Analysis of cardiovascular and inflammatory genes as risk factors for Alzheimer's disease

By

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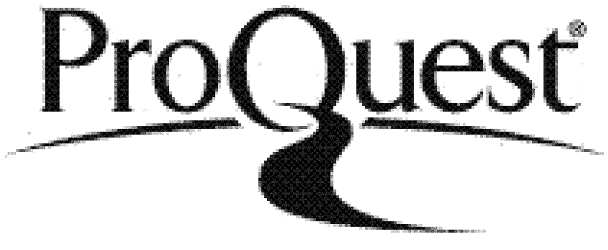

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterised by the abnormal accumulation of extracellular β-amyloid in senile plaques and the formation of intracellular neurofibrillary tangles. The mechanism of pathology is unknown: however, the formation of β-amyloid from amyloid precursor protein (APP) by proteolytic processing is considered to be an essential component of the pathway. Evidence suggests that both the cardiovascular and inflammatory systems may play a role in the neuropathology of AD.

Around 95% of all AD cases have an age of onset over 65 (late onset) and do not show Mendelian inheritance. So far only one risk factor has been identified for late onset AD, the ε4 allele of the apolipoprotein E (*APOE*) gene, which is neither necessary nor sufficient to cause the disease. At least four other loci are believed to account for the remaining genetic risk.

Four genes, connected to either the inflammatory or cardiovascular system or both, were investigated for association with late onset AD in up to 180 late onset cases and 180 age- and sex-matched controls. The I allele of *DCP1* has previously been reported to be associated with AD although no association was detected in this study. Six other polymorphisms within the gene were also studied but yielded no positive genotypic, allelic or haplotypic associations. The other genes studied, *TACR2*, a peptide receptor that maps to a region of suggestive linkage on chromosome 10, *ECE1*, a potent vasoconstrictor which also maps to a region of suggestive linkage on chromosome 1 and *PI12*, a serine protease inhibitor that can form amyloidogenic fragments, were screened for polymorphisms. Fifteen polymorphisms were discovered (five coding) with only one two-marker haplotype in *ECE1* ($p = 0.001$) and a polymorphism upstream of *TACR2* ($p = 0.05$) showing statistically significant association. Neither association retained statistical significance after adjusting for multiple testing. This study, in particular the work on *DCP1*, highlights the problems inherent in detecting genetic risk factors for complex diseases and the importance of meta-analyses.

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Outside of work, my thanks and all my love go to my best friends Joo, Humpage, Rich, Hannah, Ham and Andy. Cheers guys for all your support and all the social activities!

Mam a Dad – diolch am bob dim dros yr wyth blynedd diwethaf. Fuaswn i ddim wedi cyrraedd lle rydw i rwan heb eich cymorth a chariad. Mae hi wedi bod yn siwrna hir a dwi'n gobeithio fy mod i wedi gwneud chi'n falch ohono fi.

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Abbreviations

8OHG	8-hydroxyguanosine
ACE	Angiotensin converting enzyme
ACh	Acetylcholine
AD	Alzheimer's disease
AGE	Advanced glycosylation endproducts
AGT	Angiotensinogen
AICD	Amyloid precursor protein intracellular domain
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ASP	Affected sibling pair
βA	Beta-amyloid
COX	Cytochrome c oxidase
CTF	Carboxy terminal fragment
CNS	Central nervous system
CVD	Cardiovascular disease
ECE1	Endothelin-converting enzyme 1
EOAD	Early-onset Alzheimer's disease
ET-1	Endothelin-1
FAD	Familial Alzheimer's disease
FADFTD-GSG	French Alzheimer's Disease and Fronto-Temporal Dementia Genetics Study Groups
HDL	High density lipoprotein
HEK	Human embryonic kidney
IDE	Insulin-degrading enzyme
LD	Linkage disequilibrium
LDL	Low density lipoprotein
LOAD	Late-onset Alzheimer's disease
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MLS	Maximum lod score
MMSE	Mini-mental state examination
NFT	Neurofibrillary tangle
NICD	NOTCH intracellular domain
NIDDM	Non-insulin dependent diabetes mellitus
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drugs
OR	Odds ratio
ORF	Open reading frame
PS1	Presenilin 1
PS2	Presenilin 2
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
SAD	Sporadic Alzheimer's disease
SP	Senile plaque
TACE	Tumor necrosis factor converting enzyme
TACR2	Tachykinin receptor 2

TGN	*trans*-Golgi network
TUNEL	terminal deoxynucleotide transferase-mediated dUTP nick end labelling
VaD	Vascular dementia
VLDL	Very low density lipoprotein

Contents

Chapter 1

Introduction

1.1 Background

Alzheimer's disease (AD) (OMIM #104300) is the most common form of dementia accounting for up to 55% of all cases (Dugue *et al.* 2003) and resulting in approximately 100,000 deaths each year in the UK. Since 1900, the life expectancy in the western world has increased by over 50%, from 47 to 75, and is expected to continue rising. As age is the predominant risk factor for dementia, cases are expected to treble over the next 50 years placing an enormous strain on health services. The need for a more complete understanding of the pathogenesis of AD and other dementias leading to more effective treatments is crucial.

The disease is characterised by chronic, progressive memory loss, disorientation and aggression. In the latter stages of the disease the patients develop loss of motor, sensory and linguistic ability. While AD is a pathological diagnosis, the clinical diagnosis is made by clinical assessment and a variety of assessment schedules. Initially, a family and clinical history will be taken to eliminate other possible causes of dementia such as hypertension. The Mini-Mental State Examination (MMSE) is carried out which measures efficacy of short term memory (Folstein *et al.* 1975). Non-demented individuals score near 30 while a score of less than 26 is considered significantly low enough to warrant further testing. Finally, other cognitive tests such as the Alzheimer's Disease Assessment Scale (ADAS) can be used to further confirm the result. Diagnosis can only be confirmed at post-mortem by the presence of neurofibrillary tangles and senile plaques. However, clinical diagnosis is now highly accurate with over 90% of cases diagnosed correctly.

Once diagnosed, a case can be classified depending on age of onset and family history. Early onset AD (EOAD) is classified as the cases which display the symptoms of the disease before the age of 65. Those with onset of symptoms at the age of 65 or higher are classified as late onset (LOAD). Secondly, if the patient inherited AD in an autosomal dominant fashion it is classified as familial AD (FAD). All others are classified as sporadic cases (SAD) and account for around 95-99% of all cases; although a significant fraction of these could be due to other unknown genetic factors.

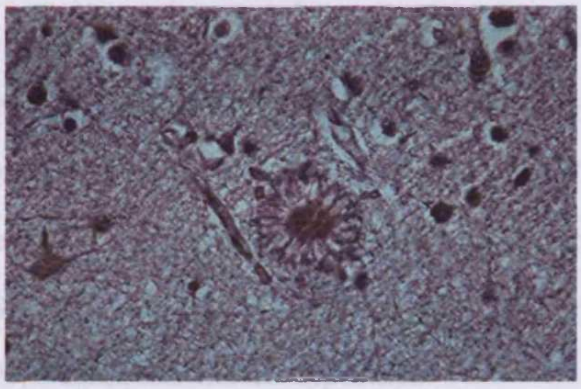
1.2. Pathology of Alzheimer's Disease

The early symptoms of mild memory loss and minimal cognitive impairment lead gradually over 5-15 years to dementia and death. The impairment involves early synaptic dysfunction (Anderton *et al.* 1998), followed by severe neuronal changes including synaptic loss, widespread neuritic dystrophy, and neuronal death. Particularly vulnerable regions include certain brainstem nuclei, basal forebrain, amygdala, hippocampus, entrorhinal cortex and the neocortex. Cholinergic input to the hippocampus and neocortex are particularly susceptible to neurodegeneration but the mechanisms behind this selectivity are unknown. Neurotransmitter deficits are likely to be factors in the memory and cognitive impairments (Holtzman and Mobley, 1991). The primary features of AD neuropathology however, are the presence of senile plaques (SP) and neurofibrillary tangles (NFT).

1.2.1 Senile Plaques

Senile plaques are star-shaped masses of aggregated extra-cellular amyloid deposits associated with neuronal and axonal injury (Figure 1.1).

Figure 1.1 Senile plaque



Bodian's silver staining of senile plaque from an AD patient.

Dystrophic neurites surround and permeate the SPs and display ultrastructural abnormalities such as numerous mitochondria, enlarged lysosomes and paired helical filaments which are very similar to NFTs. Activated microglia and reactive astrocytes are also associated with the SPs (Cras *et al.* 1990) with the former found near the central core and the latter outside the plaque with processes extending towards the core. Plaques show large variation in size (10 - >120μm) and density.

The plaques consist largely of a 39-42 amino acid peptide, β-amyloid (βA), the product of enzymatic cleavage of amyloid precursor protein (APP). βA_{1-42} is the most hydrophobic form of βA and also the most abundant in plaques. In neuronal cells βA_{1-40} is produced in greater quantities than βA_{1-42} at approximately a 9:1 ratio and it is also found in the plaques but at a much lower concentration. Other proteins found in SPs include alpha-2-macroglobulin (A2M), apolipoprotein E (ApoE), low density lipoprotein receptor related protein (LRP) and complement factors.

Immunohistochemical staining of AD patients with antibodies raised against βA gave increased detection of plaques and led to the discovery of diffuse plaques. These plaques did not display the same compact, fibrillar structure as senile plaques

and were found in areas not associated with AD degeneration e.g. caudate, cerebellum and the thalamus (Joachim *et al.* 1989; Yamaguchi *et al.* 1988). Dystrophic neurites were rarely seen associated with these plaques and they were mainly composed of βA_{1-40}. The presence of diffuse plaques in areas containing SPs and in non-demented aged individuals led to the suggestion that diffuse plaques were precursors to SPs. Work on transgenic mice and immunohistochemical work on Down's patients (Lemere *et al.* 1996) is consistent with this hypothesis.

1.2.2 Neurofibrillary Tangles

Studies of the neurons affected by degeneration in AD revealed that a large proportion of their perinuclear cytoplasm was taken up by abnormal fibres consisting of paired helical filaments (PHF), see Figure 1.2.

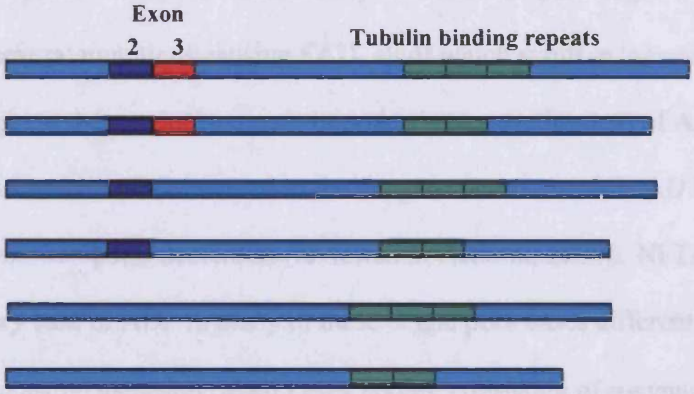
Figure 1.2 Neurofibrillary tangle

Immunohistochemical (Bielschowsky) staining of a neuron containing a neurofibrillary tangle. Taken from http://www.md.huji.ac.il/mirrors/webpath/NEURO.html

These PHF were present in cells which had lost the neuronal cytoskeleton (Hanger *et al.* 1992). Immunocytochemical and biochemical analysis suggested the PHF was composed of tau, a microtubule associated protein (Wood *et al.* 1986). Alternative splicing of exons two, three and ten produces six different isoforms

containing either three or four repeats of the tubular dimer binding site, see Figure 1.3 below (Goedert *et al.* 1989).

Figure 1.3 Tau isoforms



The six different isoforms produced by alternative splicing of exons two, three and ten of the tau gene. Splicing of exon ten produces different length repeats of tubulin dimer binding regions.

Digestion of the filaments and electrophoresis of the released tau showed an altered migration resulting from increased phosphorylation, which could be reversed by treatment with alkaline phosphatase (Litersky and Johnson, 1992). Moderately phosphorylated tau binds to microtubules with high affinity. In comparison unphosphorylated tau binds with a lower affinity and hyperphosphorylated tau with the lowest affinity of all (Kosik 1992 and Garcia de Ancos *et al.* 1993).

Several kinases have been shown to phosphorylate tau in vitro including cyclin-dependent kinase 5 (CDK5) (Lee *et al.* 2000a), glycogen synthase kinase 3β (GSK3β) (Mandelkow *et al.* 1992) and mitogen-activated protein kinase (MAPK) (Drewes *et al.* 1992). None of these kinases alone can produce NFTs (Zheng-Fischhofer *et al.* 1998). Phosphorylation of tau increases in cells transfected with *GSK3β* (Wagner *et al.* 1996) and lithium, an inhibitor of GSK3β, causes dephosphorylation of tau (Munoz-Montano *et al.* 1997). Presenilin 1, a familial AD

causing gene, binds directly to GSK-3β and tau suggesting a link to Alzheimer's (Takashima *et al.* 1998).

A considerable debate developed over which of the neuropathological features of AD, the SPs or the NFTs, was most clearly related to the pathological event. The discovery of several mutations causing FAD, all of which result in increased levels of βA gives weight to the amyloid-cascade hypothesis (see section 1.4) of AD. In addition to this, mutations discovered in the tau gene do not result in AD but in the distinctive fronto-temporal dementias (reviewed in Heutink, 2000). NFTs are not present in every case of AD. In many of these tangle poor cases different forms of neuronal cytoplasmic inclusion called Lewy bodies, consisting of α-synuclein, are seen (Hansen *et al.* 1993). The fact that mutations in tau cause a different disorder which contains no SPs and that not all AD cases contain NFTs suggests that the amyloid cascade is the most likely to be the correct hypothesis. Transgenic mice have been produced bearing the FTDP-17 mutations which produce NFTs; however they do not show an AD based pathology but a motor neuron disease phenotype (Lewis *et al.* 2000) Recent evidence however, points to a possible link between both hypotheses with the injection of βA_{1-42} into mutant tau (P301L) mice leading to a five fold increase in the number of NFTs (Gotz *et al.* 2001).

1.3 Genetics of Alzheimer's disease

Two methods of analysis have been central to the characterisation of the genetics of AD and need to be described in further detail here; linkage studies and association studies. Linkage analysis was not performed in this study and is not discussed in detail here.

Linkage Analysis

Linkage analysis is the study of a polymorphic marker and a disease to determine whether their recombination fraction (ϑ) differs significantly from 0.5 (the recombination fraction of two unlinked loci) and are therefore linked. It can be quite complex to determine the recombination fraction in large pedigrees as quite often the meiosis can be uninformative. In these cases a mathematical equation can be used, usually using a computer, to determine a lod score (Morton, 1955). The lod score (Z) is the logarithm of the odds of linkage which is determined by the likelihood that the marker is linked to the disease divided by the likelihood that it is not. This ratio needs to be worked out over a range of recombination fractions from 0 to 0.5 which can be plotted as a curve. This then used to determine the most likely recombination fraction, which will occur at the maximum Z ($\vartheta = 0$ in the case of no recombinants). The threshold for statistically significant linkage (with 5% error rate) is $Z>3$ and no linkage at $Z<-2$ with anything in between considered inconclusive. For a complete genome screen markers are selected to be approximately 5-10cM apart and the lod scores obtained are plotted against genetic distance along the chromosome (Strachan and Reid, 2000)

Multipoint linkage is the same as two-point analysis mentioned above except that more than two loci are analysed simultaneously using programs such as Genehunter (http://www.fhcrc.org/labs/kruglyak/Downloads/). This method is useful to confirm the chromosomal order of markers which can sometimes be incorrect.

Association studies

The regions of possible linkage produced by the linkage studies mentioned in the previous section can be large enough to contain hundreds of genes. Association

studies look for the disease locus both directly and indirectly and are more likely to pick up weak genetic effects (Elston, 1995; Risch and Merikangas, 1996). Whereas a linkage study looks for commonly inherited parts of the genome in affected relatives, association studies look at genotypes and their relationships with disease. Linkage studies focus on more recent, observable inheritance where relatively little recombination has occurred, association looks at the result of hundreds of recombination events, narrowing the disease region.

This is done by looking at genotypic, or sometimes allelic, frequencies in a diseased population and seeing if they differ significantly from the frequencies in a control population. Therefore the standard requirements for an association study are a large sample of carefully selected cases and controls and an easily genotyped polymorphic marker. The sample must be of sufficient power i.e. large enough, to detect the genetic effect being tested. The presence of a control sample is one of the main differences between linkage and association studies and their selection is very important.

Controls must be selected from a similar population to the cases and matched carefully for age, sex etc. Failure to do so can lead to population stratification, reducing the power of the study and possibly give false results (reviewed in Cardon and Bell, 2001). In an ideal situation family based controls are used along with a family based test such as the transmission disequilibrium test (TDT), however this is problematic for a late-onset disease such as AD. On way to avoid this problem is to use affected and unaffected sibs and the sib TDT (Spielman and Ewens, 1998) which are easier to obtain than parental genotypes.

The choice of the cases can also have an effect on the study. In a heterogenous disease with varying phenotype, disease variants within the cases can

dilute the association and reduce the power of the study. AD and vascular dementia (VaD) share many characteristics (see section 1.5) and contamination of AD association studies with VaD cases can be a problem. The history of the population selected will also affect the results, especially if the population expanded rapidly from a recent bottleneck as in Finland. In these populations linkage disequilibrium (LD), the non-random segregation of alleles, which is central to association studies, can extend over greater distances (Eaves *et al.* 1998; Gordon *et al.* 2000). In some cases this can be quite useful if, for example, a genome wide association study is to be carried out. A genome wide association study is a huge undertaking and if typing one SNP can provide information over a distance of 3cM in a particular region, then the minimum number of SNPs needing to be typed is reduced significantly.

Conversely, if the common disease-common variant hypothesis is correct in disorders such as hypertension and AD then populations such as the Finns will be of little use. This hypothesis speculates that the variants providing susceptibility to many common diseases were present in the founding populations of humans and have spread through the population because of neutral selection (Lander, 1996; Collins *et al.* 1999). These variants have now emerged as disease associated because of environmental factors creating an opportunity for the disease to be expressed e.g. increased life expectancy for AD or high sodium and calorie diets for hypertension and diabetes. It is therefore clear that a population which has been through a relatively recent bottleneck may not carry these variants and therefore a more ethnically diverse population e.g. UK may be more valid for these association studies.

When a positive association between an allelic variant and a disease is discovered, care must be taken in interpreting the results. Some false positive results are obtained because of statistical artifacts, particularly through multiple testing. As

more SNPs are analysed, the possibility of a type I error increases and the significance level must be adjusted (see chapter 7 for more details). In addition, the SNP in question may not actually be the causative variant, but merely in LD with another polymorphism which has a functional effect.

The main method of mapping a disease gene currently is to perform genome wide linkage analysis to identify regions of suggested linkage. These regions are then fine mapped using association studies often using functional information to identify genes likely to be associated with a particular disorder. With the completion of the human genome project and the position of many genes confirmed, particular genes within a region can be cherry-picked based on functional evidence to maximise efficiency.

This technique has been used successfully in the identification of *NOD2* as a risk factor in Crohn's disease (OMIM #266600), an inflammatory bowel disorder with both a genetic and environmental effect. Genome screens produced a region of suggestive linkage on chromosome 16. *NOD2* was selected for analysis within the region because of its role in the inflammatory response to microbial infection which constitutes the environmental factor of the disease. Two separate groups reported a frameshift polymorphism within the gene which showed association with Crohn's (Hugot *et al.* 2001; Ogura *et al.* 2001).

1.3.1 APP

In 1984 Glenner and Wong isolated the primary constituent of cerebrovascular plaques, the βA peptide. This work led to the discovery of APP, which was the first gene found to be associated with FAD, and the mapping of its gene to 21q21 (Goldgaber *et al.* 1987). βA was also found in SPs of patients with Downs syndrome,

where there is a chromosome 21 trisomy, along with NFTs and dementia (Glenner and Wong, 1984). These facts raised the possibility of a genetic abnormality on chromosome 21 contributing to the disease. This hypothesesis was reinforced by St George-Hyslop *et al.* (1987) when linkage studies revealed a FAD locus on chromosome 21 near the location of the APP gene.

Attempts to replicate these findings by several groups failed (Van Broeckhoven *et al.* 1987; Pericak-Vance *et al.* 1988; Schellenberg *et al.* 1988). It was only later when mutations in *APP* were found in some FAD pedigrees (Goate *et al.* 1991; Chartier-Harlin *et al.* 1991) that this work was confirmed. Gradually it has become clear that these mutations are rare even in FAD cases and so far only a small number of *APP* mutation pedigrees have been found worldwide.

All of the mutations in *APP* discovered so far cluster in three areas and have been shown to alter the proteolytic processing of the APP holoprotein (see next section). The mutations are shown in Table 1.1 below.

Table 1.1Early-onset AD mutations in *APP*

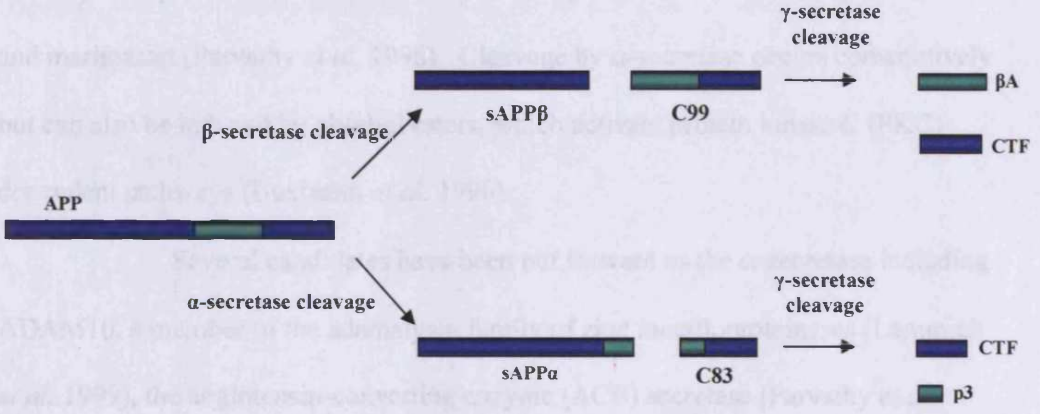
Wild Type	Mutant	Position	Reference
Lys	Asn	670	Mullan *et al.* 1992
Met	Leu	671	Mullan *et al.* 1992
Ala	Thre	713	Carter *et al.* 1992
Thr	Ile	714	Kumar-Singh *et al.* 2000
Val	Met	715	Ancolio *et al.* 1999
Ile	Val	716	Eckman *et al.* 1997
Val	Iso	717	Goate *et al.* 1991,

A list of the mutations found in the APP gene which cause familial early-onset Alzheimer's disease. Position within the amino acid shown along with the wild type and mutant genotypes

1.3.1.1 Proteolytic Processing of APP

The APP holoprotein exists as a polypeptide with a single transmembrane span. During translation it is translocated to the endoplasmic reticulum and into the secretory pathway. As it passes through the *trans*-Golgi network (TGN) it is matured by N- and O-glycosylation and tyrosyl-sulfation (Weidemann *et al.* 1989). Finally the holoprotein is cleaved by a variety of proteases known as secretases. Three distinct cleavage sites and their corresponding proteases (α,β and γ secretase) result in two distinct pathways, producing a large soluble fragment and either βA or p3 (see Figure 1.4)

Figure 1.4 Proteolytic processing of *APP*.



Processing of APP via the α and β secretase pathways. APP holoprotein is cleaved by either the α-secretase to produce sAPPβ and C99 or by the β-secretase to produce sAPPα and C83. γ-secretase then cleaves to produce either the βA peptide or p3 and a carboxy-terminal fragment (CTF).

α-secretase cleavage

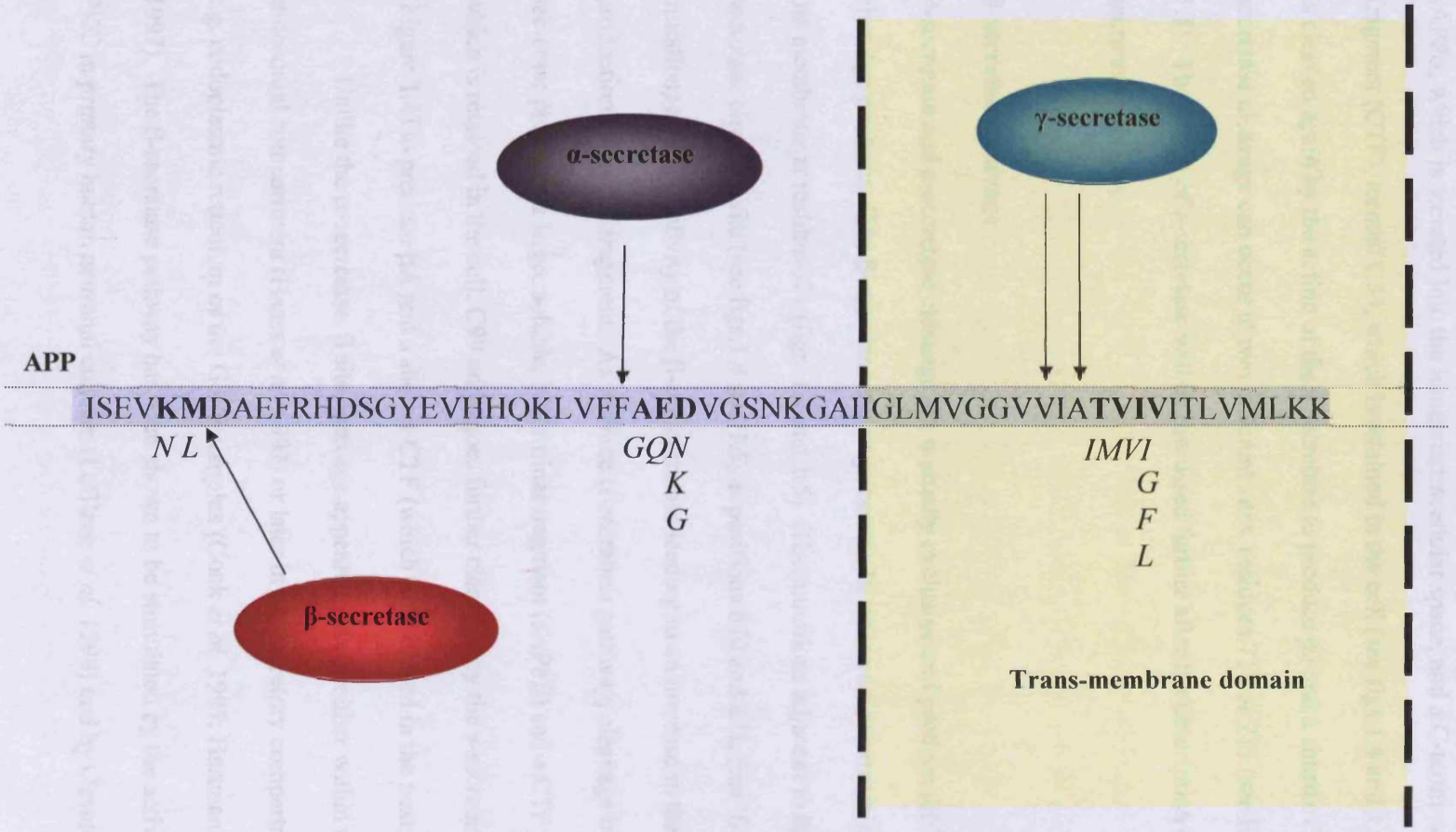
The α-secretase pathway was the first to be characterised and involves cleavage twelve amino acids N-terminal to the transmembrane domain of APP. This site is also Lys^{16} within the βA domain, thus this cleavage prevents the formation of the βA

peptide (see fig.1.5 on the following page). Mutations have been found in the α-secretase cleavage site resulting in other, related disorders e.g. Dutch type cerebroarterial amyloidosis (Levy *et al.* 1990) at residue 692, however none have been found which directly influence the production of βA in AD.

Sambamurti *et al.* (1992) and Kuentzel *et al.* (1993) both suggested that the α-secretase cleaved within the TGN in an attempt to characterise the pathway and isolate the enzyme responsible. However, recent work by Parvathy *et al* (1999), using biotinylated secretase inhibitors showed that cleavage was almost exclusively carried out on the surface of neuronal cells. The α-secretase is yet to be identified, however it is known that it is an integral membrane protein (Sisodia, 1992) and that it is inhibited by hydroxamic-acid-based zinc metalloproteinase inhibitors e.g. batimastat and marimastat (Parvathy *et al.* 1998). Cleavage by α-secretase occurs constitutively but can also be induced by phorbol esters, which activate protein kinase C (PKC) dependent pathways (Buxbaum *et al.* 1990).

Several candidates have been put forward as the α-secretase including ADAM10, a member of the adamalysin family of zinc metalloproteinases (Lammich *et al.* 1999), the angiotensin-converting enzyme (ACE) secretase (Parvathy *et al.* 1997) and tumour necrosis factor-α convertase (TNFα) (Buxbaum *et al.* 1998), based on similarities in solubilization and inhibitor profiles or work done on animal models. However, it is unclear whether these candidates are acting directly upon APP or whether they aid in the activation of a seperate α-secretase. Parvathy *et al.* (1999) shows that the inhibitor profile of the TNFα convertase is different to that of both the α-secretase and the ACE secretase and is therefore unlikely to be directly involved in the α site cleavage of APP.

Figure 1.5 βA fragment and disease causing mutations.



Amino acid sequence of βA fragment (highlighted in purple) within the APP holoprotein. Position of the mutations shown (bold), along with the mutant alleles (in italics), the position of secretase proteolytic cleavage sites and the transmembrane domain (dotted line).

Cleavage by α-secretase produces a large soluble N-terminal fragment termed sAPPα, which is secreted into the lumen/extracellular space, and a C-terminal fragment (CTF), termed C83, which is retained in the cell (see figs.1.4 and 1.5). C83 is cleaved again by the action of the γ-secretase to produce p3 and a shorter CTF. γ-secretase cleavage can occur at two different sites, residues 711 or 713 (see Figure 1.5). The action of γ-secretase will be discussed further after the description of the β-secretase pathway.

β-secretase cleavage

β-secretase and α-secretase cleavage are mutually exclusive and produce different cleavage products. The β-secretase cleavage occurs 28 amino acids extracellularly of the membrane at residue 671 (figs. 1.4 and 1.5). The mutations adjacent to the β-secretase cleavage site (see figs.1.4 and 1.5) at positions 670 and 671, (the 'Swedish' mutation) alter the activity of the β-site secretase leading to an increase in the production of the βA fragment. As with the α-secretase pathway, cleavage by β-secretase produces a large, soluble, N-terminal fragment (sAPPβ) and a CTF (C99) which is retained in the cell. C99 undergoes further cleavage by the γ-secretase (Figure 1.4) to produce βA and a shorter CTF (which is discussed in the next section).

Unlike the α-secretase, β site cleavage appears to occur either within the endosomal compartment (Haass *et al.* 1992) or later in the secretory compartments e.g. endoplasmic reticulum or the Golgi complex (Cook *et al.* 1997; Hartmann *et al.* 1997). The β-secretase pathway has been shown to be stimulated by the activation of PKC in primary human neuronal cultures (LeBlanc *et al.* 1998) and by elevating

intracellular Ca^{2+} levels in human embryonic kidney (HEK) cells (Querfurth and Selkoe, 1994).

Vassar *et al.* (1999) using expression cloning to identify genes that altered βA production in HEK 293 cells found one positive clone, significantly homologous to the aspartic protease family, that displayed all the characteristics of the β-secretase and was termed a beta-site APP cleaving enzyme (BACE). Overexpression of BACE, which has been mapped to 11q23.3 (Saunders *et al.* 1999), causes an increase β-secretase activity and inhibition of BACE by antisense oligonucleotides reduces β-secretase activity. β-secretase activity increases 10-fold in cell lines co-transfected with *BACE*, which is also termed ASP2, and *APP* carrying the Swedish mutation (Yan *et al.* 1999). They also describe another membrane bound aspartyl protease (*ASP1*) which has high homology to *BACE* and maps to chromosome 21q22. ASP1 or BACE2 cleaves APP at the β site and within the βA site adjacent to known EOAD mutations in HEK cells (Farzan *et al.* 2000). However, expression analysis of BACE2 revealed very low expression of the gene in adult rat brain, low expression in human peripheral tissues and very low or undetecTable levels in human foetal and adult brain (Bennett *et al.* 2000a). These results do not match the expected pattern for the β-secretase leading to suggestions that BACE alone cleaves APP *in vivo*.

γ-secretase cleavage

As there are at least two different positions in APP for the γ-secretase to cleave and mutations in this region of APP result in an increase in the proportion of the highly amyloidogenic fragment βA_{1-42} being produced (Ancolio *et al.* 1999). γ-secretase cleavage of APP occurs within the transmembrane domain and this led many to

believe it was a pathological event associated with βA production. It was not until 1992 that βA was shown to be secreted by cell under normal conditions (Haass *et al.* 1992 and Busciglio *et al.* 1993). The exact cellular location of γ-secretase activity remains unknown, however experiments have shown that the substrates for γ-secretase are fully N– and O-glycosylated, and therefore secretase activity must occur during or after the Golgi complex (Weidemann *et al.* 1989).

1.3.1.2 Animal models

Animal models of disease can provide valuable information about the progression and the pathology of the disease and provide a system to test therapeutic agents. As the mutations underlying EOAD have been elucidated several animal models of mutant *APP* have been produced with mixed results. Swedish mutation transgenic mice (APPswe) tend to produce high levels of total βA (Hsiao *et al.* 1995) while V717F mice (PDAPP) produce much higher quantities of βA_{1-42}, giving a higher concentration of plaques (Games *et al.* 1995) mimicking the effects of the mutations in AD patients. Neither animal model displays NFTs but their neurones do contain abnormally phosphorylated tau. Mice transgenic for the C-terminal end of *APP* (C104 and C100) show all the pathogenic hallmarks of the other lines and in addition display defects in synaptic plasticity, degeneration in the CA1 region of the hippocampus and deficits in long term potentiation (LTP) (Kammesheidt *et al*, 1992; Oster-Granite *et al*, 1996; Nalbantoglu *et al*, 1997).

1.3.1.3 Function of APP

APP gives rise to several isoforms by alternative splicing (563, 695, 714, 751 and 770 residues) and post-translational modification. Both the APP751 and the APP770 splice forms are expressed in many neuronal and nonneuronal cells, while the 695 form is expressed at high levels in neuronal cells and low levels in nonneuronal cells (Haass *et al.* 1991). The main difference between these isoforms is the lack, in *APP695*, of an exon encoding an extracellular domain with high homology to a protease inhibitor of the Kunitz-type (KPI) family (Kitaguchi *et al.* 1988). Isoform 714 also lacks this motif. The APP gene is highly conserved and has been found in all mammals studied. *Drosophila* express a partial homolog, APPL (Rosen *et al.* 1989)

Expression of *APP* can be induced by stress conditions (Dewji *et al.* 1995) and growth factors and cytokines (Goldgaber *et al.* 1989; Adler *et al.* 1991; Forloni *et al.* 1992). The promoter itself displays the characteristics of a housekeeping gene: it is very GC rich, lacks a functional TATA box and has multiple sites for the binding of stimulating protein 1 (SP1). As with the remainder of the APP gene, the promoter shows high levels of homology in other mammals, up to 80% in primates and rats (Song and Lahiri, 1998; Chernak, 1993).

The normal cellular function of APP remains unknown. Several theories have been put forward based on what is already known. Three of the isoforms contain an extracellular domain encoding a KPI and these isoforms inhibit the action of proteases such as trypsin and chymotrypsin (Sinha *et al.* 1990), while Smith *et al.* (1990) reports that they inhibit factor X1a, a peptide involved in blood clotting. APP has also been suggested to be involved in cell-cell interactions (Qiu *et al.* 1995), however none of these functions have been confirmed *in vivo*.

Recent evidence has revealed two new pathways which may lead to the identification of the function of APP. Kamal *et al.* (2000) reports that axonal transport of APP within neurones via the microtubules requires APP to complex with the light chain subunit of kinesin-1. Further work has revealed an axonal membrane compartment containing β-secretase, APP, kinesin-1 and PS1 and that the transport of this compartment is mediated by APP and kinesin-1 (Kamal *et al.* 2001). They also show that APP is cleaved within this compartment, both *in vivo* and *in vitro*, producing βA and a CTF which also results in the liberation of kinesin-1 from the complex.

The second possible pathway involves the processing of APP and signal transduction. Research has centred on the pathology associated with the βA fragment so far, because of its central role in SPs (see section 1.2.1). However, many recognised that the CTF produced by the β- and γ-secretase cleavage is potentially amyloidogenic as a result of its high hydrophobicity (see previous section for processing of APP). Antibodies raised against the CTF stain both NFTs and dystrophic neurites surrounding SPs (Arai *et al.* 1990; Yamaguchi *et al.* 1990a).

These CTFs have also been shown to be toxic to cells. PC12 cells secrete significant amounts of lactose dehydrogenase, an indicator of cell toxicity, in the presence of CTF and this sensitivity increases after differentiation into neurones. The levels of βA required for similar levels of toxicity are significantly higher (Kim and Suh, 1996; Suh, 1997). In addition, this neurotoxicity has been shown to occur in differentiated PC12 cells in the presence of CTF not containing the βA peptide or transmembrane domain suggesting the carboxy terminal sequence could play a role in the neurodegeneration of AD (Lee *et al.* 2000b). The production of the CTF involves other FAD linked proteins, the presenilins. The possible role of the CTF in signal

transduction is discussed after the role of presenilins in γ-secretase cleavage (see section 1.3.2.3)

1.3.2 Presenilins 1 and 2

A linkage study by Schellenberg *et al.* (1992) revealed a possible susceptibility gene on chromosome 14 and after further positional cloning and linkage studies a novel gene, named *presenilin 1* (*PS1*), was discovered on 14q24.2 (Sherrington *et al.* 1995). A homologous gene was also discovered on chromosome 1q44.13, in which mutations were found in a German EOAD family, and was named *presenilin 2* (*PS2*) (Levy-Lahad *et al.* 1995). Studies of both these genes have revealed up to 75 missense mutations (and one deletion) in *PS1* and three missense mutations in *PS2*. *PS2* mutations give the most aggressive and earliest AD symptoms, usually leading the patient's death by their 60s (Levy-Lahad *et al.*1995).

When they were first discovered, it was unclear how mutations in *PS1* or PS2 affected the pathology of AD. The analysis of βA production in cultured fibroblasts taken from patients with PS mutations shows that they produce higher levels of $\beta A_{1\text{-}42}$ compared to other AD patients (Scheuner *et al.* 1996). Recent work using mouse models suggest that all of these mutations act as gain of misfunction mutations and not loss (Qian *et al.* 1998), which will be detailed further in section 1.3.2.2.

Since their discovery as genes involved in early-onset familial AD, the function of presenilins had been under intense scrutiny. The presenilin holoproteins are cleaved by endoproteolysis in the endoplasmic reticulum followed by the CTF and the NTF forming stable heterodimers (Podlisny *et al.* 1997 and Ratovitsky *et al.* 1997). Homologous proteins were found to be involved in vesicle transport and in the Notch developmental pathway in *Caenorhabditis elegans* (Levitan and Greenwald,

1995). The discovery of the presenilin homolog gene, *sel-12*, in *C.elegans* provided the opportunity to make knockout models. Loss-of-function mutations in *sel-12* result in lethal defects because of the failed activation of the lin-12 receptor in the *C. elegans* Notch pathway during development, suggesting a role for the presenilins in human Notch signalling. Presenilin knockout mice show decreased levels of βA_{1-40} and βA_{1-42} and a corresponding increase in the levels of C83 and C99 (DeStrooper *et al.* 1998). Full length APP and presenilin were shown to coimmunoprecipitate, most likely interacting around the transmembrane domains of both proteins (Xia *et al.* 1997).

These findings led to two theories about the involvement of the presenilins in the activity of γ-secretase. The first hypothesis was that the presenilins were directly involved in the catalytic process. Others argued that APP did not interact (Thinakaran *et al.* 1998) and that they were involved in the regulation of the transport of the protease and/or APP. Recent work using photoactivated inhibitors (Li *et al.* 2000) and transition-state analogue inhibitors (Esler *et al.* 2000) suggests that PS1 and PS2 form the γ-secretase complex itself. Work using animal models has not been conclusive in determining the exact function of the presenilins (see section 1.3.2.2 and De Strooper *et al.* 1998). Others have used mutation analysis to study the aspartyl residues in PS1, which are vital for the function of acidic proteases. Mutation of Asp257 within PS1 does not affect the production of βA (Capell *et al.* 2000) and this is confirmed by Kim *et al.* (2001) who mutated both Asp257 and Asp385. The mutant PS1 disrupted βA trafficking which gives weight to the transport regulation hypothesis.

This work has raised the possibility of using γ-secretase inhibitors therapeutically to reduce the levels of βA produced. However, as mentioned above, the presenilins are involved in other pathways. *Drosophila* work has revealed that Notch regulated signal transduction is essential in cell differentiation during development (reviewed in Artavanis-Tsakonas *et al.* 1999). The cleavage product of Notch, NICD (Notch intracellular domain), is transported into the nucleus where it activates transcription of CBF-1 and Su(H) among others (Schroeter *et al.* 1998). The role of the presenilins in this pathway has been confirmed in various transgenic animal models and mammalian cell lines (De Strooper *et al.* 1999; Ray *et al.* 1999; Song *et al.* 1999; Struhl and Greenwald, 1999; Berezovska *et al.* 2000; Zhang *et al.* 2000). The lethality of Notch knockouts suggests this may limit the use of γ-secretase inhibitors. However, partial inhibition of PS1 (only a single allele knocked out) in a mouse model significantly reduces γ-secretase activity while maintaining the production of NICD (Zhang *et al.* 2000). See Figure 1.6 for an overview of presenilin cleavage pathways in humans.

1.3.2.1 The γ-secretase complex

The purified cellular extract that displays γ-secretase activity has a very high molecular mass, higher than that of PS1 and PS2 combined, giving the impression that the presenilins work as part of a complex (Capell *et al.* 1998; Yu *et al.* 1998). Yu *et al.* (2000) isolated a novel presenilin interacting protein which they named nicastrin. Mutagenesis of nicastrin in cell lines overexpressing APP leads to variations in βA production, suggesting a role for this protein in γ-secretase cleavage. Nicastrin has also been shown to bind the carboxy-terminal region of β-secretase

cleaved APP. Other proteins possibly involved in the secretory complex have been isolated using the yeast-two-hybrid system, including the catenins, (Zhou *et al.* 1997; Yu *et al.* 1998). α- and β-catenin immunoprecipitate with PS1, however loss of this interaction has been shown to have no effect on the action of the γ-secretase. Recent work using an immobilised inhibitor of γ-secretase showed that the presenilin heterodimers, nicastrin and C83 all bind to the inhibitor but the calsenilins and β-catenin do not, casting further doubt on their role in the complex (Esler *et al.* 2001).

Analysis of the Notch pathway in other organisms has recently revealed new components of the γ-secretase complex. Mutation analysis of developmental defects in *C. elegans* showed that *Aph-1* is involved in the targeting of Aph-2 (the nematode nicastrin ortholog) and can disrupt Notch signalling when mutated (Goutte *et al.* 2002). Similar work in *C. elegans* and the use of RNA inhibitors in *Drosophila melanogaster* has revealed that PEN-2 is also involved in the complex (Francis *et al.* 2002). Both APH-1 and PEN-2 have since been shown to be integral parts of the γ-secretase complex (Steiner *et al.* 2002; Kimberley *et al*, 2003) and are co-ordinately regulated with the presenilins and nicastrin. Current evidence suggests that APH-1 binds the 'immature' form of nicastrin, which is then transported to and binds the PS holoprotein. This complex binds PEN-2 which promotes the endoproteolysis of PS, forming the heterodimers, which in turn results in the maturation of nicastrin. The maturation of nicastrin signals a functional γ-secretase, the active site of which is the PS heterodimer (LaVoie *et al.* 2003; Takasugi *et al.* 2003). These studies show that elegantly designed genetic experiments in *C. elegans* can yield a significant amount of information about human diseases. Figure 1.6 illustrates the different presenilin cleavage pathways.

1.3.2.2 PS transgenic mice

Due to the large number of *PS1* mutations and the fact that they are the most common cause of familial EOAD, a very large number of *PS1* overexpressing transgenic mice have been produced. One feature is consistent in all of these models; an elevated ratio of βA_{1-42} as compared to βA_{1-40} in mutant *PS1* expressing mice (Borchelt *et al.* 1996; Duff *et al.* 1996; Citron *et al.* 1997). Several groups have crossed *PS1* mutant mice with *APP* mutant mice to produce double mutant transgenic mice (Duff *et al.* 1996; Borchelt *et al.* 1997; Takeuchi *et al.* 2000) and these produce the highest levels of βA of all.

