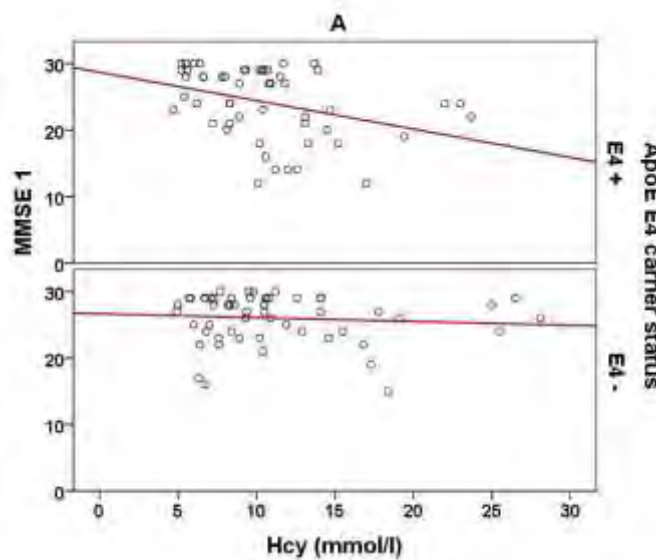
These results showed an inverse relationship between hcy and cognition, with a greater strength of association in ϵ 4 carriers compared with ϵ 4 non-carriers (see figure 5.11). The relationship between hcy and cognition as measured by MMSE₁ ($r_s=-0.41$, $p=0.003$), CAMCOG₁ ($r_s=-0.39$, $p=0.003$) and the Learning Subscale₁ ($r_s=-0.33$, $p=0.019$), was significant in ϵ 4 carriers but not in ϵ 4 non-carriers (see table 5.21).

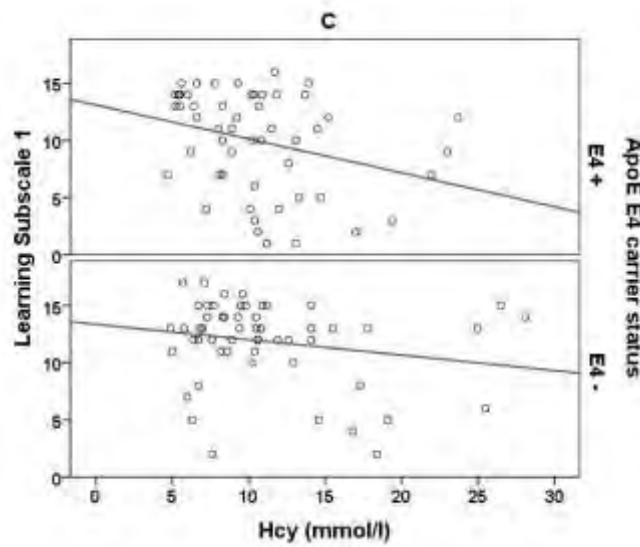
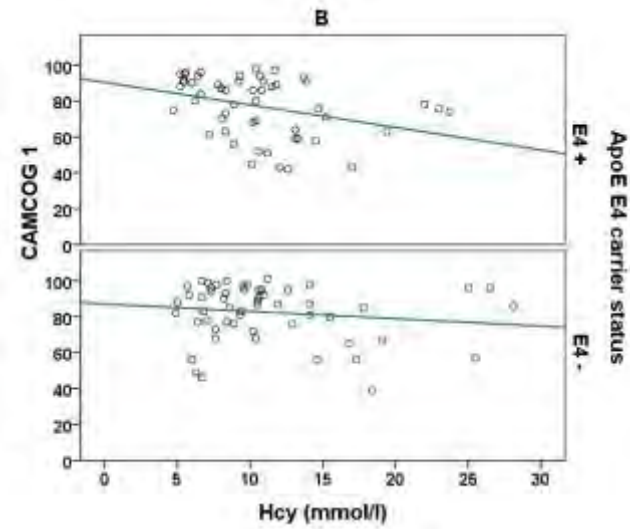
Table 5.21: Correlation between hcy concentrations and cognition as measured by MMSE₁, CAMCOG₁ and the Learning Subscale₁ in ε4 carriers vs. ε4 non-carriers

Plasma Homocysteine				
ApoE ε4 carrier status		Cognitive Test	Spearman's rho (r _s)	p value
ε4 +		MMSE ₁	- 0.41	0.003*
		CAMCOG ₁	-0.39	0.003*
		Learning Score ₁	- 0.33	0.019**
ε4 -		MMSE ₁	-0.08	0.574
		CAMCOG ₁	- 0.09	0.499
		Learning Score ₁	-0.13	0.331

**significant at the 0.01 level*
***significant at the 0.05 level*

Figure 5.11: The relationship between hcy concentrations and A) MMSE₁ B) CAMCOG₁ and C) Learning Subscale₁ scores in ApoE ε4 carriers and non-carriers





We know that hcy levels increase with age and that cognitive scores fall with age. So, could this interesting result be explained by the confounding effect of age?

To answer this question, I compared the mean ages of ApoE ε4 carrier and ε4 non-carrier groups. Table 5.22 shows the results of the independent samples t-test used in this analysis.

Table 5.22: Comparison of the mean ages between ApoE ε4 carrier and ε4 non-carrier groups.

	ε4+	ε4-	Statistical analysis between ε4+ and ε4-
Age mean (SD)	73.4 (7.9)	76 (9.7)	$t = -1.55 (107)$ $p = 0.124$

There was no statistical age difference between the groups. The association between plasma homocysteine and cognition in ApoE ε4 carriers was not confounded by the effect of age. Thus, the association between hcy and cognition is greater in ApoE ε4 carriers compared with ε4 non-carriers. In fact, this association was only present in ε4 carriers.

Regression analysis

In another analysis, I wished to determine whether plasma hcy levels could independently predict changes in cognitive scores when controlling for age and ApoE ε4 carrier status.

I used stepwise regression in a single model consisting of 3 predictor variables, hcy, ε4 carrier status and age, and cognition as measured by MMSE₁, CAMCOG₁ and the Learning Subscale₁ as the dependent variable.

Table 5.23 is a summary of the results. The variance for age and ApoE ε4 status are shown in Table 5.23a. The results showed the following:

MMSE₁

Hcy concentrations, ε4 carrier status and age accounted for 19% of the variance in MMSE₁ scores ($r^2 = 0.19$, $F(3) = 7.8$, $p < 0.001$).

Furthermore results showed age and ApoE ε4 carriers made significant predictions to the MMSE₁ score. A 1 unit increase in age and the presence of the ε4 allele resulted in a decline in the overall MMSE₁ score by 0.35 ($\beta = -0.35$, $p = 0.001$) and 0.26 ($\beta = -0.26$, $p = 0.005$) units respectively. Hcy concentrations were not significant within this model.

