Université de Montréal

Genetic associations of apolipoprotein E, alpha-1-antichymotrypsin and intronic presenilin-1 polymorphisms in Alzheimer's disease

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Genetic associations of apolipoprotein E, alpha-1-antichymotrypsin and intronic presenilin-1 polymorphisms in Alzheimer's disease

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SUMMARY

BACKGROUND: Alzheimer's disease (AD) is a neurodegenerative disease in the elderly characterized by dementia and histopathological accumulations of senile plaques and neurofibrillary tangles. Familial inheritance patterns, similarity to trisomy-21 and concordance rates in twin studies support a genetic etiology of the disease, but with multifactorial influences. Dissection of complex traits is difficult because of the possibility of reduced penetrance, genetic heterogeneity, epistasis and phenocopy effects. Gene mapping experiments have identified several AD candidate loci including APP, PS-1 and PS-2 which are involved in early-onset AD. Association studies have identified ApoE and ACT as additional AD susceptibility genes.

HYPOTHESIS AND METHOD: We tested the hypothesis that ApoE, ACT and PS-1, either alone or in interaction, are involved in AD. Polymorphisms in these genes were tested for association with AD, along with additional factors such as gender, age, gene dose, and plaque and tangle densities. In order to minimize the confounding effects of complex disease dissection, our sample consisted of autopsied-confirmed AD cases and aged non-demented control subjects from a founder population.

RESULTS: The ApoE ε4 allele was strongly associated with AD in a dose-dependent manner. However, ε4 was neither necessary nor sufficient to cause AD. SP accumulation was correlated with ε4 but not with NFT accumulation and age at onset. AD cases with age at onset > 80 had a lower frequency of ε4, suggesting an age-dependent effect of ε4. No gender effect of ε4 was seen. We found a protective effect of ε2. ACT TT genotype was weakly associated with AD risk, which is in contrast to other studies which have reported an ACT AA association, suggesting that the ACT polymorphism may be in linkage disequilibrium with a nearby functional AD mutation. We were unable to corroborate previous reports that AD risk is associated with the intronic PS-1 polymorphism.

AD risk was found to be significantly increased in individuals who were carriers of both ApoE ε4 allele and ACT TT genotype, and also in individuals who are carriers of both ACT TT and PS-1 12 genotypes, suggesting a genetic interaction between ACT and ApoE, and also between ACT and PS-1. The ACT/ApoE interaction was associated with SP accumulation. Otherwise, both putative interactions could not be correlated with disease severity indices such as onset age and SP and NFT accumulation, suggesting that these putative interactions are subtle. No interaction effect was seen between ApoE and PS-1 polymorphisms.

CONCLUSION: Our results provide statistical correlations for the involvement of ApoE, ACT and PS-1, either alone or in interaction, in AD pathogenesis. However, these polymorphisms do not adequately explain AD in our sample, suggesting that other genetic and/or environmental AD modifiers exist.

RÉSUMÉ

PROLÉGOMÈNES:
La maladie d'Alzheimer (MA) est une maladie neurodégénérative affectant les personnes agées et caractérisée par la démence menant à la mort. Parmi les caractéristiques histopathologiques, on retrouve des accumulations, dans le cerveau, de plaques séniles extracellulaires composées de peptides ß-amyloïdes (Aß) de même que des dégénérescences neurofibrillaires composées de protéines tau. Il est difficile de diagnostiquer la MA avec certitude sans autopsie. Les modèles de transmission familiale de la maladie, les similitudes avec la trisomie 21 ainsi que les études sur les jumeaux, semblent indiquer une étiologie génétique de la maladie, mais avec des influences multiples.

Les analyses de liaison ont permis d'identifier plusieurs loci candidats pour la MA, notamment le gène codant pour la protéine précurseur de l'amyloïde (APP) situé sur le chromosome 21q11.2-21, ainsi que deux gènes codant pour la préséniline, PS-1 et PS-2, situés respectivement sur les chromosomes 14q24.3 et 1q42.1 . Il y a corrélation entre des mutations dans ces gènes et la forme précoce de la MA chez les familles à l'étude. Une exception toutefois: un polymorphisme intronique de PS-1 a été associé plutôt à la forme tardive de la maladie. Les gènes APP, PS-1 et PS-2 ne sont responsables que d'une petite fraction de la totalité des cas de MA. Bien que la fonction de la protéine APP ne soit pas connue, sa modification post-traductionnelle en Aß en fait une candidate logique comme responsable de la MA. Les gènes PS-1 et PS-2, fortement homologues entre eux, codent pour des protéines transmembranaires dont la fonction est inconnue mais qui pourraient être impliquées dans la signalisation cellulaire, le transport de calcium ou l'apoptose. Une grande partie des expériences *in vitro* et *in vivo* semblent démontrer que des mutations dans ces gènes de la forme précoce de la MA seraient responsables d'une augmentation de la production du peptide

amyloidogénique Aß42(43). Cette forme plus longue du peptide s'agglutine plus rapidement en fibrilles que la forme courante Aß40. Selon l'hypothèse de la cascade amyloïde, la cause primaire de la MA serait une augmentation de l'amyloidogénèse alors que les autres manifestations de la MA, telle que la perte neuronale et la dégénérescence neurofibrillaire, seraient secondaire à ce premier phénomène.

Des études d'association ont également identifié l'apolipoprotéine E (ApoE) et l'a-1-antichymotrypsine (ACT), que l'on retrouve respectivement sur les chromosomes 19q13.2 et 14q31-32.3, comme autres gènes de susceptibilité à la MA. Le gène de l'ApoE, qui code pour un transporteur de cholestérol dans le métabolisme des lipides, est polymorphique avec trois allèles majeurs: ε2, ε3 et ε4. On sait qu'il y a une corrélation entre l'allèle et la forme tardive de la MA dans un grand nombre de populations différentes. Il a également été démontré que l'effet de l'allèle ε4 comme facteur de risque de la MA dépend du nombre d'allèles présents, de l'âge et du sexe du patient. Toutefois, ces études sont controversées, tout comme celles qui suggèrent un rôle protecteur pour l'allèle ε2 de l'ApoE contre la MA. Selon certaines études, un polymorphisme bi-allèlique du peptide signal de l'ACT est associé à la MA. La présence de l'allèle ε4 d'ApoE augmente d'autant les risques associés avec l'ACT, ce qui suggère une interaction possible entre ces 2 gènes dans la pathogénèse de la MA. D'autres études n'ont toutefois pu confirmer ces résultats. L'ApoE a pu être localisée par immunocytochimie dans les plaques et les dégénérescences neurofibrillaires des cerveaux Alzheimer et il a été montré *in vitro* qu'elle peut se lier aux peptides Aß et aux protéines tau, l'affinité de cette intéraction étant fonction de l'isoforme d'ApoE étudié. L'ACT est un réactif de phase aiguë exprimé en plus grande concentration durant l'inflammation et étroitement associé à l'Aß dans les plaques séniles des cerveaux Alzheimer. *In vitro*, il a été démontré que l'ACT accélère la formation des fibrilles de Aß42(43). Tous ces résultats représentent des preuves indirectes d'un rôle biologique de l'ApoE et de l'ACT dans la pathogénèse de la MA, probablement comme chaperons pathologiques impliqués dans les dépôts de Aß dans le neutropile.

HYPOTHÈSE ET MÉTHODES:

Des caractéristiques complexes qui présentent des paramètres de pénétrance réduite, d'hétérogénéité génétique, d'épistasie et d'effets de phénocopie, compliquent l'identification des gènes impliqués dans l'étiologie de la maladie. Pour minimiser ces difficultés, il est nécessaire de recourir à des approches non-paramétriques d'analyse génétique et d'utiliser une population fondatrice de manière à pouvoir analyser ces caractéristiques de façon méthodique. Des analyses génétiques non-paramétriques telles que des études d'association permettent de corréler le partage d'allèles entre les groupes de témoins et de cas pour un locus génétique donné avec la maladie. Cette stratégie permet de faire abstraction de paramètres tels que les interactions géniques multiples et l'environnement. Les populations fondatrices permettent l'identification de gènes reliés à des maladies complexes car elles ont subi les effets de l'isolation de leurs pools génétiques et de l'augmentation rapide de la population à partir d'un nombre limité de familles. De ces conditions résultent des populations homogènes au point de vue ethnique et dont les individus ont une plus forte probabilité d'avoir hérité, d'un ancêtre commun, un gène prédisposant à une maladie donné. Nous avons testé l'hypothèse selon laquelle ApoE, ACT et PS-1, soit indépendamment ou en intéraction, sont impliqués dans la MA. Nous avons effectué une étude d'association entre les cas et une population témoin portant sur l'ApoE, l'ACT et des polymorphismes introniques de PS-1 en utilisant la technique de PCR-RFLP chez une population fondatrice de la région du Saguenay-Lac-St-Jean, au Québec. Etant donné qu'il est difficile de diagnostiquer avec certitude la MA, les sujets ont été sélectionnés sur la base d'une autopsie pour les cas d'Alzheimer et sur des tests neuropsychométriques pour les sujets âgés non-séniles constituant le groupe témoin. Pour chacun des polymorphismes, nous avons déterminé s'il y avait un effet relié au sexe, à l'âge et au nombre d'allèles sur la maladie. Les indices morphométriques des cas autopsiés tels que l'âge d'apparition de la maladie, la densité de plaques séniles et celle des dégénérescences neurofibrillaires nous ont permi de déterminer s'il y avait une relation entre ces polymorphismes et la sévérité de la maladie.

RÉSULTATS:

L'allèle ε4 de l'ApoE est fortement associé, et de façon quantitative, avec la MA. Toutefois, l'allèle ε4 n'est ni nécessaire ni suffisant pour causer la MA ce qui suggère que sans en être la cause, l'allèle ε4 peut augmenter la susceptibilité à la MA. Les porteurs de l'allèle ont de plus grandes accumulations de plaques séniles. Un effet quantitatif de l'allèle ε4 a pu être démontré sur l'accumulation des plaques sénile mais non sur celle des dégénérescences neurofibrillaires, ni sur l'âge où se déclare la maladie, ce qui suggère un rôle direct de l'ApoE dans le processus d'accumulation des plaques séniles. Pour ce qui est de ε4, le sexe du patient n'a aucune influence dans notre échantillon. Les cas où la maladie apparait après 80 ans ont une fréquence de porteurs de l'allèle ε4 beaucoup plus faible, indiquant donc que l'effet de l'ApoE sur l'âge d'apparition de la maladie dépend de l'âge du patient. Le risque de développer la maladie diminue de façon significative chez les porteurs de l'allèle ε2 par rapport aux non-porteurs, ce qui laisse supposer que l'allèle ε2 protège contre la MA. Ce risque augmente chez les individus porteurs de l'allèle ε4 et du génotype ACT TT. L'analyse de régression indique que la combinaison des génotypes ApoE et ACT laisse prévoir une plus grande probabilité d'apparition de la MA que chaque génotype pris séparément. Le risque augmente également chez les sujets porteurs des génotypes ACT TT et PS-1 1 2, ce qui semble indiquer une interaction entre ApoE et ACT et entre ACT et PS-1. Aucune interaction n'a été observée entre ApoE et PS-1. Nous considérons que les associations positives observées peuvent être dues à un déséquilibre de liaison de loci voisins reliés à la MA ou à des effets d'hétérogénéité ethnique. Nous abordons également les fonctions possibles des protéines codées par ces gènes et leurs interactions possibles. Des études additionelles sur ces polymorphismes seront nécessaires au développement des tests fiables pour la prédisposition à la MA.

CONCLUSIONS:

L'allèle ε4 de l'ApoE semble augmenter le risque de MA et pourrait influencer la sévérité de la maladie en facilitant l'accumulation des SP. Il semble également y avoir une interaction génétique entre ApoE et ACT et entre ACT et PS-1. Nos résultats suggèrent que ces gènes pourraient

être directement impliqués dans la pathogénèse de la MA. Toutefois, on ne peut exclure un déséquilibre d'association entre ces polymorphismes et d'autres gènes voisins relié à la MA. Enfin, ces polymorphismes ne peuvent expliquer totalement la MA dans notre échantillonnage. Ceci nous porte à croire qu'il existe encore d'autres facteurs environnementaux et/ou génétiques.

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LIST OF ABBREVIATIONS

3MS:	Modified Mini-Mental State
ACT:	alpha-1-antichymotrypsin
AD:	Alzheimer's disease
ApoC1:	apolipoprotein C1
ApoC2:	apolipoprotein C2
ApoE:	apolipoprotein E
APP:	amyloid precursor protein
Aß:	amyloid-beta
CI:	confidence interval
cDNA:	complementary deoxyribonucleic acid
DNA:	deoxyribonucleic acid
ERAB:	endoplasmic reticulum-associated binding protein
FAD:	familial Alzheimer's disease
Fig.:	figure
HCHWA-D:	hereditary cerebral hemorrhagic amyloidosis- Dutch type
kDa:	kilo-Dalton
LDL:	low density lipoprotein
LRP:	low density lipoprotein receptor-related protein
MA:	maladie d'Alzheimer
mRNA:	messenger ribonucleic acid
NFT:	neurofibrillary tangles
NS:	non-significant
OR:	odds ratio
PCR:	polymerase chain reaction
PS-1:	presenilin-1
PS-2:	presenilin-2
RFLP:	restriction fragment length polymorphism
RNA:	ribonucleic acid
sAPP:	secreted amyloid precursor protein
SD:	standard deviation
SLSJ:	Saguenay-Lac-St-Jean
SP:	senile plaques
VLDL:	very low density lipoprotein
YAC:	yeast artificial chromosome

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DEDICATION

To Henny.

INTRODUCTION

Alzheimer's disease (AD) is a progressive, neurodegenerative disease characterized by gradual cognitive deterioration leading to profound dementia followed by death. AD is the most common cause of cognitive decline in the elderly (Small et al., 1997). Symptoms of the disease include gradual memory loss, disorientation, difficulty in learning, loss of language skills, impairment of judgement and personality changes. Ultimately, the symptoms lead to a total inability to take care of oneself, placing a tremendous financial and emotional burden, not only on the patient, but also on family members and other caregivers. Duration of the symptoms from onset to death is highly variable, ranging from 3 to 20 years with a mean duration of 8 years. Age at onset is also variable. Most individuals diagnosed with AD are over age 65. However, AD can occur in individuals as young as age 30. The different onset ages are loosely classified as late-onset (age at onset >65 years) and early-onset (age at onset <65 years). Affected members of families in which the disease appears to be transmitted in an autosomal dominant manner are referred to as "familial" AD cases, as opposed to the "sporadic" form, whereby no obvious inheritance pattern is observed.

Diagnosis of the disease is extremely difficult. Many conditions may cause dementia which are not associated with AD. A comprehensive clinical diagnosis normally include a complete patient health history, a family history of dementia, a physical examination, brain imaging tests such as positron emission tomography and magnetic resonance imaging, a neurological or mental status assessment and various other cognitive tests. In the case of advanced dementia in the patient, a custodial respondant provides additional information regarding progression of the symptoms. The battery of evaluations can only lead to a possible or

probable diagnosis of AD. Confirmation of AD requires an autopsy of brain tissue.

AD neuropathology is characterized by extensive neuronal loss, and excessive accumulations of extracellular amyloid senile plaques (SP) and intracellular neurofibrillary tangles (NFT). These brain lesions are predominantly localized to neo-cortex, hippocampus and amygdala, areas which are associated with memory and intellectual function. SP are mainly composed of a dense core of amyloid-beta (Aß) peptides surrounded by dystrophic neurites which can be viewed using standard staining techniques and confocal microscopy. The Aß peptides are aggregated into fibrils with a ß-pleated sheet conformation. NFT are intraneuronal inclusions of paired helical filaments composed of abnormally phosphorylated tau protein. Hyperphosphorylated tau has also been found in the 'halo' surrounding senile plaques which are normally devoid of NFT.

The causes of AD are not known but evidence exists to support, at least in part, a genetic etiology. Genetic studies have thus far identified putative causal genes Amyloid Presursor Protein (APP), Presenilin-1 (PS-1) and Presenilin-2 (PS-2) which are associated with early-onset AD, and a susceptibility gene, Apolipoprotein E (ApoE), which is associated with late-onset AD. Mutations in all of these genes, with the exception of ApoE, account for affected pedigrees with relatively restricted phenotypes or population sources. The function of these genes and their role in AD pathogenesis are currently the subject of intense investigation, as are efforts to identify other AD genes. Available evidence in AD research suggests that AD etiology is multifactorial with influences from multiple genes and environmental factors.

The first AD candidate to be discovered was a gene on chromosome 21 that codes for APP. The gene was localized by linkage analysis in pedigrees with several affected members. This gene appeared to be a logical AD candidate since APP is a precursor protein which, after post-translational modifications, yields Aß. Accumulation of Aß appears to be a critical factor in the formation of amyloid plaques. However, APP

mutations which co-segregated with early-onset families could ultimately account for only a small percentage AD cases.

Another significant genetic marker was discovered when it was shown that the epsilon-4 (ε4) allele of the polymorphic ApoE gene was overrepresented in predominantly late-onset AD cases. The ε4 isoform was also associated, in a dose-dependent manner, with an increased deposition of Aß in AD brains and an earlier age at onset. *In vitro*, the various ApoE protein isoforms behave differently in generating outgrowth and branching of neurons, and have different binding affinities to Aß. Although many lines of evidence support the hypothesis that ApoE plays a direct role in AD pathology, no conclusions have yet been firmly established since the mechanism of disease onset is unknown. Although the ApoE ε4 association appears to be a fairly robust and consistently observed result, ApoE ε4 is neither necessary nor sufficient for disease onset. This precludes the possibility of using ApoE genotype for predictive testing of AD at the present time. However, ApoE may retain its usefulness as a pre-disposing genetic marker, especially when applied in the context of a complementary diagnostic tool along with other clinical, biochemical and genetic criteria.

Alpha-1-antichymotrypsin (ACT), an injury response protein, has been implicated in AD because of its colocalization with amyloid plaques in AD brain. Additionally, a polymorphism within the signal peptide sequence of ACT was shown to be associated with AD, especially in interaction with ApoE. However, this result is controversial since several studies have not been able to corroborate this association.

The most recently identified AD genetic loci are two homologous presenilin genes, PS-1 and PS-2. The predicted protein structure of these genes suggests that they are transmembrane proteins and may be involved in cellular signal transduction or calcium channelling. Numerous mutations in PS-1 have been identified but their functional significance is unknown and thus far, with the exception of an intronic polymorphism, have not been correlated with phenotypes other than early-onset AD cases. Defects in the PS-2 gene are at present correlated

only in Volga-Germans, a small founder population with a highly restricted gene pool.

AD appears to be a complex disease with multifactorial causes. Complex traits may segregate in a non-Mendelian manner which complicates the identification of genes involved in disease etiology because of parameters such as reduced penetrance, genetic heterogeneity, epistasis and phenocopy effects. In order to minimize these difficulties, the dissection of complex traits favours non-parametric genetic analysis methods and the use of a founder population. Genetic analysis strategies such as association studies correlate allele-sharing between case and control populations for a given genetic locus and the disease phenotype. Case-control association studies do not rely on parameters such as multiple gene interaction and environment. Young founder populations are populations which have recently undergone rapid growth from a small group of original ancestors and which have remained relatively isolated. These populations are useful for the identification of genes involved in complex disease because individuals from such populations are more likely to be ethnically homogeneous and to have inherited a single disease gene from a common ancestor.

Study Objectives

We tested the hypothesis that ApoE, ACT and PS-1, either alone or in interaction, can increase AD risk by performing a case-control association study of ApoE, ACT and intronic PS-1 polymorphisms in a founder population from the Saguenay-Lac-St-Jean area of Québec. Because of the difficulties associated with the accurate diagnosis of AD, all subjects in this study were selected on the basis of autopsy-confirmed AD cases (n=131) and elderly non-demented control subjects (n=157). We intend to determine if there are additional AD risk factors associated with these polymorphisms such gender, age and gene dose. Morphometric indices such as age of onset, and NFT accumulation will allow us to determine if the polymorphisms are correlated to severity of the disease.

CHAPTER 1

LITERATURE REVIEW OF AD GENETICS

1.1 AD etiology

Several lines of evidence support the hypothesis that AD etiology has a genetic component. AD shows familial aggregation patterns, resemblance to Down syndrome and concordance rates in twin studies, that are consistent with the notion that AD has a genetic etiology, but with a strong environmental component.

Family history appears to be an important risk factor for AD. Epidemiological studies have reported that first-degree family members of patients with AD have an increased risk of developing this disorder (Breitner et al., 1988; Fitch et al., 1988; Rocca et al., 1986; Silverman et al., 1994). Although familial clustering implies a genetic etiology, it does not prove that genes are the underlying cause of AD because environmental factors, to which members of the pedigree are commonly exposed, may also be responsible. A more compelling argument is the inheritance of the disease in several family members that can be followed in large AD pedigrees. It has been shown that susceptibility to AD is determined, in part, by a major autosomal dominant allele but also by other genetic or non-genetic factors (Farrer et al., 1991). Affected members of these pedigrees are referred to as "familial" AD cases as opposed to the "sporadic" form. In the latter, transmission of the disease does not resemble Mendelian segregation of a trait and may therefore be caused by environmental factors. However, these sporadic forms may nevertheless still be genetically based since they may actually be segregating as complex

traits with reduced penetrance and in interaction with genes or environment.

Patients with Trisomy-21 (Down Syndrome) have a genetic abnormality consisting of having three copies of chromosome 21 instead of the normal two. Trisomy-21 patients have many clinical and neuropathological features, such as dementia and amyloid plaques, which are indistinguishable from those of AD. However, Trisomy-21 individuals express these features at a much earlier age than the general AD population. These observations suggest that a dose effect via overexpression of a gene product on chromosome 21 could account for AD pathogenesis.

Twin studies are important in studying the genetic contribution of a disease. Monozygotic twins have an identical genetic background and therefore, any change in disease phenotype in one twin vs. the other is best explained by some external factor such as environment. Twin studies which have attempted to evaluate the genetic component of AD have been inconclusive. Some monozygotic twins have shown concordance with AD phenotype whereas others have not. A recent study demonstrated a high concordance rate for monozygotic twins, suggesting a strong genetic component in AD (Bergem et al., 1997). In another study, 3 monozygotic twin pairs examined by detailed neuroanatomic, neuropsychologic and neuropathologic criteria remained discordant for over a decade (Kumar et al., 1991) and 8 sets of monozygotic twins with early-onset and absence of ApoE ε4 or a family history of AD remained concordant for a period of 8-18 years (Breitner et al., 1995). The observation that some monozygotic twins show incomplete concordance and wide differences in age at onset suggests that penetrance is affected by additional factors apart from age in these twins (Nee et al., 1987).

A genetic predisposition cannot be ruled out in these twin studies but the inheritance of any predisposing genetic factors would most likely have been substantially modulated by environmental determinants in disease onset. As an example, one twin study showed that older female subjects

may show a reduction or a delay of risk of AD following long-term exposure to glucocorticoid or non-steroidal anti-inflammatory drugs (Breitner et al., 1994). Other potential environmental factors have been implicated in AD, at least as modifiers, by epidemiological studies. For example, factors such as smoking, prior history of depression, positive history of arthritis, high blood pressure, alcohol abuse and level of education have been shown to affect age at onset of AD (Rao et al., 1995). Therefore it is clear that AD can be classified as a complex disease since many different factors, both genetic and environmental, either in isolation or in interaction, can cause a single phenotype.

1.2 AD is a complex disease

The term complex disease refers to any phenotype that does not exhibit classical Mendelian recessive or dominant inheritance attributable to a single gene locus. Complex disease is caused by multiple genes (or gene products) interacting with each other, or by environmental factors, or both (Fig. 1). The multifactorial influences can complicate the simple correlation between genotype and phenotype one hopes to find in genetic studies. Thus, it is possible that one genotype can correspond to several phenotypes as a result of chance, environment or gene interaction.

In complex disease, individuals with reduced penetrance who inherit a predisposing allele may not manifest the disease. Others who inherit no predisposing allele may nonetheless possess the disease phenotype as a result of random or environmental causes (phenocopy). Penetrance may depend on factors such as age, sex, and interaction with other genes.

Polygenic traits require the simultaneous presence of mutations in multiple genes. Epistasis, the interaction of two or more genes, may be required to produce the disease phenotype. Epistatic effects complicate genetic analysis because no single locus is strictly required to produce a discrete trait.

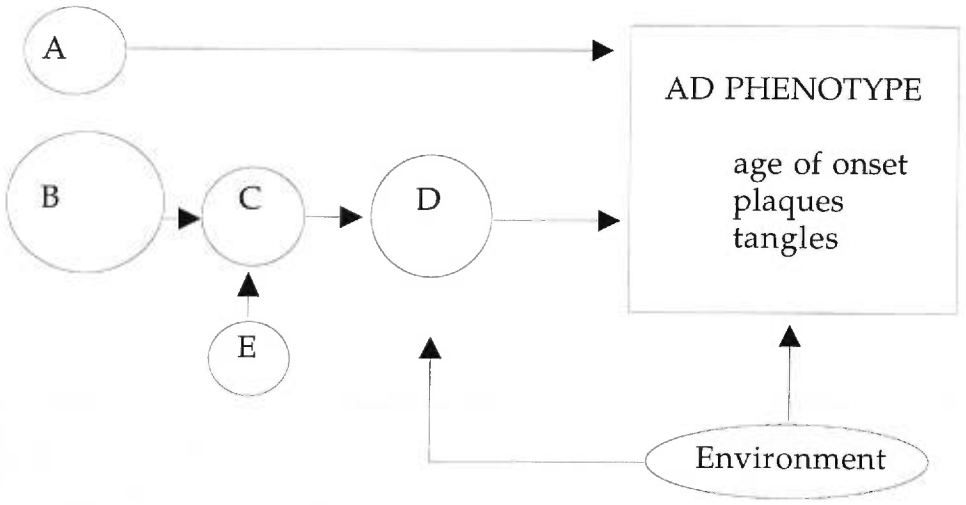

Fig 1. Cascade involving multifactorial influences in AD etiology. Gene products (A-E), as well as environment, may act alone or in interaction to produce the AD phenotype.

Conversely, one may also observe several genotypes which correspond to a single phenotype in complex disease. Locus or genetic heterogeneity arises when mutations in more than one gene give rise to the same disease phenotype. This may occur when the gene products encoded by the mutations are required for a common biochemical pathway. Locus heterogeneity can confound identification of disease-causing genes because an allele may cosegregate in some families, but not in others. Polygenic risk determinants and genetic heterogeneity may account for the variable onset and course of AD, as well as for possibly different etiologies in familial vs. sporadic AD. Apart from an apparent autosomal dominant transmission pattern in FAD, cases with typical FAD are phenotypically indistinguishable from the sporadic form of AD

(although certain clinical phenotypes of presenile FAD are sometimes different from the senile form, ie. increased incidence of myoclonies, rigidity, involontary movements, amongst others). While it is assumed that a major inherited gene defect is responsible for FAD, the sporadic AD phenocopy may ultimately be explained by environmental factors, or by different genes not involved in FAD, or both.

The interplay of many different factors produces a cascade of events which may have many different manifestations in the phenotype. In AD, phenotype characteristics such as age of onset and density of senile plaques and neurofibrillary tangles may be modified according to the severity of the disease. Barring environmental influences, the degree to which the phenotype is modified will ultimately be dependent not only on which genes, but also which variants (or polymorphisms) of these genes, participate in the cascade. Indices such as age of onset and density of senile plaques and neurofibrillary tangles can be measured quantitatively and can provide a means to gauge severity of the disease. Such indices are useful in the analysis of complex diseases because they provide information about the severity of the disease. Such information can be used for selecting the most severe forms of the disease for analysis or conversely, for identifying which genotypes are related to the disease severity.

1.3 Current strategies to identify AD genes

In order to understand disease etiology, it is necessary to understand the cascade of biochemical events which leads to disease and therefore, to know the identity of proteins participating in the cascade. Unfortunately, *a priori* knowledge of biochemical cascades leading to disease is rarely available, although certain strategies which rely on animal models and biochemical isolation techniques can generate good candidates. For example, transgenic mice with knockout or mutated genes can provide information on the *in vivo* consequences of mutating candidate genes.

The differences observed in the resulting phenotype may provide clues as to the function of candidate genes in the perturbation of the wild-type function, or generate new candidates for further analysis.

Alternatively, a statistical approach can be used to identify different gene products which contribute to the disease-causing process. This provides the possibility to understand the cascade of biochemical events which lead to disease without any *a priori* knowledge of the gene product's function. This approach is purely genetic. Genes implicated in the disease process are identified by gene mapping techniques using linkage analysis, allele-sharing methods or association studies. These genes are localized by positional cloning and characterized for mutations associated with the disease. The normal and aberrant protein functions are correlated to these gene mutations and then fitted to a model which describes the biochemical pathway leading to disease.

These gene identification strategies are based on the assumption that the recombinational rate between two genetic marker loci is proportional to the intermarker distance, ie. that alleles of a marker locus near the disease locus will recombine at a much slower rate than alleles of a marker locus that is farther away from the disease locus. Marker alleles which are very close to the disease locus will tend not to recombine during meiotic events and therefore, will tend to cosegregate with the disease locus. These alleles will be represented more often than expected and are said to be in linkage disequilibrium with the disease locus. The degree of significance of the disequilibrium is evaluated by various statistical methods of which there is a wide range of complexity.

The genetic approach is likely to be the method of choice for the identification, in step-wise fashion, of the major causal and modifier loci which contribute to complex disease cascades. The identification of a first susceptibility or modifier gene overcomes a major hurdle in the elucidation of the disease cascade, since subsequent searches for other implicated genes can be performed in samples whose genetic background is controlled for the known modifier. Recently, this approach was used to identify an additional putative AD locus on chromosome 12 by

performing a genome-wide search on families in which AD was not influenced by the known susceptibility gene, ApoE (Pericak-Vance et al., 1997). It is hoped that this iterative approach will yield all the major genes involved in the disease process.