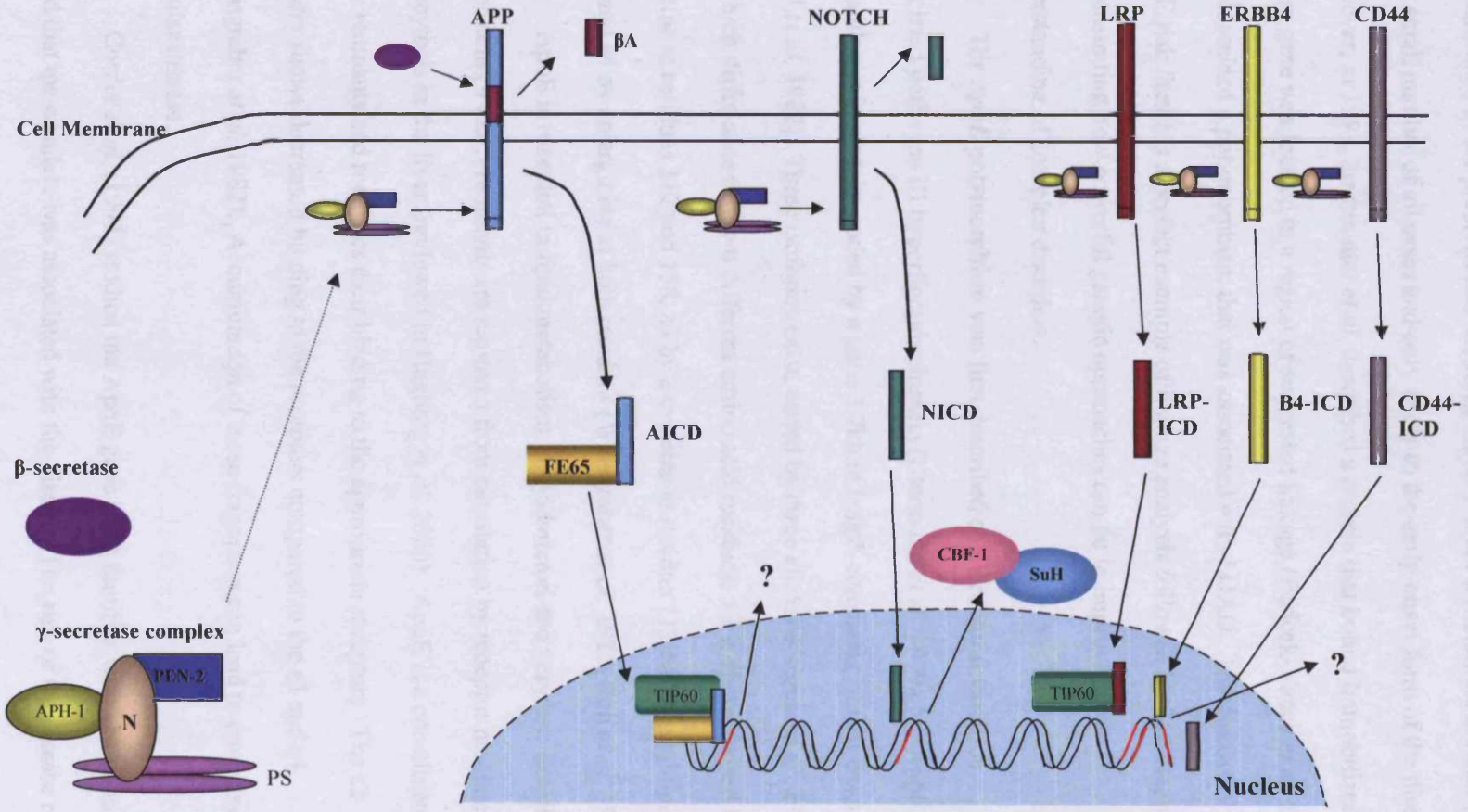
Neuronal loss is not apparent in *PS1* single transgenic mice and, despite the increased βA production, double transgenic mutant *PS1/APP* mice do not show extensive neuronal loss in the hippocampus or the prefrontal cortex (Takeuchi *et al.* 2000). An incomplete phenotype is characteristic of many animal models of neurodegenerative diseases, particularly those with an onset in later life in humans. Transgenic mouse models of both amyotrophic lateral sclerosis (reviewed in Guegan and Przedborski, 2003) and familial Parkinson's Disease (Lee *et al.* 2002) fail to show the cellular pathology seen in the human form of the diseases This could be a result of the mice not having a long enough lifespan to develop the full pathology. As with the neuronal loss, single transgenic *PS1* mice show no significant decrease in learning. Double mutants do show significant behavioural impairments, but no more so than single APP transgenics (Janus *et al.* 2000; Holcomb *et al.* 1999).

1.3.2.3 Presenilins in APP signal transduction

Presenilin cleaves the product of tumor necrosis factor-converting enzyme (TACE)-processed Notch to produce a CTF termed NICD (Schroeter *et al.* 1998). This peptide then translocates to the nucleus and activates the transcription of several genes. As the presenilins are also vital in the γ-secretase cleavage of APP, which bears a strong resemblance to Notch processing, this naturally led to the hypothesis that the cytoplasmic domain of APP could also be involved in nuclear signalling.

Analysis of the γ-secretase cleavage of both Notch and APP revealed a new cleavage site within the APP transmembrane domain, several residues carboxyl-terminal of the βA site, which is exactly analogous to the Notch cleavage site (Yu *et al.* 2001). This novel cleavage produces a fragment which has now been termed APP intracellular domain (AICD) to mirror the terminology used in the Notch pathway. The AICD is stabilised by FE65, then translocated to the nucleus (Kimberly *et al.* 2001) where it forms a complex with TIP60 (see Figure 1.6), a protein involved in gene transcription (Cao and Südhof, 2001). Recent work suggests a role for APP in phosphoinositide-mediated calcium signalling (Leissring *et al.* 2002). Presenilin mutations not only increase βA production but also alter intracellular calcium signalling pathways (Leissring *et al.* 1999) and these deficits are rescued in PS-/- cells when treated with AICD.

Figure 1.6 γ-secretase processing pathways



The role of the gamma secretase complex in the processing of transmembrane proteins. The complex is formed from PS heterodimers, APH-1, nicastrin (N) and PEN-2. Cleavage of APP, NOTCH, CD44 (Lammich *et al.* 2002), ERBB4 (Lee *et al.* 2001) and LRP (May *et al.* 2002) produces ICD fragments which translocate to the nucleus and activate transcription of genes (shown by red line). The genes activated in this manner are unknown for all of the pathways except the NOTCH pathway which activates CBF-1 and SuH. FE65 stabilises the AICD fragment for translocation. TIP60 plays a role in transcription and binds both AICD and LRP-ICD (Kinoshita *et al.* 2003)

1.3.3 Apolipoprotein E

As mentioned in the previous two sections, the *APP* and *PS* mutations account for a very small number of all cases and only apply to the early-onset form of the disease. However, in 1993, Strittmatter *et al.* described a protein that bound immobilized βA, whose gene was located in a region of suggested linkage (Pericak-Vance *et al.* 1991) and contained a polymorphism that was associated with LOAD. The discovery of the ApoE risk factor is a perfect example of linkage analysis followed by fine mapping demonstrating how powerful genetic approaches can be in improving our understanding of complex disorders.

The *ApoE* polymorphism was first described as a structural variation associated with type III hyperlipoproteinemia (Utermann *et al.* 1979a,b). ApoE is a 317 amino acid protein, coded by a gene 3.7kb in length consisting of four exons (Rall *et al.* 1982). Three isoforms exist, coded by three alleles designated ε2, ε3 and ε4 which differ at one of two different amino acid residues. ε2 is characterised by a cysteine at residues 112 and 158, ε3 by a cysteine at residue 112 and an arginine at 158 and ε4 by an arginine at both residues (Weisgraber *et al.* 1981; Rall *et al.* 1982)

ApoE is essential in lipid metabolism. Chylomicron and very low density lipoprotein (VLDL) remnants are removed from circulation by receptor mediated endocytosis in the liver (reviewed in Hagberg *et al.* 2000). ApoE is a constituent of these remnants and mediates their binding to the appropriate receptors. The ε2 isoform shows decreased binding to the receptors compared to the ε3 and ε4 (Weisgraber *et al.* 1982). Accumulation of these fragments can lead to coronary vascular disease.

Corder *et al.* (1993) studied the ApoE gene in 42 families with LOAD and found that the ε4 allele was associated with the disease. The risk of the disease rose

from 20 to 90% and the age of onset decreased from 84 to 68 as the number of ε4 alleles increased. This complemented earlier work which showed that the ε4 allele bound βA much more rapidly than ε2 and ε3 (Strittmatter *et al.* 1993). Both Talbot *et al.* (1994) and Corder *et al.* (1994) describe the protective effect of the ε2 allele from LOAD and that this effect is not merely the absence of the ε4 allele. The ApoE ε4 allele is neither necessary nor sufficient to cause AD with most ε4 carriers not developing the disease (Hyman *et al.* 1996; Myers *et al.* 1996). Another large study of 400 AD cases and 1000 controls shows an increased risk for the ε4 allele with an OR (odds ratio) of 2.7 and a protective effect for the ε2 allele (OR 0.5) (Bickeboller *et al.* 1997).

1.4 Other genetic risk factors

Linkage studies

ApoE is the only risk factor so far identified for LOAD. Early estimates suggested that ApoE accounts for 50% of the 'predicted genetic effect' in AD (Roses *et al.* 1995). However, more recent evidence places the effect in the region of 7-15% and indicated that 4 or more unknown loci, with effects at least the same size of that of the *ApoE* gene, account for the remainder (Daw *et al.* 2000).

Several genome screens have been carried out to identify regions of suggested linkage to LOAD. A two stage genome screen using sib pair analysis in 38 LOAD families showed suggested linkage across a 30cM region on chromosome 12 with a multi point MLS (maximum lod score) of 3.2 (Pericak-Vance *et al.* 1997 and 1998). Other regions of interest were found on chromosomes 4, 6 and 20. A systematic genome-wide LD map at 10cM intervals found association with LOAD on

chromosomes 1q31.1, 1q43, 10p12, 12q24, 19q13 and Xq26.1 with the chromosome 19 association occurring as a result of LD between the marker and ApoE (Zubenko *et al.* 1998). Another genome-wide LD mapping survey produced eight chromosomal regions of interest in a population of Finns; 1p36.12, 2p22.2, 3q28, 4p13, 10p13, 13q12, 18q12.1, and 19p13.3 (Hiltunen *et al.* 1999 and 2001). A complete genome screen in 292 ASPs revealed 4 regions of suggestive linkage on chromosomes 1, 9, 10 and 19 with other peaks seen on chromosomes 5, 14, 12 and 21 (Kehoe *et al.* 1999b). The whole genome was screened in a second stage (n = 451) with all the regions with a lod score in excess of 1 from the first stage being analysed in greater detail. Regions on chromosomes 1, 5, 6, 9, 10, 12, 19, 21 and X retained lod scores of greater than 1 with the highest multipoint lod of 3.9 score seen on chromosome 10 (Myers *et al.* 2000 and 2002). One of the markers (D10S1217) within the lod-1 region on chromosome 10 showed association with LOAD in an attempt to narrow the region.

Several studies have shown that the chromosome 10 region may play a role in LOAD but the results have varied slightly. A linkage study of AD patients with high plasma levels of βA_{1-42} gave a MLS of 3.93 within the same region (Ertekin-Taner *et al.* 2000). Another linkage study found linkage with D10S583, which maps to 10q but not in the same region as that found in the Myers study (Bertram *et al.* 2000). Linkage analysis using age of onset as a marker gave a MLS of 2.6 in AD patients near the region reported by Bertram *et al.* (Li *et al.* 2002).

Association studies

As genotyping methods become highly automated and cheaper the number of association studies performed in complex diseases has grown and AD is no exception. Many of the genes studied have been selected because of positional information from

linkage studies, others because of functional evidence (e.g. encoded proteins present in SPs). A search of Entrez-PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed) produces over 250 such association studies in AD since 1995 with a selection shown in Table 1.2 on the following page.

The first thing to note is the sheer number of genes in the non-exhaustive list below; over thirty genes have been studied for association with AD and the number is increasing every month. From this list 33% of the studies have not been replicated at all while only 15% have over half of their follow-up studies giving a positive association. Only 16% of the genes studied have had a meta-analysis performed on the published data of which four gave a positive result. This Table displays the difficulties in discovering a true association for a complex disease and the need for standardisation of association studies (reviewed in Daly and Day, 2001).

A2M

As can be seen from the Table, some of the genes have been investigated extensively. The most heavily genotyped is α-2-macroglobulin (A2M), a proteinase inhibitor found in SPs (Rebeck *et al.* 1995) that has also been shown to degrade βA *in vitro* (Qiu 1996). A2M also maps to chromosome 12, an area of suggestive linkage in several genome screens (see previous section) and is therefore both a good functional and positional candidate. Two different polymorphisms were analysed in two different studies both of which gave a positive result; an insertion/deletion at the splice site of exon 18 (Blacker *et al.* 1998) and isoleucine to valine change at residue 100 (Liao *et al.* 1998). The studies that have followed have mostly genotyped one polymorphism or the other, in varying populations, with mixed results. However, a

Table 1.2 AD association studies

Gene	Author	Cases	Controls	Result	Replications	Meta
5-HT6	Li *et al.* (1997)	196	271	p = 0.004	2/7	-
A2M	Blacker *et al.* (1998)	104*	-	p = 0.001	7/30	-ve
ACE	Tysoe *et al.* (1996)	141	141	na	11/17	OR 1.3
ACT	Kamboh *et al.* (1995)	225	315	p = 0.055	6/26	-
αSN	Xia *et al.* (1996)	223	149	p = 0.005	2/4	-
AR	Lehmann *et al.* (2003)	150	190	OR 2.5	0	-
AGT	Taylor *et al.* 2001	239	342	na	0	-
APBB1	Hu *et al.* (1998)	190	267	p = 0.005	1/3	-
APOC1	Kamino *et al.* (1996)			p = 0.002	3/3	-
APOD	Desai *et al.* (2003)	70	163	p = 0.013	0	-
ApoE P	Bullido *et al.* (1998)	220	264	p = 0.0004	6/15	-
BACE	Murphy *et al.* (2001)			na	1/6	-
BCHE	Lehmann *et al.* (1997)	74	104	OR 6.9	3/11	Yes
BDNF	Kunugi *et al.* (2001)	170	498	p = 0.00004	2/2	-
BH	Montoya *et al.* (1998)	357	320	p = 0.009	1/4	-
C1r	Rosenmann *et al.* (2003)			na	0	-
CA	Buxbaum *et al.* (2000)			na	0	-
CATHD	Papassotiropoulos *et al.* (1999)	102	191	p = 0.001	2/8	2% risk
CHAT	Mubumbila *et al.* (2002)			OR 3.7	0/2	-
CYP46	Kolsch *et al.* (2002)	114	144		1/2	-
eNOS	Dahiyat *et al.* (1999)	317	392		0/5	-
ICAM	Pola *et al.* (2002)	98	115	OR 3.0	0/1	-
IDE	Abraham *et al.* (2001)	133	135	na	1/3	-
IL-1a	Grimaldi *et al.* (2000)			OR 4.9	4/9	-
LRP	Lendon *et al.* (1997)				6/12	OR 1.3
NEP	Oda *et al.* (2002)	201	208	na	0/1	-
PS1	Wragg *et al.* (1996)	208	185	OR 2.0	11/21	-
STH	Conrad *et al.* (2002)	51	30	OR 11.9	0/3	-
TNFα	Collins *et al.* (2000)	266*	-	p = 0.005	3/4	-
VLDLr	Okuizumi *et al.* (1995)	404	380	OR 2.1	2/7	-

Table detailing some of the AD association studies carried out since 1995. Author of original study shown, along with population numbers used for analysis. Results of analysis shown as either p-value, OR (odds ratio) or na (no association). Replication numbers are shown as fraction of positive associations from total number of studies. The presence or lack of a meta-analysis is also shown with result where applicable. Gene acronyms are as follows 5-HT6 (5-hydroxytryptamine 6), A2M (alpha-2-macroglobulin), ACE (angiotensin-converting enzyme), ACT (alpha-1-antichymotrypsin), αSN (alpha-synuclein), AR (androgen receptor), AGT (angiotensinogen), APBB1 (APP binding protein family B member 1), APOC1 (apolipoprotein C1), APOD (apolipoprotein D), ApoE P (apolipoprotein promoter), BACE (beta site APP cleaving enzyme), BCHE (butyrylcholinesterase), BDNF (brain derived neurotrophic growth factor), BH (bleomycin hydrolase), C1r (complement component 1), CA (calsenilin), CATHD (cathepsin D), CHAT (choline acetyl transferase), CYP46 (cytochrome P450, family 46), eNOS (endothelial nitric oxide synthase), ICAM (intercellular adhesion molecule 1), IDE (insulin degrading enzyme), IL-1a (interleukin-1a), LRP (low density lipoprotein receptor related protein), NEP (neprilysin), PS1 (presenilin 1), STH (saitohin), TNFα (tumor necrosis factor α), VLDLr (very low density lipoprotein receptor). * denotes a family based association study and therefore no controls were used.

study of mRNA in individuals carrying the deletion found no aberrations in splicing (Blennow *et al.* 2000) suggesting the polymorphism does not have a functional effect.

No work appears to have been done on the functional role of the V100I polymorphism.

A meta-analysis of all studies up to 2000 shows no significant effect for either polymorphism in AD (Koster *et al.* 2000) for white and mixed ethnic populations but a significant decrease in the deletion allele is seen in Asian AD populations. A2M highlights many of the problems seen in association studies, particularly the concentration on one or two polymorphisms within one gene even if there is no evidence for a functional effect at those positions.

ACT and ACE

Both alpha-1 antichymotrypsin (ACT) and angiotensin-converting enzyme (ACE) will be discussed in more detail in sections 1.5.1, inflammation and 1.6, cardiovascular disease respectively. It should be noted here that most of the studies shown in Table 1.2 for ACE and ACT suffer from the same limitations as that for A2M; only a single polymorphism has been genotyped and these are unlikely to be the disease susceptibility polymorphisms (Rosatto *et al.* 1999; McIlroy *et al.* 2000).

ApoE -491

The epsilon variant risk factor for AD in *ApoE* has already been detailed in section 1.3.3. However recent work also suggests that *ApoE* promoter polymorphisms are associated with AD independent of ε4 status. These studies appear to show that in addition to the binding properties of the protein, the level of expression of ApoE is important in the pathogenesis of AD. Most of the studies have concentrated on two polymorphisms, -419A/T and -219A/T (Artiga *et al.* 1998), with Table 1.2 detailing the association studies performed on the former. The functional element of this

polymorphism has been studied to a greater degree than the other genes mentioned in this section. The -491 has been shown to affect the level of *ApoE* transcription (Artiga *et al.* 1998) and AD patients homozygote for the A allele have a significantly increased βA burden (Pahnke *et al.* 2003). As with all of the other genes mentioned in this section the results of the replications have been contradictory which could be down to different populations being used and partial LD between -491 and the epsilon polymorphisms (Roks *et al.* 1998). No meta-analysis has yet been carried out on these polymorphisms.

BCHE

Butyrylcholinesterase (BCHE) has also been subject to a number of association studies. BCHE activity is increased in AD patients (Perry *et al.* 1978) and cholinergic neurons are particularly vulnerable to degeneration as mentioned in section 1.2, which makes this gene a good functional candidate. In addition, the gene maps to 3q26.1 (Soreq *et al.* 1987) which is near a region of suggested linkage (Hiltunen *et al.* 2001). A polymorphism that is associated with reduced serum levels of BCHE (Bartels *et al.* 1992) gave an OR of 6.9 for LOAD rising to 12.8 in patients over 75. The K variant of *BCHE* association has not been replicated many times but a meta-analysis of all published studies shows that risk of AD is increased (OR 6.9) in patients over 75 carrying the K variant and the ε4 allele of *ApoE* (Lehmann *et al.* 2001).

CHAT

Choline acetyl transferase (CHAT) is a strong candidate, being involved in cholinergic neural transmission and mapping to the area of suggested linkage on chromosome 10 (Myers *et al.* 2000). The first published association study

(Mubumbila *et al.* 2002) looked at one polymorphism, a G/A shift at position +4, and reported a strong association. However two follow up studies have failed to replicate this finding, including a comprehensive screening of the whole gene and genotyping in 500 cases and controls (Harold *et al.* 2003; Schwarz *et al.* 2003). Haplotype analysis also failed to detect any association (Harold *et al.* 2003).

LRP

Another candidate that has been extensively studied is low density lipoprotein receptor related protein (LRP). As Table 1.2 suggests, ApoE ε4 is still the only confirmed genetic risk factor for LOAD and therefore other proteins involved in the same pathway are obvious candidates. LRP is a member of the LDL receptor family and binds at least 30 different ligands including ApoE, for which it is the major receptor, APP and A2M. LRP is a strong functional candidate and the gene lies in an area of suggested linkage (Kehoe *et al.* 1999b; Myers *et al.* 2001) on chromosome 12. Recent evidence shows that the receptor undergoes proteolytic cleavage by γ-secretase, similar to that of APP, forming an intracellular domain (May *et al.* 2002). The intracellular domain has been shown to translocate to the nucleus, in the same way as AICD and NICD (see Figure 1.6), where it regulates AICD/FE65 transcriptional activation possibly by binding TIP60 (Kinoshita *et al.* 2003)

Association studies of this gene have concentrated on a tetranucleotide repeat found 5' of the gene itself and on a polymorphism in exon 3. Many different populations have been genotyped and some have used very low numbers (Bi *et al.* 2001) which raises questions about their ability to detect any effect. However a meta-analysis has been carried out on the exon 3 studies giving an OR of 1.3 (Sanchez-Guerra *et al.* 2001) and suggests that the CC genotype is associated with disease.

Some of the other genes mentioned in Table 1.2 will be described in later sections. Many follow the same pattern of an initial strong association followed by negative studies in varying populations. Table 1.2 cannot take into account publication bias i.e. that positive results are preferentially published, which further reduces the strength of the associations. It is clear that initial association studies should carry out a comprehensive screening of the gene for polymorphisms within a carefully selected screening set. The detected polymorphisms should be genotyped in a case-control population which is large enough to detect an effect of the size anticipated. If at all possible, functional candidacy should be backed up by positional evidence from linkage studies. All of these limitations and more are reviewed in Daly and Day (2001) and described in a recent editorial in Human Genetics in advice for future submissions for publications (Cooper *et al.* 2002).

1.5 Mechanism of Pathology

It is generally accepted that the βA cascade is central to the pathogenesis of AD, however, the underlying mechanism which results in the degeneration of the neurons remains unclear. It is worth noting that there is a large substantial overlap between the mechanisms described in this section which suggests that more than one mechanism could be present. In addition, the outcome of the pathogenic mechanism i.e. the method of cell death is also under debate, with evidence for necrosis (Lassman *et al.* 1995) and apoptosis (Su *et al.* 1994) described. It is possible that both pathways are present with necrosis resulting from the initial insult causing apoptosis in neighbouring cells (Behl 2000).

As some of the genes analysed in this study are implicated in the inflammatory process it will be described in more detail than the other mechanisms

1.5.1 Inflammation

Several lines of evidence suggest that inflammation could be a crucial pathological process in AD. Inflammation is the localized tissue response to injury or infection and is a part of the immune system. The immune response to an infection or a foreign agent can be split into two different sections, the innate response and the adaptive response. The adaptive response is not as heavily implicated in AD as the innate response and therefore is not mentioned here.

The Innate Response

The innate system provides the immediate response and depends on macrophages, cytokines and the complement system. Macrophages recognise foreign cells, engulf and then destroy them using lysosomal compound such as nitric oxide (NO). They also secrete cytokines, via a signal cascade involving interleukin-1 receptor (IL-1R), which promote inflammation (reviewed in Janeway *et al.* 2001)

Inflammation is characterised by pain, redness, heat and swelling in the damaged/infected region induced by the action of cytokines. These are the external characteristics of changes occurring in the local vascular system; vasodilation, increased blood flow, increased adhesion within the blood vessels and increased vascular permeability. All of these changes combine to allow additional phagocytes to enter the area including monocytes and neutrophils, and to promote the accumulation of proteins and fluid from blood to accumulate in the surrounding

tissue. It is therefore clear that the cardiovascular system is also crucial to the immune response.

The alternative method for binding infective microorganisms involves the complement system; a series of plasma proteins that interact with each other. They coat the microorganism, allowing the binding of phagocytes which have complement receptors which act as chemoattractants, in a system known as opsonization, drawing more phagocytes to the site of infection or damage. Finally, the terminal products of the complement cascade form a structure, the membrane attack complex (MAC), which can create a pore in some microorganisms causing damage (for a review see Tomlinson, 1993).

Inflammation in AD

Transgenic mice that overexpress APP and produce βA do not always display nerve cell and synaptic loss (see section 1.3.1.2). Similar observations occur in humans where aged individuals can accumulate βA but do not develop dementia. One of the main differences between demented and non-demented brains is the presence of inflammatory markers situated around the aggregates such as activated microglia, activated astrocytes and elevated levels of cytokines and complement proteins. This suggests that the SPs provoke an inflammatory response in the brain. Inflammatory activity is significantly elevated in AD patients' brains compared to age matched controls (Dumery *et al.* 2001). The evidence appears to suggest that the innate response may play a role in AD neurodegeneration.

Activated microglia have been found in early, diffuse plaques, suggesting that they might form a part of the early response to pathogenic βA aggregation (Itagaki *et al.* 1989). Tan *et al.* (1999) showed that microglia, the central nervous system (CNS)

antigen presenting cells are activated after treatment with solubilised βA and in APPswe mice. Complement proteins have been found within SPs and the initial activating protein of the classical complement pathway C1q has been shown to bind to βA in vitro (Rogers *et al.* 1992). The MAC has also been reported in the brains of AD patients (Itagaki *et al.* 1994). Prolonged exposure to the activated complement pathway leads to chronic activation of immune cells, such as astrocytes, which in turn produce a cascade of cytokines. Transgenic mice and rats which chronically produce cytokines within the nervous system develop memory loss and atrophy of the hippocampal cholinergic input (Heyser *et al.* 1997).

Several epidemiological studies in the early 1990s show a possible protective effect for non-steroidal anti-inflammatory drugs (NSAIDS) against AD (reviewed by McGeer *et al.* 1996), however more recent studies have given mixed results (Stewart *et al.* 1997; Beard *et al.* 1998, in't Veld *et al.* 1998). A recent study showed that long term use of NSAIDs confers a relative risk for AD of 0.20 but no reduction in risk of vascular dementia (in't Veld *et al.* 2001). Experimental evidence also shows that anti-inflammatory treatments can reduce the pathogenesis of AD. Treatment of transgenic APP mice with ibuprofen results in a significant reduction of SP load, activated astrogliosis and neuritic dystrophy (Lim *et al.* 2000). Similar work shows that administering monoclonal and polyclonal antibodies raised against βA_{1-42} to immunise PDAPP mice reduces plaque burden by 83% and 91% respectively, compared to control animals (Bard *et al.* 2000).

Association Studies

As the inflammatory system is implicated in the pathogenesis of AD, several of the pathways' genes have been analysed in association studies, some of which are detailed in Table 1.2.

ACT is a serine protease inhibitor which associates with SPs (Abraham *et al.* 1990) and is expressed by astrocytes. βA has been shown to insert directly into the β-sheets found in ACT causing a conformational change which results in its alteration from an inhibitor to a substrate (Janciauskiene *et al.* 1998). The production of ACT is regulated by the cytokines IL-1 and TNF (Kordula *et al.* 2000). The gene maps to 14q32.13 which is not under any regions of suggested linkage (Rabin *et al.* 1986). The polymorphism analysed in most studies (A/T shift in the signal peptide) has no effect on plasma levels of the protein (McIlroy *et al.* 2000) and has given mixed results. A polymorphism has been recently described in the promoter region of *ACT* that alters plasma ACT levels (Morgan *et al.* 2001) however no association studies appear to have been carried out on this polymorphism so far.

Tumor necrosis factor (TNF) has also been extensively studied and also replicated in many samples with recent evidence suggesting it may be a risk factor associated with age of onset (Alvarez *et al.* 2002). TNF is a cytokine which has been shown to have significantly elevated circulating levels in AD patients (Fillit *et al.* 1991). The gene maps to 6p21, a region of suggested linkage in a recent genome screen (Blacker *et al.* 2003) and polymorphisms have been described that affect both transcription and secretion (Kroeger *et al.* 1997). Despite strong genetic evidence, the exact role of TNF in the pathology is unknown. TNF alpha converting enzyme (TACE), which cleaves TNF, was suggested as a candidate for the α-secretase (Buxbaum *et al.* 1998), however a follow up study suggests this is not the case

(Parvathy *et al.* 1999). TNF protects neurons from βA induced insult, possibly by activating anti-apoptotic and antioxidant (see next section) pathways (Barger *et al.* 1995).

The interleukin family of cytokines have also been heavily studied, including the interleukins (IL) IL-1 (Grimaldi *et al.* 2000), IL-6 (Papassotiropoulos *et al.* 1999) and IL-10 (Depboylu *et al.* 2003) all of which have produced mixed results, mainly negative. As mentioned previously the complement proteins are found within SPs and the complement component 1 (*C1r*) gene has been analysed for association but none was found (Rosenmann *et al.* 2003).

1.5.2 Other mechanisms of pathogenesis

Several other mechanisms have been described which may or may not be involved in the pathology of AD, some of the more well substantiated of which will be described here. As the genes analysed in this study do not have a direct effect on these pathways they are mentioned only briefly. The genes in these pathways have not been analysed as extensively using association studies as the inflammatory pathway.

A large amount of research has noted that βA can alter calcium homeostasis within the cell via the formation of ion pores, the potentiation of calcium channels and inhibition of the Na^+/K^+ ATPase (Mattson *et al.* 1992, Vitek *et al.* 1994, Mark *et al.* 1995). Calcium is an essential intracellular messenger for neuronal signalling particularly in learning, memory and long term potentiation (LTP). By regulating kinases and phosphotases Ca^{2+} controls a variety of cellular processes including synaptic transmission, plasticity and metabolic pathways (reviewed in Mattson and Chan, 2001). βA_{1-40} forms Ca^{2+} permeable channels in reconstituted lipid vesicles

(Lin *et al.* 1999). In addition, presenilin mutations induce elevated Ca^{2+} release from the ER in cultured PC12 cells (Chan *et al.* 2000).

Another cellular mechanism possibly involved in the pathology of Alzheimer's disease is the unfolded protein response (UPR) and proteasomal degredation pathway. The proteasome is a multicatalytic complex which is involved in the catabolic processing of ubiquinated proteins (reviewed in Checler *et al.* 2000). Ubiquitination of proteins occurs in order to regulate tightly the levels of regulatory proteins within the cell and also when they are improperly folded. The ubiquitin protein binds PS1/PS2 (Kim *et al.* 1997) and is present within NFTs (Morishima-Kawashima *et al.* 1993). A mutant form of ubiquitin with a frameshift in the carboxyl terminus, termed Ub^{+1}, is expressed in AD brains that inhibits the ubiquitin-proteosome system (Lam *et al.* 2000) which may affect βA clearance.

The human body produces reactive oxygen species (ROS) during the transfer of electrons in cellular respiration which can be highly damaging to the cell e.g. superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) (reviewed in Hayden *et al.* 2002). Mass spectrometry shows that βA produces free radicals and can inactivate oxidation-sensitive enzymes (Hensley *et al.* 1994). The activated microglia found surrounding SPs (Cras *et al.* 1990) are a large source of both NO and $O_2^{-\bullet}$ (Colton and Gilbert, 1987). Analysis of mitochondrial DNA (mtDNA), cytochrome oxidase production and morphology reveals a large number of mitochondrial defects in AD (Hirai *et al.* 2001) which can also produce ROS.

1.6 Cardiovascular Disease and AD

Cardiovascular disease (CVD) and AD are strongly linked and the idea that depleted cerebral blood flow can lead to cognitive decline has been recognised for over a century. There are two main schools of thought in the link between CVD and Alzheimer's. Some researchers believe that Alzheimer's should in fact be reclassified as a vascular disorder and not a neurological disorder (de la Torre, 2002) as nearly all risk factors for AD appear to reduce perfusion. They suggest that AD is a secondary event following an intracranial microvascular insult caused by CVD. A different theory has been put forward by some researchers that both diseases are independent but convergent as opposed to being directly linked (reviewed in Casserly and Topol, 2004). If the latter theory is correct it opens up the opportunity of treating at risk groups with anti-CVD drugs from an early age, however these drugs must be able to cross the blood brain barrier.

CVD is the most common cause of death in the western world and covers a wide range of disorders, some of which are discussed here. The reduced perfusion can also lead to problems in the inflammatory system (see section 1.5) because of its requirement for increased blood flow. Detailed below are the major causes of CVD along with evidence the links between them and AD. As to which of the two theories mentioned above the evidence supports is unclear and more work needs to be carried out using long term, longitudinal studies.

1.6.1 Atherosclerosis

Atherosclerosis is a factor in approximately 50% of all deaths in the west (reviewed in Lusis, 2000) and is characterised by the accumulation of lipids and fibrous elements intra-arterially which can become occlusive (Ross, 1993). Two of the main risk

factors for atherosclerosis are high levels of low density lipoprotein (LDL) and low levels of high density lipoprotein (HDL) (Gordon *et al.* 1977). LDL accumulates in the subendothelial matrix by interacting with matrix proteoglycans through one of its constituent's apolipoprotein B (ApoB). Once trapped, LDL undergoes modifications, including oxidation which is particularly important in the formation of the lesion. These oxidised LDLs stimulate the adjacent endothelial cells into producing pro-inflammatory molecules such as macrophage colony stimulating factor. Oxidised LDL also inhibits the production of NO, a potent vasodilator, further reducing the rate of perfusion in the vessels.

Nitric Oxide

In 1987 both Ignarro *et al.* and Palmer *et al.* showed that NO is the factor that mediates endothelial-dependent relaxation of smooth muscle cells in response to acetylcholine (ACh). NO is formed from L-arginine (Palmer *et al.* 1987) in the endothelium and then diffuses across the cell membrane to the vascular smooth muscle cells where it activates guanylate cyclase which in turn increases cyclic guanosine-3',5'-monophosphate (cGMP) production (Ignarro *et al.* 1984, Moncada and Higgs, 1993). It is cGMP that mediates the biological effects of NO e.g. control of vascular tone and blood pressure and platelet activation. Endothelial NO expression, from a gene on chromosome 7 (Wang and Marsden, 1995), is tightly regulated and can be induced under certain conditions such as sheer stress (reviewed in Davies, 1995).

In atherosclerosis impaired endothelium-dependent vasorelaxation in response to ACh is seen. Animal studies have shown that the impairment is in the L-arginine-NO pathway and not a reduced response in the vascular smooth muscle cells (Bossaller *et al.* 1987; Zeiher *et al.* 1991). NO has a number of anti-atherogenic properties including inhibiting the production of superoxide anion (Clancy *et al.* 1992) and the oxidation of LDL (Hogg *et al.* 1993)

HDLs are strongly protective against atherosclerosis, particularly because of its role in removing excess cholesterol. HDL also has antioxidant properties, preventing LDL oxidation through one of its constituents, serum paraoxanase (Shih *et al.* 2000). ApoE is also strongly implicated in atherosclerosis, with *ApoE* knockout mice developing the disease spontaneously (Zhang *et al.* 1992). Other risk factors include hypertension, smoking, high fat diets and lack of exercise (Assmann *et al.* 1999).

A strong connection is exists between AD and atherosclerosis because of a possible shared risk factor, ApoE (see section 1.3.3). A study of post-mortem confirmed AD cases found a significant increase in the degree of cerebral atherosclerosis in ε4 carriers (Kosunen *et al.* 1995). *ApoE* genotype is also associated with carotid artery thickening (Terry *et al.* 1996). The Rotterdam study showed that atherosclerotic indicators increase the likelihood of both vascular dementia and AD and that this effect is compounded in the presence of the ε4 allele (Hofman *et al.* 1997). A meta-analysis of heart disease association studies with *ApoE* showed an excess of the ε4 allele in CVD patients with an OR of 1.7 (McCarron *et al.* 1999). Other genes that have shown a positive association with both atherosclerosis and AD include angiotensinogen (AGT) (Gardemann *et al.* 1999) and ACE (Arbustini *et al.* 1995).

In addition increased cholesterol levels are strongly linked to atherosclerosis development (Steinberg, 1989) which can be caused by both environmental and genetic factors (Papassotiropoulos *et al.* 2003). Increased plasma cholesterol can lead to the formation of 'lipid rafts' in cell membranes and these have been shown to favour the localisation of APP, β-secretase and γ-secretase, which could result in increased βA production (Kojro *et al.* 2001). Animal work has shown that a high cholesterol diet can lead to increased βA immunoreactivity and the formation of extracellular plaques (Refolo *et al.* 2000).

1.6.2 Hypertension

Hypertension (OMIM #145500) or chronically elevated blood pressure is a complex disorder with both genetic and environmental factors involved. Several physiological systems contribute to the control of blood pressure and one of the most important of these is the renin-angiotensin system (RAS). Most of the genes involved in this system, which is described in more detail in section 3.1, have been analysed in relation to blood pressure. Polymorphisms in the renin gene show no association with blood pressure (Naftilan *et al.* 1989), while the M235T variant in angiotensinogen shows a strong and replicable association (Jeunemaitre *et al.* 1993; Kiema *et al.* 1996). A recent meta-analysis of 127 studies gives an OR of 1.3 and 1.6 for whites and Asians respectively for the TT genotype (Sethi *et al.* 2003) The ACE gene, which converts angiotensin I to angiotensin II, is slightly more controversial with many studies giving both positive and negative associations. These are discussed in more detail in section 1.6.4.

Several studies show that endothelium-dependent vasorelaxation to ACh is impaired in hypertensive patients (Taddei *et al.* 1993, Cardillo *et al.* 1998). However,

more recent evidence suggests that NO release is reduced in hypertension, as opposed to hypertension being caused by a NO deficiency (reviewed in Vallance, 1999).

Hypertension in dementia has been extensively studied. A 15-year longitudinal study of 70-85 year olds shows that higher systolic and diastolic blood pressure is seen in 70 year old patients that develop dementia between 79 and 85 compared to those who do not (Skoog *et al.* 1996). A follow up study of 1000 men, where the history of cardiovascular risk factors was available over a period of 20 years, shows that high 24 hour BP is associated with cognitive impairment (Kilander *et al.* 1998). This effect does not appear to be alleviated by treatment with anti-hypertensive drugs in the elderly (Prince *et al.* 1996). A recent study reports no association between hypertension and cognitive function but does find an increased risk of vascular dementia in patients with a history of hypertension with heart disease (Posner *et al.* 2002).

The same study also fails to find an association of hypertension with AD. However a longitudinal study of hypertension does report increased blood pressure in patients that go on to develop AD (Skoog *et al.* 1996). A similar study with a 25 year follow-up reports an increase in risk for AD with high blood pressure and also an increase in the number of NFTs compared to age matched controls (Petrovitch *et al.* 2000).

1.6.3 Diabetes Mellitus

Diabetes mellitus is a metabolic disorder which affects the transport of glucose into the cells via insulin, a pancreatic hormone. There are three main types of diabetes; type I (OMIM #222100) is an autoimmune disease where the insulin producing beta-cells are destroyed by the immune system and generally develops in childhood; type

II (OMIM #125853) develops in adults over 40 and is characterised by the body not being able to utilise the insulin produced which is termed insulin resistance; gestational diabetes affects pregnant women and tends to disappear post-natally (http://diabetes.niddk.nih.gov/index.htm).

Type II diabetes is considered a cardiovascular disease with patients also suffering from hypertension and abnormal levels of blood lipids, both of which are atherogenic risk factors. The incidence of cardiovascular disease in diabetics is twice that of age matched controls (Kannel and McGee, 1979). Therefore only type II, or non-insulin dependent diabetes (NIDDM), will be discussed here.

Linkage analysis has revealed up to ten different genomic regions possibly involved in type II diabetes (Hanson *et al.* 1998; Ghosh *et al.* 2000). These undiscovered genetic elements, along with possible environmental factors result in insulin resistance. In addition to insulin based defects, it appears that amylin, a cellular twin of insulin which is co-expressed and co-secreted with it, undergoes abnormal processing and begins to form fibrils within the extracellular space between the pancreas and the endothelial cells (islet) (reviewed in Hayden and Tyagi, 2002). The disease is then compounded over time as insulin resistance results in hyperinsulinaemia and the amylin fibrils aggregate forming a diffusion barrier between the cells. This lesion within the islet also produces large amounts of reactive oxygen species, through a variety of pathways, which are toxic to cells (see section 1.5.2).

Studies have been carried out to look at the association between diabetes mellitus and dementia and AD. The Rochester Epidemiology Study shows a slight increase in risk for AD in type II diabetes patients (Leibson *et al.* 1997). A recent study reports a minimal association between AD and diabetes (OR 1.3) and a stronger

association between diabetes and dementia after stroke (OR 3.4) (Luchsinger *et al.* 2001).

Another connection between diabetes and AD is through the insulin-degrading enzyme (IDE) and its possible role in degrading βA. IDE is an 110kDa neutral metalloendopeptidase found within the intracellular membrane and as a soluble protein (Qiu *et al.* 1998; Vekrellis *et al.* 2000), suggesting it can degrade both membrane bound and soluble βA. IDE can also degrade insulin, rat amylin, human amylin and TGF-α and as rat amylin cannot form fibrils the enzyme must recognise a motif within the protein and not merely the β-pleated sheet conformation (Bennett *et al.* 2000). The ability of IDE to cleave βA is reduced once the peptide has oligomerised and become insoluble (Vekrellis *et al.* 2000). Recent work on transgenic animals shows that that IDE -/- mice have a 50% reduction in βA degredation and increased levels of AICD (Farris *et al.* 2003). IDE expression in ε4 positive AD patients is reduced by 50%, suggesting a possible interaction between the two (Cook *et al.* 2003).

The gene itself lies on chromosome 10q, near markers shown to be in linkage with AD in a study of 435 families (Bertram *et al.* 2000). In addition, one marker (d10S583) which maps >195kb from IDE shows association with AD within this sample. Missense polymorphisms have been discovered in the Goto-Kakizaki inbred diabetic rats that reduce the degredation ability of IDE (Fakhari-Rad *et al.* 2000). However this data is not supported by association studies in humans from other groups. No association was seen between eight IDE polymorphisms and LOAD (see Table 1.2) in a comprehensive screen of the gene (Abraham *et al.* 2001). A similar study in 388 individuals also failed to find an association (Boussaha *et al.* 2002).

1.6.4 Vascular Dementia

The diagnosis of AD can be complicated by a closely related disorder, vascular dementia (VaD) and it was not until the 1970s that the two diseases were clarified (Tomlinson *et al.* 1970). VaD, also known as multi-infarct dementia, refers to cognitive dysfunction as a result of cerebrovascular injury. Accounting for approximately 15% of all dementia cases it is the second most common dementia in the west after AD (Dugue *et al.* 2003). A variety of different vascular lesions can result in VaD including large vessel atherosclerotic occlusions, small vessel disease and intracerebral haemorrhage associated with hypertension. It is not clear whether the number of lesions a patient has suffered is linked to VaD but it does appear that the position of the lesion is of greater importance than its size. Many patients who develop cognitive impairment associated with one destructive lesion display signs of vascular disease across the brain (reviewed in Iqbal *et al.* 2001)

Most of the risk factors for VaD are the same as those for stroke; hypertension, diabetes mellitus, high serum cholesterol and cardiac disease amongst others. These risk factors are also - along with age - shared by both VaD and AD. A few familial VaD pedigrees have been discovered. A mutation in APP at position 618 resulting in hereditary cerebral haemorrhage with amyloidosis has been described in a Dutch family (HCHWA-D) by Levy *et al.* (1990). Studies of ApoE in VaD have yielded variable results with some showing an increase in the ε4 allele in patients (Hofman *et al.* 1997; Slooter *et al.* 1997) but others have failed to replicate the result (Alafuzoff *et al.* 2000). As mentioned previously, it can be very difficult to separate VaD and AD and this could lead to contamination of the cases with AD patients.

Another reported risk factor for VaD is also implicated in AD; homocysteine levels. Homocysteine is a product of 1-carbon metabolism and high plasma levels of

which are associated with both cardiovascular and cerebrovascular disease (Perry *et al.* 1995; Graham *et al.* 1997). A study of plasma homocysteine in stroke, VaD and AD patients found an increased risk in all three groups even after adjusting for other vascular risk factors (OR 1.35, 1.44 and 1.3 respectively) (McIlroy *et al.* 2001). Elevated levels of homocysteine lead to p53 induced apoptosis in cultured hippocampal neurons (Kruman *et al.* 2000). A follow-up study shows that homocysteine also inhibits DNA repair and sensitises cells to oxidative damage-induced death (Kruman *et al.* 2002).

