AN INVESTIGATION INTO RISK FACTORS OF

ALZHEIMER'S DISEASE:

ASSOCIATION WITH PRE-CLINICAL, CLINICAL

AND AUTOPSY CONFIRMED AD

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Alzheimer’s disease (AD) is the most commonly diagnosed form of dementia in the elderly. Predominantly this disease is sporadic in nature with only a small percentage of cases exhibiting a familial trait. Whilst early-onset AD may be explained by single gene defects, most AD cases are late-onset (>65 years), and although there is no known definite cause for this form of the disease, there are several known risk factors. Of these, the ε4 allele of the apolipoprotein E gene (APOE) is a major risk factor. Unlike the pathogenic mutations in the amyloid precursor or those in the presenilins, APOE ε4 alleles increase risk for AD but are not causative, even when both copies are present. In addition to the polymorphism at the ε2/ε3/ε4 locus, which determines what type of apoE protein one inherits, polymorphisms within the proximal promoter of the APOE gene have also been identified, which are thought to alter transcription of the APOE gene. Recently a mutation in PS1, namely Glu318Gly, has also been identified as a genetic risk factor for AD. The aims of this thesis were to investigate the roles of genetic risk factors (APOE ε4, -491A/T, -219G/T and Glu318Gly) and apolipoprotein levels in clinically diagnosed and autopsy confirmed AD cases in the Australian population. Risk factors were then studied in subjective memory complainers and post-menopausal woman, to determine their value as predictors of disease and whether they alter the efficacy of oestrogen’s putative neuroprotection.

This thesis confirms and extends the association of the APOE ε4 allele with AD and its gene dosage effect on increasing this risk and decreasing the age of onset in the Australian population. In addition, the significant association of the -491A allele with AD and its association with increased levels of plasma and cerebral apoE suggest that
this polymorphism plays an important role in the development of AD. The -219T allele conferred a negligible increase in risk and was associated with decreased brain apoE levels. However, little impact on plasma apoE levels was observed. Cerebral apolipoprotein D (apoD) levels were found to be elevated in AD cases independent of apoE, suggesting that apoD levels may be related to cognitive decline and that apoD and apoE contribute to the pathogenesis of AD via separate molecular mechanisms. The APOE ε4 and the -491A allele were associated with both subjective memory impairment and a high rate of conversion to clinical endpoints. Homozygosity at both loci may play an integral role in cognitive decline and may be useful for the identification of individuals at high risk for the development of AD. The frequency of the Glu318Gly mutation was increased in familial AD individuals, suggesting that this mutation may play a role in AD similar to that of APOE ε4. Likewise, it was associated with subjects complaining of memory impairments. However, whether this mutation directly contributed to cognitive decline remains to be determined. The evaluation of the therapeutic efficacy of oestrogen on cognition and the pharmacogenetic effect of the APOE ε4 allele on oestrogen’s therapeutic efficacy requires further longitudinal studies, that utilise more sensitive measures of cognition, before firm conclusions can be reached.

This thesis presents genetic and biochemical evidence supporting the notion that regulation of apoE protein levels may contribute to the risk of AD, separate and apart from the well-known polymorphisms at the ε2/ε3/ε4 locus. Furthermore, this thesis presents data that confirms and extends the genetic association of the APOE coding and promoter polymorphisms with AD, pre-AD subjective memory complaints and a high rate of conversion to MCI or AD in the Australian population.
DECLARATION

This is to certify that

(i) The thesis comprises only my original work.
(ii) Due acknowledgements are made in the text to all other materials used.
(iii) The thesis has not previously been accepted for any other degree in this or another institution.
(iv) The thesis has been substantially accomplished during enrolment in the degree.
(v) The thesis is less than 100,000 words in length, exclusive of tables, illustrative matter and appendices.

Simon Matthew Laws
(B.Sc. Hons)
PUBLICATIONS

JOURNAL ARTICLES


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CONFERENCE ABSTRACTS

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‘Thou wouldst be loved?- then let thy heart
From its present pathway part not!
Being everything which now thou art,
Be nothing which thou art not.
So with the world thy gentle ways,
Thy grace, thy more than beauty,
Shall be an endless theme of praise,
And love- a simple duty.’
- Edgar Allan Poe
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LIST OF ABBREVIATIONS

a.a. Amino acid
ACh Acetylcholine
AChE Acetylcholinesterase
AD Alzheimer’s disease
ADAM (10/17) A disintegrin and metalloprotease
ADRDA Alzheimer’s Disease and Related Disorders Association
ApoD Apolipoprotein D (protein)
ApoE Apolipoprotein E (protein)
APOE Apolipoprotein E (gene)
εₙ (or APOE εₙ) Non-ε4 allele of the Apolipoprotein E gene
APP Amyloid precursor protein
α-APPs Alpha secreted APP
β-APPs Beta secreted APP
APP-C100 C-terminal fragments of APP
APS Ammonium persulfate
Aβ Amyloid-β-protein
BACE Beta site APP cleaving enzyme
BChE Butyrylcholinesterase
Bis N,N’-methylene-bis-acrylamide
bp Base pairs
BSA Bovine serum albumin
cAMP Adenosine 3’, 5’-cyclic monophosphate (cyclic AMP)
CAA Cerebral amyloid angiopathy
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CAD</td>
<td>Coronary Artery Disease</td>
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<tr>
<td>CAMDEX</td>
<td>Cambridge Examination for Mental Disorders of the Elderly</td>
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<tr>
<td>CAMCOG</td>
<td>Cognitive component of the CAMDEX</td>
</tr>
<tr>
<td>CCV</td>
<td>Clathrin-coated vesicles</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyl transferase</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CRGH</td>
<td>Concorde Repatriation General Hospital</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>CVLT</td>
<td>California verbal learning test</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double deionised water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxynucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DS</td>
<td>Down syndrome</td>
</tr>
<tr>
<td>DSM-IV</td>
<td>Diagnostic and Statistical Manual of Mental Disorders - Fourth Edition</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme labelled immunosorbent assay</td>
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<tr>
<td>EOAD</td>
<td>Early-onset Alzheimer’s disease</td>
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<td>EOFAD</td>
<td>Early-onset familial Alzheimer’s disease</td>
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<tr>
<td>ERα</td>
<td>Oestrogen receptor-α</td>
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<td>ERβ</td>
<td>Oestrogen receptor-β</td>
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<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
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<tr>
<td>FAD</td>
<td>Familial Alzheimer’s disease</td>
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<td>H₃PO₄</td>
<td>Phosphoric Acid</td>
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<tr>
<td>hrs</td>
<td>Hours</td>
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<td>HCR</td>
<td>Hepatocyte control region</td>
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<tr>
<td>HMG-CoA</td>
<td>3-Hydroxy-3-Methyl-Glutaryl Coenzyme A</td>
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<tr>
<td>HPH</td>
<td>Hollywood Private Hospital</td>
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<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
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<tr>
<td>cHRT</td>
<td>Combined hormone replacement therapy (opposed oestrogen therapy)</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
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<tr>
<td>HSPG(s)</td>
<td>Heparin sulphate proteoglycan(s)</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>Kilobases</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>LOAD</td>
<td>Late-onset Alzheimer’s disease</td>
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<tr>
<td>LRP</td>
<td>Low density lipoprotein receptor-like protein</td>
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<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamps</td>
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<tr>
<td>MMSE</td>
<td>Mini-mental state examination</td>
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<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microliter</td>
</tr>
<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
</tr>
<tr>
<td>MC(s)</td>
<td>Memory complainer(s)</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>MHRI</td>
<td>Mental Health Research Institute</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>min(s)</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>ml</td>
<td>Millilitres</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NFT/s</td>
<td>Neurofibrillary tangle/s</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NINCDS</td>
<td>National Institute of Neurological and Communicative Disorders and Stroke</td>
</tr>
<tr>
<td>NISAD</td>
<td>The Neuroscience Institute of Schizophrenia and Allied Disorders</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>dNTP(s)</td>
<td>Deoxynucleotide triphosphate(s)</td>
</tr>
<tr>
<td>OR(s)</td>
<td>Odds ratio(s)</td>
</tr>
<tr>
<td>ORT/ERT</td>
<td>Oestrogen/estrogen replacement therapy</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCV</td>
<td>Packed cell volume</td>
</tr>
<tr>
<td>PGE</td>
<td>Prostaglandin E</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMI</td>
<td>Post-mortem interval</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>PS1</td>
<td>Presenilin 1</td>
</tr>
<tr>
<td>PS2</td>
<td>Presenilin 2</td>
</tr>
<tr>
<td>RAVLT</td>
<td>Rey auditory verbal learning test</td>
</tr>
<tr>
<td>RNA</td>
<td>Riobonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SAD</td>
<td>Sporadic Alzheimer’s disease</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNP(s)</td>
<td>Single nucleotide polymorphism(s)</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element-binding protein</td>
</tr>
<tr>
<td>TACE</td>
<td>Tumour necrosis factor alpha converting enzyme</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline containing Tween 20</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N,N-Tetra-methylethylenediamine</td>
</tr>
<tr>
<td>TES</td>
<td>Tris-EDTA-salt buffer</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TP</td>
<td>Total protein</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(hydroxy methyl)-methylamine</td>
</tr>
<tr>
<td>Tween-20</td>
<td>polyoxyethylene (20) sorbitan monolaurate</td>
</tr>
<tr>
<td>URE</td>
<td>Upstream regulatory element</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
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<tr>
<td>w/v</td>
<td>Weight per volume</td>
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</tbody>
</table>
Dementia has been defined as the “organic loss of intellectual function” (Miller and Keane, 1978) and results in the “impairment of intellect, memory and personality but without impairment of consciousness” (Lishman, 1978). Progressive dementia is the most commonly diagnosed senile dementia with the most common manifestation being Alzheimer’s disease (AD), a progressive neurodegenerative disease that is the most commonly diagnosed cause of dementia in the elderly (Lendon et al, 1997; Gilman, 1997; Gooch and Stennett, 1996).

In 1905, a German physician named Alois Alzheimer first described the clinical symptoms and amyloid containing neuropathological lesions in the brain of a 53-year-old patient who had suffered from dementia. Although senile dementia was well recognised at this time, the 53-year-old patient under study was particularly interesting due to the expression of both an early onset dementia and the presentation of neuropathological structures known as senile plaques and neurofibrillary tangles. Thus the term Alzheimer’s disease was developed and used only for ‘presenile dementia’ or dementia occurring before the age of 65 years. Since this initial description the term has been subsequently applied to patients exhibiting amyloid neuropathy with progressive dementia usually occurring between the ages of 65 to 90 (Gilman, 1997). AD accounts for 50% of all diagnosed cases of dementia worldwide (Gooch and Stennett, 1996; Myers et al., 1996). At present AD has an incidence that doubles every 5 years between the ages of 65 and 85 years in all human populations (Finch and Cohen, 1997). It affects 10-13% of individuals over the age of 65 and 35-48% of those over the age of 80 years (Gooch and Stennett, 1996). Another interesting aspect is its higher prevalence in the
female population, even after controlling for differences in longevity (Lendon et al., 1997). The majority of AD cases are sporadic in nature with only a small percentage (5-10%) showing evidence of a familial trait (Lendon et al., 1997). Within the ageing population, the incidence of AD is increasing and as such the early identification and the development of suitable means of treatment is an ongoing goal of AD research.

1.1 CLINICAL CHARACTERISTICS AND DIAGNOSIS OF AD

The onset of AD is clinically characterised by the gradual onset and progressive decline in cognition (Small et al., 1997) and memory, often associated with abnormalities of speech and language and visuospatial dysfunction (Lendon et al, 1997; Gilman, 1997). Other symptoms that are apparent in the early stages of the disease are disturbances in judgement (Gilman, 1997). In addition, AD sufferers have difficulty learning new information and retaining it for more than a few minutes (Small et al., 1997).

The rate of cognitive decline in AD is variable, but at onset, motor and sensory functions are not affected (Small et al., 1997). Following onset of AD, the degree of cognitive impairment becomes more severe. Patients often develop gross behavioural disturbances, including paranoia, hallucinations and disturbances of motor activity (Small et al., 1997; Gilman, 1997). Other cognitive impairments that develop in the later stages of AD include the loss of voluntary ability to perform skilled motor activities (apraxia) including speech (aphasia), disorientation and executive functioning (Small et al., 1997).

The definitive diagnosis of AD can only be made on neuropathological evidence of the disease (Gilman, 1997; Gooch and Stennett, 1996; McKhann et al., 1984). Without such
evidence, only available at autopsy or following biopsy, the diagnosis of AD is based on the clinical presentation and described as probable or possible AD (McKhann et al., 1984). The clinical criterion for the diagnosis of AD is outlined by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) (McKhann et al., 1984). The clinical examination is of great importance as it provides data to fulfil inclusionary and exclusionary criteria for the diagnosis of AD. These criteria are then used to identify subgroups of AD patients, which is important for both patient care and research (McKhann et al., 1984).

Presently, with no cure for AD, the principal aim of treatment is to improve the quality of life and maximise functional performance by enhancing the cognition, mood, and behaviour of AD sufferers (Small et al., 1997). Acetylcholine (ACh) is an extremely important neurochemical that is involved in the transmission of nerve impulses from a neurotransmitter to the postsynaptic receptor (Barner and Gray, 1998). In AD sufferers the frontal cortex and hippocampus, regions of the brain strongly linked to memory and cognition, show a significant reduction in ACh due to impaired synthesis by the enzyme choline acetyl transferase (ChAT). Enzymes called cholinesterases, which include Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE), degrade ACh and thus decrease levels further. It is hoped that the inhibition of these enzymes will inhibit the intrasynaptic degradation of ACh resulting in increased ACh concentrations in the synapses allowing increased stimulation of receptors and in turn improve cognition (Barner and Gray, 1998). Thus, drugs termed Cholinesterase Inhibitors are currently the only widely recognised approved treatment for AD. These drugs decrease the ability of AChE and BChE to degrade ACh, thus keeping levels of ACh at a sufficient level, which in turn minimises the loss of cognitive function (Barner and Grey, 1998;
McKenna et al., 1997). Whilst the use of these drugs improves the quality of life of the sufferer they are by no means an effective long lasting cure for AD. This goal is at the forefront of AD research and will only be achieved when the complex causative nature of the neuropathology of AD, which gives rise to the clinical presentation of AD, is fully understood.

1.2 NEUROPATHOLOGICAL HALLMARKS OF AD

1.2.1 Macroscopic Changes

As AD progresses the loss of white and grey matter is reflected byatrophy within selected regions of both hemispheres. This atrophy is typified by the macroscopic hallmarks of AD that include widening of the sulci, narrowing of the gyri and ventricular dilation (Brun, 1983; Figure 1.1). This pathology is most evident in regions of the brain responsible for the functions of memory, language and judgement. In particular these regions include the temporal lobe, the hippocampus (the most severely effected), the post-central parietal region and to a lesser extent, the frontal lobes (Brun, 1983). These structural changes are quite severe and can be determined using neuro-imaging techniques such as Computer Tomography (CT) scans and Magnetic Resonance Imaging (MRI). These diagnostic tools are valuable for increasing diagnostic accuracy (Hecker, 1998). However, while these macroscopic changes are characteristic of AD they are not
specific for the disease and the severity is highly variable, depending on the severity/stage of the disease process.

1.2.2 Microscopic Changes

Microscopically, AD pathology is characterised by amyloid (also termed senile or neuritic) plaques (Masters et al., 1985; Beyreuther and Masters, 1997; Tokuda et al., 1996; Iwamoto et al., 1996), neurofibrillary tangles (Gilman, 1997; Gooch and Stennett, 1996), amyloid congophilic angiopathy (Grey et al., 1990; Leblanc et al., 1992; Itoh et al., 1993) and neuronal loss (Pomera, et al., 1992; Engelborghs and Deyn, 1997).

These neuropathological changes of AD have been seen to originate in the entorhinal cortex and progress to the hippocampus, a region in the brain important for memory formation (Gilman, 1997). However, the changes are not limited to these areas of the brain. The cerebral cortex in particular is affected (Gilman, 1997) as are numerous subcortical structures including the nucleus basalis of Meynert (Engelborghs and Deyn, 1997) and cerebral blood vessels (Sandbrink et al., 1995).

1.2.2.1 Amyloid plaques

Amyloid is the name given to a group of proteins which, when deposited in tissue, exhibit a fibrillar ultra-structure and a β-pleated sheet molecular configuration with an affinity for certain dyes such as Congo Red (Underwood, 1992). The amyloid plaques seen in AD (Figure 1.2) exhibit each of these properties. They differ from other amyloid deposits seen in the body through its central core protein, a small 4 kilodalton (kDa) protein called β-amyloid (Aβ; Masters et al., 1985) which is seen only in AD amyloid deposits.
These plaques are a complex aggregation, both chemically and structurally, of several different proteins. The most common of which is Aβ, which itself is derived from a larger precursor protein, termed the amyloid precursor protein (APP; Haas et al., 1997; Olesen et al., 1997). The amyloid plaques consist of a central core of radiating amyloid fibrils surrounded by dystrophic neurites and activated glial cells (Gooch and Stennett, 1997) and other associated proteins. These include APP, α1-antichymotrypsin, apolipoprotein E (apoE), immunoglobulins, and complement factors (Haas et al., 1997; Olesen et al., 1997).

Amyloid plaques exist as two major forms. The first is the classical form, which, as described above, consists of a compact Aβ deposit. The second form is termed diffuse amyloid plaques. These are an amorphous deposition of the same Aβ protein and occur in the parenchyma of AD brains to a greater extent than classical plaques (Gooch and Stennett, 1997; Sandbrink et al., 1995). These plaques are surrounded by far less dystrophic neurites and activated glial cells, than the classical plaque, suggesting that it is the classical plaque that is the primary cause of cerebral damage and resultant dysfunction (Yamaguchi et al., 1988, 1989, 1990). It has been suggested that these diffuse plaques, which may develop as a normal part of ageing, may possibly be precursors to the formation of the AD amyloid (senile) plaques (Delaere et al., 1993). Reports of transitional plaque forms and the intermingling of these different plaque types support the transitional theory (Joachim and Selkoe, 1992) and suggest that amyloid plaques are dynamic lesions that may mature over time from the benign diffuse forms to the toxic classical senile plaque.
1.2.2.2 Neurofibrillary tangles

Neurofibrillary tangles (NFTs; Figure 1.3) are the second major type of brain lesion seen in AD patients (Gilman, 1997; Gooch and Stennett, 1996) and are believed to correlate highly with the clinical symptoms and the severity of dementia (McKee et al., 1991; Arriagada et al., 1992). Although this correlation is strong it is unclear whether these structures are the initial lesion or a response to nerve cell injury (Goedert, 1993). In either case, it reinforces the belief that these alterations in the neuronal cytoskeletons are signs of neuronal dysfunction (Lee and Trojanowski, 1992; Kosik, 1994). Although NFTs may appear in the brains of healthy aged individuals they are found in greater numbers in patients with AD, primarily in regions associated with memory and other intellectual functions. Arriagada et al., (1992) found that the severity of dementia was positively related to the number of NFTs in the neocortex, but not to the degree of the deposition of senile plaque deposition. They also reported that NFTs appeared in the entorhinal cortex, CA1/subiculum field of the hippocampus and the amygdala early in the disease process.

NFTs are found inside nerve cells (Gilman, 1997), cell bodies and apical dendrites (Gooch and Stennett, 1997). They appear as paired helical filaments (Gilman, 1997; Goedert, 1993) composed of high molecular weight proteins (>200kD), which are primarily twisted fibres of the microtubule associated protein tau (MW 55-62k) (Tapiola
et al., 1997). An increased level of tau, in the cerebrospinal fluid (CSF), in the later stages of AD has been shown to be predictive of severe neurodegeneration (Tapiola et al., 1997).

*Tau* exists as six isoforms ranging in length from 352 to 441 amino acids, produced from a single gene by alternative messenger RNA (mRNA) splicing (Goedert et al., 1993). The normal function of *tau* is to stabilise microtubules against disassembly and to allow their interaction with other cellular components (Gooch and Stennett, 1997), especially the anterograde and retrograde axonal transport of proteins (Gilman, 1997). This function is regulated by the reversible phosphorylation and dephosphorylation of tau by protein kinases and phosphatases (Gilman, 1997).

The tau present in a large proportion of the NFTs is abnormally phosphorylated, promoting the formation of the paired helical filaments which measure between 8nm at their narrowest point and 20nm at their widest (Goedert, 1993). The hyperphosphorylation of tau would suggest that increased kinase or decreased phosphatase activity is apparent in AD brains (Gilman, 1997). Studies have shown that the decreased phosphatase activity may be of importance in AD (Gooch and Stennett, 1997).

### 1.2.2.3 Cerebral amyloid angiopathy

Cerebral amyloid angiopathy (CAA; Figure 1.4) is a prominent feature of AD (Premkumar et al., 1996) being present in anywhere up to 97% of AD cases (Vinters et al., 1987). CAA is characterised by the accumulation of amyloid and is localized to the leptomeninges, the small pial vessels, the

Figure 1.4: Cerebral amyloid angiopathy. (Congo Red)

Staining of amyloid in the cerebral wall (Arrow).
intracortical arterioles as well as the brain capillaries (Grey et al., 1990; Kalaria et al., 1996). CAA differs from generalised amyloidosis as it is, almost exclusively, confined to the brain. Whilst it is also seen in normal brains of the elderly it is much more severe in AD brains (Premkumar et al., 1996). An abundance of CAA is well established as a major aetiological factor in intracranial haemorrhages (Vinters, 1987). However, the actual factors responsible for its deposition are not clear. Studies have suggested that the ε4 allele of the lipid transport protein apoE is a significant factor in the development of CAA in AD. This conclusion was based on a high association between this allele and AD patients exhibiting lobar or intracerebral haemorrhage (Premkumar et al., 1996).

1.2.2.4 Neuronal loss

The loss of neurons is another neuropathological characteristic of AD. The resulting cell death is due to the interruption to normal neuronal transmission and is caused by the presence of neuritic plaques and neurofibrillary tangles (Barner and Grey, 1998). Cholinergic neurons are the predominant neurons degraded and are located in the nucleus basalis of Meynert in the substantia inominata of the basal forebrain with projections into the corpora amygdaloida and neocortex (Engelborghs and De Deyn, 1997). Other types of neurons affected are serotonergic and noradrenergic. However, the association of the cholinergic neurons with AD is of importance due to their location in areas of the brain associated with memory, higher intellectual function and consciousness (Barner and Grey, 1998; Gilman, 1997; McKenna et al., 1997).

Although the loss of the cholinergic neurons is apparent, the post-synaptic receptors remain preserved for a considerable period of time (Barner and Grey, 1998; Gilman, 1997). ACh is the neurochemical responsible for the transmission of neural impulses in the cholinergic neurons. The levels of this neurochemical decrease in AD due to its
impaired synthesis from choline, via ChAT. A decreased ChAT activity is a well-established marker for neuronal loss and the decreased levels of ACh correlates to the level of dementia (Engelborghs and De Deyn, 1997).

1.2.3 Molecular Pathology of AD

1.2.3.1 Amyloid β-peptide and the Amyloid Precursor Protein

Purification of the amyloid plaques of AD resulted in the identification of the major proteinaceous component of the plaques, the Aβ peptide (Masters et al., 1985; Tjernberg et al 1997; Gooch and Stennett, 1997; Sandbrink et al., 1995). It was subsequently determined that Aβ, which exists as a 4 kDa peptide, is a proteolytic product of a much larger precursor protein termed APP, which is a ubiquitously expressed transmembrane glycoprotein with a receptor like structure (Hartmann et al, 1996). APP is encoded for by a single gene on Chromosome 21, which undergoes alternate splicing to yield several isoforms ranging from ~100-130 kDa (Oishi et al., 1997; Selkoe et al., 1988). The full length APP (Figure 1.5) exists as a glycoprotein consisting of a single polypeptide chain of 770 amino acids. It comprises a large amino-terminal ectoplasmic domain, consisting primarily of a cysteine-rich domain, an acidic domain and a large glycosylated domain. The remainder of the protein consists of a single transmembrane spanning domain and a small cytoplasmic tail (Selkoe, 1993; Gooch and Stennett, 1997; Sandbrink et al., 1995). Aβ derives from a region at the junction of the ecto- and transmembrane domains of APP (Oishi et al., 1997). Complete sequencing of Aβ has shown that it consists of 40 to 43 amino acid residues. The N-terminal 28 residues of Aβ are extracellular and the remaining 15 residues are located within the transmembrane domain of APP (Figure 1.5; Esch et al., 1990). Amino acid residues 29-40/43, located within the trans-membrane domain, are hydrophobic in
nature (Figure 1.5) and confer on this protein the ability to self-aggregate and polymerise into amyloid fibrils (Figure 1.5; Masters et al., 1985; Beyreuther et al., 1986; Martins et al., 1991).

Figure 1.5: The amino acid sequence of Aβ and its location within APP.

(A) The Aβ peptide is composed of 40-43 amino acids (enclosed sequence), of which amino acids 1-28 are extracellular (green) and amino acids 29-40/43 are located within the transmembrane (TM) domain (red). These amino acids are hydrophobic in nature and confer on this molecule the ability to self-aggregate and polymerise (B). (Modified from Martins et al., 1991; Fig. 1)
Beta-amyloid exists in two main forms; a shorter, forty amino acid Aβ₄₀ species accounting for greater than 90% of all Aβ normally released from cells, and a longer, more pathological form, Aβ₄₂ only accounting for less than 10% of secreted Aβ. The Aβ₄₀ form appears to contribute to later phases of the disease pathology (Dovey et al., 1993; Asami-Odaka et al., 1995) whilst the Aβ₄₂ form is the predominant form found in the dense amyloid plaques of AD where it is believed to act as a “seed” protein (Iwatsubo et al., 1994; Lippa et al., 1998).

APP is proteolytically cleaved by two competing pathways, the non-amyloidogenic and amyloidogenic pathways (Selkoe et al., 2001). Regulation of the non-amyloidogenic pathway has been proposed to be via protein kinase C (PKC) mediated phosphorylation processes (Buxbaum et al., 1993; Gillespie et al., 1992; Demaerschalck et al., 1993; Slack et al., 1993; Löffler et al., 1993; Caporaso et al., 1994) or via a no-PKC mediated ‘constitutive’ pathway (Esch et al., 1990). Three major secretases (or secretase classes), that cleave APP, were postulated of which two have recently been identified. Firstly, α-secretase, for which the Tumour Necrosis Factor alpha converting enzyme (TACE/ADAM 17) and ADAM 10 are candidates (Buxbaum et al., 1993; Lammich et al., 1999). Secondly, β-secretase, postulated to be a 501 amino acid type 1 transmembrane protein termed beta-site APP cleaving enzyme [BACE] (Vassar et al., 1999; Hussain et al., 1999; Sinha et al., 1999; Yan et al., 1999; Lin et al., 2000) and finally γ-secretase (Fig. 1.6 A and B). In the non-amyloidogenic pathway (Fig. 1.6 A), α-secretase cleaves within the Aβ domain of APP, to release a soluble carboxyl-truncated form of APP termed α-APPs (Buxbaum et al., 1993; Sisodia., 1992). Alternatively, BACE cleaves near the N-terminus of Aβ and produces another soluble form of APP (βAPPs) and a potentially amyloidogenic, C-terminal fragment containing
Figure 1.6 A schematic representation of APP processing.

Two competing pathways proteolytically cleave the majority of APP (A) In the non-amyloidogenic pathway, α-secretase (TACE [ADAM 17] or ADAM 10) cleaves between residues Lys16 and Leu17 of the Aβ domain (green/red) of APP (yellow) to release APPs, thus precluding the formation of Aβ. The C-terminal proteolytic products are further cleaved by γ-secretase to yield non-amyloidogenic fragments (p3 and p7).

(B) Alternatively, in the amyloidogenic pathway, BACE cleaves near the N-terminus of the Aβ domain to generated βAPPs. The C-terminal fragment containing the whole Aβ domain is cleaved by γ-secretase to liberate the Aβ peptide, ending at residue 40 or 42.
the whole Aβ domain. Cleavage of this C-terminal fragment by γ-secretase is the final step in Aβ production (Fig. 1.6 B).

1.3 CAUSES AND RISK FACTORS FOR AD

Currently, there is no known definitive cause of AD. However, significant advances in the molecular pathology of AD as well as in depth studies of risk factors have identified possible causes of AD. By convention AD can be broken into two areas based on the age of onset of disease. Firstly, early-onset AD (EOAD), which accounts for approximately 10% of all AD cases, is characterised by an age of onset before 65 years with onsets as early as the late 20's. Secondly, late-onset AD (LOAD), which contributes the greater percentage of AD cases, is characterised by an age of onset after 65 years of age. LOAD is predominantly sporadic (SAD) in nature and is associated with a complex interaction of genetic and environmental risk factors. However, the majority of EOAD cases exhibit familial clustering and Mendelian inheritance with nearly 100% penetrance, this subset is termed familial AD (FAD). Mutations in three genes localised on chromosomes 21 (APP), chromosome 14 (Presenilin 1 or PS1), or chromosome 1 (Presenilin 2 or PS2) almost invariably result in the development of this aggressive form of AD.

1.3.1 APP Gene: Chromosome 21 locus

As discussed earlier, APP is the precursor for Aβ, which is the major protein component of amyloid plaques. The APP gene, on chromosome 21 was the first genetic locus associated with EOAD (Goate et al., 1991; Murrell et al., 1991; Chartier-Harlin et al., 1991), accounting for approximately 5% of EOAD and less than 0.5% of all AD cases.
CHAPTER ONE: INTRODUCTION

MUTATION  | NAME                  | REFERENCES           |
-----------|-----------------------|-----------------------|
K670N/M671L | Swedish Double mutation | Mullen et al., 1992 |
A692G    | Flemish mutation      | Hendriks et al., 1992 |
E693Q    | Dutch mutation        | Levy et al., 1990     |
E693G    | Arctic Mutation       | Kamino et al., 1992   |
D694N    | Iowa Mutation         | Grabowski et al., 2001 |
I716V    | Florida mutation      | Eckman et al., 1997   |
I716T    | Italian mutation      | Terreni et al., 2002  |
V717I    | London mutation       | Maruyama et al., 1991 |
V717L    | London mutation       | Murrell et al., 2000  |
V717G    | London mutation       | Chartier-Harlin et al., 1991 |
V717F    | London mutation       | Murrell et al., 1991  |
L723P    | Australian mutation   | Kwok et al., 1999     |

A = alanine; E = glutamic acid; D = Aspartic Acid; F = phenylalanine; G = glycine; I = isoleucine; K = lysine; L = leucine; M = methionine; N = asparagines; P = Proline; T = Threonine; V = valine; Q = glutamine

Table 1.1: APP mutations associated with AD

(Sandbrink et al., 1996). At present there are ten known APP mutations associated with AD (Table 1.1) which are limited to exons 16 and 17 of the 18 exon gene (Sandbrink et al., 1996; Lendon et al., 1997).

It was hypothesised that these missense mutations in the APP gene alter APP processing resulting in increased production of Aβ. Transfection experiments, in vitro, revealed that the K670N+M671L resulted in a five to eight times increase in Aβ production (Cai et al., 1993; Citron, et al., 1992). Further experiments showed that the V717 mutations (located at the C-terminus of Aβ) result in increased levels of the Aβ42 form (Suzuki, et
This evidence supports the hypothesis that mutations in APP have a direct impact on APP processing. This, in turn, suggests that other mechanisms which affect the activities of the secretases involved in APP processing might have either the same harmful effect seen with these mutations or possibly confer a protective effect against the development of AD (Sandbrink et al., 1996).

Aβ levels are also increased under conditions of APP gene dosage in patients with Down’s syndrome (DS) in which an extra autosome results in trisomy of chromosome 21 (Gilman, 1997; Sandbrink et al., 1996). It is thought that the presence of the extra chromosome containing the APP gene resulted in its elevated expression and thus increased Aβ secretion. This notion is supported by reports of increased plasma APP and increased Aβ40 deposition in DS patients and DS brains, respectively (Rumble et al., 1989; Iwatsubo et al., 1995).

1.3.2 Presenilin Genes: Chromosome 1 and 14 loci

The PS1 gene is located on the long arm of chromosome 14 and was the second locus to be associated with EOAD (Schellenberg et al., 1992) and is linked to approximately 50% of all EOAD cases. The PS1 protein, comprising 467 amino acids (Boteva et al., 1996), is encoded by the last 10 exons of this 12 exon gene, with the first 2 exons encoding the 5’-nontranslated region. Over 100 mutations have been found in the gene with the majority being missense mutations. The only non-missense mutation constitutes an inframe deletion of exon 9 with an amino acid substitution (S290C) at the splice junction of exon 8 and 10 (Perez-tur et al., 1995). Whilst the majority of these mutations are completely penetrant there are two exceptions. One exception is the I43F mutation, found in an English family, which exhibits incomplete penetrance (Rossor et
al., 1996; Hutton and Hardy, 1997). The other exception is the Glu318Gly mutation.

The pathogenicity of this mutation has been questioned since it is present in non-demented control individuals (Sandbrink et al., 1996; Reznik-Wolf et al., 1998a; Helisalmi et al., 2000). The actual role of PS1 in the development of AD is not fully understood. However, it has been suggested that PS1 participates in APP processing based on the observation that almost all PS1 mutants have an increase in both the total cellular Aβ production and the ratio of Aβ42:Aβ40 (Martins et al., 1995b; Lemere et al., 1996; Duff et al., 1996; Ishi et al., 1997; Xia et al., 1997; Murayama et al., 1999). Similarly, PS1 has been suggested to in fact be the elusive γ-secretase (Wolfe et al., 1999) and evidence has been provided that it directly interacts with the C100 fragment of APP the immediate precursor to Aβ (Verdile et al., 2000). Subsequent studies indicate that PS1 is a component of a high molecular weight complex that exhibits γ-secretase activity (Yu et al., 2000; Yang et al., 2002; Edbauer et al., 2002; Steiner et al., 2002; Takasugi et al., 2002).

After the isolation of PS1 it became apparent, due to sequence homologies, that it belonged to a family of genes. These sequence homologies led to the discovery of a second presenilin gene on the long arm of chromosome 1, the PS2 gene (Levy-Lahad et al., 1995; Sherrington et al., 1995; Rogaev et al., 1995) encoding a protein of 448 amino acids with 67% overall similarity with PS1 and 84% similarity within the transmembranous regions (Levy-Lahad et al., 1995; Sherrington et al., 1995; Rogaev et al., 1995). The discovery of this genetic locus was then detected in a group of Volga Germans (Levy-Lahad et al., 1995). Sequencing studies showed a missense mutation in codon 141 resulting in an asparagine to isoleucine substitution (N141I) (Levy-Lahad et al., 1995). Subsequently, another missense mutation discovered in a pedigree of Italian
descent with EOFAD was located in codon 239 and resulted in a methionine to valine substitution (M239V) (Rogaev et al., 1995).

Following the demonstration of the association between the majority of autosomal dominant EOFAD and the PS1, PS2 and APP genetic loci it became apparent that these genes together account for less than 10% of all AD cases. The analysis of families with LOAD resulted in the identification of a disease locus for the development of this form of AD. This disease locus was localised to chromosome 19 (Pericak-Vance et al, 1991). Subsequent studies led to the association of both sporadic and familial LOAD with the lipid transport protein apoE (Strittmatter et al., 1993), and the localisation of its gene (APOE) within the disease locus of chromosome 19.

1.4 APOLIPOPROTEIN E

ApoE is one of the most important cholesterol transport proteins, and it was initially isolated from plasma in 1973, when it was termed “arginine-rich lipoprotein” (Shore and Shore, 1973). Essentially most of the peripheral apoE is synthesised in the liver. ApoE is the major lipoprotein within the central nervous system (CNS), where it is synthesised by astrocytes (Mahley et al., 1984; Mahley, 1988). The importance of apoE in cardiovascular disease has been recognised for many years (Davignon et al., 1999). However, its importance in neurodegenerative diseases has increased markedly since the discovery of an association of APOE coding sequence polymorphisms with LOAD (Strittmatter et al., 1993; Martins et al., 1995a). The subsequent sections of this chapter will focus on the role of APOE in AD with an emphasis on its three major isoforms and on cis-acting factors that regulate its expression.
1.4.1 Polymorphic Nature of Apolipoprotein E

The human APOE gene is located on the long arm of chromosome 19 (19q13.2; Olaisen et al., 1982; Das et al., 1985) where it is linked to another apolipoprotein (apoC-I) and to an apoC-I pseudogene (Lauer et al., 1988). The APOE gene contains four exons and three introns and is approximately 3.7 kilobases (kb) in length (Paik et al., 1985; Das et al., 1985). Soon after its discovery, the polymorphic nature of the apoE protein was revealed via isoelectric focusing by Utermann and colleagues (Utermann et al., 1980; Utermann et al., 1982) and further refined by Zannis and Breslow, using two-dimensional electrophoresis (1981). The apoE protein exists in three major isoforms, denoted as apoE2, apoE3 and apoE4. After analysis of the amino acid sequences of these isoforms, single amino acid substitutions at residues 112 and 158 were found to account for their differences (Rall et al., 1982a; Rall et al., 1982b; Weisgraber et al., 1982). The most common isoform, apoE3, has a cysteine residue at 112 and an arginine at 158. The apoE4 isoform is generated when cysteine-112 is substituted by arginine, and apoE2 occurs when arginine-158 is substituted by cysteine (Figure 1.7). These three isoforms arise from three APOE alleles, denoted as APOE ε2, APOE ε3 and APOE ε4 [see Zannis et al., 1982 for clarification of nomenclature of apoE]. Other variants of apoE do exist, namely APOE ε1, ε5 and ε7, however they are extremely rare. Various combinations of any two of the three major alleles will give rise to one of six possible genotypes (APOE ε2/ε2, APOE ε3/ε3, APOE ε4/ε4, APOE ε2/ε3, APOE ε3/ε3 and APOE ε2/ε4). The distribution of the three alleles in the general Caucasian population is approximately 8%, 78%, and 14% for APOE ε2, ε3 and ε4, respectively (Utermann et al., 1980; Martins et al., 1995a). The polymorphic nature of the APOE gene is not constrained to these aforementioned single nucleotide polymorphisms (SNPs) that define the three common protein isoforms. Several SNPs have been identified in the
Figure 1.7 (Opposite): Apolipoprotein E gene.

The \textit{APOE} gene is located on the long arm of chromosome 19 at position 19q13.2 and consists of 4 exons. This figure illustrates the location of the single base changes with exon four (A) which result in single amino acid changes at residues 112 and 158, diagrammatically illustrated in the three-dimensional structure of residues 23-166 of the apoE protein (B).

proximal promoter region, which will be discussed later in greater, and sequencing of the \textit{APOE} gene has revealed as many as 21 SNPs as well as one diallelic indel and one multiallelic indel (Nickerson et al., 2000; Fullerton et al., 2000). These included the aforementioned coding and promoter polymorphism but as many as 14 had not been previously identified (Nickerson et al., 2000). The importance of these SNPs in relation to AD risk would be of interest to determine, although some may be of relatively low frequency in the population.

\textbf{1.4.2 The Biology of Apolipoprotein E}

Several organs synthesise apoE, including the liver, brain, spleen, lung, adrenal and kidney (Elshourbagy et al., 1985; Lin et al., 1986; Driscoll and Getz, 1984). Of these, the liver is the major site of apoE mRNA expression and is the source of approximately three-quarters of the circulating plasma apoE. Within the liver, the hepatic parenchymal cells are primarily responsible for apoE production (Lin et al., 1986). The brain displays the second highest level of expression of apoE mRNA (Elshourbagy et al., 1985). As a result, apoE is the preponderant apolipoprotein found in the CSF (Merched et al., 1997), particularly since CSF lacks apoB, a major cholesterol transport protein found in...
plasma. Thus, apoE is critical for lipid transport and cholesterol homeostasis within the CNS. Throughout the brain apoE mRNA has been shown, via in situ hybridisation, to be localized to glial cells, particularly astrocytes (Diedrich et al., 1991; Elshourbagy et al., 1985; Poirier et al., 1991; Stone et al., 1997), indicating that apoE is synthesised and secreted by astrocytes but not by neurons (Boyles et al., 1985; Nakai et al., 1996; Pitas et al., 1987a; Stoll et al., 1989). Neurons do, however, have receptors to endocytose apoE and the presence of the protein in the vesicles of these cells has been demonstrated (Xu et al., 1998).

The major function of apoE in the body is to mediate the clearance of lipoproteins by interacting with the low-density lipoprotein (LDL) family of receptors found on liver cells (Williams et al., 1994, Sakai and Yamamoto, 1994, Beisiegel et al., 1989). Lipoproteins exist as particles that facilitate transport of water-immiscible lipids through aqueous body fluids. ApoE-containing lipoproteins initially bind to cell-surface heparin-sulphate proteoglycans (HSPGs), and are subsequently transferred to the low-density lipoprotein receptor-like protein (LRP) or LDL receptor for endocytosis (Ji et al., 1993, Ji et al., 1994) via clathrin-coated vesicles (CCVs; Havel, 1998). Defects in apoE or its receptors cause the accumulation of lipoproteins under certain conditions; for example, familial hypercholesterolaemia is due to mutations in the LDL receptor (Brown and Goldstein, 1976).

Cells of the periphery obtain cholesterol via synthesis through a pathway initiated by 3-Hydroxy-3-Methyl-Glutaryl Coenzyme A (HMG CoA) reductase (Kandutsch and Chen, 1975) or by binding and internalising apoE-containing lipoproteins (Borensztajn and Kotlar, 1984), or in some cases both. Negative feedback mechanisms prevent excessive cholesterol and triglyceride synthesis and intake by cells, thereby maintaining
homeostasis (Brown and Goldstein, 1986). The existence of apoE-containing lipoproteins in the CSF (Roheim et al., 1979, Pitas et al., 1987b) together with the localisation of members of the LDL receptor family to brain cell plasma membranes (Holtzman et al., 1995, Pitas et al., 1987a) strongly suggests that apoE mediates the uptake and redistribution of cholesterol within the CNS, as is true in the periphery.

Several lines of evidence suggest that apoE may be involved in other specialised forms of neuronal homeostasis. ApoE may be a requirement in the mobilisation of cholesterol in the CNS, where it is required for neural plasticity. The cognate LDL and LRP receptors for apoE are also required for normal CNS plasticity (Bu et al., 1994, Moestrup et al., 1992, Rebeck et al., 1993). Increased apoE mRNA expression in astrocytes occurs in response to lesions of the perforant pathway in the entorhinal cortex (Poirier et al., 1991), one of the first areas of the brain to be affected by AD (Morris, 1997). ApoE is believed to play a role in the repair of damaged neurons by mediating the recycling of cell membrane components from the debris of damaged neurons. Neurons receiving apoE3-loaded lipoproteins show significant neurite extension and branching compared to those receiving apoE4-loaded lipoproteins. Neurons receiving apoE that is not associated with lipoproteins showed no significant effect (Nathan et al., 1994; Postuma et al., 1998). These data suggest that the effect of apoE on neurite outgrowth is isoform-specific and requires association with a cholesterol-containing lipoprotein. Studies of the bioactivity of apoE2 with respect to neuronal modelling and plasticity would be of particular interest considering the fact that apoE2 appears to offer protection against AD.
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1.5 APOLIPOPROTEIN E AND AD RISK

The analysis of families with LOAD resulted in the identification of a disease locus on chromosome 19 (19q13.2) (Pericak-Vance et al, 1991). Further studies led to the association of both familial (Strittmatter et al, 1993) and sporadic (Poirier et al., 1993) LOAD with the APOE ε4 allele. This allele was over-represented in AD cases whilst, the APOE ε2 allele was under-represented. This result has been confirmed by other studies, in various ethnic populations, involving both early and late onset AD cases (Corder et al., 1993; Lucotte et al., 1993; Mayeux et al., 1993b; Strittmatter et al. 1993; Saunders et al, 1993a; Saunders et al, 1993b; Dai et al. 1994; Houlden et al, 1994; Payami et al., 1994; Martins et al., 1995a; Adorer et al., 1995; Basun et al., 1995; Frisoni et al., 1995; Okuizumi et al., 1995; Hyman et al., 1996) [see Farrar et al., 1997 for a meta-analysis of these data]. In both the sporadic and familial forms of the disease, in Caucasians, the prevalence of the APOE ε4 allele has been shown to increase from approximately 14% in the control population to around 40% in the AD population (Poirier et al., 1993; Saunders et al., 1993b; Martins et al., 1995a). The magnitude of this change varies with different ethnicities; however, it is observed across most populations (Table 1.2). There is also an apparent gene dosage effect, according to the number of APOE ε4 alleles, in both familial (Corder et al., 1993) and sporadic (Frisoni et al., 1995) AD with risk increasing from 20% when no APOE ε4 alleles are present to 90% when two copies of the APOE ε4 allele are present (Corder et al., 1993). It has been shown that APOE ε2 alleles are associated with a delayed age of onset for AD (Chartier-Harlin et al., 1994; Corder et al., 1994; Talbot et al., 1994; West et al., 1994; Roses, 1995) even in carriers of APP mutations (St. George-Hyslop et al., 1994; Sorbi et al., 1995a; Sorbi et al., 1996). These findings have led many to suggest that the ε2 allele may offer a level of protection against the development of AD.
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<table>
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* Frequencies in percentage

Table 1.2: Apolipoprotein E (APOE) genotype and alleles frequencies in AD and control cases from different ethnic populations.