Identification of all genes implicated in the AD cascade is important for several reasons. Firstly, the identification of various components of biochemical cascades, and how they affect cellular processes, will help to further the understanding of the function of these various proteins and lead to new avenues of research. Secondly, components involved in rate-limiting steps of the cascade could become potential drug targets in therapeutic strategies to slow or stop the progression of the disease. And thirdly, these new mutations should enable more precise diagnosis for better phenotyping of cases and thus, should increase the chances of identifying novel disease genes in the pathway.

1.3.1 Parametric linkage analysis

Genetic linkage strategies attempt to estimate the genetic distance (θ) between a genetic marker and a disease gene, and the likelihood of that estimate (lod score), from the observed cosegregation of marker alleles and disease phenotypes in informative meioses within family pedigrees. Recently, the power of this type of analysis has been augmented as a result of the availabilty of abundant and highly polymorphic microsatellite markers evenly spaced throughout the human genome. Genome-wide searches using these polymorphic markers have succeeded in identifying causal genes in numerous simple monogenetic diseases. This approach is very powerful provided that one specifies the correct model. For example, linkage analysis was used successfully in locating a locus involved in early-onset familial AD (Schellenberg et al., 1992). Restriction of the affected cases to the most severe phenotype, ie. very early age at onset, was probably critical for this success. However, for presumably more complex multifactorial diseases such as late-onset AD, this approach is limited due to the many parameters which must be known, or approximated, and incorporated in the analysis.

For example, in AD, the estimation of the penetrance parameter can be confounded by the age-dependent onset of AD in which some elderly asymptomatic carriers of the disease gene are erroneously classified as non-affected. The mode of transmission of the disease must also be known. Does the expected segregation of the disease gene fit that of an autosomal dominant, autosomal recessive or other, more complex, inheritance pattern involving gene-environment interactions? This parameter is easiest to estimate in very large FAD pedigrees but the mode of transmission may be confounded in smaller pedigrees where less segregation data is available and where the disease is caused by non-genetic factors.

A high frequency of the disease-causing allele in the population may also confound linkage lod scores because it becomes more difficult to statistically prove a Mendelian segregation of the disease allele in homozygotes. For example, the high frequency of the ApoE ε4 allele had interfered with traditional linkage analysis because the weak linkage to chromosome 19q was initially regarded skeptically until the exon 4 polymorphism was shown to be correlated with AD by association studies. A high frequency of the disease gene will also increase the chance that the disease can be acquired through a presymptomatic married-in parental family member whereas a low frequency favours the probability that the disease gene is inherited from the affected parental family member. Inability to detect phenocopies can also affect the estimation of disease gene frequency.

Another factor which can compromise linkage analysis is the availability and reliability of family data. Due to the lateness of onset of the disease, genetic data is not obtainable in many parental members of affecteds in multiplex families. The classification of carriers vs. non-carriers is more susceptible to error in multiplex families if classification is based solely on clinical evaluation of living family members and retrospective evaluation of deceased members, as opposed to the more reliable evaluation by autopsy. Non-paternity may also confound analysis by presenting incoherent segregation possibilities.

1.3.2 Non-parametric methods

Because of the problem in estimating linkage parameters for complex diseases, allele-sharing or non-parametric genetic analyses were developed. Allele-sharing methods involve testing whether the affected relatives inherit a chromosomal region identical-by-descent (IBD) more often than expected under random Mendelian segregation. Compared to parametric linkage analysis, allele-sharing methods are better suited to analysis of complex traits since they assume no model for the inheritance of a trait. Excess allele sharing can be demonstrated using a non-parametric approach even in the presence of incomplete penetrance, phenocopies, genetic heterogeneity and a high frequency of the disease gene. The trade-off is that this method is less powerful than a correctly specified linkage model and may require large sample sizes. A family-based example of this method is affected sib-pair analysis (Blackwelder and Elston, 1985; Kruglyak and Lander, 1995).

Linkage disequilibrium analyses use populations rather than families to identify markers that are so close to disease genes as to be in linkage disequilibrium with these genes. Therefore, these types of analyses test whether a particular allele occurs at a significantly higher frequency among affecteds than among unaffected control individuals. Linkage disequilibrium analyses can be in the form of either association studies, haplotype sharing studies (IBD mapping) or linkage disequilibrium mapping. The latter two forms are useful in genetic fine mapping studies which attempt to narrow down a large candidate region of interest. Association studies also provide a complementary strategy for detection of disease-susceptibility genes and are usually essential once the chromosomal location of a disease-susceptibility gene has been determined by linkage because they locate the gene much more precisely. Since association studies involve population correlation, they require a carefully selected control group which is properly matched for ethnicity. These genetically similar controls are used in lieu of non-affected family members used in linkage and allele-sharing methods.

Positive associations can arise for any of three reasons, one of which is artifactual. Firstly, a positive association can arise if the associated allele is a cause of the disease. In this case, the same positive association would be expected to occur in all populations. Secondly, a positive association can arise if the associated allele is in linkage disequilibrium with the disease-causing mutation (eg. in a nearby gene on the same chromosome). And thirdly, positive association can also arise as an artifact of population admixture producing misleading associations that are not due to a relation between the disease and the alleles tested, but rather to the different population allele frequencies between cases and controls.

1.3.3 Founder populations

Association studies are more suitable in young, genetically isolated populations. These so-called founder populations are useful for genetic analyses for several reasons. Firstly, due to rapid growth from a small number of ancestors, founder populations are more likely to be ethnically homogeneous, and therefore, are less likely to cause spurious associations arising from admixture effects. Secondly, the number of different disease-causing genes is likely to be fewer in a founder population than in a large mixed population. This is of particular importance in the genetic analysis of complex disease due to the possibility of genetic heterogeneity. Thirdly, since linkage disequilibrium extends over greater distances in founder populations, the number of disease-associated alleles is likely to be fewer in such populations (Hastabacka et al., 1992; Terwilliger 1995).

1.4 Amyloid Precursor Protein

AD is neuropathologically distinguishable by dense extracellular amyloid plaques in post-mortem brain sections. Amyloid plaques are composed of a specific form of protein known as beta-amyloid (Aß) and the accumulation of Aß is considered to be a hallmark biochemical marker

for AD. The 40-43 amino acid Aß peptide is derived by post-translational modification of a larger amino acid polypeptide called amyloid precursor protein, or APP (Fig. 2). APP has several isoforms which are generated by alternative splicing. The predominant isoforms are APP695, APP751 and APP770 and contain membrane spanning regions. The APP gene is located on chromosome 21q11.2-q21 and has 19 exons. Aß is encoded by exons 16 and 17.

Five missense mutations in the APP gene have been identified that cosegregate with affected members of some early-onset families, suggesting that APP is implicated in AD (Fig. 2). Three of these mutations occur in exon 17 at codon 717 near the carboxyl terminus of Aß. The most common APP717 mutation is a valine to isoleucine substitution and was identified in at least 9 families (Goate et al., 1991; Naruse et al., 1991; Pulst et al., 1991; Karlinsky et al., 1992). The other APP717 mutations are less common and have been identified in one family each: valine to phenylalanine (Murrell et al., 1991) and valine to glycine substitutions (Chartier Harlin et al., 1991). The fourth mutation, also rare, was identified in a Swedish pedigree (Mullan et al., 1992b). It is a double mutation in exon 16 of APP and located near the amino terminal of Aß. The mutation consists of normal lysine and methionine being substituted for asparagine and leucine at codons 670/671, respectively. The fifth mutation was recently identified and consists of an isoleucine to valine substitution at APP716 in a single pedigree (Eckman et al., 1997). All APP mutations cause early onset of AD and penetrance of the disease is complete by age 67 years at the latest. Mean ages of onset for affected family members carrying these mutations range from 43-59 years.

In contrast to the above mutations which flank the extremities of Aß, two other mutations occur within the Aß sequence of APP which result in cerebral hemorrhage with, or without, dementia. A combination of cerebral hemorrhage and presenile dementia is caused by a mutation at codon 692 which gives rise to an alanine to glycine substitution at amino acid 21 of Aß (Hendriks et al., 1992) while cerebral hemorrhagic amyloidosis of the Dutch type (HCHWA-D) is caused by a substitution of glutamine for glutamic acid at codon 693 (Levy et al., 1990). Heavy Aß

deposition, but no NFT accumulations, have been observed in HCHWA-D patients, suggesting that these patients may not be "pure" AD cases.

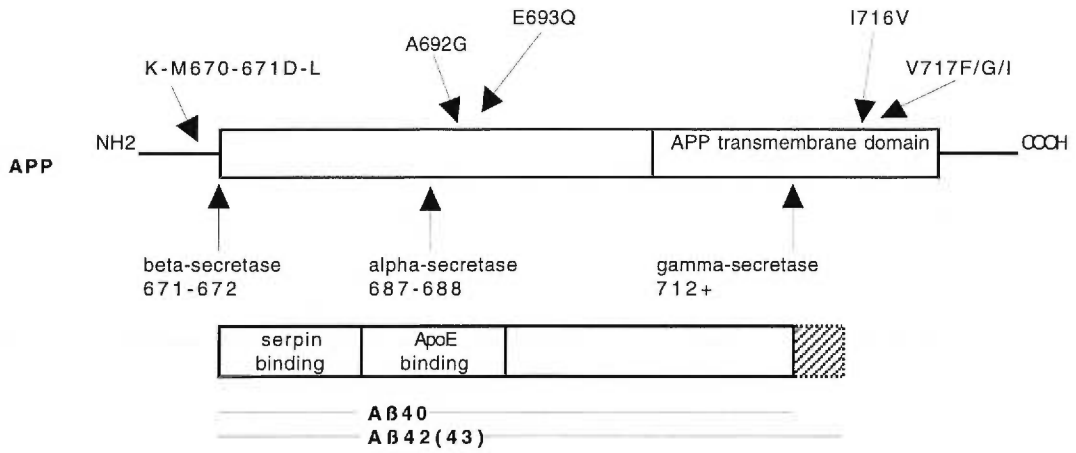

Fig 2. Structure of APP protein and Aß peptide. The sites indicated correspond to codons at which early-onset FAD-linked mutations arise, codons at which endoproteolytic cleavage of APP occur, and putative binding domains. (Diagram not to scale.)

Although linkage results obtained initially were suggestive for early-onset FAD and chromosome 21 markers (St George-Hyslop et al., 1987), subsequent testing of other early-onset and late-onset FAD pedigrees failed to corroborate an association with APP (Schellenberg et al., 1988; St George-Hyslop et al., 1990; Kamino et al., 1992). These findings suggest that the known APP mutations are an exceedingly rare cause of AD, accounting for only 5% of AD cases, and that other genetic loci are likely involved in these pedigrees. Although linkage analysis could not demonstrate cosegregation with chromosome 21 markers in numerous

late-onset and early-onset FAD families, it is possible that other mutations may exist on APP which have yet to be shown to be significant in AD, since the majority of mutation screens so far have concentrated on exons 16 and 17 of APP.

1.4.1 Role of APP in AD pathogenesis

The function of APP and its potential role in AD pathogenesis are not known. APP is a housekeeping gene that is expressed ubiquitously and has multiple alternate transcripts (Kang et al., 1987). The protein structure of APP suggests that it is an integral membrane glycoprotein with its membrane-spanning region inserted into the plasma membrane or membrane of some subset of organelles. Aß comprises 11-15 amino acids of the transmembrane domain and 28 amino acids of the extracellular domain of APP. At present, it is not known how amyloid is deposited extracellularly nor whether amyloid, or its precursor, actually play an active role in the development of AD. APP is a member of a multigene family of which APP is the only member with an Aß sequence. APP has homology to mammalian amyloid precursor-like protein (ALPL2), a protein which is present in postsynaptic compartments in the cortex and which is enriched in axon terminals of neurons, suggesting that these proteins have a potential role in neurite outgrowth or synaptogenesis (Wasco et al., 1992; Wasco et al., 1993; Slunt et al., 1994; Thinakaran et al., 1995). APP homologues have also been identified in *Drosophila* and *C. elegans* (Luo et al., 1992; Daigle and Li, 1993). Deletion of the *Drosophila* gene results in subtle behavioural deficits which are rescued by either the *Drosophila* or human genes, suggesting that there is a phylogenetic conservation of function among APP homologs and that these proteins have important cell functions.

Several arguments support the notion that Aß plays a central role in AD pathogenesis: 1) the Aß peptide is the major component of SP, and 2) all the inherited APP mutations in families with autosomal dominant inheritance of AD occur in the proximity of the Aß domain. *In vitro* experiments have demonstrated that FAD-linked APP mutations provoke an increase in Aß production. Transfected cells with the double

mutation at APP670/671, which is located adjacent to the Aß cleavage site that generates the Aß N-terminus, secrete elevated amounts of Aß (Citron et al., 1992). Cells with the mutation at APP717, which is proximal to the Aß cleavage site that generates the Aß C-terminus, overproduce a long form of Aß which is 42-43 amino acids long (Suzuki et al., 1994). Overproduction of Aß as a mechanism leading to amyloidosis is consistent with the observation that the brain pathomorphology of Trisomy-21 (Down Syndrome) individuals is indistinguishable from that of AD patients and argues for the possibility that a gene dose effect involving an over-expression of APP may explain the amyloidogenesis in both AD and Down syndrome individuals.

In neurons, the APP isoform is quickly metabolized down two possible pathways involving three unidentified proteases in normal APP proteolysis at the critical Aß site (Sisodia et al., 1990). The enzymes have not been identified and are temporarily referred to as α-, ß- and γ-secretases (Fig. 2). The first pathway involves cleavage of APP between residues 687 and 688 by an α-secretase, which is followed by cleavage at a position C-terminus of residue 712 by a γ-secretase, thus producing a 3 kDa truncated Aß peptide (Seubert et al., 1992). This pathway is not expected to be involved in AD pathogenesis since the α-secretase cleaves within the Aß fragment and does not produce an intact Aß peptide. The second pathway involves cleavage between residues 671 and 672 by a ß-secretase (Haass et al., 1992), followed by cleavage at a position C-terminus of residue 712 by a γ-secretase (Seubert et al., 1992). This pathway generates an intact 4 kDa Aß molecule. Since the locations of the five FAD-linked APP mutations are near the extremities of the Aß peptide, it follows that aberrant splicing involving ß- and γ-secretases could potentially be provoked by these mutations and thus, raises the possibility that aberrant splicing may be the mechanism of excessive Aß production in AD.

If Aß is directly involved in AD pathogenesis as a causative agent, it should be demonstrable that Aß is neurotoxic. For a long time, it was a matter of controversy in the AD research community whether Aß causes, or is simply a by-product of, neuronal degeneration and dementia. The

observations that Aß is a normally-occurring protein found in many cell types, is constitutively secreted in the cerebro-spinal fluid of humans and other mammals (Seubert et al., 1992), and that diffuse Aß staining can be found in brains of aged non-demented individuals, put in doubt the suggestion that Aß is neurotoxic. Several lines of evidence now support the hypothesis that it is the aggregation of Aß, rather than the presence of soluble Aß or non-fibrillar Aß, which is potentially neurotoxic. Additionally, the rate of Aß aggregation appears to be dependent on the different forms of the Aß peptide. The longer 42-43 amino acid form, or Aß42(43), is predicted to be more amyloidogenic than the shorter 40 amino acid form, based on the finding that the long form of Aß appears to be the major component of senile plaques (Roher et al., 1993) and has also been shown to form fibrils at a faster rate *in vitro* (Jarrett et al., 1993). The rate of Aß fibril formation has also been demonstrated to be accelerated *in vitro* as a result of the HCHWA-D mutation which is associated with families with an inherited hemmorhagic disorder characterized by Aß deposition in the cerebral blood vessels (Levy et al., 1990; Wisniewski et al., 1991). Moreover, transfected cells with the APP V717I mutation overproduce Aß42(43) peptides that readily form fibrils, possibly as a result of a longer hydrophobic C-terminus which can self-aggregate into fibrils more readily (Suzuki et al., 1994). Interestingly, transfected cells with the APP I716V mutation also produce an elevated ratio of Aß42(43), but this ratio is further increased in transfected cells with an artificial double I716V/V717I mutation (Eckman et al., 1997). These results suggest that these APP mutations cause increased production of Aß in an additive manner and provide additional support to the hypothesis that Aß accumulation is a critical factor in AD pathogenesis.

Generation of short and long forms of Aß is dependent on the cleavage site of the γ-secretase at the C-terminus of Aß (Fig 2). If γ-secretase cleaves at position 712-713, then the short 40 amino acid form of Aß is produced whereas cleavage after residue 714 produces the longer form of Aß. The mechanism for the differential cleavage is not yet clear but several possiblities exist: either different enzymes are involved in cleaving the different sites (Citron et al., 1996), or there is a reduced specificity of γ-

secretase, or there is cleavage distal to residue 714 followed by carboxypeptidase digestion of the end amino acids (Klafki et al., 1996; Tischer et al., 1996).

Additional evidence which came primarily from transgenic mouse models now strongly support the hypothesis that the formation of Aß fibrils via an accumulation of Aß is neurotoxic. Transgenic mice overexpressing mutant APP exhibit neurodegenerative effects and brain neuropathology which are similar to those found in AD (Games et al., 1995). In these mice, a PDGF-ß promoter, which targets expression preferentially to neurons of the cortex, hippocampus, hypothalamus and cerebellum of transgenic animals, drove the production of an APP mini-gene containing the FAD-linked V717F mutation. After 4-6 months, these mice developed senile plaques in the cortex, hippocampus and corpus collosum, but not in other brain regions, and plaque density in these regions increased with age. The age-dependence and neuroanatomical pattern of Aß deposition parallels that in AD. This transgenic animal model supports the hypothesis that APP expression and Aß deposition are primary events in AD neurodegeneration. However, one limitation of these mice as an animal model for AD is that they lack NFT, a hallmark characteristic of human AD pathology, but this could be due to species-specific factors in the neuronal response to injury.

Transgenic mice overexpressing K670N/M671L (APP Swedish mutation) exhibit learning and memory deficits after 9-10 months of age, but not before (Hsiao et al., 1996). These deficits were time-correlated with an increased amount of Aß, numerous amyloid plaques and Aß deposits which were localized to frontal, temporal and entorhinal cortex, hippocampus, presubiculum, subiculum and cerebellum. Moreover, the concentrations of Aß42(43) rose more markedly than did those of Aß40. Altered metabolism and production of Aß by APP variants was also demonstrated in an *in vivo* system using transgenic mice expressing a yeast artificial chromosome (YAC) containing either the entire mutant or normal APP gene. Compared with mice expressing normal APP, higher levels of both long and short Aß were detected in brains of mice expressing the Swedish APP mutation, while mice expressing the APP

V717I mutation produced elevated levels of predominantly long Aß (Lamb et al., 1997).

These accumulated findings suggest that amyloidogenesis is the first and critical step in the amyloid cascade which ultimately leads to AD. This hypothesis is referred to as the "amyloid cascade hypothesis" and predicts that all pathways of AD pathogenesis are triggered by events which make amyloid deposition more likely and that all other biochemical markers and clinical manifestations of AD occur after this first critical event. In the case of the APP pathway, these events correspond to either increased production of long Aß, or both long and short Aß. This hypothesis is further strengthened by the observation that increased production of long Aß may also be responsible for amyloidogenesis involving the other known early-onset genes, the presenilins (see below).

An alternative, but related hypothesis, is that secreted products of APP processing may play a role in AD pathogenesis by conferring protection from oxidative damage and other inflammatory insults to the brain. The observation that cerebrospinal fluid levels of secreted APP (sAPP) are significantly decreased in AD patients compared to control subjects (van Nostrand et al., 1992), and the finding that, *in vitro*, sAPP can protect neuronal cells from a variety of stresses, including glucose deprivation, EAA toxicity, and oxidative stress (Mattson et al., 1993; Schubert et al., 1993), suggest that one possible function of sAPP is to protect neurons from damage. Additionally, overexpression of APP in transgenic mice can increase the number of cortical synapses and protect against the neurotoxicity of the HIV gp120 protein (Mucke et al., 1994, Mucke et al., 1995). However, in contrast to the putative neuronal protection effects of sAPP *in vitro*, knockout mice in which the APP gene has been disrupted fail to thrive, but do not display clear evidence of neuronal degeneration except for abnormal diffuse reactive gliosis (Zheng et al., 1995). One possible explanation for this finding is that the APP deficit is being partially compensated by unknown factors in these mice.

The various pathways involving secretion of APP products are likely to be important in AD pathogenesis. The relationship between sAPP and

extracellular and intracellular Aß is not presently clear. It is not known whether altered cleavage of the Aß peptide has an effect on the amount of sAPP, the amount of intracellular Aß, or the amount of extracellular Aß deposited as SP. The effects of intracellular Aß in transgenic mice suggest that it is neurotoxic. Transgenic mice which produce non-secreted intracellular Aß via expression of an Aß minigene exhibited an unusual brain pathology consisting of marked neurodegeneration, apoptosis and gliosis in the hippocampus and cortex. Otherwise, these mice did not exhibit the usual AD characteristics such as extracellular Aß deposits, neurofibrillary tangles and dystrophic neurites. In contrast, transgenic mice which expressed secreted Aß via fusion of the Aß peptide with the N-CAM signal peptide, did not exhibit neuropathological or phenotypic alterations (LaFerla et al., 1995). This finding is important for two reasons: 1) it suggests that overproduction of intracellular Aß is indeed neurotoxic *in vivo*, but that in a secreted form, Aß peptide appears to have no effect on neurodegeneration, and 2) it suggests that intracellular Aß may mediate apoptosis possibly by overproduction of free radicals and the subsequent oxidative damage of cells. Recently, a newly-identified endoplasmic-reticulum-associated binding protein (ERAB) was shown to bind and translocate intracellular Aß to the plasma membrane in cultured cells (Yan et al., 1997). ERAB was also found to be overexpressed in neurons of AD, but not in normal, brains. These results suggest that interaction of intracellular Aß with ERAB may contribute to neuronal dysfunction in AD (Yan et al., 1997).

1.5 Presenilin genes

Apart from APP, only two other genes have been shown to cosegregate with early-onset AD. These genes are highly homologous and have been classified to a putative gene family, known as presenilins, in anticipation of additional and similar AD-causing genes. Clinical and neuropathological features of presenilin-linked individuals are non-distinctive from sporadic or late-onset AD.

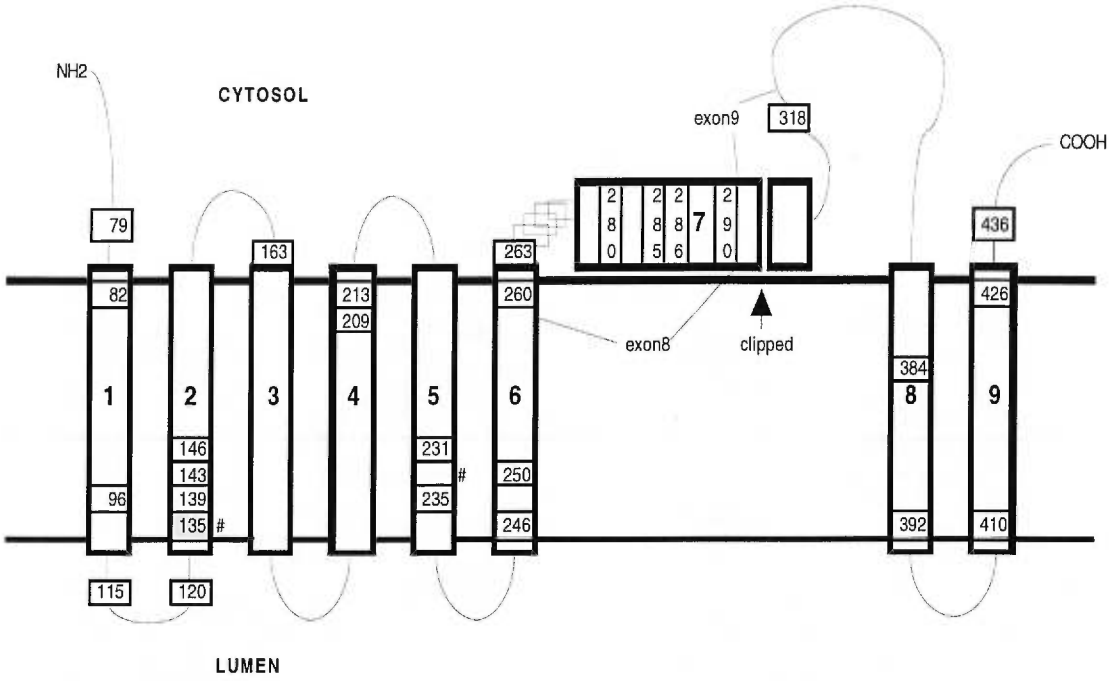

Fig. 3. Putative transmembrane structure of PS-1 (adapted from Hardy, 1997b). PS-2 protein structure is expected to be similar to that of PS-1. The residue numbers of PS-1 mutation sites are noted in rectangles. The two PS-2 mutations are indicated at homologous sites by #.

The presenilin-1 (PS-1) gene locus was identified by linkage analysis of early-onset FAD pedigrees to chromosome 14q24.3 and was subsequently confirmed in numerous other early-onset FAD pedigrees (Mullan et al., 1992a; Schellenberg et al., 1992; St. George-Hyslop et al., 1992; Van Broeckhoven et al., 1992). The gene, initially named S182, has been cloned and is predicted to encode for a 467 amino acid protein (Fig. 3)

with 7-10 hydrophobic transmembrane domains (Sherrington et al., 1995) although more recent studies suggest that PS-1 protein has 8 transmembrane domains with both the amino- and carboxyl-termini being cytoplasmic (Doan et al., 1996; Li et al., 1996). The coding region contains 10 exons numbered 3-12 (Alzheimer's Collaborative Group 1995). Alternative splicing in exon 8, and part of exon 3, is predicted to produce shorter isoforms of the protein. Alternative splicing may also produce a newly-described exon between exons 10 and 11, which is predicted to produce a 374-amino-acid isoform because it introduces a frameshift and a premature stop codon in exon 12 (Sahara et al., 1996). This mRNA species was found in liver and kidney, but not in brain, and therefore, may not be significant in AD pathogenesis. Mature PS-1 polypeptide is rapidly cleaved within the large hydrophilic loop to produce two fragments: a ~28 kDa N-terminal and a ~18 kDa C-terminal fragment (Podlinsky et al., 1997).

The presenilin-2 gene (PS-2) has been localized to 1q42.1 and was identified in Volga German pedigrees. Volga Germans are ethnic Germans who migrated to Russia in the 1760s, and later to the United States, and form a genetically distinct founder population presumed to have been descended from a common German ancestor (Bird et al., 1988). Linkage analysis had initially identified a Volga German FAD locus to chromosome 1q31-42 but the analysis was complicated by genetic heterogeneity in the sample of pedigrees (Levy-Lahad et al., 1995a).

PS-2 was subsequently cloned by virtue of its high homology to PS-1 (Levy-Lahad et al., 1995b; Rogaev et al., 1995). The proteins are 67% identical with the greatest homology, 84%, occuring in the hydrophic transmembrane region. The most divergent sequences are situated in the first hydrophilic loop at the amino end, and in the last hydrophilic loop at the carboxyl end. The great degree of homology in the transmembrane region is consistent with the hypothesis that this region is functionally important in AD. Although the PS-1 carboxyl-end loop contains many mutations and is therefore presumably of functional significance, it is curious that it would be so divergent from PS-2. One possible explanation

is that these domains in PS-1 and PS-2 affect AD pathogenesis in different ways.

More than 30 mutations in PS-1 have been identified (Fig. 3) in a variety of ethnic backgrounds (Alzheimer's Collaborative Group 1995; Rogaev et al., 1995; Campion et al., 1995; Cervenakova et al., 1995; Sherrington et al., 1995; Sorbi et al., 1995b; Tanahashi et al., 1995; Wasco et al., 1995; Chapman et al., 1996; Perez-Tur et al., 1996a; Rossor et al., 1996; Crook et al., 1997). Apart from one mutation which destroys a splice acceptor site for exon 9 (Perez-Tur et al., 1995b), all PS-1 mutations are missense mutations. Mutations have so far been identified in 7 out of the 10 exons, (4-9 and 11) with exons 5, 8, 9 and 11 accounting for about 75% of the mutations (Cruts et al., 1996). The majority of the mutations occur in the transmembrane region TM-2 and the hydrophilic loop HL-7 (Cruts et al., 1996). These regions are encoded by exons 5 and 8, respectively. The rest of the mutations are predominantly scattered in various regions of the hydrophobic domains or at the hydrophilic-hydrophobic junctions. Spacing of the mutations at roughly every 3-4 residues in TM-4, 5 and 6, and particularly in TM-2, suggests a possible interference with the α-helical structure of this domain (Alzheimer's Disease Collaborative Group 1995; Cruts et al., 1996). Ten missense mutations occur within the large hydrophilic loop near the carboxyl terminus of the protein and is encoded by the alternatively spliced exon 8. Mutation clustering here is interesting in that this domain is near the cleavage site and because of the existence of minor isoforms in which exon 8 is skipped (Cruts et al., 1996). This suggests that exon 8 may encode for an important functional domain which, when perturbed, leads to AD pathogenicity.

In contrast to the abundance of mutations identified in PS-1, only two mutations have been identified so far in PS-2: the N141I mutation in Volga German pedigrees (Levy-Lahad et al., 1995b) and the other, M239V, in an Italian pedigree (Rogaev et al., 1995). N141I and M239V are both missense mutations and correspond, respectively, to homologous sites N135 and M233 in TM-2 and TM-5 of PS-1. The N141I mutation site is also mutated at the homologous position (N135D) in PS-1 (Crook et al., 1997).