VaD and AD can co-exist and this is referred to as mixed dementia. The term mixed dementia actually refers to the presence of two or more causes of dementia however the most common form is VaD with AD. Examination of brains from pathologically confirmed AD patients found that one-third contain vascular lesions (Gearing *et al.* 1995). A study by the Canadian Consortium for the Investigation of Vascular Impairment of cognition found 76 of 149 VaD patients had mixed VaD/AD (Rockwood *et al.* 2000)

1.6.5 Shared Cardiovascular and AD Risk Factors

Some genes analysed for association in AD have also been extensively studied as cardiovascular risk factors. ApoE has already been mentioned within this section as a possible risk factor for atherosclerosis but has also shown positive association with diabetic nephropathy in both type I (Araki *et al.* 2000) and type II diabetes (Eto *et al.* 2002). Analysis of the epsilon polymorphism in hypertension has given conflicting results with two recent studies reporting an association of high blood pressure with the ε2 allele (Imazu *et al.* 2001) and the ε4 allele (Katsuya *et al.* 2002).

Endothelial nitric oxide synthase (eNOS), a gene which has shown association with AD but not been replicated (see Table 1.2), has also been studied in CAD. A polymorphic site within *eNOS* was shown to be associated with essential hypertension in patients without left ventricular hypertrophy (Nakayama *et al.* 1997). A similar study did not find an association however they did not stratify for ventricular hypertrophy (Takahashi *et al.* 1997).

As mentioned in section 1.6.2, the RAS genes are essential in blood pressure regulation and have been studied extensively in relation to hypertension, with 127 studies on angiotensinogen (AGT) alone. The *AGT* M235T polymorphism has also been genotyped in an AD case-control sample but no association was found (Taylor *et al. 2001*, see Table 1.2). However, the association studies involving the angiotensin-converting enzyme (ACE), an enzyme central to the RAS and encoded by *DCP1*, have been highly contradictory for both CAD and AD.

ACE

Studies of plasma ACE concentrations show large interindividual differences despite the fact that repeated measurements of normo-tensive subjects gives stable results (Alhenc-Gelas *et al.* 1983). A larger familial study revealed a possible genetic effect on plasma ACE activity (Cambien *et al.* 1988). Rigat *et al.* (1990) showed that an insertion/deletion (I/D) polymorphism within *DCP1* accounts for 47% of the variation in plasma ACE with higher levels found in the D/D homozygotes compared to I/I homozygotes and an intermediate level in the heterozygotes. This association was further confirmed by linkage analysis in a Caucasian European population (Tiret *et al.* 1992). Sequencing confirmed this insertion to be a 287bp *Alu* fragment found in

intron 16 which has no known functional effect including altering transcriptional levels (Rosatto *et al*, 1999).

The discovery of the *Alu* fragment and its relationship with plasma ACE levels provided a marker for disease association studies and has since been extensively used. Early work by Zee *et al.* (1992) showed an increase in the number of II genotypes in a hypertensive population. However, attempts to replicate these findings in other case control studies have given contradictory results (Schmidt *et al.* 1993; Uemura *et al.* 2000; Harrap *et al.* 1993; Morise *et al.* 1994 amongst others). Jeunemaitre *et al.* (1992) found no evidence of linkage between the ACE gene and hypertension. In contrast, a genome screen by the Framingham Heart Study found strong evidence for a locus on chromosome 17 linked to blood pressure near the ACE gene (Levy *et al.* 2000).

Left ventricular hypertrophy has also been studied extensively in relation to ACE I/D genotype and the results produced have been as mixed as those for hypertension. Schunkert *et al.* (1994) discovered an excess of the D allele in patients showing evidence of hypertrophy. This supported evidence that angiotensin II stimulated cardiac protein growth. This result was replicated by some groups (Iwai *et al.* 1994; Montgomery *et al.* 1997) but others, including the large Framingham Study, failed to find an association (Lindpaintner *et al.* 1996).

The I/D polymorphism has also been analysed in patients who have developed coronary heart disease. The first study to find an association between myocardial infarction (M.I.) and *DCP1* reported that the D/D genotype was over represented in patients in the low-risk group who had suffered a heart failure (Cambien *et al.* 1992). A subsequent attempt to replicate these findings failed to detect an association (Bøhn *et al.* 1993). However a comprehensive study comparing I/D genotypes in 4,629 M.I.

cases to nearly 6000 controls shows a slight association with the DD genotype (relative risk – 1.10) and a meta-analysis of all previous studies gives a relative risk of M.I. for the DD genotype of between 1.0 and 1.1 (Keavney *et al.* 2000).

DCP1 has been implicated in a variety of disorders and processes through the use of genetic association studies, due to its readily assayable polymorphism and the wide ranging effects of its function. Associations with the I/D polymorphism have been shown in muscle performance (Williams *et al*, 2000) and schizophrenia (Arinami *et al*, 1996). Several studies have been carried out to look at the I/D polymorphism in relation to AD and these will be discussed in more detail in Chapter 3.

Aims

This study examined the association of candidate genes with late-onset Alzheimer's disease. Four genes were studied, all of which have products related to inflammation, the cardiovascular system or both. The four genes studied were *DCP1*, *TACR2*, *ECE1* and *PI12*. Polymorphisms within these genes, reported in previously published data or detected by comparative sequencing were genotyped in UK AD case-control samples and analysed for association. The samples used were collected from Caucasians across the UK and had an age of onset over 65 years.

DCP1 encodes the ACE protein and is an essential part of the renin-angiotensin system (RAS). The gene has been extensively studied in AD: however, all previously reported work was concentrated on one polymorphism and the results produced have been contradictory. This study examined a larger number of polymorphisms in *DCP1*, previously reported in the literature, as well as the I/D polymorphism.

Tachykinin Receptor 2 (TACR2) is a receptor for the peptide neurotransmitter substance K which has a role in the nociceptive and inflammatory responses. This gene has not previously studied in AD however the gene lies in an area of suggested linkage on chromosome 10. ECE1 is an essential enzyme in the cardiovascular system, which produces endothelin-1, the most potent vasoconstrictor known. In addition to being a strong functional candidate, the gene lies in a region of suggested linkage on chromosome 3. No association studies have yet been performed on this gene for AD. The final gene studied was neuroserpin (*PI12*), a serine protease inhibitor involved in the inflammatory system and implicated in neuronal plasticity. Mutations within this gene cause a progressive neurodegenerative disorder characterised by the presence of amyloidogenic fragments within affected neurons.

Polymorphisms within this gene have been analysed in a small FAD population however no large scale association studies have been performed. Comparative sequencing of all exons, intron-exon boundaries and known or putative regulatory sequence was carried out to identify polymorphisms in the three genes above, and identified changes subsequently genotyped in the late-onset AD samples.

Chapter 2

Materials and Methods

2.1 Methods

2.1.1 Polymerase Chain Reaction (PCR)

If required, primers were designed using Oligo (v4.0) or Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and ordered from Genset or Sigma-Genosys. PCR reactions were performed on an MJ Tetrad (GRI) which is equipped with a heated lid and therefore no mineral oil overlay was required.

The reactions varied by fragment, however in general, a 12µl reaction contained 1.2µl of PCR Buffer (Qiagen), 0.2mM dNTP's, 10pmol each of forward and reverse primer, 12-18ng of template DNA and taq polymerase (Q-Taq or HotStarTaq from Qiagen). Some reactions required the addition of 2µl of Q-Sol (Qiagen) to improve the yield. For QTaq, reactions were denatured for 3 minutes (94°C), then 35 cycles at 94°C for 30s, x°C for 30s and 72°C for 30 seconds, with x being the optimal annealing temperature, which differed by reaction and was dependent on the sequence of the primers. The optimal annealing temperature was determined using the temperature gradient blocks of the MJ Tetrad which allowed several PCR reactions to be performed simultaneously at different temperatures. All samples were analysed by gel electrophoresis (see section 2.1.2) and the annealing temperature that produced the strongest and sharpest band was selected as the optimal temperature.

HotStarTaq differed by having an initial denaturing step of 15 minutes at 95°C and 45 second 95°C denaturing steps during the cycling stage. A final elongation step of 72°C for 10 minutes finished the reaction. Fragments which were particularly GC rich (>70%) were amplified using the GC-Rich PCR Kit (Roche). A 12µl reaction contained 2.4µl of GC-Rich reaction buffer, 2.4µl (1M) resolution buffer, 0.2mM dNTP's, 10pmol each of forward and reverse primer and 12-18ng of template DNA. The samples were denatured at 95°C for 3 minutes, then 31 cycles of 95°C for 30s, 58°C for 30s and 72°C for 45s. A final elongation step of 72°C for 7 minutes was carried out to end the reaction.

2.1.2 Gel Electrophoresis

Agarose (Boehringer Mannheim) was added to 0.5x TBE running buffer (National Diagnostics) to make 1.5 – 3% gels where 1g in 100ml of solution is equal to 1%. The solution was stirred gently and then heated in a microwave until the agarose dissolved. 1µl of ethidium bromide (10mg/ml) (Bio-Rad) was added to the solution after it had been allowed to cool a little, to visualise the DNA, then poured into a gel former. A glycerol based loading buffer was added to each sample and these were electrophoresed at 100v in 0.5x TBE. For increased resolution MetaPhor (Cambrex) agarose was mixed with the standard agarose to a ratio of 1:2. The fragments were visualised using a UV transilluminator and photographed using a Polaroid camera.

2.1.3 Denaturing High Performance Liquid Chromatography (DHPLC)

Polymorphisms were detected using DHPLC, a two-phase semi-automated method for variant detection. The two phases in question are liquid and solid phases consisting of PCR amplified fragments and two buffers in the former and an alkylated polystyrene-

divinylbenzene column as the latter. The column is used to detect heteroduplexes within the DNA fragments using ion-pair reverse-phase high performance liquid chromatography (Huber *et al.* 1993). The liquid phase is slowly pumped through the column where the DNA fragments bind to it, assisted by a 'bridging' chemical, triethylammonium (TEAA). The fragments are then eluted using a gradient of increasing acetonitrile concentration and heteroduplexes are detected because they are released from the column at an earlier point.

As the column is hydrophobic, TEAA is used as a bridging agent binding the negatively charged DNA molecules with its positively charged ammonium ion and the stationary phase with its alkyl chain. Both buffers which form the liquid phase contain TEAA (Transgenomic) but the second buffer also contains 25% acetonitrile and by altering the ratios of both buffers over time the concentration of acetonitrile increases. This increasing concentration of acetonitrile disrupts the hydrophobic interaction between the DNA fragments and the column resulting in their elution. Under partial heat denaturing conditions single stranded DNA will be detached sooner as they have decreased negative charges compared to a double stranded fragment. It is this phenomenon that is used to detect variants in fragments by DHPLC.

A fragment that is heterozygotic for a polymorphism will contain both alleles in a 1:1 ratio. Denaturing these fragments and slowly cooling them will result in the formation of heteroduplexes (Figure 2.1 on the following page). Once the fragment is then bound to the column, the temperature can be raised to the point of partial denaturation, and the fragment will denature around the mis-match becoming single stranded. These fragments will then be eluted from the column sooner than the homozygotes.

Figure 2.1 Heteroduplex formation.

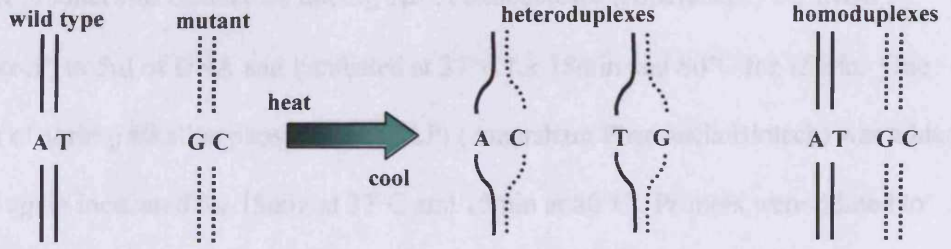


Illustration of how heating and then slow cooling of DNA fragments can produce both homoduplexes and heteroduplexes. As the fragments re-anneal, the high level of homology across the remainder of the fragment allows the possibility of mis-matching bases to align at the polymorphic site.

In this study the fragment of interest was amplified by PCR to a volume of 24µl in the LOAD screening set. The samples were then denatured at 96°C for 2 minutes and reannealed slowly by decreasing the temperature at a rate of 1°C per minute. DHPLC was performed using a WAVE™ DNA Fragment Analysis System (Transgenomic). The sample was automatically loaded onto a DNASep® column (Transgenomic) and then eluted from the column using an acetonitrile gradient in a 0.1M TEAA buffer, pH 7 at 0.9ml per minute. The gradient consisted of a mix of buffer A (0.1M TEAA pH 7, 0.1mM Na_4EDTA) and buffer B (25% acetronitrile in 0.1M TEAA pH 7). The melt profile, including the start and end points of the gradient and the optimum oven temperature, were determined for each fragment using the DHPLCMelt program available at http://insertion.stanford.edu/melt.html. In order to maximise the detection of variants the recommended temperature and T+2°C were used to analyse the fragments (Jones *et al.* 1999).

The results were displayed on a PC using the Transgenomic software as chromatograms. The peaks produced were analysed based on the number of and shapes of peaks to determine the presence of heteroduplexes.

2.1.4 Manual Sequencing

PCR product was cleaned by adding 1µl of exonuclease (Amersham Pharmacia Biotech) to 5µl of DNA and incubated at 37°C for 15min and 80°C for 15min. One unit of shrimp alkaline phosphotase (SAP) (Amersham Pharmacia Biotech) was added and again incubated for 15min at 37°C and 15min at 80°C. Primers were diluted to 25pmol/µl and labelled by incubating for 30 min at 37°C and 95°C for 3min along with 1µl of kinase buffer, 0.5µl of T4 kinase and 1.5µl of γ^{33}P dATP.

A mastermix of 3µl of cleaned template, 10.5µl of dH_2O, 2µl of thermosequenase buffer, 1µl of end-labelled primer and 1µl of thermosequenase was added to 4 tubes containing 4µl of ddATP, ddTTP, ddCTP and ddGTP respectively (all from Amersham Pharmacia Biotech). These were overlaid with mineral oil and cycled (95°C for 30s, then 30 cycles of 95°C for 30s, 60°C for 30s and 72°C for 1min.).

The reaction was stopped by adding 4µl of stop dye the samples loaded on to a 6% polyacrylamide gel and run for 1.5 to 3.5 hours at 75V and analysed by autoradiography.

2.1.5 Automated Sequencing

Automated sequencing was carried out using the BigDye kit from PE Applied Biosystems. PCR products were purified using the Qiagen PCR Purification Kit. The reaction mix, containing 5µl of clean DNA, 4µl of BigDye (Applied Biosystems) terminator mix and 3.2pmol of primer, was cycled as detailed in the BigDye manual. The reactions were cleaned by centrifugation using spin columns (Advanced Genetic Technologies) and dried down in a speed vac. Stop dye was added and the reactions electrophoresed on either the ABI 377 or the 3100 (Applied Biosystens).

For the 377 a 5% polyacrylamide gel was made using Long Ranger (Cambrex). 5ml of Long ranger was added to 18g Urea and 0.5g Molecular Sieve Dowex MR3 (Sigma) and filtered along with 5ml of 0.5xTBE using a Nalgene 245-0045 (Nalgene). 35µl of TEMED and 250µl of ammonium persulphate were added and then the solution was poured into ABI pre-formed plates then left to set for at least 2 hours.

For the 3100 the samples were prepared slightly differently. After the cycling reaction the samples were cleaned using a Sephadex G-60 (Sigma) column in 96 well plates (Millipore). After drying the sample down in the speed vac 10µl of HiDye formamide (Applied Biosystems) was added and the samples were kindly run on the ABI3100 by Nadine Norton or Hywel Williams.

For both sequencing machines the results were analysed on an iMac (Apple) using Sequence Analysis (v3.2) from Applied Biosystems. If a polymorphism was not detected within the amplified sequence the first time, it was repeated to ensure that nothing was missed. In later chapters, where the sequencing results are shown, a heterozygote is denoted by the fact that two different coloured peaks are present at the same position.

2.1.6 RFLP Assay Design

Once the polymorphisms had been detected restriction fragment length polymorphism (RFLP) assays were designed using DNAStar v4.0 (DNAStar Inc.). The MapDraw program was used which detailed any restriction enzyme cut sites that were created or abolished by the polymorphism. Enzymes were selected based upon the number of cut sites within the fragment and the cost of the enzyme. Enzymes that displayed star activity were not used.

If no restriction enzyme sites were created or the enzyme specified was unsuitable then an automated genotyping method was used (see Section 2.1.8) or a novel restriction site was created. This was done by creating a DNA primer of approximately 25bp in length which ended at the base immediately adjacent to the polymorphic site. This primer contained a mismatch base, preferably at least 2bp away from the variant, which introduced a cut site for a restriction enzyme that was suitable for use in an assay. If required, an alternative reverse primer was designed to complement the mismatch primer. A standard PCR was then carried out using the mismatch primer and its complementary reverse primer to produce a fragment containing the altered nucleotide.

2.1.7 Restriction Enzyme Digests.

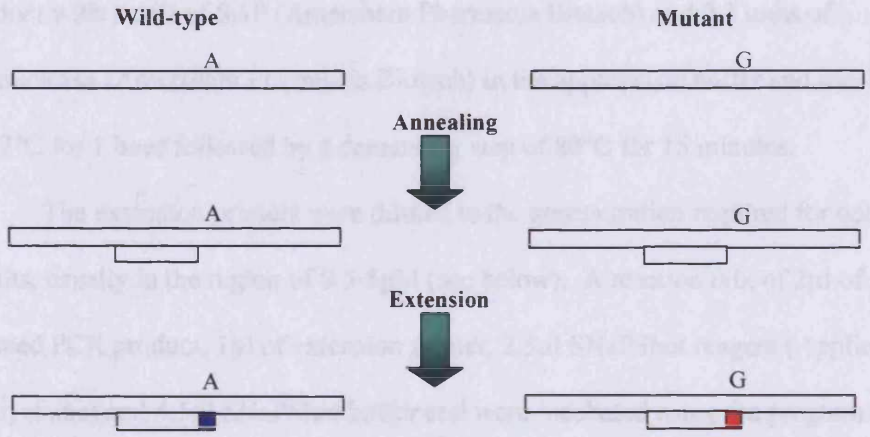
Restriction digests were carried out at the appropriate temperature, according to the enzyme, in the presence of buffer and bovine serum albumin (BSA) if required. Due to the large number of digests being carried out it was not possible to clean the PCR reactions before the digestion and therefore the enzymes were tested to ensure that they retained their activity in the presence of the PCR buffer. Digests were carried out for 16 hours and with excess enzyme to ensure complete digestion of the fragments. The enzyme was then denatured at the appropriate temperature and the samples analysed by gel electrophoresis. The enzymes used in this study along with the suppliers are detailed in section 2.2.

2.1.8 Automated Genotyping

Automated genotyping was carried out by single nucleotide primer extension using the SNaPShot system (Applied Biosystems). Primer extension uses an

oligonucleotide primer designed to anneal immediately adjacent to the polymorphic site. The primer is then extended by a single base using fluorescently labelled ddNTPs with each base being labelled with a different fluorescent dye (see Figure 2.2 below).

Figure 2.2 Primer extension



Genotyping by primer extension. PCR fragments are annealed to primers designed to bind adjacent to the polymorphic site at their 3' end. The primers are then extended by one nucleotide using ddNTPs which are labelled with four different fluorescent dyes. Depending on the genotype in the wild type and the mutant, different colour dyes will be added to the primer. These dyes are excited by a laser and the emitted light recorded by an ABI3100.

The sample can then be genotyped depending on the colour of the fluorescence produced.

In this study the case-control samples were pooled in order to reduce the amount of reagents and sample DNA used. SNaPShot can perform quantitative genotyping analysis on pooled samples. Five different pools were formed from the Cardiff MRC cases (n=96), Cardiff MRC controls (n=96), London MRC cases (n=90), London MRC controls (n=90) and the Cardiff Sib-pairs (n=114) with an equal concentration of each individual present within the pool (kindly performed by D.

Turic and D. Harold). The ratio of the peaks produced by the pooled analysis was used to determine the allele frequencies after correction using a known heterozygote.

Primers of approximately 25bp were designed with the 3' end immediately adjacent to the polymorphic site using Primer3. The polymorphic fragment was amplified by PCR for each of the pools and a known heterozygote in duplicate. Excess primers and nucleotides were removed by treatment of 10.8µl of the PCR product with 1 unit of SAP (Amersham Pharmacia Biotech) and 0.2 units of exonuclease (Amersham Pharmacia Biotech) in the appropriate buffer and incubated at 37°C for 1 hour followed by a denaturing step of 80°C for 15 minutes.

The extension primers were diluted to the concentration required for optimal results, usually in the region of 0.5-5pM (see below). A reaction mix of 2µl of cleaned PCR product, 1µl of extension primer, 2.5µl SNaPShot reagent (Applied Biosystems) and 4.5µl SNaPShot buffer and were incubated using the program shown below. This reaction was carried out an all of the fragments amplified in duplicate, providing four copies of each sample in total.

94°C 2 min

94°C 5 sec
43°C 5 sec } 25 cycles
60°C 5 sec

However, if the sample was particularly GC rich an alternative program was used (optimised by H. Williams).

96°C 10 sec
55°C 5 sec } 30 cycles
65°C 30 sec

The products were then treated with 1 unit of SAP and incubated at 37°C for 1 hour followed by denaturation at 80°C for 15 minutes. 1µl of the treated product was was then added to 9µl of HiDye formamide (Applied Biosystems) in preparation for analysis.

The finished reactions were loaded on to and run on an ABI3100 by Nadine Norton or Hywel Williams and the results analysed on an iMac using Genotyper (v2.5) from Applied Biosystems. For accurate analysis the peaks produced needed to be in the range of 200-8000. If the results produced were outside this range then the primer concentration was altered.

2.1.9 Tissue Culture

2.1.9.1 Cell Line Maintenance

B-lymphocytes were grown in T25 and T75 flasks (Nunclon) using Roswell Park Memorial Institute (RPMI) media, supplemented by 10% Foetal Bovine Serum (FBS), 1% Glycerol and 1% Penicillin/Streptomycin mix (all from Invitrogen). When they had reached confluence the cells were passaged to a concentration of 25%.

2.1.9.2 Cell Line Storage

Confluent cells were pelleted by centrifugation (2500rpm, 5min) and resuspended in 2mls of freezing media (95% FBS, 5% Dimethylsulfoxide). They were chilled at a rate of 1°C/min overnight, down to -70°C, and then stored in liquid nitrogen.

2.1.9.3 DNA Extraction from Cell Lines

The B-lymphocytes were pelleted by centrifugation (2500rpm, 5min) and lysed overnight at 55°C in 500µl of SET Buffer, 50µl of 10% SDS and 50µl of proteinase K

(20mg/ml). The digested pellet was transferred to a fresh tube, 1ml of Tris-saturated phenol added and centrifuged at 3,000rpm for 4min. Both the aqueous phase and the white interface were transferred to a fresh tube. 500µl of chloroform/isoamyl alcohol (24:1) and 500µl of Tris-saturated phenol were added and the sample spun at 3,00rpm for 4min. The aqueous phase was transferred, 1ml of chloroform added, mixed, vortexed and centrifuged (3,000rpm, 4min). Finally, the aqueous phase was transferred and 1ml of EtOH was added and allowed to precipitate the DNA at 4°C for 30min. The DNA was pelleted and resuspended in 500µl of T.E buffer.

2.1.9.4 RNA Extraction from Cell Lines

B-lymphocytes were centrifuged at 2,500 for 5 minutes. The pellet was lysed by addition of 2mls of RNAzol (Biogenesis) and pippetted gently. The lysed pellet was transferred to 1.6ml tubes, mixed vigorously and put on ice for 5 minutes. The suspension was centrifuged at 12,000g for 15min at 4°C. The colourless, aqueous, phase was transferred to fresh tubes, an equal volume of isopropanol added and stored at 4°C for 15min. The samples were then centrifuged (12,000g, 15min, 4°C) and the supernatant removed. The pellet washed in 75%EtOH, vortexed, centrifuged (8min, 7,500g, 4°C) and the supernatant removed. The pellet was dried on ice for 10 minutes and resuspended in 100µl of DEPC-treated dH_2O, 100µl of 8M NH_4OAc and 400µl of 95%EtOH.

2.1.10 Statistical Analysis

Statistical analysis of the genotypic and allelic differences between the cases and the controls was carried out using the Pearson's χ^2 test performed using Minitab™ Statistical software (v13), SPSS (v11) and the Web Chi-Square Calculator

(http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html). A threshold level of significance of 0.5 was used for individual genotyping and 0.1 for pooled genotyping. Pooled analysis that reached significance was then genotyped individually.

Conformity to the Hardy-Weinberg equilibrium in the cases and the controls was analysed using the HW program available on the college network (software written by P. McGuffin and J. Williams).

The square of the correlation coefficient (r^2) was used to determine the levels of linkage disequilibrium between polymorphisms (Hill and Weir, 1994). This was performed using a modification of the EH+ program (Zhao *et al.* 2000) available from http://www.iop.kcl.ac.uk/iop/Departments/PsychMed/GEpiBSt/software.shtml and modified by M. Hamshere and V. Moskvina.

Tests for haplotype association between LOAD and detected SNPs were performed using the EH+ software (Zhao *et al.*, 2000). A permutation test was used to obtain the empirical significance levels for differences between haplotype frequencies in cases and controls using PM+.

Multiple correction was carried out using the Bonferroni-Holm method (Holm, 1979) which uses the equation shown on the following page –

0. Order the p-values $p_{(1)} < p_{(2)} < \ldots < p_{(k)}$. Let $H_{(1)}, H_{(2)},\ldots,H_{(k)}$ be the corresponding null hypotheses.
1. Compare $p_{(1)}$ to a/k. If $p_{(1)} < a/k$ then reject $H_{(1)}$ and continue. Else stop and fail to reject any hypothesis.
2. Compare $p_{(2)}$ to a/(k-1). If $p_{(2)} < a/(k-1)$ then reject $H_{(2)}$ and continue. Else stop and fail to reject any of hypotheses $H_{(2)},\ldots,H_{(k)}$.

3. Compare $p_{(3)}$ to a/(k-2). If $p_{(3)} < a/(k-2)$ then reject $H_{(3)}$ and continue. Else stop and fail to reject any of hypotheses $H_{(3)},\ldots,H_{(k)}$.

This is continued until either a stop is reached or all of the hypotheses have been rejected. In the equation α is the threshold significance level and k is the total number of tests performed in the study.

Odds ratios (OR) were calculated using the following formula –

$OR = ad/bc$ where

	Risk Factor	No Risk Factor
Cases (count)	a	c
Controls (count)	b	d

Confidence intervals were calculated using the following formula (min, max) –

$(OR \times e^{-z\sqrt{v}}, OR \times e^{z\sqrt{v}})$

where z = 1.96 and v = variance (1/a + 1/b + 1/c + 1/d)

Power calculations were worked out using an online Power Calculator (http://calculators.stat.ucla.edu/powercalc/) available from the UCLA Department of Statistics.

2.2 Materials

Samples

The samples were recruited from both community and hospital settings and the assessment was by structured interview including scales shown to be reliable and valid in similar contexts. The assessment battery used has been demonstrated to have a positive predictive value (PPV) for NINCDS-ADRDA clinical diagnosis against the non-exclusive presence CERAD criteria for AD pathology of 0.92 (Holmes et al, 1999). Controls were also collected from general practices in the same areas as the AD patients. Controls were aged 65 years or above, of UK origin and had a normal level of cognitive functioning. The age at collection in the control sample was deliberately higher within the controls to ensure that these individuals had lived through the age of risk. Controls were screened for cognitive decline using the Mini Mental State Examination (MMSE) and a cut off score of 28 was adopted.
Several case-control samples were used during this project which are detailed below –

UK1 - 133 Caucasian AD cases (age of onset 72.5±6.5y) and 135 age- and sex-matched controls (age at collection 78.2±7.2y). In this sample 88 of the cases and 49 of the controls were samples that were also used in the Cardiff sample in Kehoe *et al.* (1999a).

UK2 - 180 Caucasian AD cases (age of onset 76.94) and 180 age- and sex-matched controls (age at collection 75.59). No overlap with any other case-control sample.

NI1 – 198 Caucasian AD cases (age of onset not available) with 209 age- and sex-matched controls. 294 samples overlap with the Belfast sample from Kehoe *et al.* (1999a).

The pooled samples used in Chapter 4 and Chapter 6 were made up from samples used in UK2 for the Cardiff and London pool, and the sibs sample used for the two stage genome screen (Kehoe *et al.* 1999b) for the sibs pool. These were only made available at the very end of my thesis which accounts for them only being used to genotype two polymorphisms.

Agarose Gel Loading Buffer

5ml Glycerol (Sigma)

0.03g Bromophenol Blue (Sigma)

0.03g Xylenecyanol (Sigma)

200µl 1M Tris-HCl pH 8.0

4.8ml Baxters dH_2O

10% Ammonium Persulphate

1g ammonium persulphate (Sigma)

10ml milliQ water

Solution stored at 4°C for no longer than 7 days.

Denaturing Polyacrylamide Gel (6%)

60ml SequaGel™-6 6% sequencing gel solution (National Diagnostics)

15ml SequaGel Complete Buffer Reagent (National Diagnostics)

600µl Ammonium persulfate

DNA Size Ladder (0.1µg/µl)

10µl 1kb ladder fragment (Invitrogen)

15µl agarose loading buffer

75µl sterile water

EDTA (0.5M)

93.06g EDTA

Make up to 500ml with dH_2O and alter to pH 8.0 with solid NaOH

Proteinase K (20mg/ml)

1g of Proteinase K in 50mls dH_2O

Restriction Enzymes

New England Biolabs – *Alu I, Ban I, BsaH I, Bsl I, Bsr I, BsrB I, BsrF I, BstN I, Ear I, Hha I, Hph I, Mse I, Msp I, Nci I, Nsi I, PflM I, PshA I, Pst I, Pvu II, Sac II*

Fermentas - *BseG I*

SET Buffer

1g Potassium bicarbonate ($KHCO_3$)

8g Ammonium chloride

200µl 0.5M EDTA

Add dH_2O to 1L

TE Buffer

10ml 1M Tris HCl

2ml 0.5M EDTA

Add dH_2O to 1L

Tris Borate EDTA (TBE) Buffer

Supplied as 10x (National Diagnostics) and diluted with dH_2O for use.

Company Contact Details

Amersham Pharmacia Biotech, Amersham Place, Little Chalfont, Bucks, HP7 9NA.

Applied Biosystems (ABI), Division Headquarters, 850 Lincoln Centre Drive, Foster City, CA 94404, U.S.A.

Bio-Rad, Bio-Rad House, Maylands Avenue, Hemel Hempstead, Herts, HP2 7TD.

DNASTAR, Inc., 1228 S. Park Street, Madison, WI 53715, U.S.A.

Genetic Research Instruments (GRI) Ltd., Gene House, Queenborough Lane, Rayne, Braintree, Essex, CM7 8TF.

Genset; 24 rue Royale, 75008 Paris, France.

Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF.

Nalgene Company, Unit 1a Thorn Business Park, Hereford HR2 6JT.

National Diagnostics, Unit 4, Fleet Business Park, Itlings Lane, Hessle, Hull, HU13 9XL.

New England Biolabs (UK), 67 Knowle Place, Wilbury Way, Hitchin, Herts, SG4 0TY.

Pharmacia Biotech, 23 Grosvenor Road, St. Albans, Herts, AL1 3AW.

Qiagen Ltd., Unit 1, Tillingbourne Court, Dorking Business Park, Dorking, Surrey, RH4 1HJ.

Sigma-Aldrich Ltd., Fancy Road, Poole, Dorset, BH12 4QH.

Transgenomic, Inc., 1620 Oakland Road, Suite D-200, San Jose, California 95131, USA.

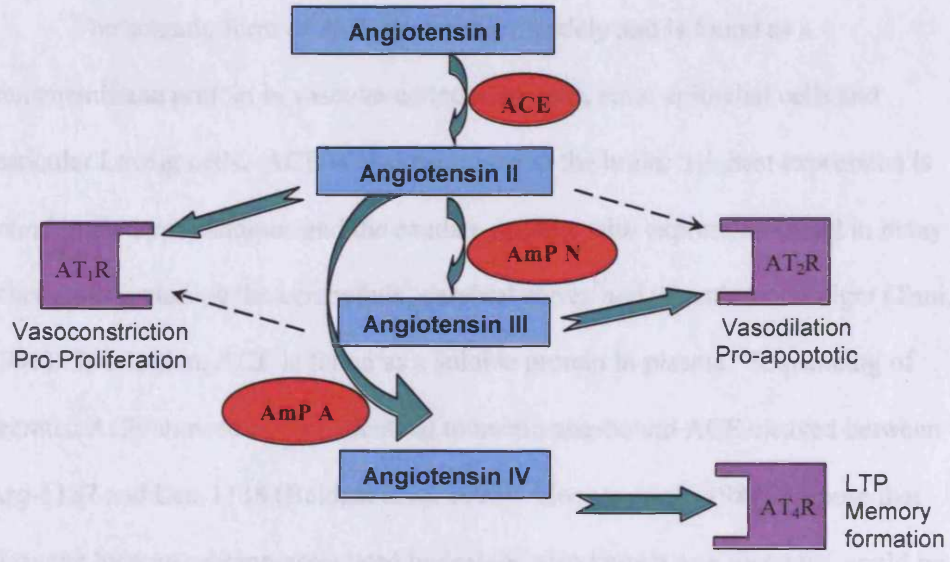
Chapter 3

Angiotensin-converting enzyme (ACE)

3.1 Background

Angiotensin-converting enzyme (ACE), EC 3.4.15.1, is a zinc metalloprotease encoded by *DCP1* on chromosome 17. The enzyme is essential in blood pressure regulation and electrolyte balance through its actions on angiotensin I and bradykinin (see Figure 3.1 below).

Figure 3.1 The Renin Angiotensin System



Schematic of the Renin Angiotensin System (RAS) and the role of ACE. Angiotensin I is formed by the cleavage of angiotensinogen by renin. ACE cleaves inactive angiotensin I to active angiotensin II (Ang II). This can be cleaved further by aminopeptidase N (AmP N) to produce angiotensin III (AngIII) or aminopeptidase A (AmP A) to produce angiotensin IV (Ang IV). Both Ang II and III bind to angiotensin receptor type –1 (AT_1R) and type –2 (AT_2R) with the preferential ligand shown by the larger arrow. Ang IV binds only to a unique receptor AT_4R. The effects mediated by each receptor are also shown (adapted from Carluccio *et al.* 2001)

ACE converts inactive angiotensin I into the potent vasopressor angiotensin II, cleaving two peptides from the carboxy terminus, and also inactivates the vasodilating peptide, bradykinin.

DCP1 encodes two isozymes with the first, somatic, form expressed in multiple tissues while the second, germline, form is expressed only in sperm. The latter isozyme is formed by alternative splicing of *DCP1*, retaining the C-terminal end of the protein (Ehlers *et al.* 1989). A high degree of internal homology is found in somatic ACE suggesting a gene duplication event has occurred (Soubrier *et al.* 1988). The fact that ACE contains only one zinc atom (Das and Soffer, 1975) makes it probable that only one of the duplicated domains is active. The testicular form of ACE is active, which suggests that the zinc atom resides in the C-terminal end of the protein and that the N-terminal region is inactive.

The somatic form of ACE is expressed widely and is found as a transmembrane protein in vascular endothelial cells, renal epithelial cells and testicular Leydig cells. ACE is also expressed in the brain. Highest expression is found in the hypothalamus and the caudate nucleus with expression found in many other areas including the cerebellum, cerebral cortex and the substantia nigra (Tani, 1991). In addition, ACE is found as a soluble protein in plasma. Sequencing of secreted ACE showed it to be identical to membrane-bound ACE cleaved between Arg-1137 and Leu-1138 (Beldent *et al.* 1993). Hooper *et al.* (1987) propose that cleavage by a membrane-associated hydrolase, also known as a secretase, could be responsible. A different mechanism was proposed by Sugimura *et al.* (1998) based around alternative splicing of ACE mRNA to produce the soluble form, however most evidence supports solubilisation by a secretase.

3.1.1 Functional Polymorphisms

As mentioned in section 1.6.5 the I/D polymorphism, the presence or absence of an *Alu* fragment in intron 16, has been extensively genotyped in relation to disease. However, it is clear from functional studies (Rosatto *et al.* 1999) and the contradictory results from association studies that the I/D polymorphism is not the functional variant of *DCP1* and several groups are now trying to isolate the underlying quantitative trait locus (QTL). Analysis of 10 polymorphisms within *DCP1* gave evidence that the major variant influencing plasma ACE levels was situated downstream of the I/D polymorphism and that the *Alu* fragment was in complete linkage disequilibrium (LD) with a large number of single nucleotide polymorphisms giving two major haplotypes (Keavney *et al.* 1998). In contrast Villard *et al.* (1996), who also used segregation-linkage analysis, reported that two ACE QTLs existed with one being in close proximity to the I/D polymorphism and the other lying 5' of the intron 16 *Alu* fragment, near the promoter. Work by Farral *et al.* (1999) using haplotypes suggested that a further 3.9kb downstream of the region described by Villard *et al.* did not exert any effect on plasma levels. McKenzie *et al.* (2001) used association, linkage and linkage adjusted for association between ACE levels and a variety of polymorphisms in an Afro-Caribbean population and reported that the QTL lay near the exon 17 polymorphism. Cladistic and linkage analysis of the 3' region in an Afro-Caribbean sample suggested a possible QTL near exon 26 (Zhu *et al.* 2000). Finally Zhu *et al.* (2001) also studied linkage and association of various ACE polymorphisms in relation to ACE concentrations and blood pressure. This work suggested two polymorphisms, exon 17 and 5'UTR (-240), accounted for the greatest proportion of the variance in ACE levels in an additive manner.

3.1.2 ACE in Alzheimer's disease

ACE is a functional candidate for AD for a variety of reasons. As mentioned previously, ACE is central to the inflammatory response through its effects on angiotensin I and bradykinin. In addition to being a potent vasopressor, angiotensin II enhances the phagocytic activity of granular macrophages (Foris *et al*, 1983). Bradykinin inhibits thrombin-induced platelet activation and stimulates tissue-type plasminogen activator (t-PA). Inflammatory processes have been strongly implicated in AD, as detailed previously (see section 1.5.1), and any alteration in the activity of ACE could lead to reduced immune response to toxic peptide aggregation.

The amyloid cascade is considered to be the key pathway in the pathogenesis of AD. All the mutations so far described increase the production of total βA or result in a modification of the ratio of $\beta A_{1\text{-}42}$: $\beta A_{1\text{-}42}$ produced. Only the α-secretase remains undescribed (see section 1.3.1.1) and is a prime candidate as a risk factor for AD. The secretase that solubilises ACE shares many properties with the APP α-secretase. Both are integral membrane proteins (Parvathy *et al.* 1997) that are stimulated by phorbol esters and both have identical inhibition profiles in the presence of zinc metalloproteinase inhibitors (Parvathy *et al.* 1998). Even if both secretases are not the same enzyme it is possible that competitive inhibition could be occurring due to the similarities in their activity profiles. ACE itself has been shown to cleave βA in vitro (Hu *et al.* 2001) and inhibit cytotoxicity. Inhibition of ACE by lisinopril blocks these effects.

Kehoe *et al.* (1999a) genotyped a case-control group of 198 AD cases and 77 controls and found an association with the I allele giving an odds ratio (OR) of 2.43. This work was confirmed by groups in London (OR 2.71) and Belfast (OR 1.82) (Kehoe *et al.* 1999a). The association remained highly significant after stratification

for ApoE genotype. Many studies of the ACE I/D polymorphism in AD have now been repeated. Some have been negative (Tysoe *et al.* 1997; Scaachi *et al.* 1998; Myllykangas *et al.* 2000), some have found an association with the I allele (Kehoe *et al.* 1999; Yang *et al.* 2000), some with the D allele (Bartres-Faz *et al.* 2000) while others suggest an effect in homozygotes against heterozygotes (Narain *et al.* 2000).

3.1.3 Aim of this study

It was clear from the contradictory results previously published that a comprehensive study of ACE polymorphisms needed to be carried out in relation to AD. Using the data from QTL analysis by several groups I analysed ACE polymorphisms spanning the entire gene in a LOAD case/control sample to look for association of other ACE polymorphisms with AD.

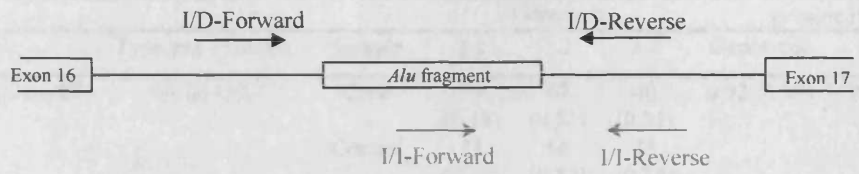
3.2 Genotyping Results

A comprehensive list of polymorphisms in the ACE genomic sequence was published by Rieder *et al.* (1999) and this was used for genotyping. The polymorphisms were analysed in association sample UK1 (see section 2.2 for details), which had a partial overlap with the sample used in the study by Kehoe *et al.* (1999a), which will be detailed further in the discussion (section 3.3). I decided to concentrate initially on genotyping the I/D polymorphism itself, to replicate the work done previously on ACE, and the two exonic polymorphisms found in close proximity to it in exons 15 and 17.

3.2.1 I/D Polymorphism

The I/D polymorphism is genotyped by a PCR based assay, which consists of two reactions. The first I/D PCR has the two primers spanning the *Alu* fragment. The size of the product depends on the genotype, being 490bp with the insertion and 193bp without. However, the results can sometimes be misleading as the reaction will preferentially amplify the shorter, D, allele in the heterozygote. This can lead to an over-representation of the D/D genotype in the results. Because of this, the second PCR (I/I PCR) has one primer within the *Alu* fragment and one outside and therefore should only work in the presence of the insertion (see Figure 3.2). The primers and conditions used are shown in Table 3.1 on the following page along with examples of the PCR fragments produced in Figure 3.3

Figure 3.2 Position of Primers for I/D Genotyping



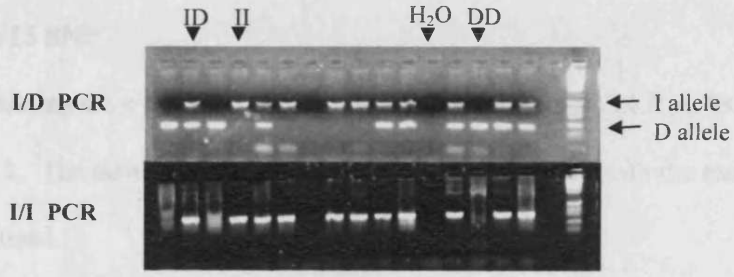
Position of both sets of PCR primers, in relation to *Alu* fragment in Intron 16 of DCP1, for genotyping the I/D polymorphism. The I/I primers, in red, only amplify in the presence of the *Alu* fragment. Not to scale.

Table 3.1 Primer details for I/D Genotyping

Section	Primer 5'-3' (For/Rev)	Taq	T (°C)	Size (bp)
I/D	CTGGAGACCACTCCCATCCTT GATGTGGCCATCACATTCGTC	QTaq	58	490/193
I/I	TGGGACCACAGCGCCCGCCAC TCGCCAGCCCTCCCATGCCCA	QTaq	61	335

Sequence of primers used for I/D genotyping. Table also shows the annealing temperature of the fragment, the Taq enzyme used and the size of the fragment produced.

Figure 3.3 I/D Genotyping



Two 2% agarose gels showing the results of the two PCRs for genotyping the ACE I/D polymorphism for the same individuals. The larger product in the I/D PCR (above) is seen with the insertion of the *Alu* fragment and the shorter in the deletion variant. The I/I PCR (below) only generates a product in the presence of the I allele.

The results of the genotyping of the I/D polymorphism in the Cardiff LOAD are shown in Table 3.2 on the following page.

Table 3.2 I/D polymorphism genotyping results

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 17	*Alu* @ -397	Case	24 (0.18)	67 (0.51)	40 (0.31)	**0.92** (0.17)	**0.77** (0.08)
		Control	23 (0.19)	66 (0.53)	35 (0.28)		

Genotype counts and frequencies (in parenthesis beneath) are shown along with the p-values generated by χ^2 tests of genotypic and allelic data compared to disease status. Value in parenthesis is the calculated chi-square. Genotype codes are as follows; 1 1 (I/I), 1 2 (I/D) and 2 2 (D/D). Position of polymorphism is shown in relation to nearest exon.