Adapted from Farrer et al., 1997 (Table 2) except † Shaw et al., 1999

1.5.1 APOE e4 and the Onset and Progression of AD

One of the clearest manifestations of the APOE e4 allele in AD is the effect it has on the modification of the age at onset of the clinical symptoms of the disease. In a study of familial late-onset AD the mean age of onset for non-e4, heterozygous e4 and homozygous e4 individuals were 84.3 years, 75.5 years and 68.8 years, respectively.
(Corder et al., 1993). The \textit{APOE} ε4 allele steadily increases risk with age, exerting its greatest effect on AD between the ages of 60-79 years and decreasing thereafter (Farrer et al., 1997; Lautenschlager et al., 1999). Additionally, the \textit{APOE} ε4 allele is associated with more rapid memory decline in non-demented individuals (Dik et al., 2000; Deary et al., 2002) and preclinical memory impairment in asymptomatic middle aged individuals (Flory et al., 2000; Caselli et al., 2001).

A pathogenic mutation in APP (APP717) invariably results in the development of AD (Maruyama et al., 1991). However, this mutation may exhibit epistatic variations with different \textit{APOE} genotypes. This is most apparent in a FAD kindred with the APP-V717I mutation, where the presence of the ε4 allele confers an earlier age of onset within the family. The presence of the ε2 allele delayed the age of onset by more than two standard deviations above the mean ages of onset for their respective pedigrees (St. George-Hyslop et al., 1994; Sorbi et al., 1995a; Sorbi et al., 1996). In more aggressive APP mutations, such as APP692 and APP693, neither the age at onset, the age at death, the occurrence of dementia or the number of strokes differ according to \textit{APOE} genotype (Haan et al., 1994). Thus the protective effect of the ε2 allele seen in the APP-V717I kindred is not observed in other APP mutations or to mutations in other AD genes, such as PS1, most probably due to the aggressive nature of these mutations. ApoE2 has been demonstrated to clear Aβ more effectively than ε4 (Yang et al., 1997). The lack of effect of the ε2 allele in cases associated with aggressive mutations may reflect the inability of apoE2 to adequately cope with the high levels of Aβ produced because of these particular mutations.

Whilst the possession of the \textit{APOE} ε4 allele appears to modify the age of onset of AD, there has been no consistent robust effect on rate of progression of the disease reported
in the literature. Various studies have reported slower (Frisoni et al., 1995; Stern et al., 1997; Poirier et al., 1997), similar (Basun et al., 1995; Growdon et al., 1996) or even faster (Lehtovirta et al., 1996a; Craft et al., 1998) rates of progression among APOE ε4 carriers. These confounding results may be due to inadequate length of follow-up periods and/or relatively small sample sizes. These factors need to be addressed before the true effect of APOE genotype on AD progression can be determined.

1.5.2 APOE ε4 and Ethnic Variation

There appears to be a role for ethnicity in the modulation of AD risk. A meta-analysis of forty studies allowed the formulation of odds ratios (OR) for AD within different ethnic backgrounds (Farrer et al., 1997). The ethnic backgrounds included in this meta-analysis were Caucasian, African-American, Hispanic and Japanese. Amongst Caucasian subjects, the risk of AD increased significantly with increasing APOE ε4 dose (ORs of 2.6, 3.2, and 14.9 for APOE genotypes ε2/ε4, ε3/ε4, and ε4/ε4, respectively), whilst ORs decreased for genotypes ε2/ε2 and ε2/ε3 (OR = 0.6). The APOE ε4-AD association was weaker amongst African-Americans and Hispanics; however there was significant heterogeneity in ORs amongst the various studies of African-Americans (Farrer et al., 1997). In Japanese populations, the APOE ε4-AD association was much stronger (ε3/ε4, OR = 5.6; ε4/ε4, OR = 33.1) than that seen in Caucasians (ε3/ε4, OR = 3.2; ε4/ε4, OR = 14.9). The frequency of the APOE ε4 allele in cognitively normal indigenous Australians (Aboriginals) was shown to be significantly higher than that of cognitively normal Caucasian Australians (0.297 vs. 0.155) (Shaw et al., 1999). However, there are few data on the prevalence of AD in Aboriginals, most likely due to the decreased life expectancy of this ethnic group. Taken together, these data suggest that across most populations, the APOE ε4 allele is a major risk factor for AD.


**Table 1.3: Odds ratios for developing AD based on APOE genotype and ethnic group.** Adapted from Farrer et al., 1997 (Table 3)

The difference in the frequency of APOE genotypes across different populations raises the possibility that the APOE ε4 allele, based on its distribution and functional properties, may be a candidate for the ‘thrifty gene’ hypothesis, a hypothesis that may account for why certain indigenous ethnic populations have a high ε4 frequency but lack the common ε4-associated increased risk for AD until they are subjected to a western diet. Corbo and Scacchi (1999) propose that populations with the highest frequency of
the \textit{APOE} $\varepsilon 4$ allele are those from a hunter-gatherer society or where food supplies are only sporadically available (e.g., Australian Aboriginals, Papuans, Polynesians and some African populations). Under these conditions, the presence of the \textit{APOE} $\varepsilon 4$ allele would be of benefit, as uptake of cholesterol would be enhanced. The chronically elevated levels of dietary cholesterol levels associated with \textit{APOE} $\varepsilon 4$ promote diseases such as coronary artery disease (CAD). Hypercholesterolemia may play a role in the mechanism by which \textit{APOE} $\varepsilon 4$ increases the risk for AD (Sparks et al., 1994). In keeping with this hypothesis, the frequency of AD would be expected to rise in aboriginal populations when they are exposed to a Western diet. This phenomenon has been suggested to explain why Africans in Nigerian and East Africa show no association of \textit{APOE} $\varepsilon 4$ with AD (Osuntokun et al., 1995; Sayi et al., 1997) whilst in African-Americans the \textit{APOE} $\varepsilon 4$-AD association has been reported (Tang et al., 1996b; Sahota et al., 1997).

\subsection*{1.5.3 \textit{APOE} $\varepsilon 4$ and Gender Variation}

A meta-analysis concluded that females were at greater risk of developing AD across all age groups and all \textit{APOE} genotypes, though most notably within \textit{APOE} $\varepsilon 4$ containing genotypes (Farrer et al., 1997; Figure 1.8). This imbalance in the risk of AD is possibly due to a rapid decline in endogenous oestrogen levels. In support of this theory are reports of decreased serum oestrogen levels in AD sufferers (Honjo et al., 1989; Yaffe et al., 2000). This hormone has been shown to reduce A\textsubscript{\textbeta} levels in cell culture (Xu et al., 1998), in blood (Gandy et al., 2001) and in the brain (Petanceska et al., 2000). Interestingly, it has been reported that the efficacy of oestrogen replacement therapy (ORT) on delaying the onset and slowing the progression of cognitive decline, is greatest in non-\textit{APOE} $\varepsilon 4$ women (Yaffe et al., 2000; Figure 1.9).
Figure 1.8: Effect of sex and age on the APOE ε4 associated odds ratios.

(Adapted from Farrer et al., 1997; Figures 2 and 3)

Figure 1.9: Effect of ERT on delaying the onset of cognitive impairment.

Adapted from Yaffe et al., 2000 (Figure 1). ERT = Oestrogen Replacement Therapy.
1.5.4 APOE ε4, Cerebral Trauma and AD

Trauma to the head has been widely implicated as a possible risk factor for AD (van Duijn, 1992; Mayeux et al., 1993a; Plasman et al., 2000), and it is reported that APOE ε4 carriers have a more severe decline in cognition following head trauma (Sorbi et al., 1995b; Teasdale et al., 1997). APOE ε4 carriers are at an increased risk for AD following such trauma (Mayeux et al., 1995; Sabo et al., 2000; Guo et al., 2000). The neuropathological mechanism for this association between APOE ε4 and post-traumatic AD is thought to involve increased accumulation of Aβ (Roberts et al., 1991; Roberts et al., 1994; Graham et al., 1995; Nicoll et al., 1995) especially in subjects who carry one or more copies of the APOE ε4 allele. This may be explained by the inability of apoE4 to adequately clear Aβ (Yang et al., 1997). Further support for this notion comes from a study of chronic neurological deficits in boxers, which demonstrated that after standardisation for number of knockouts, boxers possessing an APOE ε4 allele had more severe cognitive deficits than boxers with no APOE ε4 alleles (Jordan et al, 1997).

1.6 APOLIPOPROTEIN E AND AD PATHOLOGY

1.6.1 Apolipoprotein E and Beta-Amyloid

In 1991, Namba and colleagues first demonstrated that amyloid plaques in the AD brain showed apoE immunoreactivity. At this stage, the idea that apoE may be affecting plaque formation was purely speculative. The localisation of the APOE gene at or near the LOAD locus prompted several hypotheses to explain the apoE isoform specific observations in AD. Most of these hypotheses assume that Aβ is the primary neurotoxic
agent and that apoE is a mediator of its clearance, while others suggest that Aβ accumulation and deposition results from its direct interaction with apoE and associated proteins.

Data from initial studies indicated that purified apoE4, compared to purified apoE2 or apoE3 formed stronger, SDS resistant complexes with Aβ peptide (Strittmatter et al., 1993). Subsequent studies demonstrated that purification of apoE, which involves delipidation and denaturation, altered the function of apoE with respect to its ability to form complexes with Aβ (LaDu et al., 1995; Yang et al., 1997). With this new knowledge, further in vitro investigations were performed, and it was observed that while apoE2 bound Aβ avidly, apoE4 did not bind Aβ at all and apoE3’s affinity for Aβ lay somewhere in between apoE2 and apoE4 (Yang et al., 1997; LaDu et al., 1997). This lead to the notion, that apoE4 could be exerting its AD risk by failing to bind to Aβ and facilitate its clearance. Since apoE is a ligand for the LDL receptor family (Holtzman et al., 1995; Pitas et al., 1987b), it has been proposed that apoE2 and apoE3 complexes with Aβ interact with these receptors and are endocytosed, leading to Aβ clearance. Studies using Chinese Hamster Ovary (CHO) cells show that the formation of apoE-Aβ complexes and subsequent uptake is promoted by apoE2 and apoE3 but not apoE4 (Yang et al., 1999). This suggests an interaction between apoE and LRP for receptor-mediated uptake and degradation of Aβ that, perhaps, is impaired in APOE ε4 individuals, thus resulting in Aβ accumulation and senile plaque formation.

This observation is consistent with the increased plaque burden observed in AD patients expressing apoE4 (Beffert et al., 1996; Gearing et al., 1995; Gomez-Isla et al., 1996; Hyman et al., 1995; Nagy et al., 1995; Olichney et al., 1996; Oyama et al., 1995; Polivkoski et al., 1995; Rebeck et al., 1993; Schmechel et al., 1993; Zubenko et al., 1993;
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1994). However, this is not reflected by a more severe progression of AD in ε4 carriers, although they have on average an earlier age of onset (Farrer et al., 1997; Lautenschlager et al., 1999) and more rapid memory decline in non-demented individuals (Dik et al., 2000; Deary et al., 2002) and preclinical memory impairment in asymptomatic middle aged individuals (Flory et al., 2000; Caselli et al., 2001).

Likewise, there is no strong correlation between Aβ plaque load and the severity of cognitive decline unlike NFTs, which although not necessarily specific for AD (Giannakopoulos et al., 1997; McKee et al., 1991) do show a strong correlation. The later point may be addressed by the fact that amyloid plaques are heterogenous (Kametani et al., 1994; Lemere et al., 1996; Miller et al., 1993) and do not account for total Aβ levels in the brain. Whilst a causal link between Aβ levels and the clinical presentation of AD has not yet been proven there is evidence to suggest that Aβ levels increase in the frontal cortex prior to NFT formation and the onset of dementia (Naslund et al., 2000). Furthermore, increased Aβ levels positively correlate with increased severity of cortical Alzheimer’s type changes (Fonte et al., 2001; McClean et al., 1999).

These findings suggest an important neurotoxic role for circulating Aβ and the apoE isoform specific differences on the ability to clear it.

Whilst there is evidence in the literature to support a role for apoE in the clearance of Aβ it has also been demonstrated using mouse models that apoE, in particular apoE4, may be required for amyloid plaque formation (Bales et al., 1997; Bales et al., 1999; Holtzman et al., 2000b; Holtzman et al., 2000a). However, we have observed that aged APP transgenic mice, lacking apoE, do exhibit extensive cerebral amyloid deposits (Robertson and Martins, unpublished data) suggesting that the clearance functions of apoE versus the Aβ deposition functions are not exclusive. Additionally, the notion that
apoE could regulate APP metabolism, directly or indirectly, in an isoform-specific manner has been mooted and can thus also not be excluded (Vincent and Smith, 2001).

1.6.2 APOE ε4, Amyloid Plaques and Neurofibrillary Tangles

Several studies, have reported that amyloid plaque density is correlated to APOE genotype in a gene-dosage dependent manner, with APOE ε4 homozygous subjects demonstrating significantly increased plaque density when compared to APOE ε3/ε4 heterozygous or APOE ε3 homozygous patients (Beffert et al., 1996; Gearing et al., 1995; Gomez-Isla et al., 1996; Hyman et al., 1995; Nagy et al., 1995; Olichney et al., 1996; Oyama et al., 1995; Polvikoski et al., 1995; Rebeck et al., 1993; Schmechel et al., 1993; Zubenko et al., 1994). However, some groups have failed to confirm this association (Landen et al., 1996a; Morris et al., 1996).

Studies seeking an association between NFT density and APOE gene-dose are much less conclusive. An initial report suggested that the density of NFTs were increased in APOE ε4 homozygous patients when compared to APOE ε3 homozygous patients (Schmechel et al., 1993). Several subsequent studies supported the initial finding (Beffert et al., 1996; Nagy et al., 1995; Ohm et al., 1995; Polvikoski et al., 1995), but most studies failed to identify any relationship between NFT density and APOE genotype (Berr et al., 1994; Brat et al., 2001; Gomez-Isla et al., 1996; Morris et al., 1996; Itoh and Yamada, 1996; Landen et al., 1996a; Olichney et al., 1996; Oyama et al., 1995; Zubenko et al., 1994).
1.6.3 APOE ε4 and Cerebral Amyloid Angiopathy

Cerebral amyloid angiopathy is present in 62-97% of AD cases and is consistently present in Down’s syndrome (Vinters et al., 1987). CAA is localized to the leptomeninges, the small pial vessels, the intracortical arterioles, and the brain capillaries (Kalaria et al., 1996) and is a frequent cause of lobar cerebral haemorrhage. The association of the APOE ε4 allele with CAA was first reported after an autopsy study of LOAD cases (Schmechel et al., 1993), and other investigators have since confirmed this association (Greenberg et al., 1995; Kalaria and Premkumar, 1995; Olichney et al., 1996; Premkumar et al., 1996; Woo et al., 2002; Pfeiffer et al., 2002). Like amyloid plaque density, the APOE ε4 allele association with CAA was shown to be dose-dependent (Greenberg et al., 1995; Premkumar et al., 1996). Surprisingly, individuals with CAA-related haemorrhage (CAAH) are often carriers of the APOE ε2 allele (Nicoll et al., 1997; McCarron et al., 1998). It is hypothesised that whilst the APOE ε4 allele enhances CAA progression, possibly via its proposed inferior clearance function (Yang et al., 1999), the APOE ε2 allele predisposes to the rupture of the amyloid-laden blood vessels, potentially due to microangiopathic changes such as fibrinoid necrosis and concentric splitting of the vessel wall (Nicoll and McCarron, 2001). This is supported by data indicating that APOE ε2/ε4 individuals are at increased risk for CAAH, especially at younger ages (Nicoll et al., 1997; McCarron et al., 1998; Woo et al., 2002).

1.6.4 APOE ε4 and Hippocampal Volume

Along with the entorhinal cortex, the first region of the brain to be affected by AD, the hippocampus is one of the more vulnerable regions of the brain and exhibits severe AD
neuropathology early in the disease process (Hyman et al., 1984; Hyman et al., 1986). One clinical correlate of hippocampal dysfunction involves the loss of anterograde episodic memory, a well-known sign of AD. The loss of neurons within this region leads to atrophy, a characteristic that has been studied in detail with MRI and CT scans. These techniques have demonstrated that individuals homozygous for the APOE ε4 allele have smaller hippocampal volumes when compared to non-APOE ε4 subjects (Lehtovirta et al., 1995; Lehtovirta et al., 1996b), and this ε4 effect is dose-dependant (Geroldi et al., 2000). The effect of the APOE ε4 allele on hippocampal volume may even precede the diagnosis of AD. Data indicate that in non-demented APOE ε4 carriers, the hippocampal volume is significantly decreased (Soininen et al., 1995b; Plassman et al., 1997), and the rate of volume loss is significantly faster than when no APOE ε4 allele is present (Moffat et al., 2000). Taken together, these findings indicate that individuals possessing an APOE ε4 allele may be at greater risk for neuronal loss and in turn, show a greater rate of hippocampal atrophy, even before a clinical phenotype is present. This may in part be explained by the inadequate function of apoE4 in the repair of neuronal membranes and/or due to its poor ability to clear extracellular Aβ (Yang et al., 1997).

1.6.5 APOE ε4 and Cholinergic Function

The loss of cholinergic innervation in the cortex and hippocampus, originating from the basal forebrain nuclei, is a prominent feature of AD (Davies and Maloney, 1976; Perry et al., 1977). These neurons are crucial in memory processing and utilise the neurotransmitter ACh to function. To date, the only globally recognised treatment that has provided some benefit to AD sufferers involve a class of drugs termed acetylcholinesterase inhibitors, which maintain levels of ACh by inhibiting its
degradation by the enzyme AChE. Studies have shown that APOE ε4 carriers respond poorly to AChE inhibitors, if at all (Poirier et al., 1995; MacGowan et al., 1998; Farlow et al., 1998). The reasoning behind this selective pharmacogenetic effect is thought to be because the cholinergic system is severely damaged at an early stage in APOE ε4 carriers so that the cholinesterase inhibitors provide limited benefit for this group of patients. This notion is supported by reports indicating that the marked reduction in temporal cortex and hippocampal ChAT activity can be related to an increase in APOE ε4 copy number (Soininen et al., 1995a; Arendt et al., 1997). A similar APOE ε4 dose-dependent decrease in AChe immunoreactivity, nicotinic-binding sites and nerve growth factor (NGF) receptor density has been shown in the cortex and hippocampus of AD patients (Arendt et al., 1997; Poirier et al., 1995). Furthermore, AChe-positive cell density, in both the nucleus basalis of Meynert and the diagonal band of Broca is significantly decreased in APOE ε4 homozygous AD patients as compared to non-APOE ε4 subjects (Poirier, 1999).

1.6.6 Other Functional Impacts of APOE ε4

The aforementioned associations of apoE with the neuropathology of AD are but just a few of the putative mechanisms by which the APOE ε4 allele increases AD risk. Numerous other mechanisms, which may directly or indirectly be associated with AD neuropathology, are worth mentioning. Firstly, apoE has been illustrated to have allele-specific antioxidant activity and effects on cytotoxicity by oxidative insults and beta-amyloid peptides with apoE2 showing the greatest benefits and apoE4 the least (Miyata and Smith, 1996). Additionally, apoE2 performs best and apoE4 worst in modulating Aβ42-induced oxidation to synaptosomes (Lauderback et al., 2002). Secondly, studies in apoE knockout mice using the neuron-specific enolase promoter to express human
apoE3 or apoE4 demonstrate that human apoE isoforms have differential effects on brain function \textit{in vivo} which impinge on cognitive performance (Raber et al., 1998). Thirdly, the observed decreased activity of neurons of the nucleus basalis, in AD, was shown to be apoE4 dependent (Salehi et al., 1998). Suggesting that one mechanism by which apoE4 participates in the pathogenesis of AD is via decreasing neuronal metabolism (Salehi et al., 1998). Fourthly, membrane phospholipid metabolite alterations commonly observed in AD have been reported to be more severe in the presence of the apoE4 allele (Klunk et al., 1998). Fifthly, the ability of apoE3 and not apoE4 to block \( \alpha \)-APPs induced inflammatory reactions in microglia suggests that increased amyloidogenic processing, resulting in high levels of the non-neuroprotective \( \beta \)-APPs, could adversely affect the balance of APPs activities that determine neuronal viability, especially in individuals with the apoE4 isoform (Barger and Harmon, 1997). Finally, apoE4 transgenic mice have been illustrated to be more susceptible to focal ischemia than apoE3 mice (Xu et al., 1996). These mechanisms add to the complex nature by which the apoE4 isoform may result in increased AD neuropathology and contribute to the \( APOE \varepsilon 4 \)-AD association.

1.7 REGULATION OF \( APOE \) EXPRESSION

Transcription of the \( APOE \) gene results in the production of apoE mRNA comprising 1163 base pairs (bp) (McLean et al., 1984). Translation of the apoE mRNA leads to production of a pre-apoE-isoprotein of 317 amino acids, which contains an 18 amino acid amino terminal signal peptide (Zannis et al., 1984). The pre-apoE isoprotein co-translationally translocates across the membrane of the endoplasmic reticulum and undergoes proteolytic processing and glycosylation to generate the mature protein of 34.2kDa, comprised of 299 amino acids (Rall et al., 1982a; Figure 1.10). Approximately
30bp upstream from the transcription initiation site is a TATA box consensus sequence. This sequence is just one of numerous promoter and enhancer elements that have been identified within the APOE regulatory region and shown to be important in the regulation of apoE mRNA synthesis (Figure 1.11).

### 1.7.1 APOE Promoter and Associated Transcription Factors

ApoE synthesis is regulated by an interaction of developmental, hormonal and dietary factors. These agents act to regulate the expression and/or interactions of a number of proteins that bind to the proximal promoter region as well as to downstream elements.
involved in tissue-specific expression. The \textit{APOE} proximal promoter (-1000bp to +400bp relative to the transcription initiation site) has numerous \textit{cis}-acting positive and negative regulatory elements (Figure 1.11). Apart from proximal promoter elements, a 319bp tissue-specific regulatory element, termed the hepatocyte-specific control region (HCR), is located approximately 18kb downstream of the \textit{APOE} promoter. This element can cause high levels of both apoE and apoCI mRNA to be expressed in liver (Simonet et al., 1991; Simonet et al., 1993; Dang and Taylor, 1996; Allen et al., 1997).

The proximal promoter elements are localised within the region spanning -360/-80 and within the first intron. These regions were observed to impart a positive effect on expression (Smith et al., 1988). Within these regions, it has been elucidated that the
APOE proximal promoter contains; a GC Box transcriptional control element at -59/-45, a non-specific enhancer element at -366/-246, an upstream regulatory element (URE1) at -193/-124 and a downstream regulatory element at +44/+262 (Paik et al., 1988). Within URE1, a sequence spanning -161/-141, termed the positive element for transcription, has the ability to act alone as an enhancer element (Chang et al., 1990). This element interacts with the transcription factor Sp1 (Chang et al., 1990; Figure 1.11) that constitutively binds the GC Box motif, suggesting that Sp1 may play an important role in the basal level of expression of APOE as well as the activity of this enhancer element. Another regulatory element termed URE3 was identified at position -101/-89 and found to bind a 300kDa protein, from placental nuclear extracts, termed URE3BP (Jo et al., 1995). Subsequent studies in human HepG2 cells determined that a region within URE3 (-94/-84) bound the transcription factor BEF-1, a member of the NF-1 family of nuclear factors, and resulted in a negative effect on regulation of the gene (Berg et al., 1995; Figure 1.11). The mediation of this regulation is possibly modulated by differential phosphorylation of BEF-1, possibly via the PKC pathway (Berg et al., 1996).

Whilst several studies have examined mechanisms for the expression of APOE in the periphery little is known about its regulation in the brain. However, studies of the proximal promoter by Garcia and colleagues (1996) revealed that Adenosine 3’, 5’-cyclic monophosphate (cyclic AMP; cAMP) increased promoter activity in U87 astrocytoma cell lines but had no effect in HepG2 cells. AP-2, an astrocyte and neuron associated transcription factor whose expression can be rapidly and strongly induced by cAMP (Philipp et al., 1994), binds two regions of the proximal promoter: a -60 footprint (-74/-48) and a -117 footprint (-135/-104) (Figure 1.11). Furthermore, the astrocytoma cell line revealed three sites within the proximal promoter that bind either of the two zinc finger proteins Zic1 and Zic2 (Salero et al., 2001; Figure 1.11). These
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AP-2 and Zic1/Zic2 sites within the proximal promoter may contribute to the complex regulation of apoE in the development, degeneration and regeneration of the nervous system. Abnormalities in both AP-2 and ZIC1/ZIC2 have been shown to result in deleterious effects in mice. Homozygous disruption of the AP-2 gene results in multiple congenital defects and perinatal death (Schorle et al., 1996; Zhang et al., 1996) whilst mutant mice lacking ZIC2 gene show incomplete closure of the neural tube, producing holoprosencephaly and spina bifida (Nagai et al., 2000). In humans, mutations in the ZIC2 gene are associated with holoprosencephaly (Brown et al., 2001). It remains undetermined whether AP-2 is induced in the brain after injury, or in AD, or whether Zic proteins play a functional role in the expression of apoE in the mature brain. Further studies of these transcription factors are needed to increase our understanding of the mechanisms by which apoE plays a role in the development and repairing of the nervous system and in AD. Overall, the numerous factors that have been identified to potentially bind the APOE proximal promoter and impart some regulatory influence reflect the complex mechanisms involved in the regulation of the APOE gene.

1.7.2 Effect of Promoter Polymorphisms on Transcriptional Activity

Some of the research into the mechanisms by which APOE expression may be regulated has focused on polymorphisms within the APOE promoter. To date, several polymorphisms within the promoter region of the APOE gene have been identified (Figure 1.11). Polymorphisms at -491 (A/T transversion); -427 (T/C transition); -219 (G/T transversion - also known as the Th1/E47cs polymorphism due to its location within a potential Th1/E47 transcription factor-binding site; Lambert et al., 1998b); and a C/G transversion polymorphism at position +113 termed IE1 (due to its location within an enhancer element in intron 1; Mui et al., 1996; Artiga et al., 1998b). These polymorphisms are proposed to affect the transcriptional activity of the APOE gene in
both the periphery and the CNS. This hypothesis is supported by findings that the A to T and the T to G substitutions, at -491 and -219, respectively, resulted in a 63% decrease and a 169% increase in APOE promoter activity, respectively, in HepG2 cells (Artiga et al., 1998b) and similar changes were also reported when astrocytoma cells were studied (Artiga et al., 1998a). The mechanism by which these polymorphisms alter the level of activity of transcription is thought to involve differential binding of nuclear proteins. These single nucleotide polymorphisms may result in changes in the secondary structure of the DNA, thus altering the binding of transcription factors to neighbouring regulatory elements. Alternatively, they may affect the direct binding of transcription factors to putative transcription sites within which they are located.

1.7.3 APOE Expression in AD

The risk of sporadic AD is associated not only with the APOE e4 genotype but also with increased levels of apoE in plasma (Taddei et al., 1997), which is independent of APOE genotype. This association of increased levels is not restricted to plasma apoE but has also been reported in the frontal cortex of autopsy confirmed AD cases (Pirttila et al., 1996). However, other studies have not observed this increased level of apoE in AD cases, reporting either no change (Harr et al., 1996) or a decrease in apoE level (Bertrand et al., 1995; Beffert et al., 1999). This discrepancy may be accounted for by differences in the methods used to measure apoE i.e. bound, unbound or total apoE levels. Interestingly, APOE promoter polymorphisms, that possibly impart some regulatory influence on APOE expression, have been shown to be associated with AD risk.
Early reports suggest that apoE mRNA levels were either decreased (Poirier et al., 1990) or unchanged (Oyama et al., 1995) in the hippocampus and cortex of patients with AD. However, more recent studies have reported increased expression of apoE mRNA in the frontal and temporal cortex (Yamada et al., 1995, Yamagata et al., 2001) and the hippocampus (Zarow et al., 1998) of neuropathologically confirmed AD patients. Most data indicate that both apoE mRNA and protein are elevated, presumably due to an increase in apoE expression within reactive astrocytes, a neuropathological hallmark of AD (Dietrich et al., 1991; Shao et al., 1997; Martins et al., 2001).

Whilst overall apoE protein levels may play an important role in AD pathology, it is plausible that the combination of the ε4 genotype together with apoE levels may increase risk synergistically. Increased apoE levels might provide an explanation for the ‘incomplete penetrance’ phenomenon associated with ε4 homozygosity. However, only two studies (Lambert et al., 1998a; Growdon et al., 1999) have addressed the question of the level of apoE ε4 protein. Only the study of Lambert and colleagues (1998a) report a significant increase in the relative quantity of APOE ε4 mRNA expression in the brains of AD patients as compared to controls. Although further studies are needed it is plausible that APOE promoter polymorphisms may contribute to the elevated levels of the apoE4 isoform.

1.7.4 APOE Promoter Polymorphisms In AD

Factors that regulate apoE expression, such as selected polymorphisms in the APOE promoter, may contribute to an individual’s risk for AD. Four promoter polymorphisms have been identified and their association with AD risk has been investigated. Of these
four polymorphisms, the $-491A/T$ polymorphism has been the most thoroughly investigated and shown to have the most robust effect on $APOE$ transcription.

The association between the AA genotype at the $-491$ position of the $APOE$ promoter and the risk for AD was first reported in two Caucasian populations, one from Spain and the other from the USA (Bullido et al., 1998). The authors found that homozygosity of the $-491A$ allele was associated with an increased risk of developing AD which was subsequently confirmed in a large French cohort (Lambert et al., 1998a). These findings imply that, in addition to the qualitative effects of the $APOE$ alleles, the quantitative variation of isoform expression due to polymorphisms in the proximal promoter may contribute to AD and may help explain why some individuals who carry two $APOE\ \varepsilon4$ alleles escape AD.

Although the $-491A/T$ promoter polymorphism has been extensively studied, the remaining three polymorphisms have received less attention. The $-219T/G$ polymorphism has been studied in two French Caucasian populations and has been reported to be associated with an increased risk for AD. This genotype has been proposed to act as a modifier of $APOE\ \varepsilon4$ risk (Lambert et al., 1998a,b). However, this effect has been sought but not found in Caucasian populations from the United States (Rebeck et al., 1999). This discrepancy may be explained by the strong linkage disequilibrium that was observed between the $-219T$ and $APOE\ \varepsilon4$ alleles.

Strong linkage disequilibrium is also apparent between the $+113C$ and $APOE\ \varepsilon4$ allele. Four studies have been published to date on the association of the $+113G/C$ polymorphism and AD with conflicting conclusions. Two studies (Mui et al., 1996; Rebeck et al., 1999) conclude that, after controlling for linkage, any apparent
association between the +113C allele and AD is due entirely to linkage with the \textit{APOE} \( \varepsilon 4 \) allele. However, two other studies (Lambert et al., 1998b; Bullido et al., 2000) found an association between the +113C and AD, which remained significant after controlling for the \textit{APOE} \( \varepsilon 4 \) allele.

To date, the \(-427T/C\) polymorphism has been the subject of five investigations. Only two of these studies, one Spanish (Artiga et al., 1998a) and one French (Zurutuza et al., 2000) population, have shown evidence for an independent association between the \(-427C\) allele and AD. This finding has not been found in the remaining three studies (Lambert et al., 1998b; Bullido et al., 2000; Wang et al., 2000). However, in the studies of Artiga and colleagues (1998a) and Bullido and colleagues (2000) a common haplotype that includes both the \(-491A\) and \(-427C\) alleles has been shown to be independently associated with AD.

Regulation of the transcription of \textit{APOE} is highly complex and requires an interaction of not only the possible functional proximal promoter polymorphisms but also of the proximal and distal regulatory regions with transcription factors, to impart a net effect on \textit{APOE} expression. In turn, this complex genetic mechanism of regulation may itself be influenced by non-genetic factors such as diet and hormone levels that also impart regulatory effects, directly or indirectly. This complex interplay between genetic and environmental factors must be thoroughly considered in order to fully appreciate the role of apoE in AD. It is thus one of the major focuses of this thesis to investigate the role of these promoter polymorphisms in AD, both in terms of genetic risk and effect on apoE levels.
1.8 **HIGH RISK GROUPS**

The prevalence of AD has increased steadily over the last century as the average life expectancy of individuals has risen. It is foreseeable that the incidence of AD will also increase further as the world’s population continues to age. In Australia alone the number of individuals believed to be suffering from AD was estimated to be 135,000 (in 1998), a figure which has been predicted to more than double by the year 2030 (Henderson and Jorm, 1998). In the United States, AD is numbered the fourth leading cause of death behind heart disease, cancer and stroke (Lendon et al., 1997) and has been predicted to affect 14 million individuals by the year 2050. Whilst this social impact of the disease is immense the economic impact of the disease is also noteworthy. In the United States, the estimated cost of evaluating an individual with dementia is US$1,000 to US$1,500, and the average cost for institutionalisation (in 1998) was estimated to be US$40,000 per year. This translates into an estimated cost of US$412 billion in the United States for the year 2000. These figures alone make it imperative that both effective treatment regimes and definitive means of early diagnosis are developed. One such means of identifying predictors for the early diagnosis of AD is investigating the biological profiles of individuals, in groups thought to be at high risk of developing AD in the future, whilst monitoring their cognitive performance over a period of time. Two such groups that have been investigated in this thesis include individuals with subjective memory complaints and post-menopausal women.

**1.8.1 Subjective Memory Complainers**

The availability of effective symptomatic treatment and the prospect of further therapeutic advances have given impetus to early diagnosis, focusing on the pre-
dementia phase of AD. This pre-dementia phase includes the continuum of normal aging, memory complaints without objective impairment and mild cognitive impairment (MCI). A number of reports have been directed at conceptualising changes in brain function with ageing and the classification of pre-dementia syndromes (O’Brien et al., 1992, Caine 1993, Zaudig 1992).

Pre-dementia syndromes may be purely subjective with no objective evidence of brain dysfunction. Conversely, cognitive deficits may manifest but have no effect on functional ability and therefore not meet criteria for dementia. The relationship between memory complaint in non-demented persons and future risk of dementia has prompted numerous studies (Sunderland et al., 1986, Derouesne et al., 1989, O’Connor et al., 1990, Bolla et al., 1991, Christensen 1991, O’Brien et al., 1992, Taylor 1992, Bassett and Folstein, 1993, Grut et al., 1993, Flicker et al., 1993, Hanninen et al., 1994, Jorm et al., 1994, Gagnon et al., 1994, Schmand et al., 1996, Jonker et al., 1996, Geerlings et al., 1999, Schofield et al., 1997, Jorm et al., 1997). Subjective memory complaints in the elderly population are fairly common, with prevalence rates between 22% and 56% in community-based studies (Jonker et al., 2000). However, they are frequently associated with depressive symptoms, especially in self-referrals to memory clinics or in clinical studies (Barker et al., 1995; Derouesné et al., 1999). Early reports did not support a role for memory complaints in early diagnosis (Sunderland et al., 1986, Derouesne et al., 1989, Taylor et al., 1992, Hanninen et al., 1994). However, there is now emerging evidence that self reported memory complaints do confer greater risk of future dementia (Bolla et al., 1991, Christensen 1991, Bassett and Folstein, 1993, Geerlings et al., 1999, Schofield et al., 1997) with studies reporting that memory complaints in subjects with objective cognitive impairment were predictive for cognitive decline and dementia with odds ratios between 2.6 and 4.1 (Jonker et al., 2000). Schofield et al reported that the
risk of dementia was increased for those with objective evidence of cognitive
dysfunction (Schofield et al., 1997). Interestingly, Geerlings and colleagues (1999)
found a greater risk in memory complainers who had no apparent cognitive impairment
— they were 2.8 times more likely to develop AD than non-complainers (Geerlings et
al., 1999).

Memory complaints are an imperative criteria of MCI and many of the elderly subjects
in the above mentioned studies would fall in this diagnostic category which is
becoming, as a potential border zone between normal ageing and dementia, an
increasingly important research focus (Petersen et al., 2000). Prevalence estimates
indicate that 17% of older adults (aged 65 years or over) and 30% of the very old (aged
85 years and over) have MCI (Graham et al., 1997). Follow-up studies showed that MCI
patients develop dementia, in the majority of cases AD, at a rate of 1% to 30% per year
(Daly et al., 2000) with an average conversion rate of approximately 15% per year. In
2001 the American Academy of Neurology (Petersen et al., 2001) released consensus
criteria for MCI, which indicates the current effort to establish international accepted
clinical criteria for MCI. Next to memory complaints these non-demented patients need
to score 1.5 standard deviations or more below age-matched controls in a memory test,
need to have intact activities of daily living and a normal general cognitive function.

Development of a reliable method of risk stratification based on symptomatology and
cognitive function is needed. Combining this with the assessment of genotypic and
phenotypic markers may allow for the determination of a risk profile for AD that could
allow for accurate diagnosis many years before clinical symptoms become obvious.
Accurate early assessment of memory disorders has merit for a number of reasons. This
can provide reassurance for those with no evidence of dementia while those who do may benefit from early intervention.

1.8.2 *Post-Menopausal Women*

With the average age of menopause in women being approximately 51 years the majority of females will live approximately one-third of their adult life in a state of oestrogen deprivation. Whilst this oestrogen deprivation is widely accepted to increase the risk for elderly woman to develop osteoporosis and cardiovascular disease the most dreaded and disturbing aspect of aging is the development of cognitive decline leading to dementia. Males are more commonly affected by stroke and cardiovascular disease, whereas the age-specific prevalence of AD is higher in females (Jorm et al., 1997). Numerous studies report that the incidence of AD is also higher for women than for men and that this increased risk of AD is apparent in women across all *APOE* genotypes (Farrer et al., 1997; Figure 1.8).

This imbalance in the risk of AD has been postulated to be due to a rapid decline in endogenous oestrogen levels in post-menopausal woman. In support of this theory is the finding that serum oestrogen levels are decreased in women suffering from AD (Honjo et al., 1989; Yaffe et al., 2000). It has been suggested that the supplementation of endogenous levels, through the means of ORT (ERT = estrogen replacement therapy), may improve cognition in non-demented peri-/post-menopausal women and/or delay/prevent the onset of cognitive decline. This hypothesis is supported by findings that adverse cognitive functioning is seen after oestrogen deprivation (Farrag et al., 2002) and can be reversed to premenopausal levels with ORT (Kampen & Sherwin, 1994: Philips & Sherwin, 1992).
1.8.2.1 Mechanisms of oestrogens action on cognition and dementia

The presence of oestrogen receptors in the hypothalamus, preoptic, anterior pituitary, and CA1 region of the hippocampus (McEwan et al., 1994) has led to several mechanisms being postulated for oestrogen's effect on neuropsychological functioning. These include the modulation of neurotransmitters, potentiating neuronal remodelling, altering APP metabolism and protection against neurodegeneration.

Studies of the effects of oestrogen supplementation in ovariectomised rats demonstrated that oestrogen increased ChAT and potassium stimulated ACh release (Luine, 1985; reviewed in Gibbs and Aggarwal, 1998). Oestrogen has also been shown to prolong survival of cholinergic neurons (Honjo et al., 1992). The neuropsychological impact of oestrogen intervention is also reflected in the assessment of behavioural memory tasks in rats with those receiving oestrogen performing better in association with increased choline uptake and higher levels of ChAT in the hippocampus and frontal cortex (Simpkins et al., 1994). Oestrogen may also enhance cognitive function by promoting cholinergic activity in brain, independently or in synergy with other factors. In the hypothalamus and CA1 hippocampal pyramidal neurons oestrogen has been suggested to regulate synaptic plasticity via stimulation of axonal sprouting and dendritic spine formation (McEwan et al., 1994; Matsumoto, 1991). In comparison, ovariectomised rats have a significant decrease in dendritic spine density in CA1 pyramidal neurons (Gould et al., 1990). The mechanism by which oestrogen elicits these effects is believed to be at the genomic level where the interaction of oestrogen with its receptors, which are bound to oestrogen response elements, recruits co-activators that are involved in the regulation of the transcription of genes involved in a diversity of pathways. However, oestrogen has been suggested to also elicit a faster non-genomic response through second messengers such as G-proteins and cAMP (Wong et al., 1996). Additionally, an interaction between oestrogen and various intracellular signalling pathways has been
mooted including extracellular regulated kinase (ERK; Singh et al 1999), nuclear factor κB (NF-κB) as well as the regulation of PKC.

Oestrogen has been suggested to play a role in the metabolism of APP as demonstrated by its ability to reduce Aβ production in several studies (Jaffe et al., 1994; Green et al., 1996; Petanceska et al., 2000). Treatment of ovariectomised guinea pigs with 17β-estradiol significantly decreased Aβ levels (Petanceska et al., 2000). Oestrogen may achieve this possibly via stimulating the amount and/or activity of α-secretase. The mechanism by which oestrogen achieves this has been suggested to be via an increase in level and/or activity of PKC, in turn, promoting “regulated APP cleavage” via the non-amyloidogenic pathway. It has also been suggested that the effect of oestrogen on Aβ production may be PKC independent. In this case oestrogen has been shown to regulate ERK which in turn has been shown to lower Aβ production (Mills et al., 1997).

Oestrogen also appears to interact with range of genes involved in apoptosis and the pro-inflammatory response. Several studies have illustrated the ability of oestrogen to attenuate the neurotoxic effects of Aβ. For example, oestrogen has been illustrated to modulate the expression of the anti-apoptotic protein Bcl-xL (Pike, 1999) and protect neuronal cells from the pro-apoptotic action of mutant PS1 (Mattson et al., 1997). Aβ can activate NF-kB which is crucial in the induction of transcription of pro-inflammatory cytokines such as interleukin-1 and 6. This activation can be attenuated by the administration of oestrogen (Dodel et al., 1999). Thus the combination of oestrogen deprivation and neurotoxin accumulation may be potentially detrimental to the aging brain. Aβ is also a potent free radical generator causing oxidative damage to DNA, proteins and lipids. Oestrogen has been demonstrated to protect neurons from this Aβ induced cellular degeneration (Goodman et al., 1996). This effect has been suggested to be an intrinsic property of the molecule and is believed not to be receptor
mediated. The potent antioxidant properties of oestrogen in combination with the ability of oestrogen to cross the blood brain barrier suggests that it may have high therapeutic value in a range of neurodegenerative diseases.

**1.8.2.2 Clinical evidence of the benefits of oestrogen replacement therapy**

The high incidence of AD in post-menopausal woman has prompted numerous clinical studies to determine if ORT can delay the onset or slow the progression of cognitive decline (Kimura, 1995; Maki et al., 2000; Yaffe et al., 2000; Paganini-Hill and Henderson, 1994; Paganini-Hill and Henderson, 1996; Barrett-Connor and Kritz-Silverstein, 1993; Kampen and Sherwin, 1994; Robinson et al., 1994). Some of these studies have looked at ORT in post-menopausal healthy woman to evaluate if those on ORT perform better on measures of cognitive performance. Whilst others have studied post-menopausal woman suffering from dementia to determine whether use of ORT is more frequent in non-demented woman and also to evaluate whether or not ORT can improve cognition in those already suffering cognitive deficits.