Mutations in PS-1 are associated with very early mean age at onset ranging from 35-55 years (Van Broeckhoven 1995). Penetrance generally is complete by age 60. Families with the same PS-1 mutation tend to have affected members with similar age at onset, thus further correlating these mutations with disease onset. Disease duration appears to be short ranging from 5.8-6.8 years and suggests that the PS-1 mutations cause a relatively severe form of AD. The N141I mutation is expected to be less malignant than PS-1 mutations due to a later mean age at onset (54.9 ± 8.4 years) and a longer mean duration of disease (7.6 ± 3.2 years). A wide variation of age at onset ranging from 40 to 75 years and the lateness of onset are features which distinguish the PS-2 mutations from other early-onset FAD mutations.

Although PS-1 mutations are associated with early-onset FAD, controversial evidence has linked PS-1 to late-onset AD. Prior to the cloning of PS-1, suggestive allelic association in marker D14S52 in families with mean age at onset 60-70 years was observed using affected pedigree member method, although linkage analysis had previously excluded chromosome 1 and 14 loci (Schellenberg et al., 1993). After the cloning of PS-1, the possibility that this locus was implicated in late-onset AD was again raised when it was demonstrated that an intronic polymorphism in PS-1 was significantly associated with late-onset AD (Wragg et al., 1996). The association was subsequently confirmed by at least two groups (Higuchi et al., 1996; Isoe et al., 1996b; Kehoe et al., 1996a and 1996b) but not by others (Scott et al., 1996a; Cai et al., 1997; Mann et al., 1997b; Scott et al., 1997; Tysoe et al., 1997b). Subsequent positive associations were also not as large as in the original report.

It is estimated that the PS-1 mutations account for about 75% of all early-onset cases, making these the most common early-onset FAD mutations. The PS-2 mutations are much less frequent than PS-1 mutations since analysis of additional presenile AD families without PS-1 mutations failed to identify any new mutations beyond the two already known, although it is possible that this observation is skewed by bias in selecting only the earliest-onset families for linkage analysis (Sherrington et al.,

1996). It is important to note that early-onset FAD accounts for less than 5% of all AD cases. Therefore, APP, PS-1 and PS-2 explain a relatively minor portion of the overall AD population. The remaining portion of AD remains to be explained, either by other genes or by environment, or by both. It is also possible that other missense mutations or polymorphisms in PS-1 or PS-2 will be discovered in sporadic AD cases.

1.5.1 Role of PS-1 and PS-2 in AD pathogenesis

Although the function of the presenilins is not known, their protein structure suggests that they function as a receptor molecules, ion channels or membrane structural proteins. All identified mutations occur in amino acids that are conserved in PS-1 and PS-2, suggesting that they are likely to be functionally significant. Because PS-1 and PS-2 are highly homologous, they are expected to have similar, but not identical, functions since a mutation in one is sufficient to cause AD. Both presenilins are expressed ubiquitously including in the brain (Levy-Lahad et al., 1995b; Sherrington et al., 1995; Levy-Lahad et al., 1996). In rat and human brains, PS-1 and PS-2 mRNA are present in neurons of hippocampus, cerebral cortex, cerebellum and choroid plexus (Kovacs et al., 1996; Suzuki et al., 1996). Homozygous knockout PS-1 mice possess skeletal deformations, impaired neurogenesis and neuronal cell death and die shortly after birth, although it is not clear whether neuronal impairment or defective formation of the axial skeleton is the lethal factor in these mice (Shen et al., 1997).

PS-1 is significantly homologous to two *Caenorhabditis elegans* genes: PS-1 has 48% homology to sel-12, and 24-37% homology to spe-4 (Van Broeckhoven 31a 1995). Mutations in sel-12 produce an egg-laying defect in *C. elegans*, probably through modulation of the signalling pathway involved in cell-cell recognition (Levitan et al., 1995). The finding that normal PS-1, but not mutant PS-1, can rescue sel-12 mutants suggests a functional similarity between the two proteins and that mutations in PS-1 are functionally significant (Levitan et al., 1996). In the same study, PS-1 cDNAs with 6 different AD-linked mutations showed a reduced ability to rescue sel-12 mutants, suggesting that these mutations cause, at least, a

partial loss of function of PS-1 protein. PS-1 mutations which cause a gain of function are yet to be identified. In PS-1 null mice, expression of Notch-1 and its ligand, Delta-1, are markedly reduced in the presomitic mesoderm (Wong et al., 1997). This observation suggests that PS-1 is involved in the Notch signalling pathway that is necessary for normal somite segmentation and axial skeleton development.

The other *C. elegans* homologous protein, spe-4, is involved in cytoplasmic protein partitioning during spermatogenesis (L'Hernault et al., 1992). One hypothesis arising from this homology is that the presenilins are involved in intracellular trafficking of APP, since the latter is normally processed in the Golgi apparatus. The observation that both presenilins localize to intracellular membranes, which are presumably Golgi and endoplasmic reticulum, is consistent with this hypothesis (Kovacs et al., 1996; Culvenor et al., 1997).

Indirect evidence that PS-1 is implicated in AD pathogenesis is provided by immunohistochemical analysis of AD brain using a C-terminal polyclonal antibody which localized PS-1 antigen to senile plaques (Wisniewski et al., 1995). Subsequent *in vitro* and *in vivo* experiments suggested that presenilin mutations probably cause AD by facilitating Aß deposition. *In vitro*, cells transfected with mutant presenilin produce longer Aß and amyloid fibrils (Borchelt et al., 1996; Maury et al., 1997; Tomita et al., 1997). Plasma from individuals with PS-1 and PS-2 mutations contain an increased ratio of Aß42(43), compared with non-affected control subjects and most late-onset sporadic AD cases, suggesting that increased production of Aß42(43) is caused by presenilin mutations as opposed to being an indirect result of disease state (Scheuner et al., 1996). However, elevated amounts of Aß42(43) in roughly 10% of late-onset AD cases suggests that overproduction of Aß42(43) may also be a cause of disease in a subset of late-onset cases. These observations are consistent with the amyloid cascade hypothesis, and support the notion that early- and late-onset AD pathologies are initiated by different causative agents (genes, environment) that undergo different primary events, even though the end pathology is almost identical.

Further evidence that presenilin mutations cause an increased production of Aß42(43) is offered by the finding that brains of individuals with PS mutations have a particularly abundant amount of long Aß (Cruts et al., 1996; Lemere et al., 1996; Mann et al., 1996a; Mann et al., 1996b). Aß42(43) is the predominant peptide species deposited within plaques in AD due to PS-1 and PS-2 mutations (Mann et al., 1996a; Mann et al., 1997a). In both PS-1 and PS-2 brains, the observation that lack of Aß40 in numerous plaques that concomitantly contain Aß42(43) suggests that Aß42(43) is the peptide form that is initially deposited as plaques, with Aß40 appearing later, and only in a subset of plaques. The finding that transgenic mice overexpressing mutant PS-1 produced significantly more Aß42(43) than transgenic mice overexpressing normal PS-1 (Duff et al., 1996; Citron et al., 1997) suggests that presenilin-encoded AD follows a pathogenic route involving Aß42(43), and that mutant presenilins provoke gain of deleterious function because normal endogenous PS-1 did not prevent the increase in Aß42(43) (Duff et al., 1996). This last claim is not well-established since the authors were able to demonstrate the presence of endogenous PS-1 mRNA, but not PS-1 protein.

An alternative, or perhaps downstream, AD pathway involves apoptosis. Transgenic mice with ALG-3, the mouse homologue of PS-2, appear to be capable of inhibiting apoptosis, suggesting that defective PS-2 may be involved in initiating programmed cell death (Vito et al., 1996). The observation that full length, but not cleaved, PS-2 induces apoptosis in cultured cells suggests that cleavage of PS-2 may be an important event in preventing induction or regulation of apoptosis (Janicki et al., 1997).

1.6 Apolipoprotein E

ApoE has also been implicated in AD. The ApoE gene is located on chromosome 19q13.2 and is linked to nearby Apolipoprotein C1 (ApoC1) and Apolipoprotein C2 (ApoC2) genes. Exon 4 of the ApoE gene is

polymorphic, with three major alleles named ε2, ε3, and ε4. The three alleles encode for different polypeptide isoforms which are differentiated by two variable codons at residues 112 and 158. The ε2 variant has a cysteine at both sites, while the ε4 variant has an arginine at both sites. The ε3 variant has a cysteine at residue 112 and an arginine at residue 158. The ε3 variant is the most common isoform and is considered to be the wild-type isoform.

Genetic analysis of late-onset FAD pedigrees using traditional linkage analysis and affected pedigree member method tentatively identified the ApoE gene as a risk factor or a susceptibility locus for AD (Schellenberg et al., 1987; Pericak-Vance et al., 1991). Subsequently, genotyping of polymorphisms in exon 4 of the ApoE gene demonstrated a strong association between ApoE ε4 allele and late-onset FAD; the ε4 allele frequency was 0.52 in affected subjects compared to 0.16 in age-matched control subjects (Corder et al., 1993; Saunders et al., 1993b). The ApoE ε4 association was rapidly confirmed in late-onset AD in familial studies, autopsied cases, and in clinic- and community-based familial and sporadic cases (Rebeck et al., 1993; Mayeux et al., 1993; Betard et al., 1994; Tsai et al., 1994; Yu et al., 1994). The ApoE association is a fairly robust result. Recently, in a large meta-analysis of ApoE data from 40 research laboratories, ApoE ε4 was shown to be a major risk factor across ethnic groups, gender and all ages between 40-90 (Farrer et al., 1997).

Numerous studies have demonstrated that AD risk is dependent on ApoE gene dose. The odds ratios (OR) for developing AD, compared with ε3/ε3 individuals, range from 2.2-4.4 for subjects with one ε4 allele and from 5.1-19.3 for subjects with two ε4 alleles (Mayeux et al., 1993; Poirier et al., 1993; Corder et al., 1994; National Institute on Aging, 1996). Some studies demonstrated a difference between the OR of ε4/ε4 vs. ε3/ε3 and the OR of ε3/ε4 vs. ε3/ε3 (Yoshizawa et al., 1994; St. Clair et al., 1995; Jarvik et al., 1996; Myers et al., 1996). In other studies, the large and overlapping confidence intervals did not allow one to establish this conclusion firmly (Mayeux et al., 1993; Dai et al., 1994; Kuusisto et al., 1994, van Duijn et al., 1994a; Lehtovirta et al., 1995; Tang et al., 1996). Since AD and death are competing risks in the aged population, any

factor which decreases age at onset will increase the observed frequency of the disease. Therefore, the dose effect may, to a certain extent, be explained by the observation that age at onset decreases with increasing ε4 dose (Strittmater et al., 1993a; Mayeux et al., 1993; Lucotte et al., 1995). Each ε4 allele lowers the age at onset by 7-9 years in late-onset FAD (Strittmater et al., 1993a; Payami et al., 1994), and by 3-7 years in late-onset sporadic AD (Poirier et al., 1993). However, in another study, no dose effect of ε4 was seen (Tsuda et al., 1994).

Some studies have observed a reduced frequency of ε2 allele in late-onset AD, suggesting that ε2 may confer a protective effect for AD (Corder et al., 1994; Talbot et al., 1994; Bickeboller et al., 1997). This notion is consistent with the finding that increased ε2 allele frequency is associated with longevity (Schachter et al., 1994) and suggests that the putative protective effect of ε2 may extend to other age-related risks apart from AD. However, no protective effect of ε2 was found in African-Americans (Maestre et al., 1995; Tang et al., 1996), and the ε2 allele frequency was significantly elevated in Italian sporadic and familial AD (Sorbi et al., 1994). In a Dutch population-based study of early-onset AD, subjects with genotypes ε2/ε2 and ε2/ε3 had a higher risk for AD, while subjects with the ε2/ε4 genotype had little or no additional risk (van Duijn et al., 1995).

The risk of developing AD may be modified by gender differences in carriers of ApoE, although the results are controversial. Gender associations with AD may be confounded by differential survival of the sexes in the elderly. One study demonstrated a gender difference in familial AD subjects although the sample had very few men older than 60 years of age (Payami et al., 1996). However, no sex difference was found in one study (Corder et al., 1995a), and later confirmed in a large sample consisting of 417 AD patients and 1030 controls from a French population (Bickeboller et al., 1997). In the latter study, the ApoE ε4 allele in control subjects was less frequent in males than in females. This may be due to early selection against ε4 male carriers, since it has been shown that ApoE ε4 confers risk of ischemic heart disease in middle-aged men, but not in aged men (Van Bockxmeer et al., 1992; Kuusisto et al., 1995). The lower incidence of ApoE ε4 in the older male population may also explain, at

least in part, the higher age-specific prevalence of AD in women reported in the literature (Breteler et al., 1992). There may also be an effect of gender on the ε4 dose effect. Males who are homozygous ε4 have a younger age at onset, whereas in females, one ε4 allele is sufficient to reduce age at onset, without further reduction in risk in the presence of another ε4 allele (Payami et al., 1994; Farrer et al., 1995). This result is in contrast to another study which could not demonstrate a gender difference in age at onset (Lucotte et al., 1995).

ApoE allele frequencies vary depending on ethnicity in human populations. The ε4 allele frequency is relatively high in African-American (0.26), intermediate in American Caucasian (0.14), and low in Asian (0.07) populations (Kamboh 1994). A decrease in the population ApoE ε4 allele frequency has been correlated with an expected decrease in the prevalence of AD in the Amish (Johnson et al., 1993) and Japanese (Kawamata et al., 1994) populations. These findings reinforce the ApoE association with AD. The ApoE ε4 allele association with AD has been consistently observed in several samples of various ethnic groups, including Caucasians (Rebeck et al., 1993; Mayeux et al., 1993; Strittmater et al., 1993a; Tsai et al., 1994; van Duijn et al., 1994a; Yu et al., 1994), Japanese (Noguchi et al., 1993), and Hispanics (Maestre et al., 1995; Tang et al., 1996). The extensiveness of the association in many different ethnic groups argues against the possiblity of an artifactual correlation due to population admixture.

However, the ApoE association in African-Americans is inconclusive. Although one study showed an association with ε4 in African-Americans (Hendrie et al., 1995), another study found an increased risk for ε4 homozygotes, but there was a significantly decreased risk for ε3/ε4 individuals compared to ε3/ε3 individuals (Tang et al., 1996). Yet another study reported that the association between ApoE ε4 and AD was limited to homozygotes, but a significant increase in risk was also seen for ApoE ε3/ε4 heterozygotes (Maestre et al.,1995). If ApoE ε4 is indeed a susceptibility factor in AD, then these results imply that African-Americans have either a modifier gene, or an environmental factor, that partially protects them from the effects of the single ApoE ε4 gene dose,

but not from a presumably more deleterious double ε4 gene dose. Another intriguing and unexpected observation is that a higher prevalence of AD is not seen in African-Americans in spite of the higher ε4 allele frequency in this population.

Recent studies have indicated that the ApoE effect in AD may be age-dependent (Sobel et al., 1995; Asada et al., 1996). Studies which have stratified families based on age at onset have demonstrated that although ApoE ε4 is overrepresented in all age groups, it is most dramatic in the <70 age group (Rebeck et al., 1994; Corder et al., 1995b; Maestre et al., 1995). One study demonstrated that the OR for developing AD with ApoE ε4 is different for different age groups (ie. <59, 60-69, 70-79, 80>), suggesting that the ApoE genotype does not exert its influence with the same magnitude within the whole period at risk for AD, but mainly in the 6th and 7th decade of life (Bickeboller et al., 1997). Recently, in a study using a large sample of AD families stratified by age at onset, it was shown that ApoE ε4 exerts its maximal effect in the age group 60-65, although a considerable effect is still apparent up to age 70 (Blacker et al., 1997). Age at onset was also significantly decreased in AD patients with 2 copies of ApoE ε4 compared to patients with either one or no copies of ApoE ε4 (66.4 vs. 72.0, respectively, $p<0.001$) (Blacker et al., 1997).

1.6.1 Role of ApoE in AD pathogenesis

Human ApoE is a 34 kDa glycoprotein consisting of 299 amino acids. ApoE is primarily synthesized in the liver, but is also found in brain and various other tissues. In brain, ApoE protein is synthesized primarily in microglia and astrocytes, and is involved in lipid transport in the cerebrospinal fluid (Mahley et al., 1988). The normal biological function of ApoE is in lipid metabolism. ApoE is a plasma cholesterol-transporting protein which is responsible for clearing remnants of chylomicrons and Very Low Density Lipoproteins (VLDLs) from blood via receptor-mediated endocytosis in liver and peripheral cells. ApoE plays an important role in the redistribution of lipids between various tissues from their sites of synthesis to their sites of utilization and excretion. ApoE binds with high affinity to LDL receptor and LDL-

receptor related protein (LRP). The efficiency with which ApoE binds to its receptors is dependent on ApoE isoform. Compared to ApoE ε3 isoform, ε4 isoform shows increased catabolism of lipoproteins, higher total plasma and LDL cholesterol, whereas ε2 has the opposite effect of ε4.

The observation that ApoE immunohistochemically localizes to both senile plaques and neurofibrillary tangles in AD brains (Namba et al., 1991) implies that ApoE has a biological role in AD. The ApoE association with AD is reinforced by the observation that AD patients with ApoE ε4 allele usually have an earlier age at onset (Corder et al., 1993; Locke et al., 1995), and a more rapid progression of the disease (Bennett et al., 1995), suggesting a possible link between ApoE genotype and AD pathogenesis. However, the correlation between ApoE and the rate of disease progression has not been well-established since it has been shown that disease duration is longer in ApoE ε4 carriers vs. non-ε4 carriers (Frisoni et al., 1995). It is possible that the ε4 association found in cross-sectional studies could, at least in part, be due to this phenomenon, rather than true risk conferred by the ε4 allele. However, data in which progression of AD is not related to ε4 gene dose argues against this hypothesis (Corder et al., 1995b). Two major lines of evidence, discussed below, support the hypothesis that ApoE is directly implicated in AD pathogenesis: 1) ApoE is associated with Aß deposition which leads to SP formation, and 2) ApoE is associated with tau leading to NFT formation.

In vitro studies have shown that ApoE binds with high affinity to Aß and that the ε4 isoform binds with greater affinity and forms Aß fibrils faster than the ε3 isoform (Strittmater et al., 1993b; Sanan et al., 1994). ApoE has also been shown to bind and transport hydrophobic peptides, such as soluble Aß, in cerebrospinal fluid in a manner that would prevent fibril formation (Wisniewski et al., 1993). Additionally, amyloid load and plaque density is correlated with ε4 dose in AD brains (Rebeck et al., 1993; Schmechel et al., 1993). These findings suggest that isoform-specific differences in ApoE may accelerate Aß deposition by increasing the rate of Aß fibril formation in AD brain. However, in another study, ε3 isoform bound with greater avidity than ε4 isoform, leading to the

contrary hypothesis of ε3 clearance, rather than ε4 deposition, of Aß (LaDu et al., 1994).

Additional evidence which supports the hypothesis that ApoE plays a central and direct role in AD pathogenesis comes from the observation that in AD brain, ApoE and low-density lipoprotein-related receptor protein (LRP) colocalize to senile plaques, and the observation that anti-LRP immunoreactivity is greater in reactive astrocytes and senile plaques of AD patients compared to controls (Rebeck et al., 1993). Since LRP recognizes and binds ApoE-containing lipoproteins, it was proposed that LRP plays a role in Aß clearance in the neuropil by receptor-mediated endocytosis of ApoE-Aß complexes. Compared to ε3 isoform, the ε4 isoform may be associated with an impaired or reduced capacity for Aß clearance because of either altered ApoE-Aß interaction or lower levels of ApoE. This notion is consistent with the finding that there is a higher level of Aß burden and SP in ε4/ε4 AD individuals (Rebeck et al., 1993; Schmechel et al., 1993).

It has also been proposed that ApoE is implicated in neuronal repair based on the observation that ApoE levels dramatically increase in astrocytes after peripheral nerve injury (Muller et al., 1985; Ignatius et al., 1986; Mahley, 1988). The finding that in primary neuronal cultures, ApoE ε3 complexed with VLDL increases neurite outgrowth, whereas complexes containing ApoE ε4 are inhibitory (Nathan et al., 1994), suggests that isoform-specific differences in ApoE may have an effect on normal nerve maintenance and repair of neurons that occur during remodeling of synaptic connections. It has been proposed that AD patients with ApoE ε4 lose neuronal function as a consequence of a reduced capacity for neuronal regeneration (Poirier, 1994). This hypothesis posits that ApoE is secreted in astrocytes as a result of Aß accumulation which is presumed to be neurotoxic. The function of ApoE in this context is to scavenge and sequester cholesterol to neurons. The cholesterol is then presumably used for neurite outgrowth and synaptogenesis. An increased supply of ApoE to injured sites which contain Aß, and an increased binding affinity in ApoE ε4 isoform, may provoke an increased rate and efficacy of Aß deposition in brain. It is also

possible that the increased binding characteristic of ε4 isoform reduces the amount of freely circulating ApoE to combat the neurotoxicity of Aß. However, the observation that ApoE ε4 non-demented individuals also sometimes exhibit amyloid plaque formation argues that ApoE ε4 may promote Aß deposition independently of other features of AD (Polvikoski et al., 1995).

ApoE has also been shown to interact with tau, the major protein component of NFT. Tau protein is found in normal nerve cells and its main function is thought to be related to maintaining the integrity of the nerve cytoskeleton by directing the assembly and disassembly of microtubules. Proper microtubule assembly is essential for proper transport of nutrients and other materials to and from different regions of the neuron. This activity is mediated by a complex pathway of intracellular and extracellular signals such as phosphorylation of tau protein. Hyperphosphorylation of tau in AD affected nerve cells may cause deregulation of microtubule assembly resulting in the loss of tau's ability to bind microtubules. This deregulation may prevent proper neural maintenance and structure, and is possibly a prerequisite event for the disruption of the neural cytoskeleton, and consequent NFT formation, events which may ultimately lead to nerve cell death.

ApoE has been localized to the neuronal cytoplasm (Han et al., 1994) and is associated with neurofibrillary tangles in AD (Namba et al., 1991, Crowther 1993). *In vitro*, hyperphosphorylated tau does not complex with ApoE ε3 nor with ε4. However, non-phosphorylated tau binds with high avidity to ε3 isoform but with virtually no binding affinity to ε4 isoform (Strittmater et al., 1994). This observation has led to the hypothesis that individuals with ApoE ε4 are more at risk for developing AD due to decreased levels of ε3 isoforms, which prevents hyperphosphorylation of tau in the normal aging brain. Binding of ApoE ε3 with tau may help stabilize microtubules and the cytoskeleton, and perhaps helps maintain the structure and function of neurons. The binding of ε3 to tau might also inhibit phosphorylation of tau and thus, retard the formation of paired helical filaments involved in the development of NFT. However, in contrast to Aß, ApoE ε4 has not been

correlated with NFT density in AD brain and an *in vivo* association of tau with ApoE has not yet been demonstrated (Gomez et al., 1996).

Inflammatory events may also contribute to AD. Recent evidence has demonstrated that a secreted derivative of ß-APP (sAPP-α) can induce inflammatory reactions in microglia and enhance their production of neurotoxins, suggesting that increased amyloidogenic processing could adversely affect the balance of sAPP activities that determine neuronal viability (Barger et al., 1997). Prior incubation of sAPP-α with ApoE ε3, but not ε4, blocks its ability to activate microglia (Roses, 1996). These results suggest that ApoE ε3 may confer protection in the AD cascade resulting from an inflammatory pathway.

The observation that plasma lipoproteins from ApoE deficient mice are more susceptible to *in vitro* oxidation than lipoproteins from wild-type mice suggests that ApoE may play a role in protection from oxidative damage (Hayek et al., 1994). *In vitro* experiments demonstrate that ApoE has allele-specific effects in protecting a rat neuronal cell line from oxidative cell death, with ε2 the most effective, ε3 moderately effective and ε4 being the least effective (Miyata et al., 1996). In the same allele order, ApoE at levels found in the cerebrospinal fluid also protected cells from Aß peptides, while higher doses of ApoE led to increased cytotoxicity, particularly for ε4 isoform (Miyata et al., 1996). These results suggest that ApoE is a multifunction protein which functions not only as a lipoprotein transporter but also as a protein with antioxidant activity. The latter property may be involved in AD pathogenesis and general longevity via isoform-dependent protection from antioxidative damage in neuronal cells.

1.7 Alpha-1-antichymotrypsin

ACT is a single glycoprotein of about 68 kDa belonging to a class of proteins known as serine protease inhibitors, or serpins. ACT is

synthesized predominantly in the liver but is also found in other cell types such as astrocytes (Pasternak et al., 1989). Its target substrates include neutrophil cathepsin G and mast cell chymase, proteins which are capable of converting angiotensin I to the biologically active vasoconstrictor angiotensin II *in vitro* (Reilly et al., 1982). The ACT gene has been cloned and sequenced (Chandra et al., 1983), and maps to 14q31-q32.2, which is about 30 cM downstream from PS-1. It has 42% homology at the amino acid level to α-1-antitrypsin (PI). Both proteins are acute-phase reactants which are expressed in higher concentrations in plasma during inflammatory states or infection.

ACT was first implicated in AD by immunochemistry studies which localized the ACT protein in AD brain to amyloid deposits (Abraham et al., 1988). Antisera raised against partially purified amyloid filaments were used to screen a cDNA library expressing proteins identical, or related, to AD amyloid deposits. DNA sequencing in positive clones identified the protease inhibitor α-1-antichymotrypsin. Presence of the protein was confirmed by light and electron microscopy to be present in AD and in non-demented aged brain amyloid deposits, but not in the amyloid lesions of Creutzfeld-Jacob disease. This observation suggests that ACT may have similar roles in AD and normal aging as opposed to a general response to cerebral amyloidosis. Visualization under electron microscopy with double-labelling of ACT and Aß on purified amyloid fibers indicated that the two proteins were tightly associated in amyloid deposits. The level of ACT mRNA was enhanced in AD brain compared with control brain. The mRNA levels were highest in hippocampus and inferior temporal gyrus, two areas known to be selectively abundant in SP and NFT in AD brains. In contrast, the precentral gyrus, which is often not affected in AD, contained a low level of ACT mRNA. The increase in mRNA in affected areas of AD brain was paralleled by a corresponding increase in the level of protein. Compared with the normal brain, AD brain showed enhanced levels of soluble ACT protein in those areas that contained abundant amyloid deposits. The specificity of localization of ACT mRNA and protein and the tightnesss of association with amyloid fibers suggest that ACT plays a role in the formation of AD amyloid deposits (Abrahams et al., 1988).

ACT protein has been shown to accelerate amyloid filament formation at least ten-fold *in vitro* (Ma et al., 1994). In the same study, ApoE was also shown to promote Aß fibril polymerization. ApoE ε4 had the highest capacity for fibril formation, with ApoE ε3 and ACT being about equal, and ApoE ε2 the least, of the four categories. The rate of polymerization also depends on the form of Aß: in previous experiments by this group, Aß40 was shown to be less effective at fibril formation in the presence of ACT and ApoE. The ability of ACT and ApoE to promote fibril polymerization *in vitro* suggests that their *in vivo* function is to act as pathological chaperones that promote Aß deposition in highly specific regions of the brain (Ma et al., 1994), possibly via a mechanism in which ACT and ApoE each recognize and bind to Aß, but at different sites, and with different degrees of specificity. The region containing amino acids 1-12 of Aß closely resembles the conserved active site of serine proteases and *in vitro* experiments have identified that the region is specifically recognized and bound by ACT as a pseudosubstrate (Potter et al., 1992; Fraser et al., 1993), suggesting that this region is a natural target for protease inhibitors such as ACT. In contrast, ApoE interacts primarily with the region containing amino acids 12-28 of Aß, a sequence predicted to form the ß-pleated sheet conformation found in amyloid filaments (Wisniewski et al., 1992; Strittmater et al., 1993a).

Additional evidence linking ACT to AD was provided by the finding that a polymorphism in the signal peptide of ACT appears to be associated with a significantly increased risk for AD (Kamboh et al., 1995a). The AA genotype frequency of the ACT polymorphism was overrepresented in AD cases compared to controls (0.351 vs. 0.270, respectively; $p<0.02$). The OR for developing AD for AA vs. non-AA carriers was 1.5 (95%CI=1.1-2.1; $p=0.04$). Two studies have so far confirmed the ACT association (Thome et al., 1995; Yoshiiwa et al., 1996). However, no association nor interaction was seen in several other studies (Haines et al., 1996; Muramatsu et al., 1996; Nacmias et al., 1996; Talbot et al., 1996; Didierjean et al., 1997; Fallin et al., 1997a; Helisalmi et al., 1997; Morgan et al., 1997; Murphy et al., 1997; Tysoe et al., 1997a).

1.8 Gene interaction studies

What evidence is there to support the hypothesis of gene interaction in AD etiology? The observation that family history and ApoE genotype are good predictors of disease provide evidence which support the notion that AD risk is modified by an interaction of ApoE and other genetic loci. Logistic regression analysis of families stratified by ε4 and non-ε4 carriers in the proband indicates that ApoE genotype (p=0.001) and family history (p=0.018) were significant in the prediction of age at onset of AD, and positive family history was generally associated with earlier mean age at onset, especially in the ε4ε4 genotype (Jarvik et al., 1996). In a community-based sample of both early- and late-onset AD, the association of ApoE was stronger in subjects with a family history of AD than in those without (Perez-Tur et al., 1995a; Jarvik et al., 1996). Similar effects of ApoE genotype and family history have been demonstrated in several population-based and clinic-based samples (Jarvik and Wijsman 1994; van Duijn et al., 1994a; Farrer et al., 1995; St. Clair et al., 1995). These observations suggest an interaction of ApoE and other, currently unknown, genetic and/or environmental factors.