CAMCOG₁ and the Learning Subscale₁ Scores

Similarly, age, ApoE ε4 carrier status and hcy concentrations accounted for 18% of the variance in CAMCOG₁ scores ($r^2 = 0.18$, $F(3) = 7.4$, $p < 0.001$) and 16 % of the variance in the Learning score₁ ($r^2 = 0.16$, $F(3) = 6.7$, $p < 0.001$).

A 1 unit increase in age and the presence of the ε4 allele resulted in a 0.35 ($\beta = -0.35$, $p = 0.001$) and 0.23 ($\beta = -0.23$, $p = 0.013$) unit decline in CAMCOG₁ scores respectively. Furthermore, results showed that if a participant carried the ε4 allele and had a 1 unit increase in age, the overall Learning score decreased by 0.28 ($\beta = -0.28$, $p = 0.003$) and 0.26 ($\beta = -0.23$, $p = 0.013$) units respectively. Hcy concentrations made no significant predictions to the CAMCOG₁ and Learning Scores.

Table 5.23: Stepwise linear regression model: hcy, age and ε4 carriers and cognition as measured by MMSE₁, CAMCOG₁ and the Learning Subscale₁ scores

Dependent Variable	Predictor variable	β	p value	95% CI
MMSE ₁	• hcy	-0.07	0.501	[-0.24,0.12]
	• ε4 +	-0.26	0.005**	[-4.0,-0.73]
	• age	-0.35	0.001*	[-0.29,-0.08]
CAMCOG ₁	• hcy	-0.08	0.444	[-0.88,0.39]
	• ε4 +	-0.23	0.013**	[-13.1,-1.56]
	• age	-0.35	0.001*	[-0.9,-0.27]
Learning Subscale ₁	• hcy	-0.13	0.191	[-0.27,0.06]
	• ε4 +	-0.28	0.003**	[-3.7,-0.76]
	• age	-0.26	0.013**	[-0.21,-0.25]
*significant at the 0.01 level				
**significant at the 0.05 level				

Table 5.23a: Variance in the MMSE, CAMCOG and Learning Subscale scores

Table 5.23a	MMSE ₁	CAMCOG ₁	Learning Subscale ₁
R	0.43	0.42	0.41
r²	0.19	0.18	0.16

In this model, age and ApoE ε4 status were independently associated with cognitive decline.

In summary, the inverse relationship between hcy and cognition was present in ε4 carriers but not in ε4 non-carriers. In a regression analysis, hcy did not predict changes in cognitive scores.

5.7.7) H7: Participants with high baseline hcy concentrations will decline faster when compared to those with lower baseline hcy levels.

Neuropsychological evaluations were performed on 92 participants 1 year after the baseline examinations.

The data for this hypothesis were analysed 2 ways. First I used the paired Wilcox Signed Rank test to test the difference in median MMSE, CAMCOG and the Learning Subscale scores from the initial baseline tests (t1) to the follow-up scores (t2). Second, a simple linear regression was used to

evaluate whether high baseline hcy concentrations could predict changes in cognition within the AD sample group only.

Tables 5.24-5.26 show the difference in median scores from t1-t2 in all 3 cognitive domains.

The results showed that the median difference in CAMCOG scores from t1-t2 was significant in both control ($z=-2.2, p=0.027$) and AD participant groups ($z=-3.0, p=0.003$) i.e. CAMCOG scores were worse in AD participants but improved in control participants 1 year later.

Table: 5.24

Mdn (IQR)	All Participants	Control	AD
MMSE₁	27 (22-29)	29 (28-29)	22 (18-24)
MMSE₂	27 (21-29)	28 (28-30)	20 (16-23)
Paired Wilcox Signed Rank z (p value)	-1.4 (0.150)	-0.49 (0.63)	-1.9 (0.058)
<i>Values are given as medians with IQR in parentheses</i>			

Table: 5.25

Mdn (IQR)	All Participants	Control	AD
CAMCOG₁	84 (68-93)	92 (88-96)	67 (56-76)
CAMCOG₂	86 (60-94)	94 (89-94)	62 (49-73)
Paired Wilcox Signed Rank z (p value)	-1.3 (0.196)	-2.2 (0.027**)	-3.0 (0.003*)
<i>*significant at the 0.01 level</i>			
<i>** significant at the 0.05 level</i>			
<i>Values are given as medians with IQR in parentheses</i>			

Table: 5.26

Mdn (IQR)	All Participants	Control	AD
Learning Subscale₁	12 (8-14)	14 (13-15)	6.5 (4-10.25)
Learning Subscale₂	13 (7-15)	14 (13-15)	7 (4-10.5)
Paired Wilcox Signed Rank z (p value)	0.25 (0.801)	-1.90 (0.063)	-1.3 (0.211)
<i>Values are given as medians with IQR in parentheses</i>			

Regression analyses

In table 5.27, indices summarising the variables and statistics in a simple regression model are shown.

In AD participants only, I looked at the change in MMSE ($MMSE_2 - MMSE_1$), CAMCOG ($CAMCOG_2 - CAMCOG_1$) and Learning Subscale scores ($Learning_2 - Learning_1$) as the dependent variable, and high baseline hcy levels as the independent variable.

Results in all 3 cognitive domains were not statistically significant: high baseline hcy concentrations did not predict cognitive decline.

Table 5.27: Simple Regression Model – high hcy concentrations and change in MMSE, change CAMCOG and change Learning Subscale

Model 1	Variable	β	<i>p</i> value	95% CI
Change MMSE	• high hcy t1	-0.64	0.693	[-4.4,2.9]
Change CAMCOG	• high hcy t1	0.122	0.459	[-4.4,9.6]
Change Learning	• high hcy t1	-0.001	0.994	[-2.5,2.5]

H7 was therefore not proven, high baseline hcy did not predict changes in the MMSE, $CAMCOG_1$ and the Learning Subscale score in AD participants, 1 year later. The results however, showed that CAMCOG scores improved in controls and declined in AD participants 1 year after the baseline examination.

5.8) Summary of results

Demographic and biochemical results

- AD participants were older and had fewer years of education compared with controls.
- AD participants scored lower on all cognitive test scores compared with cognitively healthy controls.
- Participants with AD had higher plasma hcy concentrations compared with participants in the control group.
- There were a greater number of $\epsilon 4$ carriers in the AD group compared with the control group.