The association of ORT and cognitive performance has been evaluated in several observational studies of post-menopausal healthy woman. However, consensus is yet to be reached on whether the use of ORT improves cognition in healthy post-menopausal woman with studies reporting both improved cognitive performance (Kimura, 1995; Maki et al., 2000; Yaffe et al., 2000) and either no association (Paganini-Hill and Henderson, 1996; Barrett-Connor and Kritz-Silverstein, 1993) or inconclusive findings (Kampden and Sherwin, 1994; Robinson et al., 1994). Possible contributing factors for the inconclusive data is the impact of confounding factors such as depression as well as the impact of genetic factors, such as APOE genotype, which may predispose individuals to cognitive deficits.
Several trials of oestrogen intervention in post-menopausal healthy woman have been undertaken, including both randomised (Caldwell and Watson, 1952; Hackman and Galbraith, 1976; Campbell and Whitehead, 1977; Fedor-Freybergh, 1977; Sherwin, 1988; Ditkoff et al., 1991; Phillips and Sherwin, 1992) and non-randomised controlled trials (Rauramo et al., 1975). Several studies suggest that cognition improved after oestrogen intervention. However, these studies are hindered by methodological problems, such as small sample size, standardisation of neuropsychological assessment, incomplete follow-ups, and no controlling for depression. Whilst the results of these studies tend to suggest that oestrogen intervention does improve cognitive performance these methodological problems impinge on the ability to draw firm conclusions.

Whilst the data from the aforementioned studies are fraught with limitations and a lack of consensus, taken as a whole, they nonetheless suggest that oestrogen does provide some beneficial effects on cognition in normal post-menopausal women. As such several studies have been undertaken to evaluate whether women who currently take or have taken ORT are less likely to develop dementia. Of these studies, a case-control (Henderson et al., 1994) and two prospective cohort studies (Tang et al., 1996a; Kawas et al., 1997) found a statistically significant decrease in the risk of dementia in ORT users. However, other case-control studies reported mixed findings, ranging from a non-significant risk reduction (Broe et al., 1990; Paganini-Hill and Henderson, 1994; Mortel and Meyer, 1995), no difference in risk (Graves et al., 1990; Brenner et al., 1994) to a non-significant increased risk of dementia (Heyamn et al., 1984; Amaducci et al., 1986).

It should be noted that whilst all studies matched for age few were adjusted for the impact of education and none adjusted for depression. A meta-analysis of these studies was performed by Yaffe and colleagues (1998) and provides a more precise estimate of the effect of ORT on dementia risk (summarised in Table 1.4). This study concluded
### Table 1.4: Meta-analysis of oestrogen and dementia risk

(Adapted from Yaffe et al., 1997; Table 5)

<table>
<thead>
<tr>
<th>STUDY DESIGN</th>
<th>SUMMARY O.R. (95% C.I.)</th>
<th>TEST OF HETEROGENEITY (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All Studies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any dementia(^{(a-j)})</td>
<td>0.71 (0.53-0.96)</td>
<td>0.10</td>
</tr>
<tr>
<td>Any AD(^{(a-j)})</td>
<td>0.71 (0.52-0.98)</td>
<td>0.11</td>
</tr>
<tr>
<td>NINCDS-ADRDA(^{(a,c-f,h-j)})</td>
<td>0.69 (0.48-1.01)</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Case-Control Studies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any dementia(^{(a-h)})</td>
<td>0.79 (0.56-1.12)</td>
<td>0.09</td>
</tr>
<tr>
<td>Any AD(^{(a-h)})</td>
<td>0.80 (0.56-1.16)</td>
<td>0.11</td>
</tr>
<tr>
<td>NINCDS-ADRDA(^{(a,c-f,h)})</td>
<td>0.80 (0.5-1.28)</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Prospective Studies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NINCDS-ADRDA(^{(i,j)})</td>
<td>0.48 (0.29-0.81)</td>
<td>0.86</td>
</tr>
</tbody>
</table>


\(^{a}\)Heymann et al., 1984; \(^{b}\)Amaducci et al., 1986; \(^{c}\)Broe et al., 1990; \(^{d}\)Graves et al., 1990; \\
\(^{e}\)Brenner et al., 1994; \(^{f}\)Henderson et al., 1994; \(^{g}\)Paganini-Hill and Henderson, 1994; \\
\(^{h}\)Mortel and Meyer, 1995; \(^{i}\)Tang et al., 1996a; \(^{j}\)Kawas et al., 1997
that overall these studies suggest a decreased risk of dementia in ORT takers (OR 0.71) with diagnosis by NINCDS-ADRDA criteria in prospective studies showing the greatest protection (O.R. 0.48).

The epidemiological evidence that ORT may potentially prevent or delay the onset of dementia has been the catalyst for studies that wish to evaluate the use of ORT as a means for treating AD (Fillit et al., 1986; Honjo et al., 1989; Honjo et al., 1993; Ohkura et al., 1994; Asthana et al., 2001). Across these studies the intervention with oestrogen resulted in variable increases in measures of cognitive performance. However, whilst these results tend to suggest a beneficial impact of ORT as a treatment for AD they are subject to short durations of therapy and very small sample sizes (in each case the efficacy of oestrogen treatment was studied in less than 20 women with AD). Subsequent larger randomized placebo controlled trials in sample sizes of 42 (Henderson et al., 2000), 50 (Way et al., 2000) and 120 women (Mulnard et al., 2000) have attempted to address these limitations. The general consensus from these studies is that ORT has no effect on improving cognition in AD affected women, however, they do not rule out the possibility that oestrogen may be beneficial in delaying the onset of AD. Additionally, it is imperative that individuals who respond best to such prophylactic treatment are identified considering the associated side effects of hormone replacement therapy.

1.9 HYPOTHESES AND OBJECTIVES

There is growing evidence in the literature to suggest a role for polymorphisms in the APOE proximal promoter in the development of AD. The locations of these polymorphisms suggest that the mechanism by which they may increase AD risk is via
altering the expression of the gene. As such it is the aim of this thesis to address the following hypothesis:

"Polymorphisms within the \textit{APOE} proximal promoter are associated with an increased risk of AD and this risk is attributed to altered apo\textit{E} concentrations"

The specific objectives to test this hypothesis are:

1. Determine if any association exists between polymorphisms in the \textit{APOE} proximal promoter and the development of AD in a clinically diagnosed case-control cohort and an autopsy confirmed case-control cohort.

2. Determine if polymorphisms in the \textit{APOE} proximal promoter modify plasma apo\textit{E} concentrations and whether any change is reflected in cerebral concentrations.

Epidemiological evidence suggests that certain groups of individuals are at an increased risk of developing AD. These include individuals with subjective memory complaints and post-menopausal woman. The role of genetic and biological factors in these groups as well as the effect of hormone replacement will be investigated and the following hypotheses tested:

"Subjective memory complainers perform worse cognitively and will have a higher frequency of selected genetic and biological risk factors."
"Post-menopausal woman using hormone replacement therapy will perform better on measures of cognition."

"The ability of oestrogen to protect against cognitive deficits is determined by the biological profile of the individual"

The specific objectives to test these hypotheses are:

1. In a cohort of subjective memory complainers determine the association of selected genetic and biological markers with cognitive impairment.

2. Determine if hormone replacement therapy is associated with better performance on measures of cognitive decline and whether this is modified by selected genetic and biological markers.
2.1 MATERIALS

2.1.1 Consumables

2.1.1.1 PCR consumables

<table>
<thead>
<tr>
<th>ITEM</th>
<th>COMPANY</th>
<th>LOCATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq Polymerase (incl. 10x Polymerase Buffer, 25mM MgCl₂)</td>
<td>Fisher Biotech</td>
<td>Perth, W.A., Australia</td>
</tr>
<tr>
<td>dNTP’s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC19 DNA / HpaII cut DNA marker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100bp DNA marker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bromophenol Blue</td>
<td>Sigma Chemical Company</td>
<td>St. Louis, MO, USA</td>
</tr>
<tr>
<td>Xylene Cyanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ficoll type-400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All primers</td>
<td>Genset Oligos</td>
<td>Lismore, NSW, Australia</td>
</tr>
<tr>
<td>AmpliTaq Gold (incl. 10x PCR Buffer Gold, 25mM MgCl₂)</td>
<td>Roche</td>
<td>Branchburg, New Jersey, USA</td>
</tr>
<tr>
<td>dNTP’s (for use with AmpliTaq Gold)</td>
<td>Promega</td>
<td>Madison, WI, USA</td>
</tr>
</tbody>
</table>

Table 2.1: PCR consumables
## 2.1.1.2 Miscellaneous consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Company</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GENERAL CONSUMABLES</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Tris-(hydroxy methyl)-methylamine (Tris)</td>
<td>BDH Chemicals</td>
<td>Vic., Australia</td>
</tr>
<tr>
<td>• Ethylenediaminetetra-acetic acid (EDTA)</td>
<td>Australia Pty. Ltd.</td>
<td></td>
</tr>
<tr>
<td>• Polyoxyethylene (20) sorbitan monolaurate (Tween20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Ethanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Methanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Sodium Chloride (NaCl)</td>
<td>Ajax Chemicals</td>
<td>Auburn, NSW, Australia</td>
</tr>
<tr>
<td>• Boric Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Casein</td>
<td>Sigma Chemical Company</td>
<td>St. Louis, MO, USA</td>
</tr>
<tr>
<td>• Aprotinin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Leupeptin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Phenylmethylsulphonyl fluoride (PMSF)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Dimethyl sulfoxide (DMSO)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Luminol enhancer and stable peroxide solutions</td>
<td>Pierce</td>
<td>Rockford, IL, USA</td>
</tr>
<tr>
<td>• Amersham Hyperfilm-ECL film</td>
<td>Amersham</td>
<td>Buckinghamshire, UK</td>
</tr>
<tr>
<td>ITEM</td>
<td>COMPANY</td>
<td>LOCATION</td>
</tr>
<tr>
<td>------</td>
<td>---------</td>
<td>----------</td>
</tr>
<tr>
<td><strong>GENERAL CONSUMABLES (cont...)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Sodium dodecylsulphate (SDS)</td>
<td>Bio-Rad</td>
<td>Irvine, CA, USA</td>
</tr>
<tr>
<td>• Glycine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Tricine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Nitrocellulose membranes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Acrylamide (analytical grade)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• N,N’-methylene-bis-acrylamide (Bis)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• N,N,N,N,N’-Tetra-methylene diamine (TEMED)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Ammonium Persulphate (APS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Triton</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Bovine Serum Albumin (BSA) Chemical Reagent Grade</td>
<td>ICN Biomedicals Inc,</td>
<td>Aurora, OH, USA</td>
</tr>
<tr>
<td><strong>ANTIBODIES</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Monoclonal anti-human apoD (#8CD6)</td>
<td>Signet Laboratories</td>
<td>Dedham, MA, USA</td>
</tr>
<tr>
<td>• HRP-conjugated monoclonal anti-human apoD (#8BG4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• HRP-conjugated rabbit anti-goat IgG</td>
<td>Dako Corp.</td>
<td>Carpinteria, CA, USA</td>
</tr>
<tr>
<td>• Goat anti-human apoE polyclonal antibody</td>
<td>Incstar Corp.</td>
<td>Stillwater, MN, USA</td>
</tr>
</tbody>
</table>

Table 2.2: General consumables and antibodies
2.1.2 Study Participants

2.1.2.1 Clinically diagnosed AD cohort (A)

This cohort consisted of 625 Caucasian individuals, including 379 healthy non-AD individuals (average age: 77.02 ± 0.57), 200 (LOAD; 84.56 ± 0.52) and 46 sporadic EOAD (60.33 ± 1.39) patients. Of the 625 individuals, 400 were bled using standard venipuncture techniques at the Sir James McCusker Alzheimer’s Disease Research Unit [Hollywood Private Hospital (HPH), Nedlands, W.A.]. The remaining 225 individuals were sourced from two separate locations; 197 from the Centre for Education and Research on Ageing [Concord Repatriation General Hospital (CRGH), Concord, N.S.W.] and 28 from the Garvan Institute of Medical Research (St. Vincent’s Hospital, Sydney, N.S.W.). At all locations the diagnosis of dementia was made by experienced clinicians using Diagnostic and Statistical Manual of Mental Disorders - Fourth Edition criteria (DSM-IV; American Psychiatric Association, 1994) and the designation of probable Alzheimer’s disease was determined following the NINCDS-ADRDA (McKhann et al., 1984). The HPH cohort comprised 204 control and 196 AD individuals, whilst the CRGH cohort comprised 165 control and 32 AD individuals. The St. Vincent’s cohort comprised 10 control and 18 AD individuals. Of the 379 non-AD individuals examined none exhibited any evidence or history of neurological disease and all were spouses of affected subjects or volunteers from the same communities as the AD sufferers. Of the sporadic EOAD individuals, none were positive for PS1 mutations. No mutations were found either within exons 16 and 17 of the APP gene, nor were the most common PS2 mutations (N141I and M238V) detected in any of the patients.
2.1.2.2 **Clinically diagnosed AD cohort (B)**

This cohort is essentially the same as that described in Section 2.1.2.2 with slight modification. This cohort consisted of 682 individuals, of which 43 were sporadic EOAD patients (42% female, mean age 62 years, range 42-71), 191 sporadic LOAD patients (61% female, mean age 80 years, range 64-95) and 46 familial AD patients (unrelated; 46% female, mean age 65 years, range 38-93). The 402 healthy controls (50% female, mean age 75 years, range 50-92) were Australian volunteers with no apparent cognitive deficits, who were spouses of AD afflicted individuals or were recruited from the same communities as the AD patients. Individuals were ambulatory, community dwelling, and recruited from memory evaluation units as described in Section 2.1.2.1 (Perth, 436 individuals and Sydney, 246 individuals). No selection criteria other than voluntary participation were imposed. Diagnosis of dementia and the designation of probable Alzheimer's disease were determined in the same manner as described in Section 2.1.2.1. Of the 43 sporadic EOAD patients, none had any mutation within the known AD genes (APP, PS1 and PS2) and no family history of EOAD was present. Of the 46 familial AD patients (unrelated; mean age of onset, 58 ± 10 years), 4 had a mutation other than the Glu318Gly in the PS1 gene. Of the sporadic EOAD and familial AD Glu318Gly positive individuals, none were positive for other PS1 mutations. No mutations were found either within exons 16 and 17 of the APP gene, nor were the most common PS2 mutations (N141I and M238V) detected in any of the patients. The disease was considered familial if at least one additional first degree relative suffered from dementia.

2.1.2.3 **Subjective memory complainers cohort**

The subjective “memory complainers” cohort consisted of 98 individuals with memory complaints (average age: 63.4 ± 1.0) and 49 controls (61.3 ± 1.5) who complained of no
memory impairment. The 98 “memory complainers” were recruited from individuals referred to The Memory and Capacity Evaluation Unit at Osborne Park Hospital between 1996 and 1998. Additionally, advertisements were placed in local newspapers seeking people with memory complaints. Control subjects were recruited through media advertisements and by asking spouses of complainers to volunteer for the study. Selection criteria were used to determine which volunteers would be included in the study. Volunteers were excluded from the study if they; met criteria for the diagnosis of dementia (DSM-IV; American Psychiatric Association), had a prior medical history of stroke, had a Mini-Mental State Examination (MMSE) score lower that 24 (Folstein et al., 1975) or presented with depression [based on the ICD-10 algorithm within the Cambridge Examination for Mental Disorders of the Elderly (CAMDEX; Roth et al., 1986)]. Volunteers for the control group were also excluded if they had a family history of dementia to produce the best possible representative sample of a true control population.

2.1.2.4 Post-menopausal cohort

This cohort was recruited after ethics approval was provided by the University of Western Australia. A total of 330 post-menopausal woman were recruited from a database, of which we were kindly given access to by Dr. Bronwyn Stuckey (Keogh Institute for Medical Research, Perth, WA, Australia), or through advertisements to the general public. After clinical assessment (See Section 2.2.1) women were excluded from analysis if they; met criteria for the diagnosis of dementia (DSM-IV; American Psychiatric Association), had a prior medical history of stroke, had a MMSE score lower that 24 (Folstein et al., 1975), a score of less than 80 on the Cognitive component of the CAMDEX (CAMCOG) or presented with depression (based on the ICD-10 algorithm within the CAMDEX; Roth et al., 1986). Women were also removed from
analysis if they scored greater than 2 on the Subjective Memory Complaints component of the CAMDEX. Thus from the initial 330 recruited a total of 298 women were included in the final cohort for analysis. The cohort consisted of 189 women on some form of HRT (age: 63.6 ± 0.4) and 109 were taking no form of HRT (age: 64.5 ± 0.6), forming an aged matched control group. The HRT group (n = 189) consisted of 100 women taking oestrogen only (ORT; age: 63.4 ± 0.6) and 89 women were taking combined HRT (oestrogen and progesterone; cHRT or opposed oestrogen therapy; age: 63.9 ± 0.6). The ORT group was further stratified by the level of dose of oestrogen administered per day (or the transdermal equivalent), being 1.875mg (n = 6; age: 65.2 ± 1.0), 1.25mg (n = 64; age: 62.4 ± 0.7), 0.625mg (n = 28; age: 64.6 ± 1.2) and 0.3125mg (n = 2; age: 71.0 ± 0.0).

2.1.3 Brain Samples (AD and Control cases)

Pre-frontal cortex tissue (Brodmann area 9) were obtained from 60 brain specimens collected from institutes in Perth (HPH), Melbourne (University of Melbourne and the Mental Health Research Institute (MHRI)), Sydney (N.S.W. Tissue Resource Centre and The Neuroscience Institute of Schizophrenia and Allied Disorders (NISAD)) and Philadelphia (MCP-Hahnemann University, PA, USA). AD cases were neuropathologically confirmed based on established NINCDS-ADRDA criteria [n = 20; 65.6 ± 3.3 years; male:female 1.2; Post-mortem interval (PMI) = 21.5 ± 1.3 hrs] and consisted of 12 cases (HPH) with age of onset greater than 65 and 8 cases (Philadelphia) with an age of onset less than 65 years. Neuropathologically assessed control brains (3 cases from HPH; 15 from NISAD; 22 from MHRI) were from individuals with no known brain disease (n = 40; 61.2 ± 2.5 years; male:female 1.33; PMI = 26.0 ± 3.8 hrs).
2.2 METHODS

2.2.1 Clinical Assessment

The clinical assessment outlined here was utilised for the Subjective Memory Complainers and Postmenopausal cohorts (Sections 2.1.2.3 and 2.1.2.4, respectively). An experienced geriatrician clinically reviewed all volunteers and recorded medical history and completed a physical and neurological examination. A trained nurse interviewed all subjects using the CAMDEX (Roth et al., 1986). The CAMDEX includes standardised patient and informant interviews, a depression scale and a cognitive examination. The cognitive component (CAMCOG) includes tests of orientation, memory, language, praxis, attention, calculation, abstract thinking and visual perception. Scores can range from 0 to 107. A score of 80 or greater is considered sufficient to exclude dementia. The CAMCOG also includes questions that allow the computation of the MMSE. In addition, the interviews gather demographic information and recent and remote medical history.

2.2.2 Sample Collection

Blood samples were collected through standard venipuncture techniques into heparin, EDTA and serum 9ml vacutainer tubes (Interpath Services, West Heidelberg, Vic., Australia). Heparin and EDTA tubes were pre-treated with 25 μl of a prostaglandin E (PGE) solution (40μl of PGE1 Stock (500 μg/ml) and 50ml of 100% ethanol). Serum tubes were centrifuged at 1000 rpm in a Spintron GT-25 centrifuge for 5 minutes and serum was collected and stored in fresh tubes at −20°C. Heparin/EDTA (whole blood) tubes were centrifuged at 800-1000rpm in an Eppendorf 5810 R centrifuge for 10
minutes with no brake applied. Plasma was transferred to fresh tubes and packed cell volume (PCV) set aside for extraction of leucocytes. Transferred plasma was centrifuged for a further 15 minutes at 1500rpm (full brake) for isolation of platelets. Plasma was transferred to fresh tubes, leaving platelet pellet undisturbed, and stored at –20°C. Platelet pellet was washed in TBS and resuspended in 500µl TBS for storage at –20°C. For isolation of leucocytes, PCV (approximately 4mls) was mixed with approximately 3ml of 0.9% saline and 2ml of dextran solution (for one litre; 50g Dextran, 7g NaCl, 0.02% w/v Sodium azide). The dextran/saline-PCV suspension was allowed to settle at room temperature for approximately 30 minutes. The top fraction was then collected into fresh tubes and centrifuged at 1000rpm for 5 minutes. Supernatant was discarded and the pellet washed a further two times with saline. The leucocyte pellet was resuspended in foetal calf serum (CSL Pty Ltd, Parkeville, Vic., Australia) containing 10% v/v Dimethyl sulphoxide (DMSO) and stored at –80°C until required for DNA extraction or for the development of immortalized cell lines.

Stored leucocytes were centrifuged at 4000rpm for three minutes followed by the discarding of the supernatant. Cells were resuspended in 1.0ml of 0.9% NaCl followed by centrifugation at 4000rpm for 3 minutes. Washing in 0.9% NaCl was repeated until pellet was ‘clean’. After final supernatant was discarded the pellet was resuspended in 0.5ml of TES buffer pH 8.0 (0.30g Tris, 1.461g NaCl, and 0.093g EDTA made to 250ml with ddH2O), 60µl of 20% w/v SDS and 30µl of 20mg/ml proteinase K and mixed. It was then rotated slowly overnight at 37°C. An equal volume of phenol was added and then centrifuged at 13000rpm for 5 minutes and the upper layer transferred to a clean eppendorf tube. To precipitate DNA, 50µl of 3M sodium acetate and 1.0ml of 100% ethanol was added and mixed. Precipitated DNA was centrifuged at 13000rpm for 15 minutes and supernatant was discarded. The DNA pellet was resuspended in
400µl of 70% ethanol then mixed and centrifuged at 13000rpm for 5 minutes. After the DNA pellet was allowed to dry, it was resuspended in 400µl of ddH₂O and then stored frozen at -70°C.

### 2.2.3 Gel Preparation

#### 2.2.3.1 Non-denaturing polyacrylamide gel electrophoresis (PAGE)

All electrophoresis that was required for Polymerase Chain Reaction (PCR) protocols (Section 2.2.4) were performed using either the Hoefer Mini VE® system (Hoefer Pharmacia Biotech Inc., San Francisco, CA, USA) or the Bio-Rad Mini-Protean® system (Bio-Rad, Irvine, CA, USA). An 8% w/v (for APOE, -491A/T and Glu318Gly genotyping) or 12% w/v (for -219G/T genotyping) 19:1 acrylamide:N,N'-methylene-bis-acrylamide (BIS) solution was prepared by dissolving 76g acrylamide and 4g BIS (for 8%) or 114g acrylamide and 6g BIS (for 12%) in 1 litre of 1x TBE buffer (324g Tris, 165g boric acid and 28.2g EDTA made to three litres with ddH₂O, pH adjusted to 8.3 and diluted one in ten for 1x TBE). Polymerisation of the gel was achieved by the addition of ammonium persulfate (APS) and N,N,N',N'-Tetra-methylethlenediamine (TEMED) to the acrylamide mix in the volumes described in Table 2.3.

#### 2.2.3.2 Sodium dodecyl sulfate PAGE (SDS-PAGE)

All western blotting (Section 2.2.5.2) electrophoresis was performed using the Hoefer Mini VE® system (Hoefer Pharmacia Biotech Inc., San Francisco, USA). A 49.5% polyacrylamide stock solution, containing 48% (w/v) acrylamide and 1.5% (w/v) BIS, was prepared from which the stacking and resolving gels were made. Polymerisation of the gel was achieved by the addition of APS and TEMED in volumes specific for stacking and resolving gels (Table 2.4).
### CHAPTER TWO: MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Bio-Rad Mini-Protean® (Volume per gel)</th>
<th>Hoefer Mini VE® (Volume per gel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19:1 acrylamide solution</td>
<td>10 ml</td>
<td>15 ml</td>
</tr>
<tr>
<td>25% APS</td>
<td>20 μl</td>
<td>30 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>15 μl</td>
<td>22.5 μl</td>
</tr>
</tbody>
</table>

Table 2.3: Non-denaturing-PAGE gel preparation

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<thead>
<tr>
<th>Reagent</th>
<th>Volume /gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>4% Stacking gel (6.0ml/gel)</td>
<td>49.5% polyacrylamide stock</td>
</tr>
<tr>
<td>3M Tris-HCl, containing 0.3% SDS</td>
<td>1.49 ml</td>
</tr>
<tr>
<td>double deionised water (ddH₂O)</td>
<td>4 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>24 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>2.4 μl</td>
</tr>
<tr>
<td>8% Resolving gel (7.5ml/gel)</td>
<td>Polyacrylamide stock (49.5/3%)</td>
</tr>
<tr>
<td>3M Tris-HCl, containing 0.3% SDS</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>double deionised water (ddH₂O)</td>
<td>3.76 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>25 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>12% Resolving gel (7.5ml/gel)</td>
<td>Polyacrylamide stock (49.5/3%)</td>
</tr>
<tr>
<td>3M Tris-HCl, containing 0.3% SDS</td>
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<tr>
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</table>

Table 2.4: SDS-PAGE gel preparation
2.2.4 Polymerase Chain Reaction (PCR) Protocols

2.2.4.1 APOE genotyping

APOE genotyping was performed via PCR amplification in a total volume of 15μl, containing 1.0U of AmpliTaq Gold, 0.2mM of each dNTP, 2mM of MgCl₂, and 10% DMSO. Amplification occurred over 37 cycles on an Eppendorf MasterCycler Gradient. Briefly, 1 cycle of 94°C for 5 minutes, 65°C for 30 seconds and 70°C for 90 seconds; 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds and 70°C for 90 seconds; 1 cycle of 94°C for 30 seconds, 65°C for 30 seconds, 70°C for 10 minutes and 4°C for 1 minute (as described by Hixson and Vernier, 1990). The oligonucleotide primers, (P1) 5'-TCC AAg gAg CTg CAg gCg gCg CA-3' and (P2) 5'-ACA gAA TTC gCC CCg gCC Tgg TAC ACT gCC A-3', were used as described by Wenham et al (1991). The amplified 244bp product was digested for four hours at 37°C using 5.0U of the restriction enzyme Hhal (Fisher Biotech, Perth, W.A.) (restriction sequence G▲CGC). This restriction site occurs in four sites besides those that occur in the presence of 112Arg (APOE ε4 only) and 158Arg (APOE ε3 and ε4). The digested product was mixed with Ficoll loading buffer (1.5g Ficoll, 0.02g Bromophenol Blue, 0.02g Xylene Cyanol, 10 mls 1x TE buffer – 10mM Tris, 1mM EDTA) then electrophoresed in an 8% non-denaturing polyacrylamide gel in 1x TBE buffer (see Section 2.2.3.1) at 110V for one hour and stained with ethidium bromide (1.5μl of 10mg/ml ethidium bromide into 50ml of ddH₂O) for two to five minutes followed by destaining in ddH₂O for a further five to ten minutes. Gels were then visualised under UV light via a UVInc UV transilluminator, to reveal DNA fragments with electrophoretic migration patterns unique to each allele (Wenham et al., 1991; Figure 2.1), then photographed via Polaroid GelCam on Polaroid 667 (ISO 3000) film (St. Albans, Hertfordshire, England) for permanent record.
Figure 2.1: Apolipoprotein E genotyping.

A representation of a typical APOE genotyping gel produced after digested PCR product is run on an 8% non-denaturing PAGE gel.

M = Marker [pUC HpaII cut DNA marker - arrows represent fragment sizes corresponding to adjacent numbers (in base pairs)]

Black arrowheads correspond to digested PCR product (adjacent sizes in base pairs)

- Lanes 1, 14 - APOE ε2/ε2
- Lanes 4, 5 - APOE ε2/ε4
- Lanes 8, 9 - APOE ε3/ε4
- Lanes 2, 3, 12, 13 - APOE ε2/ε3
- Lanes 6, 7 - APOE ε3/ε3
- Lanes 10, 11 - APOE ε4/ε4
2.2.4.2 *APOE*-491 genotyping

The genotype of the *APOE* -491A/T polymorphism was determined via a two stage PCR amplification, using the genomic primers [P1 (-1017) 5'-CAA ggT CAC ACA gCT ggC AAC-3' and P2 (+406) 5'-TCC AAT CgA Cgg CTA gCT ACC-3'] and the nested primers [P1 (-285) 5'-TgT Tgg CCA ggC Tgg TTT TAA-3' and P2 (-512) 5'-CCT CCT TTC CTg ACC CTg TTC-3'] as described (Bullido et al., 1998). PCR was performed on an Eppendorf MasterCycler Gradient in a total volume of 20μl (genomic) or 15μl (nested), containing 1.0U of Taq Polymerase, 0.2mM of each dNTP and 1mM of MgCl₂, during 37 cycles. Briefly, 1 cycle of 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 68°C for 2 minutes; 1 cycle of 94°C for 30 seconds, 68°C for 10 minutes, 4°C for 1 minute. The amplified product of 229bp was digested for four hours at 37°C using 5.0U of the restriction enzyme *DraI* (Fisher Biotech, Perth, WA) (restriction sequence TTT\_AAA) that only digests the product in the presence of the -491A allele. The digested product was mixed with Ficoll loading buffer and then electrophoresed in an 8% non-denaturing polyacrylamide gel in 1x TBE buffer (see Section 2.2.3.1) at 110V for two hours and stained with ethidium bromide (1.5μl of 10 mg/ml ethidium bromide into 50ml of ddH₂O) for two to five minutes followed by destaining in ddH₂O for a further five to ten minutes. Gels were then visualised under UV light via a UVP\_inc UV transilluminator, to reveal DNA fragments with electrophoretic migration patterns unique to each allele (-491T = 229bp; -491A = 209bp; Figure 2.2), then photographed via a Polaroid GelCam on Polaroid 667 (ISO 3000) film for permanent record.

2.2.4.3 *APOE* -219 G/T (Th1/E47cs) genotyping

Variation at the *APOE* -219 locus was determined via PCR amplification using the forward ‘mismatched’ primer, 5'-AgA ATg gAg gAg gCT gCC Tg-3' (mismatch...
Figure 2.2: Apolipoprotein E -491 A/T promoter polymorphism genotyping.

A representation of a typical -419A/T genotyping gel produced after digested PCR product is run on an 8% non-denaturing PAGE gel.

M = Marker (100 base pair marker - arrows represent 400, 300 and 200 bp fragments)

Black arrowheads correspond to allele fragments: -491A = 229bp and -491T = 209bp

Lanes 1-3, 7, 8, 10, 12, and 13 = -491AA genotype

Lanes 4, 6, 9, and 11 = -491AT genotype

Lane 5 = -491TT genotype
Figure 2.3: Apolipoprotein E -219 G/T Promoter Polymorphism Genotyping.

A representation of a typical -219G/T genotyping gel produced after digested PCR product is run on an 8% non-denaturing PAGE gel.

M = Marker (pUC HpaII cut DNA marker - arrow represents 35 base pair fragment)

Black arrowheads correspond to allele fragments: -219G = 49bp and -219T = 31bp

Lanes 1, 3 = -219GG genotype

Lanes 2, 4 = -219GT genotype

Lane 5-7 = -219TT genotype
underlined) and the reverse primer 5'-ACT CAA ggA TCC CAg ACT Tg-3' to generate a Bstnl digestion site as described (Lambert et al., 1998b). PCR was performed on an Eppendorf MasterCycler Gradient in a total volume of 25μl, containing 1.0U of Taq Polymerase, 0.2mM of each dNTP and 1.5mM of MgCl₂, during 42 cycles. Briefly, 1 cycle of 94°C for 4 minutes; 40 cycles of 94°C for 30 seconds, 54°C for 60 seconds, 72°C for 60 seconds; 1 cycle of 94°C for 30 seconds, 54°C for 60 seconds, 72°C for 5 minutes and 4°C for 1 minute. The amplified product was digested for four hours at 65°C using 5.0U of the restriction enzyme Bstnl (New England Biolabs, Beverly, MA, USA) (restriction sequence CC▲(A/T)GG) which digests the product when a G is present at the polymorphic site. The digested product was mixed with Ficoll loading buffer and then electrophoresed in an 12% non-denaturing polyacrylamide gel in 1x TBE buffer (see Section 2.2.3.1) at 110V for one hour and 30 minutes and stained with ethidium bromide (1.5μl of 10mg/ml ethidium bromide into 50ml of ddH₂O) for two to five minutes followed by destaining in ddH₂O for a further five to ten minutes. Gels were then visualised under UV light via a UVP Inc UV transilluminator, to reveal DNA fragments with electrophoretic migration patterns unique to each allele (-219G = 49bp, -219T = 31bp; Figure 2.3), then photographed via a Polaroid GelCam on Polaroid 667 (ISO 3000) film for permanent record.

2.2.4.4 Glu318Gly (E318G) genotyping

Glu318Gly variation was determined via PCR amplification using the forward 'mismatched' primer, 5'-ATC CAA AAA TTC CAA gTA TAA TCC Tg-3' (mismatch underlined) and the reverse primer 5'-CTg ggC ATT ATC ATA gTT CTC AAg-3' to generate a Bstnl digestion site as described (Dermaut et al., 1999). PCR was performed on an Eppendorf MasterCycler Gradient in a total volume of 25μl, containing 1.0U of Taq Polymerase, 0.2mM of each dNTP and 1mM of MgCl₂, during 32 cycles. Briefly, 1
cycle of 94°C for 4 minutes; 30 cycles of 94°C for 30 seconds, 54°C for 60 seconds, 72°C for 60 seconds; 1 cycle of 94°C for 30 seconds, 54°C for 60 seconds, 72°C for 60 seconds and 4°C for 1 minute. The amplified product of 154 bp was digested for four hours at 65°C using 5U of the restriction enzyme BsmI (New England Biolabs, Beverly, MA, USA) (restriction sequence \(CC_k(A/T)GG\)) which will only digest the product when the Glu318Gly variation is present. Digested product was then electrophoresed in an 8% non-denaturing polyacrylamide gel in 1x TBE buffer (see Section 2.2.3.1) at 110V for one hour and 30 minutes and stained with ethidium bromide (1.5µl of 10mg/ml ethidium bromide into 50ml of ddH2O) for two to five minutes followed by destaining in ddH2O for a further five to ten minutes. Gels were then visualised under UV light via a UVP Inc UV transilluminator, to reveal DNA fragments with electrophoretic migration patterns unique to the presence or absence of the Glu318Gly variation (Present = 130bp, Absent = 154bp; Figure 2.4), then photographed via a Polaroid GelCam on Polaroid 667 (ISO 3000) film for permanent record.

2.2.5 Apolipoprotein Assays

2.2.5.1 Sample preparation

Approximately 250mg of tissue was dissected from the prefrontal cortex and used for measurements of apoE protein levels. Tissue was homogenised in phosphate buffered saline (PBS; pH 7.4) with 5µg/ml aprotinin, 5µg/ml leupeptin and 0.1µM phenylmethylsulphonyl fluoride. Protein concentrations in the brain homogenates were determined via the Micro BCA Pierce protein assay kit. Blanks (100µl PBS) as well as 100µl duplicates of samples and standards [bovine serum albumin (BSA); supplied with kit] were aliquoted into a 96 well microtitre plate. Reagents were prepared as per manufacturers instructions and 100µl of this colorimetric reagent was added to each
Figure 2.4: Glu318Gly (E318G) Genotyping.

A representation of a typical E318G genotyping gel produced after digested PCR product is run on an 8% non-denaturing PAGE gel.

M = 100 base pair marker (arrows represent 200 and 100 bp fragments)

Black arrowheads correspond to allele fragments: E318G neg = 154bp and E318G pos = 130bp

Lanes 1-6 = E318G -/- (i.e. mutation absent)

Lanes 7-10 = E318G +/- (i.e. mutation present)
blank, sample and standard. The plate was incubated at 60°C for 30 minutes and the absorbance read at 595nm on a Bio-Rad Model 3550 Microplate reader. Protein concentrations were calculated, with reference to the standard curve, via Microplate Manager for PC (ver. 4.0; Bio-rad, Irvine, CA, USA). The quantitation of plasma total protein levels was performed after determining total protein concentration also via the Micro BCA Pierce protein assay kits.

2.2.5.2 Western blot for Apolipoprotein E

Frontal cortex homogenates (25µg of total protein) or plasma (100µg of total protein) were diluted 1:4 with SDS sample buffer (1M Tris HCl pH 6.8, 8% w/v SDS, 4% w/v glycine), boiled for 5 minutes and proteins separated on 8-12% SDS-polyacrylamide gels (Section 2.2.3.2), using similarly treated recombinant human apoE4 (Panvera, USA) as a standard. Electrophoresis was performed at a constant current of 60mA per gel in cathode buffer (1M Tris-HCl, pH 8.25, 1M tricine and 1% w/v SDS) and anode buffer (2M Tris-HCl, pH 8.9). Separated proteins were transferred onto nitrocellulose membranes at 4°C for 12 hours, at a constant current of 250mA in 25mM Tris, containing 200mM glycine and 20% v/v 100% methanol using the Trans-Blot cell® apparatus (Bio-rad, Irvine, CA, USA). Membranes were incubated with blocking solution (PBS pH 7.4, 0.5% w/v casein, 10mM NaOH) for 45 minutes at room temperature, and then immersed in goat anti-human apoE polyclonal antibody at a dilution of 1:4000 for 2hrs in PBS pH 7.4, also at room temperature. Membranes were then washed three times in wash buffer (TBS-T; 10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.05% v/v Tween20) and immersed, at room temperature, in horseradish peroxidase-conjugated rabbit anti-goat IgG for 1 hour in TBS-T pH 8.0 (dilution 1:4000). Membranes were then washed three times before chemiluminescent visualization was carried out after 1 minute incubation with a 1:1 mix of luminal
Figure 2.5: Western blot for Apolipoprotein E in plasma.

A representation of a typical gel produced after 100 μg of total protein from plasma was resolved on an 8-12% SDS PAGE gel and immunoblotted using a goat anti-human apoE polyclonal antibody. An ~34.2 kDa band (arrowhead) corresponding to the mature apoE protein was observed in lanes 1-6. This was quantitated against recombinant human apoE4 standards of 80ng, 40ng and 10ng in lanes S1, S2 and S3, respectively. A high molecular weight marker was run in lane M and bands corresponding to molecular weights (represented by arrows and adjacent molecular weights in kDa) were observed after transfer onto nitrocellulose membranes.
enhancer and stable peroxide solution. Blots were exposed to Amersham Hyperfilm-ECL film for up to 3 minutes to achieve desired signal intensity. Developed film (Figure 2.5) was analysed using a UMAX scanner (transmission scanning at 600 dots per inch), and apoE protein concentration was quantitated using NIH Image software, version 1.62.

2.2.5.3 Measurement of Apolipoprotein D (ApoD) via ELISA

ApoD levels were quantitated in frontal cortex homogenates, as prepared in section 2.2.4.1, using a sandwich Enzyme Labelled Immunosorbent Assay (ELISA). Two antibodies, monoclonal anti-human apoD antibody (#8CD6) and an HRP-conjugated monoclonal anti-human apoD antibody (#8BG4) (Signet Laboratories, Dedham, MA, USA) were used. Microtitre high capacity binding plates (Costar, Corning Inc., Corning, NY, USA) were coated with 50µl of the monoclonal apoD antibody (#8CD6) for 1 to 2 hours at room temperature at a concentration of 4.7µg/ml (1:1000 dilution of stock antibody in TBS, pH 7.5). Wells were then washed four times with TBS-T (TBS pH 7.5, 0.1% v/v Triton) in a Bio-Rad Microtech plate washer. After the final wash 50µl of blocking solution (TBS-T with 5% w/v bovine serum albumin (BSA)) was added to each well and incubated for one hour at room temperature. Microtitre plate was then washed a further four times in TBS-T. Aliquots (50µl) of the various tissue homogenates (50µg of total protein per well), blanks and purified apoD standard (kindly donated by Dr Elizabeth Thomas, Scripps Research Institute, La Jolla, CA, USA) at concentrations of 0.05 to 1.0µg/ml were added in triplicate. Samples were allowed to incubate for 1 to 2 hours at room temperature before the plate was washed four times in TBS-T. To each well 50µl of HRP-conjugated monoclonal anti-human apoD antibody (#8BG4; 1:2000 dilution in TBS pH 7.5) was added and incubated for 1 hour at room temperature. After four washes with TBS-T and then a further two washes with TBS, to
remove bubbles, 50μl of TMB (3,3',5,5'-tetramethylbenzidine) liquid substrate system (KPL, Gaithersburg, MA, USA) to allow colour formation over a period of 5 to 30 minutes, depending on strength of signal. Colour reaction is then quenched with the addition of 50μl of 1M H₃PO₄ and absorbance read at 450nm on a Bio-Rad Model 3550 Microplate reader. Protein concentrations were calculated, with reference to the standard curve, via Microplate Manager for PC (ver. 4.0; Bio-rad, Irvine, CA, USA).

2.2.6 Statistical Analysis

The data was analysed using the statistical package ‘SPSSv10.0’ for Windows. In general categorical variables were investigated using the Pearson method for the analysis of contingency tables ($\chi^2$) or, when noted, the Fisher’s exact test (for comparisons when any cell size was less than or equal to 5). Relative risks (odds ratios) were estimated from 2X2 tables. Ninety-five percent confidence intervals were estimated for the odd ratios (CI). Continuous variables were analysed using either the Student’s t-test or the Mann-Whitney U-test, dependent on the distribution of the data. Where appropriate analysis of variance was performed with Bonferonni correction applied for multiple comparisons.
CHAPTER THREE
RISK FACTORS IN CLINICALLY DIAGNOSED AD

3.1 INTRODUCTION

The lipid transport protein apoE, encoded by the *APOE* gene on chromosome 19, is the predominant apolipoprotein found in the brain (Mahley, 1988). The ε4 allele of *APOE* is an important risk factor for the development of familial and sporadic LOAD in most populations (Strittmatter et al., 1993; Martins et al., 1995a). However, it has been suggested that the ε4 allele is neither necessary nor sufficient for the development of AD. This is because the ε4 allele is associated with only 50% of LOAD cases and that several ε4 carriers escape the disease. It has been shown that levels of apoE mRNA in the brains (Yamada et al., 1995) and levels of apoE protein in the plasma (Taddei et al., 1997) are increased among patients with AD when compared to those of controls.

In order to determine whether the increased levels of apoE are associated with any genetic alterations, several studies have looked into polymorphisms lying within the regulatory region of the *APOE* gene (Bullido et al., 1998; Artiga et al., 1998b; Lambert et al., 1998a, Lambert et al., 1998b). Two such polymorphisms, the -491A/T and the -219G/T (or Th1/E47cs) have been linked with an increase risk of developing AD. Bullido and colleagues (1998), demonstrated that a common genotype of the -491A/T promoter polymorphism, namely -491AA, was associated with an increased risk of developing AD and also that the -491A allele was associated with higher constitutive levels of *APOE* promoter activity than the -491T allele. This finding raised the possibility that the -491A allele might play a role in increasing the risk of developing AD by increasing apoE expression. Meanwhile, the -219T/G polymorphism has been
studied in two French Caucasian populations, where it has been reported that the -291T allele is associated with an increased risk for AD. This allele has been proposed to act as a modifier of APOE ε4 risk (Lambert et al., 1998a, Lambert et al., 1998b). However, this effect was not found in another Caucasian population from the United States (Rebeck et al., 1999). It has been suggested that this lack of association is due to strong linkage with the APOE ε4 allele (Rebeck et al., 1999). In this chapter, the association and relative risk of the APOE -491A/T and -291T/G promoter polymorphisms are investigated in a large Australian Caucasian population. The association of these polymorphisms with altered plasma apoE levels has also been assessed in the current study.