Segregation analysis involves fitting a general model to the inheritance pattern of a trait in pedigrees. Using a model involving the presence of a simple Mendelian factor in a background of multifactorial inheritance, one tries to estimate key parameters such as the allele frequency, penetrance, and proportion of cases explained by the Mendelian factor. Segregation analysis can be sensitive to ascertainment of families (eg. preferential inclusion of affecteds will overstate the penetrance), and may have little ability to distinguish among the many possible modes of inheritance in complex traits. It may also have difficulty in estimating the number of genes influencing a trait and in identifying penetrance associated with multiple loci.

Several studies have attempted to determine the mechanism of disease transmission using complex segregation analysis of AD families, taking into account the effects of ApoE genotype (Rao et al., 1995; Jarvik et al., 1996; Rao et al., 1996). Complex segregation analysis was used to determine whether the genetic models in families differed as a function of the proband's ApoE genotype, as would be expected if the ApoE genotype influences risk of AD. This analysis indicated that a Mendelian model of transmission was not compatible with the data for families of both ε4 and non-ε4 probands, and that the parameter estimates of the most parsimonious model are different for ε4 and non-ε4 families (Jarvik et al., 1996; Rao et al., 1996). Complex segregation analysis in a large sample of ApoE-stratified families could not explain transmission of AD by a simple genetic or non-genetic model: models postulating sporadic occurrence, no major gene effect, random environmental transmission and Mendelian inheritance were all rejected (Rao et al., 1996). Their data suggest that transmission in families with probands with at least one ε4 allele best fitted a dominant model, whereas in families lacking ε4, transmission of AD was best explained by a single gene inheritance model. However, a more complex genetic model, or multiple genetic models, may ultimately account for risk in the latter group. These segregation analyses support a genetic model in these families that includes the effects of loci other than ApoE, and that these heritable loci operate in interaction with ApoE.

Although several genes have been shown to be associated with early-onset AD, ApoE is the only locus which has so far been shown to be associated with late-onset AD. In twin studies, there was no significant difference in the rate of ApoE ε4 allele between twin pairs concordant and discordant for AD (Bergem et al., 1997), suggesting that ApoE is not the major causal locus, but is more likely to be a susceptibility locus that can modify the rate of progression of AD. ApoE is estimated to account for only half of the genetic etiology of late-onset AD (Roses et al., 1995b). Therefore, the hunt for additional genetic mechanisms in late-onset AD continues. The strong clustering of cases in a large inbred Amish pedigree in which all 6 affected late-onset AD family members had ApoE ε3/ε3 genotype suggests that a genetic form of AD different from ApoE-

related AD is operating within this pedigree (Pericak-Vance et al., 1996). The older age and the later age at onset in this pedigree is concordant with previous findings of age stratification of risk in ApoE (Corder et al., 1995b) and further suggests that the AD dementia in this Amish pedigree is not related to the ApoE gene. These types of pedigrees are suitable for genetic screening for additional AD genes.

One candidate for gene interaction with ApoE is ACT. The ApoE ε4 gene dose effect associated with AD risk appears to be modified by ACT genotype, according to a study on late-onset AD by Kamboh et al. (1995a). Compared to no copy of ApoE ε4, the OR for developing AD was higher with two copies of ApoE ε4 (OR=11.1, 95%CI=4.7-26.4; $p<0.0001$) than with one copy of ApoE (OR=3.5, 95%CI=2.4-5.0; $p<0.0001$). However, in the presence of ACT AA genotype, the OR increased to 6.4 (95%CI=3.1-13.4; $p<0.0001$) for individuals with one copy of ApoE ε4 and to 34.0 (95%CI=4.3-272.5; $P<0.001$) for two copies of ApoE ε4. Their results suggest that an interaction between ApoE and ACT is involved in AD pathogenesis.

Studies in familial early-onset AD have not yielded significant evidence for a relationship between ApoE ε4 and the risk for developing early-onset AD. ApoE ε4 allele frequencies in family members with presenilin mutations did not differ significantly from controls (Saunders et al., 1993a; Saunders et al., 1993b; Van Broeckhoven et al., 1994). In PS-1 families, ApoE genotype was found not to influence age at onset (Van Broeckhoven et al., 1994; Levy-Lahad et al., 1995c; Brice et al., 1996). Prior to the identification of PS-2, no association was found between ApoE and age at onset in Volga German families (Levy-Lahad et al., 1995a). However, the analysis was redone using only the families with the PS-2 N1411 mutation and the results indicated the possibility of a small epistatic effect of ApoE and PS-2 (Bird et al., 1996). In total, these results argue that the presenilin AD pathway probably does not involve ApoE. However, the presenilin mutations identified to date are generally associated with very early ages at onset and are presumed to be relatively severe. Therefore, it is possible that overriding factors in presenilin genes may mask the effects of subtle interactions with ApoE.

Interaction data for APP is scant due to the rarity of APP FAD pedigrees. In one early-onset AD family with the APP Swedish mutation, a younger onset age was seen in patients with ApoE ε4 although this difference was not statistically significant (Alzheimer's Disease Collaborative Group 1993). In other pedigrees with the APP717 mutation, presence of ε4 appears to lower the age at onset whilst presence of ε2 has the opposite effect (Brooks et al., 1995; Hendrie et al., 1995; Nacmias et al., 1995; Sorbi et al., 1995a).

Interestingly, an ε4 association was found in early-onset AD cases who had a family history of dementia (van Duijn et al., 1994b; Perez-Tur et al., 1995a). In the van Duijn study (1994b), ApoE ε4 homozygosity was sufficient to increase the risk of both familial and sporadic early-onset AD, regardless of family history, suggesting that ApoE ε4 homozygosity may be sufficient to increase the risk of early-onset AD in the absence of other genetic factors. In ApoE ε4 heterozygotes, an increased risk could only be shown in subjects who had a positive family history of dementia, suggesting that in ApoE ε4 heterozygotes, interaction with other genetic or environmental factors may be necessary to increase the risk of early-onset AD. Taken together, these results suggest that early- and late-onset AD may have common etiologies involving modification of the ApoE-genotype effect by family history. In another APP-linked early-onset AD pedigree, all clinically affected family members had the APP717 mutation and were ApoE ε3/ε4 while an asymptomatic carrier of the APP717 mutation, at 2 SD above the mean age at onset, lacked the ε4 allele (St George-Hyslop et al., 1994). The effect of the ApoE ε4 allele on age at onset in these pedigrees suggests a possible gene interaction between APP and ApoE.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cases and controls

The case cohort consisted of 131 autopsied AD cases from the SLSJ area of Québec (40 males, 91 females). The majority of cases were selected from amongst families with at least 2 family members with affected possible or probable AD, according to genealogies reconstructed from the SOREP database (Gauvreau et al., 1988). Age of onset was determined by informant questionnaires and medical records. The cohort had a mean age at onset of 70.8, SD=8.8 years, and was primarily composed of late-onset AD cases. Diagnostic assessment of definite AD was made by neuropathologists. A definitive diagnosis of pure presenile (onset < 65 years old) or pure senile (onset > 65 years old) AD required all of the following criteria to be met: 1) fulfilment of the NINCDS-ADRDA criteria (McKhann et al., 1984) for probable or possible AD, prior to death; 2) exclusion of other dementing non-AD disorders (ie. brains with neuropathological Lewy Body and cardiovascular disease components were excluded from the cohort); and 3) histopathological confirmation of AD. The latter was based on a modified Khachaturian scale which required the presence of at least 20 neurofibrillary tangles mm^{-3} and 10 senile plaques mm^{-3}, irrespective of age, in at least two sections of the limbic lobe and lateral neocortex, after screening for areas richest in degenerative changes on modified Bielschowsky stains. SP density measurements included both diffuse plaques and neuritic plaques. Morphometric indices including SP and NFT densities were measured by two neuropathologists and averaged to obtain the final density measures. Mean density of SP was 35.3 , SD=13.8 mm^{-3} and mean density of NFT

was 30.5, SD=12.5 mm^{-3}. DNA for all AD cases was obtained from snap-frozen brain tissue.

The control cohort consisted of 157 elderly subjects randomly selected from amongst a group of non-demented control subjects whom had previously been recruited for a population-based study on health and aging in the SLSJ area of Québec (Canadian Study of Health and Aging Working Group, 1994). The cohort included 71 males and 86 females. All control subjects in this study were screened for cognitive impairment by Modified Mini-Mental State (3MS) Examination (Teng and Chui, 1987). Subjects were classified as non-demented if they obtained a cut-off score of 78% or greater. Subjects with scores between 61-77% had to pass additional neuropsycological testing before being classified as non-demented. No subjects with 3MS test scores of less than 61% were included in this control cohort. At the time of evaluation, the mean age of subjects was 82.4, SD= 5.5 years. DNA from control subjects was obtained from blood samples.

2.2 DNA extractions

2.2.1 Brain tissue

High molecular weight genomic DNA was extracted from -80° C frozen brain tissue for use in PCR amplification. Freshly thawed brain tissue (100 mg) was homogenized in 0.5 mL of TRI-Reagent™ (Molecular Research Center) with a PELLET PESTLE™ (Kontes) mixer motor and stem directly in a 1.5 mL tube. Another 0.5 mL TRI-Reagent was added to the tube, mixed thoroughly and stored at room temperature for 5 min to permit complete dissociation of nucleoprotein complexes. Next, 0.2 mL chloroform (Fisher) was added and the mixture was incubated for 2-3 min and centrifuged at 12000 G for 15 min at 4° C. The upper aqueous phase containing mRNA was removed and 0.3 mL of 100% absolute ethanol was added to the remaining interphase and organic bottom

phase. The sample was mixed by inversion, stored at room temperature for 2-3 min and the DNA sedimented by centrifugation at 2000X G for 5 min at 4°C. The supernatant containing protein was removed. The DNA pellet was washed 3 times with 1.5 mL of 0.1 M sodium citrate (Fisher) in 10% ethanol (BDH). In between washes, the pellet was incubated in the washing solution for 30 minutes with periodic mixing and centrifuged at 2000 g for 5 minutes at 4°C. The DNA pellet was resuspended by adding 1.5 mL of 75% ethanol, stored for 10-20 min at room temperature and centrifuged at 2000 g for 5 min at 4°C. The DNA pellet was dried for 5-10 minutes under vacuum and resuspended in 0.3 mL of 8 mM sodium hydroxide (Fisher). The pellet was slowly passed through a pipet tip to ensure full solubilization of the DNA and any insoluble material was removed by centrifugation at 12000 g for 10 min at 4°C. The resulting supernatant containing DNA was transferred to new tubes and the pH adjusted to 8.4 with 0.1 M HEPES (Boerhinger Mannheim). An aliquot of the DNA was used to measure absorbance at 260 nm in order to determine its concentration. One absorbance unit equalled 50 µg double-stranded DNA/mL.

2.2.2 Whole blood

High molecular weight DNA was extracted from -80° C frozen whole blood preserved in EDTA with the WIZARD™ Genomic DNA Purification Kit (Promega) for use in PCR amplification. All spins and incubations were carried out at room temperature. The whole blood was thawed and mixed by inversion. 300 µL of the blood was pipetted into a tube containing 900 µL of WIZARD™ Cell Lysis Solution. The cells were gently lysed by incubating for 10 min with rocking followed by a 30 s spin in a microfuge at 13000 rpm. As much of the supernatant as possible was removed and the pelleted white cells were resuspended by vigorous vortexing. 300 µL of WIZARD™ Nuclei Lysis Solution and 1.5 µL WIZARD™ RNase Solution were added to the tube and mixed by inversion. The solution was incubated at 37 C for 1 h followed by addition of 100 µL of WIZARD™ Protein Precipitation Solution. The solution was then vortexed and spun at 13000 rpm for 3 min. The supernatant was carefully decanted to a clean tube containing 300 µL

isopropanol (BDH) and gently mixed until the DNA came out of solution. The solution was then centrifuged at 13000 rpm for 1 min. The pellet was washed with 300 µL 80% ethanol and centrifuged again. The supernatant was removed by decanting and the pellet allowed to air dry. 100 µL of WIZARD™ DNA Rehydration Solution was used to resuspend the DNA pellet followed by a 24 h incubation to allow for full solubilization. An aliquot of the DNA was used to measure absorbance at 260 nm in order to determine its concentration. One absorbance unit equalled 50 µg double-stranded DNA/mL.

2.3 RFLP Genotyping

2.3.1 ApoE genotyping

A portion of exon 4 of the apo E gene was amplified using PCR on extracted DNA samples from affected and control individuals according to a modified Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) protocol (Wenham et al., 1991). Two fluorescent dye-labelled primers were constructed: dye-labelled forward primer 22A= 5'(Hex)-cgcaggcccggctgggtgcggacatggagg-3' and dye-labelled reverse primer 22B= 5´-(6-Fam)-ccctcgcgggccccggcctggtacactgcca-3' (Fig. 4). Primer 22A was designed with an introduced mismatch (underlined t) in order to remove a Hha I restriction site in the amplicon. Total reaction volume was 12.5 µL overlaid with mineral oil (Sigma) and consisting of 25 ng genomic DNA, 0.6 pmoles each of forward and reverse primer, 200 uM each dNTP (Pharmacia), 2.5 mM magnesium chloride (Perkin Elmer), 4% DMSO (Fisher), 1X Taq Buffer (Perkin Elmer) and 0.5 U AmpliTaq™ DNA Polymerase (Perkin Elmer). The reactions were performed in a 96-well microplate (Gordon Technologies) on an MJ Research Model PTC-225 thermocycler with the following program: 1 cycle at 95° C for 5 min followed by 11 cycles at 96° C for 60 sec; 70° C for 60 sec; 72° C for 60 sec followed by 26 cycles at 94° C for 30 sec; 63° C for 30 sec; 72° C for 30 sec followed by 1 cycle at 72° C for 10 min.

Restriction enzyme digestion of the amplicons was done directly in the PCR microplate well using the entire PCR reaction volume and 1X NEB Buffer #4 (New England Biolabs or NEB) supplemented with 100 μg/ml acetylated BSA (NEB) and 2 U Hha I enzyme (NEB) at 37° C for 6 h.

An aliquot of 1.0 μL of crude digested sample was mixed with 0.5 μL deionized formamide (Fisher) and 0.5 μL GS350 ROX standard (ABI), denatured at 90° C for 2 min. Samples were loaded onto a 4.8% polyacrylamide gel and run on an ABI Model 377 DNA sequencer for 2 h at 3000V constant voltage. The scanned data was analyzed with ABI Genescan™ ver 2.0 fragment analysis software.

The genotype of each sample was determined by the pattern of the digested dye-labelled fragments (Fig. 5). The three different ApoE isoforms have either a cysteine or arginine at codons 112 and 158. If arginine exists at either site, then *Hha* I will cut at the corresponding site. If cysteine is present, the enzyme will not cut at that position. Therefore, the presence of allele ε2 is indicated by the presence of 2 fragments: 81 bp blue and 108 bp green corresponding to cysteine 112 and cysteine 158; allele ε3 is indicated by the presence of 33 bp blue and 108 bp green fragments corresponding to cysteine 112 and arginine 158; allele ε4 is indicated by the presence of 33 bp blue and 36 bp green fragments corresponding to arginine 112 and arginine 158. Heterozygotes have combinations of these fragment patterns (Fig. 5).

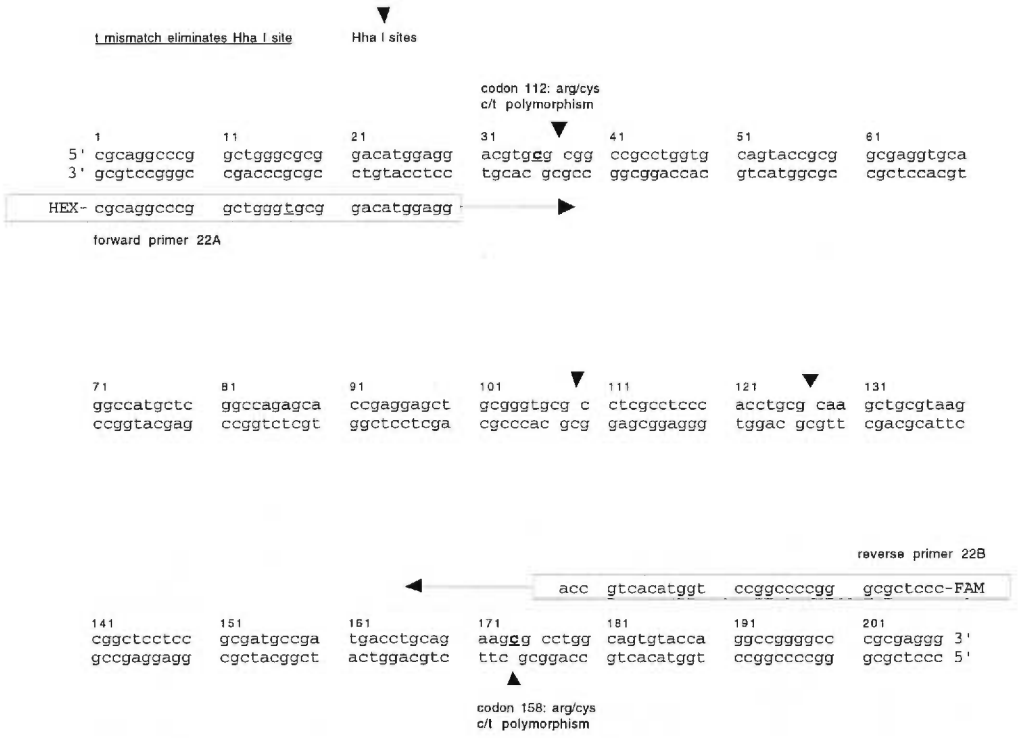

Fig. 4. The sequence of the ApoE amplicon. A region spanning 208 bp of exon 4 of the ApoE gene was amplified using PCR and subsequently digested with *Hha* I restriction enzyme. The amplified region contains two polymorphic sites at nucleotide positions 36 and 174 of the amplicon which code for amino acids at codons 112 and 158, respectively, of the mature Apo E protein. Both polymorphic sites are either t or c which code for either cysteine or arginine, respectively. In order to detect both polymorphisms simultaneously, we double-labelled the PCR product with a HEX-dyed forward primer and a FAM-dyed reverse primer. Two constitutive *Hha* I sites exist at amplicon positions 109 and 127. Another constitutive *Hha* I restriction site was eliminated by designing the forward primer with a t mismatch (underlined). The ε2 allele corresponds to cysteine at both codons 112 and 158 which in turn corresponds to t at the polymorphic restriction sites. Consequently, *Hha* I will not cut at these sites giving rise to a HEX-labelled fragment of 109 bases and a FAM-labelled fragment of 81 bases. The ε3 allele has a cysteine at codon 112 and an arginine at codon 158 which corresponds to t and c at the respective *Hha* I sites. In this case, *Hha* I will cut at codon 158 but will not cut at codon 112 giving rise to a HEX-labelled fragment of 109 bases and a FAM-labelled fragment of 33 bases. The ε4 allele corresponds to arginine at both polymorphic codons which in turn corresponds to c at the polymorphic restriction sites. Consequently, *Hha* I will cut at both these sites giving rise to a HEX-labelled fragment of 35 bases and a FAM-labelled fragment of 33 bases.

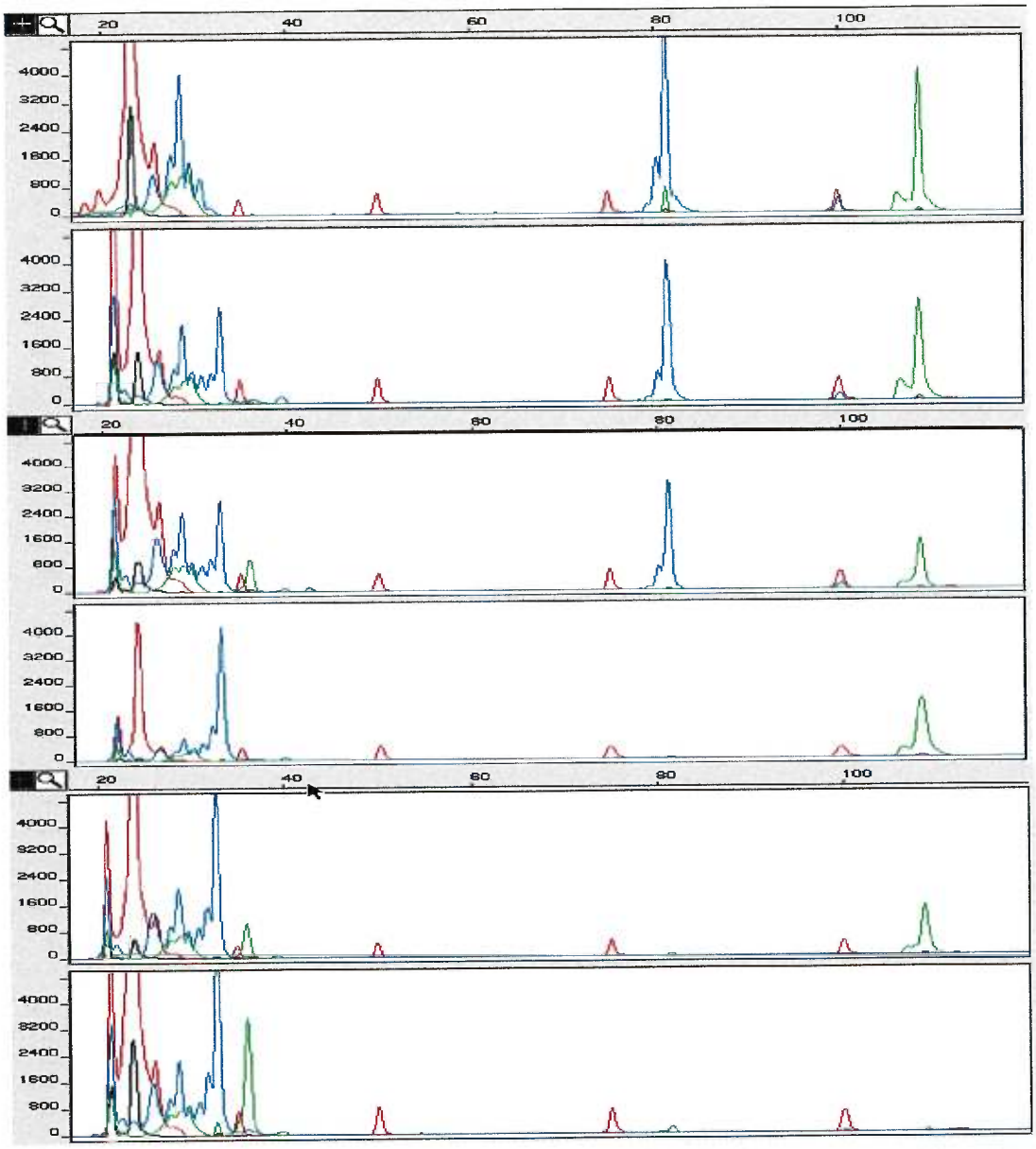

Fig. 5. Electrophoretograms showing the 6 possible ApoE genotypes. The *Hha* I digested PCR fragments were separated by electrophoresis on a denaturing polyacrylamide gel using the ABI 377 DNA Sequencer and analysed using ABI GENESCAN™ ver 2.0 software. The horizontal scale is in bp and the vertical scale is in peak height units. The multicolor peaks just prior to the 33 bp fragment represent unincorporated fluorescent primers and primer-dimers. The red peaks correspond to an internal size standard which denote sizes 35, 50, 75 and 100 bp. Allele-specific *Hha* I digestion patterns are denoted by blue and green peaks: allele ε2 by blue 81 bp and green 108 bp peaks; allele ε3 by blue 33 bp and green 108 bp peaks; and allele ε4 by blue 33 bp and green 35 bp peaks. Panels 1-6 (top to bottom, respectively) show the 6 possible ApoE genotype allele profiles: 1) ε2/ε2 ; 2) ε2/ε3; 3) ε2/ε4; 4) ε3/ε3; 5) ε3/ε4; 6) ε4/ε4.

2.3.2 ACT genotyping

The bi-allelic alanine/threonine (A/T) polymorphism located in the signal peptide sequence of the ACT gene was assayed in AD cases and controls by PCR-RFLP (Fig. 6) using the restriction enzyme *BstN* I (New England Biolabs) according to a modified protocol (Kamboh et al., 1995a). Each PCR consisted of 25 ng genomic DNA, 5 pmol each of forward primer ACT-1: 5'cagagttgagaatggaga-3' and reverse primer ACT 2.1-HEX: 5'-agattctcctcgtcaagt-3', 0.2 mM dNTP (Pharmacia), 2% DMSO (Fisher), 1X AmpliTaq™ buffer (Perkin-Elmer) and 0.5 U AmpliTaq™ (Perkin-Elmer) in a reaction volume of 12.5 µL. Reactions were overlaid with mineral oil (Sigma) and cycled in a 96-well microplate (Gordon Technologies) on an MJ Research Model PTC-225 thermocycler with the following program: 1 cycle at 94° C for 5 min followed by 35 cycles at 94° C for 60 sec; 52° C for 60 sec; 72° C for 60 sec followed by 1 cycle at 72° C for 5 min.

Restriction enzyme digestion of the amplified fragments was done directly in the PCR microplate well using the entire PCR reaction volume and 1X NEB Buffer #2 (New England Biolabs or NEB) supplemented with 100 µg/ml acetylated BSA (NEB) and 2.5 U *BstN* I enzyme (NEB) at 60° C overnight.

An aliquot of 1.0 µL of the digested sample was mixed with 0.5 µL deionized formamide (Fisher) and 0.5 µL GS350 ROX standard (ABI), denatured at 90 C for 2 min, loaded onto a 4.8% polyacrylamide gel and run on an ABI Model 377 DNA sequencer for 2 h at 3000V constant voltage. The scanned data was analyzed using ABI Genescan™ ver 2.0 fragment analysis software (Fig. 7). If the polymorphic site is a, corresponding to an alanine codon, *BstN* I digestion will produce a 79 bp HEX-labelled fragment (allele A). If the polymorphic site is t, corresponding to threonine, *BstN* I will not cut, producing a 112 bp HEX-labelled fragment (allele T).

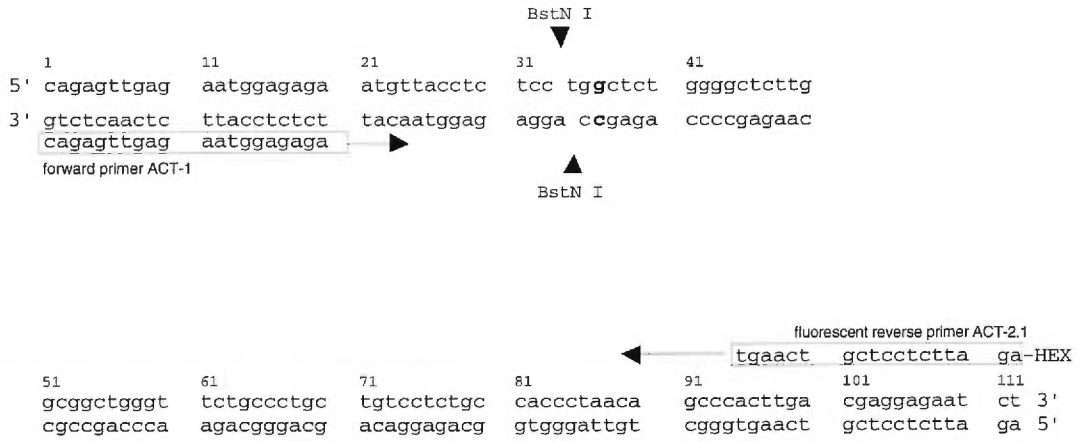

Fig. 6. The sequence of the ACT amplicon. PCR amplification with primers ACT-1 and ACT-2.1 generates a 112 bp HEX-labelled PCR product. The nucleotide at position 36 is polymorphic and can be either g or a. If g exists at this position, then digestion of the amplicon with *BstN* I restriction enzyme produces 2 fragments of 79 bp and 33 bp of which only the 79 bp fluorescently labelled fragment is visible on the gel. This nucleotide position corresponds to the codon Alanine (allele A) at position -15 in the signal sequence of the mature polypeptide. If the nucleotide at position 36 is an a, which corresponds to the codon Threonine (allele T), then *BstN* I will not cleave producing a 112 bp fluorescently labelled fragment on the gel.