Results by hypothesis

H1	<ul style="list-style-type: none"> Age and hcy were directly related i.e. as the participants' ages increased, so did their plasma homocysteine concentrations. This relationship held true for the AD and control groups analysed separately.
H2	<ul style="list-style-type: none"> Hcy was inversely related to vitamin B₁₂ and folate i.e. as the vitamin B₁₂ and folate concentrations decreased, hcy concentrations increased. The relationship between hcy and vitamin B₁₂ held true in both AD and control groups when analysed separately.
H3	<ul style="list-style-type: none"> Hcy concentrations were not associated with vascular risk factors as measured by the Co-morbid Score.
H4	<ul style="list-style-type: none"> Vitamin B₁₂ and cognition were directly related i.e. the higher the serum vitamin B₁₂ levels, the better the cognitive scores. In a group of participants with low vitamin B₁₂ levels (<258pmol/l), there was no association between vitamin B₁₂ concentrations and cognitive impairment. In a regression model controlling for age, both vitamin B₁₂ concentrations and age were independently associated with cognitive changes. As the age of the participants increased, cognitive scores were lower. With increasing vitamin B₁₂ levels, cognitive scores were higher.
H5	<ul style="list-style-type: none"> Hcy concentrations were inversely related to cognitive function in all participants as well as AD and control groups analysed separately The risk of cognitive impairment was 3.5x greater in participants with high hcy concentrations (>15mmol/l) compared to those with low hcy concentrations (<15mmol/l). In a regression analysis, hcy concentrations were not independently associated with cognitive changes - age and education were significant confounding variables. The older the participants and the fewer years of formal education, the lower the cognitive scores.
H6	<ul style="list-style-type: none"> The inverse relationship between hcy concentrations and cognition as demonstrated in H5, was significant in ApoE ε4 carriers but not in the ApoE ε4 non-carrier group. Age was not a confounding factor in this relationship. In a regression analysis, plasma homocysteine levels did not predict cognitive scores. ApoE ε4 carrier status and age were both independently associated with changes in cognitive scores.
H7	<ul style="list-style-type: none"> In the longitudinal analysis, high baseline hcy levels did not predict cognitive decline 1 year later. There were, however, significant changes in the CAMCOG scores from t₁-t₂ in both AD and control groups. Control CAMCOG scores improved and AD CAMCOG scores worsened 1 year later.

Chapter 6

Discussion

This study examined the associations and inter-relationships between plasma homocysteine, vitamin B₁₂, folate and the ApoE genotype in a group of older South Africans with or without cognitive impairment (AD), who resided in the Western Cape region of South Africa. To my knowledge, this was the first study of its kind in a South African population and one of only a few studies (see de Silva *et al.* 2005) to examine these relationships in a developing low to middle income country

In general, the nutritional status of persons in LMIC is believed to be poorer than that of developed countries, and dietary intake of vitamin B₁₂ and folate may be less. Hence it was important to establish whether the same inter-relationships applied in our context, especially given the fact that deficiencies of vitamin B₁₂ and folate are potentially modifiable.

In a broad overview of the results, plasma homocysteine levels, in this sample group, were independently associated with age and vitamin B₁₂ concentrations. Age, vitamin B₁₂ levels and ApoE ε4 carrier status independently predicted cognitive scores. Plasma homocysteine, however, was not independently associated with cognition when controlling for other confounding variables, namely age and the level of education. Nevertheless, an inverse relationship between homocysteine and cognition was seen in ε4 carriers and not in ε4 non-carriers.

Before discussing my results and hypotheses in relation to the literature, I should like to comment briefly on the demographics of the study population.

6.1) Demographic characteristics and Baseline Cognitive Scores

The Western Cape region consists largely of persons of mixed European and indigenous African ancestry. There were 2 main participant groups, cognitively healthy older persons (NINCDS-ADRDA negative) and patients with mild to moderate AD (NINCDS-ADRDA possible or probable AD).

Participants in the AD group were slightly older than those in the control group (76.6 vs 73.2). Also, the AD participants had fewer years of formal education compared with controls (8 vs 14).

Historically, older South Africans in the Western Cape born in the 1940s and 1950s, received fewer years of education. They left school at an early age to start work in order to supplement the income of large families (Callinicos 1987). A low level of education and other markers of low socio-economic status have been shown to be linked to AD and dementia (Stern *et al.* 1994, Letenneur *et al.* 1999, Ngandu *et al.* 2007). Furthermore, these factors also contribute to cognitive decline in older people (Karp *et al.* 1994, Evans *et al.* 2004).

Age itself is the most important risk factor for the development of AD (Fratiglioni *et al.* 1991, Bachman *et al.* 1993, Lindsay *et al.* 2002). Hence it is not surprising that the AD group turned out to be slightly older than the control group.

Although there were more females than males in this study, this difference was the same for both AD and control groups. Possible explanations for the female preponderance are the larger number

of older females than males in the general population and in the Western Cape in particular (Statistics South Africa 2011), and the greater willingness of women to participate in research studies compared with men (Dunn *et al.* 2004).

Cognitive function scores, as measured by MMSE, CAMCOG and the Learning Subscale, were lower in the AD group compared with controls. This, of course, is expected as the groups were largely defined in terms of their cognitive function.

6.2) Overall Biochemical and Genetic Characteristics

AD and control groups differed significantly with regards to plasma homocysteine and folate concentrations. AD participants had higher homocysteine and lower folate levels. The difference between serum vitamin B₁₂ levels in AD and control groups tended towards, but did not quite reach, statistical significance ($p=0.053$). Many other studies have shown lower vitamin B₁₂ levels in AD compared with control groups (Clarke *et al.* 1998, Bernard *et al.* 1998). There are, however, many potential confounding variables such as age and education, which I shall review later.

Concerning the ApoE $\epsilon 4$ carrier status (homozygous and heterozygous) there were more $\epsilon 4$ carriers in the AD group compared with cognitively healthy controls (57% vs 43%).

Possession of the ApoE $\epsilon 4$ allele is a well-established risk factor for late onset AD (Farrer *et al.* 1995). I have discussed the associations between AD and the $\epsilon 4$ allele in section 2.6.5 pg. 24. I shall further discuss ApoE $\epsilon 4$ and its inter-relationships with plasma homocysteine and cognition in H6.

Sandholzer *et al.* showed that ApoE $\epsilon 4$ homozygotes were 3 to 5 fold more frequent in the South African indigenous Khoi-San community compared to a European population. Many of my participants are likely to have partial Khoi-San ancestry (Quintana-Murci *et al.* 2010). It is therefore not surprising that nearly half (48%) of all participants, both AD and controls, carried an $\epsilon 4$ allele.

The $\epsilon 4$ frequency is generally believed to be higher in the indigenous African population than those of European descent. While not many people of the Nguni (black African) ancestry were included in my study, they too have allelic frequencies approaching those of the Khoi-San (Willis *et al.* 2003, Joska *et al.* 2010).