Whilst LOAD is associated with numerous genetic factors that predispose the carrier to an increased risk of developing the disease, the majority of EOAD cases are associated with mutations in the PS1 gene (Sherrington et al., 1995) on chromosome 14 which are inherited in an autosomal dominant fashion. The PS1 gene consists of 10 coding (exons 3-12) and three non-coding exons (exons 1A, 1B and 2) which specify a 467 amino acid protein predicted to contain between 6 to 9 (Sherrington et al., 1995; Lehmann et al., 1997; Doan et al., 1996; Slunt et al., 1995) transmembrane domains and to include a large hydrophilic loop region (amino acids 267-403). To date, more than 90 different mutations (Sherrington et al., 1995; Cruts et al., 1995; Hardy, 1997; Cruts and Van Broeckhoven, 1998; Kwok et al., 1997; Taddei et al., 1998; Perez-Tur et al., 1995; Rogaeva et al., 2001), mainly missense mutations, in the PS1 gene have been identified in more than 90 families of various ethnic origins (Mattila et al., 1998). These mutations are highly penetrant, resulting in an early age of onset. Patients carrying the same PS1 mutation usually display very similar ages of onset, which tend to be even closer in members of the same family (Van Broeckhoven, 1995). One exception, however, is the
CHAPTER THREE: RISK FACTORS IN CLINICALLY DIAGNOSED AD

Glu318Gly mutation, caused by an A to G transition at codon 318 in exon 9 of PS1, which exhibits a variable age of onset for AD ranging from 35-64 years (Cruts and Van Broeckhoven, 1998). The pathogenicity of this mutation has been questioned since it has been found in a number of non-demented control individuals in addition to having been found in EOAD and LOAD patients without a known family history (Sandbrink et al., 1996; Reznik-Wolf et al., 1998b; Helisalmi et al., 2000), and in familial AD (FAD) patients (Taddei et al., 1998; Forsell et al., 1997; Cruts et al., 1998). This finding has led researchers to suggest that the Glu318Gly mutation is either a rare polymorphism (Mattila et al., 1998; Aldudo et al., 1998; Dermaut et al., 1999a), a neutral mutation (Dermaut et al., 1999b), or a mutation with incomplete penetrance (Dermaut et al., 1999b) leading to the disease only in a subset of cases. In this chapter, the frequency of the Glu318Gly mutation was determined in a large Australian Caucasian population consisting of individuals with FAD, early- or late-onset sporadic AD (SAD), and age-matched controls, in an attempt to clarify the role of this mutation in Alzheimer's disease.

3.1.1 Aims

- To determine frequency of APOE, APOE -491A/T and -219T/G promoter polymorphism genotypes
- Determine if the -491A/T and -219T/G promoter polymorphisms confer independent risk for AD
- Determine the association of these promoter polymorphism with levels of plasma apoE protein
- Determine the presence/absence of the Glu318Gly mutation
- Clarify the role of this mutation in AD
3.2 MATERIALS AND METHODS

Genotyping of the *APOE* polymorphisms was determined in the clinically diagnosed AD cohort as described in Section 2.1.2.1. Briefly, 625 individuals were in this cohort, which included 379 healthy controls, as well as 200 LOAD and 46 EOAD patients. Of the 379 normal controls collected there was no individual who exhibited any evidence or history of neurological disease and they all were volunteers from the same communities as the AD sufferers. The presence/absence of the Glu38Gly mutation was determined in the clinically diagnosed AD cohort as described in Section 2.1.2.2. Briefly, this cohort consisted of 682 individuals, of which 43 were sporadic EOAD patients, 191 sporadic LOAD patients, 46 FAD patients and 402 healthy controls.

All genotypes were determined on genomic DNA extracted from blood samples as per Section 2.2.2. *APOE* genotypes were determined as per Section 2.2.4.1, according to the procedure of Hixson and Vernier (1990) using oligonucleotide primers described by Wenham and colleagues (1991). The *APOE*-491A/T promoter polymorphism genotypes were determined as described in Section 2.2.4.2, according to the procedure of Bullido and colleagues (1998). The *APOE*-219G/T promoter polymorphism genotypes were determined as described in Section 2.2.4.3, essentially as described by Lambert and colleagues (1998b). Glu318Gly screening was performed as described in Section 2.2.4.4, according to the procedure of Dermaut and colleagues (1999).

Apolipoprotein E plasma levels were determined using the western blotting technique outlined in Section 2.2.5.2. Levels were determined in a sub-set of 136 individuals from the cohort described in Section 2.1.2.1. Briefly, plasma samples from 44 control and 92 AD (75 LOAD and 17 EOAD) individuals were assayed.
3.3 RESULTS

3.3.1 Genetic Screening of Risk Factors

3.3.1.1 APOE ε4 associated with AD in Australian cohort

Apolipoprotein E genotype was determined in the 625 member cohort described in Section 2.1.2.1. Genotype and allele distribution (Table 3.1a) was similar to that published previously in the Australian population (Martins et al., 1995a) as well as that reported by a meta-analysis of numerous studies in similar Caucasian populations (see Section 1.5, Table 1.2). The APOE genotypic distribution within the control sample was close to that expected under Hardy-Weinberg equilibrium ($\chi^2$ test, $P > 0.35$). Pearson's $\chi^2$ and odds ratio analysis of the genotype distribution in Table 3.1a reveals the expected association of APOE ε4 containing genotypes with AD ($p < 0.0001$, OR 3.10, 95% CI: 2.20 – 4.35; Table 3.1b). In this cohort 51.2% of all AD cases (51.5% of LOAD cases, $p < 0.0001$, OR 3.12, 95% CI: 2.18 – 4.49; and 50.1% of EOAD cases, $p < 0.0005$, OR 2.95, 95% CI: 1.58 – 5.49) possessed an APOE genotype that included the ε4 allele. Similarly, analysis of allelic distribution revealed similar associations in combined AD ($p < 0.0001$, OR 3.00, 95% CI: 2.26 – 3.99), LOAD ($p < 0.0001$, OR 2.79, 95% CI: 2.06 – 3.76) and EOAD ($p < 0.0001$, OR 3.51, 95% CI: 2.18 – 5.66). The reported gene dosage effect of the ε4 allele (Corder et al., 1993; Frisoni et al., 1995) is clearly illustrated in Table 3.1a with AD cases accounting for 25 out of 29 or 86.2% ε4 homozygotes. This is reflected in the marked increase in the odds ratio from 3.1 to 10.61 (95% CI: 3.64 – 30.87; Fisher’s exact $p < 0.0001$) in all AD cases. APOE ε4 homozygotes are 8.15 (95% CI: 2.69 – 24.73, Fisher’s exact $p < 0.0001$) and 22.8 (95% CI: 6.70 – 77.65, Fisher’s exact $p < 0.0001$) times more likely to develop LOAD and EOAD, respectively, than non-ε4 individuals.
### (a) 

#### APOLIPOPROTEIN E

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### (b) 

#### TEST

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<td>AD vs. Ctrl</td>
<td>(p &lt; 0.0001)</td>
<td>3.12 (2.18 - 4.49)</td>
</tr>
<tr>
<td>LOAD vs. Ctrl</td>
<td>(p &lt; 0.0005)</td>
<td>2.95 (1.58 - 5.49)</td>
</tr>
<tr>
<td>EOAD vs. Ctrl</td>
<td>(p &lt; 0.0005)</td>
<td>10.61 (3.64 - 30.87)</td>
</tr>
<tr>
<td>2(e4/e4) vs. (e2/e2) (genotype freq)</td>
<td>(p &lt; 0.0001)</td>
<td>8.15 (2.69 - 24.73)</td>
</tr>
<tr>
<td>AD vs. Ctrl</td>
<td>(p &lt; 0.0001)</td>
<td>4.16 (1.46 - 12.06)</td>
</tr>
<tr>
<td>LOAD vs. Ctrl</td>
<td>(p &lt; 0.0001)</td>
<td>22.80 (6.70 - 77.65)</td>
</tr>
<tr>
<td>EOAD vs. Ctrl</td>
<td>(p &lt; 0.0001)</td>
<td>3.00 (2.26 - 3.99)</td>
</tr>
<tr>
<td>3(e4) vs. (e2) (allele freq)</td>
<td>(p &lt; 0.0001)</td>
<td>2.79 (2.06 - 3.76)</td>
</tr>
<tr>
<td>AD vs. Ctrl</td>
<td>(p &lt; 0.0001)</td>
<td>3.51 (2.18 - 5.66)</td>
</tr>
</tbody>
</table>
Table 3.1 (adjacent): Apolipoprotein E Genotype and Allele Frequency within a cohort of normal controls and clinically diagnosed AD.

APOE ε4 containing genotypes and allele are highlighted in grey. Values in italics are the relative percentages of each genotype or allele within each group (a). Frequencies were used to calculate significance and odds ratios (b).

εn represents a non-APOE ε4 allele (i.e. ε2 or ε3; e.g. εn/εn may be ε2/ε2, ε2/ε3 or ε3/ε3 whilst εn/ε4 may be ε2/ε4 or ε3/ε4)

1 Comparison of the frequency of ε4 containing genotypes (i.e. ε4/ε4 and εn/ε4) versus non-ε4 containing genotypes (i.e. εn/εn)

2 Comparison of the frequency of individuals homozygous for ε4 versus non-ε4 (i.e. εn/εn) genotypes

3 Comparison of the frequency of the ε4 allele (ascertained by calculating allele frequencies) versus the non-ε4 alleles (i.e. εn)

f Fisher’s exact test used as one or more cells was ≤ 5
3.3.1.2 The APOE -491A/T promoter polymorphism is an independent risk factor for AD

Apolipoprotein E -491A/T promoter polymorphism genotype was determined in 625 individuals from the cohort described in Section 2.1.2.1. Firstly, the data were analysed to determine possible associations of the three -491A/T polymorphism genotypes (AA, AT and TT) with development of AD (Table 3.2a). Fisher’s exact test for 2x2 contingency tables and odds ratio analysis (Table 3.2b) of the genotype distribution in Table 3.2a found that genotypes containing the -491A allele (i.e. AA or AT genotypes) were significantly associated with AD (Fisher’s exact p < 0.01) and were at a greater than 4 fold risk of developing AD when compared to -491TT individuals (OR 4.09, 95% CI: 1.40 – 11.94). Individuals homozygous for the -491A allele were at the greatest risk of developing AD (Fisher’s exact p < 0.005, OR 5.00, 95% CI: 1.70 – 14.65).

When these data were stratified on the basis of onset of AD, it was found that the -491A containing genotypes were significantly associated with LOAD (Fisher’s exact p < 0.01, OR 4.44, 95% CI: 1.32 – 14.93) but not EOAD (Fisher’s exact p = 0.50, OR 3.04, 95% CI 0.40 – 23.03). Likewise homozygosity of the -491A allele was only associated with LOAD (Fisher’s exact p < 0.005, OR 5.32, 95% CI: 1.57 – 17.99) and not EOAD (Fisher’s exact p = 0.22, OR 4.02, 95% CI: 0.53 – 30.58). Pearson’s χ² and odds ratio analysis of the allele frequencies revealed similar associations in combined AD (p < 0.0001, OR 2.14, 95% CI: 1.56 – 2.93) and LOAD (p < 0.0001, OR 2.02, 95% CI: 1.45 – 2.83) whilst the -491A allele now reached significance in the EOAD group (p < 0.005, OR 2.79, 95% CI: 1.37 – 5.66).

The association of the -491AA genotype and the -491A allele were further analysed in relation to the presence or absence of the APOE ε4 allele (Table 3.3a). Individuals with
genotypes containing the -491A allele were significantly associated with AD and were at a greater than 4 fold risk of developing AD when compared to -491TT individuals in the absence of the APOE ε4 allele (Fisher’s exact p < 0.05, OR 4.73, 95% CI: 1.09 – 20.50). Individuals homozygous for the -491A allele were at the greatest risk of developing AD in the absence of ε4 (Fisher’s exact p < 0.05, OR 5.22, 95% CI: 1.19 – 22.82). However, in the presence of the APOE ε4 allele the association of -491A containing genotypes, although conferring an increase in risk, failed to reach statistical significance (Fisher’s exact p = 0.65, O.R. = 2.00, 95% CI: 0.33 – 12.21). This was also the case in ε4 positive individuals homozygous for the -491A allele (Fisher’s exact p = 0.38, O.R. = 2.38, 95% CI: 0.39 – 12.21). Pearson’s $\chi^2$ and odds ratio analysis of -491 allele frequencies confirmed the association of the -491A allele with an increased risk for AD in individuals lacking the APOE ε4 (p < 0.05, OR 1.53, 95% CI: 1.05 – 2.22) and it also revealed a stronger significant association of the -491A with AD in the presence of the APOE ε4 allele (p < 0.005, OR 2.46, 95% CI: 1.31 – 4.62).

Table 3.2 (adjacent): APOE -491A/T Promoter Polymorphism Genotype and Allele Frequencies within a cohort of normal controls and clinically diagnosed AD.

Values in italics are the relative percentages of each genotype or allele within each group (a). Frequencies were used to calculate significance and odds ratios represented in (b).

1 Comparison of the frequency of the -491A containing genotypes versus the -491TT genotype (i.e. -491AA/AT versus -491TT)

2 Comparison of the frequency of the -491AA genotypes versus the -491TT genotype

3 Comparison of the frequency of the -491A allele (ascertained by calculating allele frequencies) versus the -491T allele

$^f$ Fisher’s exact test used as one or more cells was ≤ 5
### (a) APOLIPOPROTEIN E -491A/T

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<th>STATUS</th>
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<th>T</th>
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<td>EOAD</td>
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<td>7</td>
<td>1</td>
<td>83</td>
<td>9</td>
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<td>82.6</td>
<td>15.2</td>
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<td>90.2</td>
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### (b) TEST

<table>
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<th>Groups tested</th>
<th>p</th>
<th>O.R. (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-491A vs. -491TT (genotype freq)</td>
<td>AD vs. Ctrl</td>
<td>$p &lt; 0.01^f$</td>
<td>4.09 (1.40 – 11.94)</td>
</tr>
<tr>
<td></td>
<td>LOAD vs. Ctrl</td>
<td>$p &lt; 0.01^f$</td>
<td>4.44 (1.32 – 14.93)</td>
</tr>
<tr>
<td></td>
<td>EOAD vs. Ctrl</td>
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<td>3.04 (0.40 – 23.03)</td>
</tr>
<tr>
<td>2-491AA vs. -491TT (genotype freq)</td>
<td>AD vs. Ctrl</td>
<td>$p &lt; 0.005^f$</td>
<td>5.00 (1.70 – 14.65)</td>
</tr>
<tr>
<td></td>
<td>LOAD vs. Ctrl</td>
<td>$p &lt; 0.005^f$</td>
<td>5.32 (1.57 – 17.99)</td>
</tr>
<tr>
<td></td>
<td>EOAD vs. Ctrl</td>
<td>$p = 0.22^f$</td>
<td>4.02 (0.53 – 30.58)</td>
</tr>
<tr>
<td>3-491A vs. -491T (allele freq)</td>
<td>AD vs. Ctrl</td>
<td>$p &lt; 0.0001$</td>
<td>2.14 (1.56 – 2.93)</td>
</tr>
<tr>
<td></td>
<td>LOAD vs. Ctrl</td>
<td>$p &lt; 0.0001$</td>
<td>2.02 (1.45 – 2.83)</td>
</tr>
<tr>
<td></td>
<td>EOAD vs. Ctrl</td>
<td>$p &lt; 0.005$</td>
<td>2.79 (1.37 – 5.66)</td>
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(a) **APOLIPOPROTEIN E -491A/T**

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<th>TT</th>
<th>A</th>
<th>T</th>
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</thead>
<tbody>
<tr>
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<td>157</td>
<td>105</td>
<td>21</td>
<td>429</td>
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<td>55.5</td>
<td>37.1</td>
<td>7.4</td>
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<td>26.0</td>
</tr>
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<td>44</td>
</tr>
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<td>1.7</td>
<td>81.7</td>
<td>18.3</td>
</tr>
<tr>
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<td>Control</td>
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<td>70</td>
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<td>3</td>
<td>163</td>
<td>29</td>
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<td></td>
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<td>84.9</td>
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</tr>
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<td>235</td>
<td>17</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>88.1</td>
<td>10.3</td>
<td>1.6</td>
<td>93.3</td>
<td>6.7</td>
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</table>

(b) **TEST**

<table>
<thead>
<tr>
<th>TEST</th>
<th>Groups tested</th>
<th>p</th>
<th>O.R. (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.-491A vs. -491TT</td>
<td>AD vs. Ctrl (ε4 absent)</td>
<td>$p &lt; 0.05^f$</td>
<td>4.73 (1.09 - 20.50)</td>
</tr>
<tr>
<td></td>
<td>AD vs. Ctrl (ε4 present)</td>
<td>$p = 0.65^f$</td>
<td>2.00 (0.33 - 12.21)</td>
</tr>
<tr>
<td>2.-491AA vs. -491TT</td>
<td>AD vs. Ctrl (ε4 absent)</td>
<td>$p &lt; 0.05^f$</td>
<td>5.22 (1.19 - 22.82)</td>
</tr>
<tr>
<td></td>
<td>AD vs. Ctrl (ε4 present)</td>
<td>$p = 0.38^f$</td>
<td>2.38 (0.39 - 14.59)</td>
</tr>
<tr>
<td>3.-491A vs. -491T</td>
<td>AD vs. Ctrl (ε4 absent)</td>
<td>$p &lt; 0.05$</td>
<td>1.53 (1.05-2.22)</td>
</tr>
<tr>
<td></td>
<td>AD vs. Ctrl (ε4 present)</td>
<td>$p &lt; 0.005$</td>
<td>2.46 (1.31-4.62)</td>
</tr>
</tbody>
</table>
Table 3.3 (adjacent): *APOE* -491A/T promoter polymorphism genotype and allele frequencies stratified by the presence or absence of the *APOE* ε4 allele.

(a) Values in italics are the relative percentages of each genotype or allele within each group. Frequencies were used to calculate significance and odds ratios (b). (Presence = ε4/ε4 or ε4/ε4 genotypes and Absence = εn/εn genotypes; where εn = non-ε4 allele)

1 Comparison of the frequency of the -491A containing genotypes versus the -491TT genotype (i.e. -491AA/AT versus -491TT) in the presence or absence of the *APOE* ε4 allele

2 Comparison of the frequency of the -491AA genotypes versus the -491TT genotype in the presence or absence of the *APOE* ε4 allele

3 Comparison of the frequency of the -491A allele (ascertained by calculating allele frequencies) versus the -491T allele in the presence or absence of the *APOE* ε4 allele

Fisher’s exact test used as one or more cells was ≤ 5
TheAPOE -219T/G promoter polymorphism is not independently associated with AD.

Apolipoprotein E -219T/G promoter polymorphism genotype was determined in 625 individuals from the cohort described in Section 2.1.2.1. Data was analysed to determine the level of associations of the three genotypes (GG, GT and TT) with development of AD (Table 3.4a). Pearson’s χ² and odds ratio analysis (Table 3.4b) of the genotype distribution in Table 3.4a found that genotypes containing the -219T allele (i.e. TT or GT genotypes) were not associated with AD (p = 0.08, OR 1.41, 95% CI: 0.96 - 2.07). However, individuals homozygous for the -219T allele were at a 1.79 times greater risk of developing AD (p < 0.05, OR 1.79, 95% CI: 1.13 – 2.83) when compared to individuals homozygous for the -219G allele.

Upon stratification of the data on the basis of onset of AD, the -219T containing genotypes were found not to be significantly associated with either LOAD (p = 0.06, OR 1.48, 95% CI: 0.97 – 2.25) or EOAD (p = 0.72, OR 1.14, 95% CI: 0.56 – 2.33). However, homozygosity of the -219T allele was associated with LOAD (p < 0.05, OR 1.90, 95% CI: 1.16 – 3.11) but not EOAD (p = 0.45, OR 1.39, 95% CI: 0.59 – 3.26) when compared to homozygosity of the -219G allele. Pearson’s χ² and odds ratio analysis of the allele frequencies revealed an association of the -219T allele with an increased risk for AD (p < 0.05, OR 1.34, 95% CI: 1.07 – 1.68). When analysed on the basis of onset the -219T allele remained significant in the LOAD group (p < 0.05, OR 1.38, 95% CI: 1.08 – 1.76). However, no significant association within the EOAD group was observed (p = 0.45, OR 1.18, 95% CI: 0.77 – 1.82).

The association of the -219TT genotype and the -219T allele were further analysed in relation to the presence or absence of the APOE ε4 allele (Table 3.3a). Individuals with
genotypes containing the -219T allele were not associated with AD in the absence (p = 0.41, OR 1.22, 95% CI: 0.76 – 1.97) or the presence (p = 0.87, O.R. = 0.94, 95% CI: 0.44 – 2.03) of the APOE ε4 allele. Individuals homozygous for the –219T allele were now no longer associated with an increased risk of developing AD when the absence (p = 0.29, OR 1.38, 95% CI: 0.75 – 2.55) or the presence (p = 0.74, O.R. = 1.16, 95% CI: 0.50 – 2.69) of the APOE ε4 allele was taken into account. Analysis of –219 allele frequencies confirmed this lack of association of the –219T allele with an increased risk for AD in individuals either lacking (p = 0.30, OR 1.17, 95% CI: 0.87 – 1.59) or possessing the APOE ε4 allele (p = 0.52, OR 1.14, 95% CI: 0.77 – 1.67).
### (a) APOLIPOPROTEIN E-219T/G

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<td>AD</td>
<td>246</td>
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<td>272</td>
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<td>48.8</td>
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<tr>
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### (b) TEST

<table>
<thead>
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<th>Groups tested</th>
<th>p</th>
<th>O.R. (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-219T vs. -219GG</td>
<td>AD vs. Ctrl</td>
<td>p = 0.08</td>
<td>1.41 (0.96 – 2.07)</td>
</tr>
<tr>
<td></td>
<td>LOAD vs. Ctrl</td>
<td>p = 0.06</td>
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<tr>
<td></td>
<td>EOAD vs. Ctrl</td>
<td>p = 0.72</td>
<td>1.14 (0.56 – 2.33)</td>
</tr>
<tr>
<td>2-219TT vs. -219GG</td>
<td>AD vs. Ctrl</td>
<td>p &lt; 0.05</td>
<td>1.79 (1.13 – 2.83)</td>
</tr>
<tr>
<td>(genotype freq)</td>
<td>LOAD vs. Ctrl</td>
<td>p &lt; 0.05</td>
<td>1.90 (1.16 – 3.11)</td>
</tr>
<tr>
<td></td>
<td>EOAD vs. Ctrl</td>
<td>p = 0.45</td>
<td>1.39 (0.59 – 3.26)</td>
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<tr>
<td>3-219T vs. -219G</td>
<td>AD vs. Ctrl</td>
<td>p &lt; 0.05</td>
<td>1.34 (1.07 – 1.68)</td>
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<tr>
<td>(allele freq)</td>
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<td>p &lt; 0.01</td>
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<td>EOAD vs. Ctrl</td>
<td>p = 0.45</td>
<td>1.18 (0.77 – 1.82)</td>
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</table>
Table 3.4 (adjacent): Apolipoprotein E -219T/G (Th1/E47cs) promoter polymorphism genotype and allele Frequencies within a cohort of normal controls and clinically diagnosed AD.

(a) Values in italics are the relative percentages of each genotype or allele within each group. Frequencies were used to calculate significance and odds ratios in (b).

1 Comparison of the frequency of the -219T containing genotypes versus the -219GG genotype (i.e. -219 versus -219GG)

2 Comparison of the frequency of the -219TT genotypes versus the -219GG genotype

3 Comparison of the frequency of the -219T allele (ascertained by calculating allele frequencies) versus the -219G allele
### (a)

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<th>GT</th>
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<th>G</th>
<th>T</th>
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### (b)

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<tbody>
<tr>
<td>1. -219T vs. -219GG (genotype freq)</td>
<td>AD vs. Ctrl (ε4 absent)</td>
<td>p = 0.41</td>
<td>1.22 (0.76 – 1.97)</td>
</tr>
<tr>
<td></td>
<td>AD vs. Ctrl (ε4 present)</td>
<td>p = 0.87</td>
<td>0.94 (0.44 – 2.03)</td>
</tr>
<tr>
<td>2. -219TT vs. -219GG (genotype freq)</td>
<td>AD vs. Ctrl (ε4 absent)</td>
<td>p = 0.29</td>
<td>1.38 (0.75 – 2.55)</td>
</tr>
<tr>
<td></td>
<td>AD vs. Ctrl (ε4 present)</td>
<td>p = 0.74</td>
<td>1.16 (0.50 – 2.69)</td>
</tr>
<tr>
<td>3. -219T vs. -219G (allele freq)</td>
<td>AD vs. Ctrl (ε4 absent)</td>
<td>p = 0.30</td>
<td>1.17 (0.87-1.59)</td>
</tr>
<tr>
<td></td>
<td>AD vs. Ctrl (ε4 present)</td>
<td>p = 0.52</td>
<td>1.14 (0.77-1.67)</td>
</tr>
</tbody>
</table>
Table 3.5 (adjacent): Apolipoprotein E -219T/G (Th1/E47cs) promoter polymorphism genotype and allele frequencies stratified by the presence or absence of the APOE ε4 allele.

(a) Values in italics are the relative percentages of each genotype or allele within each group. Frequencies were used to calculate significance and odds ratios in (b).

(Presence = ε4/ε4 or ε4/ε4 genotypes and Absence = εn/εn genotypes; where εn = non-ε4 allele)

1 Comparison of the frequency of the -219T containing genotypes versus the -219GG genotype (i.e. -219TT/GT versus -219GG) in the presence or absence of the APOE ε4 allele

2 Comparison of the frequency of the -491TT genotype versus the -219GG genotype in the presence or absence of the APOE ε4 allele

3 Comparison of the frequency of the -219T allele (ascertained by calculating allele frequencies) versus the -219G allele in the presence or absence of the APOE ε4 allele
3.3.1.4 Relative risk of the APOE polymorphisms alters in aged AD cases.

The AD associated risk of the APOE ε4 allele has been reported to steadily increase with age, exerting its greatest effect on AD between the ages of 60-79 years and decreasing thereafter (Farrer et al., 1997; Lautenschlager et al., 1999). A recent meta-analysis of the -491A/T and -219T/G promoter polymorphisms has reported that the risk associated with the -219TT/TG genotypes is greatest in individuals over the age of 81 years whilst the -491AA/AT genotypes have a greater effect in those under the age of 81 years (Lambert et al., 2002). To ascertain the effect of age on the AD associated risks of both APOE and the two promoter polymorphisms the data was stratified into two groups, those aged 81 or younger and those over the age of 81 years (Table 3.6) and Pearson's $\chi^2$, or where appropriate Fisher's exact, and odds ratio analysis were performed.

The risk associated with APOE ε4 containing genotypes decreased from 3.24 (95% CI: 2.09 – 5.02; p < 0.0001) in the younger age group to 2.86 (95% CI: 1.66 – 4.94; p < 0.0001) in those over the age of 81 years. Risk associated with homozygosity for the ε4

---

Table 3.6 (adjacent): Analysis of changes in relative risk of the APOE and promoter polymorphism genotype and alleles when stratified by age.

Odds Ratios with 95% Confidence intervals (italicised in brackets) and significance levels (italicised) of APOE and promoter polymorphisms were calculated in individuals aged ≤ 81 or > 81 years of age. Promoter polymorphisms were further stratified by the presence/absence of the APOE ε4 allele.

n = non-APOE ε4 allele

* Lack of -491TT genotypes prevented analysis of relative risks in these groups

$^f$ Fisher's exact test used as one or more cells was ≤ 5
<table>
<thead>
<tr>
<th>Age at Onset</th>
<th>APOE Genotype</th>
<th>219T/C Polymorphism</th>
<th>491A/V Polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>TT/C</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>69</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>70</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>71</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>72</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>73</td>
<td>CC</td>
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<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>74</td>
<td>CC</td>
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<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>75</td>
<td>CC</td>
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<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>76</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>77</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>78</td>
<td>CC</td>
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<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>79</td>
<td>CC</td>
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<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>80</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>81</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>82</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>83</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>84</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>85</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>86</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>87</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>88</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>89</td>
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<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>90</td>
<td>CC</td>
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<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>91</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>92</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>93</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>94</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>95</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>96</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>97</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>98</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>99</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
<tr>
<td>100</td>
<td>CC</td>
<td>100 1.0 (1.18-1.10)</td>
<td>69.6 1.0 (0.68-1.08)</td>
</tr>
</tbody>
</table>

**Note:** The table shows the distribution of APOE genotypes and 219T/C and 491A/V polymorphisms across different ages at onset.
allele decreased from 15.94 (95% CI: 4.63 - 54.90; Fisher's exact p < 0.0001) to 8.00 (95% CI: 0.81 - 71.92; Fisher's exact p = 0.07) whilst analysis of allele frequencies in the two groups revealed a decrease in risk from 3.23 (95% CI: 2.25 - 4.64; p < 0.0001) to 2.39 (95% CI: 1.50 - 3.81; p < 0.0001) in those over the age of 81 years.

Analysis of the two APOE promoter polymorphisms revealed that the AD associated risk of both the -491A/T and -219T/G polymorphism was greatest in individuals over the age of 81 years. In the case of the -491A/T promoter polymorphism the risk of -491A allele containing genotypes, although not reaching significance in either age group, increased from 3.26 (95% CI: 0.91 - 11.62; Fisher's exact p = 0.07) to 6.45 (95% CI: 0.82 - 50.84; Fisher's exact p = 0.06). The limitation of this analysis is that the frequency of the -491TT genotype made it difficult to determine the level of risk conferred by -491A allele containing genotypes in this cohort of 625 individuals, as opposed to the meta analysis performed by Lambert and colleagues (2002) on 3,658 individuals from six independent subject groups. The risk associated with homozygosity of the -491A allele increased from 4.02 (95% CI: 1.12 - 14.47; Fisher's exact p < 0.05) in the younger age group to 7.87 (95% CI: 1.00 - 62.33; Fisher's exact p < 0.05) in those over 81 years. Likewise analysis of allele frequencies revealed an increase in the level of risk of 1.97 (95% CI: 1.34 - 2.90; p < 0.001) in those 81 years or younger to 2.63 (95% CI: 1.49 - 4.63; p < 0.001) in those over 81 years.

Further stratification of the -491 data by the presence/absence of the ε4 allele did not allow for a comparison of the associated risk of the -491A allele containing genotypes in the two age groups due to the lack of the -491TT allele in individuals 81 years or younger with an ε4 allele and individuals older than 81 years without the ε4 allele. However, analysis of allele frequencies revealed that in individuals with the ε4 allele the
risk associated with the -491A allele increased slightly from 2.32 (95% CI: 1.06 – 5.06; p < 0.05) to 2.85 (95% CI: 0.96 – 8.47; p = 0.07). Whilst in the absence of the ε4 allele the risk associated with the -491A allele increased from 1.38 (95% CI: 0.87 – 2.19; p = 0.16) to 2.07 (95% CI: 1.05 – 4.07; p < 0.05) in those over 81 years.

Analysis of the -219T/G promoter polymorphism data revealed that the -219T allele containing genotypes did not reach significance in either age group, as was the case when this polymorphism was analysed regardless of age (Section 3.1.3.3). However, the OR did increase from 1.29 (95% CI: 0.80 – 2.09; p = 0.29) to 1.69 (95% CI: 0.87 – 3.26; p = 0.12). Homozygosity of the -219T allele was not significantly associated with AD in the younger age group (p = 0.16), though it was significantly associated with AD in individuals over 81 years of age (p < 0.05) and the relative risk had increased from 1.5 (95% CI: 0.85 – 2.65) in the younger age group to 2.46 (95% CI: 1.12 – 5.41) in those over 81 years. Likewise analysis of allele frequencies reached significance only in the older age group (p < 0.01) and the relative risk increased from 1.24 (95% CI: 0.93 – 1.66; p = 0.15) in those 81 years or younger to 1.65 (95% CI: 1.15 – 2.38) in those over 81 years.

The -219 data was stratified further by the presence/absence of the ε4 allele. In individuals possessing the ε4 allele this analysis revealed neither a significant association for either age group nor a change in relative risk with age. In fact, the relative risks were slightly decreased when -219T allele containing genotypes [1.21 (95% CI: 0.51 – 2.92; p = 0.66) to 0.51 (95% CI: 0.09 – 2.96; Fisher’s exact p = 0.68)], -219TT homozygosity [1.48 (95% CI: 0.56 – 3.93; p = 0.43) to 0.58 (95% CI: 0.09 – 3.76; Fisher’s exact p = 0.67)] and when allele frequencies were analysed [1.27 (95% CI: 0.78 – 2.08; p = 0.33) to 0.97 (95% CI: 0.52 – 1.79; p = 0.91)]. In individuals
without the ε4 allele a change in relative risk with age was found, however, this only reached significance when allele frequencies were analysed. Relative risks slightly increased for -219T allele containing genotypes [1.07 (95% CI: 0.59 – 1.97; p = 0.81) to 1.49 (95% CI: 0.68 – 3.25; p = 0.32)], for -219TT homozygosity [0.93 (95% CI: 0.42 – 2.04; p = 0.85) to 2.56 (95% CI: 0.96 – 6.81; p = 0.06)] and for the analysis of allele frequencies [0.98 (95% CI: 0.66 – 1.44; p = 0.90) to 1.89 (95% CI: 1.18 – 3.03; p < 0.01)].

3.3.1.5 Increased frequency of the Glu318Gly mutation in familial AD (FAD)

The presence/absence of the Glu38Gly mutation was determined in a clinically diagnosed AD cohort as described in Section 2.1.2.2. In this cohort the APOE ε4 genotypic distribution within the control group (n = 402) was close to that expected under Hardy-Weinberg equilibrium ($\chi^2$ test, p > 0.5). The genotypic frequencies of the Perth (n = 201, $\chi^2$ test, p > 0.5) and Sydney (n = 201, $\chi^2$ test, p > 0.8) control sample did not differ from that expected.

As shown in Table 3.7, the Glu318Gly mutation was detected in 6 sporadic LOAD patients, in 4 FAD patients (unrelated) and in 9 control subjects. Of the 43 sporadic EOAD patients screened, none were found to have this mutation. The frequency of this mutation was highest in the FAD group (8.7%), compared with sporadic LOAD group (3.1%) and was lowest in control subjects (2.2%). When the mutation frequencies were compared by either the Pearson $\chi^2$ method or Fisher’s exact test, no significant difference between the sporadic LOAD group and control subjects was observed (Fisher’s exact p = 0.578; OR 1.42, 95% CI: 0.50 – 4.04). However, a significant difference (Fisher’s exact p < 0.05 OR 4.16, 95% CI: 1.23 – 14.09) was observed when the FAD group was compared with age-matched control subjects.
### Table 3.7: Identification of the PS1 Glu318Gly (E318G) mutation in sporadic EOAD and LOAD, familial AD and control subjects in the Australian population.

(a) Values in italics are the relative percentages of the presence of the E318G mutation within each group. Frequencies here were used to calculate significance and odds ratios in the text and (b).

EOAD = Sporadic early-onset Alzheimer’s Disease

LOAD = Sporadic late-onset Alzheimer’s Disease

* P < 0.05 compared to controls

# E318G (+) individuals in parentheses

NT = Not tested

-/-, +/- and +/- = non-APOE ε4, heterozygote ε4 and homozygote ε4, respectively

1 Comparison of the frequency of individuals with the E318G mutation with those where it is not present.

<table>
<thead>
<tr>
<th>Group</th>
<th>Subjects investigated</th>
<th>E318G Positive</th>
<th>Other PS1 mutation</th>
<th>APOE ε4 Status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[n]</td>
<td>[n(%)]</td>
<td>[n]</td>
<td>-/-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[n(#)]</td>
</tr>
<tr>
<td>EOAD</td>
<td>43</td>
<td>0</td>
<td>0</td>
<td>21(-)</td>
</tr>
<tr>
<td>LOAD</td>
<td>191</td>
<td>6 (3.1)</td>
<td>NT</td>
<td>89(3)</td>
</tr>
<tr>
<td>Familial AD</td>
<td>46</td>
<td>4 (8.7)</td>
<td>4</td>
<td>23(2)</td>
</tr>
<tr>
<td>Controls</td>
<td>402</td>
<td>9 (2.2)</td>
<td>NT</td>
<td>296(6)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TEST</th>
<th>Groups tested</th>
<th>p</th>
<th>O.R. (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E318G pos vs. E318G neg</td>
<td>LOAD vs. Ctrl</td>
<td>p = 0.58</td>
<td>1.42 (0.50 - 4.04)</td>
</tr>
<tr>
<td></td>
<td>FAD vs. Ctrl</td>
<td>p &lt; 0.05</td>
<td>4.16 (1.23 - 14.09)</td>
</tr>
</tbody>
</table>
Of the 90 or more mutations identified so far in the PS1 gene, no relationship between the inheritance of the APOE ε4 allele and age of AD onset has been shown. Glu318Gly data was stratified by the presence/absence of the APOE ε4 allele in order to test for an interaction between the Glu318Gly mutation and the ε4 allele (Table 3.7). No association between APOE genotype and the Glu318Gly mutation was observed in FAD and LOAD in either the absence (FAD: Fisher’s exact p = 0.107; LOAD: Fisher’s exact p = 0.437) or the presence of the ε4 allele (FAD: Fisher’s exact p = 0.217; LOAD: Fisher’s exact p = 1.000). Only one of the six sporadic LOAD Glu318Gly positive patients was an APOE ε4 homozygote whilst the other 23 ε4 homozygous individuals, in this and the other groups, exhibited no change at this specific PS1 locus.

The Glu318Gly mutation was detected in 4 FAD patients from 4 unrelated families who had onset of disease at the ages of late 50’s, 60, 64 and 69 years. Details of each proband’s pedigree is shown in Table 3.8. As with previous reports (Sandbrink et al., 1996; Reznik-Wolf et al., 1998b; Helisalmi et al., 2000; Forsell et al., 1997; Cruts et al., 1998) for other ethnic groups, co-segregation of this mutation with AD could not be properly tested due to the lack of sufficient family members for analysis. In each of the families in this study, with the exception of the PERTH-7 family, the proband was the only affected member available for analysis. In the PERTH-7 family, an affected brother of the proband also had the mutation (Table 3.8). The age of onset of the affected brother was 82 years compared to 60 years for the proband. This variable age of AD onset within this family with the Glu318Gly mutation indicates that the clinical effect of this mutation (compared to the fully penetrant PS1 mutations) is more likely to be modifiable by other factors.
### Table 3.8: Glu318Gly mutation analysis in familial AD pedigrees

<table>
<thead>
<tr>
<th>Family</th>
<th>Family members screened</th>
<th>AD cases screened</th>
<th>Non-AD cases screened</th>
<th>Mean age of family onset</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$</td>
<td>$n (\dagger)$</td>
<td>$n (\dagger)$</td>
<td>years ($\ddagger$)</td>
</tr>
<tr>
<td></td>
<td>[years $^*$]</td>
<td></td>
<td>[years $^{**}$]</td>
<td></td>
</tr>
<tr>
<td>PERTH-5</td>
<td>1(^{\text{g}})</td>
<td>1 (1)</td>
<td>NA</td>
<td>Late 50s (2)</td>
</tr>
<tr>
<td></td>
<td>[Late 50s]</td>
<td>[-]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PERTH-6</td>
<td>4</td>
<td>1 (1)</td>
<td>3 (1)</td>
<td>64 ± 8 (6)</td>
</tr>
<tr>
<td></td>
<td>[64]</td>
<td>[35]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PERTH-7</td>
<td>6</td>
<td>2 (2)</td>
<td>4 (1)</td>
<td>68 ± 11 (3)</td>
</tr>
<tr>
<td></td>
<td>[60 &amp; 82]</td>
<td>[81]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PERTH-8</td>
<td>3</td>
<td>1 (1)</td>
<td>2 (2)</td>
<td>71 ± 2 (2)</td>
</tr>
<tr>
<td></td>
<td>[69]</td>
<td>[40 &amp; 47]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Age of Onset

**Age at venipuncture

$\dagger$ Glu318Gly positive individuals in non-italicised parentheses

$\ddagger$ Number of AD individuals in the family used to determine mean age of onset in italicised square parentheses

$\text{g}$ Only one family member available for analysis

NA = Not available for screening
For each family, a number of unaffected relatives were available for analysis, with the exception of the PERTH-5 family where none were available for screening. As can be seen in Table 3.8, one of three unaffected relatives (two siblings and one first degree relative) screened from the PERTH-6 family has the Glu318Gly mutation. The unaffected Glu318Gly positive individual, studied at 35 years of age, may be considered as 'at risk' when compared to the mean age of onset for this family (64 ± 8 years, range 55-80 years). This is also the case with the PERTH-8 family (mean age of AD onset 71 ± 2 years, range 69-73 years), where two siblings (aged 40 and 47 years) of the proband were positive for the Glu318Gly mutation available for analysis. Thus, both individuals are still 'at risk' of developing AD. For the PERTH-7 case, 4 unaffected relatives (a brother, sister and two siblings) were screened. Only the unaffected brother was positive for the Glu318Gly mutation and his age at the time of study was 81 years. Comparison of his age to the mean age of AD onset for the family (68 ± 11 years, range 59-83 years) does not exclude him as an escapee.

3.3.2 Plasma Apolipoprotein E Protein Levels

3.3.2.1 ApoE levels are elevated in AD independent of APOE genotype

Plasma apoE levels were quantitated in 136 samples, including 44 control and 92 AD individuals, a sub-set of the clinical AD cohort described in Section 2.1.2.1. Plasma apoE levels were significantly elevated (Student’s t-test, p < 0.001) in AD [3.32 ± 0.09 µg apoE/mg Total Protein (TP)] when compared to control individuals (2.35 ± 0.06 µg apoE/mg TP; Figure 3.1a). When onset of AD was investigated (Figure 3.1b) it revealed that both LOAD (3.28 ± 0.10 µg apoE/mg TP) and EOAD (3.48 ± 0.29 µg apoE/mg TP) had significantly higher levels than controls after applying Bonferroni correction for multiple comparisons (p < 0.001). EOAD individuals tended to have higher levels of
Figure 3.1: Plasma apolipoprotein E levels in control and AD individuals.

Plasma apoE levels were determined to be elevated in AD individuals when compared to controls (A). EOAD individuals tended to have the highest levels of apoE when age of onset was investigated (B). Where appropriate Bonferroni correction for multiple comparisons was applied.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

(A) Control vs. AD individuals: * Student’s t-test t = 6.71, p < 0.001

(B) LOAD vs. EOAD vs. Control individuals: **p < 0.001
Figure 3.2: Plasma apolipoprotein E levels in control and AD individuals: stratified by presence/absence of APOE ε4 allele.

Plasma apoE levels were determined to be elevated in AD individuals when compared to controls in both the absence (A) and presence (B) of the APOE ε4 allele.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

(A) Control vs. AD individuals: * Student’s t-test $t = 2.51$, $p < 0.05$

(B) Control vs. AD individuals: ** Student’s t-test $t = 5.48$, $p < 0.0001$
plasma apoE when compared to LOAD individuals but this did not reach statistical significance.

To determine whether or not the increased plasma apoE levels seen in AD individuals was due to the increased frequency of the APOE ε4 allele, seen in this group, control and AD individuals was stratified by the absence (Figure 3.2a) or presence (Figure 3.2b) of this allele. Plasma apoE levels were found to be significantly higher in AD individuals irrespective of the absence (AD vs. Control: 3.26 ± 0.15 vs. 2.27 ± 0.06 μg apoE/mg TP, p < 0.0001) or presence (AD vs. Control: 3.36 ± 0.11 vs. 2.66 ± 0.18 μg apoE/mg TP, p < 0.05) of the APOE ε4 allele.