Both populations conformed to the Hardy-Weinberg equilibrium, with p-values of 0.66 and 0.69 for the cases and the controls respectively (see section 2.1.10). The I/I and I/D were added together and analysed against the D homozygotes to test for a dominant effect from the I allele but did not give a significant result ($\chi^2 = 0.00$, p-value = 0.98).

3.2.2 Exon 15 SNP

Primers spanning exon 15 of DCP1 were designed using Oligo v4.0 as detailed in section 2.1.1. The details are shown in Table 3.3 below along with the reaction conditions used.

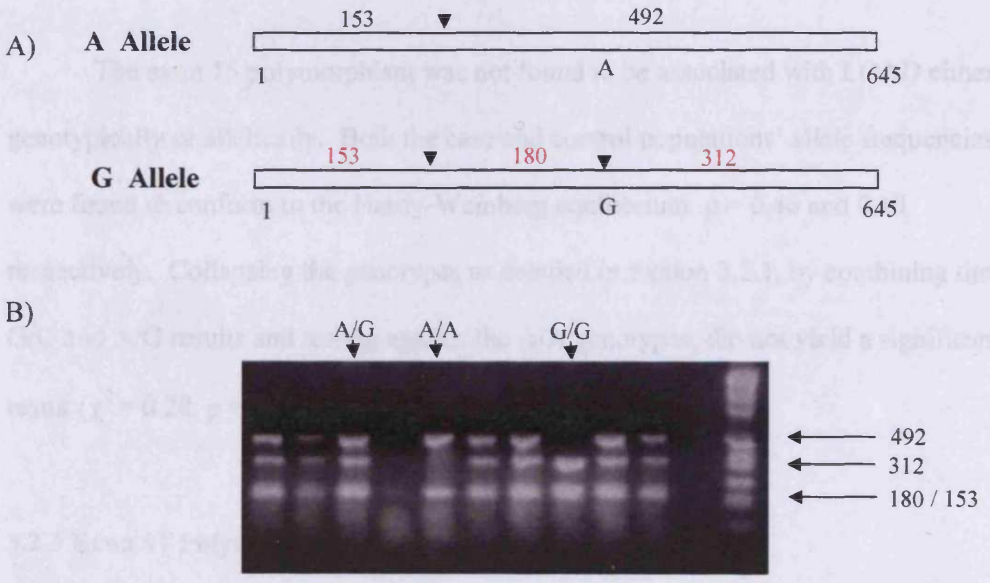
Table 3.3 – Primer Details for Exon 15 Amplification

Exon	Primer 5' –3' (For/Rev)	Taq	T (°C)	Size (bp)
15	TGCATCCAGGACGTTATCAG	Qtaq	64	645
	CGCTGTAGGTGGTTTCCATA			

Sequence for primers used in exon 15 PCR. The size of the fragment is also shown, along with the annealing temperature and the enzyme used in the reaction.

An RFLP assay for the described polymorphism was designed using DNAStar, with the G allele creating a new *Hha I* cut site. Figure 3.4 below shows the position of the cut sites in the fragments along with examples of the RFLP analysed on a 2% agarose gel. Digests were carried out at 37°C for 16 hours to ensure complete digestion as detailed in section 2.1.6.

Fig 3.4 Exon 15 RFLP Assay



A) Diagrammatic representation of *Hha I* cut sites within the ACE exon 15 fragment for the different alleles. B) Digested samples taken from the Cardiff LOAD case-control sample analysed on a 2% agarose gel. Genotypes and fragment sizes are shown.

These polymorphisms were genotyped in the Cardiff LOAD case-control sample. Statistical analysis was carried out using χ^2 tests in SPSS and Minitab, the results of which are shown in Table 3.4 on the following page. Conformation of the data to the Hardy-Weinberg equilibrium was tested as detailed in section 2.1.10 for all SNPs in this study.

Table.3.4 Results of Exon 15 Case-control analysis

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 15	A/G @ 2193	Case	22 (0.17)	67 (0.52)	39 (0.30)	**0.80** (0.17)	**0.83** (0.04)
	Ala→Ala @ 731	Control	21 (0.16)	74 (0.57)	36 (0.27)		

Results of ACE exon 15 SNP genotyping in the Cardiff case-control sample. Position of polymorphism within cDNA shown, numbered from the start of translation, and within the polypeptide. Genotype numbers are given with frequencies in parenthesis below, along with the p-value and chi-square value for genotypic and allelic association. Genotype codes are as follows; 1 1 – (G/G), 1 2 – (A/G), 2 2 – (A/A).

The exon 15 polymorphism was not found to be associated with LOAD either genotypically or allelically. Both the case and control populations' allele frequencies were found to conform to the Hardy-Weinberg equilibrium, p = 0.46 and 0.10 respectively. Collapsing the genotypes as detailed in section 3.2.1, by combining the G/G and A/G results and testing against the A/A genotypes, did not yield a significant result ($\chi^2 = 0.28$, p = 0.60)

3.2.3 Exon 17 Polymorphism

As for exon 15, primers spanning exon 17 were designed using Oligo v4.0 and optimised for PCR. The primer details and reaction conditions are shown in table 3.5 on the following page.

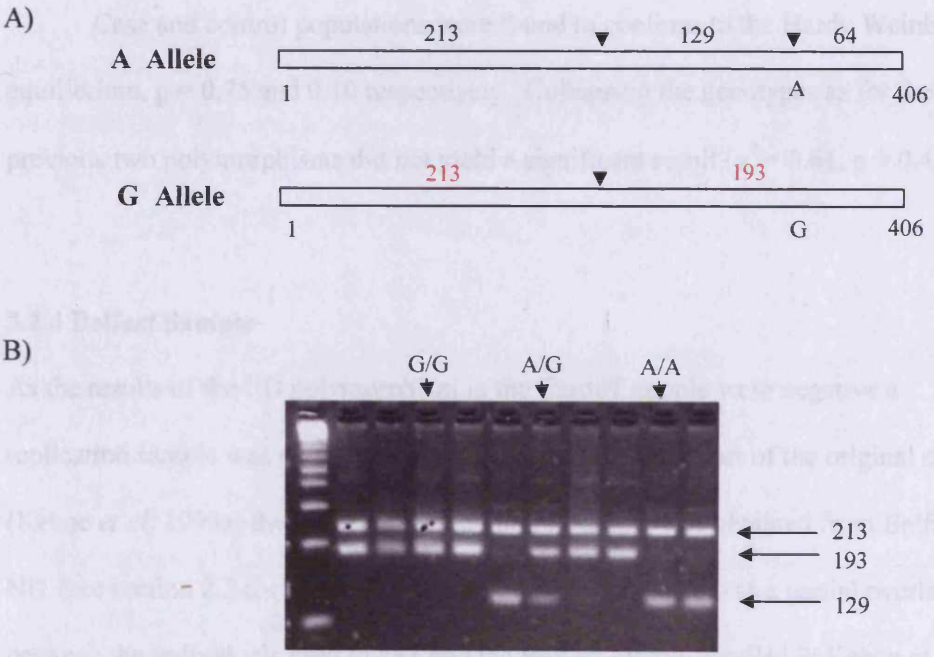
Table 3.5 – Primer Details for Exon 17 Amplification

Exon	Primer 5' –3' (For/Rev)	Taq	T (°C)	Size (bp)
17	GTGGTTTCGCCAATTTTAT GAGTCCCCTGCATCTACATA	Qtaq	57	406

Sequence for primers used in exon 17 PCR. The size of the fragment is also shown, along with the annealing temperature and the enzyme used in the reaction.

Using DNAStar it was seen that the exonic G/A polymorphism created a *BseGI* cut site within the fragment. Figure 3.5 shows the position of the cut sites within the fragments and examples of fragments of different genotypes digested. All digests were carried out for 16 hours to ensure complete digestion.

Figure 3.5 Exon 17 RFLP Assay



A) Diagrammatic representation of *BseGI* cut sites within the ACE exon 17 fragment for the different alleles. B) Digested samples taken from the Cardiff LOAD case-control sample analysed on a 2.2% agarose gel supplemented with Metaphor agarose. The 64bp fragment is not visible on this picture but is not needed for genotyping. Genotypes and fragment sizes are shown.

The polymorphism was genotyped in the Cardiff LOAD case-control sample and analysed using the same methods as for the previous polymorphisms. The results are shown in Table 3.6 below.

Table.3.4 Results of Exon 17 Case-control analysis

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 17	G/A @ 2328	Case	19 (0.19)	51 (0.51)	30 (0.30)	**0.63** (0.92)	**0.74** (0.11)
	Thr→Thr @ 776	Control	20 (0.18)	66 (0.57)	29 (0.25)		

Results of ACE exon 17 SNP genotyping in the Cardiff case-control sample. Position of polymorphism within cDNA shown, numbered from the start of translation, and within the polypeptide. Genotype numbers (and frequencies in parenthesis) are given along with the p-value and chi-square value for genotypic and allelic association. Genotype codes are as follows; 1 1 – (G/G), 1 2 – (A/G), 2 2 – (A/A)

Case and control populations were found to conform to the Hardy Weinberg equilibrium, p = 0.75 and 0.10 respectively. Collapsing the genotypes as for the previous two polymorphisms did not yield a significant result ($\chi^2 = 0.61$, p = 0.43).

3.2.4 Belfast Sample

As the results of the I/D polymorphism in the Cardiff sample were negative a replication sample was obtained for analysis. As they were part of the original study, (Kehoe *et al.* 1999a) the replication case-control sample was obtained from Belfast, NI1 (see section 2.2 for details). As with sample UK1, there was a partial overlap between the individuals used in NI1 and the Belfast sample detailed in Kehoe *et al.* (1999a). The I/D polymorphism was genotyped along with the exon 17 polymorphism. It has long been realised that the I/D polymorphism can be problematic to genotype due to the problem of preferential amplification of the D allele discussed earlier, while the exon 17 polymorphism is a straightforward RFLP.

The exon 17 was used as an alternative to I/D polymorphism genotyping in the samples available as results from Rieder *et al.* suggested that both polymorphisms were effectively in complete LD, which was confirmed by the genotyping in UK1. The results of the genotyping and analysis in the Belfast sample are shown below in Table 3.5.

Table 3.5 Results of genotyping in Belfast sample

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 17	*Alu* @ -397	Case	39 (0.26)	65 (0.44)	44 (0.30)	**0.60** (1.0)	**0.82** (0.05)
		Control	21 (0.24)	44 (0.51)	22 (0.25)		
Exon 17	G/A @ 2328	Case	18 (0.23)	38 (0.47)	24 (0.30)	**0.75** (0.56)	**0.51** (0.44)
	Thr→Thr @ 776	Control	19 (0.25)	39 (0.50)	19 (0.25)		

Results of association study between *DCP1* I/D and exon17 polymorphisms and LOAD in Belfast case-control sample along with position of the polymorphisms. For I/D polymorphism (*Alu*), 1 1 - (I/I), 1 2 – (I/D) and 2 2 – (D/D); for Exon 17 polymorphism 1 1 (G/G), 1 2 (A/G), 2 2 (G/G). Genotype counts shown with frequencies in parenthesis beneath.

As in the Cardiff sample, no association was found with LOAD either genotypically or allelically in the Belfast sample. Neither polymorphism deviated from the Hardy-Weinberg equilibrium. Collapsing the columns containing I alleles as detailed earlier did not produce a significant result ($\chi^2 = 0.54$, p-value = 0.46).

As mentioned in the introduction (section 3.1.2) Zhu *et al.* (2000 and 2001) attempted to pinpoint the functional polymorphism of ACE and highlighted three polymorphisms which either were the QTL or at least lay near possible functional polymorphisms. One was the exon 17 polymorphism which had already been analysed and the other two were an A/G near exon 26 and an A/T polymorphism in the 5' UTR. These were chosen to be analysed next along with two other SNPs. The first of these was a C/T lying within intron 9 that was selected to provide a SNP 5' of

the *Alu* fragment and would be useful in determining the levels of LD across the gene. Secondly, a recent paper by Eyries *et al.* (2000) reported a C/T mutation in exon 25 resulting in an amino acid change (P1195L) found in patients with abnormally high plasma ACE levels. All four variants were genotyped in sample UK1.

3.2.5 5' UTR polymorphism

Amplification of the 5'UTR fragment was carried out using the same primers as described by Keavney *et al.* (1998), but with slightly different conditions. The primer details are described in Table 3.6.

Table 3.6 5'UTR fragment primer details

Exon	Primer 5' –3' (For/Rev)	Taq	T (°C)	Size (bp)
5' UTR-1	ACCATGGCCTGGTGAAGAAGC* CGGCTCTGCCCCTTCTCCTGCGC*	HotStart	63	482
5' UTR-2	TGTCACTCCGGAGGCGGGAGG* GAGAAAGGGCCTCCTCTCTCT*	HotStart	61	167

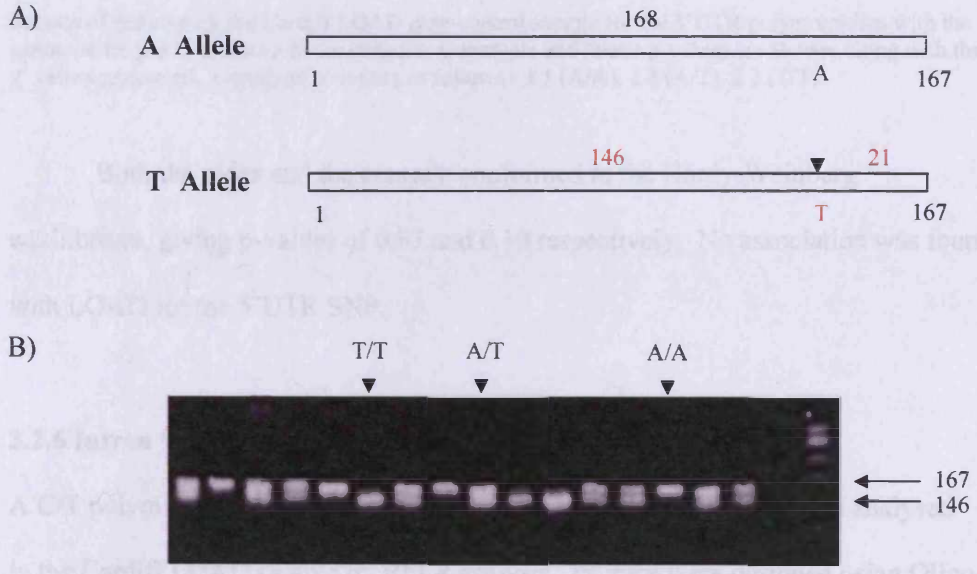
Primer details and PCR conditions for 5'UTR fragment of DCP1. Annealing temperature, size of fragment and taq used are shown.). * as described by Keavney *et al.* (1998). **Bold** base in primer denotes a mismatch primer to induce a cut site.

Initial amplification with the 5'UTR-1 primers was done using standard HotStart Taq conditions (section 2.1.1) as opposed to a touch-down reaction. The product from this reaction was diluted 1:500 and used as template for the 5'UTR-2 which included a mismatch primer designed to introduce a *XbaI* cut site for genotyping by RFLP.

A diagram of this fragment with the cut sites can be found in figure 3.6 on the following page along with some examples of different genotypes run on a 2.5% agarose gel. Due to the difference in fragment sizes being only 21bp, a third of the agarose was replaced with MetaPhor to provide improved resolution as described in

section 2.1.2. Digests were carried out at 37°C for 16 hours to ensure complete digestion.

Figure 3.6 5'UTR RFLP Assay



A) Diagram showing *XbaI* cut sites within 5'UTR-2 fragment after mutagenesis with reverse primer, as detailed in table 3.6, in the different alleles. Fragment sizes produced shown. B) Samples from Cardiff LOAD case-control sample amplified with 5'UTR primers and digested with *XbaI* and run on a 2.5% agarose gel supplemented with MetaPhor. Faint bands seen at ~500bp in some lanes are the product of 5'UTR-1, used as a template.

The entire UK1 sample was amplified and digested as detailed above. The results and analysis are shown in Table 3.7 on the following page.

Table 3.7 Results of genotyping 5'UTR polymorphism

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
5'UTR	iA/T @ -240	Case	30 (0.34)	44 (0.49)	15 (0.17)	**0.55** (1.2)	**0.76** (0.10)
		Control	32 (0.32)	56 (0.56)	12 (0.12)		

Results of genotyping the Cardiff LOAD case-control sample for the 5'UTR polymorphism with the genotype frequencies shown in parenthesis. Genotypic and allelic p-values are shown, along with the χ^2 values generated. Genotype codes are as follows - 1 1 (A/A), 1 2 (A/T), 2 2 (T/T).

Both the cases and the controls conformed to the Hardy-Weinberg equilibrium, giving p-values of 0.87 and 0.10 respectively. No association was found with LOAD for the 5'UTR SNP.

3.2.6 Intron 9 polymorphism

A C/T polymorphism, which lies 57 base pairs upstream of exon 10, was analysed in the Cardiff LOAD sample by RFLP analysis. Primers were designed using Oligo v4.0, ensuring that no secondary structures or self-priming occurred in the reaction as detailed in section 2.1.1. The sequences of the primers and the reaction conditions can be found in Table 3.8 below.

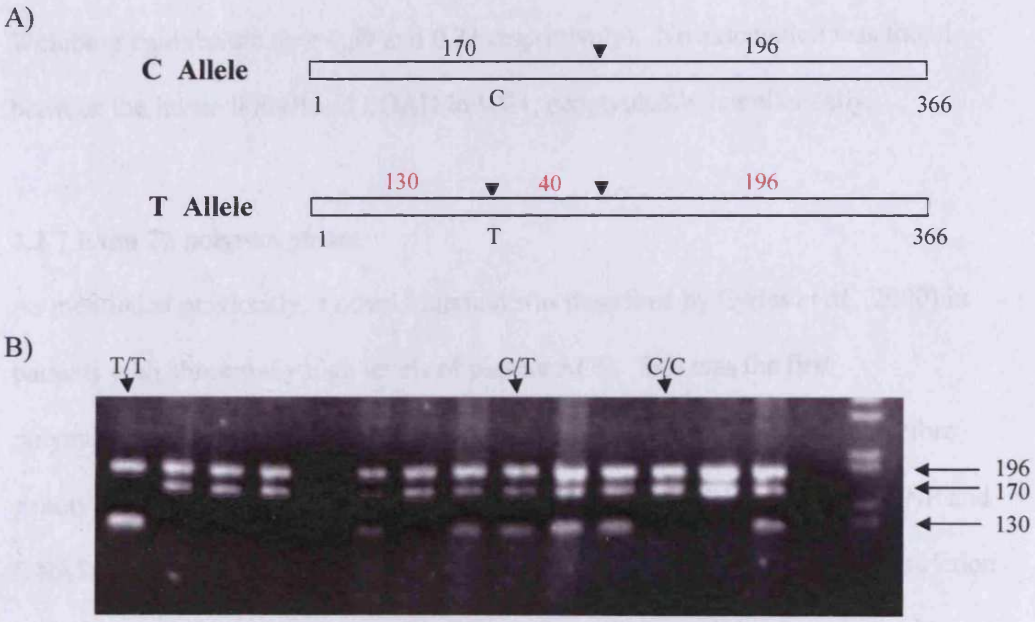
Table 3.8 Intron 9 primer details

Exon	Primer 5' –3' (For/Rev)	Taq	T (°C)	Size (bp)
Intron 9	CTAATGATGTCCCCCGCTGT GAGGCAGCTAGGATCACAGG	QTaq	61	365

Primer details and reaction conditions for amplification of fragment containing the intron 9 polymorphism. The annealing temperature, size of the fragment and the Taq polymerase used are also shown.

DNAStar was used to design an RFLP assay as detailed in section 2.1.6. The T allele was found to create a *MseI* cut site. Digests were carried out at 37°C with the recommended buffer. Figure 3.7 below shows a schematic of the fragment with the *MseI* cut sites labelled and a gel with several examples of the different genotypes analysed on a 2% agarose gel.

Figure 3.7 Intron 9 RFLP Assay



A) Diagram of intron 9 fragment, showing location of *MseI* cut sites, along with the sizes of fragments obtained after digestion. B) Samples from Cardiff LOAD case-control sample after amplification with intron 9 primers, digestion with *MseI* and run on a 2% agarose gel.

Analysis of the results can be found in Table 3.9 on the following page.

Table 3.9 Intron 9 SNP genotyping results

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 10	iC/T @ -57	Case	21 (0.21)	53 (0.52)	27 (0.27)	**0.92** (0.17)	**0.86** (0.03)
		Control	17 (0.19)	50 (0.55)	24 (0.26)		

Results of intron 9 SNP genotyping in Cardiff LOAD case-control sample. Position of SNP shown in relation to nearest exon. Genotype counts shown with frequencies in parenthesis. Genotypic and allelic association p-values are shown along with the calculated chi-square values. 1 1 – (T/T), 1 2 – (C/T) and 2 2 – (C/C).

Both the cases and the control samples' genotypes conformed to the Hardy-Weinberg equilibrium (p = 0.59 and 0.31 respectively). No association was found between the intron 9 SNP and LOAD in UK1, genotypically nor allelically.

3.2.7 Exon 25 polymorphism

As mentioned previously, a novel mutation was described by Eyries *et al.* (2000) in patients with abnormally high levels of plasma ACE. This was the first polymorphism with a definite functional effect in the ACE gene and was therefore genotyped in the LOAD sample. Primers were designed which spanned the SNP and DNAStar revealed that the C/T polymorphism abolished an existing *BsaHI* restriction site. The sequence for the primers and the optimal PCR conditions are shown in Table 3.10 below.

Table 3.10 Primer details for exon 25 PCR

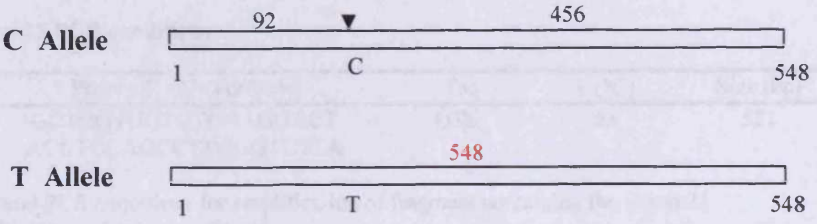
Exon	Primer 5' –3' (For/Rev)	Taq	T (°C)	Size (bp)
25	CATGTTGAGCTACTTCAAGC CCAGTGTTCCCATCCCAG	Qtaq	60	548

Primer details and reaction conditions for amplification of fragment containing the exon 25 mutation. The annealing temperature, size of the fragment and the Taq polymerase used are also shown.

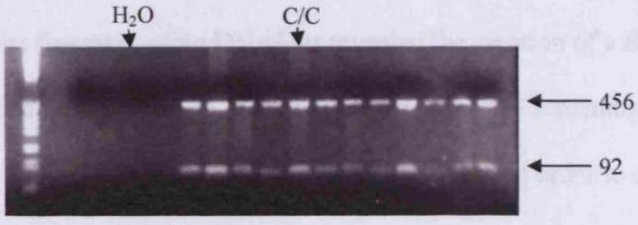
The LOAD case control sample was amplified and then digested with *BsaHI* for 16 hours as detailed in section 2.1.7. Figure 3.8 on the following page shows a diagram of the exon 25 fragment with the position of the cut site and some examples analysed on an agarose gel. Table 3.11, also on the following page, shows the results of genotyping the mutation in the case-control sample.

Figure 3.8 RFLP assay for exon 25 mutation

A)



B)



A) Diagram of exon 25 fragment, showing location of *BsaHI* cut sites, along with the sizes of fragments obtained after digestion. B) Samples from Cardiff LOAD case-control sample after amplification with exon 25 primers, digestion with *BsaHI* and run on a 1.8% agarose gel.

Table 3.11 Genotyping results for exon 25 mutation

			Genotype			p-value ($\chi 2$)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 25	C/T @ 3583	Case	114	-	-	-	-
	Pro→Leu @ 1195	Control	117	-	-		

Results of exon 25 mutation genotyping in Cardiff LOAD case-control sample. Position of polymorphism within cDNA shown, numbered from the start of translation, and within the polypeptide.

No copies of the mutation were found in either the cases or the controls and therefore no analysis was carried out.

3.2.8 Intron 25 polymorphism

Primers were designed using Primer3, as detailed in section 2.1.1, to span the region containing the polymorphism near exon 26 and optimised using the usual methods. The sequences of the primers are shown in Table 3.12 below along with the optimised PCR conditions.

Table 3.12 Intron 25 PCR conditions

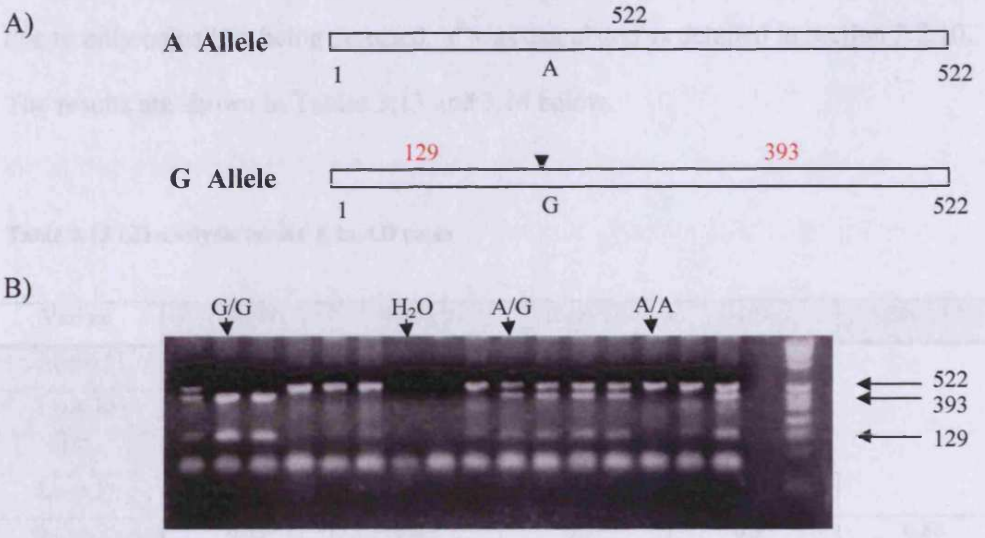
Exon	Primer 5' –3' (For/Rev)	Taq	T (°C)	Size (bp)
26	GCTGGTGGTCTGGAGGACT ACCTCCAGCCTTGGGTCTTA	QTaq	58	521

Primer sequence and PCR conditions for amplification of fragment containing the intron 25 polymorphism. Fragment size is also shown.

Analysis of the fragment using DNAStar revealed the creation of a *BsrBI* cut site within the fragment by the G allele. Several samples from the screening set were amplified and digested for 16 hours, as detailed in section 2.1.7, in order to check that the enzyme would work in the PCR buffer. The entire Cardiff LOAD case control sample was the amplified by PCR and the genotypes obtained by the *BsrBI* RFLP assay.

Figure 3.9 on the following page shows the position of the *BsrBI* cut sites within the fragment for both alleles and also samples of the different genotypes analysed on an agarose gel by electrophoresis. Table 3.23, also on the following page, shows the results for the intron 25 SNP genotyping in the case-control sample.

Figure 3.9 Intron 25 RFLP assay



A) Diagrammatic representation of *BsrBI* cut sites within the ACE intron 25 fragment for the different alleles. B) Digested samples taken from the Cardiff LOAD case-control sample analysed on a 2% agarose gel.

Table 3.12 Genotyping results for ACE intron 25 SNP

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 26	iA/G @ -6	Case	24 (0.19)	67 (0.52)	37 (0.29)	**0.96** (0.09)	**0.89** (0.02)
		Control	23 (0.19)	67 (0.54)	34 (0.27)		

Results of ACE intron 25 SNP genotyping in the Cardiff LOAD case-control sample. Position of polymorphism from the nearest exon is shown. Genotype numbers are given with frequencies in parenthesis, along with the p-value and chi-square value for genotypic and allelic association. Genotype codes are as follows; 1 1 – (G/G), 1 2 – (A/G), 2 2 – (A/A).

Both the cases and the controls conformed to the Hardy-Weinberg equilibrium, with p-values of 0.51 and 0.32 respectively. No association was found with LOAD for this polymorphism, genotypically nor allelically.

3.2.9 Linkage Disequilibrium (LD)

In order to ascertain the level of LD across the gene, the r^2 value was calculated for six of the polymorphisms. The exon 25 polymorphism was left out of the analysis due to only one allele being detected. r^2 was calculated as detailed in section 2.2.10. The results are shown in Tables 3.13 and 3.14 below.

Table 3.13 LD analysis for ACE in AD cases

Variant	5' UTR	Intron 9	Exon 15	I/D	Exon 17
Intron 9	0.42				
Exon 15	0.31	0.48			
I/D	0.32	0.82	0.97		
Exon 17	0.42	0.80	0.88	0.90	
Intron 25	0.33	0.83	0.92	0.95	0.88

Table 3.13 LD analysis for ACE in controls

Variant	5' UTR	Intron 9	Exon 15	I/D	Exon 17
Intron 9	0.52				
Exon 15	0.37	0.89			
I/D	0.38	0.89	0.98		
Exon 17	0.38	0.85	0.95	0.98	
Intron 25	0.40	0.87	0.97	0.98	0.96

Calculated r^2 values after 1000 simulations for the polymorphisms spanning *DCP1* with cases shown in table 3.12 and controls in table 3.13.

The polymorphisms showed a very high degree of LD across most of the gene with the 5'UTR polymorphism appearing to be a slight outlier. As was expected, there was nearly complete LD between the exon 15, 17 and I/D polymorphisms. These results match those previously reported by Rieder *et al.* (1999) in their study of variation within the ACE gene. LD analysis of the I/D and exon 17 polymorphism in

the Belfast replication sample gave r^2 values of 0.95 and 0.94 for cases and controls respectively

3.2.11 Haplotype Analysis

As none of the individual polymorphisms showed an association with LOAD, I decided to perform haplotype analysis to look for marker-disease association. This was carried out using EH+ as detailed in section 2.2.10. The results are shown in Tables 3.14 through 3.17 below and on the following page. As with the LD analysis, the exon 25 mutation was left out due to only one genotype being present in the sample.

Table 3.14 ACE two marker haplotypes

Markers	p-value								
1 2	0.95	**2 3**	0.30	**3 4**	0.93	**4 5**	0.21	**5 6**	0.26
1 3	0.92	**2 4**	0.67	**3 5**	0.43	**4 6**	0.76		
1 4	0.85	**2 5**	0.94	**3 6**	0.57				
1 5	0.85	**2 6**	0.89						
1 6	0.81								

Estimated p-values, after 1000 simulations, of marker-disease haplotype analysis for two marker ACE haplotypes. Polymorphisms represented in table are 1) 5'UTR, 2) Intron 9, 3) Exon 15, 4) Intron 16 *Alu* I/D, 5) Exon 17 and 6) Intron 25.

Table 3.15 ACE three Marker Haplotypes

Markers	p-value						
1 2 3	0.37	**2 3 4**	0.59	**3 4 5**	0.49	**4 5 6**	0.18
1 2 4	0.52	**2 3 5**	0.40	**3 4 6**	0.49		
1 2 5	0.89	**2 3 6**	0.26	**3 5 6**	0.15		
1 2 6	0.75	**2 4 5**	0.56				
1 3 4	0.88	**2 4 6**	0.66				
1 3 5	0.70	**2 5 6**	0.65				
1 3 6	0.66						
1 4 5	0.61						
1 4 6	0.66						
1 5 6	0.81						

Table 3.16 ACE four marker haplotypes

Markers	p-value				
1 2 3 4	0.46	**2 3 4 5**	0.58	**3 4 5 6**	0.18
1 2 3 5	0.38	**2 3 4 6**	0.37		
1 2 3 6	0.20	**2 3 5 6**	0.22		
1 2 4 5	0.66	**2 4 5 6**	0.74		
1 2 4 6	0.46				
1 2 5 6	0.84				
1 3 4 5	0.80				
1 3 4 6	0.47				
1 3 5 6	0.33				
1 4 5 6	0.71				

Table 3.17 ACE five marker haplotypes

Markers	p-value
1 2 3 4 5	0.56
1 2 3 4 6	0.30
1 2 3 5 6	0.21
1 2 4 5 6	0.84
1 3 4 5 6	0.63

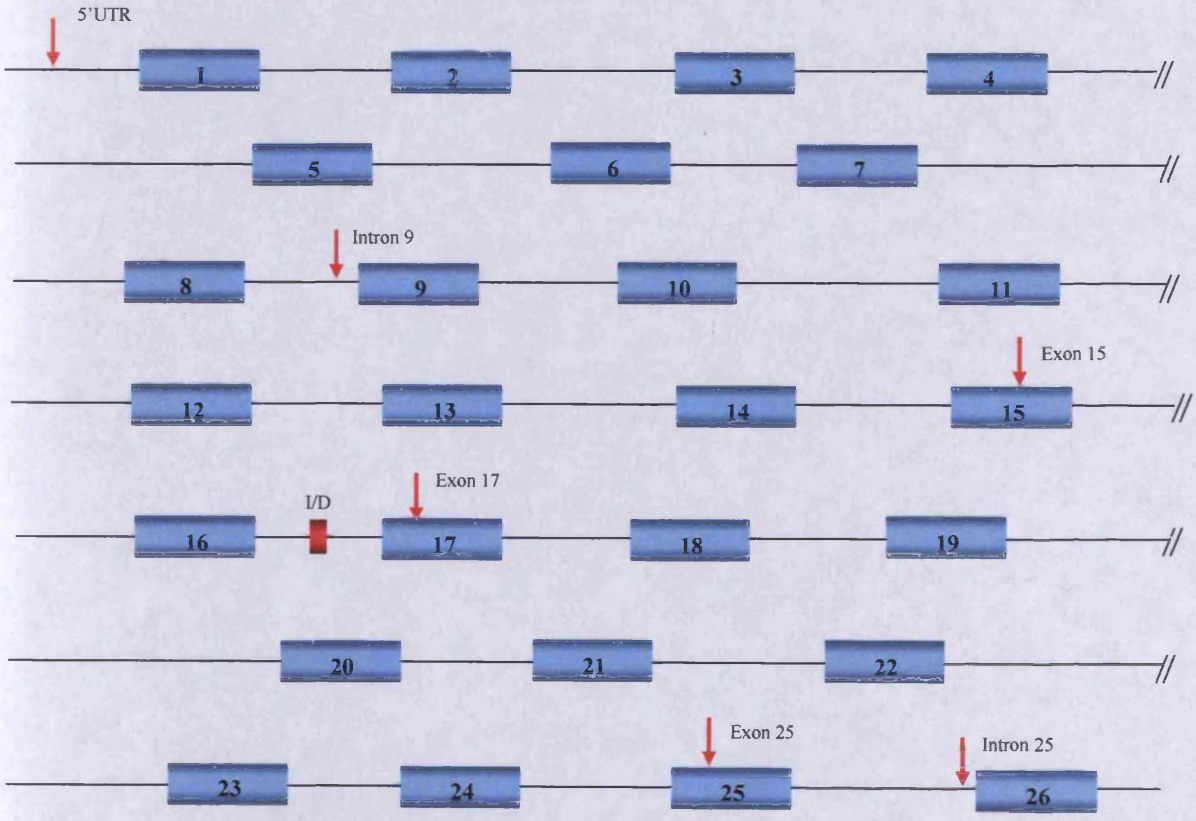
Estimated p-values, after 1000 simulations, of marker-disease haplotype analysis with table 3.15 showing three marker; 3.16 showing four marker and 3.17 showing five marker haplotypes. Polymorphisms represented in table are 1) 5'UTR, 2) Intron 9, 3) Exon 15, 4) Intron 16 *Alu* I/D, 5) Exon 17 and 6) Intron 25.

None of the haplotypes showed an association with LOAD in sample UK1

Overview of *ACE* Results

Position	Type	Sample	1 1	1 2	2 2	Assn	Allelic	HW	A1	A2
5' UTR	iA/T @ -240	Case	30	44	15	**0.55**	0.76	0.87	104	74
		Control	32	56	12			0.10	120	80
Intron 9	iC/T @ -57	Case	21	53	27	**0.92**	0.86	0.59	95	107
		Control	17	50	24			0.31	84	98
Exon 15	A/G @ 2193	Case	22	67	39	**0.80**	0.83	0.46	111	145
	Ala→Ala @ 731	Control	21	74	36			0.10	116	146
Intron 16	*Alu* @ -397	Case	24	67	40	**0.92**	0.77	0.66	115	147
		Control	23	66	35			0.69	112	136
Exon 17	G/A @ 2328	Case	19	51	30	**0.63**	0.74	0.75	89	111
	Thr→Thr @ 776	Control	20	66	29			0.10	106	124
Exon 25	C/T @ 3583	Case	114	-	-	-	-	1.00	228	-
	Pro→Leu @ 1195	Control	117	-	-			1.00	234	-
Intron 25	iA/G @ -6	Case	24	67	37	**0.96**	0.89	0.51	115	141
		Control	23	67	34			0.32	113	135

Summary of ACE genotyping in the Cardiff LOAD case-control sample. The position of polymorphisms, either within the polypeptide or from nearest exon is shown along with genotype and allele counts. Results of association shown as p-values for both allelic and genotypic analysis, and for concordance with the Hardy Weinberg equilibrium.



Genomic position of polymorphisms analysed in this chapter within *DCP1* (arrows) to illustrate level of gene coverage. I/D polymorphism shown by red block. Not to scale.

3.3 Discussion

This comprehensive study of angiotensin-converting enzyme polymorphisms has shown no association with LOAD in two separate samples. Several polymorphisms have been genotyped spanning most of the coding region of the gene and all gave p-values in excess of 0.6 genotypically.

The initial aim of this project was to confirm the association discovered by Kehoe *et al.* (1999a) and to move to functional work from there. The sample, UK1, used in this study overlapped with the one used by Kehoe, with 137 of the individuals being common to both samples (88 Cases and 49 controls). Analysis of the genotyping shows eleven discrepancies between the original genotyping and that of this study, 5 in the cases and 6 in the controls. In all of these the difference was of one allele, giving an overall error rate of less than 5%, which is not unexpected in genotyping studies but does give cause for concern. As mentioned briefly in the results section, genotyping the I/D polymorphism is not a straightforward process and consists of two PCR reactions, as the PCR which spans the *Alu* fragment is susceptible to preferential amplification of the shorter, D, allele. This is counteracted by a second PCR which only produces a fragment in the presence of the I allele. However, previous studies (Rieder *et al.* 1999) and this study have reported that there is very high, if not complete, LD between this polymorphism and two exonic polymorphisms which lie in exons 15 and 17. Both of these SNPs can be genotyped by relatively straightforward RFLPs. In this study, all of the samples which showed a discrepancy with the previous genotyping were confirmed using genotypes for one or both of these SNPs. It is possible that recombination could lead to a rare haplotype but it does not seem likely that this could have occurred in all eleven samples. This further evidence

suggests that the current genotyping is correct. These samples were regenotyped by myself and also blind genotyped by a co-worker; the genotypes again corresponded with mine (Emily Franks personal communication). Looking at the original data in Kehoe *et al.* (1999a), altering the genotypes to the results obtained here and re-running the analysis, gave an association which had in fact increased significance ($p = 0.008$). However, as the entire sample was not available for me to re-genotype this cannot be confirmed.

Because of the doubts raised by these results, it was decided to obtain the Belfast sample which was used to replicate the work of Kehoe *et al.* (1999a). As with the Cardiff sample, several of the DNA samples had been depleted and could not be replaced; therefore the entire sample could not be regenotyped. Out of the 365 in the new Belfast association sample 294 were used in the original study. From these, 25 discrepancies were discovered again, backed up by blind genotyping of the exon 17 polymorphism. As with the Cardiff data, these discrepancies did not abolish the old association. However, no association was found with LOAD in the NI1 sample.

Publication bias means that the number of negatives will be under represented in the literature; however, from the data available in published results, the evidence for an association is vague and conflicting.

In all of these studies of ACE the I/D polymorphism was the only polymorphism genotyped probably simply because this polymorphism was the first found and seemed to be functional in the sense that it could account for a substantial proportion of the variation seen in plasma ACE level (Rigat *et al.* 1990) providing a convenient marker. It is, however, generally considered that the *Alu* fragment is not the functional polymorphism, which could partly explain the contradictory results. If the functional polymorphism is in partial LD with the *Alu* fragment and is a risk factor

for LOAD then it could result in different results in different samples. Several different studies have attempted to describe the QTL within DCP1 with strong evidence for the 5'UTR, exon 17 and intron 25 SNPs being the functional polymorphisms or at least positionally close (Zhu *et al.* 2000; McKenzie *et al.* 2001; Zhu *et al.* 2001). In light of this research all three were genotyped in this study and none of these putative QTL were found to be associated with LOAD.

One other polymorphism was genotyped in this study. Despite a large number of SNPs having been discovered within the ACE gene, most have been intronic or conservative and it is difficult to explain how they could have a functional effect. Eyries *et al.* (2000) described a mutation in exon 25 which substituted a leucine for a proline at position 1195 in the peptide. It was suggested that this mutation altered the stalk region of the enzyme, allowing increased access for the, as yet unidentified, ACE secretase in turn leading to increased plasma concentrations of ACE. This mutation was found in individuals with highly elevated ACE concentrations and was therefore an excellent candidate for analysis in AD. However, no copies of the mutation were found in the cases or the controls. The paper does not detail how rare the mutation is and can therefore be assumed to be too rare for this study.

Analysing all of these polymorphisms together in a marker-disease based haplotype study did not yield any positive results. The LD results show a very high level across most of the gene resulting in most of the haplotypes being uninformative. Only the 5'UTR polymorphism shows an r^2 consistently less than 0.5 in relation to the other polymorphisms. However none of the haplotypes involving this marker show any sort of association.

There are other reasons for false positive ACE associations other than the controversy surrounding the functional polymorphism. There is evidence that the D

allele is a risk factor in vascular disease (Keavney *et al.* 2000). As vascular disease often affects patients younger than AD then the older population could be expected to have an excess of the I allele. Patients with VaD, when detected, are excluded from AD association studies which could lead to artificial inflation of the I allele in the cases. The possible effect of the D allele in cardiovascular disease is a strong confounding factor in all AD/ACE association studies. The study by Farrer *et al.* (2000) found an association with the D allele in the 66-70 year age group. However, after age stratification, the numbers available for analysis in this study are quite low which could distort the results. Also, the creation of a 66-70 year age group appears quite arbitrary as there is no particular reason why the gene should have an effect in that age range. The meta-analysis performed by Narain *et al.* (2000) suggested that overall, the I allele is a modest risk factor for AD. A second meta-analysis was recently published which includes all of the studies since 2000, reporting an OR of 1.3 for AD for the I allele (Kehoe *et al.* 2003) and an OR of 1.1 for the II genotype. The study also reports the association of AD with a haplotype spanning *DCP1*. The haplotype in question consists of three markers, two of which were genotyped in this project, the 5'UTR and exon 17 SNPs, and a third lying nearly 4kb upstream of the transcriptional start-site. Haplotype analysis of the 5'UTR (SNP1) and exon 17 (SNP5) SNPs in this study did not give a significant result (see haplotype **1 5** in table 3.14). The largest single association in the Kehoe *et al.* study (2003) comes from the 5'UTR SNP which gave an OR of 1.65 whereas similar analysis in this project was not significant ($p>0.05$, $\chi^2 = 0.91$). The power to detect an effect of that size in UK1 was 0.89 (see section 2.1.10).

Ioannidis (1998) showed that small studies with negative results will take statistically longer to be published than large positive results. A follow up study

looked at 36 different disease/gene associations and found that the initial study was often of greater effect than subsequent studies and that in many cases the effect disappeared as the data accumulated (Ioannidis *et al.* 2001). This effect can be seen clearly in ACE studies of myocardial infarction (MI). The original MI study by reported the D allele as being a risk factor with an O.R approaching 3 (Cambien *et al.* 1992). Subsequent studies gave variable results and a recent meta-analysis has estimates the O.R to be approximately 1.1 (Keavney *et al.* 2000).

In a time where the Human Genome Project has provided a massive number of SNPs and advances in technology have made high throughput genotyping accessible, care has to be taken in the design of association study to avoid false positives. The most common pitfalls being small sample size, poorly matched control groups, failure to attempt replication and over interpretation of the results (Cardon and Bell, 2001). The sample size in this study was large enough to detect an effect of the size seen in Kehoe's study (power of 0.99 to detect an OR of 2.22), although a larger sample would have been preferable and the controls were matched carefully based on age and ethnicity. A sample from Belfast was also analysed, which came from an ethnically similar population and of equal size to replicate the finding.