There was a two-fold increased risk of AD in $\epsilon 4$ carriers compared with $\epsilon 4$ non-carriers. One possible biological mechanism underlying the increased susceptibility to AD in ApoE $\epsilon 4$ carriers include less efficient clearance of amyloid from the brain parenchyma (Strittmatter *et al.* 1993). Additionally, the ApoE $\epsilon 4$ allele is associated with impaired neurite growth, cytoskeletal instability and mitochondrial dysfunction in neurones (Nathan *et al.* 1994 and 1995).

The results of this study, if extrapolated to the rest of the South African population, suggest that we will be faced with a major epidemic of AD in the future as life expectancy increases and the indigenous population with a high $\epsilon 4$ carrier frequency ages.

I shall now discuss the results with regards to the specific hypotheses (H1-H7).

6.3) H1: Age and plasma homocysteine levels were directly related i.e. as the participants' ages increased, so did their plasma homocysteine concentrations.

My results were consistent with findings from European and North American studies. Most of these studies describe a positive correlation between advancing age and plasma homocysteine concentrations, independent of vitamin status and vascular risk factors (Selhub *et al.* 1993, Nygard *et al.* 1998).

I showed an independent association between age and homocysteine levels unrelated to vitamin B₁₂ and folate status. This relationship was significant in all participants as well as in AD and control groups analysed separately. Older age was associated with higher homocysteine concentrations not only in patients with AD, but also in cognitively healthy older persons. It is possible that additional age-related factors such as deteriorating renal function and the presence of vascular disease, could explain the relationship between age and homocysteine.

Serum creatinine has been shown to be a strong determinant of plasma homocysteine concentrations in general (Bostom *et al.* 1999, Wollensen *et al.* 1999) and in older persons particularly (Brattstrom *et al.* 1994, Norlund *et al.* 1998, Chen *et al.* 2013). I had serum creatinine measurements for all the participants. Thus, as part of a post-hoc analysis, I analysed the relationship between serum creatinine and plasma homocysteine. They were, in fact, strongly correlated i.e. as the serum creatinine levels increased so did the plasma homocysteine concentrations ($r_s=0.587$, $p<0.001$).

Clinically, there were no obvious nutritional deficiencies or malabsorption syndromes present in the participants. However, subclinical or subtle B₁₂ deficiencies, other dietary deficiencies and malabsorption syndromes, many of which may also be age-related, cannot be excluded. Other lifestyle and environmental factors such as smoking (Targer *et al.* 2000), alcohol consumption (Sakuta *et al.* 2005), coffee consumption (Temple *et al.* 2000), drug interactions (Sachdev *et al.* 2004) and decreased exercise and stress, may contribute to elevated homocysteine concentrations although little is known about their associations with plasma homocysteine levels in older persons.

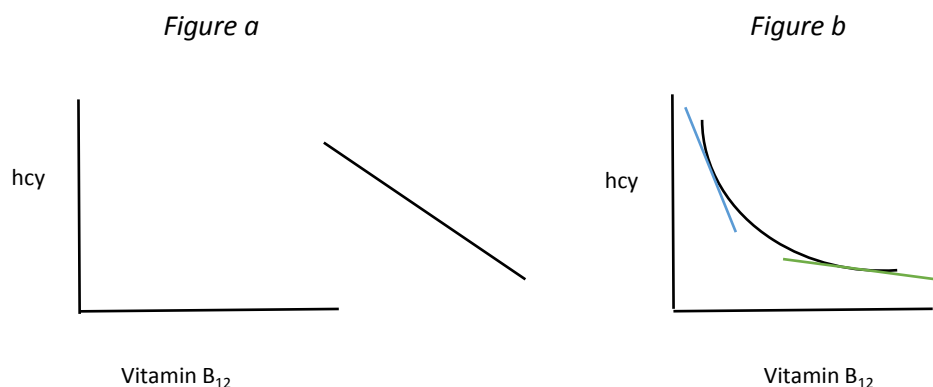
A larger epidemiological study amongst older South Africans would be useful to determine whether other factors mentioned above contribute to elevated plasma homocysteine levels with advancing age.

In summary, the findings from my study confirmed the independent association between age and plasma homocysteine previously reported in international studies. Declining renal function may partly explain this association.

6.4) H2: Plasma homocysteine concentrations were inversely related to vitamin B₁₂ and folate levels i.e. as the vitamin B₁₂ and folate concentrations decreased, plasma homocysteine concentrations increased.

The inverse relationship between vitamin B₁₂ and homocysteine has been well documented i.e. low vitamin B₁₂ levels are linked to higher plasma homocysteine concentrations (Stabler *et al.* 1988, Selhub *et al.* 1993, Ubbink *et al.* 1993, McCaddon *et al.* 1998). My findings are consistent with those reported in the literature. Homocysteine, along with measurements of other biological compounds such as methylmalonic acid (MMA) and holotranscobalamin (holoTC), probably better reflect vitamin B₁₂ status than circulating blood plasma vitamin B₁₂ concentrations (Clarke *et al.* 2004, Miller *et al.* 2006).

The relationship between vitamin B₁₂ and homocysteine was stronger in the AD group compared with cognitively healthy controls and the median homocysteine concentrations were higher in the AD group compared with the controls. This suggests that the association between vitamin B₁₂ and homocysteine is not linear (figure a) but exponential (figure b) i.e. homocysteine levels rise more rapidly when vitamin B₁₂ levels are in the lower range. A study by Vogiatzoglou *et al.* in 2009 supports this idea.