3.3.2.2 APOE genotype has no gene dosage dependent effect and negligible impact overall on plasma apoE levels

When analysing the plasma apoE data, in respect to AD-control comparisons stratified by APOE genotype, it was interesting to note that control individuals possessing the ε4 allele (2.66 ± 0.18 μg apoE/mg TP) had significantly higher (p < 0.05) levels than controls lacking the ε4 allele (2.27 ± 0.06 μg apoE/mg TP). Plasma apoE levels were investigated with respect to APOE genotype to determine whether APOE genotype imparted any effect, gene dose dependent or otherwise, on the expression of the protein in plasma. Irrespective of disease status (Figure 3.3a) the presence of an ε4 allele was associated with significantly increased apoE levels, after applying Bonferroni correction for multiple comparisons (p < 0.05). This effect did not manifest in a gene dosage dependent manner as only ε4 heterozygotes (3.26 ± 0.12 μg apoE/mg TP) and not ε4 homozygotes (3.22 ± 0.25 μg apoE/mg TP) had significantly higher levels than non-ε4 individuals (2.82 ± 0.11 μg apoE/mg TP).
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**Figure:**

- **A**
  - apoE Level (µg/mg Total Protein)
  - ε2/ε2, ε2/ε4, ε4/ε4
  - (n = 78) (n = 46) (n = 12)

- **B**
  - ε2/ε2, ε2/ε4, ε4/ε4
  - (n = 35) (n = 9) (n = 0)

- **C**
  - ε2/ε2, ε2/ε4, ε4/ε4
  - (n = 43) (n = 37) (n = 12)

**Legend:**
- Max
- Min
- Mean+SEM
- Mean
- Mean-SEM
Figure 3.3 (adjacent): Effect of APOE genotype on plasma apolipoprotein E levels.

APOE genotype imparted no gene dosage effect and negligible overall effect on plasma apoE levels irrespective of disease status (A) and likewise in control individuals (B). APOE genotype had no significant impact on levels in AD individuals (C). Where appropriate Bonferroni correction for multiple comparisons was applied.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

εn = non-APOE ε4 allele (i.e. ε2 or ε3)

(A) εn/εn vs. εn/ε4 vs. ε4/ε4: * p < 0.05

(B) εn/εn vs. εn/ε4: ** Student's t-test t = 2.62, p < 0.05
When disease status was taken into account the effect of APOE genotype was only apparent in control individuals (Figure 3.3b) with ε4 heterozygotes (2.66 ± 0.18 μg apoE/mg TP) having significantly higher (Student’s t-test, p < 0.05) plasma apoE levels when compared to non-ε4 controls (2.28 ± 0.06 μg apoE/mg TP). No control individuals in this subset were homozygous for the ε4 allele and thus no data was available to determine a gene dose dependent effect. Conversely, APOE genotype imparted no effect on plasma apoE levels in AD individuals (Figure 3.3c). Heterozygous ε4 AD individuals (3.41 ± 0.13 μg apoE/mg TP) tended to have the highest levels, however, this was not significantly different to AD individuals either homozygous for ε4 (3.22 ± 0.25 μg apoE/mg TP) or not possessing the ε4 allele (3.26 ± 0.15 μg apoE/mg TP). There was no trend towards a gene dose effect and, in fact, AD individuals homozygous for the ε4 allele exhibited a trend towards lower plasma apoE levels than non-ε4 AD individuals.

3.3.2.3 APOE -491AA promoter polymorphism genotype is associated with elevated plasma apoE levels

Plasma apoE levels in this subset of individuals were stratified by the -491 A/T promoter polymorphism genotype previously determined in Section 3.3.1.2. Initially the data was stratified on the basis of promoter polymorphism genotype irrespective of disease status or APOE genotype (Figure 3.4a). APOE -491AA promoter polymorphism genotype was associated with significantly elevated plasma apoE levels (3.42 ± 0.10 μg apoE/mg TP) when compared to the -491AT (2.39 ± 0.07 μg apoE/mg TP; p < 0.0001) and -491TT (2.45 ± 0.16 μg apoE/mg TP; p < 0.001) genotypes. However, there was no apparent gene dose dependent effect of the -491A allele observed. Within individuals possessing the APOE ε4 allele (Figure 3.4b) the -491AA genotype had significantly higher plasma apoE levels (3.41 ± 0.12 μg apoE/mg TP) when compared to 491AT/ε4
individuals (2.65 ± 0.13 μg apoE/mg TP; Student's t-test p < 0.005). No comparison could be made with -491TT individuals and no gene dose effect could be determined due to the lack of -491TT/ε4 individuals. Similar findings were observed in individuals lacking the ε4 allele (Figure 3.4c). Statistical analysis after applying Bonferroni correction for multiple comparisons revealed that -491AA/non-ε4 individuals had significantly higher plasma apoE levels (3.42 ± 0.17 μg apoE/mg TP) when compared to -491AT/non-ε4 (2.30 ± 0.08 μg apoE/mg TP; p < 0.0001) and -491TT/non-ε4 individuals (2.45 ± 0.15 μg apoE/mg TP; p < 0.005). As with previous analysis there was no indication that the -491A allele imparted a gene dose dependent impact on altering plasma apoE levels. A comparison between -491AA-ε4 carriers and -491AA- non-ε4 carriers revealed no significant difference in plasma apoE levels, suggesting an ε4 independent effect of the -491AA genotype on plasma apoE levels.

Plasma apoE data was then stratified by disease status and the impact of the APOE -491AA promoter polymorphism genotype investigated. In control individuals (Figure 3.5a) the APOE -491AA promoter polymorphism genotype was associated with significantly elevated plasma apoE levels (2.65 ± 0.14 μg apoE/mg TP) when compared to -491AT individuals (2.25 ± 0.08 μg apoE/mg TP; p < 0.05). Although -491AA control individuals tended to have higher levels than -491TT controls (2.39 ± 0.13 μg apoE/mg TP) this failed to reach statistical significance. Likewise in AD individuals (Figure 3.5b) the -491AA genotype had significantly elevated plasma apoE levels (3.51 ± 0.10 μg apoE/mg TP) when compared to the -491AT genotype (2.61 ± 0.14 μg apoE/mg TP; p < 0.0001). The low frequency of the -491TT genotype amongst AD individuals prevented meaningful statistical analysis although levels tended to be markedly decreased in the -491TT carriers (2.67 ± 0.71 μg apoE/mg TP).
Figure 3.4 (adjacent): Effect of -491A/T promoter polymorphism genotype on plasma apolipoprotein E levels.

APOE -491AA promoter polymorphism genotype was associated with significantly elevated plasma apoE levels irrespective of disease status (A) when compared to the other genotypes. However, no gene dose dependent effect was observed. The elevated levels associated with this genotype occurred in both the presence (B) and absence (C) of the APOE ε4 allele. Where appropriate Bonferroni correction for multiple comparisons was applied.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

(A) AA vs. AT vs. TT: * p < 0.0001

** p < 0.001

(B) AA vs. AT: † Student’s t-test t = 3.16, p < 0.005

(C) AA vs. AT vs. TT: ‡ p < 0.0001

§ p < 0.005
Figure 3.5: Effect of -491A/T promoter polymorphism genotype on plasma apolipoprotein E levels in AD and control individuals.

APOE -491AA promoter polymorphism genotype was associated with significantly elevated plasma apoE levels when compared to the other genotypes in both control (A) and AD (B) individuals. However, no gene dose dependent effect was observed. AD individuals with the -491AA genotype had significantly higher plasma apoE levels than control individuals with this genotype. Where appropriate Bonferroni correction for multiple comparisons was applied.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

(A) AA vs. AT vs. TT: * p < 0.05

(B) AA vs. AT vs. TT: ** p < 0.0001

AD (AA vs. AT vs. TT) vs. Ctrl (AA vs. AT vs. TT): *** p < 0.05
between AD –49IAA carriers and control –49IAA carriers revealed a significant increase in plasma apoE levels (p < 0.05) in the AD –49IAA individuals. There was no difference observed between AD and control individuals in either –491AT or –49ITT carriers.

3.3.2.4  APOE –219T/G promoter polymorphism genotype has a minor impact on plasma apoE levels

Plasma apoE levels in this subset of individuals were stratified by the –219T/G promoter polymorphism genotype previously determined in Section 3.3.1.3. Initially the data was stratified on the basis of promoter polymorphism genotype irrespective of disease status or APOE genotype (Figure 3.6a). Comparisons of levels across the three –219T/G genotypes revealed that carriers of the –219T allele (i.e. –219TT or –219GT genotypes) had slightly, yet statistically significantly, elevated plasma apoE levels (3.18 ± 0.14 and 3.07 ± 0.11 µg apoE/mg TP, respectively) compared to carriers of the –219GG genotype (2.55 ± 0.15 µg apoE/mg TP; p < 0.05). No significant difference was observed between –219TT or –219GT genotypes. These findings are in contrast to cell transfection studies (Artiga et al., 1998a, 1998b) and study of plasma apoE levels (Lambert et al., 2000) where it is suggested that the –219T allele exerts a gene dose dependent effect on lowering apoE levels.

Within individuals possessing the APOE ε4 allele (Figure 3.6b) the –219T allele containing genotypes tended to have elevated plasma apoE levels (–219TT: 3.42 ± 0.17 µg apoE/mg TP, –219GT: 3.21 ± 0.13 µg apoE/mg TP) when compared to –219GG/ε4 individuals (2.79 ± 0.38 µg apoE/mg TP), however, this failed to reach statistical significance in all comparisons. ApoE levels differed between the three genotypes in individuals lacking the ε4 allele (Figure 3.6c). Although –219TT and –219GT
Figure 3.6 (adjacent): Effect of $-219T/G$ promoter polymorphism genotype on plasma apolipoprotein E levels.

$APOE$ $-219TT$ and $-219GT$ promoter polymorphism genotypes, irrespective of disease status, were associated with significantly elevated plasma apoE levels (A) when compared to the $-219GG$ genotype. When stratified by the presence (B) and absence (C) of the $APOE$ $e4$ allele the $-219TT$ and $-219GT$ genotypes tended to have higher levels of plasma apoE, however, this did not reach statistical significance. Where appropriate Bonferroni correction for multiple comparisons was applied.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

(A) GG vs. GT vs. TT: * $p < 0.05$
genotypes tended to have a higher level of plasma apoE, statistical analysis after applying Bonferroni correction for multiple comparisons revealed no significant difference in all comparisons between \(-219\)TT (2.90 ± 0.20 \(\mu\)g apoE/mg TP), \(-219\)GT (2.97 ± 0.16 \(\mu\)g apoE/mg TP) and \(-219\)GG (2.48 ± 0.17 \(\mu\)g apoE/mg TP) genotypes in non-\(\varepsilon4\) individuals.

Plasma apoE data was then stratified by disease status and the \(APOE\) \(-219\)T/G promoter polymorphism genotypes investigated. In control individuals (Figure 3.7a) there was no statistical significance difference observed in plasma apoE levels, after Bonferroni correction for multiple comparisons, for any \(APOE\) \(-219\)T/G promoter polymorphism genotype. Control individuals with the \(-219\)GG genotype tended to have the lowest plasma apoE levels (2.10 ± 0.08 \(\mu\)g apoE/mg TP; \(p < 0.05\)) compared to the \(-219\)GT genotype (2.49 ± 0.11 \(\mu\)g apoE/mg TP) and the \(-219\)TT genotype (2.46 ± 0.10 \(\mu\)g apoE/mg TP). Analysis of AD individuals (Figure 3.7b) also revealed that there was no significant difference in plasma apoE levels between \(-219\)TT (3.46 ± 0.16 \(\mu\)g apoE/mg TP), \(-219\)GT (3.30 ± 0.13 \(\mu\)g apoE/mg TP) and \(-219\)GG (3.03 ± 0.25 \(\mu\)g apoE/mg TP) genotypes after correcting for multiple comparisons. A comparison between respective \(-219\)T/G genotypes in AD and control individuals revealed that across the board AD individuals had significantly elevated plasma apoE levels (\(-219\)GG, \(p < 0.05\); \(-219\)GT and \(-219\)TT, \(p < 0.005\)). This difference suggests that in AD individuals other factors, for example the \(-491\)AA promoter polymorphism genotype, may play a more important role in elevating plasma apoE levels than the \(-219\)T/G promoter polymorphism.

### 3.3.2.5 \(-491\)A/T alters \(-219\)T/G associated apoE levels, but not vice versa

Individuals were grouped based on the combinations of the two promoter polymorphism genotypes (Figure 3.8). This analysis was done irrespective of disease status, as further
Figure 3.7: Effect of -219T/G promoter polymorphism genotype on plasma apolipoprotein E levels in AD and control individuals.

APOE -219GG promoter polymorphism genotype was associated with significantly lower plasma apoE levels when compared to the other genotypes in control individuals (A). In AD individuals (B) this genotype also tended to have the lowest levels but did not reach statistical significance. However, there was a trend towards a gene dose dependent effect. AD individuals had significantly higher plasma apoE levels than control individuals with corresponding genotypes. Where appropriate Bonferroni correction for multiple comparisons was applied.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

AD (GG vs. GT vs. TT) vs. Ctrl (GG vs. GT vs. TT): * p < 0.05 ** p < 0.005
breakdown of values would have allowed no significantly relevant analysis to be performed. After correction for multiple comparisons, no significant impact of the –219T/G genotypes on altering levels in –491AA, –491AT or –491TT individuals were observed. On the other hand, the –491A/T genotypes was associated with significantly altered plasma apoE levels in the –219T/G genotypes. In –219TT individuals, –491AA individuals had significantly elevated levels (3.63 ± 0.17 µg apoE/mg TP) compared to –491AT individuals (2.54 ± 0.15 µg apoE/mg TP; p < 0.005). This association was also observed in –219GT individuals where those possessing the -491AA genotype (3.35 ± 0.13 µg apoE/mg TP) had significantly elevated levels compared to those individuals with the –491AT genotype (2.52 ± 0.13 µg apoE/mg TP, p < 0.01). Likewise in –219GG individuals where the -491AA genotype (3.15 ± 0.28 µg apoE/mg TP) had significantly higher levels compared to the –491AT genotype (2.13 ± 0.08 µg apoE/mg TP, p < 0.05).

Figure 3.8 (adjacent): Analysis of the combined effect of –491A/T and -219T/G promoter polymorphism genotypes on plasma apolipoprotein E levels.

Individuals were grouped based on the combinations of the two promoter polymorphism genotypes and done irrespective of disease status. –491A/T genotypes significantly altered plasma apoE levels associated with the –219T/G genotypes. However, the same did not apply for –219T/G genotypes. Where appropriate Bonferroni correction for multiple comparisons was applied.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

AA/TT vs AA/GT vs AA/GG vs AT/TT vs AT/GT vs AT/GG vs TT/TT vs TT/GT vs TT/GG
*p < 0.005 (AA/TT vs. AT/TT)
**p < 0.01 (AA/GT vs. AT/GT)
***p < 0.05 (AA/GG vs. AT/GG)
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APOE Level (ug/mg Total Protein)
3.4 DISCUSSION

3.4.1 The Genetic Risk Associated with the Different APOE Loci

3.4.1.1 APOE e4 allele and AD risk in the Australian population

Since the initial analysis of families with LOAD resulted in the identification of a disease locus on chromosome 19 (Pericak-Vance et al., 1991) and the subsequent association of the e4 allele of the APOE gene, located within (19q13.2), with an increased risk of AD (Strittmatter et al., 1993) much research has centred on APOE, its genetic association and its mechanism(s) by which it elicits this increased risk. The initial analysis of data from this chapter supports the association of APOE e4 containing genotypes with an increased risk of AD (OR 3.10; Table 3.1) which is consistent with earlier studies (Corder et al., 1993; Lucotte et al., 1993; Mayeux et al., 1993b; Strittmatter et al. 1993; Saunders et al, 1993a; Saunders et al, 1993b; Dai et al. 1994; Houlden et al, 1994; Payami et al., 1994; Martins et al., 1995a; Adorer et al., 1995; Basun et al., 1995; Frisoni et al., 1995; Okuizumi et al., 1995; Hyman et al., 1996) and reflects the common consensus that approx 50% of AD cases [(in this case 51.2% of AD (51.5% and 50.1% of LOAD and EOAD, respectively)] can be attributed to this allele [see Farrar et al., 1997 for a meta-analysis of these data]. It also supports the notion that carriage of a genotype containing an APOE e4 allele makes no dispersions between LOAD and sporadic EOAD as far as risk is concerned, with both equally afflicted (OR 3.12 and 2.95, respectively).

The suggested gene dosage effect in both familial (Corder et al., 1993) and sporadic (Frisoni et al., 1995) AD, where with increasing copy number of the APOE e4 allele the
risk of AD increases from 20% (non-\(APOE\) e4) to 90% (homozygous \(APOE\) e4; Corder et al., 1993), is evident in this study with marked overrepresentation of homozygosity in AD cases (86.2% of homozygotes) and the marked increases in relative risk with increasing copies of the e4 allele (3.1 to 10.61; Table 3.1). Once again this is the case in both LOAD and the sporadic EOAD cases examined (OR 3.12 to 8.65 and 2.95 to 22.8, respectively). What is apparent is that there is a gene dosage effect of this allele not only on risk but on age of onset of AD. The AD associated risk of the \(APOE\) e4 allele has been reported to steadily increase with age, exerting its greatest effect on AD between the ages of 60-79 years and decreasing thereafter (Farrer et al., 1997; Lautenschlager et al., 1999). In a study of familial LOAD the mean age of onset for non-e4, heterozygous e4 and homozygous e4 individuals were 84.3 years, 75.5 years and 68.8 years, respectively (Corder et al., 1993). In this study homozygosity for the e4 allele is associated with an earlier age of onset, as reflected by both a higher frequency of e4 homozygotes in the EOAD group and by the difference in relative risk, associated with e4 homozygosity, between the EOAD (22.8) and LOAD (8.65) groups. Analysis of the relative risk in individuals over the age of 81 years (Table 3.6) supports the notion that the effect of \(APOE\) e4 containing genotypes on AD decreases after the age of 79 years (3.24 to 2.86) and is most evident in the e4 homozygous cases (15.94 to 8.00).

Whilst the association of the e4 allele of \(APOE\) with AD is now well established it is universally acknowledged that although possession of the e4 allele increases risk it is not causal, as is the case of the pathological mutations of APP or PS1. This is due to the observation that there are a number of e4 carriers who do not develop disease and an even greater number of non-e4 individuals that do develop disease. The general consensus is that whilst the e4 allele can be considered the major genetic risk factor for LOAD it is neither necessary nor sufficient for the development of the disease and that
other genetic factors must also be involved in the development of this form of AD. To this end the study of Taddei and co-workers (1997), wherein an association of increased plasma apoE levels with AD was mooted, can be seen as a catalyst for research into identifying the genetic factors within the promoter region of the \textit{APOE} gene that may regulate this effect and in turn may predispose carriers to AD.

\subsection{3.4.1.2 The APOE \textit{-}491A/T promoter polymorphism is an independent risk factor for AD}

Factors that regulate apoE expression, such as selected polymorphisms in the \textit{APOE} promoter, may contribute to an individual’s risk for AD. Four promoter polymorphisms have been identified and their association with AD risk has been investigated. Of these four polymorphisms, the \textit{-}491A/T polymorphism has been the most thoroughly investigated and shown to have the most robust effect on \textit{APOE} transcription. The association between the AA genotype at the \textit{-}491 position of the \textit{APOE} promoter and the risk for AD was first reported in two Caucasian populations, one from Spain and the other from the USA (Bullido et al., 1998). The authors found that homozygosity of the \textit{-}491A allele was associated with an increased risk of developing AD which was subsequently confirmed in a large French cohort (Lambert et al., 1998a). These findings imply that, in addition to the qualitative effects of the \textit{APOE} alleles, the quantitative variation of isoform expression due to polymorphisms in the proximal promoter may contribute to AD and may help explain why some individuals who carry two \textit{APOE} \textit{e4} alleles escape AD.

The results gathered from this large Caucasian population confirms these initial associations, with \textit{-}491A containing genotype being associated with an increased risk of AD (OR 4.09; Table 3.2) and the greatest effect being seen in \textit{-}491AA individuals (OR
5.00). These findings (Laws et al., 1999) have been verified in numerous studies (Town et al., 1998; Casadei et al., 1999; Alvarez-Arcaya et al., 2001; Bullido et al., 2000; Wang et al., 2000a; Lambert et al., 2002) including post-mortem studies (Ahmed et al., 1999; Laws et al., 2002b See Chapter 4). However, some studies have failed to find an association of the -491A/T promoter with LOAD in Caucasian (Song et al., 1998; Helisalmi et al., 1999; Thome et al., 1999; Zurutuza et al., 2000) and Asian populations (Chen et al., 1999; Toji et al., 1999). The clinical significance of one of these latter studies (Thome et al., 1999) is questionable as AD individuals were compared with psychiatric inpatients rather than cognitively healthy controls. In the two studies of Asian populations (Chen et al., 1999; Toji et al., 1999) the lack of association may be accounted for by the high frequency of the -491AA genotype in the respective control groups. This point is supported by a recent post-mortem study in the Japanese population where all 47 cases were positive for the AA genotype (Yamagata et al., 2001).

When LOAD and EOAD cases were studied (Table 3.2) the association of -491A containing genotypes and -491A homozygosity was observed only in LOAD individuals. However, this lack of association can be attributed to the small EOAD population available for study which yielded only a single -491TT individual. Thus, firm conclusions on risk in EOAD individuals can not be drawn, although analysis of allele frequencies suggest that the -491A allele is associated with both EOAD (OR 2.79) and LOAD (OR 2.02) but more statistically robust in the latter.

It has been suggested by a number of studies that the association of the -491A allele with AD is due to linkage disequilibrium between the -491 locus and the APOE ε4 allele. However, the -491A allele has been shown to be associated with both APOE ε4
carriers and non-carriers, suggesting that this genotype causes an independent risk for AD (Town et al., 1998). When the data from this chapter were stratified by the presence/absence of the ε4 allele (Table 3.3) and allele frequencies analysed the -491A allele was associated with an increased risk for AD in both ε4 carriers (OR 2.46) and non-carriers (OR 1.53). Subsequent studies reported that whilst the -491-AD association could be partly accounted for by linkage disequilibrium, the association of the -491AA genotype with AD remained in non-APOE ε4 individuals (Bullido et al., 2000; Wang et al., 2000a). Log-linear and logistic regression analysis has also concluded that -491AA genotype is likely to be an independent risk factor for AD, since no interaction was detected between APOE ε4 and -491AA for modifying risk for AD (Wang et al., 2000a). Overall, these findings suggest that the -491A allele is associated with AD; however, this association can be partly accounted for by linkage disequilibrium with APOE ε4.

A recent meta-analysis of the -491A/T and -219T/G promoter polymorphisms has reported that the risk associated with the -219TT/TG genotypes is greatest in individuals over the age of 81 years whilst the -491AA/AT genotypes have a greater effect in those under the age of 81 years (Lambert et al., 2002). The limitation of this analysis in this thesis is that the frequency of the -491TT genotype made it difficult to determine the level of risk conferred by -491A allele containing genotypes in this cohort of 625 individuals, as opposed to the meta analysis performed by Lambert and colleagues (2002) on 3,658 individuals from six independent samples. As such meaningful conclusions can only be drawn on statistical analysis performed on allele rather than genotype frequencies (Table 3.6). This analysis revealed that there was a similar level of association in both age groups (OR 1.97 compared to OR 2.63 in those 81 years or below and older than 81 years, respectively).
In summary, this study has confirmed that the -491AA genotype is associated with an increased risk of AD and that this association is present both in carriers and non-carriers of the ε4 allele of APOE suggests that the association is, to some extent, independent of ε4 status. This association, although strong, is not as robust an association as that seen by the ε4 allele of APOE, which remains the most significant risk factor for LOAD. Hypothetically, it is possible that the associated risk of this promoter polymorphism and that of the allelic forms of APOE may act in conjunction with one another, thus placing individuals carrying both the APOE ε4 and -491A alleles at an even higher risk for developing AD.

3.4.1.3 The APOE -219T/G promoter polymorphism is not an independent risk factor for AD

Although the -491A/T promoter polymorphism has been extensively studied, the remaining three polymorphisms have received less attention. The -219T/G polymorphism has been studied in two French Caucasian populations and has been reported to be associated with an increased risk for AD. This genotype has been proposed to act as a modifier of APOE ε4 risk (Lambert et al., 1998a,b). However, this effect has been sought but not found in Caucasian populations from the United States (Rebeck et al., 1999). This discrepancy has been suggested to be due to strong linkage disequilibrium that was observed between the -219T and APOE ε4 alleles (Rebeck et al., 1999).

Analysis of data from this study revealed that possession of a -291T allele containing genotype was not associated with an increased risk of AD and that when broken down into LOAD and EOAD cases the lack of association remained (Table 3.4). The only statistically significant association with AD that was observed was in individuals
homozygous for the -219T allele (OR 1.79, p < 0.05) and when broken down into LOAD and EOAD cases it remained statistically significant in LOAD cases only (OR 1.90, p < 0.05). The same pattern of association was observed when allele frequency was analysed. These findings suggested that the -219T allele is only a minor risk factor for AD and that this association is associated with homozygosity of the allele in individuals with a later age of onset. This association, however, has been suggested to be due to strong linkage with the ε4 allele of APOE (Rebeck et al., 1999). To determine if this was the case the -219T allele was investigated in ε4 carriers and non-carriers (Table 3.5). As was the case with all AD cases, -219T containing genotypes were not associated with AD in carriers or non-carriers. The association of homozygous individuals with increased AD risk, seen previously, was now no longer evident as was the case when allele frequencies were analysed in carriers and non-carriers.

A recent meta-analysis by Lambert and colleagues (2002) has suggested that the association of the -219T allele with AD increased in individuals over the age of 81 years. When this cohort was stratified into the two distinct age groups (Table 3.6) analysis seems to support the conclusion of Lambert and colleagues (2002) as homozygosity of the -219T allele was associated with AD only in individuals aged over 81 years. This was also the case when allele frequencies were analysed. To this extent the lack of association in EOAD individuals in this cohort lends support to this conclusion. However, when ε4 status was taken into account homozygosity was no longer significantly associated with AD, in either ε4 carriers or non-carriers, in the over 81 year old group. The analysis of allele frequencies revealed that the association of the -219T allele remained significant in non-ε4 carriers but not in ε4 carriers. However, the low frequency of control ε4 individuals over 81 years makes analysis of the effect of both homozygosity and allele frequencies difficult and prevents any firm conclusions
being drawn. Overall, these findings lend some credence to the work of Lambert and colleagues (2002) that, in individuals over 81 years, the -219T allele does exert some independent effect on increasing risk for AD, although this is probably only minor and the majority of any association reported in the literature is due more so with linkage to the ε4 allele rather than the -219T allele itself. More studies need to be undertaken to clarify the impact that this promoter polymorphism has on AD risk.

3.4.2 Plasma ApoE Levels in AD

3.4.2.1 Plasma apoE levels are elevated in AD with negligible impact from APOE genotype

The study of Taddei and co-workers (1997) was the first study to suggest that increased plasma apoE levels were associated with AD and that this association was independent of APOE ε4 status. A subsequent study did not find this association and reported that levels of apoE were marginally decreased in serum of AD patients (Slooter et al., 1998). Only one other study has reported on plasma apoE levels in AD (Scacchi et al., 1999), where the authors concluded that there was no statistically significant difference in levels between controls and AD cases. The findings in this chapter confirms the initial study of Taddei and colleagues (1997) with plasma apoE levels being found to be significantly increased in AD individuals compared to controls (Figure 3.1a). This significant increase was consistent in both LOAD and EOAD individuals (Figure 3.1b) and when APOE genotype was taken into account remained significantly increased in AD individuals regardless of the presence or absence of the ε4 allele (Figure 3.2). It should be noted that there is considerable overlap between the AD and control data which makes the use of this data problematic for the diagnosis of AD, if it is taken as a sole diagnostic marker. Interestingly one study has suggested that apoB levels are increased in the serum of AD patients (Caramelli et al., 1999). This finding suggests
that apoE may not be the only factor in lipid metabolism to play a role in AD
disease. However, apoB is not found in the brain and as such the biological
relevance of this finding to AD needs further investigation (though an indirect effect via
the periphery cannot be ruled out) as does the biological relevance of other
apolipoproteins, in particular those that are expressed in brain tissue, such as apoD.

It has been widely reported that the \textit{APOE} genotype plays an important role in
determining the levels of apoE in plasma and/or serum in studies of normal (Smit et al.,
1988), AD (Slooter et al., 1998; Scacchi et al., 1999) and coronary heart disease (Corbo
et al., 1999; Corbo et al., 2001). The general consensus of these studies is that the \textit{APOE}
e2 allele is associated with elevated levels of apoE whilst the e4 allele is associated with
the lowest levels and the e3 allele somewhere in between. In the case of studies in AD
populations (Slooter et al., 1998; Scacchi et al., 1999) the authors suggest this as the
reason as to why levels of apoE were reported to be decreased in the serum (Slooter et
al., 1998) and plasma (Scacchi et al., 1999) of AD cases. However, in this study we
report that plasma apoE levels were significantly elevated in AD cases in both carriers
and non-carriers of the e4 allele (Figure 3.2). To determine if apoE levels were elevated
in e4 carriers the data was stratified firstly by \textit{APOE} genotype only (Figure 3.3a) and
then by \textit{APOE} genotype and disease status (Figure 3.3b, c). This analysis revealed a
marginally significant increase in plasma apoE levels in e4 heterozygotes compared to
non-e4 individuals, no difference was observed between e4 homozygotes and non-e4
individuals or e4 heterozygotes. When further stratified by disease status, e4
heterozygotes still had a marginally significant increase in plasma apoE levels in control
individuals only. Interestingly it is in AD individuals that we see the only evidence of
support for the published literature with e4 homozygotes tending to have the lowest
levels of plasma apoE. However, this was not statistically significant when compared to
non-e4 and heterozygote e4 individuals and a comparison between Figures 3.3b and
3.3c show that across all categories of ε4 carriage plasma apoE levels are increased in AD individuals. These findings suggest that APOE genotype has negligible effect on the actual levels of the protein in plasma and that other factors must be at play in modifying the levels reported in this study in AD.

3.4.2.2 The -491A/T but not the -219T/G promoter polymorphism alters plasma apoE levels

The APOE -491A/T and -219T/G promoter polymorphism are proposed to affect the transcriptional activity of the APOE gene in both the periphery and the CNS. This hypothesis is supported by findings that the A to T and the T to G substitutions, at -491 and -219, respectively, resulted in alterations in APOE promoter activity, in HepG2 cells (Artiga et al., 1998b) and U87 astrocytoma cells (Artiga et al., 1998a). These APOE promoter polymorphisms have also recently been identified to co-exist with transcription factor recognition sites (See Chapter 7, Figure 7.1), as predicted by the mapping of the APOE promoter with the TFSITES database (EMBL; Bullido and Valdivieso, 2000). Thus, it is suggested that these polymorphisms may be functional and in turn may impart any suggested susceptibility for AD via regulation of apoE levels. However, very few in vivo studies have been reported.

This thesis presents the first published data (Laws et al., 1999) from an in vivo study undertaken to investigate the impact of the -491A/T promoter polymorphism on levels of apoE, and in the case of this chapter, in plasma. Irrespective of disease status individuals with the -491AA genotype had significantly elevated plasma apoE levels when compared to -491AT and TT genotypes (Figure 3.4a) and this was constant when stratified for the presence (Figure 3.4b) and/or absence (Figure 3.4c) of the APOE ε4 allele, however, no gene dose dependent effect was observed. The same pattern of
association was observed when -491 genotype was analysed in control and AD groups (Figure 3.5), although AD individuals with the -491AA genotype had significantly elevated levels of plasma apoE when compared to control individuals with the corresponding genotype. These findings are consistent with the changes observed in transfection studies in both HepG2 and astrocytoma cells (Artiga et al., 1998a,b) and has been confirmed in an AD study in the Italian population (Scacchi et al., 2001). However, the -491AA-associated elevation in plasma apoE levels was restricted to non-ε4 individuals. In a study of the role of APOE polymorphisms in coronary heart disease Corbo and colleagues (2001) reported that whilst there was no significant difference in plasma apoE levels between the -491 genotypes there was a trend towards increased levels in -491AA individuals. Whilst, a study of the role of the -491A/T polymorphism in myocardial infarction reported no difference in levels of plasma apoE in the control population when stratified by -491 genotype (Lambert et al., 2000). In addition to this association with elevated apoE levels in plasma, the -491AA genotype has been independently associated with increased Aβ40, Aβ42 and total Aβ load in AD brains (Lambert et al., 2001). This observation is supported by previous studies that indicate that apoE is required for cerebral amyloidosis in amyloid plaque-forming transgenic mice (Bales et al., 1997; Bales et al., 1999; Holtzman et al 2000a; Holtzman et al., 2000b). This increased expression of the APOE gene in those bearing the AA genotype, reported in this study, may help to explain why a large number of AD individuals who do not posses the ε4 allele of APOE still develop AD and in turn why some ε4 carriers escape this disease.

The second APOE promoter polymorphism studied in this chapter was the -219T/G promoter polymorphism. This polymorphism when transfected into HepG2 cells (Artiga et al., 1998b) and U87 astrocytoma cells (Artiga et al., 1998a) was associated with
decreased apoE expression as opposed to the -491A allele which has been implicated in elevating apoE expression. In turn, in the control group of the myocardial infarction study of Lambert and colleagues (2000) the -219T allele was associated with decreased levels of plasma apoE and this effect of the -219T allele was dose dependent (Lambert et al., 2000). These findings coupled with the suggested association of the -219T allele with increased risk for AD, although observed to be marginal in this thesis, brings with it an interesting conundrum. The -491A allele is associated with increased risk for AD and increased apoE levels in plasma, whilst the -219T allele is associated with increased AD risk but decreased apoE levels. This suggests that over- and under-expression of apoE may be important in AD risk.

In this thesis, however, the findings of Artiga and colleagues (1998a, b) and Lambert and colleagues (2000) could not be replicated as no difference was observed in plasma apoE levels between each of the three -219T/G genotypes. In fact plasma apoE levels were found to actually be increased marginally, yet to a statistically significant degree, in individuals homozygous for the -219T allele when compared to -219G homozygotes irrespective of disease status (Figure 3.6a). However, this increase disappeared when the presence (Figure 3.6b) and absence (Figure 3.6c) of the APOE e4 allele was taken into account and no difference was observed when stratified by disease status. A possible explanation for the lack of replication of the published findings may be due to the lack of association of the -219T allele with increased AD risk in this cohort. The findings from this thesis suggest that the -219T allele has a minor association with AD in those over 81 years but has very little, if any, effect on apoE expression. This thesis also suggests that the effect of the -491A/T promoter polymorphism on apoE levels is far greater than that of the -219T/G promoter polymorphism (Figure 3.7). Further studies into the role of both promoter polymorphisms are required to address the current
conjecture in the literature and to elucidate the role of these polymorphisms and the level of apoE expression in AD. In addition the levels of apoE in plasma cannot be directly compared to intracerebral levels, as these represent two distinct pools which are separated by the blood brain barrier.

3.4.3 The Role of the Glu318Gly Mutation in Familial AD (FAD)

Recently, the PS1 mutation Glu318Gly has been reported to be a risk factor for AD (Taddei et al., 1998; Sandbrink et al., 1996; Reznik-Wolf et al., 1998b; Helisalmi et al., 2000; Forsell et al., 1997; Cruts et al., 1998). However, a number of groups have reported this variant as either a rare polymorphism (Mattila et al., 1998; Aldudo et al., 1998; Dermaut et al., 1999a), a neutral mutation (Dermaut et al., 1999b), or a mutation with incomplete penetrance (Dermaut et al., 1999b) leading to AD only in some cases, possibly in combination with other genetic and environmental factors. A number of recent findings have led some researchers to suggest a lack of pathogenicity of the Glu318Gly mutation in AD (Mattila et al., 1998; Aldudo et al., 1998; Dermaut et al., 1999a, b). Cruts and Van Broeckhoven (1998) noted that the Glu318 residue is located in the middle part of the hydrophilic loop VI, a region of the PS1 protein that is weakly conserved in human PS2, or in the presenilin-homologues Drosophila PS1 and Caenorhabditis elegans SEL-12. Location of the Glu318Gly mutation in this site would be compatible with either the absence of pathogenic effects (polymorphism) or weak pathogenic effects (reduced penetrance; Mattila et al., 1998).

To assess the frequency of the Glu318Gly mutation and to evaluate its contribution to AD in the Australian population, sporadic EOAD and LOAD cases, FAD cases and control subjects were screened. None of the 43 sporadic EOAD patients screened were
found to have this mutation. The frequency of the Glu318Gly mutation was highest in the FAD group (8.7%), compared with 3.1% in the sporadic LOAD group and was lowest in control subjects (2.2%). When the mutation frequencies were compared by the Pearson $\chi^2$ method, no significant difference between the sporadic LOAD group and control subjects ($P = 0.513$). This result for the Australian population is consistent with those of recent reports for other populations. For example Mattila and colleagues (1998) found no significant difference in the frequency of the Glu318Gly mutation between FAD patients and control subjects. However, our results showed a significant difference ($P < 0.05$) when we compared the FAD group with age-matched control subjects. This is consistent with Helisalmi and colleagues (2000) who showed that this variant was significantly increased in both familial and SAD cases. It is interesting to note that Mattila and colleagues (1998) and Helisalmi and colleagues (2000) report conflicting results for this variant in AD cases in the same Finnish population.

Upon comparing the frequency of Glu318Gly mutation in control subjects across the various ethnic populations, we found the Australian population to have the lowest frequency (2.2%; 9 carriers/402 individuals). The frequency in the control Dutch population is 4.1% (9 carriers/219 individuals; Dermaut et al., 1999a,b), in the Finnish population, 6.8% (4 carriers/59 individuals; Mattila et al., 1998) and 4.0% (10 carriers/270 individuals; Helisalmi et al., 2000), and in the Spanish population, 4.5% (4 carriers/89 individuals; Aldudo et al., 1998). The fact that the frequency of this mutation in controls is twice as high in these other populations, may be a contributing factor for the lack of association between the Glu318Gly mutation and AD in these populations. Therefore, analogous to the major genetic risk factor, the $APOE \varepsilon 4$ allele, the association between the Glu318Gly mutation and FAD may vary among different ethnic groups.
The pathogenicity of the Glu318Gly mutation has been questioned because to date, it has not been possible to show co-segregation of this mutation with AD due to the lack of sufficient family members in all reported cases. For example, the Glu318Gly mutation was first reported in a German early-onset AD patient with an age of onset of approximately 47 years and without a known family history (Sandbrink et al., 1996). This same mutation was then detected in 2 AD patients belonging to a Swedish early-onset family (Forsell et al., 1997) with onset ages of 60 and 68 years and later in a Dutch FAD patient with an age of onset of 57 years (Cruts et al., 1998). Three more patients with this mutation, from three different families, have been reported: one with an age of onset of 55 years but without a known family history (Reznik-Wolf et al., 1998b), a second with a similar age of onset (Reznik-Wolf et al., 1998b), and the third with an age of onset in the late 50’s (Taddei et al., 1998). The latter two patients had a family history of AD.

Another confounding factor has been the variable age of AD onset associated with this mutation, ranging from 35-64 years (Cruts and Van Broeckhoven, 1998). The major argument in support of the Glu318Gly mutation being non-pathogenic stems from the observation that numerous non-demented control individuals with this mutation have been identified among various ethnic populations, which have similar frequencies to their corresponding AD populations. However, we have shown that the Glu318Gly frequency is increased in the FAD group in the Australian population indicating that at least in this population this mutation may play a role in the pathogenesis of AD. Further genetic studies on other populations need to be undertaken to identify those populations that may be vulnerable to this mutation.
Functional studies have further questioned the pathogenicity of the Glu318Gly mutation. Dermaut and colleagues (1999a, b) found no difference in the level of Aβ42 in conditioned media of HEK-293 cells stably transfected with the Glu318Gly mutation compared to stably transfected cells with wild-type PS1. Unfortunately, the primary data were not displayed in the publication by Dermaut and colleagues (1999a, b) and there was no indication whether the level of Aβ42 had been compared with Aβ42 levels from HEK-293 cells stably transfected with other known PS1 pathogenic mutations. Furthermore, transfected cell lines may not be the best method for assessing whether Aβ42 levels are increased in response to the Glu318Gly mutation, as it has been shown that other PS1 mutations which are associated with varying degrees of clinical severity as determined by their ages of onset, do not exhibit corresponding differences in Aβ42 production in transfected cell lines (Citron et al 1997; Murayama et al., 1999). Further studies with skin fibroblasts from individuals with the Glu318Gly mutation need to be undertaken to determine whether this mutation results in increased Aβ42 levels as has been shown for other PS1 mutations (Martins et al., 1995b).

3.4.4 Summary

This chapter has confirmed and extended the association of the APOE ε4 allele with AD and its gene dosage effect on increasing this risk and decreasing the age of onset. In addition, the significant association of the −491AA promoter polymorphism in AD individuals and its resulting increased level of expression of the APOE gene suggest that this polymorphism plays an important role in the development of AD, both in the presence and absence of the ε4 allele of APOE. Conversely, the −219T/G promoter polymorphism confers a negligible increase in risk, only observed in those over 81 years, and has little if any impact on apoE levels.
Additionally, the PS1 mutation, Glu318Gly, was found in a high frequency in FAD individuals which was significantly increased when compared to control subjects. No interaction with the ε4 allele of APOE was observed. These findings suggest that the Glu318Gly mutation may be conceptualised as playing a role in AD analogous to that played by APOE ε4: i.e. by acting as a genetic risk factor for FAD. However, there is evidence that the association between the Glu318Gly mutation and FAD varies among different ethnic groups.
4.1 INTRODUCTION

Apolipoprotein E is one of a large family of apolipoproteins that are associated with lipoprotein complexes and are involved in lipid transport in various cell types and tissues. In humans, three major allelic variations in the \textit{APOE} gene (\(\varepsilon2\), \(\varepsilon3\) and \(\varepsilon4\)) exist, which encode three protein isoforms that differ in their biological properties (Zannis et al., 1981). Substantial evidence implicates \textit{APOE} polymorphisms in modulating the risk for AD. The previous chapter illustrated that the \(\varepsilon4\) allele is significantly over-represented in AD and is supported comprehensively in the literature (Corder et al., 1993; Poirier et al., 1993; Rebeck et al., 1993; Strittmatter et al., 1993; Martins et al., 1995a), where it appears to increase risk for AD in a gene dose-dependent manner (Corder et al., 1993). In addition to the influence of \textit{APOE} genotype recent evidence, presented both in the literature and the previous chapter, indicates that regulation of \textit{APOE} expression may also contribute to the pathogenesis of AD. Initial clues to the existence of this relationship were based on observations that plasma apoE levels (Taddei et al., 1997) and apoE mRNA levels (Yamada et al., 1995) were elevated in individuals suffering from AD and subsequently corroborated in the preceding chapter. Additionally, apoE immunoreactivity has been localized not only to the senile plaques, but also to vascular amyloid and the neurofibrillary tangles of AD (Harr et al., 1996; Poirier, 2000; Tomiyama et al., 1999). The foregoing chapter and subsequent studies have shown an association of \textit{APOE} promoter polymorphisms with increased risk of developing AD (Artiga et al., 1998b; Lambert et al., 1998a; Lambert et al., 1998b; Bullido et al., 1998; Laws et al., 1999). As illustrated in the previous chapter,
increased levels of plasma apoE are associated with the AA genotype of the -491A/T promoter polymorphism, which has been subsequently supported in the literature (Scacchi et al., 2001). However, levels of apoE in plasma cannot be directly extrapolated to intracerebral levels, as these represent two distinct pools which are separated by the blood brain barrier. Since AD is a brain disorder, it is important to determine whether apoE levels in the brain reflect the elevation observed in plasma and whether promoter polymorphisms play a role in modulating brain apoE. This chapter will endeavour to address these questions.

