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MOLECULAR GENETICS OF ALZHEIMER'S DISEASE

With special emphasis on presenilin, amyloid beta precursor protein and apolipoprotein E genes

Doctoral dissertation

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of the central nervous system and a leading cause of dementia. A part of AD is genetic in etiology and is classified as familial AD (FAD). FAD is further subdivided into early- and late-onset forms. The genes implicated on early-onset FAD comprise the amyloid beta precursor protein (APP) gene on chromosome 21, the presenilin-1 (PS-1) gene on chromosome 14 and the presenilin-2 (PS-2) gene on chromosome 1. Apolipoprotein E (ApoE) polymorphism is a major susceptibility factor especially for late-onset AD and is located on chromosome 19. It may be involved in other neurodegenerative disorders (ND) and play a role in regulating cholinergic metabolism in human brain. Alpha-1-antichymotrypsin (ACT) and the PS-1 intron 8 polymorphisms have been proposed to modify the risk associated with the ApoE ε4 allele to induce AD.

The aims of the present study were (1) to search for previously characterised APP mutations in a sample of 34 familial and 139 sporadic AD cases; (2) to perform linkage analysis to the PS-1 and the PS-2 genes in 15 AD families and to search for new PS-gene mutations in early-onset FAD families by sequencing some PS-1 exons; (3) to determine the frequency of the ApoE alleles in 94 ND and in 94 AD samples; (4) to use the activity of acetylcholinesterase (AChE) of 57 AD patients in the cerebrospinal fluid (CSF) as an index of cholinergic system and study its relationship with different ApoE alleles and (5) to study the combined effect of ApoE-ACT polymorphism in 218 and ApoE-PS-1 intron 8 polymorphism in 219 sporadic AD patients.

The well known APP mutations were not found. Linkage analysis in FAD families failed to detect linkage to the PS-1 and PS-2 genes. Instead, a missense mutation at codon Glu318Gly (glutamic acid to glycine) in the PS-1 gene was found in one FAD family. The frequency of the ApoE ε4 allele tended to increase in some ND groups: vascular dementia (35%), Parkinson's disease and dementia (38%), Lewy-body disease (28%) and frontal dementia (39%) in addition to AD (44%) as compared to controls (17%). Increased AChE activity was observed in the CSF of AD patients carrying the ApoE ε4 allele. Factors modifying the polymorphism (ACT and the PS-1 intron 8) were not associated, either alone or in combination, with the ApoE ε4 allele, and the associated risk of AD.

The APP mutations are a rare cause of AD. Well known APP mutations were not found which may partly be due to the small sample size (34 familial AD cases). The linkage study excluded the PS-1 and the PS-2 genes. This could be due to the structure of the AD families or excessively high age of onset in the affected AD patients in families. A missense mutation Glu318Gly was present in three affected AD cases and in two offspring from the same AD family but it was absent from 40 healthy controls. Whether it is pathogenic remains to be determined. Although an increased ApoE ε4 allele frequency was observed in some ND groups, only in the AD group was this statistically significant when compared to controls. Increased AChE activities in CSF suggest that the cholinergic metabolism is altered in proportion to the number of ApoE ε4 alleles in AD. This finding could have an impact on therapeutic approaches using cholinesterase inhibitors in AD. Increased risk of developing AD caused by ε4 allele and its effect were independent from ACT and the PS-1 intron 8 polymorphisms.

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Seppo Helisalmi

ABBREVIATIONS

AChE	Acetylcholinesterase
ACT	α_1 -antichymotrypsin
AD	Alzheimer's disease
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
βA4	β-amyloid
ChAT	Choline acetyltransferase
ChE	Cholinesterase
cM	centiMorgan
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
FAD	Familial AD
LOD	Logarithm of the odds
ND	Neurodegenerative disorders
PCR	Polymerase chain reaction
PS	Presenilin
RFLP	Restriction fragment length polymorphism

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by the Roman numbers **I-VI**.

I. Helisalmi S, Mannermaa A, Lehtovirta M, Ryynänen M, Riekkinen P Sr, Soininen H: Screening for amyloid beta precursor protein codon 665, 670/671 and 717 mutations in Finnish patients with Alzheimer's disease. Neurosci Lett 205:68-70, 1996

II. Helisalmi S, Mannermaa A, Lehtovirta M, Hiltunen M, Ryynänen M, Riekkinen P Sr, Soininen H: Presenilin-1 and Finnish Alzheimer's disease; linkage and mutation analysis, submitted

III. Helisalmi S, Linnaranta K, Lehtovirta M, Mannermaa A, Heinonen O, Ryynänen M, Riekkinen P Sr, Soininen H: Apolipoprotein E polymorphism in patients with different neurodegenerative disorders. Neurosci Lett 205:61-64, 1996

IV. Soininen H, Lehtovirta M, Helisalmi S, Linnaranta K, Heinonen O, Riekkinen P Sr: Increased acetylcholinesterase activity in the CSF of Alzheimer patients carrying ε4 allele. NeuroReport 6:2518-2520, 1995

V. Helisalmi S, Mannermaa A, Lehtovirta M, Ryynänen M, Riekkinen P Sr, Soininen H: No association between α_1 -antichymotrypsin polymorphism, apolipoprotein E and patients with late-onset Alzheimer's disease. Neurosci Lett 231:56-58, 1997

VI. Helisalmi S, Mannermaa A, Lehtovirta M, Ryynänen M, Riekkinen P Sr, Soininen H: Lack of association between presenilin-1 polymorphism, Alzheimer's disease and apolipoprotein E. Alzheimer's Res 3:159-161, 1997

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1. INTRODUCTION

Alzheimer's disease (AD) is the most common cause of cognitive decline in the elderly, being characterized by progressive loss of memory and other cognitive functions and changes in personality. Familial AD is genetically heterogenous and three different genes have been identified by genetic studies, amyloid beta precursor protein (APP), presenilin-1 (PS-1) and presenilin-2 (PS-2). Mutations in these genes lead to familial forms of AD. Mutations in each of these genes also result in elevated levels of amyloid beta (βA4), a proteolytic processing fragment of the APP, that is deposited in the brains of AD patients.

Apolipoprotein E (ApoE) is a glycoprotein, which is involved in the transport of cholesterol and the metabolism of lipoprotein particles. ApoE gene contains polymorphic forms and an increased frequency of the ε4 allele has been observed in AD patients when compared with control samples (Strittmatter et al., 1993). The association between the ApoE ε4 allele and other neurodegenerative disorders (ND) is controversial (Pirttilä et al., 1996a). Some studies have reported an increased ε4 allele frequency, for example, in patients with vascular dementia, Lewy-body dementia and Parkinson's disease with dementia (Frisoni et al., 1994; Harrington et al., 1994; Gearing et al., 1995). Moreover, recent research has shown that ApoE polymorphism may have impact on neuronal plasticity and regenerative capacity (Arendt et al., 1997) as well as the degree of the cholinergic deficit in AD brains (Poirier et al., 1995).

Since ApoE polymorphism is known to be a risk factor, many laboratories have sought other genetic risk factors. Alpha-1-antichymotrypsin (ACT) has been suspected to play a role in AD, because it binds the βA4 in AD brains (Abraham et al., 1988). It is proposed that a polymorphism in the signal peptide of ACT gene increases the risk of developing late-onset AD (Kamboh et al., 1995). Polymorphism in the PS-1 intron 8 has been demonstrated to increase the risk of developing late-onset AD (Wragg et al., 1996).

Linkage analysis is a commonly used method to study pedigrees with inherited diseases. The idea of this method is to follow the segregation of marker allele(s) from parents to offspring in families with affected and non-affected members. The extent of linkage is measured by defining a statistical probability, the LOD score value.

This study is a part of an extensive investigation aimed at identifying genes underlying AD. The purpose of the present study was to delineate molecular genetics of AD with patients from eastern Finland.

2. REVIEW OF THE LITERATURE

2.1 Alzheimer's disease and genes contributing to it

In western societies, AD is the major disease causing progressive dementia, characterized by gradual loss of memory, decline in other cognitive functions and decrease in functional capacity. Survival disease is variable patients with AD, and they usually die of infections, with death occurring approximately 10 years after the onset of symptoms. Histopathologically, the characteristic features seen in the brain of AD patients are the presence of senile plaques with amyloid beta accumulation, neurofibrillary tangles and cerebrovascular amyloid deposits (Selkoe 1989). Extensive neuronal damage and loss of synapses are also found in AD brain (Terry and Katzman 1983). The same kind of neuropathological changes as in AD also occur in other ND disorders and to a lesser degree in normal aging (Hardy and Allsop 1991). AD is an age-dependent disorder, its prevalence increases with advancing age. In Finland, the prevalence of moderate to severe AD is 0.3% in persons aged between 30 to 64 years, 3.6% in those aged 65 or more and 14.8% in persons aged over 85 years (Sulkava et al., 1985). Clinically, increasing age and a positive family history of dementia are the only definite risk factors for AD (Van Duijn et al., 1991a). Severe head trauma and previous depression may also increase the risk for AD (Mortimer et al., 1991;Van Duijn et al., 1991a; Jorm et al., 1991) .

AD is usually divided into early-onset ('presenile') dementia accounting for 25% of cases and late-onset ('senile') dementia using the age 65 years at onset as the cut-off age (Terry and Katzman 1983), and it is also divided into familial and sporadic forms of disease according to family history. Early-onset familial cases comprise 10% and late-onset familial cases 30% of all AD patients (Van Duijn et al., 1991a).

The clinical diagnosis of AD is based on criteria defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R, American Psychiatric Association 1987) and the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association Work Group (NINCDS-ADRDA) (McKhann et al., 1984). The NINCDS-ADRDA criteria provide guidelines for a clinical diagnosis of *probable* and *possible* AD. A diagnosis of *definite* AD can be confirmed in neuropathological examination of the brain tissue either from biopsy or autopsy material. The criteria of the Neuropathology Task Force of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Mirra et al., 1991) have been commonly used to set the neuropathologic diagnosis of AD.

Mutations in at least three different genes are responsible for early-onset FAD and show nearly 100% penetrance with autosomal dominant inheritance (Figure 1). The APP gene is located on chromosome 21 (Goate et al., 1991) and a total of six missense mutations have been described so far, all of which lead to AD. The APP gene mutations are responsible for

about 2% of all published cases of FAD (Tanzi et al., 1992) and approximately 5-20% of early-onset FAD (Tanzi et al., 1996; Campion et al., 1996).

At least 41 different mutations have been found in the PS-1 gene on chromosome 14 (Hardy 1997). All mutations, except one, are missense mutations. PS-1 mutations account for 30-50% of presenile AD families (Hutton et al., 1996).

The PS-2 gene on chromosome 1 was found using sequence homology strategy to the PS-1 (Levy-Lahad et al., 1995b; Da Silva and Patel 1997). The PS-2 gene mutations (2% of all early-onset FAD) are much rarer causes of early-onset FAD than mutations in the PS-1. Two missense mutations with incomplete penetrance have been found so far.

Polymorphism of ApoE gene has been demonstrated on chromosome 19 (Figure 1). ApoE ε4 allele has been identified as a risk factor especially in late-onset AD patients. Not all individuals with ApoE ε4 allele are afflicted by AD, even at very old ages.

Several other risk factors have also been proposed. The ACT and the PS-1 intron 8 polymorphism on chromosome 14 may act as factors that alters the risk of AD associated with the ApoE ε4 allele in late-onset AD (Kamboh et al., 1995; Wragg et al., 1996). HLA, the major histocompatibility complex in humans, consists of several polymorphic genes which are involved in regulating the immune response. Evidence has accumulated for an association of HLA-A2 allele and patients with early- or late-onset AD on chromosome 6 (Payami et al., 1997). The gene coding for the low-density lipoprotein receptor-related protein gene (LRP), which is the ApoE receptor and resides on chromosome 12, may be associated with the expression of late-onset AD (Kang et al., 1997). Mitochondrial cytochrome c oxidase (CO) gene mutations have been described to segregate with late-onset AD. Cell lines that express these mutant mitochondrial DNA molecules exhibited a specific decrease in CO activity and increased production of reactive oxygen species (Davis et al., 1997). In addition, a recent study has reported evidence for a new locus on chromosome 12 in late-onset FAD (Pericak-Vance et al., 1997). A complete genomic screen revealed four regions of interest (chromosomes 4, 6, 12 and 20), but chromosome 12 gave the strongest and the most consistent result in linkage analysis.

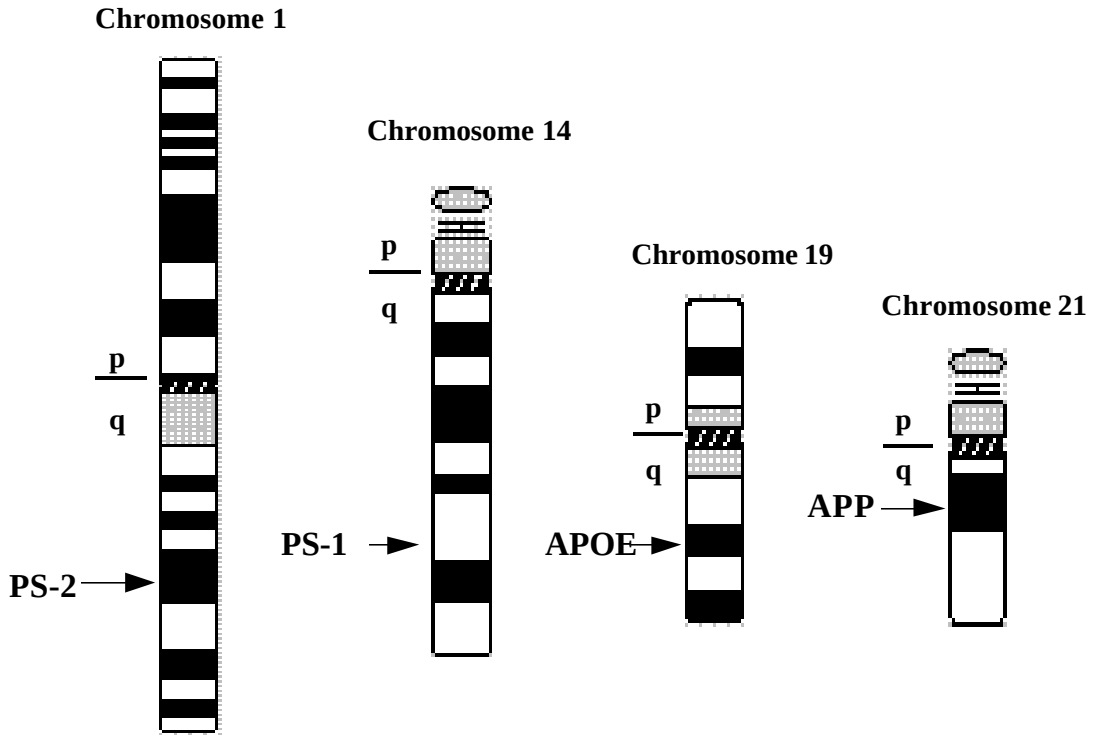

Figure 1. Ideograms of human chromosomes 1, 14, 19 and 21 showing the cytogenetic locations of the genes for presenilin-2 (PS-2), presenilin-1 (PS-1), apolipoprotein E (ApoE) and amyloid beta precursor protein (APP).