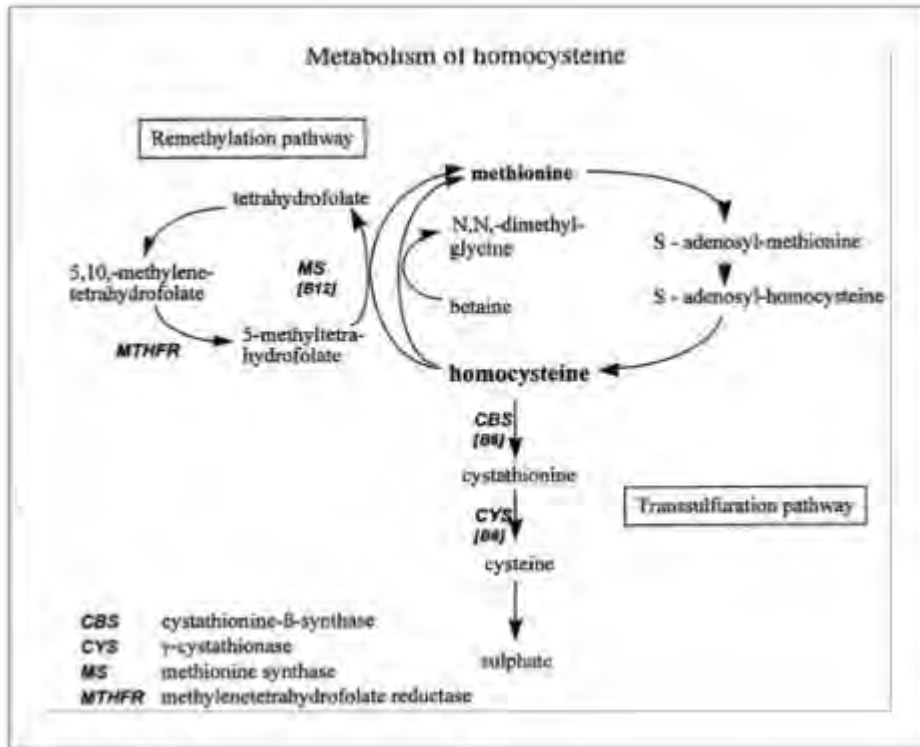


The fact that most participants had folate levels in the normal to high range (12 - >45.5nmol/l), probably reflects the fact that folic acid fortification of cereals and wheat in South Africa is mandatory. This was introduced in 2003. When the group median values were compared with the defined National Health Laboratory Service (NHLS) normal reference ranges there were no participants with low folate (<5 nmol) levels. Additionally, the widespread use of multi-vitamin supplementation by older people is likely to contribute to the excessively high folate values measured (NFCS-2005).

In a multiple regression model, vitamin B₁₂ levels, but not plasma folate, independently predicted changes in plasma homocysteine concentrations. This inter-relationship can be explained by the closely linked metabolism between vitamin B₁₂ and plasma homocysteine (see figure 6.1). Remethylation of homocysteine to methionine (which eliminates excessive homocysteine), is catalysed by methionine synthase (MS), a vitamin B₁₂- dependent enzyme. Thus, vitamin B₁₂ deficiency, occurring through reduced dietary intake would result in reduced MS activity and hyperhomocysteinaemia. Dietary lack and reduced absorption of B₁₂ could well apply to older persons in our study.

Vitamin B₁₂, folate and their associations with cognitive function are discussed later on in H4

Figure 6.1: The Metabolism of Homocysteine
 From: Doshi et al. 1999



6.5) H3: Plasma homocysteine concentrations were not associated with vascular risk factors as measured by the Co-morbid Score.

The association between elevated homocysteine levels and vascular disease has been well documented in the literature based on studies conducted mainly in Europe and North America. Plasma homocysteine is an independent risk factor for atherosclerosis as well as cardiovascular and cerebrovascular disease (Clarke *et al.* 1991; den Heijer *et al.* 1996; Selhub *et al.* 1995; Ueland *et al.* 2000). Certain vascular risk factors, such as hypertension and cigarette smoking, may work synergistically with high homocysteine levels to potentiate vascular disease (Graham *et al.* 2002, O’Callaghan *et al.* 2002). Independent associations between systolic blood pressure and homocysteine levels have also been reported (Sutton-Tyrell *et al.* 1997, Budge *et al.* 2002).

In this study, I hypothesised that higher plasma homocysteine levels would be associated with increasing numbers of vascular risk factors. There was, however, no significant relationship between plasma homocysteine and vascular risk factors as measured by the Co-morbid score. Additionally, there was no significant relationship between the Co-morbid score and cognition as measured by MMSE, CAMCOG and the Learning Subscale (post-hoc analysis MMSE $p=0.22$, CAMCOG $p=0.109$, Learning Subscale $p=0.082$).

Possible reasons for the lack of association are as follows:

- i. The Co-morbid Score was based on previous diagnoses from clinical records. I did not apply strict criteria for the diagnosis of hypertension, Type 2 Diabetes Mellitus and hypercholesterolaemia. Therefore, this was a crude measure of vascular risk factors.
- ii. The score was based on the presence or absence of risk factors. It did not account for the degree of severity or participant compliance with medication. For example, 2 participants could be assigned the same score rating with completely different severities or control of the vascular risks. For example, one participant might have a blood pressure (BP) of 220/110, a plasma glucose level of 15mmol/l, a cholesterol levels of 9mmol/l and smoke 20 cigarettes/day. Another participant might have had a marginally raised BP of 150/95, a plasma glucose of 8mmol/l, a cholesterol level of 7mmol/l and smoke 10 cigarettes/day. Both would have been assigned the same Co-morbid score.

The above hypotheses evaluated the associations between plasma homocysteine and its various determinants in this sample population. In a single stepwise regression model, vitamin B₁₂, age and serum creatinine levels independently predicted plasma homocysteine levels. These findings are consistent with the published literature (Selhub *et al.* 1993, Seshadri *et al.* 2002). Our failure to find an association between plasma homocysteine and vascular risk factors could be due to the lack of refinement of the Co-morbid score.

Large epidemiological studies with strict diagnostic criteria and measurements of severity would be more useful. In a future study we should attempt to assess the extent of cerebrovascular disease, both overt and subclinical, by quantifying the degree of white matter changes using MRI scans.

6.6) H4: Vitamin B₁₂ and folate are directly related to cognition.

Serum vitamin B₁₂ levels and age independently predicted cognitive scores.

Vitamin B₁₂

In this study vitamin B₁₂ was positively correlated with cognition as measured by MMSE, CAMCOG and the Learning Subscale scores in all study participants. These results were broadly in agreement with the majority of cross-sectional studies which demonstrated an association between low vitamin B₁₂ and cognition in older persons (Goodwin *et al.* 1983, Bernard *et al.* 1998, Lindeman *et al.* 2000). These authors agree that low vitamin B₁₂ and folate levels are associated with poorer cognitive function in older persons.

In my study the median vitamin B₁₂ concentration in AD participants was lower compared with controls (317 vs 414 pmol/l) but both median values were normal by South African NHLS laboratory standards. In a categorical analysis, there was no association between vitamin B₁₂ levels and cognitive impairment. In fact, there were very few people in this sample with traditionally defined low vitamin B₁₂ levels (<148 pmol/l) and subclinical vitamin B₁₂ deficiency (<258 pmol/l). Thus, the number of vitamin B₁₂ deficient persons may have been too small to demonstrate an association with cognition categorically. Additionally, it is also possible that serum vitamin B₁₂ measurements were not truly reflective of the intracellular vitamin B₁₂ status in my study participants. Other

markers of low vitamin B₁₂ such as elevated plasma homocysteine, methylmalonic acid (MMA) and holoTC measurements could more accurately reflect low vitamin B₁₂ status (Clarke *et al.* 2004, Miller *et al.* 2006, Obeid *et al.* 2007).