One item to note from the analysis of the ACE gene is the high LD across the region spanning from exon 10 to exon 26. This region corresponds roughly with the ancestral break point in the ACE gene described by Farral *et al.* (1999). This large section, spanning approximately 20kb is comparable to the recently described blocks of LD found across the human genome (Daly *et al.* 2001; Jeffreys *et al.* 2001). These studies show that LD is highly structured, with long stretches which are interspersed with smaller regions characterised by increased levels of recombination. This evidence has huge implications for association studies. Genotyping of only a few

polymorphisms could define all the haplotypes over a very large area. If the gene of interest lies within one of the recombination 'hot-spots' however it could lead to a larger number of SNP's to be genotyped. In the case of ACE for example, by simply genotyping the 5'UTR and the exon 17 polymorphism you will detect most haplotypes across a 25kb region. Recent work suggests another ancestral breakpoint lies downstream of the 3'UTR, approximately 2kb from exon 26 (Soubrier *et al.* 2002). This could be the other end of the LD block, followed by another region of increased recombination.

In conclusion, this study does not suggest that the ACE gene is a risk factor for LOAD, despite being an excellent functional candidate. This goes against the recent comprehensive meta-analysis (Kehoe *et al.* 2003) giving on OR of 1.3 for the I allele. However, this modest effect may be inflated due to publication bias and other factors which cannot be accounted for in a meta-analysis e.g. case and control selection in each study. Many of the 'disease' genes which have been described over the last few years will slowly be disproved as association study designs improve and genetic information increases. It is becoming increasingly clear that population sizes in the thousands are required to provide definitive association in complex genetic disorders. LD mapping based around information from the Human Genome Project will prove to be a powerful tool in discovering disease genes but trans-ethnic mapping will also be required to elucidate rare haplotypes.

Chapter 4

Tachykinin-2 Receptor (TACR2)

4.1 Background

Tachykinin-2 receptor (TACR2), also known as neurokinin-A, substance K or neurokinin-2 receptor, is a receptor for a peptide neurotransmitter of the tachykinin family. The first member of the tachykinin family to be found, substance P, was discovered 70 years ago while tachykinin-2 was first isolated in 1983 (Kimura *et al.* 1983). The final member of the mammalian tachykinin family, neurokinin-B was isolated by Kangawa *et al.* (1983).

The receptors are found primarily in the respiratory, vascular and gastrointestinal tissues (Hua *et al.* 1985) where they modulate bronchoconstriction, vascular permeability and stimulate mucus secretion (McCormack *et al.* 1989; Naline *et al.* 1989 and Saria *et al.* 1989). Work by Haley *et al.* (2001) suggests that tachykinin-2 and its receptor is also essential in the development of the lung. TACR2 is also believed to have an important role in the nociceptive response. A study involving tachykinin-2 antagonists showed that TACR2 was involved in the nociceptive response in a normal and inflamed rodent knee joint (Neugebauer *et al.* 1996). Murine knockouts of the tachykinin-2 and substance P genes showed a significant reduction in response to intense painful stimuli (Cao *et al.* 1998). The use of TACR2 agonists prevents the induction of long term potentiation (LTP), the continual stimulation of a postsynaptic neurone, in C-fibers; the neurones which convey the nociceptive response (Liu and Sandkuhler, 1997). A role has also been suggested for tachykinin-2 in the mediation of seizures and excitotoxic damage

induced apoptosis in the hippocampus, although a majority of that effect could be caused by substance P which is encoded by the same gene (Liu *et al.* 1999)

4.1.1 Gene Structure

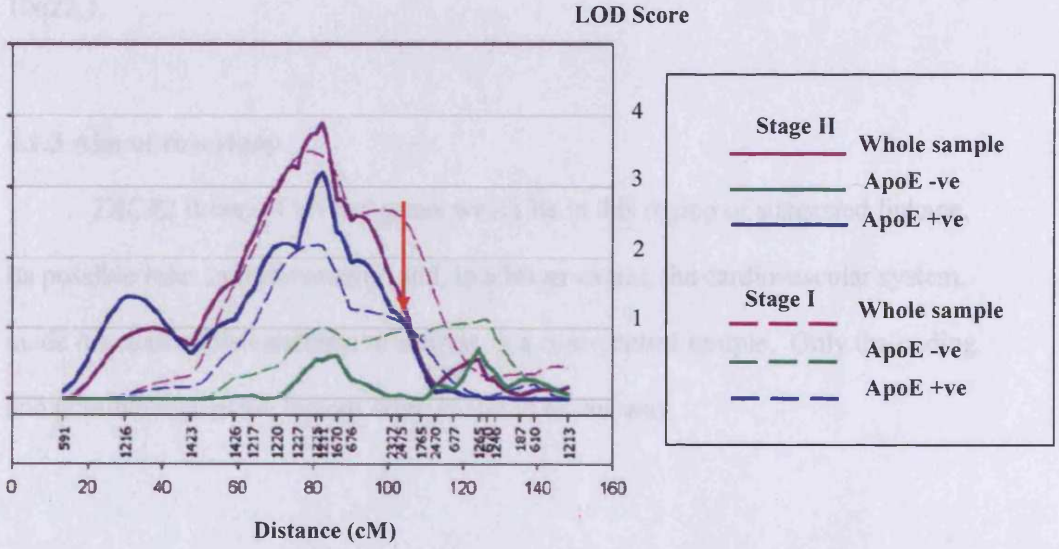
Sasai and Nakanishi (1989) were the first to deduce the protein sequence and cDNA encoded by *TACR2* from bovine and rat stomachs. Analysis of the protein sequence showed that TACR2 is a member of the rhodopsin superfamily, which are characterized by seven transmembrane domains and coupling to GTP-binding proteins as signal transducers. The human gene for TACR2 was first cloned by Gerard *et al.* (1990) after being isolated from gastric tissues. *TACR2* consists of 5 exons, with the membrane spanning regions being highly conserved between species. Studies using hybrid DNA and in situ hybridization localised the gene to chromosome 10q21 (Morten *et al.* 1991).

4.1.2 TACR2 as a candidate for Alzheimer's Disease

TACR2 is not an obvious candidate for a role in late-onset AD. The highest concentration of receptors is found in the gastric and pulmonary systems, not the brain. However, recent work by Saffroy *et al.* (2001) on rat brain using tachykinin-2 antagonists revealed the presence of binding sites for tachykinin-2. These receptor sites showed a localisation distinct from tachykinin-1 and -3 receptors and were found in the hippocampus, the thalamus and the septum. TACR2 is also implicated in the inflammatory system, with receptors being present on the surface of monocytes and an enhanced respiratory burst being seen in monocytes from rheumatoid arthritis patients after TACR2 stimulation (Lavagno *et al.* 2001). The possible role of the inflammatory response in AD has already been discussed in section 1.5.3.3.

However, the strongest evidence for *TACR2* as a candidate gene in LOAD is its position in the genome. A two-stage genome screen for LOAD (Kehoe *et al.* 1999b and Myers *et al.* 2000) implicated a region on chromosome 10 (Figure 4.1) that gave a maximum two-point LOD score of 4.85 around marker D10S1211 (82.2cM). Allele sharing in the affected sib pairs was found to be elevated in this region in both ApoE positive and ApoE negative pairs (Kehoe *et al.* 1999b)

Figure 4.1 Results of 2-stage genome screen for LOAD on chromosome 10



Graph showing results of two-stage genome screen for LOAD on chromosome 10 (Myers et al. 2000). Values on y-axis are the multi-point LOD scores with distance across the chromosome shown on the x-axis. Position of *TACR2* shown by red arrow.

Further analysis in the second stage reduced that LOD score to 2.48, but multi-point linkage analysis of the whole sample gave a LOD score of 3.83 between the markers D10S1227 and D10S1225 (Myers *et al.* 2002). The size of the peak suggested an effect in this region greater than or equal to that of the ApoE gene (Myers *et al.* 2000).

In addition, a linkage study of a population with high plama βA levels gave evidence for linkage to the same area (Ertekin-Taner *et al.* 2000). This evidence in

combination with the strength of the linkage to LOAD meant that further work was carried out to narrow the region. Fine mapping the region was carried out by analysing 44 microsatellites in the maxLOD-1 region. Four markers were significant in the stage one pools (128 AD cases, 103 controls) and two remained significant after individual genotyping (p-values of 0.011 and 0.014 for D10S1790 and D10S1647 respectively). The significant linkage of D10S1647 with LOAD was successfully replicated in a sample from Belfast consisting of 229 AD cases and 230 controls (Turic *et al.* personal communication). *TACR2* lies 200kb telomeric of this marker at 10q22.1.

4.1.3 Aim of this study

TACR2 is one of several genes which lie in this region of suggested linkage. Its possible roles in inflammation and, to a lesser extent, the cardiovascular system, made it a reasonable candidate to analyse in a case-control sample. Only the coding and possible regulatory regions were analysed in this way.

4.2 Results

4.2.1 Screening set preparation

In order to screen for polymorphisms, a screening set of AD cases was required. 15 individuals would provide sufficient power to detect polymorphisms with a frequency as low as 5% and using AD cases increases this power although this increase cannot be quantified. Samples were ordered from the European Collection of Cell Cultures (ECACC) of individuals whose age of onset was greater than 65. Their codes and ages of onset are detailed in table 4.2 below.

Table 4.1 Screening set samples for LOAD

Code	Sex	Age at Sampling
AK0113	F	83
AK0114	F	88
AK0117	F	89
AK0126	F	78
AK0736	M	67
AK0737	F	71
AK0739	F	76
AK0741	F	91
AK0742	F	72
AK0750	F	76
AK0752	F	85
AK0753	F	76
AK0755	M	78
AK0757	F	83
AK0758	F	84

Details of the individual samples ordered from ECACC to be used as a screening set for LOAD polymorphism detection. Ages at sampling and the sex of each individual shown.

The samples were received as growing cultures of lymphoblasts. The cultures were grown as detailed in section 2.1.9.1 until they reached confluence. Several flask of each culture were grown, some were frozen down and stored (section 2.1.9) while the rest had their DNA and RNA extracted (section 2.1.9). The DNA was quantified using a spectrophotometer and working dilutions were made and stored in water at

-20°C. One sample, AK0736, did not grow and was discarded from the screening set as 14 samples was still of significant power to detect polymorphisms of the frequencies expected from the linkage data (Prof O'Donovan, personal communication)

4.2.2 Primer design

The cDNA sequence for *TACR2* was obtained from Ensembl (ENSG00000075073). This sequence was analysed for homology to the unfinished high throughput genomic sequences (htgs) database using BLAST (NCBI) which pulled out a chromosome 10 clone, AC016821. This clone was lined up with the cDNA, again using BLAST, to determine the flanking sequences for each exon. Primer3 was then used to design primers spanning each exon as detailed in section 2.1.1. Gerard *et al.* (1990) described putative TATA and GC-boxes at -300 and -360 respectively. However, in order to be complete and because of the small size of the gene, primers were designed spanning 2kb upstream of the translational start site. These primers gave overlapping fragments of approximately 500bp which were labelled TP1, TP2, TP3, TP4 and TP5 with TP1 being the furthest upstream. Figure 4.2 overleaf shows the position of these primers in relation to the exons and the regulatory regions.

4.2.3 DHPLC and sequencing

PCR reactions for each fragment were optimised as detailed in section 2.1.1. The optimised conditions are detailed in Table 4.2 on the following page along with the fragment sizes and the primer sequences. After optimisation, all of the fragments were amplified in 24µl reactions for the entire screening set. The melt profiles for the

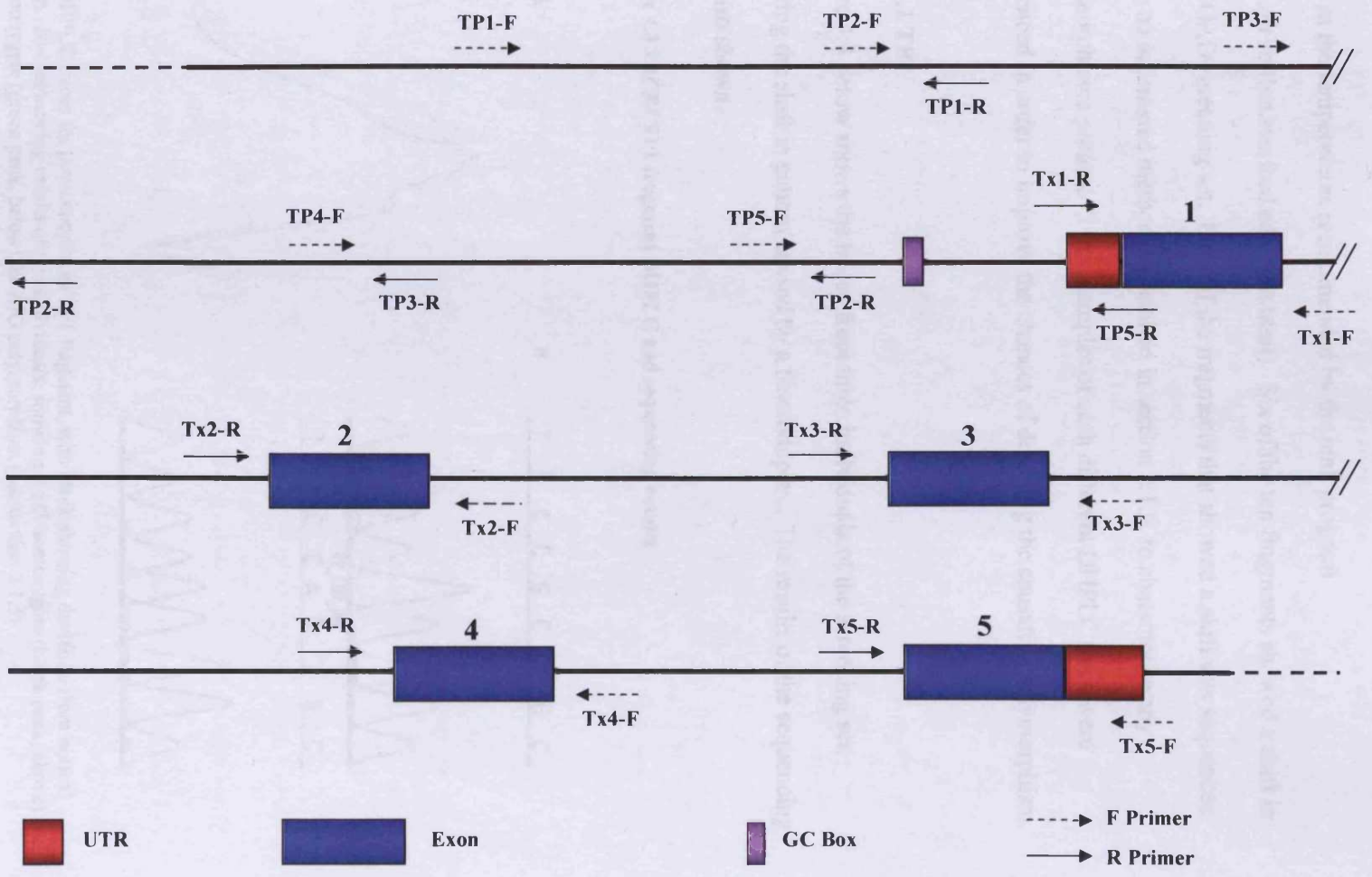
fragments were determined as detailed in section 2.1.3. Denaturing and re-annealing of the fragments was carried out to form heteroduplexes.

Table 4.1 *TACR2* PCR conditions

Exon	Primer 5' –3'	Taq	T (°C)	Size (bp)
TP1-F	GTTGTGGGGAGGAAGGAACT	HotStar	58	499
TP1-R	AACTTCTGGGTAAGTTCATCGAC			
TP2-F	CATGCTCCTCACATCTGCAC	HotStar	58	503
TP2-R	ACACACACGCATGAACCTGT			
TP3-F	CCGACTCCCGCTAATCCT	HotStar	61	515
TP3-R	GCTCTGTCTACCCCATCTGAA			
TP4-F	TGTACCAACCCCTGTGCTAA	HotStar	60	561
TP4-R	CCAGATCAGAGGAATGCACA			
TP5-F	CCTGGAGAGAGGCTGCTG	HotStar	64	504
TP5-R	TTCTGGGTCTGGAACAAAGG			
Tx1-F	GGCATGTCACCACCCTTATC	Qtaq	60	499
Tx1-R	CCTTTGTTCCAGACCCAGAA			
Tx2-F	ACACAATCCCGTGTACACTCA	Qtaq	60	501
Tx2-R	CTCTGCAGGTCGGCTGATAC			
Tx3-F	CGGCCTGAGACCGTAAG	GC-Rich	58	510
Tx3-R	GCTACCCAATGCCACCTTTA			
Tx4-F	GCCAGAGGGATTATTGTGCT	HotStar	60	499
Tx4-R	AGCTAACGGGGTCTGTGTGT			
Tx5-F	AAGGGATCCATCCCCAATAC	Qtaq	60	492
Tx5-R	ATCAAACTCACCCACGAAGG			

Sequences of the primers used for amplification of *TACR2* and its upstream region. The taq enzyme used in the optimised reaction, along with the annealing temperature and the size of the fragment produced are shown.

Figure 4.2 Primer positions for amplification of TACR2



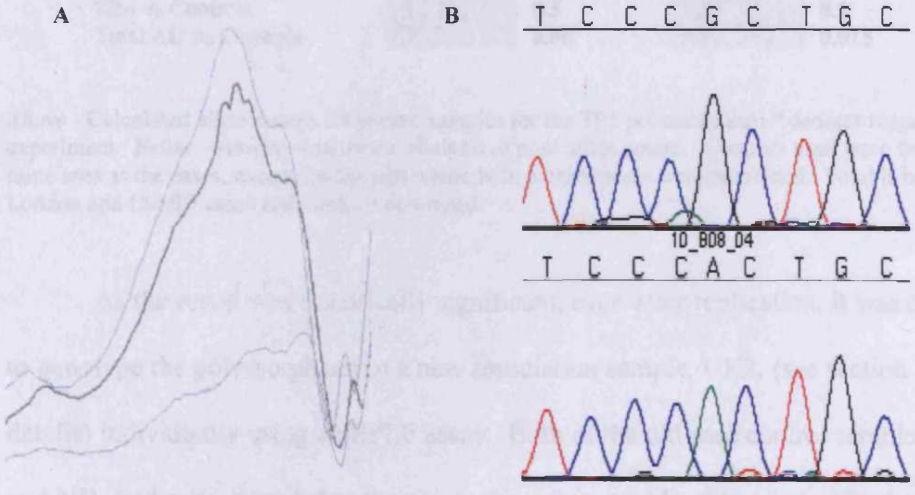
Structure of TACR2, showing positions of primers, exons and putative promoter. Primers for upstream region overlap

DHPLC analysis of all the fragments was carried out as detailed in section 2.1.3 at the temperatures recommended by the melt program (http://insertion.stanford.edu/melt.html). Six of the ten fragments showed a shift in the LOAD screening set. Each of the fragments that showed a shift was sequenced using an automated method, as detailed in section 2.1.5, to characterise any polymorphisms present. Two examples of each different DHPLC trace were sequenced in order to improve the chances of detecting the causative polymorphism.

4.2.3.1 TP1

Figure 4.3 below shows the traces from three individuals of the screening set, showing the shift in pattern caused by a heteroduplex. The results of the sequencing are also shown.

Figure 4.3 *TACR2* TP1 fragment DHPLC and sequencing results



A – DHPLC traces for three samples of TP1 fragment, with black showing deviation from normal pattern. B – sequencing results of two individuals, showing the G homozygote (black peak, above) and A homozygote (green peak, below) for A/G polymorphism (see section 2.1.5)

Sequencing revealed an A/G polymorphism which is 1855bp upstream of the transcriptional start site. Due to its distance from the gene, it was decided to analyse this polymorphism in a new pooled sample using SNaPshot (see section 2.1.8). Three pooled samples (see section 2.2) were available for analysis; Cardiff (n = 96), London (n = 90) and Sibs (a sample of LOAD diagnosed siblings, n = 114) and age matched controls from Cardiff and London. The reaction was carried with the concentration of the extension primer being 5pM (5' –TGCTGTCCTCCTGCTCCCAGGCAG – 3'). Results of the genotyping are shown in table 4.2 below.

Table 4.2 Automated genotyping results for TP1 SNP

Sample	Allele 1	Allele 2	Allele 1*	Allele 2*
Cardiff AD	97	95	100	92
Cardiff Controls	100	92	105	87
London AD	115	65	117	63
London Controls	72	108	79	101
Sibs	105	123	109	119

Test	χ^2	p-value	χ^{2*}	p-value*
Cardiff AD vs Controls	3.79	**0.05**	0.26	**0.6**
London AD vs Controls	42.9	**0.00**	16.17	**0.00**
Sibs vs Controls	1.51	**0.5**	1.97	**0.2**
Total AD vs Controls	35.5	**0.00**	5.9	**0.015**

Above - Calculated allele counts for pooled samples for the TP1 polymorphism.* denotes repeated experiment. *Below* – results of statistical analysis of pool allele counts. Controls used were from the same area as the cases, except for the sibs where both control pools were combined. Total is both London and Cardiff cases and controls combined.

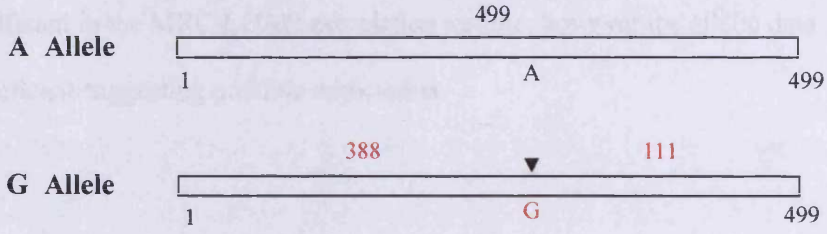
As the result was statistically significant, even after replication, it was decided to genotype the polymorphism in a new association sample, UK2, (see section 2.2 for details) individually using an RFLP assay. Both of the old case control samples, UK1 and NI1, had now degraded to the point where they could not be used. The fragment was analysed using DNAstar and the polymorphism was found to abolish a *PflMI* cut site. The enzyme was tested on samples from the screening set at the appropriate

conditions for 16 hours to test for enzyme activity within the PCR buffer and ensure complete digestion (section 2.1.7). Figure 4.4 shows a diagrammatic representation of the cut sites to show the fragment sizes produced and several samples run on an agarose gel.

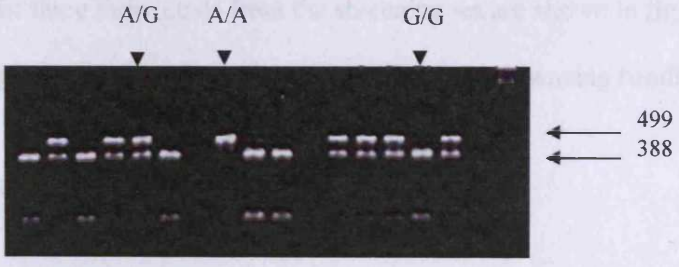
Analysis was carried out using SPSS and Minitab as detailed in section 2.1.10. The results of the association study for the TP1 polymorphism are shown in table 4.3 on the following page.

Figure 4.4 TP1 RFLP assay

A)



B)



A) Diagrammatic representation of *PflMI* cut sites within the TP1 fragment for the different alleles. B) Samples taken from the UK2 case-control sample digested with *PflMI* for 16 hours and analysed on a 2% agarose gel. Genotypes and fragment sizes are shown.

Table 4.3 Results of TP1 SNP genotyping

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 1	iA/G@ -2137	Case	32	77	57	**0.16** (3.67)	0.05 (3.77)
			(0.19)	(0.47)	(0.34)		
		Control	42	80	42		
			(0.26)	(0.48)	(0.26)		

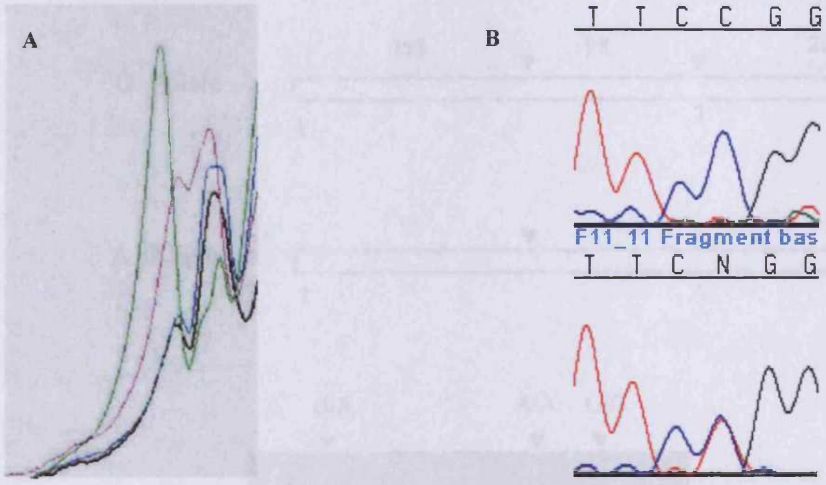
Results of TP1 SNP genotyping in the UK2 case-control sample. Position of polymorphism in relation to the nearest exon shown. Genotype numbers are given with frequencies in parenthesis below, along with the p-value and chi-square value for genotypic and allelic association. Genotype codes are as follows; 1 1 – (A/A), 1 2 – (A/G), 2 2 – (G/G).

Both samples were found to conform to the Hardy-Weinberg equilibrium with p-values of 0.51 and 0.75 in the cases and controls respectively. The genotype data were not significant in the MRC-LOAD association sample; however the allelic data were just significant suggesting possible association.

4.2.3.2 TP3

The DHPLC traces of three individuals from the screening set are shown in figure 4.5 on the following page along with the corresponding samples' sequencing results.

Figure 4.5 DHPLC and sequencing analysis of TP3

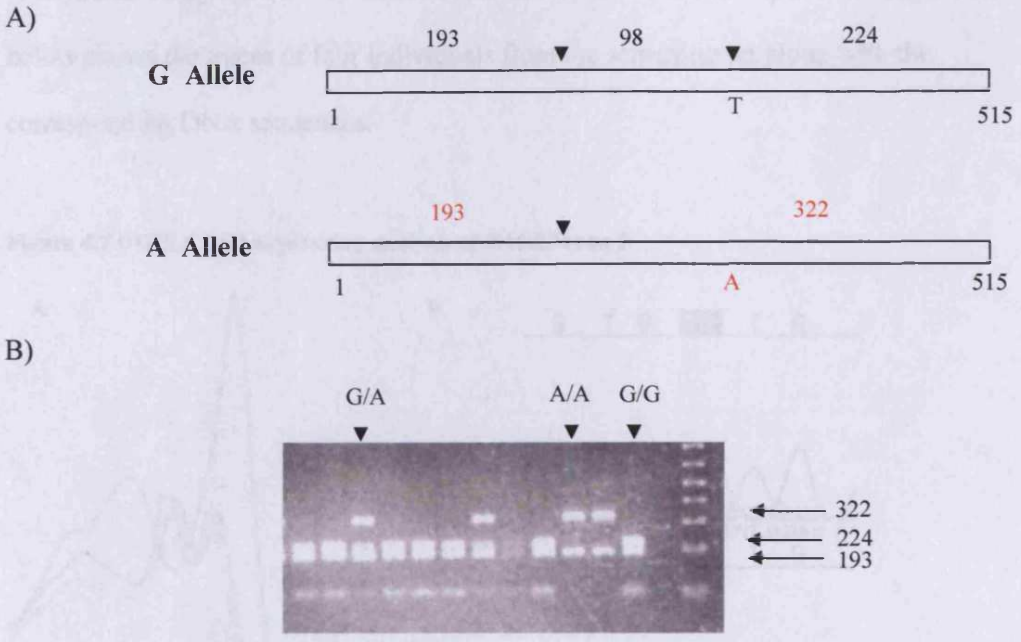


A – DHPLC traces for four samples of TP3 fragment, with green and pink showing deviation from normal pattern. B – sequencing results, showing the G homoozygote (above) and heterozygote (below) for G/A polymorphism (sequence of reverse strand shown)

The sequencing of the fragments showing different DHPLC patterns revealed a G/A SNP 894bp upstream of the transcription initiation site. In order to design an RFLP assay for this polymorphism the fragment was analysed using DNAStar. The A allele disrupts a natural *NciI* cut site. The enzyme was first tested, as detailed in section 2.2.4, and then the entire UK2 case-control sample was amplified and digested with *NciI* for 16 hours.

The position of the cut sites within TP3 are shown in Figure 4.6 below along with the fragment sizes produced after digestion. Several examples of digested samples are also shown in Figure 4.6 analysed on an agarose gel. The results of the genotyping are shown in Table 4.4 on the following page.

Figure 4.6 TP3 RFLP assay



A) Diagrammatic representation of *NciI* cut sites within the TP3 fragment for the different alleles. B) Samples taken from the UK2 case-control sample digested with *NciI* for 16 hours and analysed on a 2.5% agarose gel supplemented with Metaphor. Genotypes and fragment sizes are shown.

Table 4.4 TP3 SNP genotyping results

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 1	iG/A @ -1176	Case	120 (0.70)	43 (0.25)	8 (0.05)	**0.58** (1.01)	0.32 (0.99)
		Control	109 (0.65)	50 (0.30)	9 (0.05)		

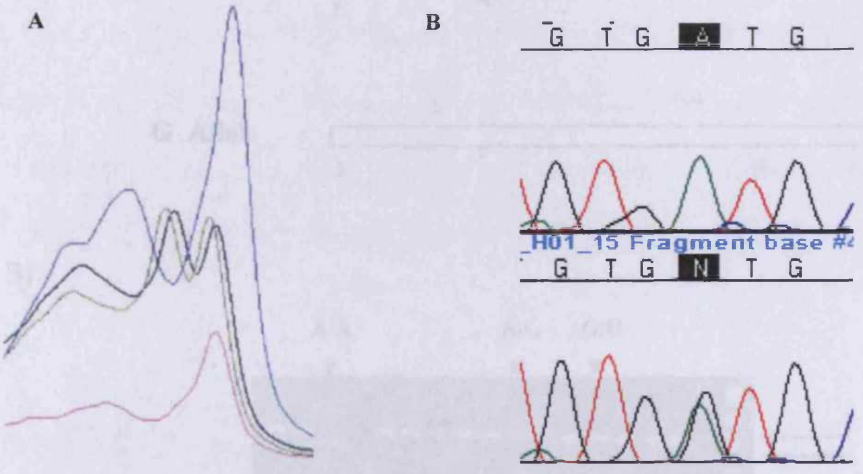
Results of TP3 SNP genotyping in the UK2 case-control sample. Position of polymorphism in relation to the nearest exon shown. Genotype numbers are given with the frequencies in parenthesis below, along with the p-value and chi-square value for genotypic and allelic association. Genotype codes are as follows; 1 1 – (G/G), 1 2 – (A/G), 2 2 – (A/A).

Neither population deviated significantly from the Hardy-Weinberg equilibrium with p-values of 0.12 and 0.31 respectively. No association was found between the G/A SNP and LOAD in the case control sample.

4.2.3.3 Exon 1

Analysis of *TACR2* exon 1 by DHPLC showed a shift in the trace patterns. Figure 4.7 below shows the traces of four individuals from the screening set along with the corresponding DNA sequences.

Figure 4.7 DHPLC and sequencing analysis of *TACR2* exon 1.

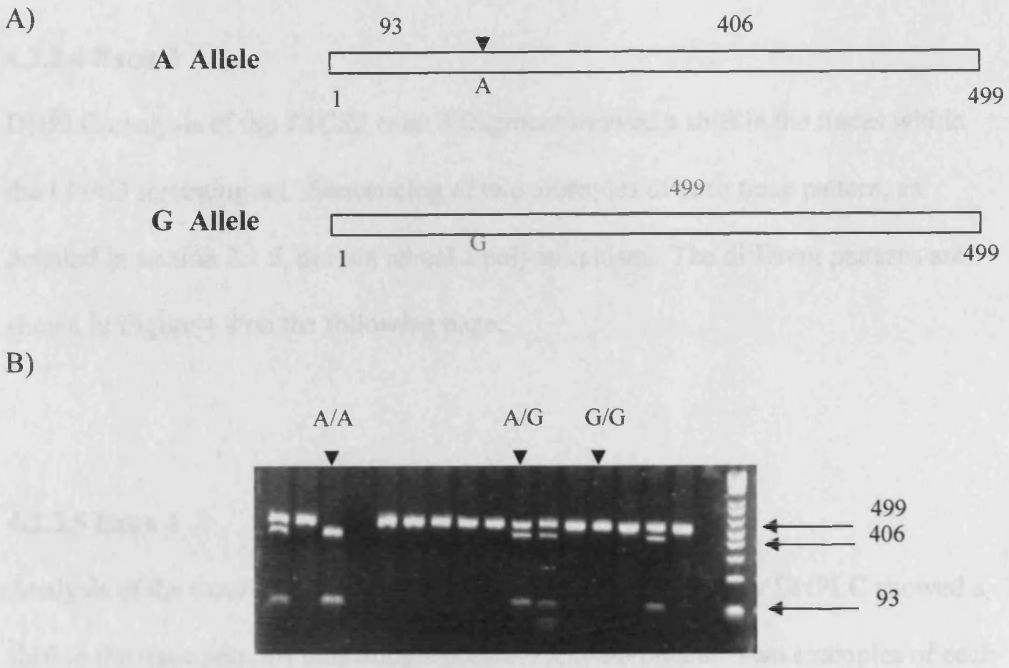


A – DHPLC traces for four samples of *TACR2* exon 1 fragment, with black and green showing deviation from normal pattern. B – sequencing results of two individuals, showing the A homozygote (above) and the G/A heterozygote (below).

The sequencing of the fragment revealed an A/G polymorphism within the coding sequence of *TACR* at nucleotide 67 of the cDNA resulting in an amino acid change (I23T). Analysis of the fragment for individual genotyping using DNAStar showed that the G allele abolished an existing *BanI* site. Figure 4.8 below shows the position of the cut site within the fragment and the sizes of the fragments produced after digestion. The enzyme was tested and then the UK2 sample was amplified and digested with *BanI* for 16 hours. Figure 4.8 also shows several individuals from the case-control sample analysed by electrophoresis on an agarose gel. The results of the

genotyping are shown in Table 4.5 below.

Figure 4.8 TACR2 exon 1 SNP RFLP assay



A) Diagrammatic representation of *BanI* cut sites within the *TACR2* exon 1 fragment for the different alleles. B) Samples taken from the UK2 case-control sample digested with *BanI* for 16 hours and analysed on a 2.5% agarose gel supplemented with Metaphor. Genotypes and fragment sizes are shown.

Table 4.5 Results of TACR2 exon 1 SNP genotyping

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 1	A/G @ 67	Case	93	44	3	**0.39** (1.87)	0.30 (1.09)
	Ile→Thre @23	Control	86	45	7		

Results of *TACR2* exon 1 SNP genotyping in the UK2 case-control sample. Position of polymorphism from start of translation in the nucleotide sequence shown along with the position of the residue within the amino acid sequence. Genotype numbers are given along with the p-value and chi-square value for genotypic and allelic association. Genotype codes are as follows; 1 1 – (G/G), 1 2 – (A/G), 2 2 – (A/A).

No association was found between the TACR2 exon 1 polymorphism and LOAD either genotypically or allelically. Both samples were found to conform to the Hardy-Weinberg equilibrium with p-values of 0.39 and 0.73 for the cases and controls respectively.

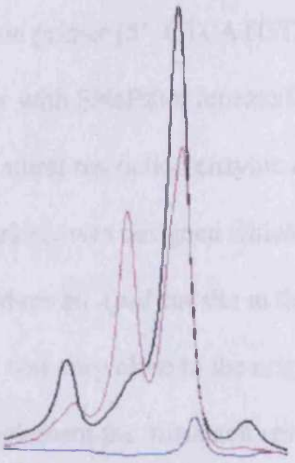
4.2.3.4 Exon 3

DHPLC analysis of the *TACR2* exon 3 fragment showed a shift in the traces within the LOAD screening set. Sequencing of two examples of each trace pattern, as detailed in section 2.1.5, did not reveal a polymorphism. The different patterns are shown in Figure 4.9 on the following page.

4.2.3.5 Exon 4

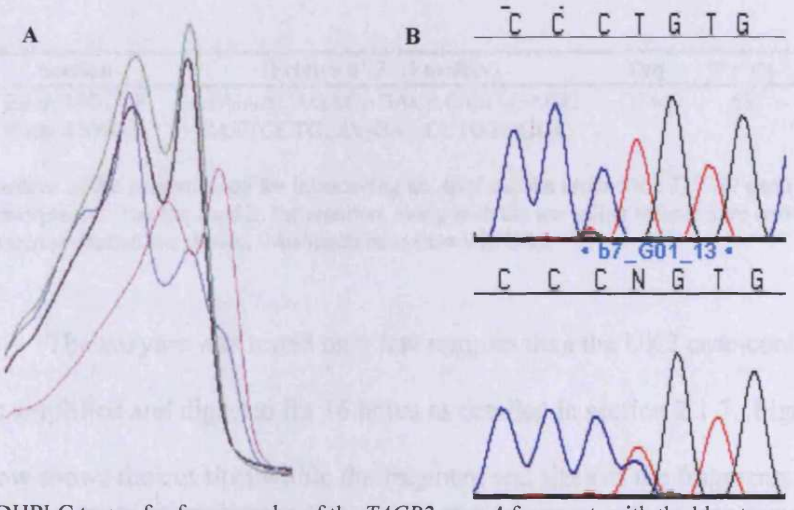
Analysis of the exon 4 fragment of *TACR2* in the screening set by DHPLC showed a shift in the trace patterns indicating a possible polymorphism. Two examples of each pattern type were sequenced using automated methods as detailed in section 2.1.5. The results of the DHPLC and the sequencing are shown in Figure 4.10 on the following page.

Figure 4.9 DHPLC results for TACR2 exon 3 fragment



DHPLC traces for two samples from the LOAD screening set of the *TACR2* exon 3 fragment, with pink showing deviation from normal the pattern.

Figure 4.10 DHPLC and sequencing analysis of TACR2 exon 4



A - DHPLC traces for four samples of the *TACR2* exon 4 fragment, with the blue trace showing deviation from the normal pattern. B – sequencing results, showing the T homozygote (above) and the heterozygote (below) for T/C polymorphism (see section 2.1.5)

Sequencing revealed a T/C polymorphism 116bp downstream of exon 4 of *TACR2*. Initially I attempted to use the automated method of genotyping, see section 2.1.8, and designed an extension primer (5' CTCATGTAGGCTGGGGGCAC 3'). However, attempts to genotype with SNaPshot repeatedly failed and therefore a RFLP assay was used. No suitable natural restriction enzyme cut sites were present at the polymorphic site. A reverse primer was designed which deliberately contained an incorrect base in order to introduce an *ApaI* cut site in the C allele, termed a mismatch primer. As the polymorphism was very close to the original forward primer a new one had to be designed to complement the mismatch reverse primer otherwise the size differences between the different fragments would not be visible on an agarose gel. The PCR conditions were then optimised using the new primers in the standard way (see section 2.1.1). The re-designed primers are shown in Table 4.6 on the below along with the optimal PCR conditions.

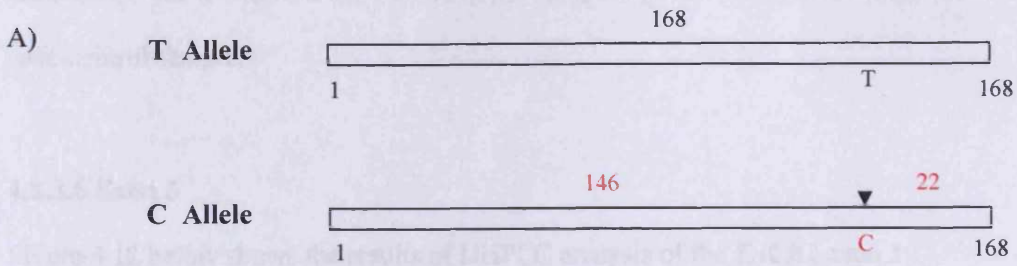
Table 4.6 TACR2 exon 4 SNP PCR conditions

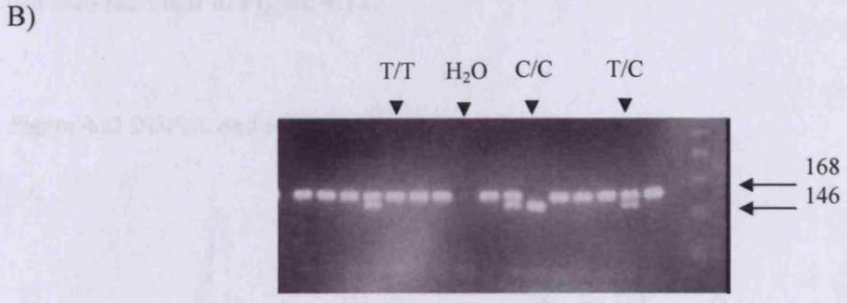
Section	Primer 5'-3' (For/Rev)	Taq	T (°C)	Size (bp)
Exon 4 SNP-F	AGGAACAGACAGACAGTGAGAGC	QTaq	63	168
Exon 4 SNP-R	CATTCCTGCCGGAACCTGTGGGC			

Sequences of the primers used for introducing an *ApaI* cut site around the *TACR2* exon 4 fragment polymorphism. The taq used in the reaction, along with the annealing temperature and the size of the fragment produced are shown. Mismatch base shown in bold.

The enzyme was tested on a few samples than the UK2 case-control sample was amplified and digested for 16 hours as detailed in section 2.1.7. Figure 4.11 below shows the cut sites within the fragment and sizes of the fragments produced. Also shown are examples of the different genotypes analysed on agarose gel. Table 4.7 on the following page shows the results of the association study.

Figure 4.11 TACR2 exon 4 SNP RFLP Assay





A) Diagrammatic representation of *ApaI* cut sites within the *TACR2* exon 4 mismatch fragment for the different alleles. B) Samples taken from the UK2 case-control sample digested with *ApaI* for 16 hours and analysed on a 2.6% agarose gel supplemented with Metaphor. Genotypes and fragment sizes are shown along with a H_2O negative control.

Table 4.7 Results of exon 4 SNP genotyping.

			Genotype			p-value ($\chi 2$)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 4	iT/C @ +116	Case	119	45	5	**0.29** (2.47)	0.10 (2.68)
			(0.70)	(0.27)	(0.03)		
		Control	101	54	7		
			(0.62)	(0.33)	(0.04)		

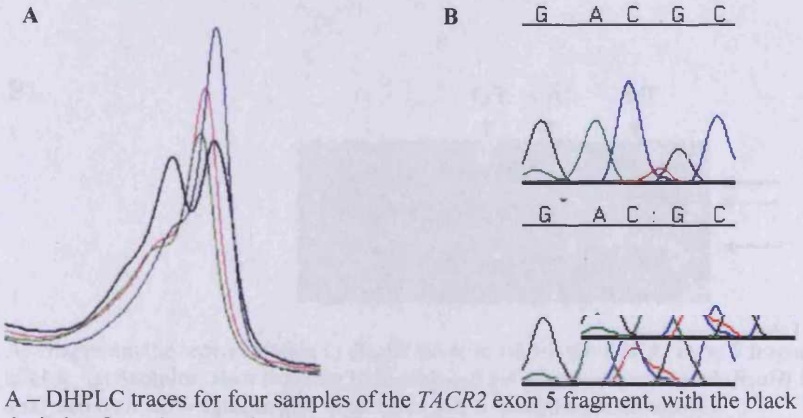
Results of *TACR2* exon 4 SNP genotyping in the UK2 case-control sample. Position of polymorphism shown in relation to the nearest exon. Genotype numbers are given with frequencies in parenthesis below, along with the p-value and chi-square value for genotypic and allelic association. Genotype codes are as follows; 1 1 – (T/T), 1 2 – (T/C), 2 2 – (C/C).

Both the cases and the controls were found to conform to the Hardy-Weinberg equilibrium (section 2.1.10), with p-values of 0.77 and 0.95 respectively. No association was found with the *TACR2* exon 4 polymorphism and LOAD in the UK2 case-control sample.

4.2.3.6 Exon 5

Figure 4.12 below shows the results of DHPLC analysis of the *TACR2* exon 5 fragment. Shifts were seen in the patterns within the screening set of LOAD patients which were sequenced using an automated method (section 2.1.5) and those results are also included in Figure 4.12.