2.2 Amyloid beta precursor protein and amyloid beta peptide

The first clue pointing to the involvement of chromosome 21 in AD came from the observation that individuals with Down syndrome, who have chromosome 21 trisomy, invariably develop the clinical and pathological features of AD if they live over 30 years (Mann et al., 1989). This supported a theory that chromosome 21 can underlie AD phenotype (Wisniewski et al., 1985; Mann 1985).

The first linkage using DNA markers was found in 1987 in autosomal dominant early-onset FAD families (St George-Hyslop et al., 1987). The gene coding for the APP was isolated and localized on chromosome 21 (Kang et al., 1987; Goldgaber et al., 1987; Tanzi et al., 1987) to the region 21q11.2-q21.1 (AD1) (Tanzi et al., 1987). Finally, sequencing of the APP gene revealed mutations in the amyloid beta (βA4) sequence and screening for mutations was initiated (Goate et al., 1991; Chartier-Harlin et al., 1991b; Hendriks et al., 1992; Mullan et al., 1992).

Native APP is a transmembrane protein of 110 to 135 kDa, with a long extracellular N-terminal segment and a short intracellular C-terminal tail (Figure 2). The important cleavage product of the APP is a 39-43 amino acid peptide, the 4 kDa βA4 peptide (Glenner and

Wong, 1984; Masters et al., 1985; Kang et al., 1987). Two-thirds of sequence of the βA4 peptide (a part of exons 16 and 17) localizes to the N-terminal region of the APP and the remaining portion of the βA4 peptide is contained within the transmembrane domain (Kang et al., 1987; Selkoe et al., 1988; LeMaire et al., 1989).

The APP gene contains 18 exons and probably represents a family of at least eight different transmembrane isoforms, which arise by alternative splicing of exons 7, 8 and 15. The APP isoforms expressed mainly by neurons (APP forms 695, 714, 751 and 770) contain exon 15, are more amyloidogenic and release much more βA4 peptide than the non-neuronal (APP forms 677, 693, 733 and 752) APP forms (Hartmann et al., 1996; Sandbrink et al., 1996a). APP751 and APP770 forms are widely expressed in different tissues, whereas the APP695 form is mainly present in the brain (Golde et al., 1990; Johnson et al., 1990). In general, the APP is expressed in mammalian tissues, including the central nervous system and peripheral tissues such as heart, muscle, kidney, and spleen (Kang et al., 1987; Goedert 1987; Oltersdorf et al., 1989).

The APP is processed by at least two pathways in all cells (Hardy, 1997). The cleavage site of α-pathway (called the α-secretase process) is within the βA4 domain between residues 687 and 688 of the APP and thus it does not yield βA4 peptide. The cleavage site of the β-pathway (called the β-secretase process) is between residues 671 and 672 of the APP and it yields βA4. In addition, the γ-pathway (called γ-secretase process) involves a cleavage in the vicinity of residue 712 of the C-terminus. In the β-pathway, the cleavage site of γ-secretase is of key importance. A short βA4 peptide (contains 1-40 amino acids) results from a cleavage site at residue 712-713, and if the cleavage site is situated at the C-terminus at residue 714, long βA4 peptide (contains 1-42(43) amino acids) is produced. Thus, the γ-pathway may be fundamental to the production of long βA4 peptide.

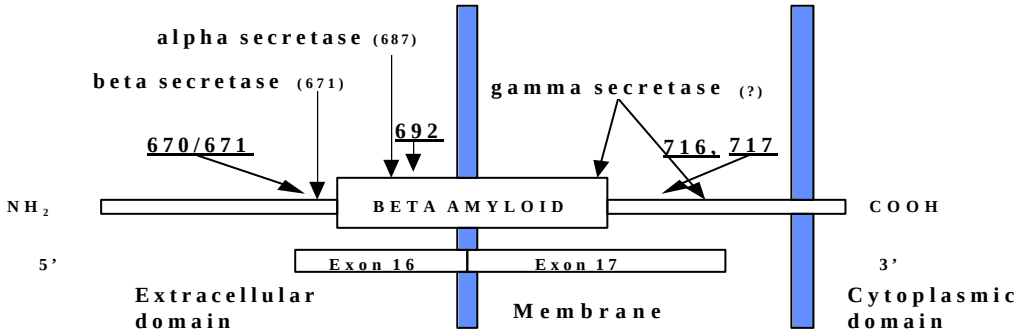

Figure 2. Domain structure of APP showing the position of the mutations causing Alzheimer's disease (underlined numbers) within amyloid beta (βA4) and secretase cleavage sites

2.2.1 Mutations of amyloid beta precursor protein gene

In 1990, the first mutation, an amino acid substitution of glutamate (Glu) to glutamine (Gln) was found within the βA4 peptide domain of APP at codon 693 ('Dutch mutation'). This mutation is responsible for hereditary cerebral hemorrhage with amyloidosis-Dutch type (HCHWA-D) (Levy et al., 1990; Bakker et al., 1991). In this disease, amyloid deposits containing the βA4 peptide are found in cerebral vessel walls and there are diffuse plaques in the brain parenchyma (Van Broeckhoven et al., 1990).

In one Dutch family, a mutation was found at codon 692 of APP ('Flemish mutation'), resulting in an amino acid substitution of alanine (Ala) to glycine (Gly) that causes an intermediate phenotype between congophilic angiopathy and AD (Hendriks et al., 1992).

A study of exon 17 of APP gene, a cytosine-thymine transition was found from affected AD individuals in a British family (Goate et al., 1991). The mutation results in a valine (Val) to isoleucine (Ile) substitution at amino acid 717 ('London mutation') that segregates with the disease. This substitution has been failed to find screening of several hundred unrelated sporadic cases or normal controls (Goate et al., 1991; Van Duijn et al., 1991b; Schellenberg et al., 1991; Chartier-Harlin et al., 1991a; Tanzi et al., 1992; Kamino et al., 1992). Following the discovery of the first mutation to be linked to AD, exon 17 has been sequenced in other early-onset AD families. Several AD families have shown to have this mutation (Table 1). Allelic variants at codon 717 have also been revealed by sequencing (Val to Ile, Val to phenylalanine (Phe) and Val to Gly substitutions) (Chartier-Harlin et al., 1991a; Murrell et al., 1991; Fujigasaki et al., 1994) the most frequent mutation being Val to Ile (Almqvist et al., 1993; Lendon et al., 1997a).

Furthermore, a clinically-silent mutation at codon 716 has been reported in a control subject with no history of cognitive decline and primary dementia (Zubenko et al., 1992). No amino acid substitution is found in this mutation. A pathogenic mutation at codon 716 ('Florida mutation') has recently been identified by Hutton and Hardy (1997).

Two Swedish early-onset AD families have been identified in which disease symptoms are caused by two base pair transversions of guanine-thymine and adenine-cytosine which resulted in lysine (Lys) and methionine (Met) being replaced by aspartic acid (Asp) and leucine (Leu) at codons 670 and 671 ('Swedish mutation'), respectively. These changes occur at two amino acids immediately before the N-terminal amino acid of the βA4 peptide (Mullan et al., 1992).

A possible sporadic missense mutation at codon 713 (Ala to threonine) and a silent mutation at codon 715 have been found in an AD patient whose onset age was 59 (Carter et al., 1992). These mutations have not been found in the general population, but they were present in her unaffected sibs and in an 88 year old unaffected aunt in this family. That makes it difficult to assess the true pathogenic nature of these mutations in AD.

A mutation at codon 665 (Glu to Asp) has been found found in an elderly subject with late-onset dementia, who fulfilled the neuropathological criteria of AD. This is the only APP mutation so far which is involved in late-onset AD. It is unclear whether this is a rare mutation, or represents a normal polymorphism (Peacock et al., 1994). APP mutations or linkage to chromosome 21 have previously been failed to find in patients with late-onset FAD (Schellenberg et al., 1988; van Duijn et al., 1991b).

Table 1. APP mutations associated with AD

Number of families	Mean age of Onset	Mutation	Reference
1		Gly 692 Ala	Hendriks et al., 1992
2	55	Lys 670 Asn Met 671 Leu	Mullan et al., 1992
		Ile 716 Val	Hutton and Hardy 1997
2		Val 717 Ile	Naruse et al., 1991
1		Val 717 Ile	Yoshioka et al., 1991
1	43	Val 717 Phe	Murrell et al., 1991
1		Val 717 Gly	Chartier-Harlin., et al 1991b
2	54	Val 717 Ile	Goate et al., 1991
1		Val 717 Ile	Fidani et al., 1992
1	48	Val 717 Ile	Karlinsky et al., 1992
1		Val 717 Ile	Yoshizawa et al., 1993
2	54	Val 717I Ile	Sorbi et al., 1993
1		Val 717 Ile	Sorbi et al., 1995
1	49	Val 717 Ile	Brooks et al., 1995
1	55	Val 717 Ile	Matsumura et al., 1996
3		Val 717 Ile	Campion et al., 1996

2.2.2 Pathogenicity of the amyloid beta precursor protein mutations

The APP mutation at codon 693 may alter the secondary structure of βA4 peptide in the cell membrane resulting in its premature deposition (Fabian et al., 1993). Cosegregation of missense mutations of the APP gene with the disease in some families with early-onset FAD have suggested that these mutations have been pathogenic (Van Broeckhoven et al., 1994a; 1995).

Mutations at codons 670/671 and 717 do not lie within βA4 but flank it, lying very close to the sites of secretase cleavage (Figure 2). Cell culture studies have elucidated the effect of the mutations upon processing of the APP. Soluble βA4 peptide (predominantly βA4 1-40 peptide) is a shorter product of cell metabolism, which is rapidly cleared, preventing plaque formation in the brain, whereas the longer peptides of 42 or 43 amino acids aggregate more rapidly into fibrils (Haass et al., 1992; Jarrett et al., 1993).

Fibroblast cell lines transfected with the Swedish APP mutation at codons 670/671 produce elevated levels of soluble βA4 peptide compared to wild-type cells (Citron et al., 1992; Cai et al., 1993; Johnston et al., 1994; Citron et al., 1994). The APP 717 mutations (Val to Ile and Val to Phe) produced up to two times longer and a more insoluble form of the βA4 peptide, which aggregated rapidly and promoted βA4 peptide deposition (Suzuki et al., 1994). The APP 717 (Val to Ile) and the APP 693 mutations did not cause any increase in secreted βA4 peptide (Murayama et al., 1991; Felsenstein et al., 1993). The APP 692 mutation led to formation of an APP molecule containing a truncated βA4 peptide and overproduced βA4 peptide (Haass et al., 1994).

AD-type neuropathology has been demonstrated in transgenic mice with APP mutations at codons 670/671 and 717 (Val to Phe), which promoted βA4 peptide depositions in the brain as well as causing memory impairment, but they lacked neuronal degeneration (Games et al., 1995; Hsiao et al., 1995).

2.3 The Presenilins

2.3.1 The presenilin-1

Since most early-onset AD families do not have mutations in the APP gene, it was expected that other AD loci might exist. In 1992, many groups presented evidence for a second locus (S182, PS-1, AD3) responsible for early-onset FAD on the long arm of chromosome 14 (St George-Hyslop et al., 1992; Van Broeckhoven et al., 1992; Mullan et al., 1992). A positional cloning strategy was used to define and isolate a candidate gene in AD families that carried coding region mutations (Sherrington et., 1995).

The PS-1 gene contains 10 protein-coding exons (the open-reading frame limited to exons 3-12 spanning approximately 75 kb) and 2 or 3 additional exons encoding the 5'-untranslated region (Clark et al., 1995b; Cruts et al., 1996). The PS-1 gene undergoes alternate splicing. Alternative splicing of exon 8 has been described (Sherrington et al., 1995; Rogaev et al., 1995). The inclusion/exclusion of codons 26-29 (Val-Arg-Ser-Gln) at the 3' end of exon 3 has also been reported (Clark et al., 1995b).

The major RNA transcripts in the PS-1 gene are 2.7 and 7.5 kb, and these are expressed in different human brain regions, skeletal muscle, kidney, pancreas, placenta, and heart (Sherrington et al., 1995). The PS-1 is a serpentine protein (467 amino acids) with six to nine transmembrane domains (TM) (Figure 3). As is with the PS-2 gene, it is also localized in the nuclear envelope, endoplasmatic reticulum and the Golgi apparatus in mammalian cells (Kovacs et al., 1996).