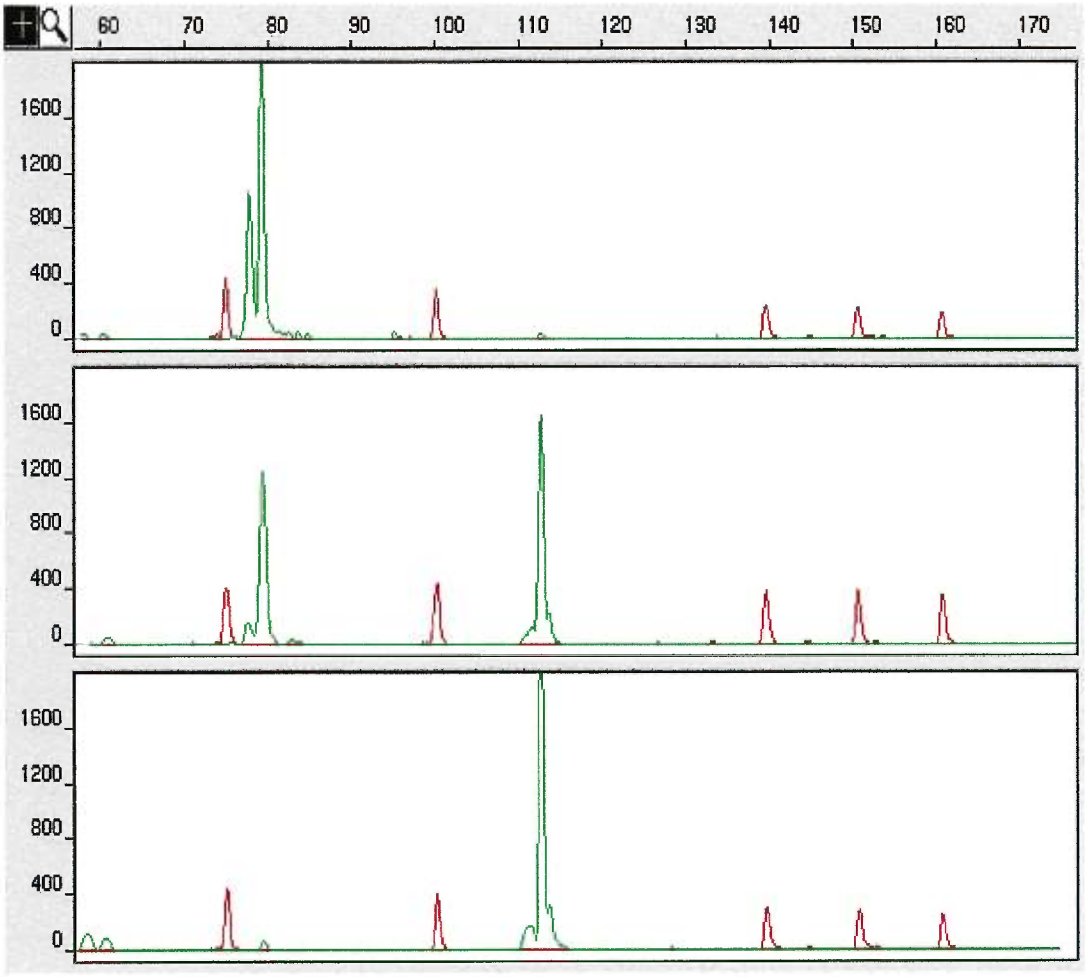

Fig. 7. Electrophoretograms showing the 3 possible ACT genotypes. The *BstN* I digested PCR fragments were separated by electrophoresis on a denaturing polyacrylamide gel using the ABI 377 DNA Sequencer. The scanned data was collected using ABI PRISM software and analysed using ABI Genescan™ ver 2.0 software. The horizontal scale is in bp and the vertical scale is in peak height units. The red peaks correspond to an internal size standard which was present in each lane and denote sizes 75, 100, 140, 150, and 160 bp. The green peaks correspond to digestion products of which the 79 bp peak denotes allele A and the 112 bp peak denotes allele T. Panels 1-3 (top to bottom, respectively) show the 3 possible ACT genotype allele profiles: 1) AA ; 2) AT; 3) TT.

2.3.3 PS-1 genotyping

The bi-allelic T/G intronic polymorphism located 3' to exon 8 of PS-1 (Fig. 8) was assayed by PCR-RFLP using *BamH*I according to a previously described protocol (Wragg et al., 1996). Genomic DNA from cases and controls was amplified using forward primer JH1 = 5'-cacccatttacaagtttagc-3' and reverse primer JH2 = 5'-cactgattactaattcaggatc-3'. The underlined nucleotides in the reverse primer create an artificial *BamH* I recognition site. Each PCR reaction consisted of 50 ng genomic DNA, 30 pmol each primer, 0.2 mM dNTP (Pharmacia), 2% DMSO (Fisher), 1X AmpliTaq™ buffer (Perkin-Elmer) and 1 U AmpliTaq™ (Perkin-Elmer) in a total reaction volume of 25 µL. Reactions were overlaid with mineral oil (Sigma) and cycled on in a 96-well microplate (Gordon Technologies) on an MJ Research Model PTC-225 thermocycler with the following program: 1 cycle at 94° C for 5 min followed by 35 cycles at 94° C for 30 sec; 45° C for 30 sec; 72° C for 30 sec followed by 1 cycle at 72° C for 3 min.

Restriction enzyme digestion of the amplicons was done directly in the PCR microplate well using the entire PCR reaction volume and 1X *BamH* I Buffer (New England Biolabs or NEB) supplemented with 100 µg/ml acetylated BSA (NEB) and 6 U *BamH* I enzyme (NEB) at 37° C for 6 h. Samples were mixed with bromophenol blue (Sigma) loading buffer and loaded onto a 3% agarose gel (Gibco) containing 0.25 µg/ml ethidium bromide (Sigma). The gel was run at 100 V for approximately 3 hr and the DNA bands were visualized under UV (Fig. 9). PCR generates a 199 bp fragment. If the polymorphic site is t nucleotide, *BamH* I does not cut resulting in a 199 bp fragment (allele 1). *BamH* I produces 2 fragments of 181 and 18 bp if the polymorphic site is g nucleotide (allele 2).

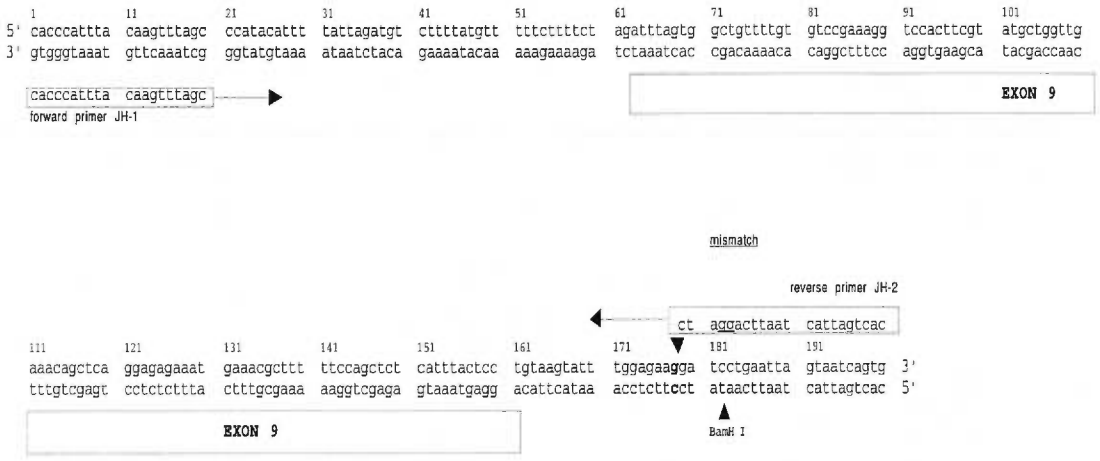

Fig. 8. Sequence of the amplicon containing the intronic PS-1 polymorphism. PCR amplification with primers JH-1 and JH-2 yields a 200 bp PCR product. A mismatch in the reverse primer generates a recognition sequence for digestion with *BamH* I restriction enzyme. Nucleotide 178 is polymorphic being either t or g. The presence of t nucleotide at this position does not create a cleavage site and therefore, a single 200 bp fragment is seen on the gel (allele 1). The presence of g nucleotide at this position creates a cleavage site for *BamH* I which produces 2 fragments of 182 bp and 18 bp (allele 2).

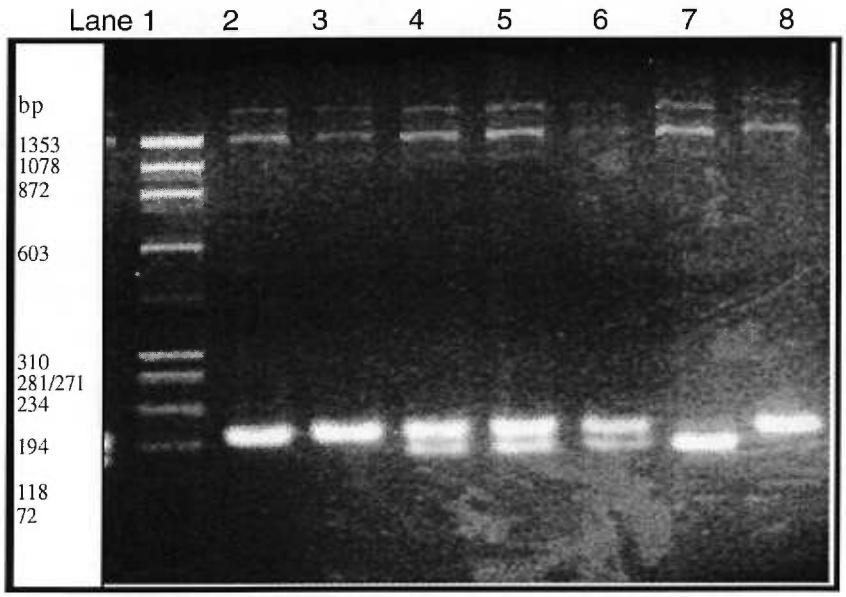

Fig. 9. Agarose gel photo of PS-1 PCR-RFLP bands. After digestion of amplified fragments with Bam H I, samples were mixed with bromophenol blue loading buffer and loaded onto a 3% agarose gel containing 0.25 ug/ml ethidium bromide. The gel was run at 100 V for approximately 3 hr followed by visualization of DNA bands under UV. Lane 1 shows a phi-x174 DNA-Hae III molecular weight marker. Lanes 2, 3 and 8 show PS-1 homozygotes with genotype 11. Lanes 4-6 show PS-1 heterozygotes with genotype 12. Lane 7 shows a PS-1 homozygote with genotype 22.

2.4 Statistical analyses

All statistical computations were performed using the SAS/STAT™ version 6 software package. Allele and genotype frequencies of the ApoE, PS-1 and ACT polymorphisms were estimated by counting alleles and calculating sample proportions. All tests were two-tailed and the statistical significance level was set at α=0.05. For multiple testing of n comparisons, we adjusted the significance level using Bonferroni correction ($\alpha < 0.05/n$).

Allele and genotype frequency differences were tested for significance using Pearson's χ^2 test or Fisher's Exact test. We used analysis of variance (ANOVA) and student t-test to test differences of means.

The risk of AD for each polymorphism was estimated by constructing logistic models in which alleles or genotypes were used to predict disease status. The strength of association between a genotype and AD was estimated as the odds ratio (OR) by logistic regression analysis. The OR estimate approximates the relative risk, ie. the risk of AD for genotype carriers divided by the risk of AD for non-carriers. An OR estimate higher than 1 indicates an increased risk, and lower than 1 indicates a decreased risk, of disease associated with the genotype. An OR estimate equal to 1 implies no association. The estimated risk was declared significant ($p<0.05$) if the Wald 95% confidence interval (95%CI) did not include 1.

CHAPTER 3

RESULTS

3.1 Analysis of ApoE, ACT and PS-1 polymorphisms

Allele and genotype frequencies for ApoE, ACT and intronic PS-1 polymorphisms are shown in Tables I, VII and X, respectively. In order to determine if allele and genotype frequencies in our case and control populations were in Hardy-Weinberg equilibrium, we used the χ^2 test for ACT and PS-1 polymorphisms. Due to the very small number of ε2ε2 and ε4ε4 genotypes, we used the Exact test to evaluate Hardy-Weinberg equilibrium for the ApoE polymorphism (Weir, 1996). Non-significant (NS) differences were found between expected and observed genotype frequencies in our control populations for PS-1 (χ^2=0.442, p=0.506 NS, df=1), ACT (χ^2=0.175, p=0.6760 NS, df=1), and ApoE (p=0.9430 NS, df=3). No significant differences were found between expected and observed genotypes in our AD case population for PS-1 (χ^2=1.99, p=0.1581 NS, df=1), but a significant difference was found for ACT (χ^2=10.47, p=0.0012 NS, df=1), and a borderline significance for ApoE (p=0.0464, df=3). These Hardy-Weinberg results permit the use of genotype and allele comparisons for PS-1, but only genotype comparisons for ACT and ApoE.

Table I. Genotype and allele frequencies for ApoE polymorphism in AD cases and controls. (Proportions are indicated in parentheses.)

	AD CASES n=131		CONTROLS n=157	
ApoE				
ε2ε2	1	(0.008)	3	(0.019)
ε2ε3	7	(0.053)	34	(0.217)
ε3ε3	36	(0.275)	86	(0.548)
ε2ε4	13	(0.099)	6	(0.038)
ε3ε4	61	(0.466)	27	(0.172)
ε4ε4	13	(0.099)	1	(0.006)
ε2	22	(0.084)	46	(0.146)
ε3	140	(0.534)	233	(0.742)
ε4	100	(0.382)	35	(0.111)

Table II. Contingency table and Odds Ratio for presence or absence of ApoE ε4 allele in AD cases and controls. (Proportions are indicated in parentheses.)

	AD CASES		CONTROLS	
ApoE	n		n	
ε4	87	(0.664)	34	(0.216)
non-ε4	44	(0.336)	123	(0.783)

ε4 vs. non-ε4: OR=7.72 (95% CI 4.49-13.29) p<0.0001

Table III. Contingency tables and Odds Ratios for individuals with ApoE ε3/ε3 genotype vs. individuals with: **A)** 1 copy of ApoE ε4 allele; and **B)** 2 copies of ApoE ε4 alleles in AD cases and controls. (Proportions are indicated in parentheses.)

	AD CASES		CONTROLS	
	n		n	
A) 1 copy ε4				
ε3/ε3	36	(0.327)	86	(0.723)
1 copy ε4	74	(0.673)	33	(0.277)

ε3/ε3 vs. 1 copy ε4: OR=5.83 (95% CI 3.25-10.47) p<0.0001

B) 2 copies ε4				
ε3/ε3	36	(0.735)	86	(0.989)
2 copies ε4	13	(0.265)	1	(0.011)

ε3/ε3 vs. 2 copies ε4: OR=30.54 (95% CI 3.84-242.95) p=0.0012

Table IV. Contingency table and Odds Ratio for presence or absence of ApoE ε2 allele in AD cases and controls. (Proportions are indicated in parentheses.)

	AD CASES		CONTROLS	
ApoE	n		n	
ε2	21	(0.160)	43	(0.274)
non-ε2	110	(0.840)	114	(0.726)

ε2 vs. non-ε2: OR=0.51 (95% CI 0.28-0.92) p=0.0241

Table V. Gender distribution of AD cases and controls by **A)** ApoE ε4 allele copies; and **B)** presence or absence of ApoE ε4. (Proportions are indicated in parentheses.)

	CASES				CONTROLS			
	MALES		FEMALES		MALES		FEMALES	
	n		n		n		n	
A) ApoE ε4 copies								
0	13	(0.325)	31	(0.341)	51	(0.718)	72	(0.837)
1	23	(0.575)	51	(0.560)	20	(0.282)	13	(0.151)
2	4	(0.100)	9	(0.099)	0	(0.000)	1	(0.012)
	p=1.000 NS				p=0.076 NS			
B) ApoE								
ε4	27	(0.675)	60	(0.659)	20	(0.282)	14	(0.163)
non-ε4	13	(0.325)	31	(0.341)	51	(0.718)	72	(0.837)
	chi-square=0.031, p=0.861 NS				chi-square=3.241, p=0.072 NS			

Table VI. Age at onset distribution of AD cases by presence or absence of ApoE ε4 allele. (Proportions are indicated in parentheses.)

Onset age	ε4 n		non-ε4 n	
≤59	11	(0.688)	5	(0.313)
60-69	28	(0.718)	11	(0.282)
70-79	38	(0.717)	15	(0.283)
≥ 80	10	(0.435)	13	(0.565)

chi-square= 6.633, p=0.085 NS

<80	77	(0.713)	31	(0.287)
≥ 80	10	(0.435)	13	(0.565)

chi-square= 6.578, p=0.010

3.1.1 ApoE

The frequency of the ApoE ε4 allele (Table I) was increased in AD cases (0.382) compared to controls (0.111). The frequency of all ApoE ε4 carriers (ie. ε2/ε4, ε3/ε4, and ε4/ε4) was significantly increased in AD cases compared to controls (χ^2=58.72, p<0.001). The largest increase was seen in the ε4/ε4 genotype; cases with this genotype were greater than 16-fold overrepresented compared to controls. The increase in ε4 allele frequency, as a proportion of total alleles, provoked a corresponding decrease in the frequency of the other ApoE alleles. Consequently, the frequencies of the ε2 and ε3 alleles in our case cohort was reduced compared to controls by 1.7-fold and 1.4-fold, respectively.

We tested the hypothesis that ApoE ε4 carriers would be more at risk for developing AD by calculating the OR for ε4 carriers vs. non-ε4 carriers in cases and controls. We pooled cases and controls into two groups representing presence or absence of ApoE ε4: individuals with at least one copy of ε4 (ie genotypes ε2/ε4, ε3/ε4, and ε4/ε4) were pooled to form the ε4 carrier group, and all individuals without ε4 (ie. genotypes ε2/ε2, ε2/ε3, and ε3/ε3) were pooled to form the non-ε4 group (Table II). The OR for developing AD was significantly increased for individuals with presence of ApoE ε4 vs. absence of this allele (OR=7.72, 95%CI=4.49-13.29; p<0.0001).

To determine if there was a dose effect of ApoE ε4 allele on susceptibility to AD, we took ε3/ε3 individuals as a reference genotype for neutral disease status, and estimated ORs for one- and two-allele doses of ApoE ε4 (Table III). Compared with ε3/ε3 individuals, the OR for developing AD was significantly increased for subjects with one ε4 allele (OR=5.83, 95%CI=3.25-10.47, p<0.0001), and further increased for subjects with two ε4 alleles (OR=30.54, 95%CI=3.84-242.95, p=0.0012) The very small number of ε4 homozygotes (n=1) in our control group implies that the latter OR estimate must be cautiously interpreted.

We stratified cases and controls into presence vs. absence of ApoE ε2 allele and estimated the OR for ε2 vs. non-ε2 carriers in order to determine if there was a protective effect of ApoE ε2 allele in our sample (Table IV). We obtained a significantly reduced OR of 0.51 (95%CI=0.28-0.92, p=0.0241).

To investigate the possibility of an AD-associated gender effect of ApoE (Table V), we stratified the AD cases by A) number of copies of ApoE ε4 allele (Table V-A); and by B) presence or absence of ApoE ε4 allele (Table V-B). We used either Fisher's Exact test or χ^2 test to determine if there were any differences in proportion between males and females in these different stratifications in both cases and in controls. Due to the small number of ε4 homozygotes in the control group, we used Fisher's Exact for cases stratified by number of ε4 copies. No significant differences between gender were seen neither for ApoE stratified by number of ε4 copies (p=1.000 NS and p=0.076 NS), nor for ApoE stratified by presence or absence of ε4 (p=0.861 NS and p=0.072 NS), in both cases and in controls, respectively.

In order to determine if there was an age-dependent effect on ApoE ε4 allele, we stratified our ApoE ε4 carrier and non-carrier AD cases into 4 age at onset groups: ≤ 59, 60-69, 70-79, and ≥ 80 years (Table VI). The frequency of carriers and non-carriers of the ApoE ε4 for each age group and was found to be similar in all age at onset groups, except for the ≥ 80 years age at onset group, which had the lowest ε4 allele frequency (0.435). When we compared the 4 age groups together we obtained only a nearly significant difference between age groups (χ^2=6.63, p=0.085). We then pooled all cases with < 80 age at onset to determine if the ≥ 80 age group was significantly different from the < 80 age group. We obtained a significant difference of ApoE ε4 frequency for this comparison (χ^2=6.58, p=0.010).

Table VII. Genotype and allele frequencies for ACT in AD cases and controls. (Proportions are indicated in parentheses.)

	AD CASES n=130		CONTROLS n=151	
ACT				
TT	43	(0.331)	31	(0.205)
AT	46	(0.354)	72	(0.477)
AA	41	(0.315)	48	(0.318)
T	132	(0.508)	134	(0.444)
A	128	(0.492)	168	(0.556)

Table VIII. Contingency table and Odds Ratio for presence or absence of ACT TT genotype in AD cases and controls. (Proportions are indicated in parentheses.)

	AD CASES		CONTROLS	
ACT	n		n	
non-TT	87	(0.669)	120	(0.795)
TT	43	(0.331)	31	(0.205)

non-TT vs. TT: OR=1.87 (95% CI 1.09-3.22) p=0.0235

Table IX. Gender distribution of ACT genotype in AD cases and controls. (Proportions are indicated in parentheses.)

	CASES				CONTROLS			
	MALES		FEMALES		MALES		FEMALES	
ACT	n		n		n		n	
AA	10	(0.250)	31	(0.344)	17	(0.254)	31	(0.369)
AT	17	(0.425)	29	(0.322)	38	(0.567)	34	(0.405)
TT	13	(0.325)	30	(0.333)	12	(0.179)	19	(0.226)
	chi-square=1.616, p=0.446 NS				chi-square=4.023, p=0.134 NS			

3.1.2 ACT

The TT genotype of the ACT polymorphism (Table VII) was overrepresented in cases (0.331) compared to controls (0.205). We tested the hypothesis that individuals with the ACT TT genotype were more at risk for developing AD. We stratified the ACT genotypes in our cases and controls into individuals with TT genotype, and individuals with AT or AA genotypes (ie. non-TT), and estimated the OR for TT vs. non-TT individuals (Table VIII). We obtained a significantly increased OR of 1.87 (95%CI=1.09-3.22; p=0.0235).

In order to determine if there was a gender effect on ACT (Table IX), we stratified ACT genotypes by gender in both cases and in controls. We used χ^2 test to see if there were any differences in proportion between males and females in these different stratifications. No significant differences between gender were seen for ACT genotype in both cases (p=0.446 NS) and in controls (p=0.134 NS).

Table X. Genotype and allele frequencies for intronic PS-1 polymorphism in AD cases and controls. (Proportions are indicated in parentheses.)

	AD CASES n=126		CONTROLS n=157	
PS-1				
11	44	(0.349)	54	(0.344)
12	67	(0.532)	80	(0.510)
22	15	(0.119)	23	(0.146)
1	155	(0.615)	188	(0.599)
2	97	(0.385)	126	(0.401)

3.1.3 PS-1

Genotype and allele frequencies for the intronic PS-1 polymorphism were similar in cases compared to controls (Table X). Using χ^2 test, no significant differences were found in genotype frequencies (p=0.793 NS) nor in allele frequencies (p=0.692) between cases and controls. In order to investigate the possiblity that a particular PS-1 genotype could confer an increased risk for AD, we estimated ORs for presence vs. absence of each PS-1 genotype. The OR values were not significant for all comparisons:
PS-1 (12 or 22) vs. 11 (OR=1.00, 95%CI=0.61-1.65, p=0.9983 NS);
PS-1 (11 or 22) vs. 12 (OR=0.87, 95%CI=0.54-1.41, p=0.5716 NS);
and PS-1 (11 or 12) vs. 22 (OR=1.35, 95%CI=0.66-2.74, p=0.4081 NS).

Table XI. ACT genotypes stratified by presence or absence of ApoE ε4 allele in AD cases and controls. (Proportions are indicated in parentheses.)

	CASES				CONTROLS			
	ε4		non-ε4		ε4		non-ε4	
ACT	n		n		n		n	
TT	29	(0.333)	14	(0.326)	4	(0.121)	27	(0.229)
AT	30	(0.345)	16	(0.372)	18	(0.545)	54	(0.458)
AA	28	(0.322)	13	(0.302)	11	(0.333)	37	(0.314)

Table XII. Contingency table and Odds Ratio for presence or absence of combined ApoE ε4 allele and ACT TT genotype in AD cases and controls. (Proportions are indicated in parentheses.)

	AD CASES		CONTROLS	
ApoE/ACT	n		n	
ε4/TT	29	(0.223)	4	(0.027)
non-ε4/TT	101	(0.777)	147	(0.973)

ε4/TT vs. non-ε4/TT: OR=10.18 (95% CI 3.46-30.0) p<0.0001

3.2 Gene polymorphism interactions

3.2.1 ApoE and ACT

Since ApoE and ACT were both individually associated with an increased risk for developing AD in our sample, we decided to test the hypothesis that there was an interaction effect of these two polymorphisms. We stratified the ACT genotypes into ApoE ε4 carrier groups: ε4 carriers (ε2/ε4, ε3/ε4 and ε4/ε4), and non-ε4 carriers (ε2/ε2, ε2/ε3 and ε3/ε3), in cases and in controls (Table XI). The TT genotype was overrepresented for both ε4 carriers (0.333 vs. 0.121), and non-ε4 carriers (0.326 vs. 0.229), in cases compared to controls, respectively. This overrepresentation was more pronounced in ε4 carriers than in non-ε4 carriers. This suggested to us that the combined presence of ApoE ε4 allele and ACT TT may be acting synergistically to increase the risk for developing AD. To investigate this possibility, we pooled the ACT TT individuals who were also ApoE ε4 carriers into one group, and ACT AT and AA individuals (ie. non-TT) who were also non-ε4 carriers into another group, and compared the relative frequency of these two groups in cases and controls (Table XII). The proportion of individuals with combined TT and ε4 genotypes vs. combined non-TT and non-ε4 genotypes was found to be significantly different in cases compared to controls (χ^2=26.05, p<0.001). The OR for individuals with TT and at least one copy of ε4 vs. individuals without this genotype (ie. combined non-TT and non-ε4) was significantly increased (OR=10.18, 95%CI=3.46-29.96, p<0.0001).

We also estimated the risk for ApoE (presence vs. absence of ε4) and ACT (presence vs. absence of TT) as individual variables taken simultaneously in the same logistic regression. We obtained significantly increased ORs for ApoE (OR=8.00, 95%CI=4.58-13.95, p<0.0001) and for ACT (OR=2.10, 95%CI=1.13-3.89, p=0.0186).

Table XIII. Contingency table and Odds Ratio for presence or absence of the combined genotypes ACT TT and PS-1 12 in AD cases and controls. (Proportions are indicated in parentheses.)

	AD CASES		CONTROLS	
ACT/PS-1	n		n	
TT/12	24	(0.192)	12	(0.080)
non-TT/12	101	(0.808)	139	(0.920)

TT/12 vs. non-TT/12: OR=2.74 (95% CI 1.30-5.78) p=0.0083

3.2.2 ACT and PS-1

We tested the hypothesis that there was an interaction between PS-1 and ACT polymorphisms. We stratified the frequencies of PS-1 genotype with ACT genotype and found that individuals with the combination of genotypes ACT TT and PS-1 12 were overrepresented in cases (0.192) compared to controls (0.080) (Table XIII). The OR for developing AD for individuals with combined ACT TT and PS-1 12 genotypes vs. individuals without these combined genotypes (ie. ACT AT or TT and PS-1 11 or 22) was significantly increased (OR=2.74, 95%CI=1.30-5.78, p=0.0083).

Table XIV. PS-1 genotypes stratified by presence or absence of ApoE ε4 allele in AD cases and controls. (Proportions are indicated in parentheses.)

	CASES				CONTROLS			
	ε4		non-ε4		ε4		non-ε4	
PS-1	n		n		n		n	
11	31	(0.369)	13	(0.310)	14	(0.412)	40	(0.325)
12	43	(0.512)	24	(0.571)	15	(0.441)	65	(0.529)
22	10	(0.119)	5	(0.119)	5	(0.147)	18	(0.146)
	chi-square=0.471, p=0.790 NS				chi-square=0.979, p=0.613 NS			

3.2.3 PS-1 and ApoE

We tested the hypothesis that there was an interaction between PS-1 and ApoE polymorphisms. We stratified the frequencies of PS-1 genotype by the presence or absence of ApoE ε4 allele in AD cases, and in controls (Table XIV). No significant differences were found between ε4 carriers and non-ε4 carriers in PS-1 genotype frequencies in cases (χ^2=0.471, p=0.790 NS) and in controls (χ^2=0.979, p=0.613 NS). We then performed logistic regression analysis taking into account the following variables in each regression: 1) presence or absence of ApoE ε4 allele, 2) presence or absence of PS-1 genotype, and 3) the interaction of variables 1 and 2. All OR values for the interaction variables of the different combinations of PS-1 genotype were non-significant: PS-1 11 vs. non-11 (OR=0.95, 95%CI=0.31-2.96, p=0.9318 NS); PS-1 22 vs. non-22 (OR=0.88, 95%CI=0.18-4.36, p=0.8762 NS); and PS-1 12 vs. non-12 (OR= 1.11, 95%CI=0.37-3.31, p=0.8565 NS).

Table XV. Mean values and standard deviation (±) for age at onset, senile plaques density and neurofibrillary tangles density in autopsied AD cases stratified by **A)** ApoE ε4 copies; and by **B)** presence or absence of ApoE ε4 allele.

	Onset age (years)		Plaques (mm^{-3})		Tangles (mm^{-3})	
A) ε4 copies						
0	72.8	(±9.8)	30.3	(±11.6)	28.0	(±11.0)
1	70.2	(±8.1)	36.0	(±13.7)	31.5	(±13.2)
2	67.6	(±7.5)	48.0	(±12.4)	33.1	(±12.2)
	p=0.1109 NS		p<0.0001		p=0.2541 NS	
B) ApoE carriers						
ε4	69.8	(±8.0)	37.7	(±14.1)	31.7	(±13.0)
non-ε4	72.8	(±9.8)	30.3	(±11.6)	28.0	(±11.0)
	p=0.0627 NS		p<0.0033		p=0.1101 NS	

Table XVI. Mean values and standard deviation (±) for age at onset, senile plaques density and neurofibrillary tangles density in autopsied AD cases stratified by presence or absence of **A)** ACT TT genotype; **B)** combined ApoE ε4 allele and ACT TT genotype; and **C)** combined ACT TT and PS-1 12 genotype.

	Onset age (years)		Plaques (mm^{-3})		Tangles (mm^{-3})	
A) ACT genotype						
TT	72.6	(±8.5)	37.3	(±13.7)	31.8	(±11.3)
non-TT	69.8	(±8.8)	34.5	(±13.8)	30.0	(±13.1)
	p=0.0851 NS		p=0.2831 NS		p=0.4538 NS	
B) ApoE/ACT						
ε4/TT	72.0	(±8.2)	40.2	(±14.1)	33.4	(±12.0)
non-ε4/TT	70.3	(±8.9)	34.0	(±13.4)	29.7	(±12.6)
	p=0.3623 NS		p=0.0310		p=0.1615 NS	
C) ACT/PS-1						
TT/12	72.0	(±9.0)	37.3	(±14.4)	33.0	(±11.5)
non-TT/12	70.3	(±8.6)	35.6	(±13.5)	30.5	(±12.2)
	p=0.3936 NS		p=0.5943 NS		p=0.3881 NS	

3.3 Morphometric comparisons

We tested the hypothesis that the presence of ApoE ε4 allele can affect disease severity by modulating age at onset and burden of plaques and tangles. Table XV shows AD cases stratified by: A) number of copies of ApoE ε4 allele; and by B) presence or absence of ApoE ε4 allele. To assess whether there were significant differences in mean age at onset, NFT density and SP density in these two stratifications, we used analysis of variance to test for dose-dependent differences (number of copies of ApoE ε4 allele) and Student t-test for ε4 carrier status differences.