Figure 4.12 DHPLC and sequencing analysis of TACR2 exon 5



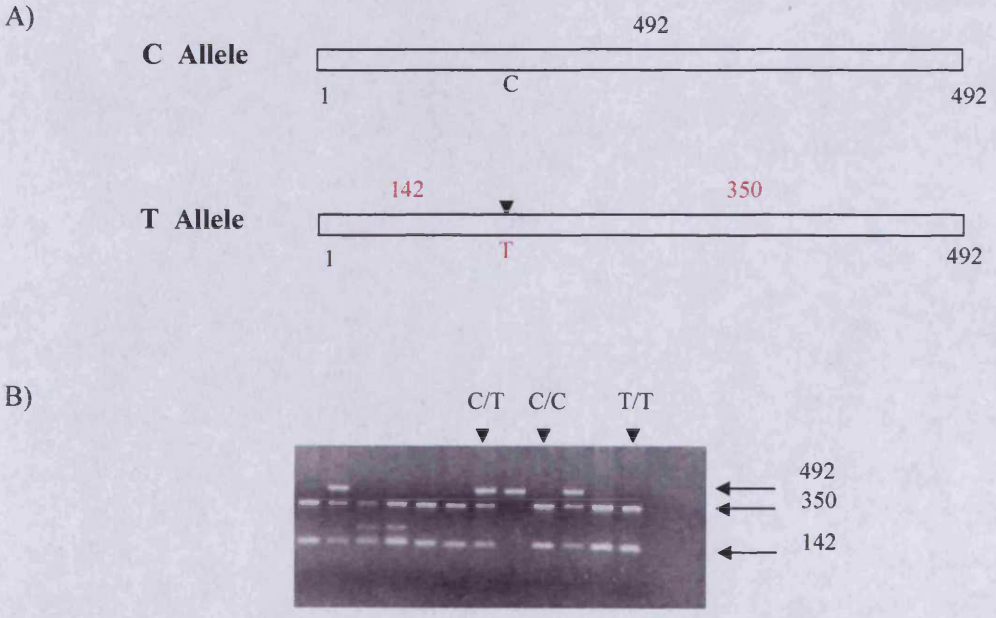
A – DHPLC traces for four samples of the *TACR2* exon 5 fragment, with the black trace showing deviation from the normal pattern. B – sequencing results of two individuals, showing a C homozygote (above) and a heterozygote (below).

Sequencing revealed a polymorphism within the coding sequence; a C/T polymorphism at position 1126 of the cDNA. This changes the amino acid at residue 376 from arginine to histidine, both basic, hydrophilic amino acids.

Individual genotyping was carried out using a RFLP assay as detailed in section 2.1.6. Analysis of the fragment showed that the T allele created a *BsaHI* cut

site which is not present in the C allele. The enzyme was tested in the PCR buffer, and then the UK2 sample was amplified and digested as detailed in section 2.1.7. Figure 4.13 below shows the position of the cut sites within the fragment and examples of the different genotypes analysed on an agarose gel.

Figure 4.13 RFLP assay for TACR2 exon 5 SNP



A) Diagrammatic representation of *BsaHI* cut sites within the *TACR2* exon 5 fragment for the different alleles. B) Samples taken from the UK2 case-control sample digested with *BsaHI* for 16 hours and analysed on a 2.2% agarose gel. Genotypes and fragment sizes are shown.

The results of the association study are shown in table 4.8 on the following page.

Table 4.8 Genotyping results for the TACR2 exon 5 SNP

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 5	G/A @1126	Case	98 (0.64)	45 (0.30)	9 (0.06)	**0.90** (0.64)	0.44 (0.60)
	Arg→His @ 375	Control	90 (0.60)	50 (0.33)	10 (0.07)		

Results of *TACR2* exon 5 SNP genotyping in the UK2 case-control sample. Position of polymorphism from start of translation shown, along with the position of the residue within the amino acid sequence. Genotype numbers are given with the frequencies in parenthesis below, along with the p-value and chi-square value for genotypic and allelic association. Genotype codes are as follows; 1 1 – (C/C), 1 2 – (T/C), 2 2 – (T/T).

No association was found between the exon 5 polymorphism and LOAD in the case-control sample, genotypically or allelically. Both the cases and the controls conformed to the Hardy-Weinberg equilibrium, with p-values of 0.22 and 0.40 respectively. An overview of all the genotyping results is shown at the end of this section.

4.2.4 LD Analysis

As for *DCP1* in section 3.2.8, the level of LD across the TACR2 gene was determined by calculating the r^2 value for the five polymorphisms. r^2 was calculated as detailed in section 2.1.10. The haplotype frequencies for the marker pairs were obtained from the marker-disease haplotype association analyses reported in section 4.2.5. The results are shown in Tables 4.9 and 4.10 on the following page.

Table 4.9 LD analysis for *TACR2* in AD cases

Variant	TP1	TP3	Exon 1	Exon 4
TP3	0.28			
Exon 1	0.13	0.05		
Exon 4	0.15	0.04	0.95	
Exon 5	0.30	0.74	0.06	0.06

Estimated r^2 values, after 1000 simulations, between markers on the TACR2 gene in the UK2 cases.

Table 4.10 LD analysis for *TACR2* in controls

Variant	TP1	TP3	Exon 1	Exon 4
TP3	0.23			
Exon 1	0.11	0.06		
Exon 4	0.11	0.07	0.96	
Exon 5	0.26	0.72	0.08	0.09

Estimated r^2 values, after 1000 simulations, between markers on the TACR2 gene in the UK2 control sample.

A high degree of LD was seen between the exon 1 and exon 4 polymorphisms in both the cases and the controls, and also between the TP3 and exon 5 polymorphisms.

4.2.5 Haplotype Analysis

Haplotypes within the TACR2 gene were analysed for disease association as detailed in section 2.1.10. The results are shown in Tables 4.11 through 4.13 below and on the following page.

4.11 TACR2 two marker haplotype results

Markers	p-value		p-value		p-value		p-value
1 2	0.12	**2 3**	0.50	**3 4**	0.45	**4 5**	0.17
1 3	0.57	**2 4**	0.13	**3 5**	0.54		
1 4	0.12	**2 5**	0.92				
1 5	0.56						

Table 4.12 TACR2 three marker haplotypes

Markers	p-value		p-value		p-value
1 2 3	0.68	**2 3 4**	0.72	**3 4 5**	0.66
1 2 4	0.23	**2 3 5**	0.86		
1 2 5	0.79	**2 4 5**	0.40		
1 3 4	0.69				
1 3 5	0.93				
1 4 5	0.62				

Estimated p-values, after 1000 simulations, of marker-disease two marker haplotype analysis with table 4.11 showing two marker and 4.12 showing three marker haplotypes. Polymorphisms represented in table are 1) TP1, 2) TP3, 3) Exon 1, 4) Exon 4 and 5) Exon 5. p-values were calculated using EH+.

Table 4.13 TACR2 four marker haplotypes

Markers	p-value		p-value
1 2 3 4	0.82	**2 3 4 5**	0.87
1 2 3 5	0.92		
1 2 4 5	0.63		
1 3 4 5	0.95		

Estimated p-values, after 1000 simulations, of marker-disease haplotype analysis. Polymorphisms represented in table are 1) TP1, 2) TP3, 3) Exon 1, 4) Exon 4 and 5) Exon 5.

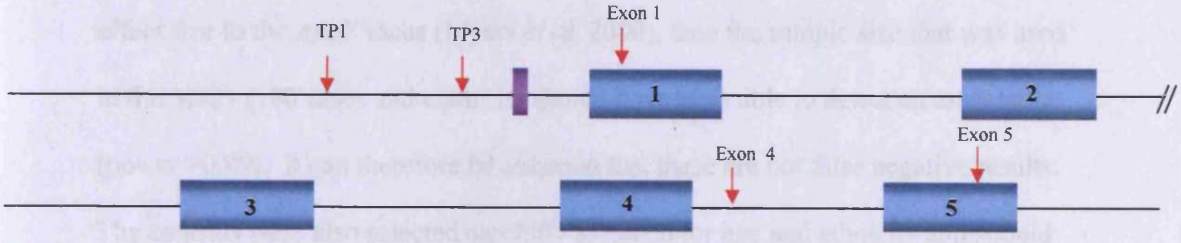
No disease association with haplotype was found in the UK2 case-control sample for the TACR2 gene polymorphisms discovered in this study.

Overview of *TACR2* Results

Position	Type	Sample	1 1	1 2	2 2	Assn	Allelic	HW	A1	A2
TP1	iA/G@ -2137	Case	32	77	57	**0.16**	0.05	0.51	141	191
		Control	42	80	42			0.75	164	164
TP3	iG/A @ -1176	Case	120	43	8	**0.58**	0.32	0.12	283	59
		Control	109	50	9			0.31	268	68
Exon 1	A/G @ 67	Case	93	44	3	**0.39**	0.30	0.39	230	50
	Ile→Thre @23	Control	86	45	7			0.73	217	59
Exon 4	iT/C @ +116	Case	119	45	5	**0.29**	0.10	0.77	283	55
		Control	101	54	7			0.95	256	69
Exon 5	G/A @1126	Case	95	48	9	**0.90**	0.44	0.22	241	63
	Arg→His @ 375	Control	90	50	10			0.40	230	70

Summary of *TACR2* individual genotyping in the UK2 case-control sample. The position of polymorphisms, either within the polypeptide or from nearest exon is shown along with genotype and allele counts. Results of association shown as p-values for both allelic and genotypic analysis, and for concordance with the Hardy Weinberg equilibrium

TACR2 SNP overview



Genomic position of polymorphisms analysed in this chapter within *TACR2* (red arrows) to illustrate level of gene coverage. Exons shown as blue rectangles and putative promoter by purple rectangle. Not drawn to scale.

4.3 Discussion

Screening of the TACR2 gene and its promoter region provided no polymorphisms that were genotypically associated with LOAD in the UK2 sample. One polymorphism, two kilobases upstream of the translational start site, did show a slight allelic association however its importance to the gene or its regulation is difficult to explain. Adjusting for multiple testing however changed the p-value required for significance to 0.0014, which is discussed in more detail in Chapter 7.

As mentioned in the introduction to this chapter (section 4.1), *TACR2* is a positional candidate for AD, lying only 200kb from a microsatellite found to be strongly associated with AD in two separate populations. In addition TACR2 has been implicated in the inflammatory response and for these reasons the gene was included in this project.

Most of the polymorphisms discovered in this gene gave negative results for association. Considering that the genome screen results suggested a gene of similar effect size to the *ApoE* locus (Myers *et al.* 2000), then the sample size that was used in this study (180 cases and controls) should have been able to detect an association (power >0.99). It can therefore be assumed that these are not false negative results. The controls were also selected carefully to match for age and ethnicity and should therefore not be affecting the results obtained in any way. One note of interest from the polymorphisms detected in this gene is the high level of non-conservative SNPs found (40%). It is rare for such a high proportion of the polymorphisms detected to alter the protein structure, even taking into account the fact that most of the intronic sequence was not screened. However, neither of these residues are conserved in the rat and mouse sequence. In addition both lie in non-critical region of the gene and not

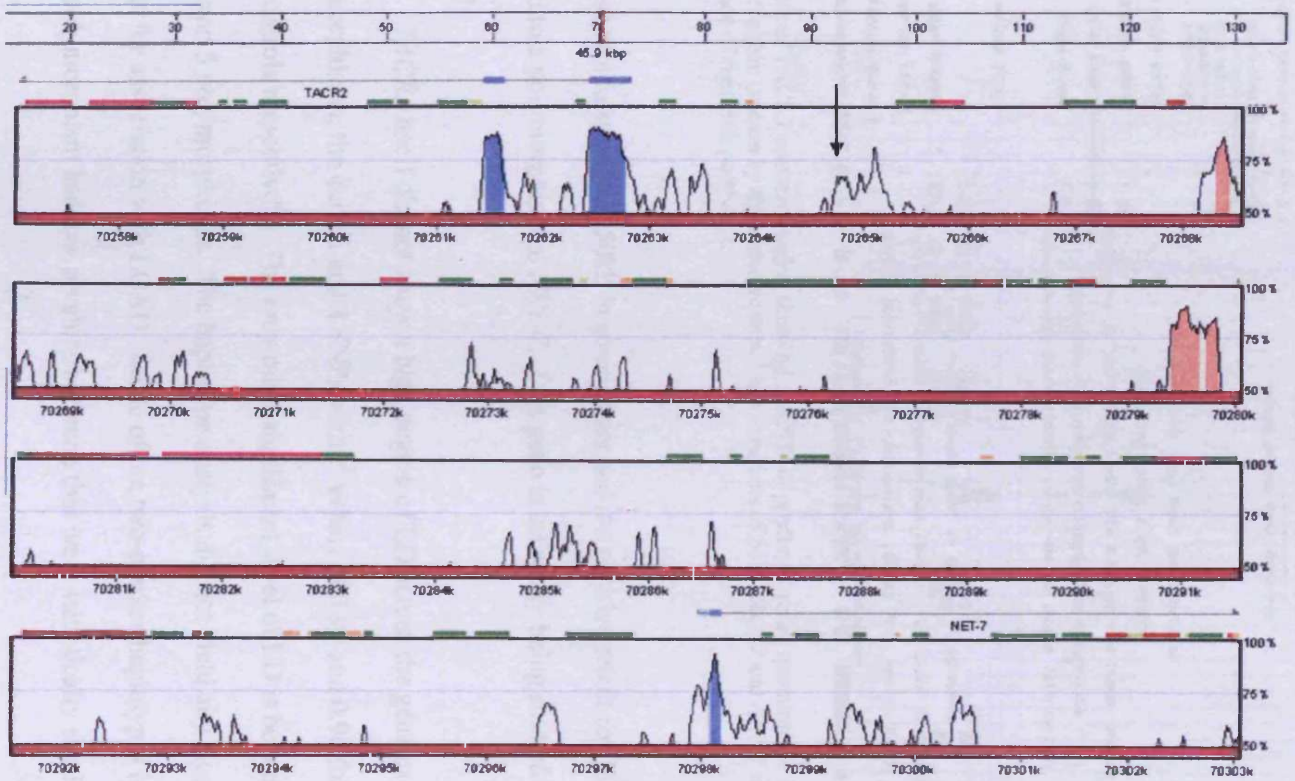
near the transmembrane domains.

One of the SNPs (TP1) did give a borderline positive result for an allelic association with the G allele being over represented in the AD cases. It was obvious from looking at the results from the SNaPShot experiments that the strong association was coming from the London sample ($p = 0.00$) as opposed to the Cardiff case-control sample ($p = 0.61$). Individual genotyping of the polymorphism by RFLP assay in the London sample confirmed this result ($\chi 2 = 13.37$, $p = 0.001$). Both populations had been collected in the same way with the same selection criteria and why they should produce such varying results is unknown. London populations are generally more diverse and this result could reflect stratification. No other polymorphisms discovered in this study show a similar discrepancy. The RFLP assay results do however show that the pooling of the samples and automated genotyping are a reliable method for association studies showing no difference in allele frequencies from the individual genotyping in the cases and 1% difference in the controls. After adjusting for multiple testing, which is discussed in more detail in Chapter 7, the result was not close to being significant.

While this result warranted further analysis of the data, no other sample was available at the time to replicate the analysis. The SNP should be genotyped in a replication sample and if it maintains its association should be studied to see what functional role, if any, it has. As mentioned earlier the polymorphism lies 2kb upstream of the translational start site in an area not generally considered to be involved in the regulation of the TACR2 gene. Analysis of the sequence surrounding the polymorphism on both Vista (http://pipeline.lbl.gov) and Genome Browser (http://genome.ucsc.edu) yielded no regulatory sequences, expressed sequence tags (ESTs) or high species homology suggesting that there is not an unknown gene in the

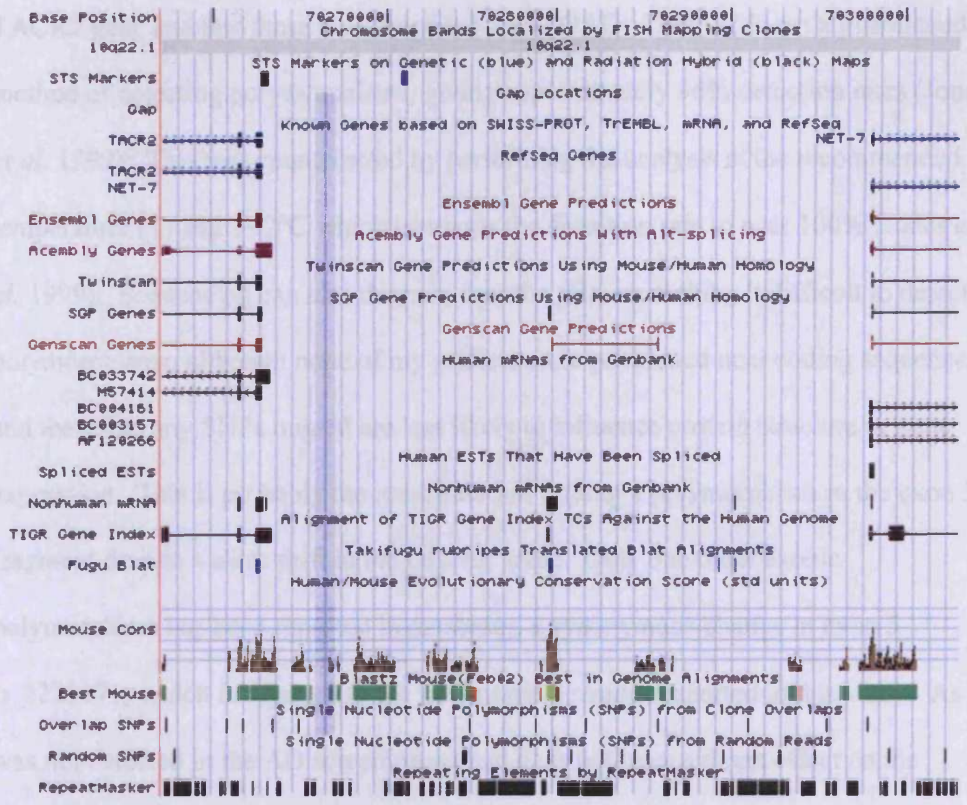
region. The results are shown in Figures 4.14 and 4.15 on the following page with both figures showing exactly the same region. The nearest known gene upstream is *NET-7*, which lies approximately 35kb upstream of the start of translation. LD has

Figure 4.14 TACR2 upstream region Vista output



Comparative sequences of human and mouse DNA performed by Vista. Level of homology, shown as a percentage on the y-axis, of the mouse and human TACR2 upstream region (x-axis). Human/mouse sequences that are >70% identical over >100bp are indicated by pink vertical bars in non-coding sequence and blue vertical bars in coding sequence. Approximate position of TP1 polymorphism shown by arrow. Nearest gene, NET-7 shown in bottom right hand of figure.

Figure 4.15 UCSC Genome Browser output



Analysis of TACR2 upstream region showing no ESTs or predicted genes spanning the TP1 polymorphism (shown by light blue vertical line). Positions of both *TACR2* and *NET-7* shown, left and right side of figure respectively).

been shown to span over 50kb in some cases and the positive result for TP1 could be linked to a polymorphism in *NET-7*. This gene is currently being studied by our group.

TACR2 itself did not show a high degree of LD across the gene except in two polymorphisms; the exon 1 and 4 SNPs with r^2 values of 0.95 and 0.96 for the cases and controls respectively. The only other significant level of LD is between the TP3 and exon 5 polymorphisms. The haplotype analysis did not yield any significant results for association with LOAD. Some of the two-marker haplotypes involving the TP1 polymorphism had low p-values but none that were statistically significant.

It is possible that other polymorphisms remain to be detected within the TACR2 gene and that these are associated with LOAD. DHPLC is not a guaranteed method of detecting polymorphisms, giving approximately 96% detection rates (Jones *et al.* 1999). This was counteracted by performing the analysis at the recommended temperature (T) and T+2°C which increases the detection rate to near 100% (Jones *et al.* 1999). Sequencing can also degrade near the primers making it difficult to detect polymorphisms; although none of my primers were positioned near coding sequence and therefore any SNPs missed are less likely to influence protein structure or gene expression. This is probably the reason for not finding a polymorphism in the exon 3 fragment despite a clear shift in the DHPLC trace. Only one other exonic polymorphism has been reported in databases, a synonymous change in exon 5 (rs2229171) which is adjacent to the polymorphic residue reported in this study. As it was not detected in the AD screening set and does not have a direct effect on the protein it is not likely to be a risk factor for AD.

Even if this gene is not associated with LOAD, and the results suggest this is likely, then the results obtained in this study are still informative for building up a SNP and/or LD map of the region.

Chapter 5

Endothelin-converting enzyme 1 (ECE1)

5.1 Background

Endothelin-converting enzyme 1, EC 3.4.24.71, catalyses the production of an active isopeptide, endothelin-1 from its inactive precursor, big-endothelin. The enzyme was isolated by Schmidt *et al.* (1994) and confirmed to be a member of the zinc metalloprotease family. ECE1 is synthesized by vascular endothelial cells and contains a single transmembrane domain, a short cytoplasmic tail and a large N-terminal domain. Endothelin-1 (ET-1) is regarded as the most potent vasoconstrictor known at present with an essential role in a number of pathophysiological situations including chronic heart failure (Omland *et al.* 1994) and hypertension (Clozel *et al.* 1993). The other identified isopeptides are endothelin-2 and endothelin-3 which, along with ET-1, are ligands for endothelin-A (ET_A) or endothelin-B (ET_B) receptor. ETA receptors mediate vasoconstriction and are located on vascular smooth muscle cells while ETB receptors, located on vascular endothelial cells, mediate vasodilation (Arai *et al.* 1990b; Sakurai *et al.* 1990).

Targeted disruption of ET-1 has also been found to have implications in embryogenesis, specifically during the development of neural crest derived tissues and the heart (Kurihara *et al.* 1994). Neural crest cells are a migratory population of cells, originating at the dorsal lip of the neural fold, which differentiate into a wide variety of cells including neurons, epidermal melanocytes and much of the bone and connective tissue of the head and neck (Jessel and Melton, 1992). Further work by

Clouthier *et al.* (1998) using ET_A deficient mice shows that expression of *goosecoid*, a transcription factor, is downstream of ETA activation.

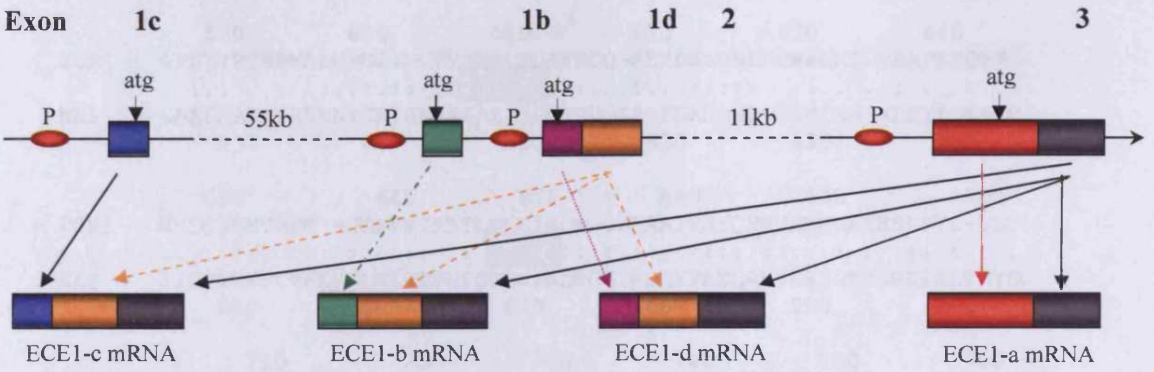
Studies on pulmonary blood flow in response to ductal constriction have revealed a role for nitric oxide (NO). Following chronic constriction a decrease in NO activity is seen alongside an increase in ET-1 mediated vasoconstriction (Black *et al.* 1998). Results of several studies suggest that NO and ET-1 regulate each other via an autocrine feedback loop (Boulanger and Luscher 1990; Redmond *et al.* 1996; McMullan *et al.* 2001).

Originally considered to be a highly specific endopeptidase, recent evidence has shown that ECE1 can cleave a variety of peptides (Hoang and Turner, 1997; Johnson *et al.* 1999). Using mutagenesis of big-endothelin, Johnson *et al.* (2002), have shown that the S_1 site of ECE1 will bind to practically any amino acid placed in the P_1 position of big-endothelin and cleave that substrate. These results suggest that ECE1 could have a wide-ranging role in the cell.

5.1.1 Gene

Using a combination of cell hybrids and fluroresence in situ hybridisation (FISH), the gene encoding ECE1 has been mapped to 1p36 (Albertin *et al.* 1996 and Matsuoka *et al.* 1996). Since its discovery, 4 isoforms of ECE1 have been discovered which differ in their N-terminal sequences (Schmidt *et al.* 1994; Shimada *et al.* 1995; Valdenaire *et al.* 1999). The structure and organisation of the ECE1 gene is shown in Figure 5.1 on the following page.

Figure 5.1 ECE1 genomic structure



Genomic organisation of the four isoforms of ECE1. Upper half shows the distance between and positions of the alternatively spliced first three exons. The other 16 exons are identical in all variants. Promoters for each shown by red circle. The mRNAs produced by each splice variant are shown in the lower half of the diagram. Arrows have been used to show how the isoforms are assembled.

Four separate promoter regions produce four different mRNA's with the sequence from exon 3 onwards being common to each isoforms. This variation in the N-terminal sequence determines whether the enzyme is expressed at the cell surface or within the TGN (the b-isoform) (Azarani *et al.* 1998). ECE1 shows high homology to other members of its enzymatic family, particularly neprilysin (NEP) and endothelin-converting enzyme 2 (ECE2). The highest homology is seen between the C-terminal sequences, especially the sequences relating to zinc binding and catalysis (see Figure 5.2 on the following page).

5.1.2 ECE1 in Alzheimer's Disease

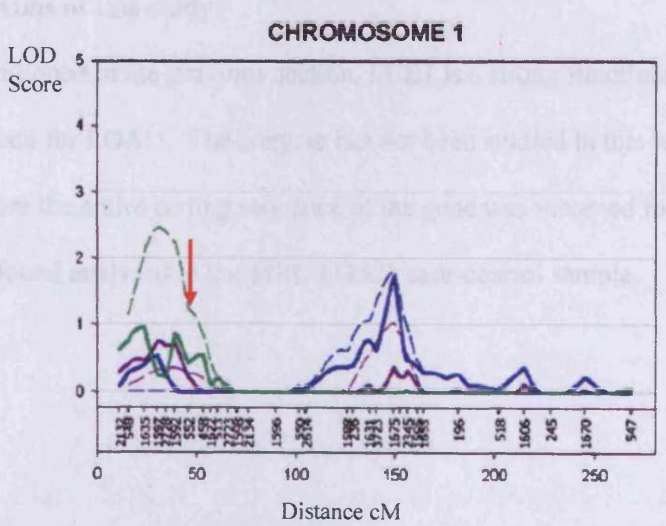
ECE1 is a good positional candidate and an excellent functional candidate for study in AD. As mentioned in section 1.3.4, a recent two-stage genome screen by this department described several areas of suggested linkage in the genome to LOAD (Kehoe *et al.* 1999b and Myers *et al.* 2002). *ECE1* lies beneath one of these peaks at 1p36 as shown in Figure 5.3 on the following page.

Figure 5.2 ECE1 and NEP homology

```
          590       600       610       620       630       640
ECE1   APFYTRSSPKALNFGGIGVVVGHELTHAFDDQGREYDKDGNLRPWWKNSSVEAFKRQTEC
       . :.. . :  .:.:.:: :.:::::::.:::::::..::.:::  ::.. . : .  ...:
NEP    GAFFSAKRPAYMNYGAIGFVIGHEITHGFDDQGRQFDKNGNLVDWWQEMTKEKYLDKAKC
          590       600       610       620       630       640

          650       660       670       680       690       700
ECE1   MVEQYSNYSVN--GEPVNGRHTLGENIADNGGLKAAYRAYQNWVKKNGAEHSLPTL-GLT
       ...:::::.:.  :  .:: .: :::::::::.: :: ::: :....: :  :: : .
NEP    IIDQYSNYTVKEVGLKLNGVNTQGENIADNGGIKEAYYAYQAWTHRHGEEARLPGLEKYS
          650       660       670       680       690       700

            710       720       730       740       750       760
ECE1   NNQLFFLGFAQVWCSVRTPESSHEGLITDPHSPSRFRVIGSLSNSKEFSEHFRCPPGSPM
         :::.:. :..::.:   :. .  . :  :.:.::::::: .:: .:::.  :.:: ::::
NEP    PRQLFWLSAANTWCAVYRNEAIKLRITTGFHAPGRFRVIGPMSNMEEFASDFKCPMGSPM
          710       720       730       740       750       760

          770
ECE1   NPPHKCEVW
       :: .:::.::
NEP    NPDKKCKVW
          770
```

Alignment of the C-teminal ends of the ECE1 (top) and NEP (bottom) protein sequences shown with residue number carried out on Swiss Prot (www.ebi.ac.uk). Zinc binding sites shown by blue boxes, active site by a red box and motif critical to enzyme maturation by a green box. Two dots indicate identical amino acids while one dot notes amino acids from the same sub-group e.g. hydrophobic.

Figure 5.3 Area of suggested linkage on chromosome 1



Results of two-stage genome screen for LOAD on chromosome 1 (Myers *et al.* 2002), with LOD score on the y-axis and cM distance on the x-axis. Position of *ECE1* shown by a red arrow.

ECE1s role as a potent vasoconstrictor makes it an essential part of the cardiovascular system. The cardiovascular system's possible role in AD has been discussed extensively in other sections of this project (see section 1.6) e.g. the probability of dementia rising significantly in people who have suffered a stroke. The enzyme may have a role in the inflammatory response, also strongly implicated in AD (see section 1.5.3), by cleaving bradykinin – a stimulator of tPA (Hoang and Turner 1997). Both ECE1 and ECE2 show increased levels of expression in neurones and astrocytes following neuronal injury (Nakagomi *et al.* 2000) suggesting that the endothelins may be playing a role in communication.

In addition, ECE1 shows homology with NEP, 39% at the amino acid level rising to 54% in the C-terminal end (MacLeod *et al.* 2001). NEP has been observed within amyloid plaques (Sato *et al.* 1991) and has also been shown to cleave βA at five different positions with high affinity (Howell *et al.* 1995; Takaki *et al.* 2000).

5.1.3 Aims of this study

As mentioned in the previous section, ECE1 is a strong functional and positional candidate for LOAD. The enzyme has not been studied in this way previously therefore the entire coding sequence of the gene was screened for polymorphisms and those found analysed in the MRC LOAD case-control sample.

5.2 Results

5.2.1 Exon Amplification

Primers were designed using Primer 3 to span all the exons of ECE1, including the b, c and d isoforms and their corresponding promoters. Fragment sizes were kept under 500bp, where possible, for optimal polymorphism detection. PCR conditions were optimised and the primer details along with the corresponding reaction conditions are shown in Tables 5.1 and 5.2 below.

Table 5.1 – Primer details for ECE1 exons 1-13

Exon	Primer 5'-3' (For/Rev)	Taq	T°(C)	Size
1c	CCAGGGACTTCAGAACAACC GTCTCGGCAAGGTCTGGA	HotStart	61	710
1bd	AAGAGGCGGGGTAGGTAGG CTCGGGTCTTGGGGTCTC	HotStart	61	595
1	GACACCCGAGACCGAGAC CTGCTAGGGGCTTCAGTTTG	GC Rich	58	374
2	AGGTGAGGGGAGACTGGTG GGAGTCCGTGCTGCATTT	HotStart	63	322
3	CCGGGTCTTGGTCTATCTCA GCTGAATCTGTGGGAACCAG	HotStart	61	454
4	TCTGCCAAGCTCCTCCTTTA CCGTCCAGTTCCAAATCTGT	HotStart	61	451
5	CGCACACTCGCTCTCTCTC CACTCCGACAGGCAGATGT	HotStart	63	405
6	CTGGAAATAACCCAGGCTCA CAGGGTCACAGAGCAAGTCA	HotStart	61	383
7	GGCTCCCCTAGCTTCAAAGA CTCTGTCCAGCCCTTCTGAG	HotStart	61	406
8	GTGGACTGGGCATAACAACC CCACCAACTCTCGTCCAGTG	HotStart	61	401
9	AGAGGGCTGTCTGTGAGAGG TGCATCCTCATTCCATTTCA	HotStart	63	423
10	TTGGCATAAGCACTCCTTCC TAGGGAGCAGTAACCCACCA	HotStart	62	399
11	GCCACAACAAGTGTGACCTC TGTGAAGTCCCAGCACAGAG	HotStart	61	388
12	AAAATTAACCGAGCGTGGTG GGTGCCTGTTGCATTTACCT	HotStart	61	414
13	CCTTTTCTAAGGGGGCATTT GGATGGGAGTCAGGGAAGAC	HotStart	56	366

Primer sequences for exons 1-13 of ECE1. Also detailed are the optimal annealing temperatures (T), the taq enzyme used and the size of the fragment produced.

Table 5.2 Primer details for ECE1 exons 14-19

Exon	Primer 5'-3' (For/Rev)	Taq	T°(C)	Size
14	CACAGTGAGGGTGCAATGAA	HotStart	61	354
	AAGGCCAAGAGAAGAGCTGA			
15	GGAAAAGAATTGGGGTAGGG	HotStart	63	352
	CTAGGCGACAGAACGAGACC			
16	CACGGAGATTACAGGCATGA	HotStart	63	389
	CGCAATGACTGAAGAGGACA			
17	CTCTACGGACAGGCATCTGG	HotStart	60	402
	CACTCACAGGGACCTCGTCT			
18	TCTGGGCTCTTGAAACATCA	HotStart	60	398
	AGGCTGGAGCAGAAGGTGTA			
19	ACGTTGAGAGCCAACACCAT	HotStart	58	468
	TCCCAAAGTGCTGGGATTAC			

Primer sequences for exons 1-13 of ECE1. Also detailed are the optimal annealing temperatures (T), the taq enzyme used and the size of the fragment produced in the amplification.

The fragments spanning exons 1d and 1c and their respective promoters needed to be larger than 500bp in order to have annealing temperatures less than 70°C as promoter regions are very GC rich.

5.2.2 DHPLC and Sequencing

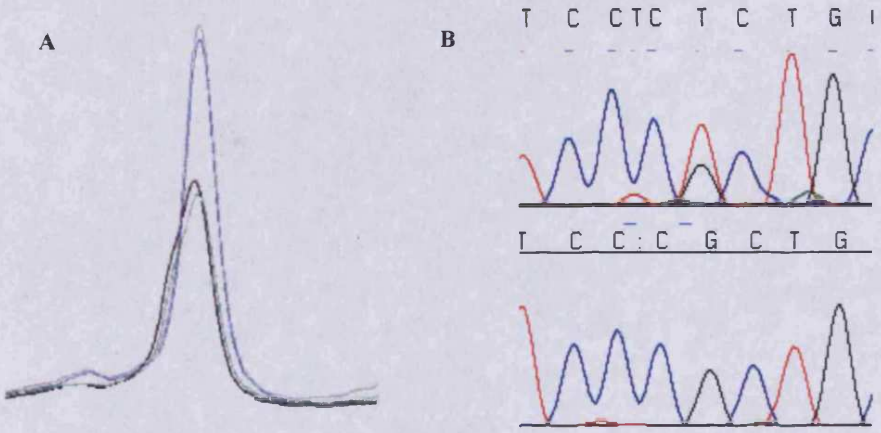
The same screening set was used for polymorphism detection as the one detailed in 4.2.1. All of the fragments were amplified using all 14 samples in 24µl reactions and then denatured and reannealed as detailed in section 2.1.3. Melt profiles for the fragments were determined (section 2.1.3).

Eight of the 21 fragments showed a significant shift. Two fragments could not be analysed by DHPLC, 1bd and 1c, despite repeated attempts and redesigning of the primers and were not studied. In order to characterise any polymorphisms, two samples of each trace pattern were sequenced using BigDye as detailed in section 2.1.5. Each of the fragments that showed a shift will now be discussed in turn.

5.2.3 Exon 1

The DHPLC traces of four samples are shown below along with examples of the sequencing (Figure 5.4).

Figure 5.4 DHPLC and Sequencing results for *ECE1* exon 1



A – DHPLC traces for four samples of exon one fragment, with black and green traces showing deviation from homozygote pattern. B – sequencing results, showing heterozygote (above) and homozygote (below) for A/C polymorphism (sequence of reverse strand shown).

Sequencing revealed a C/A polymorphism 20bp upstream of exon 1b. DNAStar was used to design an RFLP assay to genotype the SNP in the UK2 case-control sample. *SacII* was found to cut once in the C allele which was abolished in the A allele. The fragment was digested as detailed in section 2.1.7 for 16 hours to ensure complete digestion. The digests failed, even in control DNA and it was decided to try an automated genotyping technique on pooled samples. A new primer was designed for use with the SNaPshot system of genotyping (5'-CATCCCATCCCGGCCACCCGGGC-3') and ordered from Sigma-Genosys. Despite repeated attempts the genotyping of the *ECE1* exon 1 polymorphism could not be carried out which is explained in more detail in section 4.3.

5.2.4 Exon 8

The DHPLC trace for three samples from the screening set is shown below (Figure 5.5). The sequencing results were not available to include here as they were lost.

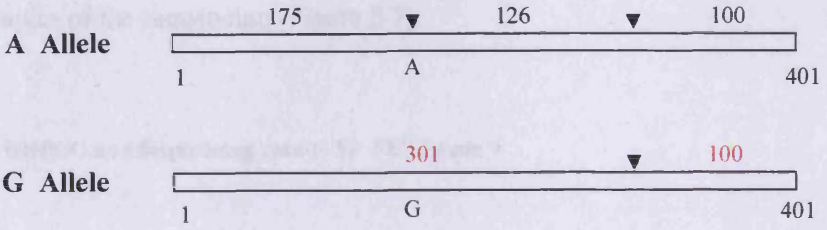
Figure 5.5 DHPLC results for *ECE1* exon 8

DHPLC traces of three samples from the LOAD screening set with the blue sample showing a different pattern.

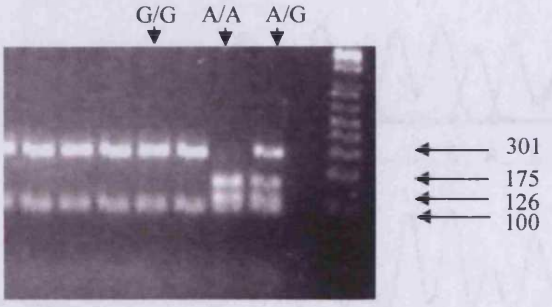
Sequencing revealed an A/G polymorphism 15bp downstream of exon 8. Analysis with DNAStar showed that *PvuII* cut twice in the A allele but one of these sites was abolished in the G allele. An RFLP assay was carried out on the MRC-LOAD sample with digests being carried out for 16 hours to ensure completion. Figure 5.6 on the following page displays the position of the cut sites within the fragment and examples of the different genotypes analysed on an agarose gel, supplemented with MetaPhor agarose. The results of genotyping in the case-control sample and the analysis are shown in Table 5.4 on the following page.

Figure 5.6 – Exon 8 RFLP Assay

A)



B)



A) Diagrammatic representation of *PvuII* cut sites within the ECE1 exon 8 fragment for the different alleles. B) Digested samples taken from the UK2 case-control sample analysed on a 2.2% agarose gel supplemented with Metaphor agarose. Genotypes and fragment sizes are shown.

Table 5.4 Results of Exon 17 Case-control analysis

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 8	iA/G @ +15	Case	142 (0.82)	29 (0.17)	3 (0.01)	**0.17** (3.59)	0.14 (2.18)
		Control	118 (0.74)	40 (0.25)	2 (0.01)		

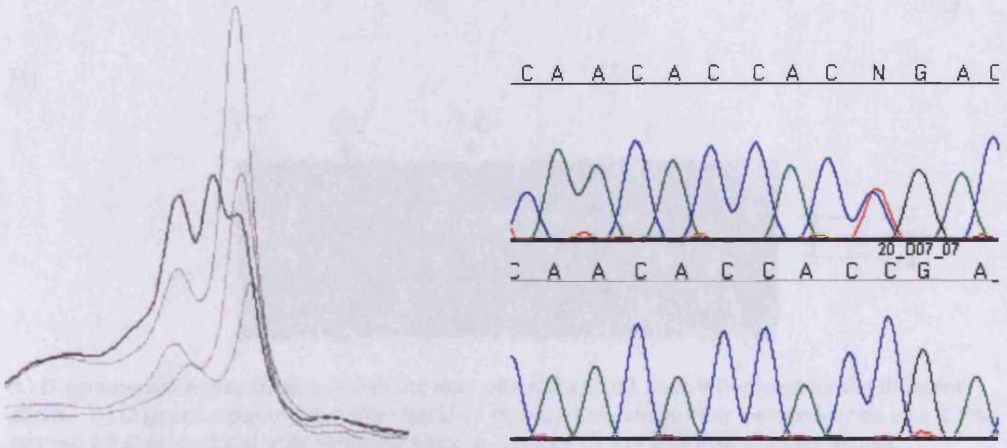
Results of ECE exon 8 SNP genotyping in the UK2 case-control sample. Position of polymorphism from the nearest exon is shown. Genotype numbers are given along with the p-value and chi-square value for genotypic and allelic association. Frequencies shown in parenthesis beneath genotype counts. Genotype codes are as follows; 1 1 – (G/G), 1 2 – (A/G), 2 2 – (A/A).

Both case and control samples were found to be in Hardy-Weinberg equilibrium, p-values of 0.30 and 0.49 respectively. There was no significant difference between the frequencies in the cases and controls either genotypically or allelically.

5.2.5 Exon 9

The DHPLC trace for four samples from the screening set are shown below along with examples of the sequencing (Figure 5.7).

Figure 5.7 DHPLC and Sequencing results for *ECE1* exon 9

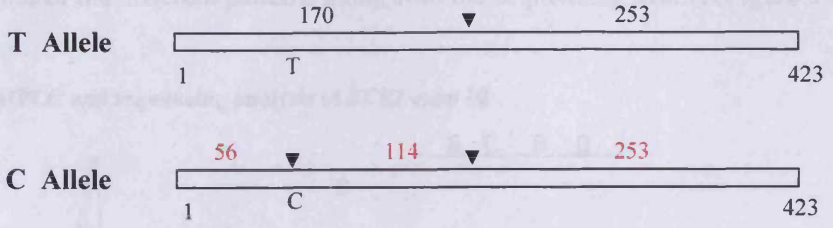


Left – DHPLC traces of three samples from the LOAD screening set with the black sample showing a different pattern. *Right* – sequencing results of two individuals from the screening set with a heterozygote C/T above and a homozygote for the C allele below.

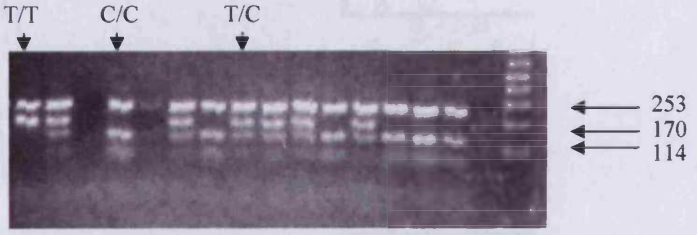
Sequencing revealed a polymorphism within exon 9. This is a synonymous change, retaining the threonine residue at position 385 of the b isoform. Analysis with DNAStar revealed a *BslI* cut site is created by the polymorphism. The positions of the cut sites within the fragment are shown in Figure 5.8 on the following page. Digests were carried out for 16 hours with the appropriate buffer and run out on a 2% agarose gel. Examples of the different genotypes are shown in Figure 5.8. Table 5.5 shows the results of genotyping the polymorphism in the UK2 case-control sample and the statistical analysis.

Figure 5.8 – Exon 9 RFLP Assay

A)



B)



A) Diagrammatic representation of *BslI* cut sites within the ECE1 exon 9 fragment for the different alleles. B) Digested samples taken from the UK2 case-control sample after electrophoresis on a 2.5% agarose gel supplemented with Metaphor agarose. Genotypes and fragment sizes are shown.

Table 5.5 *ECE1* exon 9 SNP genotyping results

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 9	c/t @ 1155	Case	10 (0.06)	71 (0.43)	83 (0.51)	**0.20** (3.23)	0.35 (0.86)
	Thr→Thr @ 385	Control	12 (0.08)	52 (0.34)	91 (0.59)		

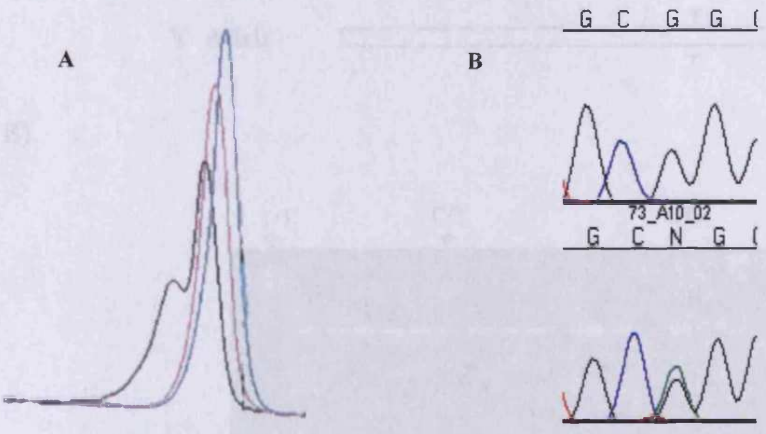
Results of ECE exon 9 SNP genotyping in the UK2 case-control sample. Position of polymorphism within cDNA shown, numbered from the start of translation, and within the polypeptide. Genotype numbers are given along with the p-value and chi-square value for genotypic and allelic association. Frequencies are shown in parenthesis beneath the genotype counts. Genotype codes are as follows; 1 1 – (T/T), 1 2 – (T/C), 2 2 – (C/C).