In a post-hoc categorical analysis, using a median split of vitamin B₁₂ concentrations, a contingency table analysis showed that vitamin B₁₂ levels <350 pmol were more closely related to cognition than the previous categorical analysis using a vitamin B₁₂ cut-off <258pmol/l ($p= 0.14$ vs $p= 0=0.074$). This supports the supposition that larger numbers could improve the statistical significance of the result.

In a multiple regression analysis, vitamin B₁₂ levels and age both independently predicted changes in cognitive scores.

Biologically, cognitive impairment as a result of low vitamin B₁₂ levels could be mediated by the effects of elevated plasma homocysteine or MMA which are both neurotoxic (Carmel 1996). They have both also been associated with brain atrophy and white matter damage (Vogiatzoglou *et al.* 2008, Smith and Refsum 2009). Clinical intervention trials would be needed to determine whether vitamin B₁₂ supplementation improves the signs and symptoms in early AD. Future studies with more accurate measurements of vitamin B₁₂ status (holoTc and MMA) could indicate that the traditional vitamin B₁₂ cut-off value, used by South African laboratories, are too low. Treatment with vitamin B₁₂ at a higher level than is currently indicated (up to 300pmol/l) may slow the neurological effects of vitamin B₁₂ deficiency including brain atrophy (Smith *et al.* 2010).

Folate

Folate was inversely related to plasma homocysteine concentrations in all participants as well as in the AD and control group. This is in keeping with the literature (Clarke *et al.* 1998, Seshadri *et al.* 2002). Folate concentrations were related to the CAMCOG and Learning Subscale scores but not to the MMSE score. Median folate concentrations were lower in the AD group compared with controls (25 vs 36 nmol/l). These findings are in keeping with studies that demonstrate that low folate levels are associated with poorer cognition (Clarke *et al.* 1998, Ramos *et al.* 2005, Ravaglia *et al.* 2005, Kim *et al.* 2008).

Biologically, low folate levels could cause cognitive impairment through 1) the toxic effects of elevated plasma homocysteine concentrations, 2) lack of folate (a precursor of purines and pyrimidine bases) leads to defective DNA and neuronal death (Kruman *et al.* 2000). Deficiency of folate is also linked to cerebral atrophy through vascular and nonvascular mechanisms such as altered neurotransmitter metabolism (Snowdon *et al.* 2000).

In summary, based on my findings and recommendations from overseas studies, vitamin B₁₂ and folate are beneficial to cognitive health. Thus it may be worthwhile supplementing vitamin B₁₂ especially in older people with and without symptoms of deficiency. This is because, 1) older persons are more susceptible to subclinical deficiency and 2) South African foods are fortified with folic acid. High folate concentrations could potentially mask underlying vitamin B₁₂ deficiency and potentiate cognitive impairment (Smith *et al.* 2009 and 2012, Selhub *et al.* 2009).

6.7) H5: Plasma homocysteine concentrations were inversely related to cognitive function. However they did not predict cognitive test scores in a regression model when controlling for age.

The association between elevated homocysteine levels and Alzheimer's disease established in the 1990s originated from studies which demonstrated an association between plasma homocysteine concentrations and atherosclerosis and cerebrovascular disease (Perry *et al.* 1995; Bots *et al.* 1997, Seshadri *et al.* 2008). Vascular risk factors are also a risk factor for Alzheimer's disease and, by association it is therefore plausible that homocysteine may be a risk factor for Alzheimer's disease. This hypothesis has been evaluated at length over the last decade in retrospective and prospective studies (Clarke *et al.* 1998, McCaddon *et al.* 1998, Seshadri *et al.* 2002, Ravaglia *et al.* 2005).

The following facts are generally accepted regarding plasma homocysteine concentrations:

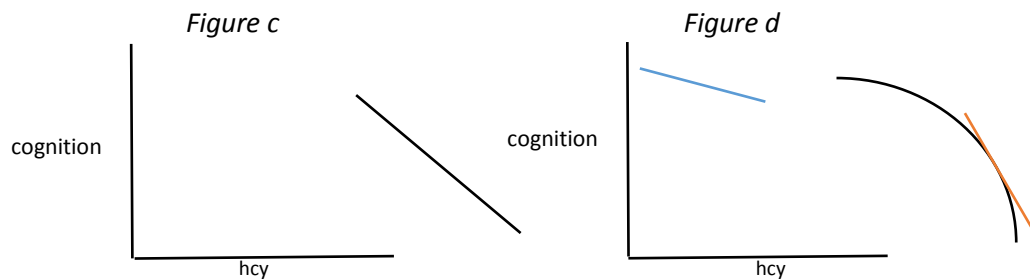
- i. Plasma homocysteine levels increase with age (H1).
- ii. Plasma homocysteine levels are higher in AD patients compared with controls.
- iii. Plasma homocysteine is an independent risk factor for AD (controlling for cofounders of age, sex, education and vascular disease).
- iv. Elevated plasma homocysteine levels at baseline predict faster cognitive decline.

In keeping with the findings of studies abroad, this study, in a continuous analysis, demonstrated an inverse association between plasma homocysteine concentrations and cognition as measured by the MMSE, CAMCOG and Learning Subscale scores. There were more AD participants with higher homocysteine concentrations compared to cognitively healthy controls (12 vs 5). The odds of having AD in individuals with high homocysteine concentrations was 3.5-fold greater compared with low homocysteine levels (<15mmol/l). These results were in keeping with the majority of case-control studies (Clarke *et al.* 1998, McCaddon *et al.* 1998, Selley *et al.* 2002, Gallucci *et al.* 2004, Linnebank *et al.* 2010).

I should point out that the cut-off value used to split plasma homocysteine concentrations into high and low groups in the categorical analysis was based on the reference ranges used by the South African "Path Care" laboratory. The laboratory based these cut-offs on the normal distribution of a European population (Fiekerstrand *et al.* 1993, Clarke *et al.* 1998). It has been suggested that a plasma homocysteine reference range should be established by individual laboratories to reflect their specific regional population with specific limits set for children, adults and the elderly (Refsum *et al.* 2004). Additionally, South Africa has mandatory folate fortification and therefore the upper limit of the plasma homocysteine reference range would generally be lower; in some cases up to 25% lower than that of a non-fortified population (Jacques *et al.* 1999, Refsum *et al.* 2004). The median homocysteine concentration in my study sample was 10.2mmol/l in all participants, 10.6mmol/l in the AD group and 9.3 mmol/l in controls. In the AD group, I would have expected a higher median plasma homocysteine concentration based on literature findings. However, this value may still represent a mild-moderately elevated concentration in the South African population.

I used regression models to determine whether the relationship between homocysteine and cognition still held true if I controlled for possible confounders, viz. age and education. (Age and education were both related to cognitive function). Plasma homocysteine concentrations did not predict changes in MMSE, CAMCOG and the Learning Subscale scores when controlling for age and years of education. However, in a post hoc analysis, high levels of homocysteine (>15 mmol/l) did predict changes in the CAMCOG and Learning Subscale scores.