For cases stratified by number of ε4 copies (Table XIII-A), mean onset age and NFT density were not significantly different ($p=0.1109$ NS and $p=0.2541$ NS, respectively). Mean SP density was significantly different between number of ε4 copies ($p<0.0001$). Using Tukey's Studentized Range Test and Bonferroni correction ($\alpha < 0.05/3 < 0.0167$) for multiple comparisons, we were able to determine a significant difference between 0 and 2 copies ($p<0.0001$), and also between 1 and 2 copies ($p=0.0025$) of ε4. However, the difference between 0 and 1 copy of ε4 did not reach the corrected threshold of significance ($p=0.0243$).

For cases stratified by presence or absence of ε4 allele (Table XIII-B), the age at onset was reduced in ε4 carriers compared with non-ε4 carriers but this difference did not reach the $\alpha=0.05$ level of significance ($p=0.0627$ NS). Both SP and NFT mean densities were increased in ε4 carrier cases compared with non-ε4 carrier cases. However, these differences were found to be significant for SP ($p=0.0033$) and non-significant for NFT ($p=0.1101$ NS).

We also tested the hypotheses that A) ACT alone, B) the interaction of ApoE and ACT and C) the interaction of ACT and PS-1, can affect disease severity by modulating age at onset and burden of plaques and tangles (Table XVI). We stratified the AD case cohort by presence or absence of:

A) the ACT TT genotype, regardless of ApoE genotype; B) the combination of ACT TT genotype and ApoE ε4 allele; and C) the combination of ACT TT genotype and PS-1 12 genotype. To assess whether there were significant differences in mean age at onset, NFT density and SP density in these different stratifications, we used Student t-test for all comparisons.

No significant differences were found for mean onset age (p=0.0851 NS), SP density (p=0.2831 NS) and NFT density (p=0.4538 NS) in cases stratified by presence or absence of ACT TT genotype (Table XIV-A).

For cases stratified by the presence or absence of the combination of ACT TT genotype and ApoE ε4 allele (Table XIV-B), mean age at onset was not significantly different (p=0.3623 NS). The mean densities of both SP and NFT were increased in individuals who were TT/ε4, although these differences were significant only for SP density (p=0.0310).

For cases stratified by the presence or absence of the combination of genotypes ACT TT and PS-1 12 (Table XIV-C), no significant differences were found in mean onset age (p=0.3936 NS), SP density (p=0.5943 NS) and NFT density (p=0.3881 NS).

CHAPTER 4

DISCUSSION

4.1 ApoE associations

4.1.1 ApoE ε4 is associated with increased AD risk

Our data show a 3.4-fold increase in the ApoE ε4 allele frequency in cases compared to controls, suggesting that there is a strong association of this allele with AD. The OR for developing AD is significantly increased in individuals who carry at least one copy of the ApoE ε4 allele vs. individuals who do not possess the ε4 allele (OR=7.72, 95%CI=4.49-13.29; $p<0.0001$) which suggests that ε4 carriers possess greater than 7 times risk for developing AD than individuals without ε4. All genotypes containing an ε4 allele are in excess in cases compared to controls, suggesting that the presence of at least one copy of ε4 is sufficient to cause a susceptibility to AD. However, the ε4 allele, in itself, is not sufficient to cause AD, since 34% of our case cohort do not possess the ε4 allele but have the disease, and 22% of our control cohort possess the ε4 allele but do not have the disease. Therefore, ApoE ε4 is neither necessary, nor sufficient, to cause AD. Logically, this result also implies that AD pathogenesis requires other factors in addition to ApoE in our sample. Thus, our data suggest that ApoE is a susceptibility, as opposed to a causal, AD locus. The AD-associated excess ApoE ε4 allele frequency in our sample population from the SLSJ area of Québec confirms and corroborates the ApoE ε4 association with AD obtained in numerous other studies using different populations. (Corder et al., 1993; Mayeux et al., 1993; Rebeck et al., 1993; Saunders et al., 1993b; Tsai et al., 1994; Yu et al., 1994).

Morphometric data in our autopsied AD brains suggest a link between ApoE genotype and AD pathogenesis. In our AD cases, mean SP density was significantly increased in ApoE ε4 carriers compared to non-ε4 carriers, suggesting that the presence of the ε4 allele not only increases the susceptibility to AD, but also increases the severity of the disease by increasing the SP burden. Mean age at onset was decreased in ApoE ε4 carriers in our sample, but since this result was borderline non-significant at the α=0.05 significance level, we are not able to corroborate findings in other studies in which AD patients with ApoE ε4 allele had an earlier age at onset (Corder et al., 1993; Locke et al., 1995). Although mean NFT density was increased in ε4 carriers, this difference was not significant. This result, taken in isolation, suggests that NFT accumulation may not be involved in AD pathogenesis.

4.1.2 Dose-dependent effect of ApoE ε4

Compared with ApoE ε3/ε3 individuals, the OR for developing AD was significantly increased for subjects with one ε4 allele (OR=5.83, 95%CI=3.25-10.47, p<0.0001), and further increased for subjects with two copies of the ε4 allele (OR=30.54, 95%CI=3.84-242.95, p=0.0012). The five-fold increase in the point estimate OR value for homozygote ε4 individuals compared to heterozygote ε4 individuals suggests a dose-dependent relationship between ApoE ε4 allele and AD risk. Specifically, AD risk appears to increase with increasing number of ε4 alleles. Although a dose effect trend is apparent, some reservations concerning the data must be noted. The very small number of ε4 homozygotes (n=1) in our control group (which produced a very wide confidence interval estimate for two copies of ε4) and the overlapping confidence intervals in ε4 heterozygotes and ε4 homozygotes do not allow us to establish the dose effect firmly.

An ε4 dose effect has been reported in numerous previous studies (Mayeux et al., 1993; Poirier et al., 1993; Corder et al., 1994; Yoshizawa et al., 1994; St. Clair et al., 1995; Jarvik et al., 1996; Myers et al., 1996; National Institute on Aging, 1996). However, in other studies, a dose effect of ε4

allele could not be firmly established also as a result of large and overlapping confidence intervals in the data (Mayeux et al., 1993; Dai et al., 1994; Kuusisto et al., 1994, van Duijn et al., 1994a; Lehtovirta et al., 1995; Tang et al., 1996; Bickeboller et al., 1997). Given that ApoE is a susceptibility factor that explains only a portion of AD etiology, it is reasonable to assume that other genetic loci are involved in the disease and acting either in isolation or in interaction with ApoE. Thus, it is possible that the ambiguity of ε4 dose effect in some studies may be due to a non-homogeneous genetic background specific to the sample under study.

We were also able to demonstrate a dose effect in our SP morphometric data. In AD brain, plaque density was highest in ε4 homozygotes, intermediate in ε4 heterozygotes, and lowest in individuals without ε4. The SP densities were significantly different between the ε4 carrier states, suggesting that there is a dose-dependent effect of ε4 on SP accumulation. An ε4 dose effect could not be demonstrated for either age at onset, nor for NFT densities, although a trend of association was apparent: onset age decreased, and NFT density increased, with increasing number of ε4 alleles, but these differences did not reach the α=0.05 level of significance. A dose effect of ApoE ε4 allele which is present in SP accumulation, but not in age at onset and NFT accumulation, suggests that ApoE may be biologically implicated in the SP accumulation pathway, but to a lesser degree, or not at all, in pathways which affect age at onset and NFT accumulation. This interpretation is consistent with our finding that age at onset and SP accumulation are correlated, or borderline-correlated, with presence or absence of ApoE ε4 allele, but that only SP accumulation is correlated in a dose-dependent manner. One caveat to be noted is that SP density in our brain cohort was measured without differentiating between neuritic and diffuse plaques. Therefore, the individual contribution of these different plaque sub-types cannot be determined.

At least one previous study has failed to correlate ApoE with NFT density in AD brain (Gomez et al., 1996). The dose effect on age at onset has not been firmly established in previous studies. Age at onset decreases with increasing ε4 dose in some studies (Mayeux et al., 1993;

Strittmater et al., 1993a; Lucotte et al., 1995; Blacker et al., 1997) while in others, no dose effect of ε4 was seen (Tsuda et al., 1994). Each ε4 allele lowered the age at onset by 7-9 years in late-onset FAD (Strittmater et al., 1993a; Payami et al., 1994) and by 3-7 years in late-onset sporadic AD (Poirier et al., 1993), suggesting that ε4 modulation of age at onset also depends on genetic background. The dose effect may to a certain extent be explained by the fact that AD and death are competing risks in the aged population. Any factor which decreases age at onset will increase the observed frequency of the disease. Therefore, the age range and the genetic background of the sample will be important considerations in the observed associations.

4.1.3 Protective effect of ApoE ε2

We found a protective effect of ApoE ε2 allele in our sample. The OR for carriers of the ε2 allele vs. non-carriers of this allele had a significantly decreased risk (OR= 0.51, 95%CI=0.28-0.92, p=0.0241). This result, however, must be interpreted cautiously for several reasons: 1) the increase in ε4 allele frequency, as a proportion of total alleles, provoked a concomitant and similar decrease in both non-ε4 alleles, suggesting that both these alleles may be neutral in their effect on AD, and 2) due to the relative rarity of this allele, sample size is a limiting factor in the statistical analysis. Compared to the consistently observed association between ε4 and AD, the association between ε2 and AD is still unclear. Some studies have observed a reduced frequency of ε2 allele in late-onset AD suggesting that ε2 may confer a protective effect for AD (Corder et al., 1994; Talbot et al., 1994; Bickeboller et al., 1997). However, no protective effect of ε2 was found in African-Americans (Maestre et al., 1995; Tang et al., 1996) and the ε2 allele frequency was significantly elevated in an Italian sample of sporadic and familial AD (Sorbi et al., 1994). Moreover, subjects with genotypes ε2/ε2 and ε2/ε3 had a higher risk for AD in a Dutch population-based study of early-onset AD, whereas subjects with the ε2/ε4 genotype had little or no additional risk for AD (van Duijn et al., 1995).

One factor which may confound interpretations regarding the role of ε2 in AD is that the protective effect of this allele may extend to other age-related risks, not just AD. This notion is consistent with the finding that increased ε2 allele frequency is associated with longevity (Schachter et al., 1994), and also, with hyperlipidemia (Davignon et al., 1988). Therefore, differential survival in ε2 carriers may represent a selection bias in the population and thus, may compromise observed associations in some studies. While the effects of ε2 on differential survival, and how these may specifically modify AD risk are still unclear, then this implies that pooling of genotypes by number of ε4 alleles poses a potential risk for enrichment of ε2 allele in certain pools, which may mask some types of interaction. Arguably, this risk is likely to be small since the ε2 allele is so rare. Nevertheless, a better demonstration of the protective effect of ε2 would be to use pedigrees in which the transmission of the allele in affected and non-affected individuals can be followed.

4.1.4 Lack of gender effect of ApoE

In our sample, there was no significant gender difference in ApoE-associated AD risk in cases nor in controls. The lack of gender difference in the control group ensures that this polymorphism is not gender-specific in the general population. This premise was necessary to establish since a lack of difference in allele frequency between gender in cases does not necessarily imply a lack of gender-specific risk in this group if there is a gender-specific overrepresentation of the risk-associated allele in the general population. Since no gender-specific risk was apparent in our cases and controls, then this suggests that the lack of sex-matched cases and controls in our sample is not likely to be problematic. Nevertheless, to be prudent and precise, all reported OR estimates in this study were corrected for gender in anticipation of unexpected, or subtle, gender differences.

Analysis of gender-specific risks in AD is complicated by the generally observed phenomenon of a disproportionate number of affected females (Breteler et al., 1992). In previous reports, a gender difference was found in familial AD subjects, although the sample had very few men older

than 60 years of age (Payami et al., 1996), but no sex difference was found in two other studies (Corder et al., 1995a; Bickeboller et al., 1997). Gender may also play a role in the ε4 dose effect. Previous studies have suggested that males who are homozygous ε4 have a younger age at onset, whereas in females, one ε4 allele is sufficient to reduce age at onset, without further reduction in the presence of another ε4 allele (Payami et al., 1994; Farrer et al., 1995). These results are in contrast to another study which showed that age at onset did not differ in males and females (Lucotte et al., 1995). Association studies into gender-based differences need to take into account the prevalence of gender-specific risks in the population. For example, gender-based associations in AD may be influenced by early selection against ε4 male carriers since it has been shown that ApoE ε4 confers an additional risk of ischemic heart disease in middle-aged men, but not in aged men (Van Bockxmeer et al., 1992; Kuusisto et al., 1995). The lower incidence of ApoE ε4 in the older male population may also explain, at least in part, the higher age-specific prevalence of AD in women reported in the literature (Breteler et al., 1992). Alternatively, the increased prevalence in women may also be in part due to longer survival in women after onset of dementia or to estrogen effects on AD development which could override the ε4 allele effects.

4.1.5 Age-dependent effect of ApoE ε4

Recent studies have indicated that the ApoE effect may be age-dependent (Sobel et al., 1995; Asada et al., 1996). In studies which have stratified families based on age at onset, the OR for developing AD with ApoE ε4 is different for different age groups, suggesting that the ApoE genotype does not exert its influence with the same magnitude within the whole period at risk for AD. Our sample also demonstrated an age-dependent ApoE ε4 effect. We stratified our AD cases into 4 age at onset groups (≤ 59; 60-69; 70-79; ≥ 80) and found that the oldest group, with age at onset ≥ 80 years, had the lowest frequency of ε4 carriers. Our finding that the ≥ 80 age group was significantly different from the pooled < 80 age group suggests that the putatively deleterious ε4 effect is minimal in very old AD patients.

The observation that ε4 carriers are reduced in the ≥ 80 age at onset group can be explained by age-censoring, such that the very elderly ε4 carriers have already died of AD, and are consequently no longer represented in the ≥ 80 age group. This interpretation is consistent with the notion that ApoE ε4 induces susceptibility to AD and also, possibly, influences the rate of progression of the disease. Our data which show that ApoE ε4 exerts its maximal effect between the ages 60-80 years corroborate previous findings which demonstrated an increased ε4 frequency in the 60-70 or the 60-79 age at onset groups (Rebeck et al., 1994; Corder et al., 1995b; Maestre et al., 1995; Bickeboller et al., 1997). Recently, in a study using a large sample of AD families stratified by age at onset, it was shown that ApoE ε4 exerts its maximal effect in the age group 60-65, although a considerable effect is still apparent up to age 70 (Blacker et al., 1997).

The biological reason for the age-dependent effect is not known but is possibly due to age-dependent protein levels of ApoE. In a cross-sectional study, ApoE-genotype effects on plasma ApoE concentration were found to differ in both males and females, with the variance due to ApoE genotype decreasing in the older ages, suggesting that the ApoE-genotype effects on plasma level of ApoE protein diminishes with age (Zerba et al., 1996). Additionally, it has been shown that the ε4 allele effects on total cholesterol and triglycerides change between longitudinal exams and may be age-dependent (Jarvik et al., 1997). These results could explain the variable age effects of ApoE with respect to AD risk.

The age-dependent effect of ApoE has several implications. Firstly, the use of ApoE genotype in genetic testing will be more feasible in certain age groups given that knowledge of the patient's age may have a significant impact on the accuracy of AD diagnosis and prediction. However, further understanding of additional gene/environment interactions in AD are needed before such testing can become truly reliable. Secondly, if age censoring by ApoE ε4 is not responsible for the lower frequency of this allele in older age at onset AD groups then it is clear that other genes may be implicated in these groups. Since the

majority of AD cases occur after age 70, it will be important to search for these unidentified AD genes using age stratification strategies.

4.2 ACT associations

4.2.1 ACT is associated with increased AD risk

Our data show that the TT genotype of the ACT polymorphism is overrepresented in cases compared to controls, suggesting that the ACT TT genotype might be a positive contributing factor in susceptibility to AD. Accordingly, a significantly increased OR (1.87, 95%CI=1.09-3.22, p=0.0235) was obtained for stratified ACT TT vs. non-TT genotypes. This result suggests that the risk for developing AD is increased in homozygous T individuals as compared to individuals with AA or AT genotype. However, our OR estimate has a 95% confidence interval which is close to 1.0 which is the threshold at which individuals possess an equal chance of having, as not having, the disease. Therefore, our result also suggests that while the ACT TT genotype confers a risk for AD, it is probable that this risk is small.

4.2.2 ACT interaction with ApoE

A higher frequency of the TT genotype was also observed in cases compared with controls, in both the ApoE ε4 and non-ε4 carrier groups, and this overrepresentation was more pronounced in ε4 carriers than in non-ε4 carriers. This result suggests that the TT genotype is associated with the disease regardless of ApoE ε4 status, although the combined presence of both ACT TT genotype and at least one allele of ApoE ε4 appears to be acting additively to increase the risk for developing AD. The proportion of individuals who were combined TT and ε4 carriers vs. combined non-TT and non-ε4 individuals was found to be significantly different in cases than in controls ($p<0.001$). The OR was also higher for individuals with combined TT genotype and at least one copy of ε4

(OR=10.18, 95%CI=3.46-30.00, p<0.0001) than for individuals with ApoE alone (OR=7.72, 95%CI=4.49-13.29, p<0.0001). These results suggest that there is an additive interaction between ApoE and ACT and that the risk for developing AD is increased in individuals with combined ApoE ε4 and TT genotypes. However, the overlapping confidence interval estimates warrant caution in our interpretation and suggest that any interaction between ApoE and ACT is likely to be small.

The ACT genotype, though its effect as a modifier for AD is minor, nevertheless provides additional information which can slightly increase the predictive value for risk of developing the disease. The logistic regression model generated with the presence of at least one copy of ApoE ε4 allele vs. non-ε4 produced a model concordance rate of 66% (p<0.0001) when applied to our data set. This result suggests that presence of the ε4 allele of ApoE is a good predictor of disease status. However, the presence of ε4 is not sufficient to fully predict disease status since the model is wrong, or cannot decide, in 34% of attempts. In the case of ACT TT vs. non-TT, the logistic regression model was able to predict disease status in 45% (p=0.0047) of the data set, suggesting that the TT genotype, taken alone, is a less accurate predictor of disease status than ApoE ε4 allele. When both polymorphisms are taken simultaneously in the regression, the ORs increase slightly for ApoE (OR=8.00, 95%CI=4.58-13.95, p<0.0001) and ACT (OR=2.10, 95%CI=1.13-3.89, p=0.0186), and the predictive value of the regression model to predict disease status increases to 70% (p<0.0001). These results suggest that the ability to predict disease status is greatest when both genotypes are used, as opposed to only one. Consistent with the small effect of ACT genotype, the increase in predictive ability from ACT is also small, certainly in comparison with ApoE genotype as a predictor of disease status, which is more substantial.

Several studies have produced contradictory results for the involvement of ACT in AD. Kamboh et al (1995a) were the first to report an association of the A allele in AD cases, especially in interaction with ApoE ε4 allele. Their data showed that the AA genotype was overrepresented in AD cases compared to controls (0.351 vs. 0.270, respectively; p<0.02) and the

OR for developing AD for AA vs. non-AA carriers was 1.5 (95%CI=1.1-2.1, p<0.04). Their data also showed that the ApoE ε4 gene dose effect associated with AD risk appears to be modified by ACT genotype. For ApoE, the OR for developing AD with one and two copies of ApoE ε4, compared to no copies of ApoE ε4, was 3.5 (95%CI=2.4-5.0; p<0.0001) and 11.1 (95%CI=4.7-26.4; p<0.0001), respectively. However, in the presence of ACT AA, the OR increased to 6.4 (95%CI=3.1-13.4; p<0.0001) and to 34.0 (95%CI=4.3-272.5; p<0.001) for one and two copies of ApoE ε4, respectively. Two studies have confirmed the ACT AA allele association and ApoE ε4 interaction (Thome et al., 1995; Yoshiiwa et al., 1996) but neither association nor interaction are seen in several other studies in American Caucasian, Italian, Japanese and English Caucasian populations (Haines et al., 1996; Muramatsu et al., 1996; Nacmias et al., 1996; Talbot et al., 1996; Didierjean et al., 1997; Fallin et al., 1997a; Helisalmi et al., 1997; Morgan et al., 1997; Murphy et al., 1997; Tysoe et al., 1997a).

Our results are in conflict with these reports. In our sample, we have demonstrated an interaction between ACT and ApoE ε4, and the interaction involves the T allele, not the A allele. Only one other study showed an association with the T allele, whereby a non-significant trend of association was seen in the frequency of the ApoE ε4 allele which increased from 0.265 in AA, to 0.366 in AT, and to 0.382 in TT genotypes of AD cases (Nacmias et al., 1996). The variability of the results in these different ACT studies suggests that the effect of the ACT polymorphism is subtle and may depend on genetic background. This implies that careful selection of the sample will be important factors in studies which attempt to demonstrate an ACT association with AD. As would be expected in a complex disease, false positives in the affected cases, genetic heterogeneity and population admixture will all be critical factors in the proper dissection of AD genetic etiology. The use of autopsied AD cases from a founder population in our study is expected to minimize the confounding effects of such factors. The advantages of such a population will be discussed in detail later.

Due to the apparently subtle effects that ACT has in AD, it is possible that any interaction with other AD factors such as ApoE will also be minimal, and may be reflected in modulatory effects such as minor differences in age at onset. One example is the ApoE modulation of APP in some early-onset AD families in which presence of ε4 appears to lower the age at onset, whilst presence of ε2 has the opposite effect in pedigrees with APP 670/671 or 717 mutations (Alzheimer's Disease Collaborative Group, 1993; Brooks et al., 1995; Hendrie et al., 1995; Nacmias et al., 1995; Sorbi et al., 1995).

Along these lines, we tested the hypothesis that the ACT TT genotype, either in isolation or in interaction with ApoE, can increase the severity of AD by looking for changes in phenotype which are associated with the TT genotype, such as a decreased age at onset or an increased SP or NFT accumulation. No significant differences were found for morphometric comparisons involving onset age or NFT density in ACT TT vs. non-TT individuals, suggesting that while the ACT TT genotype may be associated with an increased risk for AD, this risk does not appear to manifest in a lower age at onset nor in increased tangle accumulation. It is possible that the subtle effects of ACT may not be measurable by these particular morphometric indices. Cases who were carriers of both TT genotype and at least one copy of ε4 had higher mean densities of SP and NFT but the differences were significant only in SP ($p=0.0310$). Our data suggest that ACT TT genotype, in interaction with ApoE, is not only associated with an increased risk for AD but also may increase the severity of AD by modulating SP accumulation.

4.3 PS-1 associations

4.3.1 Lack of PS-1 association with AD risk

No significant differences were found between the frequency of alleles and genotypes in cases compared with controls in the intronic PS-1

polymorphism, suggesting that this polymorphism is not associated with AD in our sample. In previous studies, allele-1 of the intronic PS-1 polymorphism was found to be associated with late-onset AD (Wragg et al., 1996). The association was confirmed by at least two groups (Kehoe et al., 1996a; Kehoe et al., 1996b; Higuchi et al., 1996; Isoe et al., 1996b), but not by others (Scott et al., 1996a; Cai et al., 1997; Mann et al., 1997b; Tysoe et al., 1997b). A positive association of this polymorphism with late-onset AD is not to be expected, given that all other mutations in the PS-1 gene have been exclusively associated with very early-onset AD families. However, since some presenilin mutations result in a later age at onset, it is reasonable to allow for the possibility that some presenilin mutations, identified or yet-to-be-identified, can potentially cause late-onset AD. For example, novel mutations in PS-1 may yet be detected due to ascertainment bias for very early-onset AD cases in the initial PS-1 mutation reports. The higher and more variable age at onset in families with PS-2 mutations (onset age > 70 years in some cases) compared to families with PS-1 mutations, in spite of extensive homologies in the two proteins, also supports the possibility that PS-1 mutations will be found to be involved in late-onset AD. Furthermore, if genetic heterogeneity is presupposed, then it is possible that the effects of intronic PS-1 may be specific to other populations, but not our own. More and larger genetic and epidemiological studies are needed to establish the role of PS-1 in late-onset AD.

4.3.2 PS-1 interaction with ACT

The overrepresentation of AD cases with the combination of genotypes ACT TT and PS-1 12 compared to controls ($p=0.006$) suggests an interaction between these genotypes. We tested this hypothesis by estimating the OR for developing AD in TT/12 vs. non-TT/12 carriers which was found to be significantly increased (OR=2.74, 95%CI=1.30-5.78, $p=0.0083$). Our result suggests that there is a genetic interaction between the PS-1 and ACT polymorphisms, and that individuals with the genotype combination ACT TT/PS-1 12 are at an elevated risk for AD compared to individuals without this genotype. Additional evidence for the potential interaction between ACT and PS-1 was recently reported by

Wang et al. (unpublished data from Am. Soc. Hum. Genet. meeting, 1997). Stratification of their PS-1 data by ACT genotype showed that the risk associated with PS-1 allele-1 was confined to ACT A carriers only ($p=0.038$) and similarly, the risk associated with ACT A allele was confined to PS-1 allele-1 carriers only ($p=0.004$). Two-site haplotypes carrying ACT A and PS-1 allele-1 were also significantly increased in cases compared to controls ($p=0.009$).

Although our OR data support the possibility of a genetic interaction between PS-1 and ACT, our morphometric data suggest that age at onset, SP density and NFT density are not correlated with such an interaction. One possible explanation is that the putative interaction is not involved in pathways which modulate age at onset and SP and NFT density. Additionally, a genetic interaction between ACT and PS-1 would necessitate the involvement of PS-1 and therefore would likely produce linkage disequilibrium of PS-1 alleles with the disease. However, since no differences were found in PS-1 allele frequencies between AD cases and controls, it is reasonable to assume that any interaction effect between PS-1 and ACT is minimal, and that the presence of both ACT and PS-1 are simultaneously required to produce this minor effect. Additionally, since we have already shown an increased risk for AD in individuals with ACT TT genotype, we cannot exclude the possibility that the ACT TT genotype is the major contributor of the increase in risk observed in the putative PS-1/ACT interaction.

4.3.3 Lack of PS-1 interaction with ApoE

We stratified our AD cases by the presence or absence of ApoE ε4 allele and found no significant differences between PS-1 genotypes ($p=0.790$), suggesting that there is no interaction effect between PS-1 and ApoE in our sample. This result is consistent with previous studies which could not find an influence of ApoE genotype on age at onset in PS-1 families (Van Broeckhoven et al., 1994; Levy-Lahad et al., 1995c; Brice et al., 1996). Therefore, our results reinforce the notion that ApoE is not likely to be involved in the same AD biochemical pathway as PS-1. However, since ApoE has a relatively strong association with AD, it is possible that the

ApoE effect may be masking any subtle effects PS-1 may have in late-onset AD.

4.4 Case-control selection

Given the many difficulties inherent in genetic dissection of complex traits, the chances of identifying susceptibility genes can be increased by strategic selection of case and control subjects. Two possible strategies exist: 1) narrowing the definition of a disease, and 2) restricting the patient population. Therefore, association studies of complex diseases should be done in relatively homogeneous populations, which allows one to work with a trait that is more nearly Mendelian in its inheritance pattern, and one that is more likely to be homogeneous within a sample. On the other hand, artifactual associations can arise as result of case ascertainment bias. Therefore, important factors to consider in the selection of cases and control subjects will include clinical vs. autopsy phenotyping, age at onset, family history of AD, severity of disease, homogeneity of ethnic background, and exposure to risk-disposing environmental factors.

4.4.1 Narrowing the definition of a case

In order to identify genes implicated in complex diseases such as AD, proper classification of a "case" is crucial. Cases must be distinguished from non-affected controls as much as possible so as to minimize the effects of including false positives and false negatives in the sample. False positives, in the context of complex diseases, can arise not only from non-AD phenotypes, but also from AD phenotypes caused by different genes or environmental factors. The inclusion of false positives in our case sample population is undesirable and would have the effect of further diluting the disease gene one is seeking to detect.

The use of an autopsied case cohort in our association study represents several advantages. The problem of false positives arising from non-AD phenotypes is largely circumvented in this study through the use of autopsied histopathologically-confirmed AD cases which have been classified as relatively "pure" AD cases. The less desirable option of using clinical criteria prior to death for AD diagnosis is sometimes necessary but represents certain risks in misclassifying cases. A major problem is the differentiation of AD from other dementias. In the absence of criteria that denote other forms of dementia (eg. vascular dementia), the clinical diagnosis defaults to dementia of the Alzheimer type. The diagnostic tools used for clinical AD classification include neuro-imaging scans and neuro-psychological testing. Although these tools are becoming more reliable and standardized, AD cases classified in this manner are at best "possible" or "probable" AD until the diagnosis can be confirmed at autopsy with biochemical markers in brain tissue. It has previously been estimated that clinical criteria for the diagnosis of AD have an inaccuracy rate of approximately 10%, compared with autopsy diagnosis (Haines, 1991; Rao et al., 1994). However, since the pathobiology of AD is yet to be elucidated, the definite diagnosis of AD remains problematic even in autopsied cases. It is widely accepted that the presence of SP and NFT in the brain, along with pre-death symptoms of dementia, are hallmark indications of AD. However, diffuse non-Congophilic amyloid plaques are sometimes seen in cognitively unimpaired individuals, suggesting that these individuals have died of some other predisposing ailment before AD can progress to the end of its course or that amyloid plaque formation may also be a normal physiological condition in aging. If the latter explanation were true, this could have the potentially undesirable effect of diluting the disease allele in the case sample. Similarly, NFT formation is not always specific to AD, but can also occur in healthy aged brains and in other neurodegenerative diseases such as amyotrophic lateral sclerosis, Pick's disease, progressive supranuclear palsy, and Down syndrome.