No significant association was found between the exon 9 polymorphism and LOAD in our sample, genotypically or allelically. Both the cases and the controls conformed to the Hardy-Weinberg equilibrium with p-values of 0.31 and 0.24 respectively.

5.2.6 Exon 10

The traces of three different samples from the LOAD screening set are shown below with examples of the different patterns along with the sequencing results (Figure 5.9).

Figure 5.9 DHPLC and sequencing analysis of *ECE1* exon 10

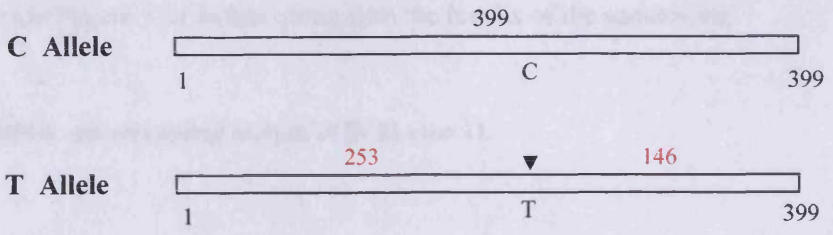


A – DHPLC analysis of three individuals from the LOAD screening set for ECE1 exon 10 with the black sample showing a different pattern. B – sequencing of two samples from the screening set with the homozygote C above and the heterozygote C/T below (reverse sequence shown).

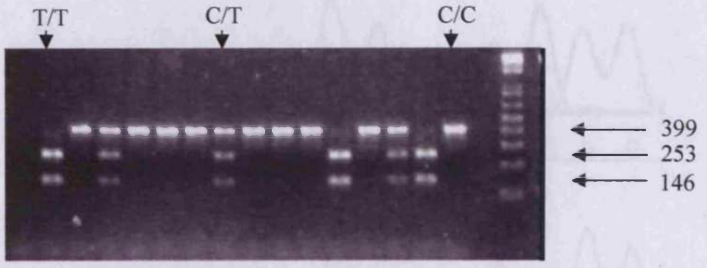
Automated sequencing revealed a C/T polymorphism 5 base pairs upstream of exon 10 of the ECE1 gene. Analysis of the fragment with for a RFLP assay using DNAStar revealed the SNP introduced a *PstI* cut site. The assay was tested on a random selection of samples from the screening set by digesting for 16 hours at the recommended conditions; then carried out on the entire UK2 sample. Figure 5.10 on the next page shows the position of the *PstI* cut site in the fragment along with examples of the different genotypes analysed on an agarose gel. The results of analysing the polymorphism in the UK2 case-control sample are shown in Table 5.6 on the following page.

Figure 5.10 *ECE1* exon 10 RFLP assay

A)



B)



A) Diagrammatic representation of *PstI* cut sites within the ECE1 exon 10 fragment for the different alleles. B) Samples taken from the UK2 case-control sample digested with *PstI* for 16 hours and analysed on a 2.2% agarose gel. Genotypes and fragment sizes are shown.

Table 5.6 Results of ECE1 exon 10 SNP genotyping

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 10	iC/T @ -5	Case	81 (0.47)	76 (0.45)	14 (0.08)	**0.19** (3.30)	0.27 (1.24)
		Control	92 (0.56)	57 (0.35)	15 (0.09)		

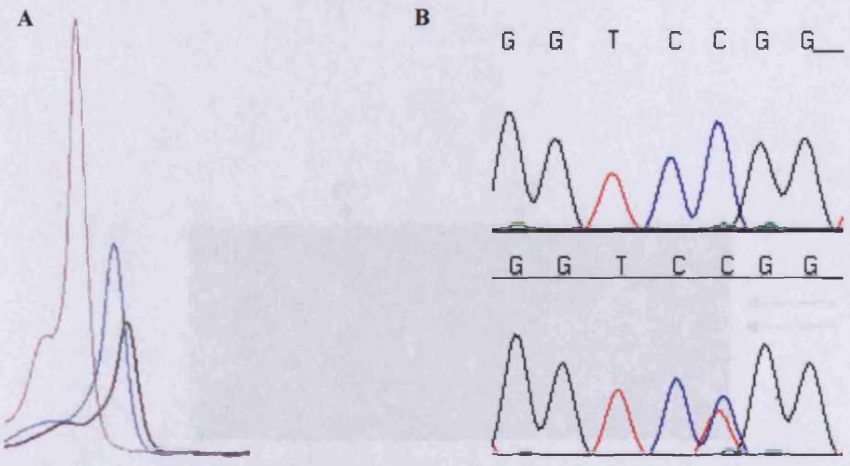
Results of ECE exon 10 SNP genotyping in the UK2 case-control sample. Position of polymorphism in relation to the nearest exon shown. Genotype numbers are given along with the p-value and chi-square value for genotypic and allelic association. Frequencies are shown in parenthesis beneath the genotype counts. Genotype codes are as follows; 1 1 – (C/C), 1 2 – (C/T), 2 2 – (T/T).

Neither genotypic nor allelic frequencies showed association with LOAD in the case control sample. Both samples conformed to the Hardy-Weinberg equilibrium with p-values of 0.51 and 0.17 for the cases and the controls respectively.

5.2.7 Exon 11

The traces of three individuals from the LOAD screening set for exon 11 of the ECE1 gene are shown in Figure 5.11 below along with the results of the sequencing.

Figure 5.11 DHPLC and sequencing analysis of ECE1 exon 11.

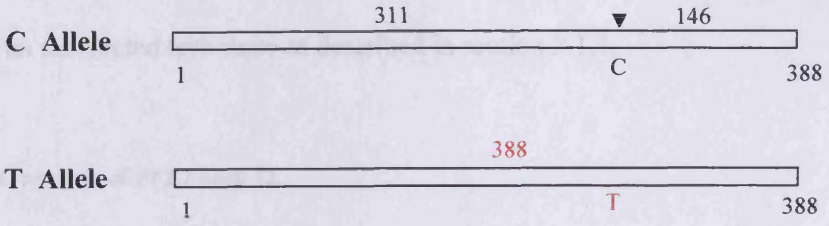


A – DHPLC analysis of three individuals from the LOAD screening set for ECE1 exon 11 with the black sample showing a different pattern. B – sequencing results from two individuals from the screening set with the heterozygote C/T below and the homozygote for the C allele above (see section 2.1.5).

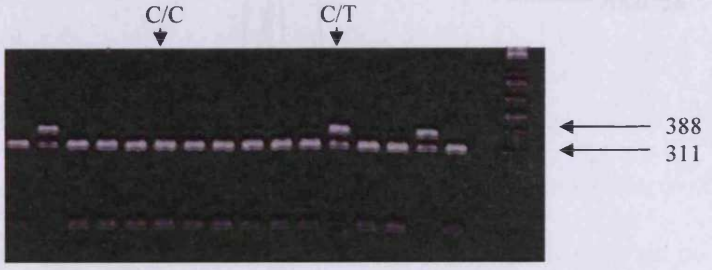
Sequencing revealed an intronic C/T polymorphism 150bp upstream of exon 11 of *ECE1*. Again it was decided to carry out individual genotyping of this polymorphism in the UK2 sample. An RFLP assay was designed as detailed in section 2.1.6 using DNAStar. A natural *MspI* cut site is abolished in the presence of the T allele. The enzyme was tested by digesting for 16 hours at the recommended conditions to ensure it worked in the PCR buffer. Figure 5.12 on the following page shows the position of the cut site within the fragment along with several examples digested and analysed on an agarose gel. The RFLP assay was performed on the whole UK2 sample and the results are shown below in Table 5.7.

Figure 5.12 *ECE1* exon 11 RFLP assay

A)



B)



A) Diagrammatic representation of *MspI* cut sites within the *ECE1* exon 11 fragment for the different alleles. B) Samples taken from the MRC LOAD case-control sample digested with *MspI* for 16 hours and analysed on a 2.5% agarose gel supplemented with Metaphor agarose. Genotypes and fragment sizes are shown.

Table 5.7 Results of *ECE1* exon 11 SNP genotyping

			Genotype			p-value ($\chi 2$)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 11	iC/T @ -150	Case	-	17 (0.1)	153 (0.9)	**0.35** (0.32)	0.58 (0.30)
		Control	-	14 (0.09)	156 (0.91)		

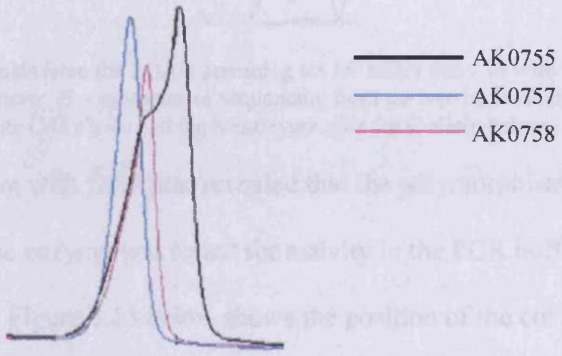
Results of ECE1 exon 11 SNP genotyping in the UK2 case-control sample. Position of polymorphism in relation to the nearest exon shown. Genotype numbers are given, with the corresponding frequency in parenthesis below, along with the p-value and chi-square value for genotypic and allelic association. Genotype codes are as follows; 1 1 – (C/C), 1 2 – (C/T), 2 2 – (T/T).

Both the cases and the controls conformed to the Hardy-Weinberg equilibrium, with p-values of 0.49 and 0.58 respectively. No association was found between the exon 11 SNP and LOAD.

5.2.8 Exon 12

DHPLC analysis of the fragment containing exon 12 showed a shift in the trace patterns as seen below in Figure 5.13. Two samples of each trace pattern were sequenced using an automated technique as described in section 2.1.5.

Figure 5.13 DHPLC analysis of *ECE1* exon 12.



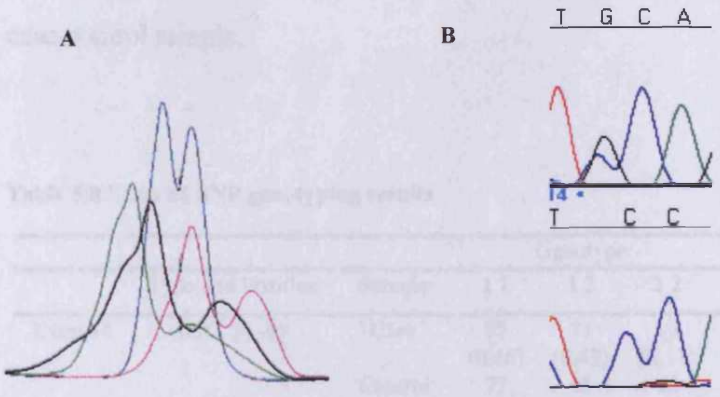
Examples of DHPLC traces for the *ECE1* exon 12 amplification fragment. Black trace (AK0755) shows different pattern to the other two samples.

Despite repeated sequencing, no polymorphisms could be detected within the fragment, possibly due to the position of the polymorphism or an error in the DHPLC, which is described in more detail in Chapter 4.3.

5.2.9 Exon 14

The traces of four individuals from the LOAD screening set for exon 14 of the ECE1 gene are shown in Figure 5.14 on the following page, along with the results of the sequencing. Automated sequencing revealed a C/G polymorphism 99bp upstream of exon 14.

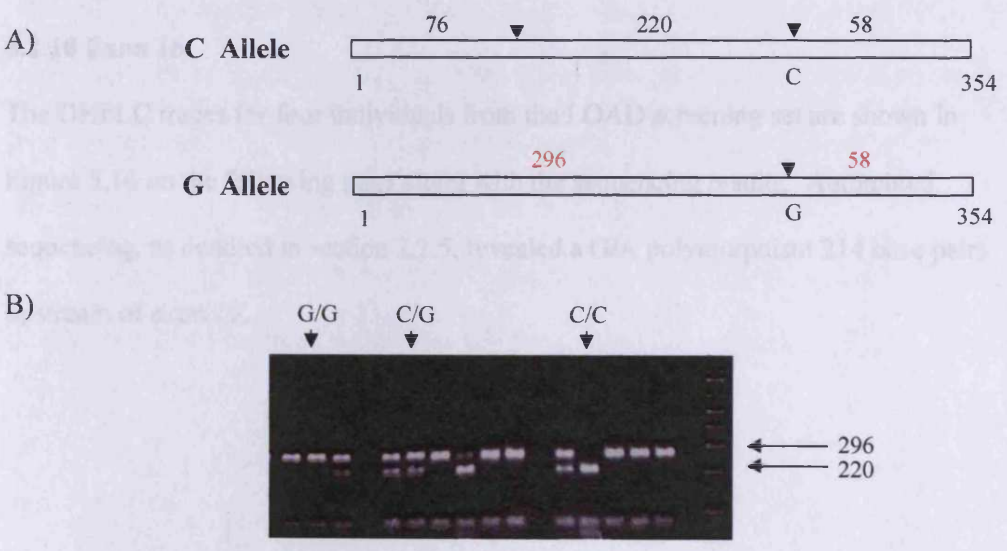
Figure 5.14 DHPLC and sequencing analysis of *ECE1* exon 14.



A – DHPLC analysis of four individuals from the LOAD screening set for ECE1 exon 14 with the black sample showing a different pattern. B – examples of sequencing from the two individuals from the screening set with the heterozygote C/G above and the homozygote for the C allele below.

Analysis of the fragment with DNAStar revealed that the polymorphism introduced a *BstnI* cut site. The enzyme was tested for activity in the PCR buffer to ensure it digested completely. Figure 5.15 below shows the position of the cut sites within the fragment, along with examples of the different genotypes analysed on an agarose gel.

Figure 5.15 *ECE1* exon 14 RFLP assay



A) Diagrammatic representation of *BstNI* cut sites within the *ECE1* exon 14 fragment for the different alleles. B) Samples taken from the UK2 case-control sample digested with *BstNI* for 16 hours and analysed on a 2.5% agarose gel supplemented with Metaphor agarose. Genotypes and fragment sizes are shown.

Table 5.8 below shows the results of genotyping this polymorphism in the entire UK2 case-control sample.

Table 5.8 Exon 14 SNP genotyping results

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 14	iC/G @ -99	Case	77 (0.46)	71 (0.43)	19 (0.11)	**0.87** (0.27)	0.60 (0.28)
		Control	77 (0.49)	65 (0.41)	16 (0.10)		

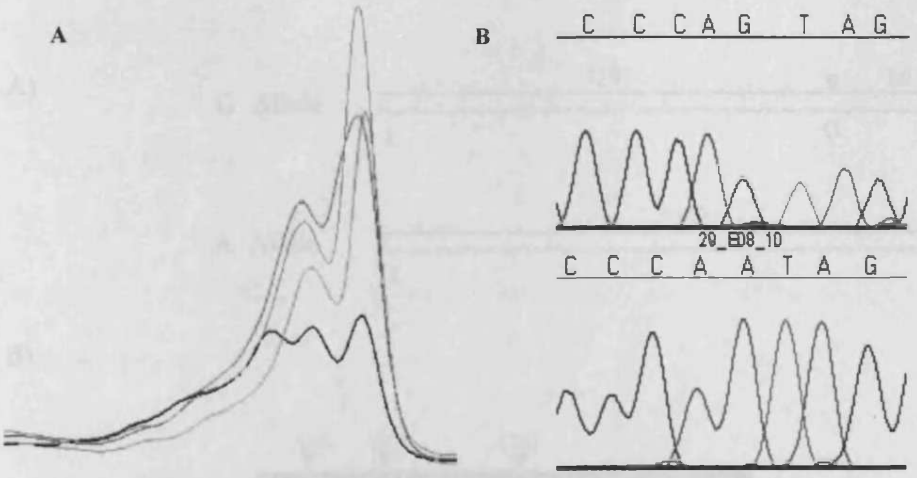
Results of the *ECE1* exon 14 SNP genotyping in the UK2 case-control sample. Position of polymorphism in relation to the nearest exon shown. Genotype numbers are given along with the p-value and chi-square value for genotypic and allelic association. Genotype codes are as follows; 1 1 – (G/G), 1 2 – (C/G), 2 2 – (C/C).

No association was found with LOAD and the exon 14 polymorphism in the case control sample, genotypically nor allelically. Both populations conformed to the Hardy-Weinberg equilibrium, which was tested for as detailed in section 2.1.10, with p-values of 0.67 and 0.68 for the cases and the controls respectively.

5.2.10 Exon 16

The DHPLC traces for four individuals from the LOAD screening set are shown in Figure 5.16 on the following page along with the sequencing results. Automated sequencing, as detailed in section 2.1.5, revealed a G/A polymorphism 214 base pairs upstream of exon 16.

Figure 5.16 *ECE1* exon 16 DHPLC and sequencing analysis

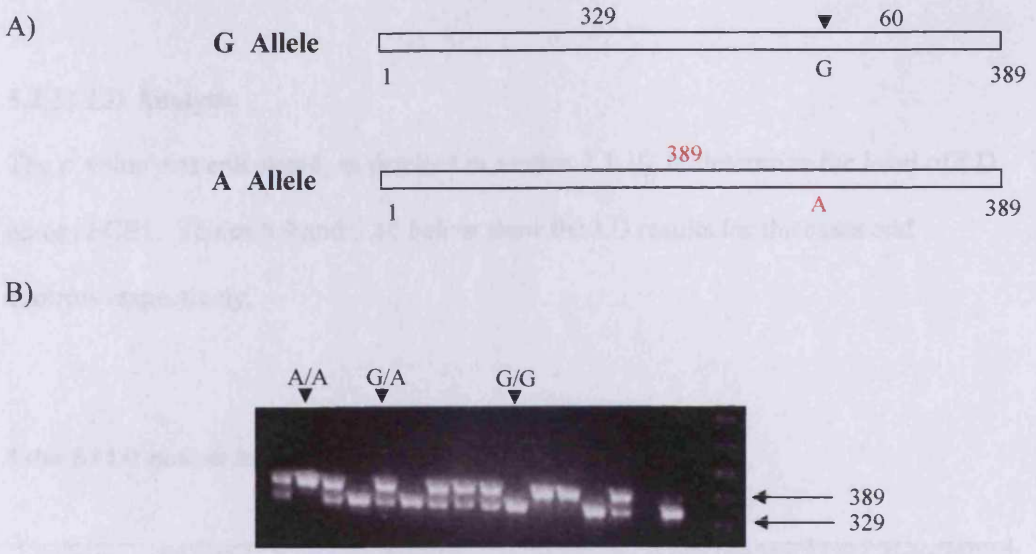


A – DHPLC analysis of four individuals from the LOAD screening set for *ECE1* exon 16 with the black and blue samples showing different patterns. B –sequencing of two individuals from the screening set with the G homozygote above and the A homozygote below.

The sequence of the fragment was analysed using DNAStar for the possibility of genotyping using a restriction fragment length assay. DNAStar revealed that the shift to an A nucleotide abolished a natural *BsrI* cut site. The enzyme was purchased and tested by digestion for 16 hours to ensure that *BsrI* worked within the PCR buffer. Figure 5.17 on the following page shows a diagrammatic version of the amplified fragments with the positions of the cut sites in the different alleles. Also shown are several samples from the case-control sample amplified, digested and analysed by electrophoresis on an agarose gel to illustrate the different genotypes.

Table 5.9 on the following page shows the results of genotyping the UK2 case control sample for the exon 16 SNP by RFLP assay and the statistical analysis.

Figure 5.17 *ECE1* exon 16 RFLP assay



A) Diagrammatic representation of *BsrI* cut sites within the *ECE1* exon 16 fragment for the different alleles. B) Samples taken from the UK2 case-control sample digested with *BsrI* for 16 hours and analysed on a 2.5% agarose gel supplemented with Metaphor agarose. Genotypes and fragment sizes are shown.

Table 5.8 Exon 16 SNP genotyping results

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Exon 16	iG/A @ -214	Case	44 (0.30)	72 (0.49)	31 (0.21)	**0.33** (2.25)	0.06 (2.56)
		Control	47 (0.33)	57 (0.41)	37 (0.26)		

Results of the *ECE1* exon 16 SNP genotyping in the UK2 case-control sample and statistical analysis. Position of polymorphism is shown in relation to the nearest exon. Genotype numbers are given, with frequencies in parenthesis below, along with the p-value and chi-square values for genotypic and allelic association. Genotype codes are as follows; 1 1 – (G/G), 1 2 – (G/A), 2 2 – (A/A).

No association was found between the *ECE1* exon 16 SNP and LOAD in the case-control sample genotypically. Allelically, there was a slight over representation of the G allele in the AD cases but it was not statistically significant. Both the cases

and the controls conformed to the Hardy-Weinberg equilibrium with p-values of 0.33 and 0.14 respectively.

5.2.11 LD Analysis

The r^2 value was calculated, as detailed in section 2.1.10, to determine the level of LD across ECE1. Tables 5.9 and 5.10 below show the LD results for the cases and controls respectively.

Table 5.9 LD analysis for *ECE1* in AD cases

Variant	Exon 8	Exon 9	Exon 10	Exon 11	Exon 14
Exon 9	0.00				
Exon 10	0.00	0.78			
Exon 11	0.01	0.02	0.02		
Exon 14	0.03	0.52	0.61	0.03	
Exon 16	0.02	0.32	0.34	0.07	0.48

Table 5.9 LD analysis for *ECE1* in AD controls

Variant	Exon 8	Exon 9	Exon 10	Exon 11	Exon 14
Exon 9	0.00				
Exon 10	0.00	0.82			
Exon 11	0.01	0.01	0.02		
Exon 14	0.01	0.41	0.46	0.02	
Exon 16	0.01	0.34	0.38	0.03	0.52

Calculated r^2 values after 1000 simulations for the polymorphisms spanning *ECE1* with cases shown in table 5.8 and controls in table 5.9. All values shown to two decimal places.

Only the exon 9 and 10 polymorphisms showed a high degree of LD in either population. An overview of the results presented in this chapter is shown at the end of this section.

5.2.12 Haplotype analysis

Analysis was carried out as detailed in section 2.2.10 to investigate if any haplotypes showed association with LOAD. The results of the analysis are shown in Tables 5.10 through 5.13 below and on the following page.

Table 5.10 ECE1 two marker haplotypes

Markers	p-value								
1 2	0.52	**2 3**	0.59	**3 4**	0.53	**4 5**	0.70	**5 6**	0.62
1 3	0.19	**2 4**	0.58	**3 5**	0.23	**4 6**	0.56		
1 4	0.20	**2 5**	0.44	**3 6**	0.10				
1 5	0.46	**2 6**	0.51						
1 6	0.51								

Estimated p-values, after 1000 simulations, of marker-disease haplotype analysis for two marker ECE1 haplotypes. Polymorphisms represented in table are 1) Exon eight, 2) Exon nine, 3) Exon ten, 4) Exon eleven, 5) Exon 14 and 6) Exon 16.

Table 5.11 ECE1 three marker haplotypes

Markers	p-value	Markers	p-value	Markers	p-value	Markers	p-value
1 2 3	0.56	**2 3 4**	0.79	**3 4 5**	0.46	**4 5 6**	0.57
1 2 4	0.50	**2 3 5**	0.71	**3 4 6**	0.14		
1 2 5	0.48	**2 3 6**	0.70	**3 5 6**	0.001		
1 2 6	0.78	**2 4 5**	0.55				
1 3 4	0.34	**2 4 6**	0.36				
1 3 5	0.20	**2 5 6**	0.13				
1 3 6	0.11						
1 4 5	0.49						
1 4 6	0.18						
1 5 6	0.41						

Estimated p-values, after 1000 simulations, of marker-disease haplotype analysis for three marker ECE1 haplotypes. Polymorphisms represented in table are 1) Exon eight, 2) Exon nine, 3) Exon ten, 4) Exon eleven, 5) Exon 14 and 6) Exon 16.

Table 5.12 ECE1 four marker haplotypes

Markers	p-value	Markers	p-value	Markers	p-value
1 2 3 4	0.48	**2 3 4 5**	0.81	**3 4 5 6**	0.03
1 2 3 5	0.63	**2 3 4 6**	0.55		
1 2 3 6	0.81	**2 3 5 6**	0.11		
1 2 4 5	0.51	**2 4 5 6**	0.17		
1 2 4 6	0.39				
1 2 5 6	0.33				
1 3 4 5	0.30				
1 3 4 6	0.11				
1 3 5 6	0.02				
1 4 5 6	0.35				

Table 5.13 ECE1 five marker haplotypes

Markers	p-value
1 2 3 4 5	0.68
1 2 3 4 6	0.44
1 2 3 5 6	0.15
1 2 4 5 6	0.17
1 3 4 5 6	0.04
2 3 4 5 6	0.18

Estimated p-values, after 1000 simulations, of marker-disease haplotype analysis for four (table 5.12) and five (table 5.13) marker ECE1 haplotypes. Polymorphisms represented in table are 1) Exon eight, 2) Exon nine, 3) Exon ten, 4) Exon eleven, 5) Exon 14 and 6) Exon 16.

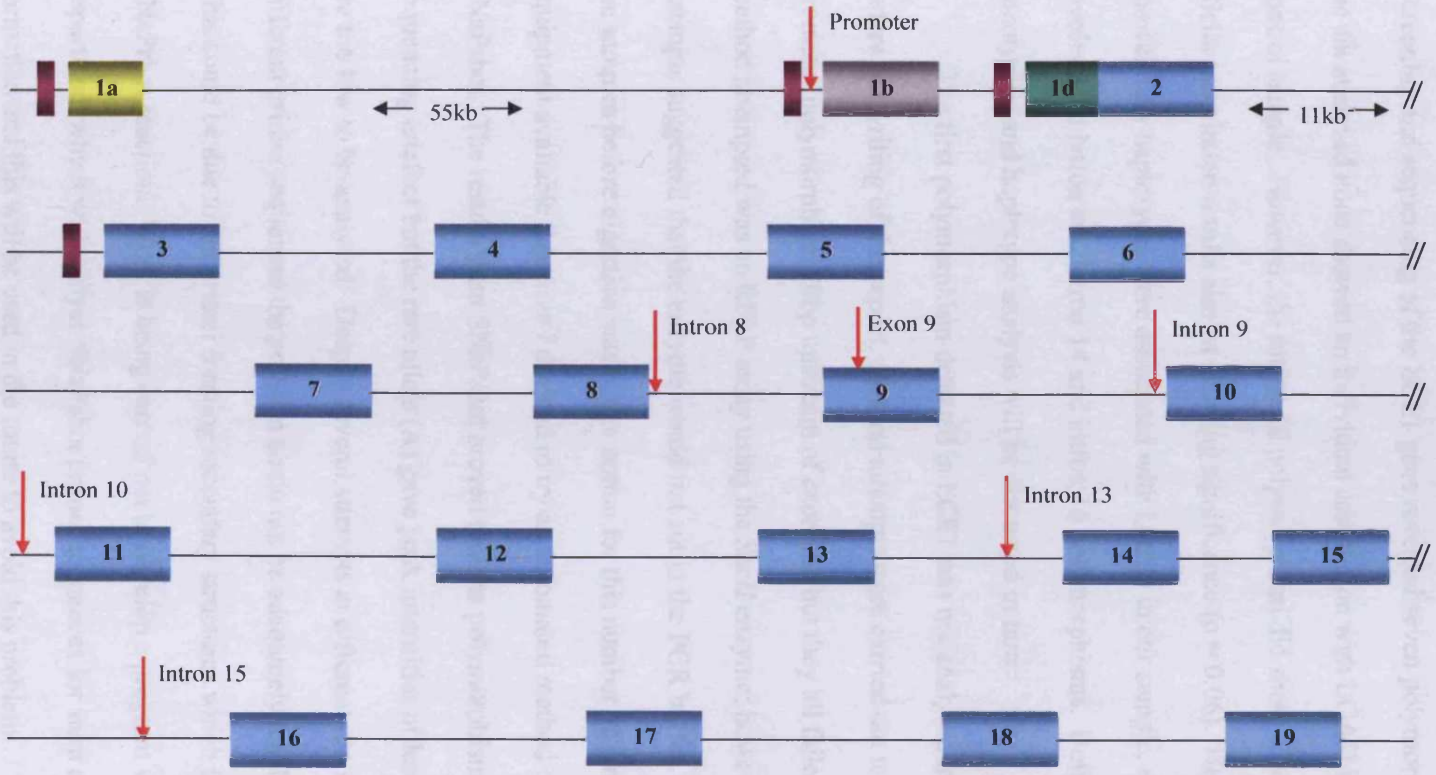
Four haplotypes gave a positive result for association with LOAD with the three marker haplotype around the exon ten, 14 and 16 polymorphisms giving a very significant result.

Overview of ECE1 Genotyping Results

Position	Type	Sample	1 1	1 2	2 2	Assn	Allelic	HW	A1	A2
Promoter	iA/C @ -20	Case	-	-	-					
		Control	-	-	-					
Intron 8	iA/G @ +15	Case	142	29	3	**0.17**	0.14	0.30	313	35
		Control	118	40	2			0.49	276	44
Exon 9	C/T @ 1155	Case	10	71	83	**0.20**	0.35	0.31	91	237
	Thr→Thr @ 385	Control	12	52	91			0.24	76	234
Intron 9	IC/T @ -5	Case	81	76	14	**0.19**	0.27	0.51	238	104
		Control	92	57	15			0.17	241	87
Intron 10	iC/T @ -150	Case	-	17	153	**0.35**	0.58	0.49	17	323
		Control	-	14	156			0.58	14	326
Intron 13	iC/G @ -99	Case	77	71	19	**0.87**	0.60	0.67	225	109
		Control	77	65	16			0.68	219	97
Intron 15	iG/A @ -214	Case	44	72	31	**0.33**	0.06	0.33	160	134
		Control	47	57	37			0.14	151	164

Summary of *ECE1* individual and pooled genotyping in the UK2 case-control sample. The position of polymorphisms, either within the polypeptide or from nearest exon is shown along with genotype and allele counts. Results of association shown as p-values for both allelic and genotypic analysis, and for concordance with the Hardy Weinberg equilibrium.

***ECE1* gene and SNP position overview**



Genomic position of polymorphisms (red arrows) analysed in this chapter within *ECE1* to illustrate level of gene coverage. The exons are shown as blue rectangles and alternatively spliced first exons shown in yellow, grey and green. Position of promoters shown as purple rectangles. Not drawn to scale

5.3 Discussion

Screening and sequencing of the ECE1 gene revealed seven polymorphisms and from the six analysed none showed an individual association with LOAD in our UK2 case-control sample. However, the intron 16 polymorphism did show a trend with the allelic association results almost showing significance ($p = 0.06$). Haplotype analysis showed four haplotypes were associated with LOAD in our sample, all of which involved the intron ten, intron 14 and intron 16 polymorphisms. Both the individual genotyping and haplotype analysis will be discussed in turn.

The first polymorphism detected in ECE1 was not analysed in the UK2 sample before the writing of this report. Several attempts were carried out to genotype the promoter polymorphism 20bp upstream of exon one but they all failed. The first method attempted was an RFLP assay using the *SacII* enzyme; however repeated attempts suggested that the enzyme would not cut in the PCR buffer. Purification of the samples before digestion was not an option for this number of samples with the equipment available therefore I decided to try an automated method of genotyping, SNaPshot. The results from SNaPshot proved that the polymorphism was not a sequencing artefact but the rare allele (A) gave peak intensities of less than 100 which are too low to be analysed. Despite several attempts at different run settings and different primer sequences the problem could not be adequately resolved in time. This could be due to the primer forming secondary structures which inhibits the SNaPshot reaction. Work is being carried out to develop a program within the department which will analyse SNaPshot primer sequences for stem and loop formation and this will be used in the future to avoid this problem.

All of the other polymorphisms detected in this gene were genotyped with no difficulties by a RFLP assay. Only one exonic polymorphism, in exon nine, was found but it did not alter the amino acid sequence of the protein. The intron ten polymorphism lies close enough to exon ten to have a possible effect on its splicing. None of the other SNPs have an obvious effect on the gene however it is the intron 16 which gives the most interesting result with a p-value of 0.06 for its allelic association. This polymorphism has a slight over representation of the G allele in the AD patients. Lying over 200 base pairs from the nearest exon it is difficult to give a functional reason why it could be related to AD; however it could lie in a previously undescribed regulatory region or possibly be involved in the formation of a secondary structure within the mRNA. However, after adjusting for multiple testing the result is nowhere near statistically significant. This is discussed in further detail in Chapter 7. One other fragment needs to be mentioned in this section. The DHPLC results for the exon twelve fragment suggested that a polymorphism was present. Nothing could be detected by sequencing suggesting it was either a DHPLC artefact or, more likely, that the polymorphism lies very close to one of the primers and is therefore in a region where the sequencing is of a lower quality. Time constraints meant that alternative primers were not designed and I concentrated on the SNPs that had already been discovered.

As reviewed by Daly and Day (2001) many case-control studies give contradictory results and this could be down to poor study design. One of the factors involved is the testing of only a single polymorphism within a gene which is of dubious functional relevance. The study of several polymorphisms by haplotype analysis can give a much clearer account of what is truly occurring in the disease state. Analysis of *ECE1* showed four haplotypes which were associated with LOAD.

All four contained the intron ten, intron 14 and intron 16 polymorphisms, with the most significant association found in the three marker haplotype containing only these SNPs (OR 1.73, confidence interval 1.13-2.67). The three marker haplotype showed a significant shift towards the 2 2 2 (T/C/A) genotype with a rise in frequency from 0.20 to 0.27. The two marker haplotype containing the intron ten and 16 polymorphisms shows a trend towards association suggesting that the majority of the effect seen in the three marker haplotype result is generated by these two markers. As all of the remaining associated haplotypes have higher p-values than the positive three marker haplotype they can be considered to be positive purely because they contain the same markers and are therefore not of direct interest. As was mentioned previously, the intron ten SNP lies close enough to the exon for it to have an effect on splicing. The intron 16 polymorphism could lie in partial LD with an unknown/undetected functional polymorphism; explaining it's near significance at the allelic level. The same could also be said of the intron 14 polymorphism. This haplotype needs to be genotyped in a replication sample to see if it remains significant, unfortunately no replication sample was available for use before the completion of this study. The NI1 sample used in Chapter 3 had been used and degraded to a point where the population numbers were too low to be useful. However, this haplotype did lose significance after adjusting for multiple testing which is discussed in more detail in Chapter 7.

Other polymorphisms do exist in this gene; since completing this work Funke-Kaiser *et al.* (2003) reported, amongst others, a C/T transition in exon 17 which alters the protein sequence (A677V). This non-conservative change lies close enough to the intron 16 polymorphism to be of interest. The DHPLC traces for exon 17 did not shown an obvious shift in this study. It is possible that DHPLC failed to detect the

polymorphism or the polymorphism was not present in our screening set. The work of Funke-Kaiser *et al.* (2003) also reported a functional effect for a polymorphism within the ECE-1b promoter region, increasing its activity. This polymorphism, along with another promoter SNP, was also shown to be associated with blood pressure in hypertensive women. As PCRs could not be optimised for the alternatively spliced exons these polymorphisms were missed in the initial screening. If the polymorphisms in the study by Funke-Kaiser are a risk factor for hypertension and cardiovascular disease they might be found at lower frequencies in an older population which could also explain why they were missed in this study. These polymorphisms were not analysed because of time-constraints but should be genotyped in the MRC-LOAD case-control sample considering the results obtained in this study.

As mentioned in section 5.1, ECE1 is a good functional candidate for AD due to its role in both the cardiovascular and inflammatory systems. Other studies have given further possible roles for ECE1 in LOAD. Eckman *et al.* reports that ECE1 cleaves the amyloid beta peptide *in vitro* (2001) and that βA levels are increased in *ECE1* knockout mice (2002). Therefore, ECE1 can be added to a growing list of enzymes, including ACE, IDE and NEP, which have been shown to degrade Aβ either *in vivo* or *in vitro*. βA might therefore require several different enzymes to ensure adequate clearing of the peptide and prevent aggregation or these similar enzymes have a wide substrate range and their cleaving of βA is merely a side effect.

Endothelin-1 has recently been implicated in the phenomenon known as the 'French paradox'. It has been noted that coronary heart disease related deaths occur much less frequently in France compared with the United Kingdom, despite both countries having similar levels of saturated fat in the average diet. The main

difference in diets between the two is that France has a much higher consumption of alcohol and in particular wine. Work by Corder *et al.* (2001) shows that polyphenol extracts from red wine decrease ET-1 synthesis. The mechanism involved was not described but alterations were seen in tyrosine-kinase signalling in endothelial cells after treatment. The polyphenolic compounds found in red wine do resemble inhibitors of the tyrosine-kinase family inhibitors structurally. Other studies have confirmed this effect (Khan *et al.* 2002) and shown that red wine extracts also induce nitric oxide synthase (iNOS) and COX-2 (Diebolt *et al.* 2001). This reduction in ET-1 could be a result of a decrease in ECE1 activity. The tyrosine-kinase signalling pathway, via Raf-MEK1/2, is involved in regulating ECE1 expression, along with the RAS-RAF and PKC pathways (Orzechowski *et al.* 2001). Thrombin is a regulator of both ECE1 and eNOS via the MEK/MAP and the Rho/ROCK pathways respectively (Eto *et al.* 2001). Recent work by MacLeod *et al.* (2002) suggests that the kinase involved is CK-I or a similar kinase.

This evidence could be of particular interest in AD. The possible role of oxidative free radicals in AD pathology has been mentioned in section 1.5.4. Red wine has long been known as a free radical scavenger and Bastianetto *et al.* (2000) shows that red wine extracts can protect hippocampal neurons against free radical insult. Work by Jang and Surh (2003) has also shown that a polyphenolic compound found in black grape skin can protect neurons against beta-amyloid induced cell death. Whether this protective effect is true remains to be seen and also further investigation is required to see if the effect comes from free radical scavenging or alterations to the cardiovascular system through ECE1 or a combination of both.

The cardiovascular system, as discussed in section 1.6, is complicated and regulated by a large number of genes. It is therefore conceivable that a large number

of common polymorphisms could combine their effects on the system. This could also give an explanation why polymorphisms in cardiovascular genes do not give consistent results in association studies as it may require alterations in multiple genes to give a pathogenic effect. In particular, as mentioned previously, several enzymes now have suggested roles in the degredation of βA. It is possible that all of these enzymes provide a redundancy and that alteration in the activity of one enzyme would not cause a reduction in peptide clearance. However, several common polymorphisms that could reduce the activity of ECE, ACE, IDE and the α-secretase might be enough to cause a pathological cascade. Unfortunately, in this study a different population was used in the analysis of *ACE* and *ECE1* and therefore the analysis of a possible synergistic effect cannot be investigated. If further genotyping does reveal an association with an ECE1 polymorphism it might be interesting to look at the different alleles in combination with the two common haplotypes seen in ACE (see section 3.3) and disease status.

Chapter 6

Neuroserpin

6.1 Background

Neuroserpin (Accession number NP_005016) is a serine protease of the serpin family. It was first identified in chickens as an axonally secreted protein of the root ganglion neurons (Stoeckli *et al.* 1989). Chicken neuroserpin was purified and sequenced giving a 1230 nucleotide open reading frame (ORF) (Osterwalder *et al.* 1996). The native chicken protein is a 55kDa glycoprotein formed after the cleavage of a 16 amino acid signal peptide. In 1997 Schrimpf *et al.* cloned human neuroserpin and mapped the gene, PI12, to chromosome 3q26 using fluorescence *in situ* hybridisation. Homology between human and chicken neuroserpin is 78% at the nucleotide level and 80% at the protein level. A homology of between 21 and 35% is seen with the other members of the serpin family, including protease nexin-1 (PN-1), plasminogen activator inhibitor-1 (PA-1), α_1-antichymotrypsin (ACT) and angiotensinogen (AT). It is expressed in neurons during embryogenesis and in adults, particularly in the choroid plexus, the Purkinje cells of the cerebellum and parts of the hypothalamus and the hippocampus (Hastings *et al.* 1997). Due to its axonal secretion and brain localisation, neuroserpin is thought to be involved in the regulation of neuronal plasticity and synapse formation.

Tissue culture studies show that neuroserpin is an inhibitor of tissue plasminogen activator (tPA) (Hastings *et al.* 1997; Osterwalder *et al.* 1998) and plasmin, but not other related proteinases such as thrombin (Osterwalder *et al.* 1998). The serine protease tPA, converts plasminogen into an active protease, plasmin

(Hoylaerts *et al.* 1982). Activity of tPA in neural tissue correlates with neurite outgrowth and migration (Krystosek and Seeds, 1984), suggesting a role in plasticity. tPA is also induced in the hippocampus during long-term potentiation (LTP) with knockout tPA mice showing deficiencies in Schaffer collateral and mossy fiber pathway LTP (Frey *et al.* 1996; Huang *et al.* 1996). A study by Tsirka *et al.* (1995) showed that tPA deficient mice were resistant to neuronal degeneration induced by excitotoxic damage and to seizures.

Berger *et al.* (1999) showed that depolarisation of cultured hippocampal neurons, by exposure to KCl, results in upregulation of neuroserpin mRNA. They also delineated the promoter region of the gene to a sequence of 125bp immediately 5' to exon 1. This region contains at least two *cis*-acting positive regulatory elements and a CAAT box. Previous work by Berger *et al.* (1998) showed a zif/268 binding site at the 5' end of intron 1. This is believed to be involved in the transcriptional regulation of neuroserpin as transcription of zif/268 led to complete suppression of neuroserpin activity. zif/268 is expressed highest immediately following depolarisation (Christy *et al.* 1988) and decreases to background levels over the next 24 hours. This evidence suggests that neuroserpin is closely regulated following depolarisation allowing tPA to carry out its function in learning and memory.

6.1.1 Disease Mutations

In 1999, Davis *et al.* described a new disease termed familial encephalopathy with neuroserpin inclusion bodies (FENIB). The two Caucasian families presented with cognitive decline including attention and concentration deficits, response regulation difficulties and impairment in visuospatial skills. Memory was also affected but not as severely as that in AD patients. One of the families had an age of onset

approximately 20 years younger and with more aggressive symptoms including epilepsy. Both families displayed neuronal inclusion bodies in the cerebral cortex and the substantia nigra which were composed mainly of neuroserpin.

Genetic analysis of these families revealed two mutations, S49P and S52R, with the latter being found in the more severely affected family. The disease displays an autosomal dominant mode of inheritance. As part of its inhibitory function, neuroserpin undergoes a conformational change which opens the five-stranded sheet of the molecule. These mutations alter the conformation so the opening is permanent, leading to the reactive loop of one neuroserpin molecule inserting into the opened sheet of another – a pathogenic aggregation. The serine at 52 is critical for the conformational change, explaining why the mutation at that residue leads to a more severe neurodegeneration.

6.1.2 Neuroserpin in AD

The positional evidence for neuroserpin is not as strong as that for the other genes analysed in this study. A small peak is seen in the first stage of the genome screen (Kehoe *et al.* 1999b) at 3q25 however it is not large enough to be considered 'suggestive linkage' (Lander and Kruglyak, 1995). Several lines of evidence do make neuroserpin a good functional candidate for AD. A recent study by Joseph Buxbaum (personal communication to Professor M O'Donovan) shows 150 genes that have their expression significantly altered in AD cases. One of these genes is neuroserpin, which shows a just over two-fold reduction in expression. I selected this gene for analysis from the large list due to its role in the cardiovascular system.

As mentioned previously, neuroserpin is an essential part of the cardiovascular system and is able to protect neurons from ischaemia induced apoptosis. In addition,

its role as an inhibitor of tPA means the enzyme has a direct effect in synaptic plasticity and memory formation. The possible role of the cardiovascular system in AD has already been discussed in section 1.6 and combining this with a role in memory formation and the observed transcriptional alteration makes neuroserpin a strong functional candidate for AD.

In a similar way to AD, aggregation of neuroserpin leads to a neuropathological disorder. Familial studies of this disease have lead to the description of two novel mutations. These mutations have been analysed in a small early-onset AD group. From seventeen index cases no copies of either mutation were discovered (The French Alzheimer's Disease and Fronto-Temporal Dementia Genetics Study Groups, 2000). They did report several new polymorphisms in the gene but none were found in significantly different frequencies to unaffected controls. No study of these mutations has so far been carried out on the late-onset form of AD.