This could be explained in two ways. Firstly, the CAMCOG and Learning Subscale scores are more sensitive tools for detecting early changes in cognition compared with the MMSE score (Hobson *et al.* 2012, Clarke CM *et al.* 1999). Secondly, the result again supports the hypothesis that the inverse association between plasma homocysteine and cognition is not linear (figure c) but rather exponential (figure d) i.e. cognition declines more rapidly with elevated plasma homocysteine concentrations.



6.8) H6: The relationship between plasma homocysteine concentrations and cognition was significant in $\epsilon 4$ carriers but not in the $\epsilon 4$ non-carrier group.

The modifying effects of ApoE $\epsilon 4$ status on the relationship between homocysteine and cognition has been researched but has not, in my opinion, been adequately explained. A small number of studies have examined the effects of ApoE $\epsilon 4$ status on the relationships between 1) vitamin B₁₂ and cognition and 2) plasma homocysteine and cognition (Brown *et al.* 2011, Feng *et al.* 2009, Vogiatzoglou *et al.* 2013). The results of these studies indicate that the ApoE $\epsilon 4$ carrier status strengthens the associations between vitamin B₁₂, plasma homocysteine and cognition (Elias *et al.* 2007, Vogiatzoglou *et al.* 2013).

In this study sample, without controlling for covariate factors, the inverse relationship between plasma homocysteine and cognition was accentuated in ApoE $\epsilon 4$ carriers relative to the same correlation in all participants (H5). The relationship between plasma homocysteine and cognition, in fact, did not exist in ApoE $\epsilon 4$ non-carriers. This result is in keeping with findings by Elias *et al.* In their study, they demonstrated a higher magnitude of association between plasma homocysteine and cognitive performance within ApoE $\epsilon 4$ carriers relative to ApoE $\epsilon 4$ non-carriers. When controlling for covariate factors, they observed no significant relationship between cognition and plasma homocysteine in ApoE $\epsilon 4$ non-carriers (Elias *et al.* 2007).

Although the biological mechanisms by which the $\epsilon 4$ allele strengthens the relationship between plasma homocysteine and cognition are not clear, it has been suggested that environmental factors could perpetuate cognitive impairment in those who are genetically predisposed to developing the disease. Individuals with the ApoE $\epsilon 4$ genotype may be more vulnerable to other risk factors for cognitive impairment such as plasma homocysteine (Kivipelto *et al.* 2008). Biologically, I hypothesized that the stronger relationship between plasma homocysteine and cognition in $\epsilon 4$ carriers could be mediated by abnormal A β metabolism. ApoE $\epsilon 4$ carriers have defective

mechanisms of A β clearance (Holtzman *et al.* 2000) and plasma homocysteine concentrations could increase amyloid deposition in the brain and also render neurones more susceptible to damage by A β peptides (Obeid *et al.* 2006; Stanger *et al.* 2009, Ho *et al.* 2001). Thus, the ApoE ϵ 4 genetic predisposition coupled with the neurotoxic effects of plasma homocysteine, could result in increased levels of amyloid in the brain and more damage to neurones relative to ϵ 4 non-carriers.

It has also been demonstrated that the ApoE ϵ 4 status strengthens the relationship between vitamin B₁₂ and cognition (Vogiatzoglou *et al.* 2013). In my study, post-hoc analyses showed that ApoE ϵ 4 allelic status did not strengthen the relationship between vitamin B₁₂ and cognition. In fact, there was no relationship between vitamin B₁₂ and cognition when analysed in ϵ 4 carriers and ϵ 4 non-carriers separately.

It would be promising if a risk factor such as plasma homocysteine could be modified to reduce the adverse effects on cognition in individuals with a genetic predisposition to AD. This would be especially important in South Africa given the high ApoE ϵ 4 allelic frequency in the indigenous population.

6.9) H7: high baseline plasma homocysteine levels did not predict cognitive decline 1 year later.

In this study we did not show that high baseline plasma homocysteine levels predicted cognitive decline 1 year after the initial assessment. However, the median CAMCOG scores in both AD and control groups were significantly different between the baseline and follow-up time point. In the AD group, scores were significantly lower 1 year later compared to the control group whose scores improved over the same period.

Studies reporting cognitive decline with high baseline homocysteine concentrations had long follow-up periods or have followed a cohort of patients with rapidly declining cognitive scores. Seshadri and Zylberstein *et al.* had followed their dementia-free cohort over a period of 8 and 35 years respectively, whilst the studies of Annerbo and Oulhaj *et al.* followed MCI participants and their conversion to Alzheimer's disease over 6 and 9.5 years respectively (Seshadri *et al.* 2002, Zylberstein *et al.* 2011, Annerbo *et al.* 2006, Oulhaj *et al.* 2010). Our study looked at potential cognitive decline 1 year later which was most likely too short to demonstrate any significant change.

The MMSE, though widely used, is an insensitive measure of early cognitive changes and is of limited value in measuring the progression of Alzheimer's Disease in less than 3 years due to the individual variability of score (Clarke CM *et al.* 1999). The CAMCOG scores decreased in participants with AD over a period of 1 year and improved in cognitively healthy controls. This was expected for the AD participants and could be explained by practice/learning effects in the controls.

Hobson *et al.* demonstrated that the CAMCOG was more sensitive in detecting cognitive impairment compared with the MMSE score (Hobson *et al.* 1999). Another study showed that the orientation and memory (learning) subscale of the CAMCOG was the best predictor of conversion to AD, but that global MMSE and CAMCOG scores were poor predictors (Conde-Sala *et al.* 2012).

In summary, the follow-up period was probably too short to adequately test H7.

Study limitations and recommendations for future research

There are several factors which could account for the discrepancies found in this study relative to overseas literature findings.