At least 41 different FAD mutations in the PS-1 gene have been identified in more than 50 unrelated families (Cruts et al., 1996; Hardy, 1997; Lendon et al., 1997a). Most mutations lie in exons 5 (TM 1-2) and 8 (TM 6-7) (Cruts et al., 1996). Mutations in these two clusters of the PS-1 gave significantly different ages at onset when compared with each other. Patients with mutations in TM 6 and 7 clusters have a higher mean age of onset than those with mutations in the clusters around TM 1 and 2 (Rossor et al., 1996). All except one of the PS-1 gene mutations are missense mutations, changing a single amino acid. The splice acceptor site mutation is the exception in exon 9 of the PS-1 gene, which results in an in-frame deletion of this exon, and a mutation causing substitution of serine by cysteine at codon 280 at the splice junction between exon 8 and 10 (Perez-Tur et al., 1995). Furthermore, one PS-1 gene mutation in an English family has been described with incomplete penetrance (Rossor et al., 1996). This mutation resulted in a isoleucine to valine at codon 141 of the PS-1 gene and mean age at onset of 55 years in two FAD patients. The mutation was also present in a healthy individual aged of 68 years.

The known FAD gene mutations are distributed throughout the PS-1 gene, most of them in predicted TM domains. Furthermore, all FAD missense mutations occur in amino acids that are conserved in the PS-1 and the PS-2 (Sherrington et al., 1995; Levy-Lahad et al., 1995b; Rogaev et al., 1995).

2.3.2 The presenilin-2

The presenilin-2 gene (PS-2, STM2, AD4) was found and isolated based on its homology to the PS-1 gene (Levy-Lahad et al., 1995b,c). Genetic linkage of AD with marker D1S479 and a missense mutation causing substitution of asparagine by isoleucine at codon 141 (Asn141Ile) were identified in chromosome 1 of the Volga German families (Levy-Lahad et al., 1995b,c).

The Volga Germans (seven families) originate from one German family that first emigrated to the Volga valley in Russia in the 1760s and later to the USA (Bird et al., 1988). Linkage of AD to chromosomes 14 and 21 have been excluded in these families (St George-Hyslop et al., 1992; Schellenberg et al., 1992). The common ethnic origin of these families have suggested that FAD in these kindreds has been the result of a common founder.

The PS-2 gene contains 10 protein-coding exons and two additional exons encoding the 5'-untranslated region (Levy-Lahad et al., 1996). The transcripts of the PS-2 gene (2.4 to 2.8 kb) are extensively expressed in many tissues (Levy-Lahad., 1995b), but show more extensive splicing than the PS-1 gene (Prihar et al., 1996). The PS-2 isoform missing exon 8 has been described (Sherrington et al., 1995; Rogaev et al., 1995). Alternative splicing of in-frame acceptor sites in introns 9 and 10 (Levy-Lahad et al., 1996) and in-frame omissions of exon 8 and simultaneous omissions of exon 3 and 4 have been reported (Prihar et al., 1996).

Two PS-2 FAD missense mutations (Asn141Ile and substitution of methionine by valine (Met239Val)) have been shown in eight unrelated pedigrees (Cruts et al., 1996; Hardy, 1997; Lendon et al., 1997a). The reported mutations of the PS-2 gene are located in different codons to those found in the PS-1 gene. The unique feature of the PS-2 gene mutations is their incomplete penetrance. Individuals carrying the mutation may remain healthy (Levy-Lahad et al., 1995b; Sherrington et al., 1996).

The PS-2 is also a serpentine protein (448 amino acids) with six to nine TM. The intron-exon structure is very similar to that of the PS-1 and 67% identity at the amino acid level and possibly has a similar function as the PS-1.

Evidence for specificity of function for both presenilins derives from the presence of non-homologous regions in these molecules: hydrophilic N-termini and the large hydrophilic loops (HL-6) between transmembrane domains six and seven in a seven TM model. These two regions are located on the same side of the membrane and are not divergent, but the HL-6 loop in the PS-2 is smaller than in the PS-1 (Levy-Lahad et al., 1995b; Clark et al., 1995b).

2.3.3 Effects of APP to the presenilins

In FAD families with the PS-1 gene mutation, the mean age of onset is earlier (45 years; range 29 to 62 years) than in families with the PS-2 gene mutation (52 years, range 40 to 88 years) and in the APP gene mutation linked families (50 years; range 43 to 62) (Lendon et al., 1997a).

Increased amounts of secreted βA4 peptide have been observed in *in vitro* cell lines transfected with the PS-1 or the PS-2 gene mutations (Scheuner et al., 1996). Based on studies of the Volga German families, the total amount of short or long βA4 peptides are significantly lower in the case of the PS-2 mutation compared to the PS-1 (Mann et al., 1997).

In vivo experiments have suggested that mutant PS-1 proteins alter the proteolytic processing of APP at the C-terminus of βA4 to favor deposition of long βA4 (Lemere et al., 1996). Transgenic mice carrying the human PS-1 mutation have twice as much soluble mouse long βA4 peptide in their brains compared with normal mice (Citron et al., 1997; Borchelt et al., 1996; Duff et al., 1996).

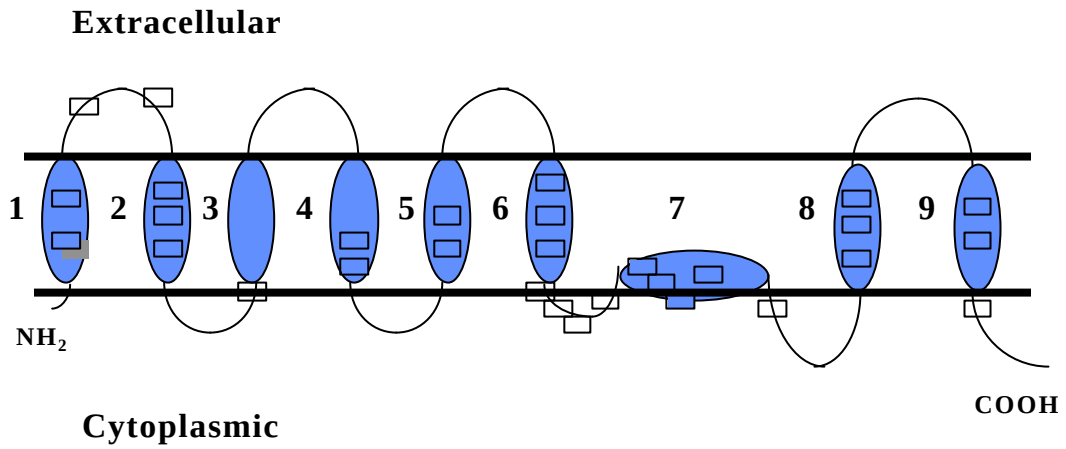

Figure 3. Putative transmembrane TM domain structure of PS-1 (modified from Li and Greenwald, 1996). The clusters of mutations (boxes) between and in the TM domains (1-9) (elliptic symbols) are distributed throughout the PS-1, most of them in TM 1, 2, 6 and 7. The number of mutations is not definitive, only showing approximately the sites of the mutations.

2.4 Apolipoprotein E

The absence of linkage to chromosome 21, but weak linkage to chromosome 19 were the earliest reported genetic studies on late-onset FAD (Pericak-Vance et al., 1991). Later, numerous studies have reported an association between the ApoE locus and AD in familial and sporadic cases.

The affected pedigree member (APM) method, with information collected on affected individuals, has been used because of the uncertainty about the exact mode of inheritance and the variability in age at onset of AD (Week et al., 1988; Week et al., 1992). The APM method detects deviations from independent segregation of disease phenotype and marker alleles. Eventually, linkage studies using multipoint analysis have mapped the late-onset AD gene (denoted as AD2 locus) on chromosome 19. The association of FAD at the ApoCII gene, which is also located in the same region of chromosome 19 as the ApoE, has pointed to

the effect of a suspectibility gene rather than a primary causative gene (Schellenberg et al., 1987).

ApoE is a glycoprotein, containing 299 amino acids, with a relative molecular mass of 34200 Da (Mahley 1988; Wiesgraber et al., 1994a,b). There are three major isoforms of ApoE (E2, E3 and E4) that are the products of three allelic forms (ε2, ε3 and ε4) of this single gene, which is located on the long arm of chromosome 19 (Emi et al., 1988). The various combinations of these alleles give rise to six different genotypes, of which the most common is ApoE E3/3 (Lehtimäki et al., 1990; Hallman et al., 1991). The three isoforms differ by the interchange of cysteine (Cys) and arginine (Arg) residues at positions 112 and 158 of the mature ApoE (Figure 4). ApoE consists of two independently folded structural domains of which the amino terminal domain (residues 1-191) is a stable globular structure containing the receptor binding function, and the carboxyl terminal domain (residues 216-299) is helical, less stable, and contains the lipoprotein binding functions (Wiesgraber et al., 1994a,b).

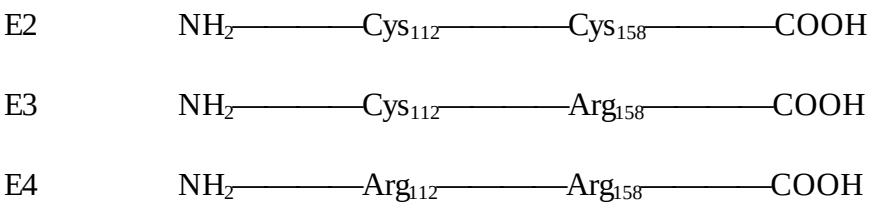

Figure 4. Polymorphism in ApoE

ApoE is involved in the mobilization and redistribution of cholesterol during neuronal growth and after injury (Mahley 1988; Poirier et al., 1991). It is also involved in many other functions in humans: nerve regeneration (Handelmann et al., 1992; Siest et al., 1995), immunoregulation (Siest et al., 1995), the activation of several lipolytic enzymes (Siest et al., 1995).

ApoE is synthesized primarily by the liver and the brain, but also by other tissues including astrocytes, macrophages and monocytes (Siest et al., 1995) and ApoE genotype is involved in lipid transport in cerebrospinal fluid and its concentrations (Mahley 1988; Lehtimäki et al., 1995; Pirttilä et al., 1996b). Neurons do not produce ApoE, but express low-density lipoprotein receptor-related protein (LRP) (the ApoE-binding receptor), by which ApoE can be internalized (Rebeck et al., 1993). When ApoE was complexed with other lipoproteins, it stimulated neurite outgrowth by delivery of cholesterol. Upon addition of free ApoE to ApoE-enriched lipoprotein complexes, it increased neurite branching and promoted neurite extension away from the cell body (Handelmann et al., 1992). In general, ApoE-containing high density lipoprotein promoted neurite outgrowth and was a ligand for the LRP (Fagan et al., 1996; Narita et al., 1997).

ApoE binds avidly to synthetic and soluble βA4 peptide in cerebrospinal fluid (Wisniewski et al., 1993). Oxygen-mediated binding of βA4 peptide to the lipoprotein-binding domain of ApoE E4 created a sodium dodecyl sulfate (SDS)-resistant complex (Strittmatter et al., 1993). In particular, ApoE E4 bound βA4 peptide more rapidly and over a narrower pH range than ApoE E3. ApoE E4 also associated with βA4 peptide to form novel monofibrils that precipitated to form dense structures (Sanan et al., 1994; Yamada et al., 1994). ApoE E4 does not bind to tau protein in vitro, unlike ApoE E2 and E3 (Wiesgraber et al., 1994a,b). It is possible that the interaction between ApoE E3 and tau protein serves as protection against tau phosphorylation and tangle formation (Strittmatter et al., 1994).

Landen et al. (1996) have demonstrated in 44 neuropathological AD samples and in 29 age-matched controls that the ApoE ε4 allele has not correlated with the number of senile plaques or neurofibrillary tangles in patients with AD. A few studies have suggested that the ApoE ε4 allele has been associated with increased accumulation of βA4 peptide in AD brains (Schmechel et al., 1993; Rebeck et al., 1993; Pirttilä et al., 1997) and even in brains of elderly nondemented subjects (Polvikoski et al., 1995). Furthermore, the ApoE ε4 allele has also been reported to be associated with an increase in the βA4 burden in DS patients (Hyman et al., 1995). There is increasing evidence for a role of ApoE in the pathogenesis of AD. ApoE is detectable by immunohistochemistry in senile plaques, neurofibrillary tangles, and cerebrovascular amyloid in AD brain (Namba et al., 1991). Distinct binding properties of ApoE isoforms to βA4 peptide (Strittmatter et al., 1993) and tau protein (Strittmatter et al., 1994) have suggested ways in which ApoE might mediate its action.

ApoE allele frequencies are highly variable in different population. Chinese, Japanese and Mayan Indians have higher ε3 (0.81-0.91) and lower ε4 (0.07-0.09) allele frequencies than other populations (Hallman et al., 1991; Kamboh et al., 1991). Blacks from Africa, the USA and inhabitants of New Guinea have the lowest ε3 (0.49-0.71) and the highest ε4 (0.21-0.40) allele frequencies (Kamboh et al., 1989; 1990; Hallman et al., 1991; Hendrie et al., 1995). The ε4 allele frequency is also higher in northern Europe than in southern Europe (Gerdes et al., 1992). The frequencies of ApoE ε2 allele is 0.04, ε3 is 0.77 and ε4 is 0.19 in Finnish youths aged from 3 to 18 years (Lehtimäki et al., 1990) and 0.04, 0.73 and 0.23 respectively, in the population aged from 20 to 55 years (Louhija et al., 1994).