6.1.3 Aim of this study

In this study I screened the gene and its promoter region for novel polymorphisms. These polymorphisms, and some of the previously described SNPs, were analysed in the MRC-LOAD case-control sample for association.

6.2 Results

6.2.1 Gene amplification

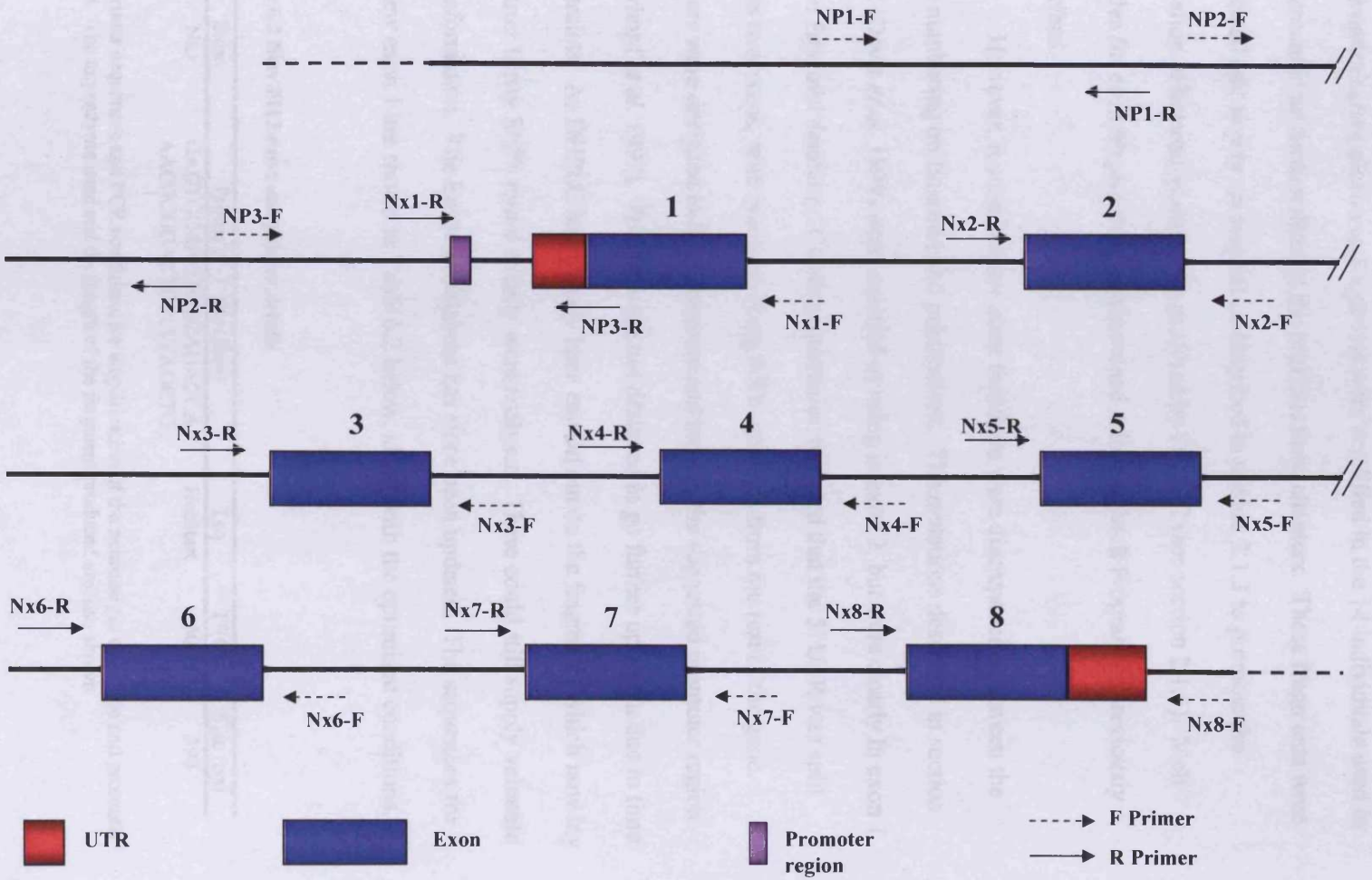
The cDNA for neuroserpin was obtained from Ensembl (ENSG00000075073) then analysed for homology to the hstg database using BLAST (NCBI) which pulled out the draft sequence of chromosome 3. This fragment was aligned with the cDNA, again using BLAST, to determine the flanking sequences for each exon. Using Primer3, primers were designed which spanned all of the exons and up to 1.5kb upstream of the translational start site to ensure complete coverage of the gene. All of the fragments were kept to sizes below 550bp for DHPLC analysis and the promoter fragments were designed with overlapping segments. The position of the primers is shown in Figure 6.1 on the following page. Conditions for the PCRs were optimised as detailed in section 2.1.1 and the primer sequences and conditions are shown in Table 6.1 below.

Table 6.1 Primers and PCR conditions for neuroserpin

Exon	Primer 5'-3' (For/Rev)	Taq	T°(C)	Size (bp)
Np1	TCACCAGGCAGTTCTCTGAA GCATCAAGGCCACATTTTCT	HotStart	60	474
Np2	AAGACAGGGCTTCTGGAACA TGAACTAGCACCACTAGGCATT	HotStart	58	540
Np3	GGGTGCAGTGACATTTTCAA ATGGCTTCCTCAGGGAAAGT	HotStart	60	510
Nx1	GGGTTTCCTAATCCTTCTATCCA CCTCCCAACATATCCTTCCA	HotStart	58	493
Nx2	GCAAAAGGAACAAGGAGGAA GGAAAGCCTGGCACTTTTC	HotStart	60	450
Nx3	GCCCAAATTAGATTGACAAGGA ATCTTCTGGTCCCCCTTGAT	HotStart	60	369
Nx4	GGGTGACACAGGAACCTCAT TGGTGATTTGTGAGATTTTGTTG	HotStart	60	362
Nx5	AACACTTTGTTCTTGTTCCAGGT GCTAGATTTATTTGGCAAGTGG	HotStart	60	296
Nx6	TAGAAAATTCCGGCTGGTTC TCCATTTAACAAAAGGGCAAA	HotStart	60	314
Nx7	GGCATAGATGGTTTTGAGGAA CCTGGAACTCCTGAAGTGCT	HotStart	60	414
Nx8	TTTGTTCACCCCCTCTTTTG TGGAGTACCTGTCTCACCTCA	HotStart	60	541

The primer sequences and PCR conditions for amplification of the neuroserpin gene exons and promoter region. The taq enzyme used and the length of the fragment produced are also shown.

Figure 6.1 Primer positions for amplification of PI12



Structure of TACR2, showing positions of primers, exons and promoter region. Primers for upstream region overlap

6.2.2 Polymorphism detection

After optimisation all eleven fragments were amplified in the 14 individuals used in the screening set as described in the previous three chapters. These fragments were denatured and slowly reannealed as described in section 2.1.3 to promote the formation of heteroduplexes then analysed by DHPLC (see section 2.1.3). Melt profiles for each fragment were determined using the Melt Program as previously described.

However, it soon became clear that there were discrepancies between the exon numbering on Ensembl and publications. The mutations described in section 6.1.2 (Davis *et al.* 1999) were reported as being in exon 2, but were clearly in exon 1 on the Ensembl database. Closer examination revealed that the 5' UTR was split across two exons, with one exon lying 60kb upstream from the rest of the gene. Primers were designed to flank the exon and include the suggested promoter region (Schrimpf *et al.* 1997). Primers were not designed to go further upstream due to time constraints. As DHPLC had already been carried out on the fragments which now lay in intron 1, any SNPs found already were analysed. These could still supply valuable LD information. The Ensembl database has since been updated. The sequences for the new exon 1 are shown in Table 6.2 below, along with the optimised conditions.

Table 6.2 New PI12 exon one primer details

Exon	Primer 5'-3' (For/Rev)	Taq	T°(C)	Size (bp)
Nx1	GAGTGGAAGTGGCAGAGCA AACGCGGACTCTTTTAGCTG	HotStart	60	390

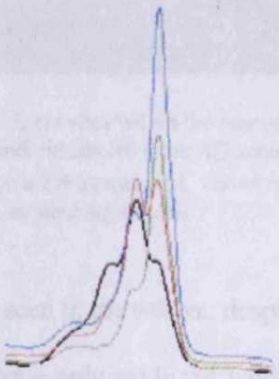
The primer sequences and PCR conditions for amplification of the neuroserpin exon one and promoter region. The taq enzyme used and the length of the fragment produced are also shown

From this point forwards, the exons will be referred to as exons 1-9, with the numbering in Table 6.1 being discarded. For convenience however, NP1, 2 and 3 will remain the same. Five fragments from the twelve showed a shift in DHPLC traces. For each of these fragments, two examples of each different DHPLC trace was sequenced using the automated method detailed in section 2.1.5, to characterise the possible polymorphism. All of these fragments and their SNPs will now be discussed in order starting with the upstream sections and any statistical analysis will be included.

6.2.3 NP1

Figure 6.2 shows the variants produced by DHPLC analysis for this fragment. Sequencing analysis revealed a T/C transition 1114 base pairs upstream of the translational start site. The sequencing results for this fragment were lost and cannot be shown here.

Figure 6.2 NP1 fragment DHPLC analysis

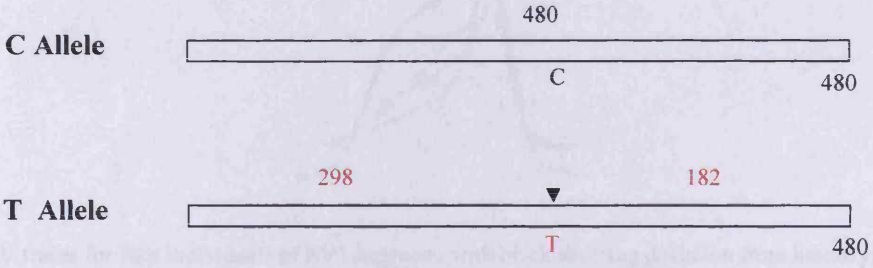


DHPLC traces for four individuals of NP1 fragment, with black, pink and green showing deviation from homozygote pattern.

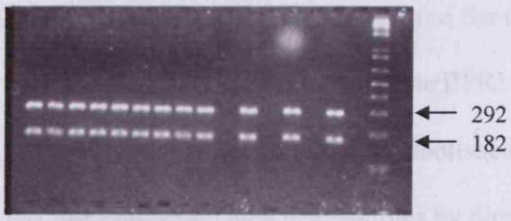
Analysis of the fragment using DNAStar, as detailed in section 2.1.6, revealed that the C variant abolished an existing *NsiI* cleavage site. The enzyme was purchased and tested on a small number of samples by digesting for 16 hours, to ensure complete cleavage, at the recommended temperature. No heterozygotes were seen in the first few samples digested and so twelve individuals from the screening set was amplified and digested. Figure 6.3 below shows the results of the RFLP assay.

Figure 6.3 NP1 RFLP analysis

A)



B)



A) Diagrammatic representation of *NsiI* cut sites within the neuroserpin NP1 fragment for the different alleles. B) RFLP analysis of twelve individuals from the AD screening set for the NP1 SNP, digested with *NsiI* for 16 hours and analysed on a 2% agarose gel. Genotypes and fragment sizes are shown. No heterozygotes seen, contradicting the sequencing results.

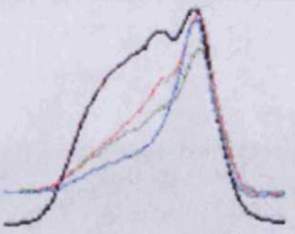
No heterozygotes were seen in the twelve, despite their presence in the sequencing results. As the enzyme only cut in the wild-type, it acted as an internal control for the purposes of its activity. The only explanation was that the SNP was

not real and merely a sequencing artifact. No further analysis was carried out on this polymorphism.

6.2.4 NP3

DHPLC traces for the NP3 fragment from four individuals in the AD screening set are shown in Figure 6.4 below. No sequencing traces were available to show here.

Figure 6.4 NP3 DHPLC analysis



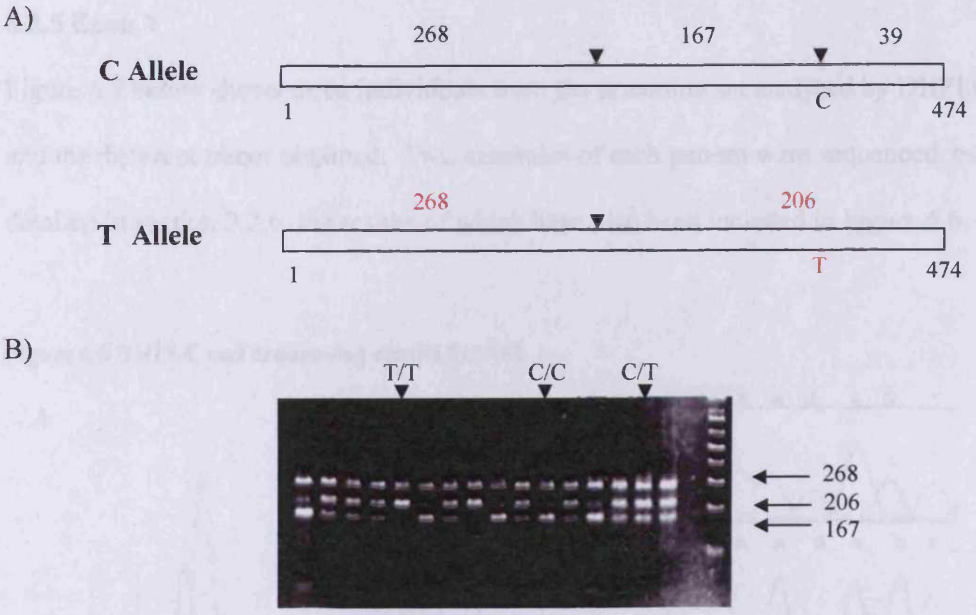
DHPLC traces for four individuals of NP3 fragment, with black showing deviation from homozygote pattern.

Sequencing revealed a C/T transition 863 bp upstream of the translational start site. The fragment was analysed using DNAStar for a possible RFRLP assay as detailed in section 2.1.6. Analysis revealed that the T allele abolished an existing *HpyCH4III* cut site. The enzyme was tested on a few samples by digesting for 16 hours, to ensure complete cleavage. After ascertaining that the RFLP assay worked correctly, the entire UK2 case control sample (see section 2.2) was amplified by PCR and digested at the appropriate temperature for 16 hours.

After digestion the samples were analysed on a 2.5% agarose gel, supplemented with Metaphor. Figure 6.5 on the following page shows the cut sites for *HpyCH4III* within the NP3 fragment and the sizes of fragments obtained after digestion. Also included is a photograph of some of the UK2 samples after

electrophoresis. The genotypes were then analysed as detailed in section 2.1.10. The results of the genotyping are shown in Table 6.3, also on the following page.

Figure 6.5 NP3 RFLP assay



A) Diagrammatic representation of *HpyCHIII* cut sites within the neuroserpin NP3 fragment for the different alleles. B) Samples taken from the MRC LOAD case-control sample digested with *HpyCHIII* for 16 hours and analysed on a 2.5% agarose gel supplemented with Metaphor. Genotypes and fragment sizes are shown

Table 6.3 NP3 genotyping results

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
NP3	iC/T @ -863	Case	59	66	30	**0.48** (1.46)	0.22 (1.49)
			(0.38)	(0.43)	(0.19)		
		Control	68	59	25		
			(0.45)	(0.39)	(0.16)		

Results of neuroserpin NP3 SNP genotyping in the UK2 case-control sample. Position of polymorphism shown in relation to the nearest exon. Genotype numbers are given with frequencies in parenthesis below, along with the p-value and chi-square value for genotypic and allelic association. Genotype codes are as follows; 1 1 – (C/C), 1 2 – (T/C), 2 2 – (T/T).

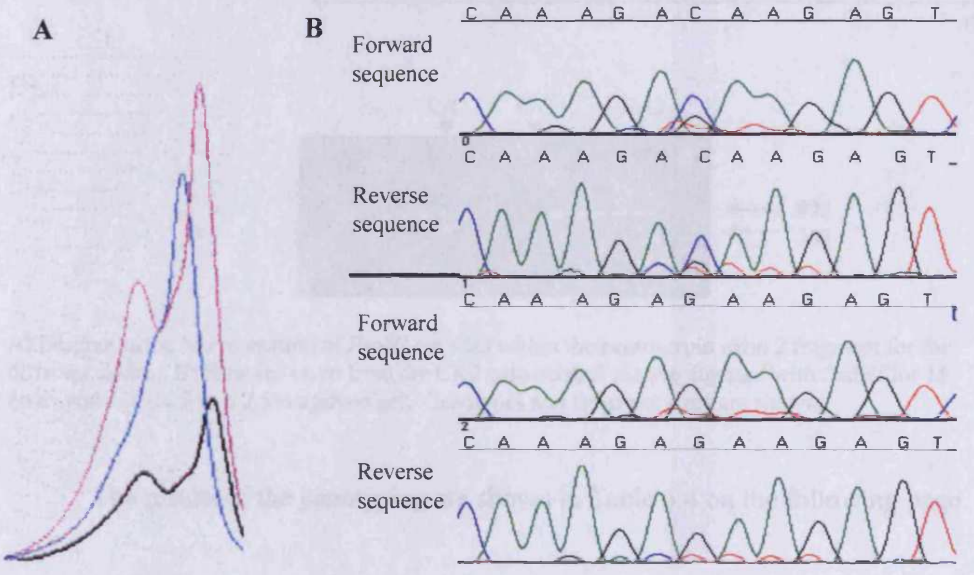
No association was found between the NP3 polymorphism and LOAD in the UK2 case control sample; genotypically nor allelically. Both the cases and the

controls conformed to the Hardy-Weinberg equilibrium as did all others analysed in this chapter, unless specifically mentioned.

6.2.5 Exon 2

Figure 6.7 below shows three individuals from the screening set analysed by DHPLC and the different traces obtained. Two examples of each pattern were sequenced, as detailed in section 2.2.6, the results of which have also been included in Figure 6.6.

Figure 6.6 DHPLC and sequencing results for Nx2



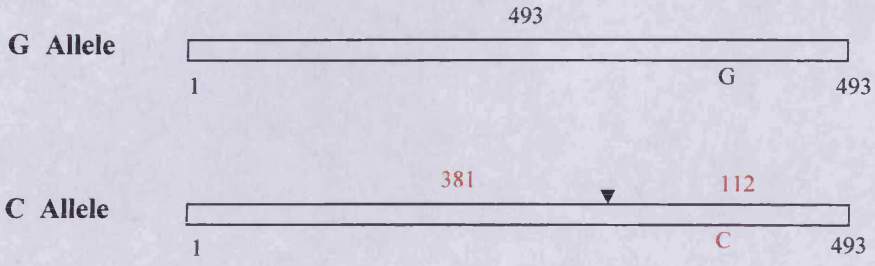
A - DHPLC traces for three samples of the Nx2 fragment, with the pink and black traces showing deviation from the normal pattern. B - sequencing results of two individuals (both forward and reverse) from the screening set, showing the C homozygote (above) and the G homozygote (below).

Sequencing revealed a G/C polymorphism within exon 2 of neuroserpin, corresponding to residue seven within the polypeptide, which gave a non-synonymous amino acid change (F7L). The fragment was analysed using DNAStar which showed that the C allele created a *PsaHI* cut site. The enzyme was tested to ensure it digested

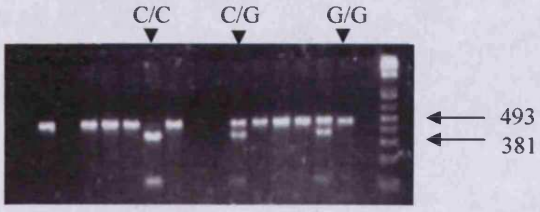
in the PCR buffer, and then the whole UK2 sample was amplified and digested for 16 hours as detailed in section 2.1.7. Figure 6.7 below shows the position of the *PsaHI* cut site and the fragment sizes produced in the RFLP assay. Also shown are some examples of the different genotypes after electrophoresis.

Figure 6.7 Nx2 RFLP assay

A)



B)



A) Diagrammatic representation of *PsaHI* cut sites within the neuroserpin exon 2 fragment for the different alleles. B) Samples taken from the UK2 case-control sample digested with *PsaHI* for 16 hours and analysed on a 2.5% agarose gel. Genotypes and fragment sizes are shown.

The results of the genotyping are shown in Table 6.4 on the following page.

Table 6.4 Nx2 genotyping results

			Genotype			p-value (χ2)	
	Type and Position	Sample	1 1	1 2	2 2	Genotypic	Allelic
Nx2	G/C @ 22	Case	132	29	3	**0.12** (4.25)	0.08 (3.16)
			(0.80)	(0.18)	(0.02)		
	F7L	Control	141	22	-		
			(0.87)	(0.13)	(0.00)		

Results of the neuroserpin exon 5 SNP genotyping in the UK2 case-control sample. Position of polymorphism from start of translation shown, along with the position of the residue within the amino acid sequence. Genotype numbers are given along with the p-value and chi-square value for genotypic and allelic association. Genotype codes are as follows; 1 1 – (G/G), 1 2 – (G/C), 2 2 – (C/C).

No association was found between the Nx2 polymorphism and LOAD in the UK2 case-control sample, although the allelic p-value was bordering on significance.

6.2.6 Neuroserpin mutations

I decided not to pursue the mutations previously described in the introduction (Davis *et al.* 1999) as a quick digestion of the screening set with *MnlI* revealed no copies of the S49P mutation were present (results not shown). This suggested that the mutation was too rare for my study as I was looking for a common polymorphism of small effect. As the same was probably true of the S52R mutation and because of time constraints it was decided to concentrate on the SNPs that I had detected.

6.2.7 Nx4

Figure 6.8 on the following page shows the DHPLC traces of three individuals from the LOAD screening set showing a shift in the pattern. Two individuals for each trace pattern were sequenced, the results of which are also shown in figure 6.9.

Figure 6.8 DHPLC results for Nx4

DHPLC traces for three samples of the Nx4 fragment, with the pink and black traces showing deviation from the normal pattern.

Sequencing of the fragments revealed a C/T polymorphism within exon four. This nucleotide lies 576bp from the start of translation and therefore affects S192, although the polymorphism does not alter the amino acid. I decided to carry out the genotyping using the SNaPshot system (see section 2.1.8), in order to conserve DNA from the case-control sample, and therefore a primer was designed leading up to the polymorphic site using Primer3. The same pooled samples were used as those described in 4.2.3.1 and the reaction was carried out using the standard method with the primer at a concentration of 5pM (5'- AGTATTTTCAGGCCTAAACTG -3'). In order for analysis of the results to be performed, the fluorescent peak readouts obtained in the reaction must give results over 200. Lower peaks become difficult to read because of background noise produced by the reaction. Despite repeated attempts, with varying conditions, too many of the peaks for the pooled samples were below 200. The heterozygote standards (which are used to correct the results) were high enough, however from my results it appears that the T allele is quite rare in the

normal population and therefore its peak height will always be low in a pooled sample. This is explained in further detail in 6.3.

A summary of the results from the genotyping is shown at the end of the results section.

6.2.8 LD analysis

LD analysis was carried out between the two genotyped polymorphisms as detailed in section 2.1.10. The level of LD between the two polymorphisms was low, giving an r^2 value of 0.21 and 0.19 in the cases and the controls respectively, after 1000 simulations.

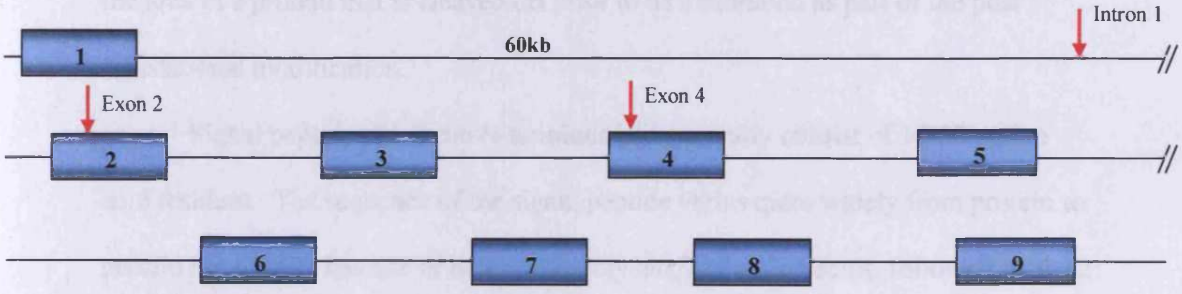
6.2.9 Haplotype analysis

In order to have complete analysis of the gene, haplotype analysis was also carried out on the two polymorphisms to determine any possible marker-disease associations. Only one haplotype is possible with two SNPs and after 1000 simulations a p-value of 0.313 was obtained ($\chi^2 = 2.58$). There is no association between the neuroserpin haplotype reported here and LOAD in the MRC case-control sample.

Overview of Neuroserpin Results

Position	Type	Sample	1 1	1 2	2 2	Assn	Allelic	HW	A1	A2
Intron 1	iC/T @ -863	Case	59	66	30	**0.48**	0.22	0.14	184	126
		Control	68	59	25			0.05	195	109
Exon 2	G/C @ 22	Case	132	29	3	**0.12**	0.08	0.35	293	35
	F7L	Control	141	22	0			0.36	304	22
Exon 4	C/T	Case	-	-	-	-	-	-	-	-
	Ser→Ser	Control	-	-	-			-	-	-

Summary of neuroserpin genotyping in the MRC LOAD case-control sample. The position of polymorphisms, either within the polypeptide or from nearest exon, is shown along with genotype and allele counts. Results of association shown as p-values for both allelic and genotypic analysis, and for concordance with the Hardy Weinberg equilibrium.



Genomic position of polymorphisms analysed in this chapter within neuroserpin (red arrows) to illustrate level of gene coverage. Not shown to scale.

6.3 Discussion

No association was found genotypically, allelically or haplotypically with LOAD in neuroserpin. However, this statement has to be made from a very limited number of results as only two polymorphisms were completely genotyped in 160 cases and controls. The polymorphism detected in exon 2 (F7L) did come close to association with a trend towards increased numbers of C alleles in AD cases. This finding should certainly be replicated in another sample although it would only take a few C/C homozygotes to be found in the controls for the result to lose significance because of the low frequency of the rare allele. This polymorphism lies within the signal peptide, the area of a protein that is cleaved off prior to its maturation as part of the post translational modification.

Signal peptides lie at the N-terminus and generally consist of 16-30 amino acid residues. The sequence of the signal peptide varies quite widely from protein to protein but usually has one or more positively charged amino acids, followed by 6-12 hydrophobic residues. The role of the signal peptide also varies from protein to protein and in the case of neuroserpin is required for its maturation and transportation to the cell surface prior to secretion (Osterwalder *et al.* 1996; Schrimpf *et al.* 1997). Figure 6.10 on the following page shows the signal peptide sequence and the position of the polymorphism within.

Figure 6.10 Neuroserpin signal peptide

Amino acid sequence of the neuroserpin signal peptide, 16 residues in length. Hydrophobic residues are shown in red and polymorphic residue in italics.

As can be seen from the figure, the polymorphic site lies at the centre of the hydrophobic core which is essential for signalling. Changes in the recognition of the signal peptide can lead to the protein not crossing the ER membrane into the lumen. However there are two reasons why this probably does not occur in this case. Firstly, the residue is not conserved in chicken neuroserpin (Schrimpf *et al.* 1997), suggesting it can be changed without altering function; and secondly the amino acid substituted in the polymorphism is also hydrophobic and would therefore maintain the essential core of the peptide. Both of the exonic polymorphisms, F7L and S192, have both previously described by the French Alzheimer's Disease and Fronto-Temporal Dementia Genetics Study Groups (2000) (FADFTD-GSG) as mentioned in section 6.1.3.2. They reported no significant differences between the 17 EOAD cases and 26 unaffected controls. The results of this study show that for the F7L polymorphism, the frequency of the C allele is approximately 10% and the FADFTD-GSG may have had difficulty seeing differences between the cases and the controls even though they were using a family study. Early results of the S192 genotyping in this project suggest that it too may have a low frequency for the rarer allele.

The main disappointment in this chapter is the failure to genotype the S192 polymorphism before running out of time. The FADFTD-GSG (2000) report that this polymorphism is in complete LD with the F7L SNP, however this analysis was only

done on a small sample which can result in over estimation of LD, especially if using the D' statistic, but the method used by the FADFTD-GSG is not mentioned in the study. It is therefore possible that a different result could have been obtained for this SNP. The results obtained from SNaPshot were frustrating. The results are produced as a spike of fluorescence that is directly correlated to the number of alleles present in the pooled sample. As with all luminescent and fluorescent technologies there is a background level which needs to be surpassed to provide accurate results. That cut-off point for this system is 200 and you need the peaks for both alleles to be above this value. These values can then be compared to the values produced by a single heterozygote sample and a correcting equation applied to work out the allele numbers in the pooled samples. In my study the rarer allele peak heights were less than 100, but the more common allele produced peaks in excess of 3000. Bringing up the peaks to a readable level could well have pushed the more common allele over the upper limit (8000) of the system. It could be that with more time the system could have been optimised further. Another option would have been to develop a mismatch primer to introduce a cut site, as analysis with DNAStar did not reveal a suitable RFLP assay.

Any importance the NP3 polymorphism may have had was lost when it was realised it lay in intron one and not near the promoter region. Despite this, it does emphasise the importance of checking several databases before embarking on a gene screening project. As the Human Genome Mapping Project nears completion, hopefully the frequency of this kind of error will reduce.

Another point to note from the polymorphism screening of neuroserpin is how well conserved the gene appears to be. Looking at the fragments spanning exons,

only 2 from 9 (22%) showed a shift in DHPLC. This is low in comparison to the other genes analysed in this study (TACR2 60% and ECE1 42%).

Neuroserpin is quite a different candidate to the other genes studied in this report. Its candidature was not based on linkage work or a previous association but on the results of unpublished microarray work. It also had a neurodegenerative disorder, quite distinct from AD, already linked to mutations found within the gene. Microarrays are a very powerful technology which will be able to provide vast amounts of information. Indeed, one of the main limiting factors at the moment is the analysis of the data provided. However, it is also important to analyse the data correctly. In the case of neuroserpin, the genetic analysis available suggests it may not be associated with AD despite a two-fold decrease in expression. This could be a result of the neurones which express neuroserpin having already degenerated in the brain of this AD patient. What is required is an early stage AD patient, to show the initial changes in gene expression that occurs at the beginning of the disease. This type of sample will always be difficult to obtain as the circumstances required are quite specific. Also, the post-mortem interval can be quite long, adding to the variation in analysis.

As a functional candidate, neuroserpin is quite strong with roles in inflammation and neuronal plasticity as was described in section 6.1.3.2. However, its candidature is both enhanced and reduced by the presence of a distinct neurodegenerative disease caused by mutations within the gene. The neurodegeneration caused by the mutations show that defects within this gene can result in memory loss and neuronal loss. This is tempered by the fact that so far no two diseases have been found to be caused by different mutations within a single gene.

In conclusion, the analysis of neuroserpin in AD requires further work. The remaining SNP needs to be genotyped and the exon 1 polymorphism requires replication in a large sample. Only when this has been carried out can a definitive picture of the genes' role in AD be produced. Microarray work will provide important results for complex diseases and good targets for analysis in association studies but these must be obtained from well planned experiments with the correct donor background.

Chapter 7

Conclusions

This project is dramatically different to that which was originally planned. As the ACE association was not replicated in this study, the work moved from characterising this association to a general study of cardiovascular/inflammatory genes in LOAD. This in turn created a project that yielded a large number of results that can be analysed in a multitude of different ways and that therefore requires careful planning.

In total, 17395bp (4968 coding) were screened for polymorphisms (not including fragments which failed to amplify) yielding 15 SNPs; an average of 1 SNP every 1160bp. A further 7 SNPs were obtained from databases and publications for analysis. Three fragments which showed a shift in DHPLC failed to yield a polymorphism after sequencing, *TACR2* exon 3, *ECE1* exon 12 and a fragment within intron 1 of the neuroserpin gene. It is unclear how DHPLC yields false positives and most evidence suggests that the sequencing was not of sufficient quality to detect the polymorphisms, especially if they lay near the primers. However it has been suggested that lower fidelity taq enzymes can introduce nucleotides to the 3'-hydroxyl termini of blunt ended fragments, especially if the fragments in question are small and GC-rich (Liu *et al.* 2002).

Several of the polymorphisms reported in this study are present on dbSNP (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Snp). The references for each one are shown in table 7.1 on the following page. Other SNPs have been reported within the genes analysed and could be used in further analysis, particularly the polymorphisms in ECE1 as mentioned in section 5.3.

Table 7.1 SNPs Database references

Gene	Position	Description	dbSNP Ref.
ECE1	Exon 1	iA/C @ -20	-
	Exon 8	iA/G @ +15	rs212522
	Exon 9	**T385T**	rs3026890
	Exon 10	iC/T @ -5	-
	Exon 11	iC/T @ -150	-
	Exon 14	iC/G @ -99	-
	Exon 16	iG/A @ -214	rs6665399
TACR2	Promoter	iA/G@ -2137	-
	Promoter	iG/A @ -1176	-
	Exon 1	**I23T**	rs5030920
	Exon 4	iT/C @ +116	-
*	Exon 5	**R375H**	rs2229170
PI12	Intron 1	iC/T @ -863	-
	Exon 2	**F7L**	-
	Exon 4	**S192S**	-

Reference numbers on dbSNP (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Snp) for the polymorphisms detected in this study along with their position and effect. Exonic polymorphisms shown in bold, (-) denotes that the SNP was not present in the database.

Across all four genes, no single polymorphism out of 20 detected yielded a statistically significant result for genotypic or allelic association with LOAD. However, borderline significance was seen in a couple of allelic studies and some highly significant results in the haplotype analysis. Taking into account all of the genotypic and haplotypic analyses, 177 different analyses were carried out in this study, which raises the question of multiple testing.

When performing a statistical test, the probability of rejecting the null hypothesis (H_o) when it is true is the same as the significance level (α), usually 0.05. This means the probability of not rejecting the true H_o is $1-\alpha$. If *n* multiple tests are carried out, the probability of not rejecting all *n* null hypotheses when all are true is $(1-\alpha)^n$. Therefore the probability of rejecting at least one H_o when all are true is $1-(1-\alpha)^n$ which is also known as the experimental error rate (EER) (Bender and Lange, 2001). So, in this study, with 177 tests the EER is 0.99989 and therefore it is almost certain to contain at least one false positive, with the expected number of false

positives being approximately nine (177 x 0.05). The EER is quite limited in its use in biological studies, therefore the maximum EER (MEER) has been developed which is the probability of rejecting at least one true H_0 irrespective of which and how many of the other null hypotheses are true.

The most parsimonious method of adjusting for multiple testing is the Bonferroni correction (Bland and Altman, 1995) which consists of dividing the significance level by the number of tests to provide an adjusted significance level e.g. 0.05/177 = 0.0003, therefore only associations with p-values less than 0.0003 should be considered significant. However, the Bonferroni method's simplicity gives low power to detect association and therefore should generally only be used when test numbers are lower than ten. The Holm method (Holm, 1979), for example, is an improvement on the Bonferroni method which provides the power but retains the simplicity by allowing the error rate to be controlled at the α level. It therefore provides more opportunities to reject H_0 providing more power. For a description of the method see Chapter 2.

Adjusting for multiple testing is considered essential in clinical trials and is incorporated into experimental guidelines (The CPMP Working Party on Efficacy of Medical Products, 1995 from Bender and Lange, 2001). Its role in association studies and how it should be carried out however is controversial. Perneger (1998) argues that correcting for multiple testing simply increases the likelihood of type II errors i.e. accepting the null hypothesis when the opposite is true, and that the study-wide error rate is largely irrelevant in biological studies. In a scathing response, Bender and Lange (1999) accuse Perneger of assuming the Bonferroni adjustment is the only method for multiple testing corrections and of ignoring the MEER which is a more accurate measure than study-wide error rates.

Following a report by Unoki *et al.* (2000) of an association between a SNP and bronchial asthma after studying 33 polymorphisms in 14 different genes, Boehringer *et al.* (2000) suggest that multiple testing correction should be applied. They argue that every gene studied is independent and therefore represents an individual null hypothesis but correction needed to be applied when markers from the same region are analysed because they may all be detecting the same signal. Therefore, for the three SNPs studied in TBXA2R the adjusted significance level is 0.05/3 = 0.0167 and as the lowest p-value obtained is 0.03 the reported association was a type I error.

However, Nyholt (2001) suggests that study wide adjustments are the more correct method. In this case the Bonferroni-corrected significance level of 0.05/33 = 0.0015 should be used to take all of the genotyped SNPs into consideration. He also suggests that in cases of high LD within a region that Bonferroni corrections are too conservative and that looking at the number of regions studied will be more valid e.g. using the asthma example above, a significance level of 0.05/14 = 0.0036 if the markers within the genes were in complete LD.

Another study by Emahazion *et al.* (2001) looks at 60 SNPs from a variety of genes for association with AD including 12 from previously reported positive associations. Several new positive associations are discovered but after adjusting for multiple testing every single one becomes non-significant. Even ApoE, a well validated risk factor, is barely significant after correcting for multiple testing. This study highlights a major stumbling block in case-control studies if this correction needs to be applied in every case. It is likely that most reported associations would not retain their significance after adjustment and this problem will become more prevalent as high-throughput methods increase the rate of genotyping. It is becoming

clear that only repeated replications can properly discern true associations from type I errors.

Therefore, for this study, assuming that all of the genes studied were independent, the lowest adjusted p-values are 0.01 for neuroserpin, 0.0014 for TACR2 and 0.0008 for ACE and ECE1. After correction none of my results remain statistically significant but one result remains interesting; the haplotype which gave a p-value of 0.001 in ECE1. This haplotype, containing the intron ten, 14 and 16 polymorphisms, deserves further genotyping in another sample. Since completing this project the MRC-LOAD sample has expanded to over 400 cases and controls and this haplotype should also be genotyped in the complete sample.

The work by Emahazion *et al.* (2001) also highlights some other pitfalls in association studies, which I have already touched upon in section 3.3. Large deviations were seen in the allele frequencies of the controls between studies which is one of the main causes of false positive results in this type of study. When designing an association study, the selection of controls needs to performed very carefully to prevent any form of stratification between the cases and the controls.

This and other concerns were raised in an editorial by Cooper *et al.* (2002) as guidelines for researchers and editors. These guidelines include i) that the disease should already have been proved to be heritable, ii) the candidate gene should be fully justified either positionally, functionally or both, iii) the controls should be carefully matched, iv) if the work is not confirming an existing hypothesis then an independent replication sample should be genotyped and, v) confidence intervals and odds ratios should be used to determine the effect size and adjustments for multiple testing should be used where necessary. This study has fulfilled all of these guidelines except iv,

where replication of the positives was not carried out due to time constraints and the lack of a suitable sample being available.

As mentioned above control selection is very important in the design of association studies. In the case of AD, case selection can be just as important as accurate diagnosis can often be difficult when faced with other dementias, in particular vascular dementia. In fact, this overlap between the two diseases can result in the diagnosis of mixed dementia. Mixed dementia is diagnosed when either pre-existing AD is complicated by a stroke which leads to cognitive decline or when AD develops after a stroke which in itself caused a cognitive decline (reviewed in Dugue *et al.* 2003). This need for accuracy is accentuated in my study as two of the genes analysed have critical roles in the cardiovascular system. The role of the ACE I/D polymorphism in cardiovascular disease has been discussed at length in chapter 3 while ECE1 catalyses the production of the most potent vasoconstrictor presently known. Without careful selection criteria, it would be possible for any positive effect seen in either gene to be linked to vascular dementia and not AD. In the case of this study however, cautious diagnosis has ensured that, to our knowledge, the case sample is not contaminated with VaD patients. As mentioned in section 2.2, the scales used to diagnose AD give a predictive value of 0.91 (Holmes *et al.* 1992) and exclusion criteria included the presence of any other dementia.

Of the four genes studied, ECE1 has provided the most interesting results, especially considering that no association studies have previously been carried out on this gene and AD. Functionally it is a good candidate and also has some positional evidence from a linkage study carried out within our own department (Kehoe *et al.* 1999b). ACE is a slightly more complicated story with a long history of both positive and negative replications. In our sample there is no trace of an association with

LOAD neither genotypically or haplotypically, as has been suggested in a recent paper (Kehoe *et al.* 2003). Many of the ACE association studies typify many of the problems highlighted by Cooper *et al.* (2002) and Ioannidis *et al.* (2001) which result in false positive results; over-manipulation of the data, small sample size, no correcting for multiple testing and poor selection of controls. However, some of these studies do provide solid positive associations in well designed experiments and because of this the role of ACE in AD remains unclear. As mentioned previously in this project, it could be that several polymorphisms in enzymes that degrade Aβ or affect the cardiovascular system are required to have a pathogenic effect. It is unfortunate that this hypothesis could not be tested in this study due to different samples being used for ACE and ECE1.

TACR2 yielded one possible significant result which did not survive correction for multiple testing. Interestingly, if the results of genotyping the London samples was analysed alone it was significant even after adjusting for multiple testing. As mentioned in section 4.3, this could be a result of the London samples coming from a more heterogenous population. As more individuals have now been added to the London sample it would be interesting to see if this effect is still present. Neuroserpin proved to be the least interesting of all the genes studied. The level of conservation appears to be high within the gene as a relatively low number of SNPs were found in comparison to the other three genes. One non-synonymous SNP did yield quite a low p-value but as the allele frequencies involved are very low this result could easily disappear in a larger sample.

Where this project should go next has already been discussed to some extent in chapters' three to six. As is true of all association studies, any positive results should be genotyped in a separate sample for replication. This is the priority for the

ECE1 haplotype that showed a positive association. If the result holds up after replication then functional work should be undertaken to determine the cause of the association. ECE1 levels should be determined in individuals with the associated haplotype and compared to the general population. This could be done using allele specific expression, which was recently used to show that a haplotype in COMT is associated with reduced expression (Bray *et al.* 2003) If this association is a true positive then a reduced level of ECE1 activity would be expected. Eckman *et al.* (2002) have shown ECE1 knockout mice have increased levels of βA and the same could be expected in a transgenic mouse carrying the associated haplotype. It would also be important to determine whether these polymorphisms cause the functional effect or merely reside near a QTL. Methods similar to those used in the ACE studies, trans-ethnic fine mapping for example, could be used.

TACR2 and neuroserpin do not require much further work, merely finishing the genotyping of the polymorphisms that couldn't be optimised. However in my opinion TACR2, despite being an excellent positional candidate, is not likely to be associated with LOAD. The work should then move to other genes within the candidate region on chromosome 10 as many remain to be analysed. Neurserpin genotyping should also be completed, and I feel that this gene will also prove to be unconnected to LOAD and there are many other differentially expressed genes to be studied. Microarray work yields a very large amount of results and false positives will be found. In addition a reduction in gene expression will be seen as a secondary effect of the neurodegeneration which can confound the results obtained from microarrays.

ACE, in this sample, is not associated with LOAD and remains quite controversial. During and since this study several more papers have been published

giving both positive and negative results. This study is a comprehensive look at the gene and no further work should be carried out until the QTL is discovered. If ACE does have a role in AD I believe it will be a small effect and possibly dependent on mutations in other genes of the cardiovascular system.

This study highlights the difficulties of discovering genetic associations with complex disorders and in particular AD. As mentioned in Chapter 1, many genes have been analysed exhaustively in relation to AD (see Table 1.2) but ApoE is still the only recognised risk factor. For many genes these problems will remain until the functional variant is properly characterised. In this study positional evidence was used to back up functional candidature for two of the genes but the regions involved can still contain hundreds of genes. Within these regions, genes can be 'cherry-picked' based upon functional relevance to increase the likelihood of detecting association. However, ApoE had no obvious link to AD when it was first discovered showing that the connections are not always obvious and making cherry-picking more difficult. This will improve as functions are assigned to genes and the pathological pathway in AD is elucidated. Until then positive associations must be replicated in different populations with carefully selected controls to reduce the number of spurious results produced.

URL List

http://www.iop.kcl.ac.uk/iop/Departments/PsychMed/GEpiBSt/software.shtml - EH+

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed – Entrez-PubMed

http://insertion.stanford.edu/melt.html - DHPLCMelt Program

http://genome.ucsc.edu – Genome Browser

http://www.md.huji.ac.il/mirrors/webpath/NEURO.html - Hebrew University Faculty of Medicine

http://diabetes.niddk.nih.gov/index.htm - National Institute of Diabetes and Digestive Kidney Disease Website

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM – Online Mendelian Inheritance in Man

http://calculators.stat.ucla.edu – Power calculator

http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi - Primer3

http://pipeline.lbl.gov - Vista

http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html - Web Chi-Square Calculator

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