The problem of false positives arising from AD phenotypes due to multiple genetic and/or environmental factors is considerably more difficult to avoid. This problem is particularly insidious due to the

lateness of onset of AD because the probability of accumulating phenotypically similar, but genetically distinct, AD traits increases with time. The finding that APP and presenilin genes cosegregate with early-onset, but not late-onset, AD pedigrees offers compelling evidence to suggest that different genes are involved in early- vs. late-onset AD. Currently, the classification of these two AD types is arbitrarily separated by the onset age of 65: early-onset AD is prior to this age and late-onset AD is after this age. This classification can pose problems if cases with distinct genetic etiologies overlap around age 65 and if the diagnosis were based solely on age at onset. Because of the possibility of genetic heterogeneity in the two groups, misclassification along these lines will reduce the probability of having a homogeneous trait in the sample.

One example is the suggestion that the effects of ACT may be more pronounced in early-onset, opposed to late-onset, AD. One study showed that age at onset was lowered in the combined presence of ApoE ε4 allele and ACT AA genotype, but only among AD patients with onset age < 65 (Talbot et al., 1996). In another study, a positive trend of association between ApoE ε4 allele and ACT T carriers was seen in a sample which included a subset of AD patients from early-onset families with PS-1 M146L and PS-2 M239V mutations with a mean onset age of 52.5 ± 2.6 years (Nacmias et al., 1996). These results are in contrast to results obtained by another group which demonstrated no association with ACT genotype in very late-onset dementia with mean onset age > 84 years in a community-based aged population (Tysoe et al., 1997b). These findings raise the possibility that the effects of ACT may be limited to earlier-onset cases, although this notion is not incompatible with the possibility that ACT can accelerate the pathogenic process resulting from distinct genes which cause dementia in the late-onset form of AD.

Misclassification of cases may also occur if genetic heterogeneity is involved in familial vs. sporadic AD. The observation that the effect of ApoE is stronger in subjects with a family history of AD than in those without (Perez-Tur et al., 1995a; Jarvik et al., 1996) suggests that gene interaction with ApoE is dependent on genetic background (eg. dependent on interactions with other genes involved in familial, but not

in sporadic, AD). Therefore, pooling of familial and sporadic AD in study samples is contraindicated because of the risk of genetic heterogeneity. Accordingly, pooling of familial and sporadic AD cases may account for the variation of associations seen in ACT studies which were conducted in community- and clinic-based non-autopsied samples (Haines et al., 1996; Muramatsu et al., 1996; Talbot et al., 1996; Didierjean et al., 1997; Fallin et al., 1997a; Morgan et al., 1997; Murphy et al., 1997; Nacmias et al., 1996; Tysoe et al., 1997a). These samples potentially include a mixture of affected cases with both familial and sporadic forms of AD. Furthermore, the subtle action of ACT implies that the effects of ACT and ApoE interaction could easily be masked if the sample were genetically heterogeneous.

One common strategy to reduce the chances of picking up secondary causal genes in a polygenic trait is to narrow the selection of the affecteds to the most severe forms of the disease phenotype. In the specific case of AD, this approach was used with success in the identification of the PS-1 gene (Mullan et al., 1992a; Schellenberg et al., 1992; St. George-Hyslop et al., 1992; Van Broeckhoven et al., 1992). Case selection in these studies was limited to individuals with very early-onset ages. However, there is no guarantee that genes identified using this strategy will be the same as ones implicated in less severe phenotypes, or that these genes will be relevant in a wider population. In spite of these caveats, genes identified in this manner may nevertheless provide new clues to a potentially overlapping pathological mechanism involved in alternate phenotypes.

False negatives in association studies are slightly less impactful than false positives but may cause the potential disadvantage of contaminating the control group. Since the selection criteria of our control subjects did not include autopsy-confirmation for absence of AD, then this poses the potential risk of including false negatives in our control cohort. However, the screening of our control subjects for signs of dementia using a standardized cognitive test (3MS) reduces this risk considerably. Having said this, we cannot exclude the possibility that our aged control subjects will subsequently develop AD, given the variability and lateness of onset in AD, even though these controls were non-demented at the

time of evaluation. Autopsy confirmation of all control subjects would have been a prohibitively expensive option and entails the possibility of a reduced sample size due to lack of participation or availability of subjects. A further consideration in our selection protocol is the possibility of ascertainment bias which is relevant to ApoE associations: individuals with cardiovascular disease are more likely to be excluded from the case series due to autopsy evaluation, but such individuals may not be excluded in the control series. This is problematic because cardiovascular disease risk is associated with ApoE genotype.

The control group used in this study was not age-matched nor sex-matched with cases. This can be problematic if the alleles of the polymorphisms being studied are dependent on gender or on age. One disadvantage, however, in using controls which are matched for both age and sex is that this imposes more stringent conditions on the selection of case and control individuals and therefore, tends to significantly lower the sample size. The use of a larger sample has the benefit of facilitating the drawing of conclusions from the data by virtue of an increase in power in the statistical analysis. A large sample size is especially critical for non-parametric association studies and for the purposes of stratification of data. Although some gender differences have been previously reported for ApoE and ACT, no gender differences were found in our sample, suggesting that any observed differences in the allele frequencies for ApoE and ACT polymorphisms between cases and controls are not likely to be caused by gender differences. Nevertheless, all OR values have been sex-adjusted to further reduce the possibility of interference from subtle gender-specific differences. Our cases and controls were not matched for age in that the mean age of the control subjects (82.4 ± 5.5 years) at the time of sampling is older than the mean onset age of cases (71.5 ± 8.2 years). This is less problematic than having a younger control group because it reduces the possibility that the disease will appear in the control individual after the time of sampling. However, the higher mean age of the control group implies the possibility of an enrichment of alleles which are associated with longevity in this group, such as the ApoE ε2 allele.

4.4.2 Narrowing the patient population

To reduce the chance of including heterogeneous AD genes in our sample, the strategy employed in this study was to restrict the patient population by using a young, isolated founder population. Our cases and controls were selected from a common geographical location, the Saquenay Lac-St-Jean area of the province of Québec (Gauvreau et al., 1988). This population has several important features. Firstly, it is a population which has remained relatively isolated from the rest of the province of Québec. The residents of the Charlevoix-SLSJ area are descendants of a few French individuals who emigrated to Québec in the 17th century. Poor transportation and communication access restricted the flow of people into and out of this region. The population also grew very rapidly between 1880 and 1940 with unusually large numbers of children per family, thus producing a population bottleneck. These factors ensured that the gene pool remained restricted and ethnically homogeneous. These so-called founder population characteristics imply that the positive associations observed in our results are not likely to be caused by admixture effects in our cases and controls.

The second important feature of the SLSJ population is the availability of a databased registry for detailed and accurate reconstruction of geneologies of individuals living, or who were living, in this region. Information from civic and parish records for the SLSJ region have been archived into a computerized database dating back to 1842, in collaboration with SOREP, a human genetics research consortium involving Université de Laval, McGill University and Université du Québec à Chicoutimi. Genealogies of most of the cases can be traced back to a common ancestor 5-10 generations ago. This allows for the possibility to select related cases who are likely to have longer stretches of linkage disequilibrium between alleles. More importantly, the disease-causing gene in such founder populations is likely to have been inherited from a common ancestor and therefore reduces the chance that polygenic influences would mask potential associations. In effect, this strategy maximizes the chances of detecting a positive association with a disease gene which is identical-by-descent with a mutated gene inherited from a

common ancestor. One caveat however is that the genes found in these restricted samples may not actually be involved in disease etiology in other populations due to between-population locus heterogeneity. One example is the association of the early-onset PS-2 mutation, N141I, in the Volga German founder population. Apart from the latter population, this mutation is otherwise absent in the general AD population. Similarly, it is possible that the interactions and associations we have found in our data set will not be applicable to other populations.

If AD etiology has an environmental component, then the use of the SLSJ founder population in our study possibly represents an additional advantage. Given the isolation to a common geographical location for this population, it is reasonable to assume that members of this population would be commonly exposed to similar environmental factors with potential AD risk effects. Therefore, the exposure to these environmental factors would be normalized in such a population and provides a relatively neutral background with respect to the effects of environment. This assumption is valid for gross and pervasive environmental factors such as air-borne pollutants, toxins or metals present in the drinking water, and broad lifestyle phenomena such as certain types of diet, but is less applicable if the environmental factor were more sporadic such as viral infection, smoking or alcoholism. Since genes can interact with environment, normalization of environmental factors to the greatest degree possible means that there is one less variable to contend with in the genetic analysis.

4.5 Linkage disequilibrium and admixture effects

The positive associations we have found must be carefully interpreted with respect to their significance in relation to the disease. The most desirable explanation for a positive association is that the allele in disequilibrium with AD is directly implicated in the pathogenic biochemical pathway that leads to disease onset. In this case, the allele's

causal relation to AD is established by the encoding of a mutation which produces a functionally defective protein that has a critical biological role with regard to disease onset or progression. However, two alternate explanations, which are not mutually exclusive, could also account for a positive association: 1) the polymorphism is in linkage disequilibrium with the true AD-causing mutation, and 2) the association is artifactual.

4.5.1 Linkage disequilibrium

If a positive association is due to linkage disequilibrium, then the true disease-causing locus resides either within the gene containing the polymorphism being assayed or in a nearby gene on the chromosome. The locus may be in a coding sequence or may also be in non-coding regions which have regulatory or transcriptional signficance such as promoters, enhancer domains, splice recognition sites and repeat expansion domains. Correlation of a random polymorphism (ie. one not associated with a direct biological role) with a disease trait will depend on the relative distance of the polymorphism to the disease locus and the frequency of the associated allele. Consequently, the correlation may depend on linkage disequilibrium characteristics in the chromosomal region containing the disease locus, which may vary depending on the particular population being studied. The more homogeneous the population, the greater the linkage disequilibrium distances are expected to extend.

For example, the ε4 allele of ApoE has been shown to be associated with AD in numerous populations. However, in spite of the robustness of the ApoE association with AD, one cannot preclude the possibility that a nearby gene in linkage disequilibrium with ApoE may be the true causative agent in AD, since it is possible that such a gene could produce similar correlations through ApoE. This notion is supported by the finding that a polymorphism in ApoC1, which is immediately downstream from ApoE, is also associated with AD (Poduslo et al., 1995). However, these results are in contrast to negative results found in linkage studies with ApoC2 which is also adjacent to ApoE (Pericak Vance et al., 1991; Yu et al., 1994; Kamino et al., 1996). ApoE, ApoC1 and

ApoC2 are tightly grouped together in a gene cluster. The proximity of these genes suggests that they should be in tight linkage disequilibrium with each other, and that alleles in one should be good surrogates for another. However, this is not the case.

Since the power to detect linkage is reduced by genetic heterogeneity, then these conflicting observations may be explained by the fact that an allele may occur more often in affected individuals within a pedigree but explains only a minor proportion of the variance of a complex trait. This allele will therefore be less effective at predicting disease status and consequently, it will be possible to detect an association with an allele without being able to detect linkage. This is an inherent weakness of linkage analysis methods when applied to complex diseases. Therefore, these observations argue that AD is genetically heterogeneous. They also argue in favour of the notion that the ApoE polymorphism may be directly involved in AD pathogenesis, as opposed to the notion that an AD causal gene is in linkage disequilibrium with ApoE. This is because a functionally significant polymorphism is more likely to cosegregate with the disease and therefore, is also more likely to be present in an affected sample and be subsequently correlated with the disease in association tests. On the other hand, a positive association which relies on linkage disequilibrium of a genetically heterogeneous trait may produce a positive association in one sample, but not in another. Similarly, a positive association for a given polymorphism in the gene cluster may be present in one sample but not another.

Alternatively, the incongruous results for this gene cluster imply at least several other possible explanations: 1) an ApoE genetic interaction occurs with ApoC1, but not with ApoC2, in AD, and 2) recombinational hotspots or recent polymorphisms may be disrupting linkage disequilibrium in this region. A deeper understanding of the interactions between ApoE and non-ApoE genetic factors will increase the chances of identifying other AD genes in genetic studies by reducing the possibility of pooling families with different genetic models in the same sample. The other lesson from these findings is that a variety of approaches is required for the proper dissection of complex traits.

Our results which show that the TT genotype is associated with AD as opposed to the AA genotype, as reported in other studies (Kamboh et al., 1995a), also argues against the notion that the ACT polymorphism has a direct biological role in AD. It is improbable that a deleterious gene product would give rise to disease susceptibility in one population but confer protection in another, unless interaction with other genetic or environmental factors is presupposed. It is more reasonable to assume that the association is related by linkage disequilibrium to another disease susceptibility mutation elsewhere, either within the ACT coding sequence, or in a nearby gene. As discussed earlier, linkage disequilibrium with different alleles is possible if the two populations have sufficiently different haplotypes in the ACT chromosomal region. This possibility is enhanced given the founder property of our population.

The contradictory correlations in several ACT studies also reinforce the possibility that the ACT polymorphism is in linkage disequilibrium with a nearby mutation which causes susceptibility to AD. It is possible that this mutation exists either within, or in the vicinity of, the ACT gene, but that the bi-allelic ACT polymorphism is not sufficiently polymorphic to be able to detect an association. This notion is consistent with the finding from one study which showed that a microsatellite marker allele, named A10 allele, near the ACT gene was overrepresented in ApoE ε4-carrier AD cases compared to controls, even though no significant difference was seen in the frequency of the ACT polymorphism between their cases and controls (Morgan et al., 1997). In that study, AD cases with 2 copies of ApoE ε4 and at least 1 copy of the microsatellite A10 allele had a significantly increased OR of 5.96 (95%C1 2.65-13.56, $p<0.001$), suggesting that there is an important contributory AD risk factor, which interacts with ApoE, that is in linkage disequilibrium with a locus in the vicinity of the ACT gene. Recently, however, another study reported that no association was found for AD and the A10 allele, either overall, or when restricted to ApoE ε4 carriers (Gaskell et al., 1997, unpublished results from Am. Soc. Hum. Genet. meeting). Since linkage disequilibrium decays with time in proportion to the recombination fraction between

loci, one possible explanation for the differences seen in the two samples is that the populations in each study are at different stages of linkage disequilibrium decay at the susceptibility locus. Linkage disequilibrium due to admixture, selection bias or genetic drift between unlinked loci are expected to decay very rapidly while linkage disequilibrium between closely linked loci will decay much more slowly. Without prior information about the probability of artifactual linkage disequilibrium in a sample, it is impossible to discern if the associated marker allele is truly linked to a disease allele. This underscores the importance of doing association studies involving complex diseases on samples which are properly controlled for admixture and other disequilibrium artifacts (see section below on admixture effects). In this regard, founder populations are particularly suitable for such association studies.

The mixed results for an intronic PS-1 polymorphism association in the literature suggest that the polymorphism *per se* is unlikely to influence the risk of developing AD, but nevertheless allow for the possibility that the polymorphism may be in linkage disequilibrium with a nearby mutation that is biologically relevant to late-onset AD. Previous evidence suggesting an allelic association in a microsatellite marker in the vicinity of PS-1 (ie. marker D14S52) in families with mean age at onset 60-70 years supports this hypothesis (Schellenberg et al., 1993). Based on our own data, we cannot reject this hypothesis. Although our results suggest that the PS-1 intronic polymorphism, by itself, is not involved in AD pathogenesis, it is possible that this polymorphism is not sufficiently polymorphic to detect an association with a locus in linkage disequilibrium with the intronic PS-1 polymorphism. Analysis of this region with polymorphic microsatellite markers would be more amenable to testing this hypothesis.

The extent of linkage disequilibrium is different for different populations, and is difficult to measure. In general though, linkage disequilibrium is expected to be more extensive in young founder populations than in large, mixed populations. The SLSJ population is a young founder population with a relatively stable founder effect. Recent attempts based on mathematical models to measure linkage

disequilibrium in this population indicate that rare mutations sampled among first settlers are maintained at levels close to the starting frequencies, even with new migrations into the gene pool (Labuda et al., 1997). While longer linkage disequilibrium sharing in founder populations may aid in the detection of disease loci by providing many surrogates for the disease marker in genetic analysis experiments, it may also represent a disadvantage in some aspects.

For example, the statistical evidence we have found for a genetic interaction between ACT and PS-1 may be due to extended linkage disequilibrium effects. Individually, ACT and PS-1 are plausible AD candidates based on the available evidence in the literature, but the close proximity of these genes, which are 30 centiMorgans apart, may be confounding the distinct and relevant effects of each gene. Potentially overlapping linkage disequilibrium effects from each gene may explain or, alternatively, obscure associations in certain markers and in certain populations. These effects are particularly problematic in founder populations where linkage disequilibrium extends over great distances. Linkage disequilibrium may also be abnormally conserved by reduced recombination in the region as a result of gross mutations such as deletions. For these reasons, a genetically heterogeneous sample containing a subset of individuals carrying an AD causal gene mutation in PS-1 could conceivably display weak linkage with ACT as a result of overlapping linkage disequilibrium encompassing the region containing the ACT and PS-1 genes. This will tend to increase the chance of observing a misleading association, especially if the markers are not very polymorphic. The other possibility that the putative interaction suggests is that the observed linkage disequilibrium is related to sharing provoked by a disease gene located within the ACT/PS-1 two-site haplotype. The observation from a recent study that the two-site haplotype containing ACT A and PS-1 allele-1 is significantly increased in cases compared to controls ($p=0.009$) reinforces this notion (Wang et al. unpublished data from Am. Soc. Hum. Genet. meeting, 1997). Haplotype analysis using case family members in our sample should resolve this issue, or at least, rule out certain sharing possibilities.

4.5.2 Admixture and other artifactual associations

Positive associations may arise as a result of population admixture. In ethnically mixed populations, a trait present at a higher frequency in an ethnic group will show positive association with an allele that also happens to be more common in that group. Therefore, misleading associations may arise which are not due to a relation between the disease and the alleles tested, but rather, to the different allele frequencies between affected and control subjects. If an association can only be found in large mixed populations, but not in homogeneous populations, then this raises the possibility that admixture or other population artifacts best account for the observed associations. As discussed above, our sample, which is derived from a founder population, is not expected to be problematic in terms of admixture. The population bottleneck effect produced by rapid growth of this population within a restricted geographical area ensures that members of this population are ethnically homogeneous.

Population admixture may account for some of the contradictory results seen in previous studies on ACT association with ApoE and AD. ACT and ApoE allele frequencies vary depending on ethnic background. The allele frequencies of ApoE ε4 (0.256 vs. 0.156) and ACT A (0.786 vs. 0.484) are significantly higher in Nigerian Black than in Caucasian populations, respectively (Davignon et al., 1988; Kamboh et al., 1997b). Given that ApoE ε4 and ACT are putative risk factors for AD, this is a surprising finding since the prevalence of AD is comparable, or lower, in Blacks than in Caucasians. One study found that there was no association between AD and ApoE ε4 in Nigerian Blacks (Osuntokun et al., 1995), while other studies in African-Americans showed similar or weaker association of ApoE ε4 and AD than that reported for Caucasian populations (Hendrie et al., 1995; Maestre et al., 1995; Tang et al., 1996). Given the wide variation of ACT allele frequencies in different ethnic groups, it is logical to assume that the observed unstable associations in AD may be explained, to a certain degree, by the effects of population admixture, if ethnically matched samples were not used. American samples may be particularly susceptible to admixture effects because of

potential inter-racial ambiguities in the classification of Caucasian and African-American individuals. It is therefore possible that admixture could ultimately account for the unusual associations found in these studies given the close genetic interaction of African-Americans and Caucasians over several centuries. Accordingly, ApoE ε4 findings in African-Americans need to be confirmed in large epidemiological studies with stringent classifications of ethnicity. In terms of ApoE and AD, the unresolved issue of ε4 allele frequencies in Blacks appears to be the exception to the rule, since the highly reproducible association of ApoE in many different ethnic groups including Caucasians, Hispanics and Asians, argues against the possibility that admixture is a major factor in these associations (Mayeux et al., 1993; Noguchi et al., 1993; Rebeck et al., 1993; Strittmater et al., 1993a; Tsai et al., 1994; Van Duijn et al., 1994a; Yu et al., 1994; Maestre et al., 1995; Tang et al., 1996). The ApoE association is therefore better explained by a direct biological involvement of ApoE or a nearby gene in linkage disequilibrium with ApoE.

Inter-study differences in ethnicity may also explain our finding of an ApoE ε4 interaction with the ACT TT genotype. In contrast to association studies which have linked the ACT AA genotype to AD (Kamboh et al., 1995a; Thome et al., 1995; Yoshiiwa et al., 1996), our study suggests that it is the ACT TT genotype which is the risk-associated genotype. It is possible that AD susceptibility in our population is specific to the T allele in ACT as a result of allelic heterogeneity of ACT in AD. If ACT operates through an interaction with other genetic loci in AD pathogenesis, then it follows that different genetic backgrounds arising from ethnic differences could potentially modify the specificity of the "disease" allele in ACT. A similar explanation may also account for the finding, in a Japanese sample, that the frequency of the AA genotype is significantly increased in cases compared to controls (0.200 vs. 0.080, respectively; p=0.05), but in subjects lacking ApoE ε4 (Muramatsu et al., 1996). This finding is in contrast to results reported by Kamboh et al. (1995a) whereby the A allele is associated with increased risk in subjects with ApoE ε4. The hypothesis that differences in genetic background as a result of ethnicity may account for the different patterns of association is consistent with the observed ethnic variations in ACT allele, such that

the A allele frequency in Japanese is lower (0.34) than in Caucasians (0.50) (Muramatsu et al., 1996). Although the specific allelic associations differ, the core argument that ACT genotype is associated with AD remains intact. The association of different ACT alleles, depending on the ethnic population, also lends support to the hypothesis that it is a mutation or polymorphism in linkage disequilibrium with the ACT polymorphism that is implicated in AD, rather than the ACT polymorphism itself.

To avoid admixture effects, one possible strategy for selecting a proper control group that is perfectly matched for ethnic ancestry in association studies is to use an internal control for allele frequencies consisting of genotyping not only affected individuals, but also their parents. Alleles in the non-affected parent, which are not also transmitted to the affected child, provides an artificial control that is well matched for ethnic ancestry. This method is called the affected family-based control or the haplotype relative risk method, and can be applied to the genotypes or to the alleles. In the opinion of Lander and Shork (1994), such internal controls should be routinely used. Parental DNA is also useful for constructing multi-marker haplotypes which can be more informative than studying single markers one at a time, especially in isolated populations where only a limited number of trait-causing chromosomes may be present.

Artifactual associations may also arise as a result of gender-dependent differences in allele frequencies. Although our data failed to provide any evidence of gender-dependent allele frequencies in ACT, previous studies have suggested that ACT allele frequencies may be gender-specific. One study demonstrated that the frequency of ApoE ε4 allele was lower in the AA genotype compared to the other two ACT genotypes in women, but not in men, and that the distribution of the A allele between ApoE ε4 and non-ApoE ε4 carriers was different in Caucasian women, but not men (Kamboh et al., 1997b). Barring the possibility that the observed differences may have arisen due to differences in survival between the sexes, this finding may have implications for the observed higher incidence of AD in women than in men. ACT and ApoE polymorphisms in AD will need further study across gender groups in order to determine

the relevance of this non-random association and underscores the need to control for parameters which may be sensitive to allele frequency variation.

Another possible source of artifactual association is selection pressure on the sample population. Differences in allele frequencies between cases and controls may arise as a result of differences in selection pressure exerted on one group, but not the other. Factors which permit non-random selection will possibly perturb allelic equilibrium and include non-random mating or migration of the population, mutation, or other unknown external selection forces. A selection bias will skew the expected distribution of genotypes towards a non-random distribution and thus, will provoke the situation where genotypes are no longer in Hardy-Weinberg equilibrium. In our control cohort, ApoE, ACT, and PS-1 genotypes are in Hardy-Weinberg equilibrium, suggesting that there are no selection biases in this cohort. In our case cohort, PS-1 genotype is in Hardy-Weinberg equilibrium, but ApoE and ACT genotypes are not. The lack of equilibrium in ApoE and ACT cases may be explained by the above selection factors or by a selection bias arising simply from case ascertainment bias. Although it is impossible to distinguish these various factors, it is probable that the latter is responsible, since there is an inherently strong external selection bias in case ascertainment, particularly if the polymorphism is in some way implicated in the disease being selected for. Since selection bias influences allelic frequencies in our sample, we are forced to compare genotypes, but not alleles, for the ACT and ApoE polymorphisms.

In order to corroborate potentially artifactual associations, one strategy is to perform a transmission disequilibrium test (TDT) once a tentative positive association is obtained. This test has the premise that a parent heterozygous for an associated allele and a non-associated allele should more often transmit the associated allele, rather than the unassociated allele, to an affected child. The transmission disequilibrium test must be applied to a new sample from the same population because individuals from the original sample used for testing association necessarily has an excess of the associated allele. In AD studies, however, the use of the

haplotype relative risk control and the transmission disequilibrium test is not always feasible due to the lateness of onset of the disease making confirmed diagnosis of both parent and child difficult. For this reason, we have opted for a standard case-control design for our association study.

4.6 Putative Protein Functions

Do the ApoE, PS-1 and ACT polymorphisms have a biological role in AD pathogenesis? For reasons discussed already, a positive association with a polymorphic allele establishes a correlation between a possible candidate locus and AD in two populations, but does not necessarily imply that the candidate locus has a biological role in the disease. It is important to note that even if linkage disequilibrium and admixture effects are sufficiently precluded as explanations in a positive association, an association which argues in favour of a biological role for the associated allele is still only a statistical correlation. Ultimately, biochemical studies are needed in order to firmly corroborate and to prove the statistical findings. It is beyond the scope of this association study to corroborate our findings with biochemical proofs. However, our data provide intriguing points of departure for further investigation into possible biological scenarios of gene interaction in AD.

Although association studies can be performed for any random DNA polymorphism, they are most meaningful when applied to functionally significant variations in genes having a direct biological relation to the trait. A clear example is ApoE. Although ApoE ε4 is now well established as an epidemiological risk factor in AD, a statistical association of the ε4 allele with the disease does not necessarily imply that ApoE isoforms have a direct causal relation to the disease. However, the dose-dependent ε4 allele association with AD and the robustness of the association in many different ethnic groups are strong arguments in support of a biological involvement of ApoE isoform protein function in the AD pathogenic cascade. If ApoE were biologically relevant in AD, it is likely

that ApoE would modulate susceptibility to AD via a mechanism which is dependent on ApoE isoform since the polymorphism impinges directly on changes in the amino acid coding sequence of the ApoE protein. The ε2 isoform has cysteine at both codons 112 and 158, the ε4 isoform has arginine at these codons, and the ε3 isoform has a cysteine at codon 112 and an arginine at codon 158. The different hydrophobicities and charge configurations of these isoforms may have a direct impact on the secondary and tertiary structures of these proteins which may, in turn, impact on the specificity of their function.

The observation that ApoE ε4 is neither necessary nor suffficient to cause AD suggests that ApoE ε4 is probably not a causal factor in AD but rather, is a susceptibility factor which modulates the effects of some prior causal event. Therefore, models which attempt to explain the biological role of ApoE in AD pathogenesis must also explain how aberrant protein function sometimes, but not always, mediates disease onset. One possible explanation is to posit an epistatic effect whereby other genetic or environmental factors are required to induce disease onset. Also, in spite of the fact that an increased ε4 allele frequency is associated with AD, it is important to note that one cannot exclude the possiblity that it is the absence of ApoE ε2 or ε3 protein, as opposed to presence of ε4, that is the risk-determining factor in AD.

Since the ACT polymorphism is located in the signal peptide sequence of the ACT protein, it is possible that the polymorphism is directly responsible for aberrant protein function. Signal peptides direct nascent proteins to lysosomal compartments where the signal sequence is cleaved and the protein is post-translationally modified. The alanine-to-threonine amino acid change in the signal peptide at the polymorphic site may alter the recognition sequence, hydrophobicity or conformational structure of the signal peptide and may thereby alter transport of the ACT polypeptide to, and its processing within, the endoplasmic reticulum (Randall et al., 1989). This deregulation can potentially increase the secretion rate and amount of the ACT protein.

Any role that the PS-1 intronic polymorphism is to have in AD will potentially involve aberrant splicing of PS-1 mRNA due to the polymorphism's location in a non-coding region and to its proximity to the exon 8 splice donor site. The locations of the known PS-1 mutations appear to be distributed in a non-random fashion throughout the PS-1 protein, suggesting that domains containing these clusters of mutations are functionally important in AD. Nine of the missense mutations occur within the last hydrophilic loop near the carboxyl terminus of the protein which is encoded by the alternatively spliced exon 8. Mutation clustering here is suggestive in that this domain is near the cleavage site and because of the existence of minor isoforms in which exon 8 is skipped (Cruts et al., 1996). This suggests that exon 8 may encode for an important functional domain which, when perturbed, leads to AD pathogenicity.