2.4.1 Apolipoprotein E polymorphism and Alzheimer's disease

The first association between dementia and the ApoE ε4 allele was observed in 1989 by Shimano and coworkers, who found an increased ε4 allele frequency in multi-infarct dementia patients compared to controls (Shimano et al., 1989). The relationship between late-onset AD and ε4 allele frequency was reported in 1993 (Corder et al., 1993; Strittmatter et al., 1993). Later, many studies have reported increased ε4 allele frequencies in late-onset AD and in sporadic cases of AD, but the association is stronger in familial AD (Corder et al., 1993;

Payami et al., 1993; Saunders et al., 1993c). An increased ApoE ε4 allele frequecy has also been shown in early-onset AD (Van Duijn et al., 1994). The risk of developing AD seems to be allele dose dependent. Individuals carrying two ε4 alleles are at higher risk and have an earlier onset of disease than those with one or no ε4 alleles (Corder et al., 1993). In AD families containing an APP mutation, an increasing number of ε4 alleles decrease the age of onset in affected patients (Hardy et al., 1993; Sorbi et al., 1993; St George-Hyslop et al., 1994; Levy-Lahad et al., 1995a). However, in FAD families with linkage to chromosome 1 or 14, the presence of the ε4 allele do not affect the age at onset (Van Broeckhoven et al., 1994b; Levy-Lahad et al., 1995a). In general, the ε2 allele at the ApoE locus may be protective against AD (Corder et al., 1994; Talbot et al., 1994). Studies on centenarians have been revealed that ε2 allele had an impact on longevity, and the frequency of ε4 allele is lower in these individuals (Schächter et al., 1994; Louhija et al., 1994; Van Bockxmeer 1994). Thus, the risk associated with ApoE ε4 allele in AD would seem to be age-dependent (Sobel et al., 1995; Rebeck et al., 1994). A significant difference between gender has also been observed. The ApoE ε4 allele frequency is higher in women than in men, especially aged 60 to 70 years (Payami et al., 1996). Furthermore, neuronal reorganization (neuronal degeneration and plastic dendritic remodeling) is affected severely in a number of subcortical areas of AD patients carrying the ApoE ε4 allele (Arendt et al., 1997).

2.4.2 Apolipoprotein E in different neurodegenerative disorders

To establish the specific role of ApoE in AD, it is essential to determine the extent of the association of the ε4 allele with types of dementia other than AD. The US National Institute on Aging and the Alzheimer's Association regard ApoE genotyping as an adjunctive diagnostic test for AD, since patients with AD are more likely to have an ApoE ε4 allele than patients with other forms of dementia. However, it is not clear whether ApoE ε4 allele is more strongly associated with AD than other dementias. It has been suggested that ApoE is of little value in distinguishing AD patients from other demented patients (Slooter et al., 1996).

A study from Japan has suggested that ApoE ε4 allele frequency has also increased in multi-infarct dementia (Noguchi et al., 1993). They have reported a significantly higher ε4 allele frequency for AD and multi-infarct dementia patients than for nondemented controls. A few other studies have also confirmed an increased ε4 allele frequency in multi-infarct dementia (Frisoni et al., 1994; Harrington et al., 1994; Slooter et al., 1996), while two studies from Finland have failed to show any significant association between the ApoE and clinically diagnosed VAD (Pirttilä et al., 1995; Pirttilä et al., 1996; Sulkava et al., 1996a) (Table 2). The possible coexistence of AD and VAD must be kept in mind (Pirttilä et al., 1995), when interpreting the data in a clinically diagnosed series pending neuropathological confirmation. The varying ε4 allele frequencies in VAD might be due to difficulties in assessing the diagnosis.

AD, VAD and mixed AD-VAD are responsible for up to 90% of all dementias in different series (Katzman et al., 1988). Many studies have reported an association of increased ε4

allele frequency with coronary artery disease (Frisoni et al., 1994; Harrigton et al., 1995; Lehtinen et al., 1995; Slooter et al., 1996). AD patients carrying an ε4 allele have been significantly more often severe coronary sclerosis than AD patients without the ε4 allele, but no association between ApoE genotypes and the extent of atherosclerosis has been found in cerebral vessels (Kosunen et al., 1995).

Two studies have reported no association between ε4 allele frequency and ischemic cerebrovascular disease (Couderc et al., 1993; Saunders et al., 1993b), whereas a study by Pedro-Potet and coworkers have suggested that the ε4 allele could also be a genetic marker predisposing to cerebrovascular disease (Pedro-Botet et al., 1992).

Lewy body dementia (LBD), also known as diffuse Lewy body disease, has been described as a common cause of dementia in the elderly (Byrne et al., 1989; Dickson et al., 1989; Perry et al., 1989; Hansen et al., 1990 Perry et al., 1990). After AD, LB disease is the most common amyloidogenic disease within the central nervous system. Unlike AD, LB disease shows few or no cortical neurofibrillary tangles. In addition to ubiquitin immunopositive Lewy bodies, patients with LB disease often display concomitant AD changes in their brain. LB patients generally present with progressive dementia, including impairment of memory and other cognitive abilities, as well as extrapyramidal signs. Patients with Lewy bodies but no AD neuropathology (the strict criteria of Lewy body disease) do not show an increased frequency of the ApoE ε4 allele (Galasko et al., 1994; Lippa et al., 1995). A few studies have confirmed an increased ε4 allele frequency (St Clair et al., 1994; Bétard et al., 1994; Harrington et al.,1994) (Table 2).

A proportion of PD patients generally develop dementia when their disease has lasted for a longer period of time. The prevalence of dementia also increases with increasing age in PD. The pathogenesis has been attributed to concomitant AD, cortical and subcortical Lewy bodies, and medial nigra degeneration (Rinne et al., 1989). That is why if dementia in PD were due to concomitant AD, the ε4 allele frequency would be higher in demented PD patients (PDD) than in nondemented PD patients and controls. Both AD and PD are age related and have some common risk factors, such as family history of dementia and depression (Treves et al., 1993; Koller et al., 1994). In addition to dopaminergic, serotonergic and cholinergic deficits, the AD pathology coexistent with PD might explain the dementia in PDD patients (Perry et al., 1985; Ruberg et al., 1986; Paulus et al., 1991). Therefore, it is not surprising that PDD patients also show a high ε4 allele frequency. An increased ε4 allele frequency has been demonstrated in a few studies in PDD (Arai et al., 1994; Gearing et al., 1995), but not in all investigations (Marder et al., 1994; Koller et al., 1995). However, several studies have not found any increase in the ε4 allele frequency in PD patients without dementia (Hughes et al., 1993; Marder et al., 1994; Schneider et al., 1995).

No increase in ApoE ε4 allele frequency has been found in familial amyloidotic polyneuropathy, Down's syndrome, amyotropic lateral sclerosis (Guam dementia), Huntington

disease, or Parkinson's disease without dementia (Table 2). However, in young mothers of DS children with a meiosis II error, ApoE ε4 allele frequency is significantly higher than in older mothers with a meiosis II error (Avramopoulos et al., 1996).

There are three other amyloidogenic diseases: Pick's disease, corticobasal degeneration and progressive supranuclear palsy. In the studies of Schneider et al., (1995) these disorders showed increased ε4 frequencies when compared with controls. The same mechanism as in AD, tau-positive inclusions, seems also to occur in these disorders.

Table 2. The allele frequencies of ApoE in different neurodegenerative diseases

Disease	e2	e3	e4	Ce4	**References**
AD+CAA	0.02	0.61	0.37	0.15	Premkumar et al., 1996
AD+CVD	0.04	0.45	0.51	0.12	Bétard et al., 1994
ALS	0.07	0.77	0.16		Mui et al., 1995
ALS/PD	0.04	0.92	0.04		Waring et al., 1994
CBD			0.34	0.14	Schneider et al., 1995
CHAA			0.40		Greenberg et al., 1995
CLBD	0.04	0.54	0.42	0.14	Pickering-Brown et al., 1994
CJD			0.33	0.17	Amouyel et al., 1994
			0.10	0.15	Saunders et al., 1993a
CVD			0.12	0.06	Isoe et al., 1996
DLBD	0.12	0.81	0.07	0.14	Galasko et al., 1994
	0.00	0.88	0.13	0.09	Lippa et al., 1995
DS	0.10	0.68	0.22	0.10	Hardy et al., 1994
	0.19	0.73	0.08	0.14	Pickering-Brown 1994
	0.12	0.79	0.10		Van Gool et al., 1995
			0.09	0.15	Saunders et al., 1993a
FAPN			0.17	0.15	Saunders et al., 1993a
HC	0.10	0.73	0.17	0.15	Harrington et al., 1994
LA			0.17	0.14	Schneider et al., 1995
LBD+AD	0.08	0.45	0.47	0.12	Bétard et al., 1994
	0.03	0.68	0.29	0.14	Galasko et al., 1994
	0.04	0.61	0.35		St Clair et al., 1994

	0.06	0.58	0.36	0.15	Harrington et al., 1994
	0.14	0.64	0.22	0.09	Lippa et al., 1995
LBD/PA	0.18	0.57	0.25	0.09	Lippa et al., 1995
MSA			0.25	0.14	Schneider et al., 1995
PD	0.06	0.85	0.09	0.08	Arai et al., 1994
	0.07	0.80	0.13		Marder et al., 1994
	0.09	0.81	0.10	0.15	Harrington et al., 1994
	0.06	0.79	0.15		Koller et al., 1995
			0.14	0.14	Schneider et al., 1995
			0.15	0.13	Whitehead et al., 1996
PDD	0	0.68	0.32	0.08	Arai et al., 1994
	0.02	0.91	0.07		Marder et al., 1994
	0.12	0.73	0.15		Koller et al., 1995
	0.02	0.58	0.40	0.14	Gearing et al., 1995
			0.13	0.11	Egensperger et al., 1996
			0.25		Slooter et al., 1996
			0.10	0.13	Whitehead et al., 1996
Pick			0.33	0.14	Schneider et al., 1995
PSP			0.24	0.14	Schneider et al., 1995
	0.07	0.79	0.13	0.10	Tabaton et al., 1995
Stroke	0.08	0.74	0.19		Pedro-Botet et al., 1992
	0.06	0.85	0.09		Couderc et al., 1993
VAD	0.04	0.75	0.21	0.09	Noguchi et al., 1993
	0.21	0.71	0.08	0.12	Bétard et al., 1994
	0	0.54	0.46		Frisoni et al., 1994
	0.05	0.82	0.13	0.09	Kawamata et al., 1994
	0	0.90	0.10		Mahieux et al., 1994
	0.12	0.63	0.25	0.15	Harrington et al., 1994
	0.07	0.62	0.32	0.22	Pirttilä et al., 1995
	0.06	0.61	0.34	0.22	Pirttilä et al., 1996a
			0.21	0.06	Isoe et al., 1996
	0.08	0.76	0.16	0.13	Sulkava et al., 1996
			0.24		Slooter et al., 1996
			0.21	0.11	Treves et al., 1996

AD=Alzheimer's disease;
ALS/PD=amyotrophic lateral sclerosis/Parkinsonism-dementia of Guam;
CAA=cerebral amyloid angiopathy;
CBD=corticobasal degeneration;
CHAA=cerebral hemorrhage associated amyloid angiopathy;
CLBD=cortical Lewy body disease;
CJD=Creutzfeldt-Jakob disease;
Cε4=ε4 in control group;
CVD=cerebrovascular disease without dementia
(D)LB(D)=(diffuse) Lewy-body (disease);

DS=Down's syndrome;
FAPN=familial amyloidotic polyneuropathy;
HC=Huntington's chorea;
LA=lobar atrophy;
LBD/PA=Lewy body disease with pathologic aging
MSA=multisystem atrophy;
PD=Parkinson's disease;
PDD=Parkinson's disease with dementia;
PSP=progressive supranuclear palsy;
VAD=Vascular dementia

2.5 Cholinergic metabolism and apolipoprotein E

2.5.1 Cholinergic system and Alzheimer's disease

Acetylcholine (ACh), the transmitter released by cholinergic neurons, is synthesized by choline acetyltransferase (ChAT) and catabolized by acetylcholinesterase (AChE), the major component of cholinesterase (ChE) (Figure 5). The nonspecific cholinesterase, buturylcholinesterase (BChE), which is lower concentrations in the brain tissue than AChE, may also possess the capacity to hydrolyze ACh (Atack et al., 1986). Many different subtypes of muscarinic receptors, for example, M1 and M2, have been described. The M2 receptor subtype is a presynaptic and M1 receptor subtype is a postsynaptic receptor (Mash et al., 1985).

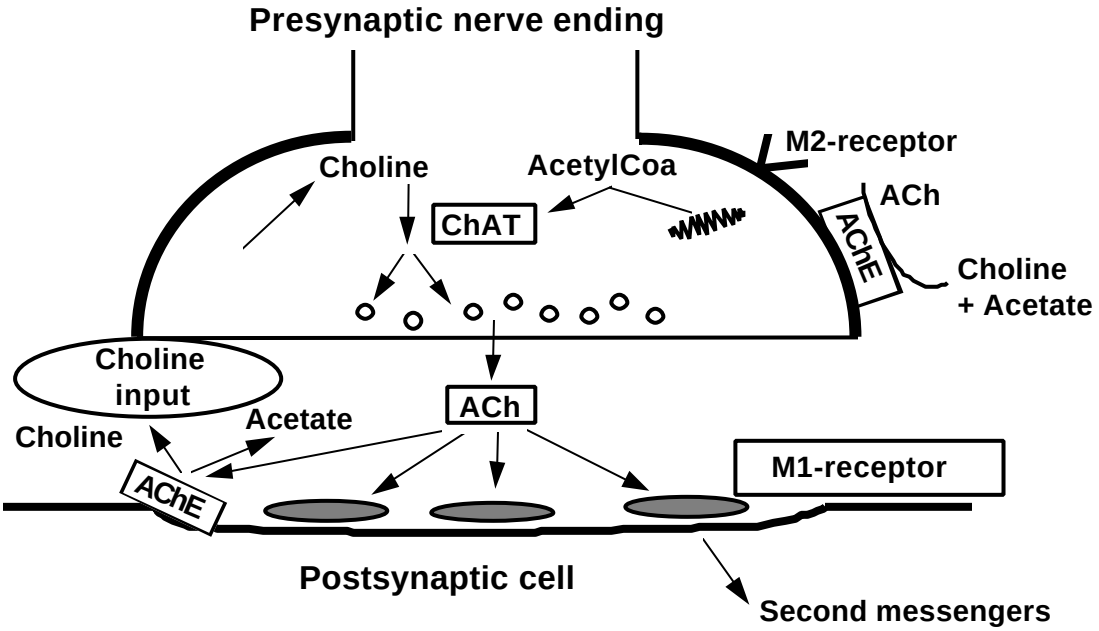

Figure 5. Cholinergic synapse. AChE = acetylcholinesterase, AcetylCoA = acetylcoenzyme A; ChAT = cholineacetyltransferase.