Another apolipoprotein that is synthesised in the brain and which has been previously associated with AD is apolipoprotein D (apoD), although its exact role is unclear. Whilst apoD is a component of high-density lipoprotein complexes, it is a member of the lipocalin superfamily of lipid transport proteins. Increased apoD mRNA and/or protein expression has been detected in the CSF, hippocampus, and selected cortical cell populations of AD subjects (Belloir et al., 2001; Kalman et al., 2000; Terrisse et al., 1998) and in the brains of an Alzheimer’s transgenic mouse model (PDAPP transgenic mice; Thomas et al., 2001b). However, unlike other apolipoproteins, such as apoE, apoA-1, and apoJ, the cortical senile plaques and neurofibrillary tangles observed in AD are immunonegative for apoD (Belloir et al., 2001; Calero et al., 2000; Harr et al., 1996; Kalman et al., 2000; Namba et al., 1991). It has recently been demonstrated that apoD protein level are elevated in prefrontal cortex and other select regions from subjects with other neurological disorders (Thomas et al., 2001a; Thomas et al., 2002). Since the AD brain clearly exhibits altered apoE levels it is important to determine whether apoD levels are also altered in the prefrontal cortex of subjects with AD and whether there is a relationship between apoD levels and either APOE genotype and/or apoE levels.
CHAPTER FOUR: RISK FACTORS IN AUTOPSY CONFIRMED AD

4.1.1 Aims

- To determine frequency of APOE coding and promoter polymorphism genotypes in AD brain
- Determine the association of these promoter polymorphism with levels of apoE protein in the frontal cortex of AD and control cases
- Determine levels of apoD protein in the frontal cortex of AD and control cases
- Determine relationship between apoD and apoE levels and/or APOE genotype

4.2 MATERIALS AND METHODS

Sections of prefrontal cortex (Brodmann area 9) from brain samples, described in Section 2.1.3, were used for studies of APOE and APOE promoter polymorphism genotypes and apoE and apoD protein levels. Briefly these samples included 20 neuropathologically confirmed (ADRDA criteria) AD cases and 40 age, sex and PMI matched neuropathologically assessed control brains from individuals with no known brain disease.

Brain samples underwent sample preparation as described in Section 2.2.5.1 for use in apoE and apoD quantitation. ApoE levels in the brain tissue were determined using the western blotting technique outlined in Section 2.2.5.2. ApoD protein quantification was undertaken using the Sandwich ELISA techniques described in Section 2.2.5.3. All genotypes were determined on DNA extracted from brain tissue using the same protocol as described in Section 2.2.2. APOE genotype was determined as per Section 2.2.4.1, APOE -491 A/T promoter polymorphism genotypes were determined as described in Section 2.2.4.2, and the APOE -219G/T promoter polymorphism genotypes were
determined as described in Section 2.2.4.3, essentially as described by Lambert and colleagues (1998b).

4.3 RESULTS

4.3.1 Genetic Profile of Brain Specimens: Confirmation of APOE ε4 and -491A AD Association

Genotypic distribution of both APOE, -491A/T and -219T/G genotypes were consistent with the distribution predicted by the Hardy-Weinberg equilibrium (APOE: $\chi^2$ test; $p = 0.9$; -491: $\chi^2$ test; $p = 0.31$; -219: $\chi^2$ test; $p = 0.95$). As expected, Pearson's $\chi^2$ analysis of the frequency of APOE ε4 containing genotypes and ε4 allele frequencies revealed a strong association with AD ($p < 0.01$; OR 4.90, 95% CI: 1.55 – 15.49 and $p < 0.01$; OR 3.40, 95% CI: 1.40 – 8.25, respectively; Table 4.1). Homozygosity of the ε4 allele indicated a marked increase in relative risk (OR 8.29, 95% CI: 0.65 – 104.9) however this failed to reach significance (Fisher's exact test $p = 0.13$).

No significant association of -491A containing genotypes or homozygosity of the -491A allele with AD was observed after statistical analysis via Fisher's exact test (Table 4.2). Additionally, analysis of the -491A allele frequencies also failed to reveal an association with AD (Fisher's exact test $p = 0.055$) although odds ratio analysis suggested otherwise (OR 3.20, 95% CI: 1.02 – 10.08). When tested via the Pearson's $\chi^2$ method it did reach significance ($\chi^2 = 4.269$, $p = 0.0388$). It was observed that all ε4 homozygotes were also homozygote for the -491A allele. No significant association was observed when the -219T/G promoter polymorphism was investigated (Table 4.3).
Table 4.1: APOE genotype and allele frequency in controls and autopsy confirmed AD cases.

APOE ε4 genotypes and allele are highlighted in grey. Values in italics are the relative percentages of each genotype or allele within each group (a). Frequencies were used to calculate significance and odds ratios in (b).

εₙ represents a non-APOE ε4 allele

1 Comparison of the frequency of ε4 containing genotypes (i.e. ε4/ε4 and εₙ/ε4) versus non-ε4 containing genotypes (i.e. εₙ/εₙ)

2 Comparison of the frequency of individuals homozygous for ε4 versus non-ε4 (i.e. εₙ/εₙ) genotypes

3 Comparison of the frequency of the ε4 allele (ascertained by calculating allele frequencies) versus the non-ε4 alleles (i.e. εₙ)

f Fisher’s exact test used as one or more cells was ≤ 5
Table 4.2: *APOE* -491A/T Promoter Polymorphism Genotype and Allele Frequencies in Controls and Autopsy Confirmed AD Cases.

Values in italics are the relative percentages of each genotype or allele within each group (a). Frequencies here were used to calculate significance and odds ratios represented (b).

1. Comparison of the frequency of the -491A containing genotypes versus the -491TT genotype (i.e. -491AA/AT versus -491TT)

2. Comparison of the frequency of the -491AA genotypes versus the -491TT genotype

3. Comparison of the frequency of the -491A allele (ascertained by calculating allele frequencies) versus the -491T allele

n/a - Absence of -491TT genotype in AD group prevented calculation of Odds Ratio’s

\[ \chi^2 = 4.269, p = 0.0388 \]
### Table 4.3: APOE -219T/G (Th1/E47cs) promoter polymorphism genotype and allele frequencies in controls and autopsy confirmed AD cases.

(a) Values in italics are the relative percentages of each genotype or allele within each group. Frequencies here were used to calculate significance and odds ratios (b).

1 Comparison of the frequency of the -219T containing genotypes versus the -219GG genotype (i.e. -219 versus -219GG)

2 Comparison of the frequency of the -219TT genotypes versus the -219GG genotype

3 Comparison of the frequency of the -219T allele (ascertained by calculating allele frequencies) versus the -219G allele

\(^{1}\) Fisher’s exact test used as one or more cells was ≤ 5
4.3.2 Apolipoprotein Levels

4.3.2.1 Apolipoprotein E levels: Increased in the frontal cortex of AD cases

Levels of apoE in the frontal cortex of AD cases were significantly increased over that of control cases (Student’s t-test, t = 6.796, p < 0.0001) in AD (368.4 ± 24.4 ng apoE/mg TP) when compared to control individuals (231.6 ± 7.5 ng apoE/mg TP; Figure 4.1a). This was consistent in both APOE e4 carriers (AD 368.4 ± 30.6 ng apoE/mg TP vs. controls 219.9 ± 14.7 ng apoE/mg TP; Student’s t-test, t = 4.123, p < 0.001; Figure 4.1b) and non-carriers (AD 368.6 ± 43.5 ng apoE/mg TP vs. controls 236.1 ± 8.7 ng apoE/mg TP; Student’s t-test, t = 4.882, p < 0.001; Figure 4.1c). When onset of AD was investigated (Figure 4.2) it revealed that both LOAD (408.2 ± 33.3 ng apoE/mg TP) and EOAD (308.8 ± 23.9 ng apoE/mg TP) had significantly higher levels than controls after applying Bonferroni correction for multiple comparisons (p < 0.001 and p < 0.05, respectively). LOAD individuals had significantly higher levels of apoE when compared to EOAD individuals (p < 0.01).

Figure 4.1 (adjacent): Apolipoprotein E levels in frontal cortex tissue from control and autopsy confirmed AD cases (Stratified by APOE e4).

Apolipoprotein E levels were determined to be elevated in AD individuals when compared to controls (A). This was consistent when stratified by the presence (B) and absence (C) of the APOE e4 allele.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

(A) Control vs. AD individuals: * Student’s t-test t = 6.796, p < 0.0001

(B) Control vs. AD individuals: ** Student’s t-test t = 4.123, p < 0.001

(C) Control vs. AD individuals: *** Student’s t-test t = 4.882, p < 0.001
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**A**

**apoE Level (ng/mg Total Protein)**

- **Ctrl** (n = 40)
- **AD** (n = 20)

**B**

**DISEASE STATUS**

- **Ctrl** (n = 11)
- **AD** (n = 13)

**C**

- **Ctrl** (n = 29)
- **AD** (n = 7)
Figure 4.2: Apolipoprotein E levels in control and autopsy confirmed AD cases (Stratified by age of onset).

Apolipoprotein E levels were significantly elevated in the frontal cortex of both LOAD and EOAD cases when compared to controls. This was seen to a greater extent in LOAD cases, significantly more so than in EOAD cases.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

Control vs. LOAD vs. EOAD: * p < 0.001, ** p < 0.05, *** p < 0.01

Bonferroni correction for multiple comparisons was applied.
When all cases were combined and stratified by the gene dose of the \textit{APOE} e4 allele (Figure 4.3a) no significant difference was observed. However, a trend towards increased apoE levels with increasing \textit{APOE} e4 copy number was observed ($e_n/e_n$: 261.9 ± 13.8 ng apoE/mg TP, $e_n/e4$: 288.2 ± 22.3 ng apoE/mg TP, $e4/e4$: 384.7 ± 107.2 ng apoE/mg TP). When AD and control cases were stratified by $\varepsilon$4 gene dose (Figure 4.3b and c) neither a significant difference nor a trend towards one was observed. Cases homozygous for \textit{APOE} $\varepsilon$4 were removed from analysis to allow for multiple comparisons. AD individuals were observed to have significantly elevated ($p < 0.001$) apoE levels regardless of being non-$e$4 (368.6 ± 115.2 ng apoE/mg TP) or heterozygote $e$4 (352.6 ± 94.5 ng apoE/mg TP) when compared to control cases (control n/n: 236.1 ± 8.7 ng apoE/mg TP, control n/e4 217.5 ± 16.1 ng apoE/mg TP).

4.3.2.2 Apolipoprotein E Levels: Evidence of gene dose dependent effect of the -491A and -219G alleles

In all cases, there was a trend towards a gene dosage effect of the -491A allele on increasing apoE levels (-491AA: 295.7 ± 14.9 ng apoE/mg TP, -491AT: 260.9 ± 24.8 ng apoE/mg TP, -491TT: 166.4 ± 9.1 ng apoE/mg TP; Figure 4.4a) though after correcting for multiple comparisons only -491AA and -491TT cases were significantly different ($p < 0.05$). Likewise, in control cases a similar trend was evident (-491AA: 250.7 ± 8.6 ng apoE/mg TP, -491AT: 218.0 ± 11.7 ng apoE/mg TP, -491TT: 166.4 ± 9.1 ng apoE/mg TP; Figure 4.4b). After applying Bonferroni correction, significance was only seen between -491AA and -491TT cases ($p < 0.005$), whilst significance between -491AA and -491AT ($p = 0.07$) and between -491AT and -491TT ($p = 0.09$) cases was not observed. In AD cases, no trend was apparent although AD cases had significantly elevated levels when compared to controls with analogous genotypes (-491AA: 360.5 ± 27.2 ng apoE/mg TP; $p < 0.001$, -491AT: 400.2 ± 60.4 ng apoE/mg TP; $p < 0.001$; Figure 4.4c)
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**APOE LEVEL**

**(n = 36)**

**APOE STATUS**

- **ε4/ε4**
- **ε4/ε4**
- **ε4/ε4**

**A**

**APOE STATUS**

- **ε4/ε4**
- **ε4/ε4**
- **ε4/ε4**

**B**

**APOE STATUS**

- **ε4/ε4**
- **ε4/ε4**
- **ε4/ε4**

**C**

**APOE STATUS**

- **ε4/ε4**
- **ε4/ε4**
- **ε4/ε4**

**Legend:**

- **Max**
- **Min**
- **Mean**
- **Mean+SEM**
Figure 4.3 (adjacent): Effect of APOE genotype on apolipoprotein E levels in frontal cortex tissue from control and autopsy confirmed AD cases.

No difference in Apolipoprotein E levels was observed between different gene doses of the APOE ε4 allele in combined (A), control (B) and AD (C) cases. AD cases had significantly elevated apoE levels compared to control cases with the same ε4 gene dose (ε4/ε4 cases were excluded in multiple comparisons due to n = 1).

Where appropriate Bonferroni correction for multiple comparisons was applied.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

ε_n = non-APOE ε4 allele (i.e. ε2 or ε3)

AD ε_n/ε_n vs. Control ε_n/ε_n: * p < 0.05

AD ε_n/ε4 vs. Control ε_n/ε4: * p < 0.05
Figure 4.4 (adjacent): Effect of -491A/T genotype on apolipoprotein E levels in frontal cortex tissue from control and autopsy confirmed AD cases.

A trend towards a gene dose effect of the -491A allele associated with elevated apoE levels was observed when all cases were combined (A). Likewise, this trend was observed when control cases were analysed (B). However, no trend was observed in AD cases (C). AD cases had significantly elevated apoE levels compared to control cases with the same -491A gene dose.

Where appropriate Bonferroni correction for multiple comparisons was applied.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

(A) All cases: AA vs. AT vs. TT: * p < 0.05

(B) Control Cases: AA vs. AT vs. TT: ** p < 0.005

AD (AA vs. AT vs. TT) vs. Ctrl (AA vs. AT vs. TT): *** p < 0.001
When all cases were combined, there was no significant difference observed between -219T/G genotypes however -219GG cases tended to have the highest level of apoE in the frontal cortex (-219GG: 312.1 ± 34.7 ng apoE/mg TP, -219GT: 262.4 ± 14.0 ng apoE/mg TP, -219TT: 265.6 ± 15.9 ng apoE/mg TP; Figure 4.5a). Likewise, in control cases neither a significant differences nor a trend towards one was evident (-219GG: 229.7 ± 13.0 ng apoE/mg TP, -219GT: 227.4 ± 11.7 ng apoE/mg TP, -219TT: 245.2 ± 13.9 ng apoE/mg TP; Figure 4.5b). However, in AD cases (Figure 4.5c) a trend towards increasing apoE levels was evident with increasing dose of the -219G allele. Cases with the -219GG genotype (509.8 ± 37.9 ng apoE/mg TP) had significantly elevated apoE levels compared to -219GT (340.3 ± 19.8 ng apoE/mg TP; p < 0.005) and -219TT (292.9 ± 30.3 ng apoE/mg TP; p < 0.001) cases. Additionally, AD cases had significantly increased apoE levels when compared to control cases with corresponding genotypes (p < 0.001 for all genotypes).

Figure 4.5 (adjacent): Effect of -219T/G genotype on apolipoprotein E levels in frontal cortex tissue from control and autopsy confirmed AD cases.

No significant difference in apoE levels was observed between -219 T/G genotypes in combined cases (A). Likewise, no significant difference was observed when control cases were analysed (B). A trend towards a gene dose effect of the -219G allele associated with elevated apoE levels was observed in AD cases (C). AD cases had significantly elevated apoE levels compared to control cases with the same -219G gene dose. Where appropriate Bonferroni correction for multiple comparisons was applied.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

(C) AD Cases: GG vs. GT vs. TT: * p < 0.005, ** p < 0.001
AD (GG vs. GT vs. TT) vs. Ctrl (GG vs. GT vs. TT): *** p < 0.001
CHAPTER FOUR: RISK FACTORS IN AUTOPSY CONFIRMED AD

** apoE Level (ng/mg Total Protein) **

(A) ** -219T/G STATUS 
- GG (n = 17) 
- GT (n = 29) 
- TT (n = 14) 

(B) ** -219T/G STATUS 
- GG (n = 5) 
- GT (n = 9) 
- TT (n = 6) 

(C) ** -219T/G STATUS 
- GG (n = 12) 
- GT (n = 20) 
- TT (n = 8) 


![Box plots showing apoE levels for different genotypes and statuses.](image-url)
4.3.2.3 Apolipoprotein D levels are increased in the frontal cortex of AD cases:

No relationship with APOE genotype or levels

Apolipoprotein D levels in the frontal cortex of AD cases were significantly increased compared to control cases (AD: 0.218 ± 0.029 µg apoD/mg TP, Controls: 0.117 ± 0.011 µg apoD/mg TP; Student’s t-test, t = 3.89, p < 0.0005; Figure 4.6a). When AD and control cases were stratified by the presence and absence of the APOE e4 allele this observed elevation in apoD levels was consistent in the presence of the e4 allele (0.245 ± 0.039 µg apoD/mg TP vs. 0.098 ± 0.022 µg apoD/mg TP; Student’s t-test, t = 3.254, p < 0.005; Figure 4.6b). In the absence of the e4 allele AD cases tended to have higher levels of apoD but this failed to reach significance (0.166 ± 0.040 µg apoD/mg TP vs. 0.125 ± 0.012 µg apoD/mg TP; Student’s t-test, t = 1.341, p = 0.189; Figure 4.6c). When onset of AD was investigated (Figure 4.7) there was no significant difference in apoD levels between LOAD (0.195 ± 0.032 µg apoD/mg TP) and EOAD cases (0.252 ± 0.058 µg apoD/mg TP) though both were significantly elevated compared to control cases (LOAD: p < 0.05, EOAD p < 0.005), after correcting for multiple comparisons.

Figure 4.6 (adjacent): Apolipoprotein D levels in frontal cortex tissue from control and autopsy confirmed AD cases (Stratified by APOE e4).

Apolipoprotein D levels were elevated in AD individuals when compared to controls (A). This was consistent when stratified by the presence of the APOE e4 allele (B). Whilst a non-significant increase was observed in AD cases compared to controls in the absence of the APOE e4 allele (C).

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

(A) Control vs. AD individuals: * Student’s t-test t = 6.796, p < 0.0001

(B) Control vs. AD individuals: ** Student’s t-test t = 4.123, p < 0.001
CHAPTER FOUR: RISK FACTORS IN AUTOPSY CONFIRMED AD

 apoD Level (μg/mg Total Protein)

0.05  0.15  0.25  0.35  0.45  0.55

A

Ctrl (n = 40)
AD (n = 20)

B

Ctrl (n = 11)
AD (n = 13)

C

Ctrl (n = 29)
AD (n = 7)

DISEASE STATUS

Mean±SEM

Max

Min
Figure 4.7: Apolipoprotein D levels in control and autopsy confirmed AD cases (Stratified by age of onset).

Apolipoprotein D levels were significantly elevated in the frontal cortex of both LOAD and EOAD cases when compared to controls. There was no statistical difference between LOAD and EOAD cases.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

Control vs. LOAD vs. EOAD: * p < 0.05, ** p < 0.005

Bonferroni correction for multiple comparisons was applied.
When all cases were combined and stratified by the gene dose of the *APOE* ε4 allele (Figure 4.8a) no significant difference in apoD levels was observed with increasing *APOE* ε4 copy number (ε₄/ε₄: 0.133 ± 0.012 µg apoD/mg TP, ε₄/ε₄: 0.182 ± 0.031 µg apoD/mg TP, ε₄/ε₄: 0.153 ± 0.072 µg apoD/mg TP). When AD and control cases were stratified by ε4 gene dose (Figure 4.3b and c) neither a significant difference nor a trend towards significance was observed. After removing homozygote ε4 cases from both groups, to allow for multiple comparisons, heterozygote ε4 AD individuals (0.255 ± 0.044 µg apoD/mg TP) had significantly elevated apoD levels compared to heterozygote ε4 control cases (0.101 ± 0.077 µg apoD/mg TP; p < 0.005). Similarly, AD cases that lacked the ε4 allele (0.166 ± 0.040 µg apoD/mg TP) tended to have higher levels of apoD compared to controls also lacking the ε4 allele (0.125 ± 0.064 µg apoD/mg TP) however this failed to reach statistical significance after applying Bonferroni correction for multiple comparisons.

To determine if any correlation existed between apoD levels and apoE levels in the frontal cortex of AD and control cases a Pearson Product Moment correlation analysis of experimental data versus demographic data (age and PMI) and apoD versus apoE levels was carried out using an assumed straight-line curve fit. This analysis revealed that no correlation existed between apoD and apoE levels in the frontal cortex in either control or AD cases (r² = 0.006 and 0.000, respectively; Figure 4.9). Additionally, there was no significant difference in apoD levels between male and female subjects and there was no correlation between apoD levels and age or PMI.
Figure 4.8 (adjacent): Effect of APOE genotype on apolipoprotein D levels in frontal cortex tissue from control and autopsy confirmed AD cases.

No difference in Apolipoprotein E levels was observed between different gene doses of the APOE ε4 allele in combined (A), control (B) and AD (C) cases. AD cases heterozygous for the ε4 allele had significantly elevated apoE levels compared to control cases with the same ε4 gene dose (ε4/ε4 cases were excluded from multiple comparisons due to n = 1).

Where appropriate Bonferroni correction for multiple comparisons was applied.

Plasma apoE levels expressed as mean ± Standard Error of Mean (SEM)

εn = non-APOE ε4 allele (i.e. ε2 or ε3)

AD εn/ε4 vs. Control εn/ε4: * p < 0.005
Figure 4.9: Correlation analysis of apoE and apoD levels in frontal cortex tissue from control and autopsy confirmed AD cases.

Correlation between apoD and apoE levels in control cases (A) and AD cases (B).

(A) Control Cases: $R^2 = 0.032$

(B) AD Cases: $R^2 = 0.000$
4.4 DISCUSSION

4.4.1 Apolipoprotein E Levels

4.4.1.1 Apolipoprotein E levels: Increased in the frontal cortex of AD cases

The ε4 allele of APOE is universally recognised as the major genetic risk factor for the development of late-onset AD, with as many as 50% of individuals affected with AD bearing an APOE ε4 allele (Strittmatter et al., 1993; Corder et al., 1993; Martins et al., 1995a). However, the ε4 allele is not sufficient to develop AD, since some individuals possessing two APOE ε4 alleles still escape AD. In the previous chapter the -491A/T polymorphism in the regulatory region of the APOE gene was found to be associated with an increased risk for developing AD independent of that conveyed by the ε4 allele of APOE (Laws et al., 1999). This confirmed a previous report (Bullido et al., 1998) and both have been supported in a subsequent study (Wang et al., 2000). In the previous chapter plasma apoE levels were also shown to be elevated in AD, confirming the work of Taddei and colleagues (1997), and that the AA genotype of the -491A/T promoter polymorphism may contribute to this elevation independent of ε4 status (Laws et al., 1999). This has been subsequently verified in the literature (Scacchi et al., 2001).

In this chapter, apoE levels in the frontal cortex of AD cases were shown to be significantly elevated over that measured in control cases. This finding is supported in the literature by reports that mRNA is increased in the frontal and temporal cortex (Yamada et al., 1995; Yamagata et al., 2001) as well as in the hippocampus (Zarow and Victoroff, 1998) of neuropathologically confirmed AD cases. It is also supported by findings of increased soluble levels in the cerebellar cortex and increased bound apoE
levels in the frontal cortex of AD brains (Pirtilla et al., 1996). Biological levels of apoE in brain tissue is a subject over which much conjecture remains, with varying reports on the levels of soluble, bound and total apoE reported. As such, larger studies, incorporating a larger pool of both late- and early-onset AD, need to be performed to elucidate the significance of the findings in this current study.

4.4.1.2 Increased apoE levels in AD cases: No association to APOE genotype

The mechanism(s) by which the APOE ε4 allele affects AD onset in an allele-specific manner is not completely understood. There is accumulating evidence to indicate that the apoE4 protein is less efficient, functionally, than the other apoE isoforms (Miyata and Smith, 1996; Yang et al., 1997; Yang et al., 1999) and some evidence has indicated that the APOE ε4 genotype is associated with lower levels of the protein in plasma (Smit et al., 1988; Slooter et al., 1998; Scacchi et al., 1999; Corbo et al., 1999; Corbo et al., 2001). However, in the previous chapter no difference could be attributed to APOE genotype. Additionally, studies have shown that apoE mRNA levels in AD brains are increased in subjects carrying the ε4 allele (Yamagata et al., 2001) whilst others have shown no correlation (Harr et al., 1996; Terrisse et al., 1998).

In this study we report a non-statistically significant trend towards an increase in apoE expression in the prefrontal cortex in individuals homozygous for the ε4 allele. However, this effect is more likely to be a result of the AA genotype at the -491 APOE promoter locus as this genotype was found to be present in all AD subjects homozygous for the ε4 allele. Additionally, the previous chapter (Laws et al., 1999) and a subsequent study (Scacchi et al., 2001) observed the -491AA genotype to be associated with altered levels of apoE in plasma [and now, as reported in this chapter, brain tissue (Laws et al., 2002b)]. The ε4 allele of APOE is also correlated with increased amyloid plaque density
in a gene-dosage dependent manner (Beffert et al., 1996; Gearing et al., 1995; Gomez-Isla et al., 1996; Hyman et al., 1995; Nagy et al., 1995; Olichney et al., 1996; Oyama et al., 1995; Polivkoski et al., 1995; Rebeck et al., 1993; Schmechel et al., 1993; Zubenko et al., 1994) not unlike the -491A allele (Lambert et al., 2000) which has been associated with increased apoE levels. As such, further studies of the effect of the ε4 allele on altering apoE levels needs to be undertaken to address this paradigm and the current conjecture in the literature.

4.4.1.3 Increased apoE levels in AD cases: Gene dose dependent effect of both the -419A and -219G alleles

As illustrated in the preceding chapter and subsequent reports (Laws et al., 1999; Scacchi et al., 2001), the correlation of increased apoE levels in brain with the -491AA genotype of the 491A/T promoter polymorphism extends and confirms the data associating the -491 AA genotype with elevated plasma apoE levels. These results are also consistent with the reports of the effect of the -491A allele on increasing APOE transcriptional activity (Artiga et al., 1998a; Artiga et al., 1998b; Bullido et al., 1998). As such, it presents a plausible explanation for the current findings in the frontal cortex. The findings in this chapter tend to suggest that the -491A allele exerts this action in a gene dosage-dependent manner. This effect was not observed in AD cases, possibly due to the well recognized several-fold up-regulation of apoE expression in reactive astrocytes in AD (Diedrich et al., 1991), which may mask this effect due to case-to-case differences in the level of activation. However, to determine if this is the case, or whether a gene-dosage effect is present, much larger numbers need to be analysed to overcome the expected the high frequency of the -491AA genotype, as seen in this study. Longitudinal studies sampling the CSF of patients may prove to be a more effective alternative for achieving a definitive answer to this question.
The in vitro studies of Artiga and colleagues (1998a, 1998b) lend credence to the findings of a gene dosage effect of the -219G allele on increasing apoE levels in the frontal cortex of AD cases. No effect of this allele was observed in control cases nor was it observed to have a marked effect in plasma, as illustrated in the previous chapter. As eluded to in the previous chapter the -219T allele is suggested to be associated with an increased risk of AD and lowered apoE levels whilst the -419A is linked with increased AD risk through its increasing of apoE levels. In combination, this suggests that either extreme of apoE levels may impart an increased risk for disease. The -219T allele was also shown in the previous chapter and subsequent studies (Rebeck et al., 1999) to be subject to strong linkage with the e4 allele. This suggests that whilst the -219T/G promoter polymorphism's role in altering APOE expression can not be discounted its role in increasing AD risk is not substantiated in this thesis. As such, further studies need to be undertaken to clarify its role in AD.

Thus, in addition to the APOE genotypes, due to polymorphisms affecting residues 112 and 158, the levels of apoE in the brain, as well as the factors that up regulate its expression, may also play a role in the pathogenesis of AD. This observation is supported by an independent line of investigation suggesting that apoE is required for the formation of cerebral amyloid plaques (Bales et al., 1997; Bales et al., 1999; Holtzman et al., 2000a; Holtzman et al., 2000b). These studies illustrated that, in the absence of apoE, APP717 transgenic mice did not form the characteristic pathology of this mouse model. Pathology was only seen to be evident in the presence of apoE. Thus, it is plausible that increased apoE levels may result in increased cerebral amyloid deposition. This hypothesis is supported by the -491AA genotype being shown to be associated with increased Aβ40, Aβ42 and total Aβ load in AD brains (Lambert et al., 2000). No studies have investigated the -219T/G promoter polymorphism with respect
to Aβ load. It is possible that if apoE levels could be regulated it may represent an effective means of delaying the onset of, or diminishing the level of, cerebral amyloidosis. In turn, this could slow the rate of cognitive decline seen in AD.

4.4.2 Apolipoprotein D

A growing number of studies have demonstrated increases in apoD level in a variety of human neurological diseases (Terrisse et al., 1998; Kalman et al., 2000; Belloir et al., 2001; Thomas et al., 2001a; Thomas et al., 2002). In this chapter, we observed an increase in apoD protein levels in prefrontal cortex of subjects with AD. These findings are in agreement with previous studies that have shown increased apoD expression in other regions of the brain in AD subjects. Increased mRNA and/or protein expression has been observed in the CSF and hippocampus of AD subjects (Terrisse et al., 1998) and the entorhinal cortex (Belloir et al., 2001) of AD subjects. Intense apoD immunostaining has also been shown in astroglial cells and pyramidal neurons of the temporal cortex of AD subjects, but no quantitative differences were observed by immunoblotting when compared to age-matched controls (Kalman et al., 2000). The region-specific nature of the increased apoD levels has led to the hypothesis that apoD is a marker for brain regions that undergo neuropathology as a component of various human neurological disorders (Thomas et al., 2002). The prefrontal cortex is the brain region most notably associated with the cognitive function and cognitive impairment is one of the most important clinical features of AD (Cummings, 2000). Hence, increases in apoD expression detected in the prefrontal cortex of AD in this study may be related to the cognitive deficits observed in these patients (Assal and Cummings, 2002; Chung and Cummings, 2000).
ApoE has been implicated in the pathology of AD since inheritance of the APOE ε4 allele was first shown to be an important risk factor for the development of LOAD (Corder et al., 1993; Poirier et al., 1993; Rebeck et al., 1993; Strittmatter et al., 1993) and has become a central molecule of focus for AD studies. Apolipoprotein J (apoJ; clusterin) is a glycoprotein, which, like apoD, is associated with high-density lipoproteins. In this chapter the apoD levels in the frontal cortex were not correlated with APOE genotype. This is in contrast to a previous study that showed a correlation between apoD levels in the CSF and hippocampus of AD subjects with inheritance of the ε4 allele (Terrisse et al., 1998). One possible explanation for this discrepancy may be region specific differences in increases in apoD levels. However, there is little literature published that would allow for firm conclusions to be drawn. Thus, further studies are required to address this discrepancy in the literature. Interestingly, CNS levels of apoJ, an apolipoprotein that has also been implicated in the pathogenesis of AD (Choi-Miura and Oda, 1996; Lidstrom et al., 1998), has been reported to be significantly decreased in subjects with two ε4 alleles (Harr et al., 1996).

Recent reports have now implicated apoD as another potentially important apolipoprotein in AD. The putative neuroprotective, compensatory functions of apoD in the CNS are consistent with proposed functions for apoE and apoJ, in the pathology of AD (Calero et al., 2000; Poirier, 2000). However, it is likely that apoD plays a distinct role from the other apolipoproteins in the pathology of AD. The cortical senile plaques and neurofibrillary tangles observed in AD are immunoreactive for apoE and apoJ, as well as other apolipoproteins (Calero et al., 2000; Harr et al., 1996; Namba et al., 1991). It is thought that these apolipoproteins are involved in the distribution and/or clearance of amyloid beta, in particularly apoE (Yang et al., 1997; Yang et al., 1999). In contrast, the cortical amyloid plaques and neurofibrillary tangles are immunonegative
for apoD (Belloir et al., 2001; Harr et al., 1996; Kalman et al., 2000). It has been suggested that several members of the apolipoprotein family interact with Aβ deposits in senile plaques through a common amphipathic alpha-helical domain (Harr et al. 1996). ApoD does not contain such a domain (rather is composed primarily of antiparallel β-sheets) and does not associate with Aβ. ApoD may have a unique effect in AD via a different mechanism(s) of action. In the current study apoD levels were not correlated with previously ascertained apoE levels in the same cohort of AD subjects (Laws et al., 2002b). This is additional support for the notion that these lipoproteins have independent functions. In summary, while apoE is a major factor in the pathogenesis of AD, the role played by apoD warrants further investigation.

4.4.3 Summary

This chapter has confirmed the association of increased apoE levels with AD reported in the previous chapter. In addition, the association of the -491A allele with increased levels of apoE in a gene dosage dependent manner suggest that this polymorphism plays an important role in the development of AD, both in the presence and absence of the ε4 allele of APOE. Additionally, the -219G allele is also associated with increasing apoE levels in a gene dose dependent manner however no effect of this polymorphism on AD risk was seen, a similar finding to that of the previous chapter.

Furthermore, this chapter reports that apoD levels are elevated in the frontal cortex of AD cases independent of both APOE genotype and apoE levels. This finding suggests that elevated apoD levels may be related to the cognitive decline observed in AD patients and that apoD and apoE contribute to the pathogenesis of AD by different molecular mechanisms.
5.1 INTRODUCTION

Dementia is defined as the loss of intellectual function (Miller and Keane, 1978) and is typically accompanied by impairment of intellect, memory and personality (Lishman, 1978). The most frequent cause of cognitive decline in late life is AD (Lishman, 1978; Lendon et al., 1997) which accounts for 50% of all dementia worldwide, and its incidence doubles every 5 years between age 65 and 85. The prevalence of the disease grows exponentially with ageing, from 5% at age 65 to approximately 40% at age 80. The recent availability of effective symptomatic treatment of AD and the prospect of further therapeutic advances have given impetus to the early diagnosis of the disease. The relationship between memory complaints in non-demented persons and future risk of dementia has prompted numerous studies (Sunderland et al., 1986, Derouesne et al., 1989, O’Connor et al., 1990, Bolla et al., 1991, Christensen 1991, O’Brien et al., 1992, Taylor 1992, Bassett and Folstein, 1993, Grut et al., 1993, Flicker et al., 1993, Hanninen et al., 1994, Jorm et al., 1994, Gagnon et al., 1994, Schmand et al., 1996, Jonker et al., 1996, Geerlings et al., 1999, Schofield et al., 1997, Jorm et al., 1997; Clarnette et al., 2001).

Currently definitive diagnosis of AD requires histopathological examination of the brain (McKhann et al., 1984). Without neuropathological data, the diagnosis of “probable AD” is based on clinical assessment (McKhann et al., 1984). Familial Alzheimer
disease cases with early onset (i.e. prior to age 65) are often associated with highly penetrant autosomal dominant mutations in genes coding for PS1, PS2 or APP. The more common form of the disease, LOAD, has been associated with genetic risk factors rather than deterministic mutations; the APOE ε4 allele is a major genetic risk factor that has been extensively characterised (Martins et al., 1995a). Numerous other phenotypes and genotypes have subsequently been linked to AD and studied in the preceding chapters, including the APOE ε4 allele, levels of the apoE protein (Taddei et al., 1997; Laws et al., 1999; Laws et al., 2002b), polymorphisms within the APOE promoter (Bullido et al., 1998; Artiga et al., 1998b; Lambert et al., 1998a; Lambert et al., 1998b) and the PS1 mutation Glu318Gly (Aldudo et al., 1998). Whilst the ε4 allele has previously been linked to preclinical memory impairment in asymptomatic middle aged individuals (Flory et al., 2000; Caselli et al., 2001) the other aforementioned factors have not been evaluated for their value as markers of preclinical memory impairment.

This chapter aims to determine whether the AD molecular markers, namely; APOE genotype, APOE –491A/T promoter polymorphism genotype, the Glu318Gly mutation and/or increased plasma apoE levels, are associated, either singly or in combination, with subjects complaining of memory loss. The association of the genetic susceptibility factors studied in this chapter with cognitive decline may provide a starting point for the formulation of criteria for an early, preclinical, diagnosis of AD.
5.1.1 Aims

- To determine frequency of APOE, -491A/T promoter polymorphism genotypes and the Glu318Gly PS1 mutation
- Determine levels of apoE protein in the plasma of ‘Memory Complainers’ and control cases
- Determine the association of these markers with Subjective Memory Complaints and level of ‘conversion’ to clinical endpoints after 3 years.

5.2 MATERIALS AND METHODS

The Subjective ‘Memory Complainers’ cohort described in Section 2.1.2.3 was utilised to study the role of genetic and biological markers studied herein. Briefly, this cohort included 98 individuals with subjective memory complaints (age 63.4 ± 1.0 years) and 49 age, gender and education matched (Stern et al., 1994; De Ronchi et al., 1998) controls (age 61.3 ± 1.5 years). This cohort was assessed clinically for a period of up to 3 years. Clinical assessment was performed as described in Section 2.2.1. Briefly, an experienced geriatrician clinically reviewed all volunteers and recorded medical history and completed a physical and neurological examination. A trained nurse interviewed all subjects using the CAMDEX (Roth et al., 1986). Those with dementia (DSM-IV; American Psychiatric Association, 1994), a MMSE score lower that 24 (Folstein et al., 1975), or prior history of stroke were excluded. Clinical endpoints, referred to as converters in this study, for follow-ups were based on the diagnosis of either MCI (Peterson et al., 1999) or AD (McKhann et al., 1984).
Leukocyte DNA was extracted from peripheral blood samples as described in Section 2.2.2. *APOE* genotype was determined using the PCR protocol as described in Section 2.2.4.1. The genotype of the *APOE* -491A/T polymorphism was determined as described in Section 2.2.4.2 and the presence of the Glu318Gly PS1 mutation was determined as described in Section 2.2.4.4. The quantitation of plasma apoE protein levels was performed as described in Section 2.2.5.2 on plasma samples prepared as described in Section 2.2.5.1.

## 5.3 RESULTS

### 5.3.1 Baseline Data from Individuals with Subjective Memory Loss

#### 5.3.1.1 *APOE* and -491A/T genotypes in subjective memory loss

Clinical assessment (Table 1) showed that subjects with subjective memory complaints had significantly lower MMSE (27.6 ± 0.2 vs. 28.8 ± 0.2; *p* < 0.005, *z* = -3.25,) and CAMCOG (96.3 ± 0.6 vs. 101.5 ± 0.5; *p* < 0.001; *z* = -5.53) scores when compared to controls. Initial analysis of plasma apoE protein levels revealed no significant difference between controls and memory complainers (*p* = 0.64, *z* = -0.47; Table 5.1). Genotypic distribution of both *APOE* and -491A/T genotype were consistent with the distribution predicted by the Hardy-Weinberg equilibrium (*APOE*: $\chi^2$ test; *p* = 0.79; -491: $\chi^2$ test; *p* = 1.0). Pearson’s $\chi^2$ and odds ratio analysis of *APOE* genotype and allele frequencies (Table 5.2) revealed that subjects with memory complaints had a significant increase in the frequency of *APOE* e4 containing genotypes (*p* < 0.05; OR 2.35, 95% CI: 1.11 – 4.97) and the e4 allele (*p* < 0.05; OR 2.05, 95% CI: 1.09 – 3.87). With increasing copies of the *APOE* e4 allele a corresponding trend for decreased CAMCOG and MMSE.
Table 5.1: ApoE levels, MMSE and CAMCOG scores in age and education matched memory complainers and controls.

MCs = Memory Complainers; Education = Age at leaving school; MMSE = Mini Mental State Examination (x/30); CAMCOG = Cognitive component of the Cambridge Examination for Mental Disorders of the Elderly (x/107); ApoE levels reported as µg/mg Total Protein; All values are mean ± SEM (standard error of the mean). Mann-Whitney U-Test was used for comparisons of means of MMSE, CAMCOG and apoE Levels.
## APOE Genotype

<table>
<thead>
<tr>
<th></th>
<th>( \varepsilon_n/\varepsilon_n )</th>
<th>( \varepsilon_n/\varepsilon_4 )</th>
<th>( \varepsilon_4/\varepsilon_4 )</th>
<th>( \varepsilon 2 )</th>
<th>( \varepsilon 3 )</th>
<th>( \varepsilon 4 )</th>
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<tr>
<td>** Controls**</td>
<td>n = 49</td>
<td>36 (73.5)</td>
<td>11 (22.4)</td>
<td>2 (4.1)</td>
<td>7 (7.1)</td>
<td>76 (77.6)</td>
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<tr>
<td><strong>CAMCOG</strong></td>
<td></td>
<td>102.2 ± 0.5</td>
<td>100 ± 1.6</td>
<td>97.5 ± 2.5</td>
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<td><strong>MMSE</strong></td>
<td></td>
<td>29.1 ± 0.2</td>
<td>28.2 ± 0.3</td>
<td>26.5 ± 2.5</td>
<td></td>
<td></td>
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<tr>
<td><strong>MCs</strong></td>
<td>n = 98</td>
<td>53 (54.1)</td>
<td>37 (37.8)</td>
<td>8 (8.2)</td>
<td>9 (4.6)</td>
<td>134 (68.4)</td>
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<td><strong>CAMCOG</strong></td>
<td></td>
<td>96.9 ± 0.8</td>
<td>96.9 ± 0.7</td>
<td>89.5 ± 3.3</td>
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<td><strong>MMSE</strong></td>
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<td>28.2 ± 0.3</td>
<td>27.3 ± 0.3</td>
<td>25.4 ± 0.9</td>
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<td></td>
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</table>

## (b)

<table>
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<tr>
<th>TEST</th>
<th>Groups tested</th>
<th>( p )</th>
<th>O.R. (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \varepsilon_4 ) vs. ( \varepsilon_n/\varepsilon_n )</td>
<td>MCs vs. Ctrl</td>
<td>( p &lt; 0.05 )</td>
<td>2.35 (1.11–4.97)</td>
</tr>
<tr>
<td>( \varepsilon_n/\varepsilon_4 ) vs. ( \varepsilon_n/\varepsilon_n )</td>
<td>MCs vs. Ctrl</td>
<td>( p = 0.181^f )</td>
<td>2.72 (0.55–13.54)</td>
</tr>
<tr>
<td>( \varepsilon_4 ) vs. ( \varepsilon_n )</td>
<td>MCs vs. Ctrl</td>
<td>( p &lt; 0.05 )</td>
<td>2.05 (1.09–3.87)</td>
</tr>
</tbody>
</table>
Table 5.2 (adjacent):  APOE genotypes and their impact on cognitive scores in memory complainers and controls.

APOE ε4 containing genotypes and allele are highlighted in grey. Values in italic parentheses are the relative percentages of each genotype or allele within each group (a). Frequencies here were used to calculate significance and odds ratios (b).

MCs = Memory Complainers; MMSE (Mini Mental State Examination; x/30) and CAMCOG (Cognitive component of the Cambridge Examination for Mental Disorders of the Elderly; x/107) are reported as mean ± SEM (standard error of the mean).

εn represents a non-APOE ε4 allele (i.e. ε2 or ε3; e.g. εn/εn may be ε2/ε2, ε2/ε3 or ε3/ε3 whilst εn/ε4 may be ε2/ε4 or ε3/ε4)

1 Fisher's exact test used as one or more cells was ≤ 5

1 Comparison of the frequency of ε4 containing genotypes (i.e. ε4/ε4 and εn/ε4) versus non-ε4 containing genotypes (i.e. εn/εn)

2 Comparison of the frequency of individuals homozygous for ε4 versus non-ε4 (i.e. εn/εn) genotypes

3 Comparison of the frequency of the ε4 allele (ascertained by calculating allele frequencies) versus the non-ε4 alleles (i.e. εn)

Bonferroni correction for multiple comparisons was applied in the analysis of the effect of ε4 allele on CAMCOG and MMSE scores within groups.

Controls: CAMCOG: n.s.d.