- I. This study was an observational case-control study. Although this type of study is less expensive and requires less time compared with longitudinal cohort studies, it is difficult to disentangle cause and effect in the population studied. I was not able to determine whether participants with AD developed low vitamin B₁₂ and high homocysteine levels as a consequence of poor dietary intake, which is common in older people, or whether these abnormalities preceded the onset of the disease. The literature indicates that low vitamin B₁₂ and elevated plasma homocysteine levels are, in fact, risk factors for AD. They therefore precede the advent of the disease.
- II. This was not a large epidemiological study, but rather a small sample of participants in the Western Cape. The results therefore reflect demographic characteristics and associations specific to this region and are not necessarily representative of the South African population.
- III. As indicated before, I used the most inexpensive and readily available methods for assessing vitamin B₁₂ and plasma homocysteine concentrations. Ideally, however, more sensitive methods of quantifying intracellular vitamin B₁₂ levels (holoTC, MMA) may have generated more significant results. In addition, it is recommended that fasting or meal-standardised measurements for plasma homocysteine should be used, as protein intake has been shown to cause variations in the plasma homocysteine concentrations on a day-to-day basis (Thirup *et al.* 1999). I used non-fasting plasma homocysteine concentrations in this study. A more standardised protocol for blood taking, following a fasting period, may have produced more significant findings.
- IV. The Co-morbid score was a crude measure of vascular disease. An interesting dimension to this study would be to relate plasma homocysteine levels to cerebrovascular disease by quantifying the extent of WMHs in the brain on MRI scans.
- V. The longitudinal part of this study was too short to observe any change in cognitive scores. It also suffered from a high attrition rate. The solution to this would have been to have larger numbers in the beginning of the study and a longer follow-up period.

Despite these shortcomings, this study is important because it is the first to examine the relationships between plasma homocysteine, vitamin B₁₂, folate, ApoE ε4 status and Alzheimer's disease in Southern Africa. The results of this study have largely agreed with the literature findings in European and North American studies.

In South Africa, we have the unique combination of a high background ApoE ε4 allelic frequency as well as an ageing population with a generally poor nutritional status. For all these reasons, it would be helpful for both our and the world's ageing population, to further explore the risk factors for Alzheimer's disease and especially the inter-relationships between vitamin B₁₂, folate, the ApoE gene, plasma homocysteine and cognitive function.

A further study with larger participant numbers and a sample more representative of the diverse ancestral background of the South African population, would better be able to describe Alzheimer's disease and its associations and risk factors in our context.

Conclusion

I hope my study, despite its limitations, will serve as the basis for continued future research on Alzheimer's disease in South Africa. I should like to continue to be involved in work of this nature.

In addition to poor access to health care facilities, there are still strong social stigmata regarding Alzheimer's disease. The latter are especially pronounced in traditional communities. There is a lack of knowledge about the disease not only amongst communities and the families and carers with Alzheimer's disease, but also amongst health professionals. The disease is under-reported and under-diagnosed in South Africa because of these factors and also because the burden of chronic infections related to HIV has received a greater health priority.

While we cannot change the fact that the population will age and that the genetic predisposition to Alzheimer's disease is non-modifiable, it is important for South Africans to be aware of potentially modifiable risk factors for cognitive impairment. Although I am aware that there still is a long way to go in identifying methods that could modify disease outcome or slow cognitive decline, I hope that my study findings will assist in this process.

Appendix

Clinical Diagnostic Criteria

Appendix A

NINCDS / ADRDA (Alzheimer's disease)

McKhann G et al. Neurology 1984; 34: 939-944

0. Negative

1. Possible

- I. Presence of a dementia syndrome, in absence of other neurological, psychiatric or systemic disorders capable of causing dementia, but with atypical features, such as variations in the onset, presentation or clinical course of the illness.
- II. Presence of a second systemic disease or brain disorder sufficient to produce dementia, but not considered to be *the* cause of the dementia.
- III. Single, gradually progressive, severe cognitive deficit (e.g. Worsening amnesic syndrome), in the absence of another identifiable cause.

2. Probable

- I. Dementia, established by history & clinical examination, and documented with, or confirmed by, cognitive or neuropsychological tests e.g. MMSE (<23), CAMCOG (<80).
- II. Deficits in 2 or more areas of cognition.
- III. Progressive worsening of memory and other cognitive functions.
- IV. No disturbance of consciousness.
- V. Age of onset > 40; usually > 65.
- VI. Absence of systemic disorders or other brain diseases [or psychiatric disorders] that could in themselves account for the progressive deficits in memory & cognition.

3. Definite

- I. Probable AD on clinical criteria.
- II. Histopathological evidence (biopsy, autopsy).

Supportive evidence for the diagnosis of Probable AD:

- I. Progressive deterioration of specific cognitive functions such as language (aphasia), motor skills (apraxia), and perception (agnosia);
- II. impaired activities of daily living and altered patterns of behaviour;
- III. family history of similar disorders, particularly if confirmed neuropathologically; and
- IV. laboratory results of:
 - a) normal lumbar puncture as evaluated by standard techniques;
 - b) normal pattern or non-specific change in EEG, such as increased slow wave activity, and
 - c) evidence of cerebral atrophy on CT with progression documented by serial observation.

Clinical features consistent with the diagnosis of Probable AD:

- I. Plateaus in the course of progression of the illness.
- II. Associated symptoms of depression, insomnia, incontinence, delusions, illusions, hallucinations, catastrophic verbal, emotional or physical outbursts, sexual disorders and weight loss.
- III. Other neurological abnormalities in some patients, especially those with more advanced disease, including motor signs such as increased motor tone, myoclonus or a gait disorder.
- IV. Seizures in advanced disease.
- V. CT normal for age.

Features that make the diagnosis of Probable AD uncertain or unlikely include:

- I. Sudden apoplectic onset.
- II. Focal neurological signs such as hemiparesis, sensory loss, visual field deficits, and incoordination early in the course of the illness.
- III. Seizures or gait disturbances at the onset or very early in the course of the illness.

Appendix B

DSM-IV diagnostic criteria for dementia of the Alzheimer's type

Diagnostic and Statistical Manual of Mental Disorders, 4th ed., American Psychiatric Association, 1994; p142.

- A. The development of multiple cognitive deficits manifest by both
 - (1) memory impairment
 - AND
 - (2) one (or more) of the following cognitive disturbances:
 - (a) aphasia (language disturbance)
 - (b) apraxia (impaired ability to carry out motor activities despite intact motor function)
 - (c) agnosia (failure to recognize objects despite intact sensory function)
 - (d) disturbance in executive functioning (i.e. planning, organization, sequencing, abstracting).
- B. The cognitive deficits in criteria A1 and A2 each cause significant impairment in social or occupational functioning and represent a significant decline from a previous level of functioning.
- C. The deficits do not occur exclusively during the course of a delirium.

Appendix C

Mild Cognitive Impairment (MCI)

Petersen RC *et al. Neurology* 2001; 56: 1133-42

- I. Memory complaint, preferably corroborated by an informant.
- II. Objective memory impairment.
- III. Normal general cognitive functioning.
- IV. Intact activities of daily living.
- V. Not demented.

Appendix D

Deterioration Cognitive Observee (DECO)

Ritchie et al. 1992

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 E

Geriatric Depression Scale (GDS)

Brink et al. 1982

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 |

Scoring System: Yes=1, No=0

GDS >7 = depression

Appendix F

The Bristol Activities of Daily Living Scale (BADLS)

Buck et al. 1996

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 and/or back to front []

- and/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 []

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