The best documented changes in activities of transmitter enzymes in AD patients are found in the cholinergic system. The first reported changes in reductions of the ChAT and AChE activities have been observed in the neocortex, hippocampus and many other brain regions in autopsy samples of AD (Bowen et al., 1976; Davies and Maloney 1976; White et al., 1977; Perry et al., 1977a,b). A significant deficit of ChAT in AD has been found in the frontal, parietal cortical and temporal cortical regions, hippocampus and amygdala, the decrease being the most severe in younger AD patients (Bowen et al., 1976; Davies and Maloney, 1976; Perry et al., 1977a,b; Rossor et al., 1984; DeKosky et al., 1985; Perry et al., 1986; D'Amato et al, 1987; Reinikainen et al, 1988).

The reduction in ChAT activity and the number of senile plaques in the cortex are correlated with the loss of neurons in the nucleus of Meynert (Candy et al., 1983; Arendt et al., 1985; Rinne et al., 1987). Demonstration by immunohistochemical methods of both ChAT and AChE in the senile plaques further has emphasized the involvement of cholinergic neurons in AD (Perry et al., 1986; Armstrong et al., 1986). In addition, the staining of AChE in the cortex has been observed to be profoundly reduced in patients with AD (Perry et al., 1986).

At the molecular level, changes in ChE and particularly in AChE in the AD brain are mainly due to loss of the predominant membrane-associated G4 molecular form (Atack et al., 1986; Fishman et al., 1986; Younkin et al., 1986). The tetrameric G4 form is the major AChE component in both brain tissue and cerebrospinal fluid (CSF) (Atack et al., 1986; Younkin et al., 1986; Sirviö et al., 1987). In the AD brain, the loss of AChE is possibly limited to the membrane-bound G4 form, whereas the soluble enzyme remains unchanged (Younkin et al., 1986). The CSF reflects evidently the release of the soluble form G4 from the brain into the CSF (Greenfield et al., 1984). Thus it is understandable, that AChE in CSF of AD patients is either unaltered or reduced in comparison to changes in brain tissue (Soininen et al., 1981; Elble et al., 1987; Reinikainen et al., 1988).The decrease is, however, slight, even in the most severe cases (Soininen et al., 1981; Reinikainen et al., 1988). During aging, AChE activities in the CSF remain unchanged (Reinikainen et al., 1988) or increase (Elble et al., 1987).

Post-mortem brain tissue is often used to examine the neurotransmitter systems in ND diseases. Brain tissue can be used for both quantitative assays and qualitative methods. However, functional activity of neurotransmitter neurons cannot generally be assessed in post-mortem tissue samples (Bowen and Davison 1986; Neary et al., 1986; Hardy et al., 1987). Examination of CSF is widely used in studying the biochemical background of organic diseases of the central nervous system (CNS). Some of the measured markers show age-related changes. For example, AChE may increase during aging (Palmer et al., 1984). Furthermore, ApoE levels in CSF are lower in AD patients than in control subjects (Lehtimäki et al., 1995).

2.5.2 Relation of cholinergic deficit to apolipoprotein E

Two studies have shown that the degree of the deficit of ACh-containing neurons in AD brains is related to the number of ε4 alleles present (Poirier et al., 1994; Soininen et al., 1995). These post mortem studies have suggested that AD patients carrying the ε4 allele might represent one subgroup with a severe cholinergic deficit. The ChAT deficit has been reported to be most pronounced in the frontal cortex of AD patients with the ApoE ε4/ε4 genotype (Soininen et al., 1995). Poirier et al. (1994) have reported a decrease of ChAT proportional to the number of ε4 alleles in the post mortem hippocampus and the temporal cortex of AD patients. Poirier et al. (1995) have also showed that analysis of clinical responses to tacrine, an acetylcholinesterase inhibitor, have suggested a different response to the drug among AD patients with the ApoE ε4 allele compared to those without ε4 allele. Pathological data have also demonstrated that the number ApoE ε4 alleles has showed an inverse relationship with residual brain choline acetyltransferase activity and nicotinic receptor binding sites in AD patients. It has been suggested that AD patients with ApoE ε4 allele, respond differentially to cholinomimetic-based therapies like tacrine, being at a greater risk for loss of their ACh synthetic capacities and therefore less capable of responding to drug (Poirier et al. 1995).

2.6 Other suspectibility factors in Alzheimer's disease

2.6.1 Alpha-1-antichymotrypsin polymorphism

Additional genetic factors may be involved in the manifestation of the AD disease. α_1-antichymotrypsin (ACT) protein levels in plasma and CSF have been observed to be elevated in AD patients, and therefore ACT has been proposed as a biochemical marker for AD (Randall and Hardy 1989; Matsubara et al., 1990). ACT is intimately associated functionally within the βA4 peptide in AD brains (Abraham et al., 1988; Abraham et al., 1990) and ACT appears to promote fibril formation of the βA4 peptide, and thus the deposition of amyloid (Ma et al., 1994; Eriksson et al., 1995; Das and Potter 1995).

ACT protein belongs to the class of serine proteinase inhibitors (serpins) and is encoded by a gene on chromosome 14 (Rabin et al., 1986), some 30 cM from the PS-1 gene (Haines et al., 1996). A polymorphism has been described in the signal peptide sequence of ACT nearly equal frequencies in the general population (Kamboh et al., 1995). This bi-allelic polymorphism of the ACT gene has been described to cause an amino acid exchange Ala (A) to Thr (T) at codon 15 in the signal peptide region (Faber et al., 1993; Kamboh et al., 1995).

This polymorphism has been stated to confer a significant risk for AD. Patients with the combination of ACT/AA phenotype and ApoE ε4 allele were reported to have a two to three fold increased risk for AD compared to controls (Kamboh et al., 1995). Some studies have confirmed this finding (Thome et al., 1995; Yoshiiwa et al., 1996), but other studies have failed to confirm any association between ACT and ApoE ε4 allele (Haines et al., 1996, Müller et al., 1996, Nacmias et al., 1996; Fallin et al., 1997; Didierjean et al., 1997). Furthermore, gender and age-specific associations between ACT and ApoE polymorphisms

have been identified in the general population of Caucasians and Nigerian Blacks. In Caucasian women and Nigerian women, the frequency of ApoE ε4 allele was significantly lower in ACT/AA phenotype than in men (Kamboh et al., 1997b).

2.6.2 The presenilin-1 intron 8 polymorphism

Mutations in the PS-1 gene accounts for the majority of familial early-onset AD. Instead, its role is opposite to the PS-1 intron 8 polymorphism in late-onset AD. Association of an intronic polymorphism situated in 3' of exon 8 of the PS-1 gene and late-onset AD has been proposed. Homozygocity of allele 1 of the intron 8 polymorphism has been described to be associated with a doubling of the risk for late-onset AD compared with heterozygocity of alleles 1 and 2 or homozygocity of allele 2 (Wragg et al.,1996). This association has been observed in both sporadic and familial cases of late-onset AD (Higuchi et al. 1996, Kehoe et al. 1996), but other studies have not found any association between the PS-1 intron 8 polymorphism and late-onset AD (Scott et al., 1996, Pérez-Tur et al., 1996; Tysoe et al., 1997; Cai et al., 1997; Lendon et al., 1997b). The opposite result has been described by Aldudo et al. (1997) in Spanish late-onset AD patients. Homozygocity of allele 2 was associated with a threefold increased risk for AD, and 4.5-fold increased risk in patients carrying no ApoE ε4 allele.

Furthermore, an increased frequency of allele 1 has been reported to be associated with the PS-1 intron 8 polymorphism in AD families with the PS-1 or the PS-2 mutations, but not with APP mutation or in late-onset AD patients (Sorbi et al., 1997).

This polymorphism occurs within the intron between exons 8 and 9 of the PS-1 gene and therefore, it is very unlikely to cause any biological effect. Three different explanations have been suggested for the association: first, the polymorphism may be biologically relevant to the disease, second, it is in linkage disequilibrium with biologically relevant variability elsewhere in the PS-1 gene or third, the polymorphism is in disequilibrium with genetic variability in another adjacent gene (Wragg et al., 1996).

Exon 8 of the PS-1 gene is alternately spliced in some tissues and a mutation in the acceptor site in the same intron has been associated with early-onset AD in a family through the loss of exon 9 (Pérez-Tur et al., 1995). In addition, exon 8 is the site of the most prominent cluster of mutations leading to early-onset AD (Cruts et al., 1996).

2.7 Linkage analysis

2.7.1 Meiosis

In meiosis, homologous chromosomes pair up and exchanges (crossing-over) between homologous chromosomes regularly occur (chiasma formation). The result of crossover is an

exchange of material between two chromatids of homologous chromosomes (genetic recombination). After meiosis, four gametes are formed. Assuming that one crossover event has occurred, two of the gametes will contain nonrecombinant and two will contain a recombinant chromosome arm (Figure 6). The distribution of chromosomes during meiosis explains the separation (segregation) of traits according to Mendelian laws (Ayala and Kiger Jr 1984; Singer and Berg 1991).

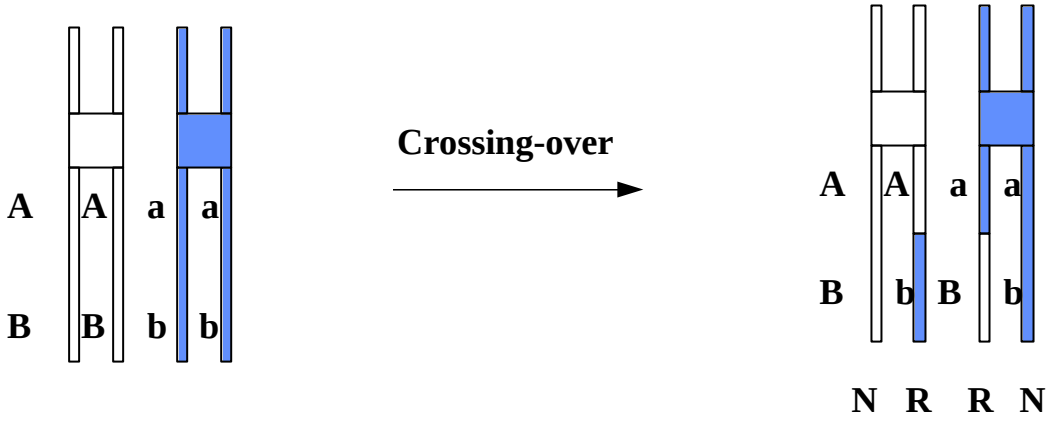

Figure 6. Genetic recombination between two homologous chromosomes. Two of the gametes are nonrecombinant (N) and two are recombinant (R) after crossing over.

2.7.2 The principle of linkage analysis

Linkage refers to two or more genes being inherited together as a result of their location on the same chromosome (i.e., the principle of linkage analysis is to measure the cosegregation of disease in a family with marker loci). That depends on the distance between their loci. The closer they lie next to each other, the more frequently they will be inherited together (i.e., they are linked). When loci are very close together, the recombination event rarely occurs. If loci lie further apart, recombination between them is more frequent. The frequency of recombination can be used as a measure of the distance between gene loci. The recombination fraction or recombination frequency (theta) refers to the probability that a gamete produced by a parent is a recombinant of two loci. In the case of four gametes, only two out of four are affected by a crossover (Figure 6). That is why the recombination fraction can be at the most 50% (theta=0.5). Crossing over events occur randomly along a chromosome and a crossing over for two loci close together is rare (theta is almost zero). The value of theta increases when the distance between loci increases until it reaches 50% (theta=0.5) for loci far apart on the same chromosome or loci on different chromosomes (Ott 1991; Dracopoli et al., 1995).

2.7.3 LOD score value in linkage analysis

Genetic distance is measured in centiMorgans (cM), where 1 cM is approximately the distance between two loci that on average show 1% recombination (theta=0.01). This is

different from the physical distance, which is given as the number of DNA base pairs lying between the two loci. The extent of linkage is measured by defining a LOD score. We have two opposite assumptions for the linkage of two neighboring loci: for linkage or against linkage, and the ratio of the two altenatives is referred to as the odds. The logarithm of this ratio (odds) is called the LOD score (logarithm of the odds), i.e., ten base logarithm of the probability ratio of the hypotheses. Generally, the closer the marker is to the disease gene, the greater the extent of cosegregation. As a result of close loci there is a smaller theta value and a bigger LOD score. A lod score of 3 is considered as a proof for linkage and thus ten base logarithm of the odds ratio is 1000/1. Exclusion of linkage is defined as a lod score of -2 and the ratio is 1/100 (Dracopoli 1995) (Figure 7).