MMSE: εn/εn > εn/ε4 (p = 0.09) > ε4/ε4 (p = 0.18) < εn/εn (p < 0.01)

MCs: CAMCOG - εn/εn > ε4/ε4 (p < 0.005) < εn/ε4 (p < 0.005)

MMSE scores: εn/εn > εn/ε4 (p = 0.14) > ε4/ε4 (p < 0.05) < εn/εn (p < 0.005)
scores was observed. After correcting for multiple comparisons this trend did not reach significance for either CAMCOG or MMSE scores in controls, however, controls homozygous for the ε4 allele scored significantly worse than non-ε4 controls (p < 0.01). In memory complainers this trend was more evident with ε4 homozygotes scoring significantly worse in the CAMCOG [εn/ε4 > ε4/ε4 (p < 0.005) < εn/εn (p < 0.005)] and MMSE [εn/ε4 > ε4/ε4 (p < 0.05) < εn/εn (p < 0.005); Table 5.2]. Across all genotypes control individuals tended to perform better on tests of cognition, although this was only statistically significant in non-ε4 cases [Mann-Whitney U-Test; CAMCOG p < 0.001 (z = -4.67), MMSE p < 0.05 (z = -2.25)].

This cohort was examined to determine whether -491A/T promoter polymorphism genotype was associated with memory complaints. Fisher’s exact test and OR analysis of -491A/T genotype frequencies (Table 5.3) revealed that subjects with memory complaints were more likely to have -491A containing genotypes (Fisher exact p < 0.05; OR 8.62, 95% CI: 0.94 – 79.36) whilst homozygosity of the -491A allele yielded a stronger association (Fisher exact p < 0.05; OR 10.56, 95% CI: 1.13 – 99.11). Pearson’s χ² and odds ratio analysis of allele frequencies suggests that the presence of at least one copy of the -491A allele conferred a 2 fold increased risk of memory complaints (p < 0.05; OR 1.98, 95% CI: 1.11-3.52). The -491A allele had no apparent effect on cognitive performance in either the control or memory complainers groups, although -491TT cases tended to perform slightly better. Across all genotypes control individuals tended to perform better on tests of cognition, this was statistically significant in -491AA [Mann-Whitney U-Test; CAMCOG p < 0.001 (z = -4.67), MMSE p < 0.05 (z = -2.25)] and -491AT [Mann-Whitney U-Test; CAMCOG p < 0.001 (z = -4.67), MMSE p < 0.05 (z = -2.25)] cases. In both controls and memory complainers, there was a trend towards increasing plasma apoE protein levels as the -491A allele copy number
increased however this trend did not attain statistical significance. Likewise, comparisons of apoE levels in controls and memory complainers with corresponding -491A/T promoter polymorphism yielded no significant difference.

To determine whether the -491A allele distribution was different between APOE ε4 carriers and non-carriers, the -491 genotypes were stratified by the presence/absence of the APOE ε4 allele (Table 5.4). In both APOE ε4 carriers and non-carriers the memory complainers group had an increased frequency of the -491A allele. This increased frequency was significant in APOE ε4 non-carriers (p < 0.05; OR 2.46, 95%CI 1.20-5.04) but failed to reach significance in carriers possibly due to the low frequency of APOE ε4 carriers in this group. Fisher exact test analysis of genotype frequencies revealed that although memory complainers tended to have an increased frequency it just failed to reach significance (-491AA/AT vs. TT: Fisher exact p = 0.08; -491AA vs. -491TT: Fisher exact test p = 0.07). This comparison in ε4 carriers was not undertaken due to the absence of -491TT individuals. Comparisons of genotype and allele frequencies within groups revealed no significant difference in distribution within memory complainers or controls in relation to the presence/absence of the APOE ε4 allele.

The memory complainers group was then classified on the basis of a family history of dementia (Table 5.5) so as to determine potential differences in the patient sample regarding the investigated polymorphisms in memory complainers with a positive family history (n = 58) for dementia and those without (n = 40). Pearson’s χ² and OR analysis of allele and genotype frequencies revealed that both APOE ε4 containing genotypes and ε4 allele frequencies had the strongest association with memory
### APOE −491 Genotype

<table>
<thead>
<tr>
<th></th>
<th>A/A</th>
<th>A/T</th>
<th>T/T</th>
<th>Allele Freq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>n = 49</td>
<td>25</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(51.0)</td>
<td>(40.8)</td>
<td>(8.2)</td>
</tr>
<tr>
<td>CAMCOG</td>
<td>101.0 ± 0.9</td>
<td>102.3 ± 0.6</td>
<td>100.5 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>MMSE</td>
<td>28.7 ± 0.3</td>
<td>28.7 ± 0.2</td>
<td>29.3 ± 0.7</td>
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</tr>
<tr>
<td>apoE Level</td>
<td>2.49 ± 0.14</td>
<td>2.29 ± 0.26</td>
<td>1.27 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>MCs</td>
<td>n = 98</td>
<td>66</td>
<td>31</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
<td>(67.3)</td>
<td>(31.6)</td>
<td>(1.0)</td>
</tr>
<tr>
<td>CAMCOG</td>
<td>96.1 ± 0.8</td>
<td>96.6 ± 0.9</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>MMSE</td>
<td>27.7 ± 0.3</td>
<td>27.4 ± 0.2</td>
<td>29</td>
<td></td>
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<tr>
<td>apoE Level</td>
<td>2.34 ± 0.12</td>
<td>2.13 ± 0.15</td>
<td>1.15</td>
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### (b)

<table>
<thead>
<tr>
<th>TEST</th>
<th>Groups tested</th>
<th>$p$</th>
<th>O.R. (95% C.I.)</th>
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<tbody>
<tr>
<td>$^1$AA/AT vs. TT (genotype freq)</td>
<td>MCs vs. Ctrl</td>
<td>$p &lt; 0.05^f$</td>
<td>8.62 (0.94–79.36)</td>
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<tr>
<td>$^2$AA vs. TT (genotype freq)</td>
<td>MCs vs. Ctrl</td>
<td>$p &lt; 0.05^f$</td>
<td>10.56 (1.13–99.11)</td>
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<tr>
<td>$^3$A vs. T (allele freq)</td>
<td>MCs vs. Ctrl</td>
<td>$p &lt; 0.05$</td>
<td>1.98 (1.11–3.52)</td>
</tr>
</tbody>
</table>
Table 5.3 (adjacent): *APOE* -491A/T polymorphism genotypes and their effect on apoE levels and cognitive scores in memory complainers and controls.

Values in italic parentheses are the relative percentages of each genotype or allele within each group (a). Frequencies here were used to calculate significance and odds ratios represented (b).

MCs = Memory Complainers; MMSE (Mini Mental State Examination; x/30), CAMCOG (Cognitive component of the Cambridge Examination for Mental Disorders of the Elderly; x/107) and apoE levels (µg/mg Total Protein) are reported as mean ± SEM (standard error of the mean).

1 Fisher's exact test used as one or more cells was ≤ 5

1 Comparison of the frequency of the -491A containing genotypes versus the -491TT genotype (i.e. -491AA/AT versus -491TT)

2 Comparison of the frequency of the -491AA genotypes versus the -491TT genotype

3 Comparison of the frequency of the -491A allele (ascertained by calculating allele frequencies) versus the -491T allele

Fisher's exact test used as one or more cells was ≤ 5

Bonferroni correction for multiple comparisons was applied in the analysis of the effect of the -491A allele on apoE levels and CAMCOG and MMSE scores - no significant effect of -491A on scores or levels was observed within the control or memory complainer groups.
### (a)

<table>
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<tr>
<th>APOE-ε4</th>
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<th>-491 Genotype</th>
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<th></th>
<th></th>
<th>Allele Freq</th>
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<td>Absent</td>
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<td>AT</td>
<td>TT</td>
<td>A</td>
<td>T</td>
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<td>49</td>
<td>23</td>
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<td></td>
<td></td>
<td>(47.2)</td>
<td>(41.7)</td>
<td>(11.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCs</td>
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<td>37</td>
<td>15</td>
<td>1</td>
<td>89</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(69.8)</td>
<td>(28.3)</td>
<td>(1.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
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<td>AA</td>
<td>AT</td>
<td>TT</td>
<td>A</td>
<td>T</td>
</tr>
<tr>
<td>Controls</td>
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<td>5</td>
<td>0</td>
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<td>5</td>
</tr>
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<td>(61.5)</td>
<td>(38.5)</td>
<td>(0.0)</td>
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<td>16</td>
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<td>(64.4)</td>
<td>(35.6)</td>
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### (b)

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<th>TEST</th>
<th>Groups tested</th>
<th>p</th>
<th>O.R. (95% C.I.)</th>
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</thead>
<tbody>
<tr>
<td>1-491A vs. 491TT (genotype freq)</td>
<td>MCs vs. Ctrl (ε4 absent)</td>
<td>$p = 0.08^f$</td>
<td>6.50 (0.70–60.76)</td>
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<tr>
<td>2-491AA vs. 491TT (genotype freq)</td>
<td>MCs vs. Ctrl (ε4 absent)</td>
<td>$p = 0.07^f$</td>
<td>8.71 (0.90–83.88)</td>
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<tr>
<td>3-491A vs. 491T (allele freq)</td>
<td>AD vs. Ctrl (ε4 absent)</td>
<td>$p &lt; 0.05$</td>
<td>2.46 (1.20–5.04)</td>
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<tr>
<td></td>
<td>AD vs. Ctrl (ε4 present)</td>
<td>$p = 0.69^f$</td>
<td>1.10 (0.36–3.36)</td>
</tr>
</tbody>
</table>
Table 5.4: APOE -491A/T distribution in APOE ε4 carriers and non-carriers

(a) Values in italics are the relative percentages of each genotype or allele within each group. Frequencies here were used to calculate significance and odds ratios (b).

MCs = Memory Complainers; Presence = εn/ε4 or ε4/ε4 genotypes; Absence = εn/εn genotypes (where εn = non-ε4 allele)

1 Comparison of the frequency of the -491A containing genotypes versus the -491TT genotype (i.e. -491AA/AT versus -491TT) in the absence* of the APOE ε4 allele

2 Comparison of the frequency of the -491AA genotypes versus the -491TT genotype in the absence* of the APOE ε4 allele

3 Comparison of the frequency of the -491A allele (ascertained by calculating allele frequencies) versus the -491T allele in the presence or absence of the APOE ε4 allele

* Not calculated in the presence of the ε4 allele due to lack of -491TT genotype in both controls and MCs

f Fisher’s exact test used as one or more cells was ≤ 5
**CHAPTER FIVE: RISK FACTORS IN SUBJECTIVE MEMORY COMPLAINTS**

(a)  

<table>
<thead>
<tr>
<th>APOE</th>
<th>Genotype Frequency</th>
<th>Allele Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\varepsilon_n/\varepsilon_n$</td>
<td>$\varepsilon_4$</td>
</tr>
<tr>
<td>+ve history (58)</td>
<td>27 (0.47)</td>
<td>31 (0.53)</td>
</tr>
<tr>
<td>-ve history (40)</td>
<td>26 (0.65)</td>
<td>14 (0.35)</td>
</tr>
</tbody>
</table>

-491  

<table>
<thead>
<tr>
<th></th>
<th>AA/AT</th>
<th>TT</th>
<th>A</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ve history (58)</td>
<td>57 (0.64)</td>
<td>1 (0.36)</td>
<td>94 (0.81)</td>
<td>22 (0.19)</td>
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<td>-ve history (40)</td>
<td>40 (1.00)</td>
<td>0 (0.00)</td>
<td>69 (0.86)</td>
<td>11 (0.14)</td>
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(b)  

<table>
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<th>TEST</th>
<th>Groups tested</th>
<th>$p$</th>
<th>O.R. (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\varepsilon_4$ vs. $\varepsilon_n/\varepsilon_n$ (genotype freq)</td>
<td>+ve history vs. Ctrl</td>
<td>$p &lt; 0.005$</td>
<td>3.18 (1.40–7.20)</td>
</tr>
<tr>
<td></td>
<td>-ve history vs. Ctrl</td>
<td>$p = 0.38$</td>
<td>1.49 (0.60–3.70)</td>
</tr>
<tr>
<td>$\varepsilon_4$ vs. $\varepsilon_n$ (allele freq)</td>
<td>+ve history vs. Ctrl</td>
<td>$p &lt; 0.01$</td>
<td>2.39 (1.22–4.66)</td>
</tr>
<tr>
<td></td>
<td>-ve history vs. Ctrl</td>
<td>$p = 0.54$</td>
<td>1.28 (0.58–2.80)</td>
</tr>
<tr>
<td>$\AA/\AT$ vs. $\ TT$ (genotype freq)</td>
<td>+ve history vs. Ctrl</td>
<td>$p = 0.13^f$</td>
<td>5.07 (0.55–46.93)</td>
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<tr>
<td></td>
<td>-ve history vs. Ctrl</td>
<td>$p = 0.09^f$</td>
<td>NC</td>
</tr>
<tr>
<td>$A$ vs. $T$ (allele freq)</td>
<td>+ve history vs. Ctrl</td>
<td>$p = 0.18$</td>
<td>1.69 (0.78–3.67)</td>
</tr>
<tr>
<td></td>
<td>-ve history vs. Ctrl</td>
<td>$p &lt; 0.05$</td>
<td>2.51 (1.16–5.43)</td>
</tr>
</tbody>
</table>
Table 5.5: Summary of *APOE* polymorphisms in memory complainers (stratified by family history of dementia)

Values in italic parentheses are the relative percentages of each genotype or allele within each group (a). Frequencies here were used to calculate significance and odds ratios represented (b).

+ve/-ve history corresponds to individuals being/not-being first degree relatives of AD sufferers.

Significance values and odds ratio's ascertained after comparison to control group NC – value can not be calculated

1 Fisher's exact test used as one or more cells was $\leq 5$

1 Comparison of the frequency of $\varepsilon 4$ containing genotypes (i.e. $\varepsilon 4/\varepsilon 4$ and $\varepsilon a/\varepsilon 4$) versus non-$\varepsilon 4$ containing genotypes (i.e. $\varepsilon a/\varepsilon a$)

2 Comparison of the frequency of the $\varepsilon 4$ allele (ascertained by calculating allele frequencies) versus the non-$\varepsilon 4$ alleles (i.e. $\varepsilon a$)

3 Comparison of the frequency of the -491A containing genotypes versus the -491TT genotype (i.e. -491AA/AT versus -491TT)

4 Comparison of the frequency of the -491A allele (ascertained by calculating allele frequencies) versus the -491T allele
complainers that had a family history of dementia (Genotype freq: p < 0.005; OR 3.18, 95% CI: 1.4 – 4.72, Allele freq: p < 0.01; OR 2.39, 95% CI: 1.22 – 4.66). However, the reverse was seen with the –491A/T polymorphism. In this analysis memory complainers without a family history of dementia had the strongest association with the –491A allele (p < 0.05; OR 2.51, 95% CI: 1.16 – 5.43) whilst it was no longer significantly associated with those who had a family history of dementia.

Homozygosity for both the -491A and APOE ε4 alleles occurred in only seven of the 147 individuals (4.8%), similar to the 5% reported in Chapter 3 (Laws et al., 1999). This genetic combination was enriched within the “memory complainers” group, which contained 6 out of 7 of these individuals or 6.1% of “memory complainers” (OR = 7.68; 95% CI = 0.9-65.87; \( \chi^2 = 4.65; p = 0.03 \)). The data were analysed to determine whether these “dual homozygotes” had elevated apoE levels. Plasma apoE levels were found to be significantly higher in these individuals (p < 0.01, t = 2.90; Table 5.6) and they performed significantly worse than controls in both the MMSE (p < 0.01, z = 2.62; Table 5) and CAMCOG (p < 0.01, z = 2.78; Table 4). Individuals who complained of memory decline and had two copies of the -491A and APOE-ε4 alleles showed significantly higher apoE levels than complainers with other genotypes (p < 0.001, t = 3.57). Furthermore, these individuals also tended to have lower MMSE and CAMCOG scores (Table 5.6) when compared to complainers of other genotypes, although this difference was not statistically significant.

5.3.1.2 The PS-1 mutation Glu318Gly and subjective memory loss

Four of the 98 cases with memory complaints (4.1%) were Glu318Gly positive, whereas this mutation was not observed in any of the 49 control individuals (Table 5.7). Glu318Gly positive memory complainers had significantly lower CAMCOG scores
Table 5.6: ApoE levels and MMSE and CAMCOG scores in individuals who are homozygous for both the APOE ε4 and the −491A alleles

Values shaded in (a) are those used in the comparisons of mean scores for CAMCOG, and MMSE and ApoE levels in (b). Mann-Whitney U-Test was used for comparisons of means of MMSE and CAMCOG scores and apoE Levels.

* These individuals lack dual homozygosity for APOE ε4 and −491A

MCs = Memory Complainers; MMSE, CAMCOG and apoE levels are reported as mean ± SEM (standard error of the mean).
than controls (p < 0.05, z = - 2.486) and tended to score worse on the MMSE though this did not reach statistical significance. There was no significant difference between carriers and non-carriers within the memory complainers group although carriers tended to score slightly less.

5.3.2 Percentage Conversion to Clinical Endpoint after 3 Years

Participants in the memory study were followed by trained geriatricians, psychiatrists and neuro-psychologists over a period of 3 years. Over this period individuals who met the criteria for the diagnosis of either MCI (Peterson et al., 1999) or AD (McKhann et al., 1984) were classified as individuals who had reached the clinical endpoint for this study. These individuals are henceforth referred to as “converters”, as having “converted” from subjective memory complaints to clinically determined memory impairment or AD. Overall, 14.3% (14 out of 98) of memory complainers were converters whilst no individual from the control group was found to have sufficient decline for conversion (Table 5.8). Individuals homozygous for the APOE ε4 allele showed a conversion rate of 37.5% at 3 years, whilst 13.5% and 10.8% of heterozygous ε4 and non-ε4 individuals, respectively, converted. Analysis of the level of conversion in -491A carriers revealed that 16.7% of -491A homozygotes converted compared to 9.7% of -491AT whilst the single -491T homozygote individual did not convert. Combining homozygosity of the ε4 and -491A alleles revealed that six memory complainers possessed this profile and 50% were diagnosed with AD after 3 years. Four memory complainers possessed the Glu318Gly mutation and only one converted; this individual was also one of the six individuals homozygous for both the ε4 and -491A alleles and the sole individual to possess all three risk markers. ApoE level did not have a significant impact on conversion with no significant difference observed between
(a)

<table>
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<tr>
<th></th>
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<th>MMSE</th>
<th>CAMCOG</th>
</tr>
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<tr>
<td>Controls</td>
<td>49</td>
<td>28.8 ± 0.2</td>
<td>101.5 ± 0.5</td>
</tr>
<tr>
<td>GLU318GLY (+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GLU318GLY (-)</td>
<td>49</td>
<td>28.8 ± 0.2</td>
<td>101.5 ± 0.5</td>
</tr>
<tr>
<td>MCs</td>
<td>98</td>
<td>27.6 ± 0.2</td>
<td>96.3 ± 0.6</td>
</tr>
<tr>
<td>GLU318GLY (+)</td>
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<td>27.3 ± 1.4</td>
<td>94.0 ± 3.8</td>
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<tr>
<td>GLU318GLY (-)</td>
<td>94</td>
<td>27.6 ± 0.2</td>
<td>96.4 ± 0.6</td>
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(b)

<table>
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<tr>
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<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu318Gly</td>
<td>MCs vs. Controls</td>
<td>p = 0.193f</td>
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<tr>
<td>MMSE</td>
<td>MCs(Glu318Gly+) vs. Controls</td>
<td>p = 0.24 (z = -1.19)</td>
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<tr>
<td></td>
<td>MCs(Glu318Gly+) vs. MCs(Glu318Gly-)</td>
<td>p = 0.88 (z = -0.15)</td>
</tr>
<tr>
<td>CAMCOG</td>
<td>MCs(Glu318Gly+) vs. Controls</td>
<td>p &lt; 0.05 (z = -2.49)</td>
</tr>
<tr>
<td></td>
<td>MCs(Glu318Gly+) vs. MCs(Glu318Gly-)</td>
<td>p = 0.51 (z = -0.67)</td>
</tr>
</tbody>
</table>

Table 5.7: Cognitive scores for GLU318GLY (+) and GLU318GLY (-) controls and memory complainers.

Values shaded in (a) are those used in the comparisons of mean scores for CAMCOG and MMSE and analysis of the frequency of the Glu318Gly mutation (b).

MCs = Memory Complainers; MMSE (Mini Mental State Examination; x/30) and CAMCOG (Cognitive component of the Cambridge Examination for Mental Disorders of the Elderly; x/107) are reported as mean ± SEM (standard error of the mean).

f Fisher’s exact test used as one or more cells was ≤ 5

Mann-Whitney U-Test was used for comparisons of means of MMSE and CAMCOG
### Table 5.8: Converter demographics

Percentage conversion for each genetic risk factor (a) and baseline (not endpoint) cognitive scores and apoE levels for converters compared to non-converters and controls (b).

* Mann-Whitney U-Test used for the analysis of cognitive scores and apoE levels in Converters vs. Non-Converters and Converters vs. Controls.

Converters: individuals who converted to the clinical endpoint of MCI or AD diagnosis.

Non-converters: individuals (all participants) who did not convert to clinical endpoints.
converters and non-converters or between converters and controls. Baseline cognitive performance had more influence however with scores significantly lower in the converters groups when compared to non-converters (MMSE: \( p < 0.005, z = -3.06 \); CAMCOG: \( p < 0.005, z = -3.37 \)) and controls (MMSE: \( p < 0.001, z = -3.58 \); CAMCOG: \( p < 0.001, z = -4.36 \)).

5.4 DISCUSSION

5.4.1 APOE and -491A/T Loci in Individuals with Subjective Memory Complaints

Cognitive assessment determined that memory complainers had significantly lower scores on the CAMCOG and MMSE. However, it must be noted that none of the subjects included in the study reached the threshold for diagnosable dementia at baseline. After following this cohort for 3 years, 14.3% of memory complainers converted to the clinical endpoints of this study. Of these converters, subjects were either diagnosed with MCI (Peterson et al., 1999) or AD (McKhann et al., 1984). This level of conversion compared to the lack of controls converting supports the notion that subjective memory complainers are a high risk group for cognitive decline and dementia (Jonker et al., 2000; Geerlings 1999).

The \( APOE \) \( e4 \) allele was significantly over represented amongst individuals with memory complaints, and a trend towards a gene dosage effect on global measures of cognition was evident at baseline. Individuals homozygous for the \( e4 \) allele showed a conversion rate of 37.5% at 3 years, emphasising that the \( APOE \) \( e4 \) allele is the most
significant marker for cognitive decline. The data are also consistent with a recent report showing an association between the APOE ε4 allele and memory decline in non-demented individuals (Dik et al., 2000; Deary et al., 2002) and preclinical memory impairment in asymptomatic middle aged individuals (Flory et al., 2000; Caselli et al., 2001). When memory complainers were stratified by family history the ε4 allele exhibited its strongest association in individuals with a positive family history of dementia. A previous cross-sectional analysis of clinical data from this cohort (Clarnette et al., 2001) showed no association of APOE ε4 with memory complaints. A high proportion of control individuals, in this study, with a relative suffering from AD, can explain this lack of association. Thus, for this study further controls were recruited and individuals with AD relatives were excluded from analysis to provide a better representation of a control population.

This chapter presents the first data in the literature looking at the possible association of the -491A/T promoter polymorphism with cognitive impairment in a pre-clinical cohort. Analysis of -491 genotype and allele frequencies revealed a significant association of the -491A allele with the “memory complainers” group with 15.4% of -491A homozygotes converted compared to 9.7% of -491AT whilst the -491T homozygote individual did not convert. The -491A allele had no apparent effect on cognitive performance in either the control or memory complainers groups. However, in both groups, there was a trend towards increasing plasma apoE protein levels as the -491A allele copy number increased. As opposed to the ε4 allele, -491A was associated with individuals without a family history of dementia. This finding suggest that the -491A allele, and subsequently apoE levels, may be an important predictive factor in individuals without a family history of dementia, whilst the APOE genotype was associated with a positive family history. The data also suggests that the -491A allele
confers an APOE ε4-independent risk for an association with memory complaints, consistent with the independent effect on AD risk reported in the preceding chapters and in the literature (Laws et al., 1999, Wang et al., 2000). When the combination of the −491A and APOE ε4 alleles was investigated among “memory complainers” a relationship between these two polymorphisms was observed. It is interesting to note that among the seven individuals in this study who were homozygous for both alleles; six were “memory complainers”. However, this profile represents only a small proportion of individuals (7 out of 147 in this study), and therefore these findings must be tested in a much larger population before definitive conclusions can be drawn. The six “memory complainers” with homozygosity of both the APOE ε4 and −491A alleles showed relatively low cognitive scores. Furthermore, it should be noted that one individual excluded from this study, at baseline, due to the clinical diagnosis of probable AD was also homozygous for both the −491A and the APOE ε4 allele.

Another biological factor that has shown promise as a potential marker for AD is plasma apoE protein levels. The levels of this protein have been previously reported to be increased in AD (Taddei et al., 1997), and this increase may be partially attributed to the presence of the −491A allele (Laws et al., 1999), which has also been independently associated with increased Aβ40, Aβ42 and total Aβ loading in AD brains (Lambert et al., 2001). A recent report has shown that CSF apoE levels are elevated in AD subjects (Fukuyama et al., 2000), providing further support for its role as an important clinical marker for this disease. However, other studies have failed to replicate this finding (Landen et al., 1996b; Lehtimaki et al., 1995; Pirtilla et al., 1996). Whilst consensus on this issue is yet to be reached, it is interesting to note that studies have shown that apoE deficiency virtually abolishes or at least delays significantly the cerebral amyloidosis in amyloid plaque-forming transgenic mice (Bales et al., 1997; Bales et al.,
1999). In the current study, plasma apoE levels were not found to be significantly elevated among “memory complainers”. However, levels were significantly higher in the sub-group of individuals who were homozygous for both the APOE ε4 and the –491A alleles. Although there was no consistent elevation of plasma apoE levels among all “memory complainers” this is to be expected as only a subset of memory complainers will convert to AD. As such, the merits of this biological marker should not be discounted. It is also plausible that apoE levels may play an important role in the rate of disease progression and might indirectly alter the efficacy of therapeutic interventions (Hone et al., 2002), in addition to predisposing an individual to developing AD. However, whilst the sample size employed in this study needs to be increased, and more follow-ups required, to obtain robust data it should be mentioned that no significant difference was observed between apoE levels in converters, a more homogeneous group, compared to either controls or all ‘non-converters’.

One limitation of the baseline data from this study is that the association of the markers studied could not be related to specific cognitive impairments due to the use of global measures of cognition, in the form of the MMSE and CAMCOG. Whilst these tests are still widely used to aid clinical diagnosis they are also acknowledged to be insensitive to early cognitive deficits (Pasquier, 1999) and fraught with limitations (Huppert et al., 1995; Cullum et al., 2000; Jacquim-Gadda et al., 2000). At baseline the CAMCOG, and to a lesser extent the MMSE, were sufficient to detect differences in cognitive performance between subjective memory complainers and controls. However, these global measures of cognition were not sensitive enough to allow for meaningful longitudinal data to be interpreted as the degree of decline observed by these tests, over the 3 year period, was minimal. It has been suggested that domain-specific measures of cognition are more effective for the prediction cognitive decline than global measures of
cognition (Fowler et al., 1997; Swainson et al., 2001). As such the limitation of the cross-sectional study at baseline was addressed through the use of more sensitive tests in combination with the CAMCOG and MMSE. The use of these sensitive tests, including tests of verbal learning and memory, such as the California Verbal Learning Test (CVLT; Delis et al., 1987), allowed the identification of the sub-group of individuals, in this thesis referred to as ‘converters’, which was not possible on the basis of global measures exclusively. This finding is supported by reports that suggest that the early detection of isolated memory deficits requires sensitive neuropsychological tests (De Jager et al., 2002) which can detect slight cognitive impairments even in individuals who are showing normal cognitive performance on the MMSE and CAMCOG.

**5.4.2 Association Between the Presenilin-1 Mutation Glu318Gly and Complaints of Memory Impairment**

Four of the memory complainers carried the Glu318Gly mutation of PS1 (4/98; 4.1%) compared to none in the corresponding control group. The frequency, of the Glu318Gly mutation in the memory complainers group, is marginally increased, though not statistically so, when compared to that reported in the previous chapter in a larger control populations from Australia (2.8%; Taddei et al., 2002) and Holland (3.2%; Dermaut et al., 1999b). Furthermore, in the preceding chapter the frequency of Glu318Gly is increased in FAD in the Australian population (Taddei et al., 2002). However, this value of 4.1% for the memory complainers group is not increased when compared to control groups in the Finnish (6.8%) and the Spanish (5.3%) populations (Aldudo et al., 1998). Of the four memory complainers positive for the Glu318Gly mutation only one reached the clinical endpoint of this study.
The CAMCOG scores of the four Glu318Gly carriers were lower than the scores recorded for the control subjects. However, it is unclear at this stage whether this result was directly influenced by the presence of this mutation or is due to the presence of other risk factors such as the APOE ε4 and -491A alleles. This ambiguity arises as CAMCOG scores in Glu318Gly carriers and non-carriers, within the memory complainers group, were not significantly different. This lack of difference in cognitive scores between Glu318Gly carriers and non-carriers indicates that other genetic or environmental factors may be involved in the non-Glu318Gly carriers although more sensitive neuropsychological tests are also warranted. However, whether they play any role in promoting cognitive decline in the four Glu318Gly carriers remains to be determined by follow-up studies. Coupled with this is the fact that the sole Glu318Gly converter was also homozygous for the ε4 and -491A alleles. It is unclear as to whether or not this is a cumulative effect of the three risk factors or whether the Glu318Gly mutation plays no role in this conversion.

5.4.3 Summary

This chapter presents data that suggests that both the ε4 allele of APOE and the -491A allele are associated with both subjective memory impairment and a high rate of conversion to MCI and/or AD. At baseline these markers were associated with trends towards inferior cognitive performance on global measures of cognition. Homozygosity at both the loci may play an integral role in cognitive decline and may be useful for the identification of individuals at high risk for the development of AD. The data also suggest that whilst partial linkage exists, the APOE ε4 and -491A alleles can independently exert risk.
The frequency of the PS1 mutation, Glu318Gly, is increased (4.1%, 4/98) in subjects complaining of memory impairment when compared to controls (0%, 0/49) in the Australian population. However, whether this mutation, *per se*, directly contributes to cognitive decline remains to be determined. Larger longitudinal studies utilising more sensitive measures of cognitive performance need to be undertaken before the true predictive value of the genetic and biological factors, studied in this chapter, can be determined.
6.1 INTRODUCTION

Oestrogen deprivation is widely accepted to be associated, in elderly woman, with increasing the risk for the development of osteoporosis and cardiovascular disease. However, the most dreaded and disturbing aspect of aging is the development of cognitive impairment. Males are more commonly affected by stroke and cardiovascular disease, whereas the age-specific prevalence of AD is higher in females (Jorm et al., 1997). With the average age of menopause in women being approximately 51 years the majority of females will live approximately one-third of their adult life in a state of oestrogen deprivation. When this is coupled with the numerous studies reporting that the incidence of AD is highest in women and that this increased risk of AD is apparent in women across all APOE genotypes (Farrer et al., 1997) it suggests that this imbalance in the risk of AD is due to a rapid decline in endogenous oestrogen levels. In support of this theory is the finding that serum oestrogen levels are decreased in AD sufferers (Honjo et al., 1989; Yaffe et al., 2000).

It has been suggested that the supplementation of endogenous oestrogen, through the means of ORT, may improve cognition in non-demented peri-/post-menopausal woman and/or delay/prevent the onset of cognitive decline or slow the progression of dementia. This hypothesis is supported by findings that adverse cognitive functioning is seen after oestrogen deprivation (Farrag et al., 2002) and can be reversed to premenopausal levels
CHAPTER SIX: HRT, APOE ε4 AND GLOBAL MEASURES OF COGNITION

with ORT (Kampen & Sherwin, 1994: Philips & Sherwin, 1992). The literature is inundated with numerous clinical studies that attempt to determine if ORT can delay the onset or slow the progression of cognitive decline. Some of these studies have looked at ORT in post-menopausal healthy woman to evaluate if those on ORT perform better on measures of cognitive performance. Whilst others have studied post-menopausal woman suffering from dementia to determine whether use of ORT is more frequent in non-demented woman and also to evaluate whether or not ORT can improve cognition in those already suffering cognitive deficits. Consensus is yet to be reached on whether the use of ORT improves cognition in healthy post-menopausal woman with studies reporting both improved cognitive performance and either no association or inconclusive findings (Kimura, 1995; Maki et al., 2000; Yaffe et al., 2000; Paganini-Hill and Henderson, 1994; Paganini-Hill and Henderson, 1996; Barrett-Connor and Kritz-Silverstein, 1993; Kampden and Sherwin, 1994; Robinson et al., 1994). Possible contributing factors for the inconclusive data is the impact of confounding factors such as a variety of environmental factors, different forms of HRT and the impact of genetic factors, such as APOE genotype, which may predispose individuals to cognitive deficits.

Yaffe and colleagues (2000) report that cognitive impairment was delayed, to the greatest extent, by ORT use in women not possessing the APOE ε4 allele. Women possessing the ε4 allele and not taking ORT had the most rapid decline to impairment whilst the use of ORT in ε4 carriers retarded decline to a similar level to that seen in non-ε4 women not on ORT (see Figure 1.9). However, Tang and co-workers (1996a) showed that oestrogen’s benefit was independent of APOE gene status. This putative pharmacogenetic effect (the ability of genetic loci to modulate the responsiveness of a drug) of the ε4 allele of APOE may explain why ORT has differential effects on cognition. It is this theory of ‘responders’ and ‘non-responders’ coupled with the
reported side-effects of HRT (i.e. increased risk of breast and endometrial cancer) that gives impetus to discerning which women will benefit from hormone supplementation as a means of delaying cognitive decline in later life.

This chapter aims to determine whether the use of HRT (in the form of oestrogen only or oestrogen plus progesterone supplementation) is associated with an improved level of cognitive function in cognitively healthy post-menopausal woman. It will also endeavour to address whether APOE genotype has a pharmacogenetic effect on the cognitive efficacy of HRT. The dosage level of ‘oestrogen only’ replacement therapy will also be investigated to determine if cognitive performance is related to oestrogen dose.

6.1.1 Aims

- Determine if the use of any form of HRT is associated with improved cognitive performance and whether it is modified by the APOE e4 allele.
- Determine if the form of supplementation (ORT or cHRT) has an effect on cognitive performance and whether it is modified by the APOE e4 allele.
- Determine if the dose of ‘oestrogen only’ supplementation is associated with altered cognitive performance and whether it is modified by the APOE e4 allele.

6.2 MATERIALS AND METHODS

The post-menopausal cohort described in Section 2.1.2.4 was utilised to study the role of HRT on cognitive performance and the pharmacogenetic effect of the APOE e4
allele. The initial cohort of 330 post-menopausal women was assessed clinically as described in Section 2.2.1. Briefly, an experienced geriatrician clinically reviewed all volunteers and recorded medical history and completed a physical and neurological examination. A trained nurse interviewed all subjects using the CAMDEX (Roth et al., 1986). At this stage exclusion criteria described in Section 2.1.2.4 was put in place so that a cognitively healthy cohort was utilised.

In brief, the analysed cohort consisted of 298 women of whom 189 were on HRT [age: 63.6 ± 0.4, with 100 on ORT age: 63.4 ± 0.6 and 89 on cHRT (oestrogen and progesterone) age: 63.9 ± 0.6] and 109 were not taking HRT (age: 64.5 ± 0.6). The ORT group was further stratified by dose of oestrogen administered per day [1.875mg (n = 6) age: 65.2 ± 1.0, 1.25mg (n = 64) age: 62.4 ± 0.7, 0.625mg (n = 28) age: 64.6 ± 1.2, 0.3125mg (n = 2) age: 71.0 ± 0.0]. Leukocyte DNA was extracted from peripheral blood samples as described in Section 2.2.2. APOE genotype was determined using the PCR protocol as described in Section 2.2.4.1.

6.3 RESULTS

6.3.1 No Effect of HRT on Global Measures of Cognition.

Within the post-menopausal cohort APOE genotype distribution was consistent with that predicted by Hardy-Weinberg equilibrium ($\chi^2$ test; p = 0.49). Initial stratification was via use/non-use of HRT (irrespective of type and APOE e4 status) and separately by the presence or absence of the e4 allele (irrespective of HRT status; Table 6.1). HRT
Table 6.1: Baseline demographics for the post-menopausal cohort. (Stratified by HRT use and APOE ε4 allele)

Global measures of cognition were determined in age, education and higher education matched HRT users/non-users (also matched for ε4 carriage) and APOE carriers/non-carriers. No significant difference in global measures of cognition was observed between HRT users and non-users or between ε4 carriers and non-carriers.

HRT = Hormone Replacement Therapy; Education = Age at leaving school (years); High-Ed = Number of years of higher education; MMSE = Mini Mental State Examination; CAMCOG = Cognitive component of the Cambridge Examination for Mental Disorders of the Elderly; All values are mean ± SEM (standard error of the mean).

* Presence (pos.) / absence (neg.) of the ε4 allele irrespective of HRT status.

Mann-Whitney U-Test was used for comparisons of means. Pearson’s $\chi^2$ test, used for comparison of APOE ε4 frequency.

1$^{\epsilon 4}$ Allele Freq  Non HRT vs. HRT: $p = 0.59 \ (\chi^2 = 0.29)$

2CAMCOG  Non HRT vs. HRT: $p = 0.53 \ (z = -0.63)$

$\epsilon 4$ vs. Non-$\epsilon 4$: $p = 0.73 \ (z = -0.35)$

3MMSE  Non HRT vs. HRT: $p = 0.39 \ (z = -0.87)$

$\epsilon 4$ vs. Non-$\epsilon 4$: $p = 0.28 \ (z = -1.08)$
Table 6.2: Global measures of cognition in HRT users/non-users (Stratified by APOE e4 allele)

Global measures of cognition were determined in age, education and higher education matched HRT users/non-users stratified by the presence/absence of the APOE e4 allele. HRT use did not significantly increase performance on global measures of cognition in e4 carriers or non-carriers. The presence of the e4 allele had no significant bearing on scores when HRT use was controlled for.

All values are mean ± SEM (standard error of the mean).

Mann-Whitney U-Test was used for comparisons of means.

Effect of HRT use:  
- e4 neg. (Non HRT vs. HRT) CAMCOG $p = 0.70$ ($z = -0.39$) 
- MMSE $p = 0.50$ ($z = -0.68$)  
- e4 pos. (Non HRT vs. HRT) CAMCOG $p = 0.63$ ($z = -0.48$) 
- MMSE $p = 0.55$ ($z = -0.59$)  

Effect of e4 Allele:  
- Non HRT (e4 pos vs. e4 neg) CAMCOG $p = 0.98$ ($z = -0.03$) 
- MMSE $p = 0.57$ ($z = -0.56$)  
- HRT (e4 pos vs. e4 neg) CAMCOG $p = 0.70$ ($z = -0.39$) 
- MMSE $p = 0.36$ ($z = -0.92$)
users and non-users were matched for age at venipuncture, age at leaving school and years of higher education. The frequency of the ε4 allele was similar in both groups (HRT: 14.0% vs. Non HRT: 15.6%; p = 0.59, $\chi^2 = 0.29$). Likewise, ε4 carriers/non carriers were similarly matched. Comparisons of global measures of cognition, via the Mann-Whitney U Test, between HRT and non-HRT groups revealed no significant difference in levels of performance on either the MMSE ($p = 0.39, z = -0.87$) or the CAMCOG ($p = 0.53, z = -0.63$). The presence of the ε4 allele did not impart any effect on global measures of cognition either (MMSE: $p = 0.28, z = -1.08$; CAMCOG: $p = 0.73, z = -0.35$).

HRT users and non-users were then stratified by the presence or absence of the ε4 allele of APOE (Table 6.2). This was done to determine if there was any impact of the ε4 allele on the efficacy of HRT on global measures of cognition and to determine if there was a difference in the level of performance within HRT groups attributable to ε4 possession. Whilst the use of HRT was associated with marginally elevated mean scores on the CAMCOG in both ε4 carriers and non-carriers this was negligible and by no means a significant improvement in performance (ε4 pos: $p = 0.63, z = -0.48$; ε4 neg: $p = 0.70, z = -0.39$). Furthermore, HRT use was not associated with increased scores on the MMSE in either ε4 carriers ($p = 0.55, z = -0.59$) or non-carriers ($p = 0.50, z = -0.68$). Likewise, within HRT groups the lack of an ε4 allele made no difference to the level of performance on either the CAMCOG (Non HRT: $p = 0.98, z = -0.03$; HRT: $p = 0.70, z = -0.39$) or MMSE (Non HRT: $p = 0.57, z = -0.56$; HRT: $p = 0.36, z = -0.92$).
6.3.2 No Effect of ORT or cHRT on Global Measures of Cognition.

The post-menopausal cohort was placed into three groups based on the absence of HRT or the use of either ORT or a combination of oestrogen and progesterone (cHRT; Table 6.3). This was done to determine whether global measures of cognition differed between the HRT groups (ORT, cHRT and Non-HRT) and whether opposing ORT with progesterone changed the level of performance compared to unopposed ORT. Each group was matched for age, age at leaving school, years of higher education and ε4 frequency.

The comparison of mean CAMCOG scores between the three groups revealed that post-menopausal women using ORT tended to perform better on the CAMCOG than non-HRT women, whilst cHRT users tended to perform the worst. However, this trend was marginal and not significant after correcting for multiple comparisons. The closest to statistical significance that was seen after correcting for multiple comparisons (Bonferroni) was between ORT and cHRT users, where ORT users tended to score better but did not reach statistical significance (p = 0.47). Similarly there was no statistically significant difference in performance on the MMSE between the three groups, once again the trend was for women on cHRT to score marginally worse than women on ORT or women not taking HRT.

The three groups were then stratified by the presence or absence of the ε4 allele of APOE (Table 6.4) so as to determine if there was any impact of the ε4 allele on the efficacy of either ORT or cHRT on global measures of cognition and to determine if there was a difference in the level of performance within these groups attributable to ε4 possession. One way ANOVA with post-hoc analysis, using Bonferroni correction for
Table 6.3: Effect of HRT type on global measures of cognition

Global measures of cognition were determined in age, education, higher education and APOE ε4 matched HRT users/non-users stratified by the type of HRT used. Women on ORT tended to perform the best however there was no statistically significant difference in the performance on global measures of cognition between the three groups.

ORT = Oestrogen Replacement Therapy; cHRT = Combined Hormone (oestrogen + progesterone) Replacement Therapy; All values are mean ± SEM (standard error of the mean).

Pearson’s χ² test used for comparison of APOE ε4 frequency.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age</th>
<th>Education</th>
<th>High-Ed</th>
<th>CAMCOG²</th>
<th>MMSE³</th>
<th>ε4 Allele Freq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non HRT</td>
<td>109</td>
<td>64.5 ± 0.6</td>
<td>15.3 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>99.3 ± 0.4</td>
<td>29.0 ± 0.1</td>
<td>15.6%</td>
</tr>
<tr>
<td>ORT</td>
<td>100</td>
<td>63.4 ± 0.6</td>
<td>15.1 ± 0.3</td>
<td>2.2 ± 0.2</td>
<td>99.9 ± 0.4</td>
<td>28.9 ± 0.2</td>
<td>14.5%</td>
</tr>
<tr>
<td>cHRT</td>
<td>89</td>
<td>63.9 ± 0.6</td>
<td>14.9 ± 0.4</td>
<td>2.1 ± 0.2</td>
<td>99.0 ± 0.4</td>
<td>28.8 ± 0.2</td>
<td>13.5%</td>
</tr>
</tbody>
</table>

¹ε4 Allele Freq

- Non HRT vs. ORT: \( p = 0.75 \) (\( χ² = 0.10 \))
- Non HRT vs. cHRT: \( p = 0.55 \) (\( χ² = 0.35 \))

Bonferroni correction for multiple comparisons was applied in the analysis of the effect different HRT types (or lack of HRT) on CAMCOG and MMSE scores.