There is circumstantial biochemical evidence to support a direct biological involvement of ApoE, ACT and PS-1 in AD. Studies have shown that ApoE immunohistochemically localizes to both senile plaques and neurofibrillary tangles and that ε4 dose is correlated with amyloid load and plaque density in AD brain (Namba et al., 1991; Rebeck et al., 1993; Schmechel et al., 1993). *In vitro* studies have demonstrated that purified ApoE binds to Aß, and that the ε4 isoform binds with greater affinity and forms Aß fibrils faster than the ε3 isoform (Strittmater et al., 1993b; Sanan et al., 1994). Recently, it has been shown that the level of ε4 mRNA expression is relatively increased in AD compared to control brain, suggesting that genetic variability in the neural expression of ApoE levels may contribute to disease risk (Lambert et al., 1997). These, and our own results, which show that SP accumulation is increased in AD brains in a dose-dependent manner with respect to ε4 allele support, but cannot prove, that there is a direct relationship between ApoE and SP accumulation. Abraham et al. (1988) have shown that ACT protein, like ApoE, is expressed in AD brain and shows co-localization with amyloid plaques. Their data show that the two proteins are tightly associated in amyloid deposits. The levels of ACT mRNA and protein were enhanced in AD brain and predominantly localized to hippocampus and inferior temporal gyrus, two areas known

to be selectively abundant in SP and NFT in AD brains. ACT has also been shown to accelerate polymerization of Aß into amyloid filaments *in vitro*. Although Aß42(43) can assemble into amyloid-like filaments spontaneously, filament formation was accelerated at least ten-fold in the presence of ACT protein (Ma et al., 1994). PS-1 has been shown to colocalize to SP and to NFT *in vivo* (Wisniewski et al., 1995; Levey et al., 1997). In yeast cells, PS-1 protein was shown to bind with APP in a two-hybrid interaction assay, suggesting that PS-1 is directly involved in Aß metabolism (Waragai et al., 1997). Therefore, these accumulated findings suggest that Aß is the common link between ApoE, ACT and PS-1.

A large body of evidence from several lines of investigation supports the hypothesis that the aggregation of Aß is neurotoxic. *In vitro* experiments have demonstrated that APP, PS-1 and PS-2 mutations cause an increased production of Aß42(43), a peptide form which aggregates into amyloid fibrils faster than the shorter Aß40 peptide form (Levy et al., 1990; Wiesniewski et al., 1991; Citron et al., 1992; Jarrett et al., 1993; Roher et al., 1993; Suzuki et al., 1994; Borchelt et al., 1996; Maury et al., 1997). These findings were corroborated *in vivo* using transgenic mice overexpressing FAD-linked APP or presenilin mutations. These mice displayed increased Aß42(43) production and brain neuropathology similar to that found in AD (Games et al., 1995; Duff et al., 1996; Hsiao et al., 1996; Citron et al., 1997; Lamb et al., 1997). Moreover, individuals with PS-1 and PS-2 mutations produce increased amounts of Aß42(43) in plasma (Scheuner et al., 1996) and in brain SP deposits (Cruts et al., 1996; Lemere et al., 1996; Mann et al., 1996a; Mann et al., 1996b; Mann et al., 1997a). These accumulated findings support the so-called amyloid cascade hypothesis, whose premise is that amyloidogenesis is the first and critical step in the cascade which ultimately leads to AD, and that all other biochemical and clinical manifestations of AD occur after this first critical event. This hypothesis therefore predicts that all causes of AD should have in common that they make amyloid deposition more likely. The biochemical interaction of ApoE, ACT and PS-1 with Aß is consistent with the amyloid cascade hypothesis.

Our data suggest two possible gene interactions involved in AD: 1) between ACT and ApoE, and 2) between ACT and PS-1. These putative interactions may be mediated by other molecules or may involve, for example, a direct physical contact between ACT and ApoE proteins. The observation that there exists two binding sites on Aß, one for ApoE and another for ACT, provides suggestive evidence that interaction between these molecules is physically coordinated. The region containing amino acids 1-12 of Aß closely resembles the conserved active site of serine proteases and *in vitro* experiments have identified that the region is specifically recognized and bound by ACT as a pseudosubstrate, suggesting that this region is a natural target for protease inhibitors such as ACT (Potter et al., 1992; Fraser et al., 1993). In contrast, ApoE interacts primarily with an adjacent region containing amino acids 12-28 of Aß, a sequence predicted to form the ß-pleated sheet conformation found in amyloid filaments (Wisniewski et al., 1992; Strittmater et al., 1993a). These results suggest that the amyloid-associated proteins, ACT and ApoE isoforms, each recognize and bind to Aß to promote fibril formation, but at different sites and possibly with different degrees of specificity.

The putative interaction between ACT TT and PS-1 12 is more difficult to fit into a biological model for several reasons. Firstly, the effect of any biological interaction is likely to be small since we did not observe any allelic association with PS-1 and AD. Furthermore, our OR estimates suggest that the modifier effects of ACT and PS-1 are subtle. This is not an unexpected finding. Nearly all mutations identified so far in early-onset AD, including APP, PS-1 and PS-2, are missense mutations. The one exception is the mutation which destroys a splice acceptor site for exon 9 in PS-1. Except for the loss of exon 9 which codes for part of a hydrophilic loop, the mutant protein is otherwise unaltered, since the reading frame remains intact for the rest of the protein (Perez-Tur et al., 1995b). Missense mutations most likely cause disease by a change or gain of protein function, rather than by a total loss of function. These gene modifications are therefore predicted to translate into subtle alterations of protein function. This prediction is consistent with the lateness of onset of AD, since an extremely deleterious mutation is not expected to

manifest so late in life. This prediction is also consistent with a complex disease etiology whereby other gene or environment interactions are required to modify an initial subtle genetic defect to a critical threshold prior to disease onset.

Secondly, although we did see an association with the ACT TT genotype, the heterozygote PS-1 12 genotype is overrepresented in cases at the expense of the 11 and 22 homozygote genotypes, which are presumably protective or neutral in AD. It is difficult to explain how a heterozygote condition would be more pathogenic than either homozygote conditions, since a deleterious mutation on one allele should also be deleterious when it is present in both alleles. One possible explanation is to posit a dominant gain of function, whereby a protein's activity is abnormally amplified by a synergistic association of two different isoforms. Conversely, it is also possible that the different isoforms dimerize to produce an inactive dimer protein. In spite of these caveats, it is nevertheless possible that these putative interactions have a biological basis, albeit small, according to our data.

If ApoE, ACT and PS-1 have a biological role in AD, what is the mechanism involved? Based on the available evidence in the literature the following models are proposed as speculative possibilities for the biological roles of ApoE, ACT and PS-1: 1) pathological chaperone model, 2) amyloid clearance model and 3) neural protection models.

4.6.1 Pathological chaperone model

If the amyloid cascade hypothesis is correct, then neural injury in AD arises as a result of the accumulation of neurotoxic Aß. Most cell types express Aß but mature plaques and neuronal death occur only in highly restricted regions of AD brains (Haass et al., 1992; Seubert et al., 1992; Suzuki et al., 1994). The question then arises: how does Aß localize to these regions? One model which has been proposed posits the role of ApoE as a pathological chaperone to explain altered Aß deposition in AD brains. According to this model, nerve injury induces ApoE secretion in astrocytes. The role of ApoE is to scavenge and sequester cholesterol.

Neuronal uptake of the ApoE-lipoprotein complex is followed by intracellular release of free cholesterol, which presumably is used for neurite outgrowth and synaptogenesis. Aß accumulation, which is neurotoxic, leads to an increased supply of ApoE to these injured sites. As a result of not only the binding, but also the rate binding, of ApoE with Aß, more senile plaques are formed. The ε4 isoform is expected to increase the rate of Aß deposition since it has a higher rate and avidity of binding to Aß compared to the ApoE ε3 isoform (Strittmater et al., 1993b). Under the same model, the differential binding of ApoE to Aß may also explain the mechanism by which ε2 confers protection from AD. Since the ε2 isoform binds with the least avidity to Aß, then this isoform would be expected to produce the slowest rate of Aß deposition. It is also possible that the increased binding characteristic of ε4 isoform reduces the amount of freely circulating ApoE to combat the neurotoxicity of Aß.

The hypothesis that ApoE promotes Aß deposition in brain is supported by compelling *in vivo* evidence from recent work on transgenic mice expressing AD-linked V717F mutant APP (PDAPP) that were crossed with mice with targeted disruption of ApoE (Bales et al., 1997). This experiment found higher Aß immunoreactivity in hippocampus and neocortex of $PDAPP^{+/+};ApoE^{+/+}$ mice compared to $PDAPP^{+/+};ApoE^{-/-}$ mice, suggesting that ApoE is directly implicated in Aß deposition in brain. Moreover, intermediate amounts of Aß in $PDAPP^{+/+};ApoE^{+/-}$ hemizygous mice suggest a dose-dependent effect of ApoE in Aß deposition.

In interaction with ApoE, ACT may also play a role as a pathological chaperone. The ability of ACT and ApoE to promote fibril polymerization *in vitro* raises the possibility that their *in vivo* functions are to act as pathological chaperones that promote Aß deposition to highly specific regions of the brain (Ma et al., 1994). This hypothesis is supported by the observation that ACT mRNA and protein expression is increased in areas of AD brain prone to filamentous amyloid deposition and by the observation that ApoE ε4, which has higher binding affinity to Aß, is associated with higher plaque burden in carriers of this allele (Rebeck et al., 1993; Schmechel et al., 1993). However, the observation

that ACT protein is present in AD and non-demented aged brain amyloid deposits, but not in the amyloid lesions of Creutzfeld-Jacob disease, suggests that ACT may not be specifically involved in AD as a general response protein to amyloidosis but rather, to general aging effects (Abraham et al., 1988).

Our results suggest an interaction between ACT and PS-1. What is the putative *in vivo* relationship between ACT and PS-1, if any? As discussed earlier, many lines of evidence now suggest that mutations in PS-1 are directly involved in increasing production of Aß42(43) whose deposition eventually leads to AD (Borchelt et al., 1996; Duff et al., 1996; Mann et al., 1996a; Scheuner et al., 1996; Citron et al., 1997; Maury et al., 1997). Therefore, one possible consequence of interaction between ACT and PS-1 is a synergistic bi-modal acceleration of Aß deposition: Aß42(43) peptide is overproduced by mutant PS-1, which is then chaperoned by mutant ACT, at an accelerated rate, to form fibrils. The mechanism by which PS-1 increases production of Aß42(43) is still unknown. PS-1 has homology to a *Caenorhabditis elegans* gene, spe-4, which is involved in cytoplasmic protein partitioning during spermatogenesis (L'Hernault et al., 1992; Van Broeckhoven, 1995). One hypothesis is that the presenilins are involved in intracellular trafficking of APP, which is normally processed in the Golgi apparatus. The observation that PS-1 localizes by *in situ* hybridization to intracellular membranes, which are presumably Golgi and endoplasmic reticulum, is consistent with this hypothesis (Kovacs et al., 1996; Culvenor et al., 1997). If the polymorphism in the ACT signal peptide alters translocation into the endoplasmic reticulum, then ACT interaction with PS-1 may play a role in increasing the secretion rate and amount of the ACT protein in AD brain. This increase in ACT is then postulated to increase the pathogenic chaperoning of Aß to senile plaques. However, our data which show that SP density is not significantly correlated with ACT TT/PS-1 12 does not support this hypothesis.

The recent finding that a newly-identified endoplasmic-reticulum associated binding protein (ERAB) binds and translocates intracellular Aß to the plasma membrane suggests that the interaction of intracellular Aß

with ERAB may contribute to neuronal dysfunction in AD (Yan et al., 1997). It is possible that ApoE may be involved in the putative ERAB pathway since ERAB protein shows homology to short-chain alcohol dehydrogenases involved in cholesterol biosynthesis, such as hydroxysteroid dehydrogenase and acetoacetyl-CoA reductase. The evidence is very preliminary, but further investigations on ERAB may provide models to explain the mechanism which links intracellular Aß to the extracellular Aß seen in amyloid deposits.

4.6.2 Amyloid clearance model

An alternative model which has been proposed is that ApoE plays a role in Aß clearance in the neuropil by receptor-mediated endocytosis of ApoE-Aß complexes. This model is based on the observation that ApoE and LDL receptor-related protein (LRP) colocalize to senile plaques in sporadic AD brain (Rebeck et al., 1993). LRP recognizes and binds ApoE-containing lipoproteins. Since Aß binds to the lipoprotein binding site of ApoE, which is different from the site which is available for binding of the ApoE receptor, it is conceivable that ApoE-Aß complexes are cleared from the neutropil via the same route as that used for ApoE-lipoprotein complexes, that is, via receptor-mediated endocytosis. Differential efficacy of Aß clearance would therefore be dependent on isoform-specific receptor affinity (or possibly ApoE levels) since the ApoE LRP/LDL receptor-binding site also contains the polymorphic site responsible for ε4 isoform (Mahley et al., 1988). This model is compatible with the observation that ApoE ε3 from cultured cells more readily complex with Aß compared to ε4 isoform, and supports the hypothesis that ApoE ε3 isoform may play a role in Aß clearance (LaDu et al., 1994). The recent finding that the amino terminal of Aß contains an ApoE binding domain is consistent with this model (Weisgraber et al., 1996). The location of the ApoE binding domain also possibly explains why α-secretase proteolysis of APP is not amyloidogenic, since this produces an amino-truncated Aß17-42 peptide lacking the ApoE binding domain.

It is reasonable to assume that mutations in LRP may also cause differential receptor affinity which, in turn, could lead to reduced Aß

clearance. The observation that a bi-allelic polymorphism in exon 3 of LRP is associated with late-onset AD, particularly among AD cases with positive family history of senile dementia, supports this notion (Kang et al., 1997). Additionally, a polymorphism in the upstream region of LRP is associated with AD in an American and a French population (Wavrant et al., 1997). However, this finding is tenuous because no association for this region was found in other studies (Clatworthy et al., 1997; Fallin et al., 1997b).

According to the amyloid clearance model, it is possible that ACT plays a protective role similar to the one proposed for ApoE ε3 whereby binding of ApoE ε3 to Aß mediates clearance of the Aß peptide. In this regard, ACT possibly functions as an injury response protein that is activated by the neurotoxic effect of Aß accumulation. It has been shown that plasma ACT increases in response to inflammation, suggesting a potential role for ACT, as in ApoE, as an injury response protein with specific functions in neurons. The observation that ACT amount is increased in cerebrospinal fluid and plasma in AD patients compared to controls is consistent with this speculation (Randall et al., 1989; Matsubara et al., 1990). The hypothesis that ACT is involved in AD in a pathway involving inflammation response is also supported by the fact that ACT has 42% homology at the amino acid level to α-1-antitrypsin protease inhibitor (Chandra et al., 1983). Both inhibitors are acute-phase reactants whose plasma concentrations increase under inflammatory states or during infection.

4.6.3 Neural protection models

ApoE may also be implicated in a mechanism of AD pathogenesis involving the production of another hallmark AD biochemical marker, NFT. In contrast to SP which is found in extracellular deposits, NFT is localized to intracellular neurons. The suggestion that ApoE may be implicated in NFT formation in AD is supported by the observations that ApoE has been localized to the neuronal cytoplasm (Han et al., 1994) and is associated with NFT in AD (Namba et al., 1991). The following model has been proposed to explain how ApoE interacts with tau, the major

protein component of NFT. Hyperphosphorylation of tau can result in the loss of tau's ability to bind microtubules and is seen as a prerequisite event for the disruption of the cytoskeleton and consequent NFT formation. Hyperphosphorylated tau does not complex with ApoE ε3 nor with ε4. Since non-phosphorylated tau binds with high avidity to ε3 isoform, but has virtually no binding affinity to ε4 isoform, then this suggests that ApoE ε4 individuals are more at risk for developing AD due to decreased levels of ε3 isoforms which normally prevent hyperphosphorylation of tau in the normal aging brain (Strittmater et al., 1994). Binding of ApoE ε3 (or possibly also ε2 given its putative protective role) with tau could stabilize microtubules and the cytoskeleton, and perhaps help maintain the structure and function of neurons. The binding of ε3 to tau might also inhibit phosphorylation of tau and thus, retard the formation of paired helical filaments involved in the development of NFT.

However, the results from our study do not support the notion of a biological involvement of ApoE in NFT formation. Although NFT density was increased in a dose-dependent manner with ε4, these increases did not reach the level of significance.

ApoE may also play a role in maintenance and repair of neurons that occur during remodeling of synaptic connections. In primary neuronal cultures, ApoE ε3 complexed with VLDL increases neurite outgrowth, whereas the complex containing ApoE ε4 is inhibitory (Nathan et al., 1994). Therefore, it is possible that AD patients with ApoE ε4 have a reduced capacity for neuronal regeneration, and consequently, loss of neuronal function, due to loss of neurite outgrowth and synaptogenesis (Poirier et al., 1994). ApoE has been implicated in neuronal repair based on the observation that ApoE levels dramatically increase after peripheral nerve injury (Muller et al., 1985; Ignatius et al., 1986; Mahley, 1988; Poirier, 1994). ApoE as a neuronal repair agent is also consistent with the finding that the risk of developing AD is increased in persons with head trauma, but only in carriers of at least one ApoE ε4 allele (Mayeux et al., 1995; Jordan et al., 1997). However, ApoE ε4 individuals who are not demented exhibit diffuse amyloid plaque formation,

suggesting that ApoE ε4 may promote Aß deposition independent of other features of AD (Polvikoski et al., 1995). In light of this observation, deposition of Aß may be a benign process in aging brain, or may be a general response to neural injury or stress. Proper examination of the role of Aß deposition in AD has been difficult because of lack of information regarding the temporal progression of SP formation in AD. Usually the only available source of information is the autopsied AD brain which displays mature plaques in a static time frame.

The observation that plasma lipoproteins from ApoE deficient mice are more susceptible to *in vitro* oxidation than lipoproteins from wild-type mice suggests that ApoE may play a role in protection from oxidative damage (Hayek et al., 1994). *In vitro* experiments demonstrate that ApoE has allele-specific effects in protecting a rat neuronal cell line from oxidative cell death, with ε2 the most effective, ε3 moderately effective, and ε4 being the least effective (Miyata et al., 1996). In the same allele order, ApoE at levels found in the cerebrospinal fluid also protected cells from Aß peptides, while higher doses of ApoE led to increased cytotoxicity, particularly for ε4 (Miyata et al., 1996). These results suggest that ApoE is a multipurpose protein which functions not only as a lipoprotein transporter but also as a protein with antioxidant activity. The latter property may be involved in AD pathogenesis and general longevity via isoform-dependent protection from antioxidative damage in neuronal cells.

The hypothetical role of ACT as a neural injury response protein also fits under the neural protection model. Interaction between ACT and ApoE may amplify injury response or protection from oxidative damage. It is interesting to note that PS-2 is highly homologous to murine ALG-3, a protein whose putative function is to inhibit apoptosis in mice (Vito et al., 1996). Since PS-1 and PS-2 are 67% homologous, these proteins are presumed to have similar functions. It is possible that normal PS-1 may play a role in preventing, while mutant PS-1 may play a role in initiating, programmed cell death. This hypothesis is consistent with the finding that apoptotic induced oxidative stress and intracellular calcium levels are greatly increased in PC12 cells expressing mutant PS-1 L286V,

compared to control cell lines and lines overexpressing wild-type PS-1 (Guo et al., 1997).

4.7 The future

To date, ApoE is the only locus shown to increase susceptibility to AD. Our data which show that ApoE ε4 allele is neither necessary, nor sufficient, to cause AD, supports the hypothesis that although ApoE can account for about half of the genetic etiology of AD, additional genetic mechanisms exist. Segregation analysis of ApoE genotype in AD families are also consistent with the hypothesis of an interaction between genes and other biological factors affecting disease susceptibility (Jarvik et al., 1996; Rao et al., 1996). Therefore, further genetic modeling studies are needed to consider the joint effects of ApoE and other putative AD loci such as ACT, PS-1, PS-2 as well as novel modifier candidates, as they become identified. For example, recent efforts to screen for new genes or modifiers in a founder population pedigree in which AD etiology is not seemingly related to ApoE ε4 have yielded preliminary, but suggestive, new AD loci (Rimmler et al., unpublished results Am. Soc. Hum. Genet. meeting, 1997). The totality of these studies will provide valuable additional knowledge about genetic modifiers linked to AD which will allow better phenotyping of AD subjects in the future. In turn, better and more informative sampling will increase the chances of identifying new AD susceptibility genes in future linkage and association studies.

As would be expected in complex diseases, the multiple action of AD genes and other modifiers, and the lateness of onset of the disease, are likely to produce subtle effects when looking at single genes in isolation. Therefore, traditional gene mapping methods which have been used successfully to detect mutant genes whose effects are more pronounced and straight-forward will not be adequate. The use of less traditional approaches which reduce the number of parameters in the statistical analysis, but increase the parameters in phenotyping of the sample, may

be more effective at uncovering new AD genes. For example, methods such as IBD mapping in founder populations may increase the chances of detecting disease genes by looking for large regions of haplotype sharing that result from a slow decay of linkage disequilibrium around the disease locus. Candidate loci within a chromosomal interval which are identified in this manner will then need to be screened for association of potential functional mutations by standard mutation detection techniques such as single-strand-conformational-polymorphism (SSCP) assays and direct sequencing. Depending on the size of the region, this step can be quite time-consuming. Association studies of large numbers of polymorphisms contained within the large newly-identified interval may provide a faster means to narrow the interval to a workable size for direct sequencing. This strategy relies on the assumption that polymorphisms which are very close to the disease gene will be identical-by-descent and therefore, will produce strong positive associations, even if the polymorphisms are not related to a functional mutation. Moreover, these polymorphisms will likely reveal interaction effects between multiple chromosomal intervals if more than one gene is responsible for the disease.

Future experiments will also have to focus on generating transgenic animals with multiple transgenes to evaluate the effects of gene interaction in AD. For example, transgenic mice with mutant APP and PS-1 will allow one to study the effects of each gene alone, and in interaction, and may provide insights into the biochemical mechanism involved in the rate of production, and the trafficking, of Aß. As an alternative method to investigating discrete AD genes, potential new candidate intervals containing several genes may be investigated directly for functional significance using *in vivo* libraries. Recently, this approach was used successfully to demonstrate the functional significance of a gene on 21q22 associated with Down syndrome (Kola et al., 1997; Smith et al., 1997). As applied to the detection of AD genes, the technique would involve producing transgenic mice with YAC sequences containing the AD-linked chromosomal interval, and screening for mice with behavioural deficits associated with AD such as memory loss. Due to a natural phenomenon of YAC fragmentation during the transfer

procedure, the time required for selection of an associated mouse phenotype with the causal sub-interval is considerably shortened. This approach will be useful for rapid screening of potential AD following linkage and association studies, and will also be useful as a complementary method to traditional positional cloning. One caveat is that this approach assumes that the disease pathways in human and mouse are similar. Although this is likely to be true for highly conserved protein components in the pathway, it is less likely that this assumption will hold true for complex pathways involving multiple components from genes and environment.

The ultimate objective of identifying AD genes is to obtain targets for therapies and treatment of the disease. Prior to this step, reliable predictive and diagnostic tests are needed to determine if the patient has the disease. Biochemical analysis is currently the diagnostic tool of choice but this method has its limitations concerning availability of tissue samples, cost, time and accuracy of diagnosis. Molecular diagnostics opens a whole new field of possibilities for improving these limitations. For complex diseases such as AD, the availability of genetic information regarding causal and modifier genes implicated in the disease offers new ways to accurately test for disease status. Even in the last few years, significant advances have been made in AD genetics. We now have at our disposal several early-onset loci (APP, PS-1 and PS-2) and one susceptibility locus for late-onset AD (ApoE). More loci are certainly on their way. Although treatments such as tacrine and donepezil hydrochloride can alleviate some of the symptoms of AD, no cure is yet available since the biochemical causes of AD are not known. This raises the ethical consideration concerning the justification or the usefulness of genetic testing if no cure is yet available.

Since early-onset AD genes account for less than 5% of all AD cases, the use of these genetic markers for diagnostic testing does not have a wide applicability. The other known AD modifier, ApoE, accounts for a larger portion of AD cases, but also has substantial limitations, in that this genetic marker is neither necessary nor sufficient to cause onset of the disease. At the present time, the use of ApoE genotyping for diagnostic or

predictive testing of AD is not recommended by the American College of Medical Genetics/American Society of Human Genetics Working Group on ApoE and Alzheimer Disease (1995) and more recently, this policy statement was reiterated by the National Study Group (Post et al., 1997). According to these groups, which are composed of leading AD geneticists, policy experts and ethicists, the feasibility of ApoE in predictive testing of AD is presently hindered by the fact that only 25-40% of carriers with one ε4 allele will develop AD, and by the fact that non-carriers of ε4 are still at risk (American College of Medical Genetics, 1995). It has been calculated that ε4 homozygote non-demented individuals have a 30% lifetime risk of developing AD (Breitner 1996). Moreover, the ε4 allele is not exclusively correlated with AD in demented individuals. Other causes of dementia have been associated with ε4 such as Lewy body dementia, Pick's disease and perhaps vascular dementia (Noguchi et al., 1993; Frisoni et al., 1994; Galasko et al., 1994; Harrington et al., 1994; Schneider et al., 1995; Isoe et al., 1996a). Therefore, even though ε4 is most likely associated with AD in the majority of demented cases, the possibility of other dementing and treatable causes cannot be ruled out (American College of Medical Genetics, 1995; Bird 1995). It has been suggested that ApoE testing could be used as an adjunct to other tests in individuals with dementia (Roses 1995a; National Institute on Aging 1996). Demented homozygote ε4 persons have a 90-95% chance of being confirmed AD at autopsy, suggesting that ApoE genotyping has a high predictive diagnostic value in certain restricted contexts (Roses 1995b; Saunders et al., 1996). ApoE testing may also be useful in predicting responses to AD drug treatments such as tacrine (Poirier et al., 1995), or in predicting disease in conjunction with other diagnostic tools such as longitudinal testing for reduced glucose metabolism in brain using positron emission tomography (Reiman et al., 1996). Due to the age-dependent action of ApoE, such tests may be further refined if used in specific age groups (Sobel et al., 1995; Asada et al., 1996; Bickeboller et al., 1997; Blacker et al., 1997).

There is every chance that new polymorphisms or mutations associated with AD can provide complementary information to ApoE genotype for the purposes of AD diagnosis. For example, the interaction of ApoE with

other genes such as ACT may increase the ability to predict AD. However, any associations with these putative gene interactions must be thoroughly confirmed and corroborated in epidemiological and biochemical studies before predictive testing using these new markers can begin. In this regard, ApoE is a good case study. Serious discussion about the prospect of using ApoE in predictive testing began only once it was realized that the association of the ApoE ε4 allele was a highly reproducible result in many different populations, and that this association was backed up by biochemical evidence linking ApoE in a direct biological role with AD. However, in spite of, or perhaps because of, this large body of investigative effort, many questions regarding ApoE-related susceptibility in AD remain unanswered, or are unclear, such as the effects of other genetic factors, environment, head trauma, age, and sex. In the case of the ACT and the intronic PS-1 polymorphisms, we are still a long way off if we are to use ApoE as a benchmark. Conflicting reports of their association with AD and their putative interaction effects pose serious caveats, and preclude their usefulness in genetic testing at the present time.

CONCLUSION

Human cell physiology is extremely complex and dynamic. Taking a reductionist and perhaps simplistic perspective, all cellular processes are predetermined by genes which encode for all components, and consequently all interactions of these components, in biochemical pathways. The only possibility for modification comes from outside the organism in the form of environmental influences. Many pathways exist constitutively for normal cell function but many other dormant pathways are activated only in response to specific stimuli such as injury. Defects in these pathways may increase susceptibility to disease by perturbing a critical balance in cellular function or by reducing the cell's efficacy to respond to injury. These defects may manifest in the phenotype in many different ways depending on the point of entry in the pathway. Philosophically speaking, all biochemical pathways are a result of the cell's desire to maintain or restore balance in response to outside stimuli. The difference between causal and susceptibility genes then becomes a matter of magnitude of response. The so-called causal genetic defect is simply a defect which cannot be rescued by an alternate pathway. Since the repertoire of available pathways will always be dependent on the genetic background of the individual, and to a certain extent, the environment, then so will the efficacy of the response.

Given the complex nature of AD, we strategized that by using a founder population to search for genetic associations, we would maximize our chances of detecting gene effects, whether in isolation, or in interaction, in a background of possible reduced penetrance, genetic heterogeneity, epistasis and phenocopy effects. In addition, the use of a founder population reduces the occurrence of spurious associations caused by population admixture effects. Using this approach, we report the following:

- We have demonstrated that the ApoE ε4 allele is strongly associated with AD and that this association is dose-dependent on number of ε4 alleles. However, ε4 is neither necessary nor sufficient to cause AD, suggesting that ε4 can increase susceptibility to AD, but is not causal. Carriers of ε4 allele had a significantly higher plaque accumulation and this, in a dose-dependent manner, suggesting that ApoE may be implicated a pathological pathway involving SP accumulation. Age of onset and NFT accumulation could not be demonstrated to be associated with ε4, suggesting that these are factors which are not modulated by ApoE genotype. No gender effect of ε4 was seen in our sample. AD cases with age at onset > 80 had a significantly lower frequency of ε4 carriers, suggesting that the effects of ApoE are age-dependent. The reduced risk associated with the ApoE ε2 allele suggests that this allele is protective against AD.

- We have demonstrated that the ACT TT genotype is weakly associated with AD risk which is in contrast to other studies which have reported an ACT AA association. For this reason, we have argued that the A/T polymorphic site of ACT is not directly responsible for AD, but may be in linkage disequilibrium with a functional mutation nearby to this polymorphism.

- We were unable to corroborate previous reports that the intronic PS-1 polymorphism is associated with AD.

- We have demonstrated that AD risk is significantly increased in individuals who are carriers of both ApoE ε4 allele and ACT TT genotype, and in individuals who are carriers of both ACT TT and PS-1 12 genotypes, suggesting a genetic interaction between ACT and ApoE and also, between ACT and PS-1. The ACT/ApoE interaction was associated with an increased SP accumulation. Otherwise, both putative interactions could not be correlated with disease severity indices such as onset age and SP and NFT accumulation, suggesting that these putative interactions are subtle. No interaction effect was seen between ApoE and PS-1 polymorphisms.

- Our results provide statistical correlations for the involvement of ApoE, ACT and PS-1 in AD pathogenesis but do not allow us to determine if these genes have a direct biological role in AD. Moreover, these polymorphisms do not adequately explain AD in our sample, suggesting that other genetic or environmental AD modifiers exist.

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