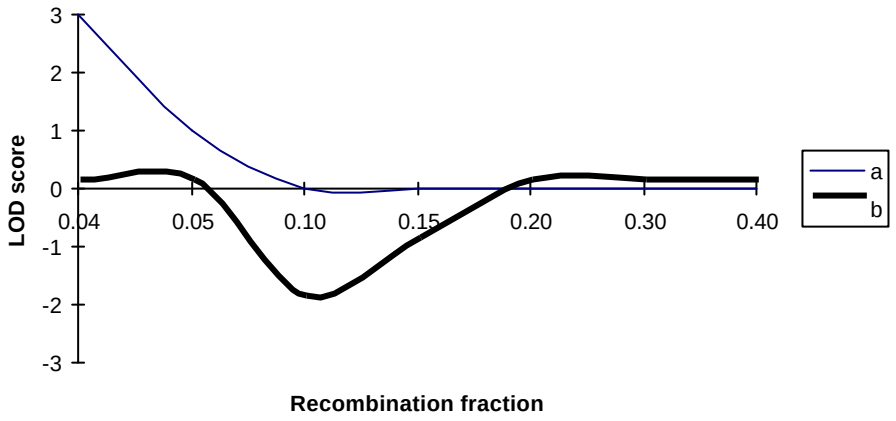

Figure 7. LOD score curves for close linkage (a) and no linkage (b)

2.7.4 Penetrance models

There are over 5500 disorders in humans that are known to be caused by defects in a single gene (McKusick, 1992). For several diseases, the predisposing genotype is necessary and sufficient for expression of the disease. In such cases, the disease is said to be fully penetrant. Penetrance can range from 0 to 100% phenomenon with respect to expression of a mutant gene. If a disease is defined as being 90% penetrant, then 90% of those with the mutant genotype will express the disease. The penetrance of a gene is the proportion of individuals showing the expected phenotype. Expressivity is the degree to which the phenotype is manifested (in penetrant individuals). For example, Duchenne muscular dystrophy has a penetrance equal to 1. That means all of those who will inherit the predisposing genotype will manifest the symptoms. Sometimes there may be clinically normal individuals who can be inferred to have a genotype for the disease, upon examination of pedigree information. Such individuals are defined as being nonpenetrant.

Huntington disease and familial AD are examples of reduced penetrance. It is often associated with diseases that have variable age at onset (i.e., age-dependent penetrance). The causes of nonpenetrance are numerous and often poorly understood (Dracopoli et al., 1995).

2.7.5 Simulation analysis

Prior to linkage analysis and collection of DNA from family members, it is reasonable to evaluate families for their power to detect linkage. Simulation analysis is useful for determining whether pedigrees contain enough information to detect linkage and for deciding which portion of a pedigree should be typed next to increase the information for a linkage analysis (Ott, 1991). A certain genetic model and appropriate penetrance parameters are assumed. The identified data set and simulating marker data are taken into account, assuming a given heterozygosity in consideration of which family members are available for study. The disease-trait phenotype can also be simulated. When sufficient information according to power studies has been observed to detect linkage, laboratory analysis can started.

3. AIMS OF THE STUDY

The present study was designed to investigate the molecular genetics of AD in eastern Finland. The etiological basis of AD is still unclear, but a part of AD can be attributed to genetic factors. The objective of this study was to investigate the role of genetic factors using both sporadic and familial AD patients.

The specific aims were:

1. To screen for the APP mutations at codons 665, 670/671 and 717 in familial and sporadic AD patients (**I**).

2. To perform linkage analysis on chromosome 14 and 1 in 15 FAD pedigrees and to examine in detail regions of the PS-1 gene by sequencing (**II**).

3. To study ApoE polymorphism (**III**) and to determine, AChE activity in the CSF in probable AD patients using this as a marker of the cholinergic system (**IV**).

4. To study α_1 -antichymotrypsin polymorphism in different ApoE genotypes in late-onset sporadic AD patients (**V**).

5. To study the PS-1 intron 8 polymorphism in different ApoE genotypes in late-onset sporadic AD patients (**VI**)

4. SUBJECTS, MATERIALS AND METHODS

4.1 General description of study subjects

This study was performed during 1995-1997. All patients and controls were investigated in the Department of Neurology in Kuopio University Hospital (Lehtovirta, 1996a,b) and genetic analysis was performed in collaboration with the Unit of Clinical Genetics, Department of Gynaecology and Obstetrics and Chromosome and DNA Laboratory of Diagnostic Services in Kuopio University Hospital. This study was approved by Ethics Committee of Kuopio University Hospital and Kuopio University and collecting the data from siblings and relatives for pedigree study was approved by the Ministry of Social Affairs and Health in Finland. All patients and control subjects in the following studies originate from eastern Finland and the demographic data of the patients and controls in the studies I-VI is given in Table 3.

4.1.1 Subpopulations of the present study

Study I

The APP gene mutations were screened for in 34 patients with FAD and 139 patients with sporadic AD. Of 34 FAD cases, there were 16 early-onset cases with a mean age at onset of 56 years and 18 late-onset cases with mean age at onset of 74 years. The clinical diagnosis of sporadic probable or possible AD was made using the NINCDS-ADRDA criteria (McKhann et al., 1984). AD was considered familial if at least two first degree relatives had dementia.

Study II

Linkage analysis was performed to chromosomes 14 and 1 in 15 FAD pedigrees (35 affected out of 184 subjects) (see II Table 1). AD families were identified in the Department of Neurology and affected individuals underwent a comprehensive clinical evaluation during which the clinical diagnosis of probable AD was made using the NINCDS-ADRDA criteria, as described in Lehtovirta et al., (1996a,b), and available records for deceased individuals were also studied (see AD pedigrees in Appendix).

Furthermore, most of the reading-frame exons of the PS-1 gene were sequenced in two AD families selected according to age at onset <60 years (families 3 and 16), in 7 FAD patients (age at onset 45-64 years) and 40 healthy controls.

Study III

The ApoE genotypes and allele frequencies were examined of a total of 248 subjects. There were 94 patients with probable AD diagnosed using NINCDS-ADRDA criteria (McKhann et

al., 1984), 29 patients with vascular dementia diagnosed according to DSM-III-R criteria (American Psychiatric Association, 1987), 15 patients with idiopathic Parkinson's disease (PD), 8 patients with PD and dementia, 9 patients with clinically diagnosed Lewy body variant of AD, 9 demented patients with predominantly frontal symptoms, 24 patients with Down's syndrome, and 60 non-demented controls.

Study IV

The ChE and AChE activities were measured in the CSF of 60 patients with probable AD according to the NINCDS-ADRDA (McKhann et al., 1984) and in 11 elderly controls. The AD patients were participants in a longitudinal study, and CSF samples were collected at diagnostic examinations. All patients had undergone an extensive evaluation and the 11 healthy controls had been examined the neurological ward.

Studies V and VI

ACT and the PS-1 intron 8 polymorphisms were performed in the same research material. Affected sporadic AD individuals underwent a comprehensive clinical evaluation, during which the clinical diagnosis of probable AD was made using the NINCDS-ADRDA criteria. AD samples and controls were split into three subgroups according to their ApoE genotypes: ALL, ApoE 23, 33 and ApoE 24, 34, 44. Genotype and allele frequencies of ACT and the PS-1 intron 8 polymorphisms were determined in different subgroups.

Table 3. Demographic data of the patients and controls in the studies I-VI

	Groups	Number	Age (years)	Male/Female
Study I	AD	139	65±9	54/85
	FAD	16	56±5¤	8/8
	FAD	18	74±6¤	6/12
Study II	AD	35	69±8#	12/23
	Controls	30	72±6	9/21
Study III	C	60	69±8	28/32
	AD	94	72±8	41/53
	VAD	29	76±5	12/17
	PD	15	71±6	8/7
	PDD	8	73±3	6/2
	LB	9	75±7	3/6
	FD	9	68±8	3/6
	DS	24	35±11	12/12
Study IV	AD 2ε4	12	67±9	6/6
	AD 1ε4	27	72±7	18/9
	AD 0ε4	21	67±9	10/11
	Controls	11	63±10	4/7
Study V	AD	218	78±9	71/147
	Controls	101	71±10	16/85
Study VI	AD	219	78±9	72/147
	Controls	104	71±10	16/88

¤#Age at onset in FAD patients (see 4.2.)

4.2 Extraction of genomic DNA

Genomic DNA was isolated by using a phenol-chloroform-isoamyl alcohol method (Vandenplas et al., 1984). In brief, DNA was extracted from 5-10 ml peripheral venous blood with EDTA. Blood cells were lysed in water and in 0.1% Nonidet P-40 solution and centrifuged at 2500 rpm for 15 min at 20^0C. White cell pellet was lysed in 100 mM sodium chloride, 25 mM EDTA (pH 8.0), 10% sodium dodecyl sulphate and 500 µg/ml proteinase K at 37 ^{0}C overnight. DNA was purified by phenol-chloroform-isoamyl alcohol extractions and

centrifuged at 2500 rpm for 15 min at 20 ^{0}C. DNA was precipitated with 2 volumes 99% ethanol and 1/10 volume of 3 M sodium acetate. Finally, DNA was collected and dissolved in TE-buffer. The concentration was determined by spectrophotometry.

Genomic DNA from paraffin-embedded tissues in postmortem AD cases was isolated as described by Isola et al., (1994). In brief, five to ten 10 µm sections were deparaffinized in eppendorf tubes (3 x 1 ml xylene for 5 min each, 3 x 1 ml 99% ethanol for 10 min each and 2 x 1 ml 70% ethanol for 5 min each). After air drying, samples were suspended in 500 µl extraction buffer and proteinase K (data not shown) and were incubated at 55^{0}C overnight. Additional proteinase K was added during 48 h (data not shown). DNA was purified and precipitated as mentioned earlier, and glycogen (0.1 mg/l; Sigma) was added as a carrier to increase the volume of the pellet. Finally, DNA was collected, dissolved and determined as described above.

4.3 Gene analyses

4.3.1 Amyloid beta precursor protein mutation (exons 16 and 17) determinations

Amplification of exon 16 was carried out in a polymerase chain reaction (PCR) using primers in flanking introns (Lannfelt et al., 1993) (Table 4 and 5). The reaction volume of 25 µl contained 25 pmol of each primer, 200 mM of each dNTP (Promega), approximately 100 ng of genomic DNA in 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.1 % v/v Triton X-100 and 0.052 % v/v dimethyl sulfoxide (DMSO) to uncoil secondary DNA structures. DNA polymerase (Taq DNA Polymerase, Promega, Madison, WI) was used 0.5U/sample. PCR incubation was performed in a programmable thermal controller apparatus (PTC-100; MJ Research, Inc.).

PCR amplification of the exon 16 generated a fragment of 278 bp. 12.5 µl PCR product was digested overnight at 37^{0}C in a 20 µl incubation volume using 12U/sample BglII (New England Biolabs, Beverly,MA) enzyme according to conditions recommended by the manufacturer into a fragment of size 139 bp in case of APP665, and with MboII (New England Biolabs, Beverly,MA) into fragments of sizes 162 bp and 116 bp in case of APP670/671 in subjects without mutations. Patients with APP mutation at codon 665 produced 278 bp and 139 bp fragments, and APP mutation at codons 670/671 produced 278 bp, 162 bp and 116 bp fragments, respectively.

After cleavage, electrophoresis was carried out on 1.8 % agarose gel. The gels stained with ethidium bromide and photographed. We included a sample with the APP670/671 mutation as a positive control.

Checking the BglII restriction site in the exon 16 was verified by sequencing using intronic primers according to the sequencing kit (70170 Sequenase™ PCR product sequencing kit, Amersham, USB™) following the manufacturer's protocol.

PCR amplification of exon 17 was carried out using intronic primers (Goate et al. 1991) (Table 4 and 5). The reaction buffer and PCR machine was the same as in amplification of exon 16, except that 100-500 ng genomic DNA and 1.0U/sample of the thermostable recombinant enzyme DNA polymerase (DynaZyme™II, Finnzymes Oy) was used.

PCR products (15 µl) were incubated overnight at 50^0C in a 20 µl volume using 8U/sample BclII (New England Biolabs, Beverly,MA) enzyme according to conditions recommended by the manufacturer. PCR amplification of the exon 17 generated a fragment of 319 bp, which was undigested with BclI in subjects without mutation. Patients with APP mutation at codon 717 produced 319 bp, 199 bp and 120 bp fragments. After cleavage, samples were handled as with exon 16.

4.3.2 Analysis of the PS-1 exons

Primers reported in Hutton et al., (1996) were used to amplify exons 4-9 and 11 of the PS-1 gene (Table 4 and 5). Cycle sequencing was performed in early-onset FAD families (families 3 and 16) (II Table 1), in 7 FAD patients (age at onset 45-64 years) and 40 controls on ABI PRISM 310 DNA analyser and ABI Prism GeneSequencing 2.1.1. Software (Perkin-Elmer) utilizing DNA Sequencing Kit (dRodamine Terminator Cycle Sequencing Ready Reaction with AmpliTaq DNA Polymerase FS, PE Applied Biosystems), as described in detail in study II.

4.3.3 Determination of apolipoprotein E allelic forms

ApoE genotypes were analyzed using PCR as described (Hixson and Vernier 1990, Tsukamoto et al., 1993) with slight modifications and primers as in Tables 4 and 5. The amplification reaction volume of 50µl contained 25 pmol of each primer, 400 ng of genomic DNA in the same reaction buffer and the same enzyme (1.5U/sample) and PCR machine as in exon 16.

PCR amplification of the ApoE generated a fragment of 299 bp, which was digested with HhaI (New England Biolabs, Beverly,MA). Eighteen microliters of PCR product were digested with 8U of HhaI (New England Biolabs, Beverly,MA) at 37^0C for at least 3 hours. Digested DNA fragments were analyzed with a 0.5 mm 10% nondenaturing polyacrylamide gel, containing 5% glycerol (the Protean II™ vertical slab gel apparatus; Bio-Rad, Richmond,CA). Electrophoresis time was 120 min at 400 V. Separate DNA fragments were visualized by ethidium bromide staining.

The fragment sizes from polymorphic HhaI sites after cleavage were as follows: the homozygote E2/E2 sample contained 91 bp and 83 bp HhaI fragments, the E3/E3 91 bp, 48 bp and 35 bp and the E4/E4 72 bp, 48 bp and 35 bp fragments, respectively. The heterozygote E3/E2 sample contained 91 bp, 83 bp and 48 bp HhaI fragments, the E4/E2 91 bp, 83 bp, 72 bp and 48 bp and the E4/E3 91 bp, 72 bp and 48 bp, respectively. The heterozygote E4/E2 sample, which contained many restriction fragments was used as a control.