²CAMCOG:

- Non HRT vs. ORT: \( p = 0.89 \)
- Non HRT vs. cHRT: \( p = 1.00 \)
- cHRT vs. ORT: \( p = 0.47 \)

³MMSE:

- Non HRT vs. ORT: \( p = 1.00 \)
- Non HRT vs. cHRT: \( p = 0.87 \)
- cHRT vs. ORT: \( p = 0.63 \)
Table 6.4: Effect of HRT type on global measures of cognition (Stratified by APOE ε4 allele)

The type of HRT regimen used did not significantly alter performance on global measures of cognition in ε4 carriers or non carriers. The presence of the ε4 allele had no significant bearing on scores when HRT type was controlled for. All values are mean ± SEM. Bonferroni correction for multiple comparisons was applied in the analysis of the effect different HRT types on CAMCOG and MMSE scores.

Effect of HRT type: ε4 neg.(Non HRT vs ORT vs cHRT) CAMCOG/MMSE: $p = 1.00$

\[
\begin{align*}
\text{ε4 pos.} (\text{Non HRT vs ORT}) & \quad \text{CAMCOG:} & p = 1.00 \\
\text{ε4 pos.} (\text{Non HRT vs cHRT}) & \quad \text{CAMCOG:} & p = 1.00 \\
\text{ε4 pos.} (\text{cHRT vs ORT}) & \quad \text{CAMCOG:} & p = 0.62 \\
\text{ε4 pos.} (\text{Non HRT vs ORT vs cHRT}) & \quad \text{MMSE:} & p = 1.00
\end{align*}
\]

Mann-Whitney U-Test was used for the analysis of the effect of the ε4 allele (when HRT type was controlled for) on CAMCOG and MMSE scores.

Effect of ε4 Allele: ORT (ε4 pos vs. ε4 neg) CAMCOG $p = 0.51$ ($z = -0.66$) MMSE $p = 0.22$ ($z = -1.22$)

\[
\begin{align*}
\text{cHRT (ε4 pos vs. ε4 neg)} & \quad \text{CAMCOG:} & p = 0.77 (z = -0.30) \\
& \quad \text{MMSE:} & p = 0.92 (z = -0.11)
\end{align*}
\]
multiple comparisons, revealed that the performance of non-\(\varepsilon 4\) women on both the CAMCOG and MMSE was basically unchanged (\(p = 1.00\), all test combinations) although women on ORT did have a marginally higher mean score on the CAMCOG. Likewise, in \(\varepsilon 4\) carriers there was negligible difference across all scores on the measures of global cognition (CAMCOG - Non HRT vs ORT, cHRT: \(p = 1.0\), ORT vs cHRT \(p = 0.62\); MMSE - all test combinations: \(p = 1.00\)) although once again the CAMCOG scores of women on ORT were marginally higher. Furthermore, comparisons of mean scores within the groups, using the Mann-Whitney U test, revealed that the lack of an \(\varepsilon 4\) allele made no difference to the level of performance on either the CAMCOG (ORT: \(p = 0.51\), \(z = -0.66\); cHRT: \(p = 0.77\), \(z = -0.30\)) or MMSE (ORT: \(p = 0.22\), \(z = -1.22\); cHRT: \(p = 0.92\), \(z = -0.11\)).

### 6.3.3 ORT Dose has no Differential Effect on Global Measures of Cognition.

Post-menopausal women who were taking ORT seemed, although marginally, to perform the best on global measures of cognition. As such women that were taking oestrogen and progesterone (cHRT) were removed from the cohort at this point and the remaining women on ORT were sub-divided into different groups based on equivalent dosages of ORT taken per day (Table 6.5). This was done to determine whether scores on global measures of cognition differed according to the level of ORT taken. Each group was matched for age, age at leaving school, years of higher education and \(\varepsilon 4\) frequency.

Mean performances on both the CAMCOG and MMSE seemed to improve marginally with increasing dosage of oestrogen per day. Individuals taking either 1.875 or
1.25mg/day tended to perform marginally better than women not taking HRT whilst women taking the lower doses (0.625 and 0.3125mg/day) seemed not to benefit at all from this level of ORT. However, statistical analysis of this data revealed that this trend did not approach significance in either the CAMCOG \( (p = 1.0, \text{ all comparisons}) \) or the MMSE \( (p = 1.0, \text{ all comparisons}) \) after utilising Bonferroni correction for multiple comparisons.

All groups were then stratified by the presence or absence of the \( e4 \) allele of \textit{APOE} (Table 6.6) to determine if there was any impact of the \( e4 \) allele on the efficacy of different doses per day of ORT on global measures of cognition. Likewise, to determine if there was a difference in the level of performance within these groups attributable to possession of the \( e4 \) allele of \textit{APOE}. Women on doses of 1.875 or 0.3125mg/day, who carried an \( e4 \) allele, were removed from analysis due to insufficient numbers. After correcting for multiple comparisons there was no significant difference in the performance on the CAMCOG or MMSE between different doses in non-\( e4 \) women (CAMCOG/MMSE: \( p = 1.0, \text{ all comparisons} \)). Although women on the two higher doses (1.875 and 1.25mg/day) had marginally higher mean scores on the CAMCOG. Similarly in \( e4 \) carriers, women on a dose of 1.25mg/day had a marginally higher mean performance on the CAMCOG, however, statistical analysis revealed that scores on both the CAMCOG and MMSE were not significantly different (CAMCOG: \( p = 1.0, \text{ all comparisons} \); MMSE: Non HRT vs ORT\(_{1.25} \) \( p = 1.00 \), Non HRT vs ORT\(_{0.625} \) \( p = 0.28 \), ORT\(_{1.25} \) vs ORT\(_{0.625} \) \( p = 0.27 \)). Furthermore, analysis of mean scores within each dose group revealed that the lack of an \( e4 \) allele had no bearing on the level of performance on the CAMCOG (ORT\(_{1.825} \): \( p = 0.14, z = -1.46 \); ORT\(_{1.25} \): \( p = 0.91, z = -0.11 \); ORT\(_{0.625} \): \( p = 0.51, z = -0.66 \)) or MMSE (ORT\(_{1.825} \): \( p = 0.49, z = -0.69 \); ORT\(_{1.25} \): \( p = 0.33, z = -0.98 \); ORT\(_{0.625} \): \( p = 0.29, z = -1.06 \)).
Table 6.5: Effect of ORT dose on global measures of cognition

Global measures of cognition were determined in ORT users (stratified by the dose of HRT) and age, education, higher education and APOE ε4 matched non HRT users. Women on ORT₁₈₇₅ or ORT₁₂₅ tended to perform the best however there was no statistically significant difference in the performance on global measures of cognition between different dosages.

ORTₙₐₜₜ = Oestrogen Replacement Therapy dosage in mg/day; All values are mean ± SEM.

Pearson’s χ² test / Fisher’s exact test were used for comparison of APOE ε4 frequency.

¹ε4 Allele Freq \( \chi^2 = 0.34 \) \( P = 0.56 \)

²CAMCOG: All tests: \( p = 1.00 \)

³MMSE: All tests: \( p = 1.00 \)
<table>
<thead>
<tr>
<th>Group</th>
<th>APOE ε4</th>
<th>N</th>
<th>CAMCOG</th>
<th>MMSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non HRT</td>
<td>Present</td>
<td>32</td>
<td>99.0 ± 0.4</td>
<td>28.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>77</td>
<td>99.4 ± 0.4</td>
<td>29.0 ± 0.1</td>
</tr>
<tr>
<td>ORT&lt;sub&gt;1.875&lt;/sub&gt;</td>
<td>Present</td>
<td>1</td>
<td>103</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>5</td>
<td>100.0 ± 0.7</td>
<td>29.4 ± 0.4</td>
</tr>
<tr>
<td>ORT&lt;sub&gt;1.25&lt;/sub&gt;</td>
<td>Present</td>
<td>19</td>
<td>100.4 ± 0.9</td>
<td>29.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>45</td>
<td>100.2 ± 0.7</td>
<td>29.0 ± 0.2</td>
</tr>
<tr>
<td>ORT&lt;sub&gt;0.625&lt;/sub&gt;</td>
<td>Present</td>
<td>8</td>
<td>99.1 ± 2.4</td>
<td>28.0 ± 0.7</td>
</tr>
<tr>
<td></td>
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<td>20</td>
<td>99.0 ± 0.9</td>
<td>28.8 ± 0.3</td>
</tr>
<tr>
<td>ORT&lt;sub&gt;0.3125&lt;/sub&gt;</td>
<td>Present</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>2</td>
<td>98.0 ± 1.0</td>
<td>28.5 ± 1.5</td>
</tr>
</tbody>
</table>
Table 6.6 (adjacent): Effect of ORT dose on global measures of cognition (Stratified by APOE ε4 allele)

Type of HRT used did not significantly alter performance on global measures of cognition in ε4 carriers or non carriers. The presence of the ε4 allele had no significant bearing on scores when HRT type was controlled for. All values are mean ± SEM

* Not included in analysis of dosages due to insufficient numbers

** Not included in analysis of dosages or ε4 effect due to insufficient numbers

Bonferroni correction for multiple comparisons was applied in the analysis of the effect different HRT dosages on CAMCOG and MMSE scores.

Effect of HRT type: ε4 neg. (Non HRT vs All ORT doses) CAMCOG/MMSE: p = 1.00

ε4 pos. (Non HRT vs All ORT doses) CAMCOG: p = 1.00

ε4 pos. (Non HRT vs ORT1.25) MMSE: p = 1.00

ε4 pos. (Non HRT vs ORT0.625) MMSE: p = 0.28

ε4 pos. (ORT1.25 vs ORT0.625) MMSE: p = 0.27

Mann-Whitney U-Test was used for the analysis of the effect of the ε4 allele (when HRT type was controlled for) on CAMCOG and MMSE scores.

Effect of ε4 Allele: ORT1.825 (ε4 pos vs. ε4 neg) CAMCOG p = 0.14 (z = -1.46) MMSE p = 0.49 (z = -0.69)

ORT1.25 (ε4 pos vs. ε4 neg) CAMCOG p = 0.91 (z = -0.11) MMSE p = 0.33 (z = -0.98)

ORT0.625 (ε4 pos vs. ε4 neg) CAMCOG p = 0.51 (z = -0.66) MMSE p = 0.29 (z = -1.06)
6.4 DISCUSSION

6.4.1 HRT Use was not Associated with Improved Performance on Global Measures of Cognition

The association of ORT and cognitive performance has been evaluated in several observational studies of post-menopausal healthy woman. However, consensus is yet to be reached on whether the use of ORT improves cognition in healthy post-menopausal woman. This chapter reports no significant differences in levels of performance on either the MMSE or the CAMCOG between HRT users and non-users in a cohort of cognitively healthy post-menopausal women. This finding was also the case in other studies (Kimura, 1995; Maki et al., 2000; Paganini-Hill and Henderson, 1994) whilst inconclusive findings have also been reported (Kampden and Sherwin, 1994; Robinson et al., 1994). However, there is still a large body of evidence that suggests that there is an increased risk of developing AD in women who have not taken HRT and that in post-menopausal women the use of HRT is associated with improved cognitive performance on a range of measures (Kimura, 1995; Maki et al., 2000; Yaffe et al., 2000).

Possible contributing factors for the inconclusive data is the impact of confounding issues such as depression as well as the impact of genetic factors, such as the ε4 allele of the APOE gene, which has been associated with memory decline in non-demented individuals (Dik et al., 2000; Deary et al., 2002), preclinical memory impairment in asymptomatic middle aged individuals (Flory et al., 2000; Caselli et al., 2001) and a high degree of association with subjective memory complaints and conversion to MCI.
or AD (as reported in the previous chapter, Laws et al., 2002a). However, in this cognitively healthy cohort (devoid of subjective memory complainers), there was no observable differences on global measures of cognition between carriers and non-carriers of the *APOE* ε4 allele.

A putative *pharmacogenetic* hypothesis for the effect of the ε4 allele of *APOE* on the therapeutic efficacy of HRT has been put forward, in which a theory of ‘responders’ and ‘non-responders’ to the benefits of HRT has been postulated. This theory is supported by Yaffe and colleagues (2000) whose study reports the cognitive benefits of ORT but suggests that these benefits, although still present in ε4 carriers, is greatest in those not possessing the *APOE* ε4 allele. This chapter however, does not observe any therapeutic benefits of HRT, on global measures of cognition, in relation to the presence or absence of the ε4 allele. In fact no significant difference in the level of cognitive performance was observed in either ε4 carriers or non-carriers, although CAMCOG scores were marginally higher in HRT users in both ε4 and non-ε4 women. Likewise, within HRT groups the lack of an ε4 allele made no difference to the level of global cognitive performance.

Whilst the findings from this section of analysis does not support the notion of improved cognition amongst HRT takers it must be noted that no differentiation was made between the use of unopposed oestrogen therapy (oestrogen only or ORT) and opposed (oestrogen plus progesterone or cHRT). The inclusion of women using these substances in combination with ORT in studies assessing the benefits of oestrogen on memory has previously been criticised as a confounding factor (Yaffe et al., 1998). There is significant literature to support the notion that oestrogen only therapy may be beneficial for improving cognition, as opposed to cHRT. This is supported by profound
neurobiological evidence of oestrogen's neuroprotective role. As such further analysis was undertaken in which HRT use was separated into ORT and cHRT.

6.4.1.1 Neither ORT nor cHRT improved cognitive performance: No differential effect of ORT vs. cHRT.

There is strong epidemiological evidence to support the theory that ORT is beneficial in improving the cognitive performance in healthy post-menopausal women (Henderson et al., 1996; Robinson et al., 1994; Steffens et al., 1999; Jacobs et al., 1998) and this is further supported by numerous neurobiological benefits attributed to oestrogen. However, numerous post-menopausal women, in particular non-hysterectomised women, take a progestogen in conjunction with oestrogen supplementation (sometimes referred to as opposed oestrogen therapy, in this chapter it is referred to as combined HRT or cHRT) to protect against any potential deleterious effects of oestrogen. It has been postulated that progestins may mitigate the cognitive benefits of oestrogens via anxiolytic, sedative or mood altering effects (Halbreich, 1997) and epidemiological studies have suggested a detrimental effect on cognitive performance (Paganini-Hill and Henderson, 1996) and a greater rate of decline (Rice et al., 2000). However, progesterone may itself offer beneficial neurological properties (McEwen and Woolley, 1994) and it has been reported that there is a positive correlation between endogenous levels of pregnanediol on enhanced perceptual and working memory functioning (Graham and Glasser, 1985). As such the lack of controlling for its use in epidemiological studies of oestrogens cognitive benefits is a common criticism (Yaffe et al., 1998).

In this chapter, the comparison of mean CAMCOG scores between non HRT, ORT and cHRT revealed that post-menopausal women using ORT tended to perform better on the CAMCOG than non HRT women, whilst cHRT users tended to exhibit the lowest
scores. However, this trend was marginal and not significant after correcting for multiple comparisons. Similarly there was no statistically significant difference in performance on the MMSE between the three groups. Once again the trend was for women on cHRT to score marginally worse than women on ORT or women not taking HRT. This lack of association was not due to differential therapeutic efficacy in relation to the ε4 allele of APOE, as the performance of non-ε4 women on both the CAMCOG and MMSE was basically unchanged although women on ORT did have a marginally higher mean score on the CAMCOG. Likewise, in ε4 carriers there was negligible difference across all scores on the measures of global cognition although once again the CAMCOG scores of women on ORT were marginally higher. Whilst these findings are far from conclusive the trend indicates that the therapeutic efficacy of ORT use and cHRT use may differ. As such there is a need to differentiate between opposed and unopposed oestrogen use in epidemiological studies of the therapeutic efficacy of ORT.

6.4.1.2 Dose of oestrogen had no differential effect on cognitive performance

There is evidence to suggest that the therapeutic efficacy of ORT in delaying the onset of preclinical cognitive decline and AD is dose dependent (Paganini-Hill & Henderson, 1996). Tang and colleagues (1996a) suggest that one standard dose of conjugated oestrogen is therapeutically effective. The ORT users were stratified by four levels of daily dosage of oestrogen only. This was done to determine whether performance on global measures of cognition differed according to the daily dose of oestrogen. Mean performances on global measures improved marginally with increasing daily oestrogen dose. Post-menopausal women taking either 1.875 or 1.25mg/day tended to perform marginally better, though not statistically so, than women not taking ORT. This finding, although not significant tends to support the theory of a dose dependent benefit of ORT. Interestingly, women taking lower daily doses (0.625 and 0.3125mg/day) seemed to
perform equally well or less than that of women not taking ORT. There was no impact of the e4 allele of APOE observed with both carriers and non-carriers alike performing to similar levels and exhibiting the same marginal trend towards increased cognitive performance with increasing daily ORT dose.

Firm conclusions on the data presented in this chapter can not be drawn. However, there is a general marginal trend towards supporting the hypothesis that HRT, in particular ORT, is beneficial for increasing cognitive performance. No effect of the e4 allele of APOE on the therapeutic efficacy of HRT, or ORT, was observed. A longitudinal study of this cohort is required to truly elucidate the efficacy of oestrogen on cognitive performance. However, limitations in this cross-sectional study need to be addressed in order to gain maximum benefit from future studies with this cohort.

6.4.2 Rationale for Inconclusive Findings: Study Limitations.

On first glance the findings presented here suggest that the therapeutic efficacy of ORT in cognitively healthy post-menopausal women is, at best, inconclusive. Nevertheless, the general consensus in the literature is for a role for ORT in enhancing cognitive performance and possibly curtailing the insidious decline towards cognitive impairment and AD (Henderson et al., 1996; Robinson et al., 1994; Steffens et al., 1999; Jacobs et al., 1998). However, numerous methodological limitations have also been associated with a majority of studies investigating the therapeutic efficacy of ORT. This calls into question the ability to draw firm conclusions on the benefits of ORT. These limitations include inadequate methodological controls; such as controlling for the form of oestrogen preparation, the use of progestins, the duration of therapy, the dosage of oestrogen and endogenous oestrogen levels; and limitations set by employing crude
global cognitive assessments in place of domain specific measures of cognition. Some of these limitations can be seen to have manifested to some degree in the present study even though attempts were made to exclude them. On retrospection, inadequate controlling for endogenous oestrogen [by either measurement of circulating levels or differentiation of naturally, surgically or chemically induced menopause (Nappi et al., 1999)], length of treatment (Paganini-Hill & Henderson, 1996; Tang et al., 1996a) and the preparation of oestrogen (Troy et al., 1994) may have impinged on the findings. Furthermore, the use of the MMSE and the CAMCOG for assessing cognitive performance may explicate the inconclusive findings.

The earliest stage of cognitive decline towards AD is believed to manifest itself in the form of episodic memory loss, followed by a progressive decline in the domains of semantic memory, attention, language, executive function, visuo-spatial, perceptual abilities and abstract thinking (Perry et al., 2000; Elias et al., 2000). Global measures of cognition such as the CAMCOG and MMSE have been shown to be sensitive enough to discriminate between individuals with AD and cognitively intact individuals and even, as seen in Chapter 5, subjective memory complainers from controls. However, they are reported to be insensitive for the detection of differences in levels of cognitive performance amongst cognitively healthy individuals (Pasquier, 1999). The previous chapter is a case in point; the use of crude global measures of cognition (in this case CAMCOG and MMSE scores) was not sufficient for discerning a decline towards, or conversion to, MCI or AD. Rather the use sensitive domain-specific cognitive measures were required.

There is a significant body of literature supporting oestrogen’s cognitive benefit in specific cognitive domains. A prime example of this is the numerous studies of
oestrogen's effects on verbal learning and memory. Lifetime levels of oestrogen appear to influence verbal functioning (Smith et al., 1999) whilst oestrogen treatment of AD affected women has been shown to enhance verbal memory (Asthana et al., 1999; Asthana et al., 2001). Verbal learning and memory performance has been shown to be at its greatest, in pre-menopausal women, during the high oestrogen level follicular phase of the reproductive cycle (Hampson, 1990) whilst ORT in young girls with Turner's syndrome (Ross et al., 2000) and in transsexual men (Miles et al., 1998) resulted in improved performance on verbal memory and tasks requiring verbal abilities.

These studies suggest that further follow up studies utilising this post-menopausal cohort would be best served with the inclusion of measures of verbal learning and memory. Tests that may be appropriate for use in such studies include the California Verbal Learning Test (CVLT; Delis et al., 1987) or the Rey Auditory Verbal Learning Test (RAVLT; Rey, 1964). Additionally, there is evidence to suggest that oestrogen may effect other domains of cognition such as working memory (Berman et al., 1997; Shaywitz et al., 1999) and visual learning and memory (e.g. visuo-spatial functions; Gibbs, 1999; Luine et al., 1998; Duka et al., 2000). The inclusion of sensitive neuropsychological assessments, of some or all of these domains, along with addressing other methodological limitations should allow more meaningful conclusions to be drawn from future longitudinal studies with this cohort.

6.4.3 Summary

Whilst there was a marginal trend towards supporting the notion that HRT (in particularly ORT) may provide cognitive benefits, the data presented in this chapter is far from conclusive and can not allow for either firm conclusions to be drawn on the
therapeutic efficacy of ORT or either the supporting or the rejecting of the stated hypotheses. However, the lack of conclusive findings in this study, on retrospection, may be due to the limitations discussed herein. The identification of these limitations will allow future longitudinal follow-up studies, which use sensitive measures of cognition, to take place. Adopting these strategies should allow meaningful conclusions to be drawn. Such studies are paramount for determining whether or not ORT yields benefits in cognitively healthy post-menopausal women and whether or not the APOE ε4 allele confers a pharmacogenetic effect on oestrogen’s therapeutic efficacy.
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7.1 DEGREE OF AD RISK VARIES BETWEEN APOE LOCI

The genetic association of the ε4 allele of the APOE gene with AD is definitely the strongest identified to date. Since the initial analysis of families with LOAD resulted in the identification of this disease locus (Pericak-Vance et al., 1991) and the initial association of the ε4 allele with an increase risk of AD (Strittmatter et al., 1993) the overwhelming consensus in the literature is that the APOE ε4 allele is the major genetic risk factor for this form of AD (Corder et al., 1993; Lucotte et al., 1993; Mayeux et al., 1993b; Strittmatter et al. 1993; Saunders et al, 1993a; Saunders et al, 1993b; Dai et al. 1994; Houlden et al, 1994; Payami et al., 1994; Martins et al., 1995a; Adorer et al., 1995; Basun et al., 1995; Frisoni et al., 1995; Okuizumi et al., 1995; Hyman et al., 1996). This thesis presents data (Chapter 3) that supports this association and reflects the common consensus that approx 50% of AD cases can be attributed to this allele [see Farrer et al., 1997 for a meta-analysis of these data]. The suggested gene dosage effect in both familial (Corder et al., 1993) and sporadic (Frisoni et al., 1995) AD is also clearly evident in this study with marked over-representation of homozygosity in AD cases and the marked increases in relative risk with increasing copies of the ε4 allele. Additionally, the APOE ε4 allele has been reported too be associated with memory decline in non-demented individuals (Dik et al., 2000; Deary et al., 2002) and preclinical memory impairment in asymptomatic middle aged individuals (Flory et al., 2000; Caselli et al., 2001). The data presented in Chapter 5 are consistent with these recent reports and illustrate that pre-clinical AD individuals who possess the APOE ε4
allele will convert to MCI or AD at a much higher rate than any other single genetic loci.

In conclusion, the ε4 allele of APOE remains the major genetic risk factor for AD and also a major risk for cognitive decline leading to AD; however, it is also widely accepted in the literature that it is not deterministic, unlike the highly penetrant PS1 and APP mutations that result in the development of an autosomal dominant form of EOAD. Thus, interest has turned to other genetic risk factors which include the polymorphic promoter loci reported in this thesis.

7.1.1 APOE -491 Promoter Locus and AD Risk

The initial association of the –491A allele with the development of AD (Bullido et al., 1998; Lambert et al., 1998a) or conversely the protective effect of the –491TT genotype has been verified in clinical (Town et al., 1998; Chapter 3: Laws et al., 1999; Casadei et al., 1999; Alvarez-Arcaya et al., 2001) and post-mortem studies (Ahmed et al., 1999; Chapter 4: Laws et al., 2002b). However, some studies have reported no association of the –491A/T promoter with LOAD in Caucasian (Song et al., 1998; Helisalmi et al., 1999; Thome et al., 1999; Zurutuza et al., 2000) and Asian populations (Chen et al., 1999; Toji et al., 1999). As eluded to in Section 3.4.1.2 the clinical significance of one study (Thome et al., 1999), where AD individuals were compared with psychiatric inpatients instead of cognitively healthy controls (a possible confounder of effect sizes), may be brought into question or at the least its validity for comparison to the larger body of literature where healthy controls are used. In the two studies of Asian populations (Chen et al., 1999; Toji et al., 1999) the lack of association may be accounted for by the high frequency of the –491AA genotype in the respective control
groups. This point is supported by a recent post-mortem study in the Japanese population where all 47 cases were positive for the AA genotype (Yamagata et al., 2001).

It has been suggested that the -491A allele is not independently associated with AD but is rather associated due to linkage disequilibrium with the APOE ε4 allele. However, the -491A allele has been shown to be associated with both APOE ε4 carriers and non-carriers, suggesting that this genotype causes an independent risk for AD (Town et al., 1998). Subsequently studies report that whilst the -491-AD association could be partly accounted for by this linkage it still remained in non-APOE ε4 individuals (Bullido et al., 2000; Wang et al., 2000). Log-linear and logistic regression analysis has also concluded that -491AA genotype is likely to be an independent risk factor for AD, since no interaction was detected between APOE ε4 and -491AA for modifying risk for AD (Wang et al., 2000). Findings presented in Chapter 3 (Laws et al., 1999) tend to support the notion of an independent effect as a significant association remained even after controlling for carriage of the APOE ε4 allele.

What is evident in the literature is that both the -491AA genotype and the -491A allele have a common propensity for an increased frequency in the non-Asian AD population. Overall, frequencies are enriched in AD individuals in all ethnic groups (Table 7.1) with the exception of one Chinese (Chen et al., 1999) and one Japanese cohort (Toji et al., 1999). It is also apparent that there is a variable frequency of this genotype in AD (0.63 – 0.93; mean 0.78), but variability is even greater in control populations (0.50 – 0.96; mean 0.67). In the Caucasian studies that utilise healthy controls, this variability is somewhat attenuated in both AD (0.67 – 0.87; mean 0.77) and control (0.5 – 0.77; mean 0.64) subjects. It is apparent that partial linkage disequilibrium does exist between the
### Table 7.1: Summary of –491A/T APOE promoter polymorphism genotype and allele frequencies in AD patients and controls.

<table>
<thead>
<tr>
<th>Country</th>
<th>n</th>
<th>AA</th>
<th>AT</th>
<th>TT</th>
<th>A</th>
<th>T</th>
</tr>
</thead>
<tbody>
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<td>C</td>
<td>AD</td>
<td>C</td>
<td>AD</td>
<td>C</td>
</tr>
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<td>0.96</td>
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</table>

¹Bullido et al., 1998; ²Lambert et al., 1998a; ³Song et al., 1998; ⁴Town et al., 1998; ⁵Roks et al., 1998; ⁶Helisalmi et al., 1999; ⁷Laws et al., 1999; ⁸Thome et al., 1999; ⁹Casadei et al., 1999; ¹⁰Toji et al., 1999; ¹¹Chen et al., 1999; ¹²Ahmed et al., 1999; ¹³Bullido et al., 2000; ¹⁴Wang et al., 2000a; ¹⁵Zurutuza et al., 2000; ¹⁶Alvarez-Arcaya et al., 2001
-491A and APOE ε4 alleles and that this linkage does account for part of the association of -491 with AD. The degree of linkage disequilibrium varies from population to population and this, combined with the variable frequency of the -491AA genotype between populations, may account for the lack of association of the -491A/T promoter polymorphism with AD in some studies.

Additionally, this thesis reports (Chapter 5; Laws et al., 2002a) that the -491A allele is significantly over-represented in a pre-clinical cohort of “memory complainers”, though not to the same degree as the APOE ε4 allele. This is the first data in the literature looking at the possible association of the -491A/T promoter polymorphism with cognitive impairment in a pre-clinical cohort. Although the -491A allele was over-represented it had, unlike the ε4 allele, no apparent effect on global cognitive performance, as measured by the CAMCOG and MMSE. However, it did impart a gene dosage effect on the rate of conversion to clinical endpoints. Homozygosity at both loci, although representing only a small proportion, was associated with the greatest rate of conversion suggesting that this dual homozygosity may play an integral role in cognitive decline and may be useful for the identification of individuals at high risk for the development of AD. However, these findings must be tested in a much larger population before definitive conclusions can be drawn.

Overall, these findings suggest that the -491A allele is associated with an increased risk for the development of AD; however, a proportion of this association can be accounted for by partial linkage disequilibrium with APOE ε4. Likewise, this thesis suggests that this genetic locus may have some merit for the identification of at risk individuals, especially in combination with the ε4 allele of APOE. In conclusion, the -491A allele is largely an independent contributor of risk for AD, although not to the same degree as
the ε4 allele, a conclusion supported by the recent meta-analysis performed by Lambert and colleagues (2002).

### 7.1.2 APOE -219 Promoter Locus and AD Risk

Although the -491A/T promoter polymorphism has been extensively studied, the remaining three polymorphisms have received less attention. The -219T/G polymorphism has been studied in two French Caucasian populations where the -219T allele has been reported to be associated with an increased risk for AD. This allele has been proposed to act as a modifier of APOE ε4 risk (Lambert et al., 1998a; Lambert et al., 1998b). However, this effect has been sought but not found in Caucasian populations in the United States (Rebeck et al., 1999) and in the Australian population studied in Chapter 3. The findings from the current study suggest that whilst at face value the frequency of the -219T allele is over-represented in the AD population it is due to linkage with the ε4 allele of APOE. This conclusion is made on the basis of when the population was stratified by the ε4 allele the association with AD disappeared completely, a finding that is supported by the study of Rebeck and colleagues (1999) and subsequently in a large French case-control study (Zurutuza et al., 2000). A subsequent meta-analysis (Lambert et al., 2002, summarised in Table 7.2) of populations from Finland, United Kingdom, France, United States [(two cohorts separate from that of the aforementioned study of Rebeck and colleagues (1999)] and Spain found no association of the -219TT genotype or -219T allele in one of the US populations and in the Spanish population. In all other populations and when all populations were pooled, the -219TT genotype and -219T allele were over-represented in the AD groups. However, no APOE ε4 independent association could be determined,
CHAPTER SEVEN: DISCUSSION

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Table 7.2: Summary of –219T/G APOE promoter polymorphism genotype and allele frequencies in AD patients and controls.

Adapted from Lambert et al., 2002 (Table 4) except ¹Chapter 3 and ²Zurutuza et al., 2000. Data from the study of Rebeck et al., 1999 not included in table as genotype frequency data (sufficient for inclusion) was not presented in the publication.
until only individuals over the age of 81 years were studied. When this analysis was also performed in the population studied in this thesis the first associations of the -219TT genotype and -219T allele were evident; however, only analysis of the allele frequency remained significant when stratified by ε4 status and then only in the absence of the ε4 allele. These findings and the observations of Lambert and colleagues (2002) are supported by the recent study of Heijmans and colleagues (2002) where the -219TT genotype was associated with an increased risk for AD in a population based cohort of subjects aged 85 years or greater. Overall, the findings of this thesis support the notion that in individuals over 81 years the -219T allele does exert some independent effect on increasing risk for AD (Lambert et al., 2002; Heijmans et al., 2002), although this is probably only minor and the majority of any association reported in the literature is due more so with linkage to the ε4 allele rather than the -219T allele itself. More studies need to be undertaken to clarify the impact that this promoter polymorphism has on AD risk.

7.1.3 Future Directions – Genetic Studies

Whilst the ε4 allele of APOE and the -491A allele have been shown in this thesis and in the literature to be independent risk factors for AD, it may be of interest to perform haplotype analysis in a larger population to determine if the presence of the -491A and ε4 on the same chromosome places the individual at greater risk. This would be of interest especially in -491AT/ε4 heterozygote individuals. A recent study examining APOE variation at the sequence haplotype level observed 22 biallelic polymorphisms and 31 haplotypes within a 5.5-kb stretch of DNA, including the four exons of the gene and some upstream sequence (Fullerton et al., 2000). Seven of the 22 variants occurred within the sequence 5' of the transcriptional start site and thus have the potential, like
that hypothesised for the -491 and -219 loci, to alter levels of \textit{APOE} transcription. As such analysis of the validity of these novel variants may be of interest. Additionally, continued longitudinal study of the "memory complainers" cohort with more sensitive and specific measures of cognitive function along with these additional loci warrants further investigation.

\section*{7.2 \textbf{Apolipoprotein E Levels in AD}}

\subsection*{7.2.1 Impact of Promoter Loci on ApoE Levels}

The initial association of the -491A allele with an increased risk for developing AD (Bullido et al., 1998; Lambert et al., 1998a) implied that quantitative variation of isoform expression due to such polymorphisms may contribute to AD risk. This contribution would be in addition to the qualitative effects of the \textit{APOE} alleles. The polymorphisms studied in this thesis (-491A/T and -219G/T) and those not examined (-427T/C and +113C/G) have been proposed to affect the transcriptional activity of the \textit{APOE} gene in both the periphery and the CNS. This hypothesis is supported by findings that the A to T and the T to G substitutions, at -491 and -219, respectively, resulted in altered promoter activity in HepG2 cells (Artiga et al., 1998b) and astrocytoma cells (Artiga et al., 1998a). The mechanism by which these polymorphisms alter the level of activity of transcription is thought to involve differential binding of nuclear proteins. These single nucleotide polymorphisms may result in changes in the secondary structure of the DNA, thus altering the binding of transcription factors to neighbouring regulatory elements. Alternatively, they may affect the direct binding of transcription factors to putative transcription sites within which they are located. The predictive mapping of transcription factor binding sites, through the TFSITES database (EMBL), to each of the
Figure 7.1: Location of predicted transcription factor sites in the APOE promoter.

Transcriptional regulatory elements (*Paik et al., 1988; *Jo et al., 1995) and related transcription factors (*Chang et al., 1990; Berg et al., 1995; Garcia et al., 1996; Salero et al., 2001), promoter polymorphism locations (*Artiga et al., 1998b; Lambert et al., 1998b and *Mui et al., 1996) and TFSITES (EMBL) mapped predicted transcription factor sites (in parentheses) for promoter polymorphisms as described by Bullido and Valdivieso (2000)

four promoter polymorphisms (Bullido and Valdivieso, 2000; Figure 7.1) has lent credibility to this latter hypothesis and puts forward the notion that these promoter polymorphisms are functional in nature.

In vitro cell studies in astrocytoma (Artiga et al., 1998a) and HepG2 (Artiga et al., 1998b) cell lines have provided strong support for this notion. However, very few in
vivo studies have been reported. The first such study is reported in this thesis (Chapter 3; Laws et al., 1999). In this study, the individuals with the –491AA genotype had significantly elevated plasma apoE levels regardless of APOE genotype and AD status. While apoE levels were increased in controls with the –491AA genotype, these levels were higher in AD individuals with the same genotype. This finding has also been recently confirmed in an Italian population (Scacchi et al., 2001). The findings from these studies are consistent with the changes observed in transfection studies in both HepG2 and astrocytoma cells (Artiga et al., 1998a; Artiga et al., 1998b). Additionally this thesis presents the first data on the effect of the -491A allele on cerebral expression of apoE (Chapter 4; Laws et al., 2002b) suggesting that the -491A allele imparts a gene dosage effect on brain levels of apoE in the frontal cortex (Laws et al., 2002b). In addition to its association with elevated apoE levels in both plasma and brain, the -491AA genotype has been independently associated with increased Aβ40, Aβ42 and total Aβ load in AD brains (Lambert et al., 2001). This observation is supported by previous studies that indicate that apoE is required for cerebral amyloidosis in amyloid plaque-forming transgenic mice (Bales et al., 1997; Bales et al., 1999; Holtzman et al 2000a; Holtzman et al., 2000b).

The –219T allele when transfected into HepG2 cells (Artiga et al., 1998b) and U87 astrocytoma cells (Artiga et al., 1998a) was associated with decreased apoE expression as opposed to the -491A allele which has been implicated in elevating apoE expression. In turn, in the control group of the myocardial infarction study of Lambert and colleagues (2000) the -219T allele was associated with decreased levels of plasma apoE and this effect of the -219T allele was dose dependent (Lambert et al., 2000). In this thesis, however, the findings of Artiga and colleagues (1998a) and Lambert and colleagues (2000) could not be replicated with respect to plasma apoE levels (Chapter
3). However, in Chapter 4 the -219T allele was associated with decreasing apoE levels in a gene dose dependent manner, supporting the earlier reports (Artiga et al., 1998a; Lambert et al., 2000), although no effect of this polymorphism on AD risk was seen. These findings coupled with the suggested association of the -219T allele with increased risk for AD, although observed to be marginal in this thesis, brings with it an interesting conundrum. The -491A allele is associated with increased risk for AD and increased apoE levels in plasma, whilst the -219T allele is associated with increased AD Risk but decreased apoE levels. This suggests that over- and/or under-expression of apoE may be of importance to AD risk.

7.2.2 ApoE Levels: Too Much or Not Enough?

This thesis presents data (Chapter 4: Laws et al., 2002b) that suggests that apoE levels in the frontal cortex of AD cases are significantly elevated over controls. This finding is supported by reports that mRNA is increased in the frontal and temporal cortex (Yamada et al., 1995; Yamagata et al., 2001) as well as in the hippocampus (Zarow and Victoroff, 1998) of neuropathologically confirmed AD cases. This is further supported by increased soluble levels in the cerebellar cortex and increased bound apoE levels in the frontal cortex of AD brains (Pirtilla et al., 1996). However, other studies have not observed this increased level of apoE in AD cases, reporting either no change (Harr et al., 1996) or actually a decrease in apoE level (Bertrand et al., 1995; Beffert et al., 1999). This discrepancy may be accounted for by differences in the methods used to measure apoE (i.e. bound, unbound or total apoE levels; western blot versus ELISA) or may in fact represent two biological extremes of a breakdown in apolipoprotein homeostasis that may, in both cases, result in the development of disease. Thus, larger
studies, incorporating a larger pool of both late- and early-onset AD cases, need to be performed to elucidate the significance of the findings in this current study.

If the main mechanism by which apoE4 conveys risk for AD is via its deficient clearance of Aβ compared to apoE2 and apoE3 and increased apoE levels are associated with an increased risk for AD it raises the paradox that increased levels of apoE2 and apoE3 may be beneficial. However, apoE levels are also increased in non-ε4 AD individuals (Taddei et al., 1997) suggesting that over-expression of apoE may impinge on the ability of the more proficient isoforms ability to clear Aβ, potentially by down-regulation of associated receptors such as LRP. A case in point where increased apoE levels has overridden the beneficial aspects of the apoE2 and apoE3 isoforms is reported by Miyata and Smith (1996) who illustrates that whilst physiological levels of apoE protected cells from Aβ peptides, higher doses of apoE led to increased cytotoxicity. Whether this is the case for the apoE-Aβ clearance hypothesis remains to be elucidated. The study of Miyata and Smith (1996) also illustrates that the role of apoE in the pathological process of AD is not limited to this putative clearance/deposition model but also in protection against oxidative stress, Aβ cytotoxicity and also, as reported by Lauderback and colleagues (2002), Aβ42-induced oxidation of synaptosomes.

The findings of Miyata and Smith, in regards to the protective effect of apoE2 isoform being overridden by elevated apoE levels, taken together with; (a) reports of both reduced (Bertrand et al., 1995; Beffert et al., 1999) and elevated (Pirtilla et al., 1996; Laws et al., 2002b) levels of apoE in AD brains, and (b) APOE promoter polymorphism that are associated with both increasing (-491A/T; Artiga et al., 1998a; Artiga et al., 1998b; Laws et al., 1999; Scacchi et al., 2001; Laws et al., 2002b) and decreasing (-219T/G; Artiga et al., 1998a; Lambert et al., 2000; Chapter 4) its expression, gives rise
to the notion that both the under-expression and over-expression of apoE in the brain may be of importance to AD. Thus, the normal functions of apoE may be impinged by the two extremes of apoE levels. This hypothesis, diagrammatically represented in Figure 7.2, is not unheard of for a biological system where disruption of homeostasis results in disease phenotypes; a prime example of which is seen in diabetes where the lack of insulin results in Type 1 diabetes whilst too much insulin results in the development of Type 2 diabetes.

The regulation of the transcription of $\textit{APOE}$ is highly complex and requires an interaction, not only of the possible functional proximal promoter polymorphisms studied in this thesis but also, of the proximal and distal regulatory regions with transcription factors to impart a net effect on $\textit{APOE}$ expression. In turn, this complex genetic mechanism of regulation may itself be influenced by non-genetic factors such as diet and hormone levels, which also impart regulatory effects directly or indirectly. This complex interplay between genetic and environmental factors must be thoroughly considered in order to fully appreciate the role of apoE in AD.

7.2.3 Future Directions – Testing the Hypothesis and Beyond

The presentation, in this thesis, of a putative biphasic model for the effect of apoE levels on increasing AD risk requires considerable investigation. Investigation, in an in vivo system, to determine whether there is a dose response effect of apoE level on the normal functioning of apoE needs to be undertaken. For example, testing this hypothesis on the ability of apoE to bind and clear Aβ would be of worth. Likewise, would be confirming the original studies of Miyata and Smith (1996) and extending this to include other cytotoxic insults. Transfection of the promoter polymorphisms, in particularly the -491
Figure 7.2: Putative Biologic Model for Effect of APOE Levels on AD Risk.

APOE Level

High

Normal

Low

Chapter Seven: Discussion

Increasing AD Risk

- Also associated with elevated Aβ load
- Possibly mediated by APOE-2 and APOE-3
- Down-regulation of LDL receptor family
- Decreased clearance of Aβ
- e9: increased oxidative effect of Aβ
- Normal Functioning

Impaired Functioning (high APOE level)

Impaired Functioning (low APOE level)

- Also associated with elevated Aβ load
- Possibly mediated by APOE-2 and APOE-3
- Insufficient APOE to bind all Aβ
- Decreased clearance of Aβ
- e9: increased oxidative effect
- Normal Functioning

Impaired Functioning (high APOE level)

Impaired Functioning (low APOE level)
locus, in addition to the three *APOE* alleles into cell lines and antagonising these cells with cytotoxic insults, such as Aβ or free radicals, may allow for the level of protection afforded by different variations of isoform expression, to be determined.

Hormone levels may also impart regulatory effects, directly or indirectly, that may modify the complex genetic mechanism of regulation of apoE expression. A prime example in the literature is oestrogen. It has been reported that estradiol up-regulates apoE gene expression by increasing levels of apoE mRNA in the polysomal translating pool (Srivastava et al., 1997) and region specific up-regulation of apoE by oestrogen (although in non physiological concentration) has also been reported (Levin-Allerhand et al., 2001). Additionally, the neuroprotective role for oestrogen in global ischemia is apoE-dependent (Horsburgh et al., 2002). These studies also suggest that each of these mechanisms is largely mediated by oestrogen receptors. As such it would be of interest to determine a dose response of oestrogen on apoE levels expressed by apoE producing cells, for instance astrocytes, that may also be transfected with the *APOE* coding and promoter polymorphisms. Likewise, the knocking out of oestrogen receptors, or transfection of the oestrogen-receptor alpha (ERα) and beta (ERβ) polymorphism may also be of interest, as would antagonising these cells with the previously mentioned cytotoxic insults.

7.3 CONCLUSION

This thesis presents data that confirms and extends the genetic association of the *APOE* coding and promoter polymorphisms with AD, pre-AD subjective memory complaints and a high rate of conversion to MCI or AD in the Australian population. Through these findings, and the presentation of the first data to suggest a role for the -491A/T
polymorphism in altering peripheral and cerebral apoE level, the work presented within this thesis has contributed significantly to our understandings of the mechanisms behind the association of the \textit{APOE} \textepsilon4 allele, \textit{APOE} promoter polymorphisms and apoE protein levels with AD. It is thus hoped that this thesis has made both a novel and important contribution to the AD literature and that it may assist in the understanding of why some individuals develop Alzheimer's disease.

\textit{The End}


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CHAPTER EIGHT: REFERENCES