4.3.4 Determination of alpha-1-antichymotrypsin allelic forms

A α_1- antichymotrypsin polymorphism was analysed using primers as in Table 4 and 5. The reaction volume of 50 µl contained 10 pmol of each primer, approximately 50-100 ng of genomic DNA in the same reaction buffer and the same enzyme (1.25U/sample) as in amplification of exon 17 without DMSO.

PCR amplification of the ACT generated a fragment of 124 bp. Ten microliters of PCR product was digested with 10U/sample BstNI (New England Biolabs, Beverly,MA) enzyme overnight at 60^0C in a 15 µl incubation volume according to the conditions recommended by the manufacturer. The ACT allele 1 (denoted as T(threonine) according to biochemical phenotype) was observed a single distinctive band of 117 bp. The ACT allele 2 (denoted as A(alanine) according to biochemical phenotype) was characterized by two fragments of 84 bp and 33 bp (Kamboh et al., 1995). Digested DNA fragments were run on a 2% agarose gel and analysed as for exon 16. The heterozygote sample (alleles 1 and 2) was used as a control.

4.3.5 Determination of the PS-1 intron 8 allelic forms

A common polymorphism of PS-1 gene was identified within the intron 3' to exon 8 in primers as in Wragg et al., (1995) (Table 4 and 5). The reaction volume of 25 µl contained 10 pmol/µl of each primer, approximately 100 ng of genomic DNA in the same reaction buffer and the same enzyme (0.5U/reaction) as in amplification of exon 17 without DMSO.

PCR amplification of the PS-1 intron 8 generated a fragment of 199 bp. Ten microliters of PCR product was digested with 5U/sample BamHI (New England Biolabs, Beverly,MA) enzyme overnight at 37^0C in a 15 µl incubation volume according to conditions recommended by the manufacturer. Polymorphic fragments of 199 bp (allele 1) and 182 bp (allele 2) were produced. After cleavage, products were run on a 3% agarose gel and analysed as described for exon 16. The heterozygote sample (alleles 1 and 2) was used as a control.

4.4 Determination of acetylcholinesterase activity

The CSF samples were taken by lumbar puncture at 8-10 h after the patients had rested overnight. The specimens were immediately frozen and stored at -70ºC until analyzed. The

ChE and AChE activities were measured using the colorimetric method of Ellman et al. (Elman et al., 1961). The total protein level was determined by the method of Lowry et al. (Lowry et al., 1951).

4.5 Linkage analysis

For linkage analysis, FAD families were classified into three groups (A, B, C) by mean age of onset (see study II Table 1). Mean age of onset in affected patients of families in groups A, B and C were <60 years, 60-70 years and >70 years, respectively.

FAD families were evaluated for their power to detect linkage using the software LINKAGE/SLINK (v2.65) with 2-point autosomal data and program MSIM (Ott 1989, Weeks et al., 1990). Linkage analysis was performed with the LINKAGE v.5.2 package according to Lathrop et al. (1984).

PCR amplification of microsatellite markers D1S479 on chromosome 1 (Gyapay et al., 1994), D14S43 (2E12B) on chromosome 14 (Sharma et al., 1991) and D14S77 (AFM218zh4) on chromosome 14 (Gyapay et al., 1994) was carried out from genomic DNA, with some modifications (Table 4 and 5). Electrophoresis was performed using nondenaturated PAGE for markers D1S479 and D14S43 or ABI Prism 310 DNA analyzer for marker D14S77 (Perkin-Elmer) and ABI Prism GeneScan 2.0.2. Software, in conditions described in study II.

4.6 Statistical methods

Statistical calculations were made with a microcomputer using the SPSS (version 6.1.2) software for windows package (studies **III-VI**).

In study **III**, one-way analysis of variance (ANOVA) followed by Duncan post-hoc analysis was used to test differences in clinical data between different groups. Chi-square test was used to determine differences between the genotype frequencies across the study groups, and Z-statistics for ε4 allele frequencies.

In study **IV**, in addition to ANOVA, analysis of covariance adjusted for age, duration and MMSE scores was used to study the effect of confounding factors among AD patients, such as age, duration of the disease and severity of dementia. A two-tailed Pearson correlation test was used.

In study **V** and **VI**, differences in allele and genotype frequencies between study groups were analysed using chi-square test and risk for odd ratios were calculated. The ACT and intronic genotypes in patients and controls were also tested against Hardy-Weinberg equilibrium.

The level of significance was set at $p<0.05$ in all studies.

Table 4. Primers for PCR amplification of the genomic DNA in studies I-VI

Fragment	Primers	Product size (bp)
Exon 16		278
F(orward)	5' CTTCAGGCCTAGAAAGAAGT 3'	
R(everse)	5' GGTGAACCAGAGTTAATAGG 3'	
Exon 17		319
F	5' CCTCATCCAAATGTCCCCGTCATT 3'	
R	5'GCCTAATTCTCTCATAGTCTTAATTTCCAC 3'	
D14S43		155-193
F	5' TGGAACACTCAGGCGA 3'	
R	5' CCAGAGCCACTTTCTAC 3'	
D14S77		203-249
F	5' GCGTGAGTCACTGTGCC 3'	
R	5'CAGACAGAAATTAACCAGAGTTGAA 3'	
D1S479		102-126
F	5' ACCCATTGCCACCATC 3'	
R	5' GGGGAGATTTGGACTGG 3'	
Exon 4		360
F	5' TAACCGTTACCTTGATTCTG 3	
R	5' CCACACTGGCTTTGAGAATA 3'	
Exon 5		260
F	5' GTGGTAATGTGGTTGGTGAT 3'	
R	5' CCCAACCATAAGAAGAACAG 3'	
Exon 6		230
F	5' TCTGTACTTTTTAAGGGTTGT 3'	
R	5' ACTTCAGAGTAATTCATCANCA 3'	
Exon 7		350
F	5' GGAGCCATCACATTATTCTA 3'	
R	5' GAGATGAGGAAAGAAAACAC 3'	
Exon 8		400
F	5' CACCCATTTACAAGTTTAGC 3'	
R	5' GATGAGACAAGTNCCNTGAA 3'	
Exon 9		240
F	5' TGGCTTGTTGTTGTCTATGC 3'	
R	5' AAGACGATAAAAACATTGCT 3'	
Exon 11		300
F	5' AAGAGTGACCAACTTTTTAAT 3'	
R	5' GTGTGGCCAGGGTAGAGAACT 3'	
ApoE		299
F	5'GCACGGCTGTCCAAGGAGCTGCAGGC3'	
R	5' GGCGCTCGCGGATGGCGCTGAG 3'	
ACT		124
F	5' CAGAGTTGAGAATGGAGA 3'	
R	5' TTCTCCTGGGTCAGATTC 3'	

Intron 8		199
F	5’ CACCCATTTACAAGTTTAGC 3’	
R	5’ CACTGATTACTAATTCAGGATC 3’	

Table 5. *PCR incubation conditions in studies I-VI*

		Pre-denaturation		*Denaturation*		*Annealing*		*Extension*		*Extra extension*	
	Cycles	T(C^0)	Time (min)	T(C^0)	Time (min)	T(C^0)	Time (min)	T(C^0)	Time (min)	T(C^0)	Time (min)
Exon 16	30	94	2	94	0.5	55	1	72	1	72	5
Exon 17	30	94	10	94	0.5	55	0.5	72	3	72	10
ApoE	35	96	5	96	2	60	2.2	73	2.5	73	10
ACT	30	94	5	94	1	55	1.75	72	2	72	5
Intronic	30	94	2	94	0.5	45	0.5	72	0.5	72	3
D14S77	30	94	5	94	0.5	55	0.5	72	0.6	-	-
D14S43	35	96	5	94	1	57	0.75	72	0.75	-	-
D1S479	30	94	3	94	0.5	57	0.5	72	2	-	-
Exon 4	35	96	2	96	0.5	50	0.75	73	0.75	-	-
Exon 5	“	“	“	“	“	52	0.75	“	“	-	-
Exon 6	“	“	“	“	“	50	0.5	“	“	-	-
Exon 7	“	“	“	“	“	50	0.5	“	“	-	-
Exon8	“	“	“	“	“	50	0.5	“	“	-	-
Exon 9	“	“	“	“	“	49	0.75	“	“	-	-
Exon 11	“	“	“	“	“	54	0.75	“	“	-	-

“ the same as the former; -not used

5. RESULTS

5.1 Amyloid beta precursor protein gene mutations in exons 16 and 17

PCR-RFLP analysis was used as a detection method for screening mutations at codons 665, 670/671 and 717. APP gene mutations were not found. The correct BglII restriction site was found for the APP665 of genomic DNA. A 278 bp PCR fragment of exon 16 was sequenced for checking and determining the exact nucleotide site for the APP 665 mutation.

5.2 Linkage analysis

Significant linkage was not found in any FAD families (II Table 2). Simulation analysis gave the lowest absolute lod-score as -6.04 and the greatest absolute lod-score as 4.29 with θ=0.10 and in 500 replicates through all families.

A microsatellite marker of D14S77 locus significantly excluded five FAD families (1, 4, 8, 10 and 12) on chromosome 14, and a microsatellite marker of D1S479 locus significantly excluded four FAD families (5, 11, 13 and 14) on chromosome 1 (II Table 1). Results of microsatellite marker from D14S43 locus did not display evidence for or against linkage in any FAD families. In all group A families, total lod-scores were 0.50, in B families -4.44 and in C families -9.63 with marker D14S77, and -0.17, -2.29 and -7.05 with marker D1S479 on chromosome 1, respectively. The highest lod-score (0.43) on chromosome 14 was in early-onset FAD family 16 (II Table 2).

5.3 Analysis of the PS-1 exons

The PS-1 mutation was found in FAD family age at onset <60 years in affected patients (II Figure1). Three affected AD patients, II-3, II-5 and II-7, and two healthy individuals, III-6 and III-8, of this family carried the same mutation in exon 9 of the PS-1 gene. This mutation changed adenine to guanine (GAA to GGA) in the last nucleotide triplet (codon) of exon 9 in the PS-1 gene. At the amino acid level, this missense mutation caused a substitution of glutamic acid to glycine at codon 318. This mutation was absent in another FAD family age at onset 52 years, in seven FAD patients (age at onset 45-64 years) and 40 healthy controls.

Exons 4-8 and 11 of the PS-1 gene did not show any changes in exon coding region in affected AD patients and controls.

5.4 Apolipoprotein E polymorphism in AD

The ApoE genotypes and allele frequencies were examined of a total of 248 subjects. There were 94 AD, 29 VAD, 15 PD, 8 PDD, 9 LB, 9 FD, 24 DS and 60 non-demented controls.

The studied groups did not differ significantly in sex distribution, but the groups differed significantly in age ($p<0.00001$): the DS patients were significantly younger than other groups ($p<0.05$). The control patients were younger than AD, FD and VAD patients, and the VAD patients were older than FD and AD patients ($p<0.05$).

The AD and FD patients had a significantly earlier age of dementia onset than VAD patients ($p<0.05$). No significant differences were found in age of onset or duration of the disease between the other groups. The duration of parkinsonian symptoms was 6±3 years in the PD group and 9±3 years in the PDD group.

Significant differences in the ApoE genotype and ε4 allele frequencies were observed only in AD patients, which differed from C ($p<0.01$), PD and DS groups ($p<0.05$) (Figure 8). The AD patients with two ε4 alleles had an earlier disease onset (64±8 years) than the other subgroups (70±10 years for AD with one ε4, 71±7 years for AD with no ε4, $p<0.05$).

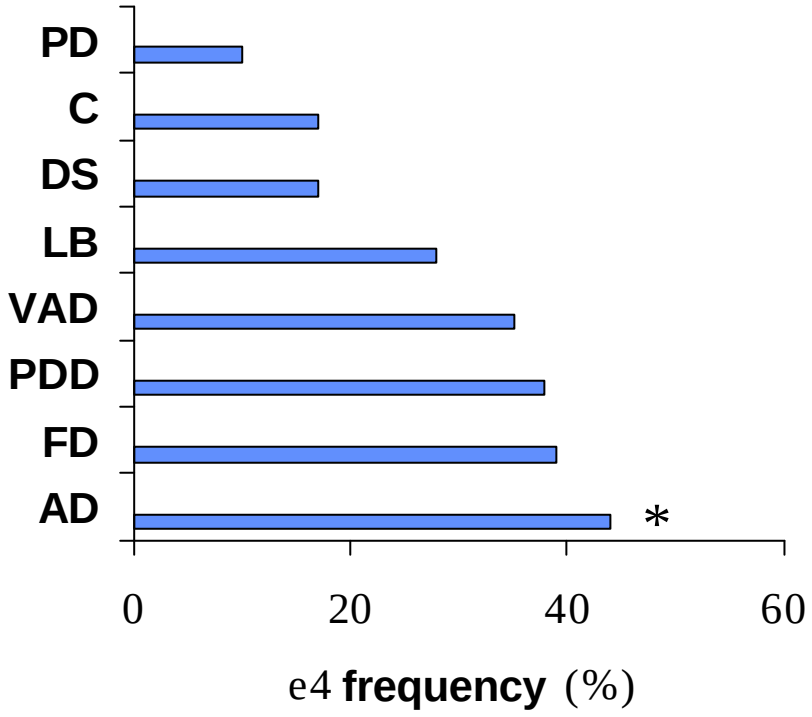

Figure 8. Frequency of ApoE ε4 allele in different ND groups and controls. *AD patients differed significantly from controls, PD and DS groups.

5.5 Determination of acetylcholinesterase activity

AD patients were divided to subgroups according to ApoE alleles: two ε4 alleles, one ε4 allele and no ε4 allele (IV Table 1). The four study groups (three AD subgroups and controls) differed significantly in age ($p<0.05$), the AD patients with one ε4 allele were older than controls and AD patients with no ε4 allele. The AD subgroups did not differ significantly in age at dementia onset, duration of disease, or in clinical severity as assessed by the MMSE scores ($p>0.05$) (IV Table 1).

The ChE (31.1±9.5 versus 29.8±8.3 nmol/ml/min) and AChE (26.1±8.6 versus 24.2±6.7 nmol/ml/min) activities for all AD patients combined into one group did not differ significantly from controls ($p>0.05$). However, different subgroups and controls showed a significant group effect in ChE and AChE activities ($p<0.0001$ for both). The AD patients with two ε4 alleles had higher ChE and AChE activities than controls and AD patients with one or no ε4 alleles ($p<0.05$). In addition, the AD patients with one ε4 allele had significantly elevated ChE and AChE activities compared to the AD patients with no ε4 allele ($p<0.05$) (Figure 9). The protein levels did not differ significantly across the controls and AD subgroups ($p>0.05$). Although there were significant differences in age among the study groups, age did not correlate significantly with the ChE or AChE activities in the whole study population or separately in AD patients or controls. Including age, duration of dementia and MMSE scores as covariates, the group differences remained highly significant ($p<0.0001$), but the effect of these factors was not significant ($p>0.05$).

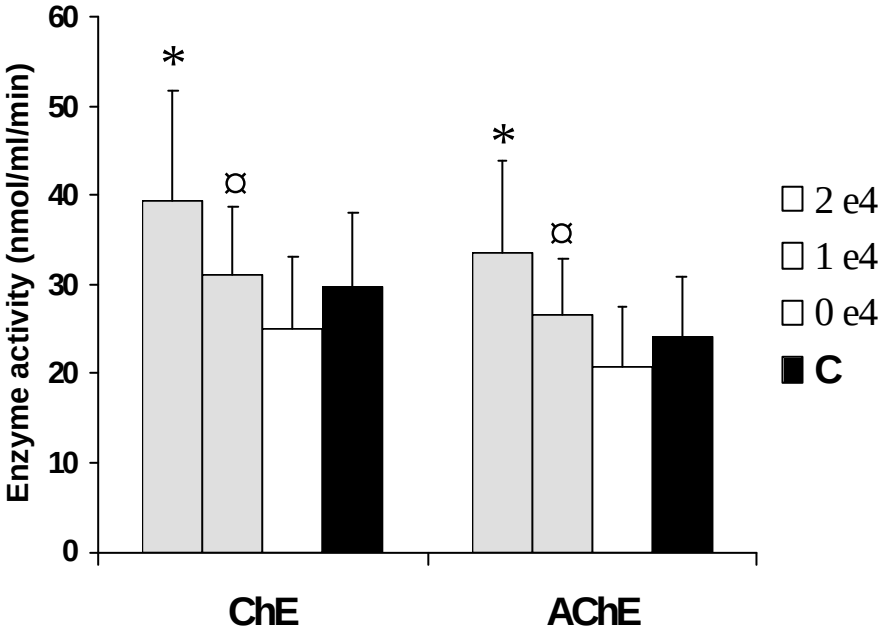

Figure 9. ChE and AChE enzyme activities in AD patients with two, one or no ApoE ε4 allele and in controls (C). Results are expressed as mean±S.D. *differs from all other groups; ¤differs from AD patients with no ε4 allele.

5.6 Alpha-1-antichymotrypsin and the PS-1 intron 8 polymorphisms

ACT and the PS-1 intron 8 polymorphisms were performed in the same research material. AD samples and controls were split into three subgroups according to their ApoE genotypes: ALL, ApoE 23,33 and ApoE 24, 34, 44. Genotype and allele frequencies of ACT and the PS-1 intron 8 polymorphisms were determined in different subgroups.

No significant differences were found between different subgroups and controls either in the genotype or allele frequencies. The ApoE ε4 allele was significantly increased in the AD group compared to controls in both ACT and the PS-1 intron 8 ($p=0.00002$ and $p<0.00001$, respectively).

The age distribution of males and females was similar ($p=0.18$ and $p=0.15$). Instead, AD patients and controls differed significantly in the gender distribution ($p=0.0018$ and $p=0.00097$). The number of females was high in the control group. Furthermore, some AD patients were significantly younger than control subjects ($p<0.00001$ and $p<0.00001$).

5.6.1 Alpha-1-antichymotrypsin polymorphism

The frequencies of ACT/AA genotypes between different subgroups and controls remained similar ($p=0.48$ for ALL, $p=0.60$ for APOE 23/33 and $p=0.52$ for APOE 24/34/44). In ApoE subgroup 23/33, the ACT/AA genotype frequency was slightly increased in AD compared to the controls (0.18 versus 0.14). Using the pooled data of the ACT AT/TT genotypes as a reference, the ACT/AA genotype was not associated with any increased risk of AD compared to controls: for ALL $p=0.98$; for ApoE 23/33 $p= 0.57$ and for ApoE 24/34/44 $p=0.69$.

Statistics were also calculated after adjustment for the age in AD group and controls ($p=0.10$). The ApoE ε4 allele was still significantly increased in the AD group compared to controls ($p=0.00001$). The frequencies of ACT/AA genotypes between different subgroups and controls remained similar ($p=0.52$ for ALL, $p=0.18$ for ApoE 23/33 and $p=0.71$ for ApoE 24/34/44).

5.6.2 The PS-1 intron 8 polymorphism

The frequencies of the intronic 11 genotype between different subroups and the controls did not differ compared to intronic 22 genotype ($p=0.46$ in ALL, $p=0.35$ in ApoE 23/33 and $p=0.44$ in ApoE 24/34/44). With the pooled data of the intronic 12/22 genotypes as a reference, the intronic 11 genotype was not associated with an increased risk of AD compared to controls ($p=0.74$ in ALL, $p=0.53$ in ApoE 23/33 and $p=0.75$ in ApoE 24/34/44).

The frequency of allele 1 was slightly lower compared to allele 2 in the AD group as well as controls in all subgroups. The frequencies of the ApoE ε4 allele remained similar between the AD patients and controls (p=0.97) in different PS-1 intron 8 genotypes (VI Table I).

Statistics were also analysed after adjustment for age in the AD group and controls (p=0.11). The ApoE ε4 allele was still significantly increased in the AD group compared to controls (p=0.00002). The frequencies of ACT/AA genotype between subgroup ALL and controls remained similar (p=0.725).

6. DISCUSSION

6.1 Research material

One strength of this study was that all patients with dementia and also the controls underwent a careful diagnostic procedure. AD and control subjects were collected in the hospitalized selected material. Thus it cannot be compared directly with the results of population-based studies.

In study **I**, well known APP mutations were not found which may partly be due to a low number of FAD cases (16 patients), who fulfilled criteria of age of onset with pathogenic mutations occuring in exons 16 and 17 of APP gene. This certainly reduced the possibility of identifying mutations in this study.

In study **II**, most FAD families were collected in a restricted area of eastern Finland and therefore, especially, the number of early-onset FAD families (two families) was low. The affected patients of these FAD families were applicable to mutation analysis of PS-1 exons.

In study **III**, the sample size of AD group (94 patients) was only high enough to evaluate the frequency of ε4 allele compared to controls.

In study **IV**, the number of AD patients (57 patients) was high enough to evaluate enzyme activities between different subgroups and healthy controls.

In studies **V** and **VI**, a number of AD patients (218 patients in ACT and 219 patients in PS-1 intron 8) was two times higher than in control group (101 and 104, respectively). This may affect the power of the study to detect an association between AD patients and controls.

In screening for APP mutations, the determination of ApoE genotypes and ACT and intronic polymorphism, the use of the PCR method and RFLP analysis are very reliable, fast and widely used and recognized techniques.

6.2 Amyloid beta precursor protein gene (exons 16 and 17)

Screening for APP codons 665, 670/671 and 717 using PCR-RFLP method did not reveal these mutations. Although rare mutations in the APP coding sequence have been found in affected members of ethnically diverse pedigrees with early-onset FAD, overall, these APP mutations are responsible for only a small proportion (<1%) of all cases of AD and about 2-3% of early-onset FAD (Tanzi et al., 1994; Baringa, 1995). Mutations of exons 16 and 17 in APP gene have not been found in late-onset FAD and in sporadic AD patients (Schellenberg et al., 1988; van Duijn et al., 1991b; Lannfelt et al., 1994).

We studied one of three possible APP mutations at codon 717 (Val to Ile), because this is the most frequent of all APP mutations. A total of 288 familial AD patients (i.e. 164 early-onset AD including the families with APP717 (Val to Ile) mutations and 124 late-onset AD cases), 300 sporadic and 614 normal controls have been determined for APP717 Val to Ile mutation (van Broeckhoven, 1995). Nevertheless, this mutation has been found in less than 20 families all over the world.

We studied the APP670/671 double-mutation, because it has been identified in Swedish families as a cause of early-onset FAD (Mullan et al., 1992; Lannfelt et al., 1993; Lannfelt et al., 1994).

Peacock and coworkers (1994) found the APP mutation at codon 665 in a patient with late-onset AD. We wanted to study this mutation, because our study contained 18 late-onset FAD cases. It is unknown whether this mutation is rare and how it is involved in the developing of AD.

A rapid and simple method was developed to analyse the APP mutation at codon 665 using a BglII digestion of genomic DNA and the same primers that Lannfelt et al. (1993) used for the detection of the double APP670/671 mutation. cDNA has earlier been used as starting material for analysing this mutation (Peacock et al., 1994). The exact nucleotide site for the APP665 mutation was determined by sequencing a part of exon 16 in APP gene.

6.3 Linkage analysis

A linkage study of FAD families was done to the PS-1 and the PS-2 genes, because previously well known APP mutations were excluded on chromosome 21, and evidence for linkage of an early-onset FAD was described on chromosome 14 in a family of Finnish descent (Haltia et al., 1994). Instead, sib-pair analysis was not used because of the low number of affected patients in families.

Highly polymorphic dinucleotide microsatellite markers at loci D14S43 and D14S77 were used to analyse a region of the PS-1 gene (Cruts et al., 1995, Clark et al., 1995a), because these gene loci are in the proximity of the PS-1 gene (0.09 cM from D14S77 locus and 1.78 cM from D14S43 locus) and these markers are used in linkage studies of the PS-1 gene.

Polymorphic dinucleotide microsatellite marker at locus D1S479 was used in the study of the PS-2 region, because significant evidence for linkage to AD had previously been observed in the Volga German kindreds with this marker (Levy-Lahad et al., 1995b,c).

Simulation analysis through all FAD families was performed in 500 replicates. The results suggested that the more replicates are simulated, the more accurate evaluation is attained to the power of the linkage in families (Ott 1989; Weeks et al., 1990).

In this study, FAD families were divided into three different subgroups according to the age at onset to analyse possible differences between groups in linkage analysis. FAD families in groups B (age of onset in affected AD patients 60-70 years) and C (age of onset >70 years) exclude linkage more clearly than families in group A (age of onset <60 years) either in the PS-1 or the PS-2 (see study II Table 2). This result partly confirms studies that the PS-1 and PS-2 genes are linked to early-onset AD. However, the main problems of our study were a low number (two families) of early-onset FAD families and the structure and the small size of these families, especially the number of affected was limited (only 1-3 affected) and often they were from the same generation. These factors certainly weakened the power of linkage analysis.

6.4 Analysis of the PS-1 exons

We found a family with mutation at codon 318 of the PS-1 gene. In this study, age at onset in affected FAD patients with mutation at codon 318 were 50, 58 and 57 years (current age), II-3, II-5 and II-7, respectively. Current ages in at-risk individuals with this mutation were 43 and 36 years, III-6 and III-8, respectively (see study II Table 1). In general, in the FAD families with the PS-1 gene mutation the mean age of onset was 45 years with a range from 29 to 62 years. Sandbrink and coworkers found this same mutation at codon 318 in a German patient with an age of onset at 47 years, but without any known familial background of dementia (Sandbrink et al., 1996b). Furthermore, this mutation was found in two Swedish AD patients with onset at 60 years and 68 years, (Forsell et al., 1997). We concluded that this mutation could be pathogenic leading to early-onset FAD in this family, because it was absent in 40 healthy controls.

Only this missense mutation is known in exon 9 of the PS-1 gene (Hutton et al., 1996; Lendon et al., 1997a; Hardy 1997). A splice site mutation is another known mutation in this exon, resulting in an in-frame deletion of exon 9 and an amino acid substitution at codon 290 (serine to cysteine) at the splice junction between exon 8 and 10 (Pérez-Tur et al., 1995). Furthermore, the proteolytic cleavage site of the PS-1 protein has been localized in exon 9 in codon 292 (Podlisny et al., 1997).

It is thought that the absence of frame-shift and nonsense mutations suggest that AD results from a gain of function (e.g. inactivation of normal PS allele) rather than loss of function (Hardy 1997). Mutation at codon 318 may lead to AD pathology indirectly through APP processing. Analysis of beta amyloid production in human fibroblasts and in plasma from individuals carrying the PS-1 or the PS-2 mutations has shown that PS mutations lead to an increased amounts of soluble amyloid beta (Scheuner et al., 1996). Furthermore, transgenic mice carrying human PS-1 mutation have twice as much soluble mouse amyloid beta in the brain when compared with normal mice (Citron et al., 1997). The importance of the PS-1 mutation at codon 318 remains to be determined.

6.5 ApoE polymorphism in AD

Since ApoE plays a crucial role in the plastic response and synaptogenesis in brain tissue and the plastic response is dramatically reduced in AD patients carrying the ApoE ε4 allele (Poirier et al., 1994; Arendt et al., 1997); it was of interest to study whether the ApoE ε4 allele frequency was altered in dementias other than AD. The present study we found increased ApoE ε4 allele frequencies also in other dementias (VAD, PDD, LB and FD), but only that in AD patients versus control subjects reached statistical significance. This was due to the fact that case numbers in the ND groups were too low and thus no discussion of the role of ε4 allele effect is feasible for these groups.

The number of cases in DS group (n=24) was high enough to analyse the effect of ApoE ε4 allele. Some DS patients develop the characteristic features of AD and dementia, whereas others do not become demented despite living into their sixth and seventh decades. However, a determination of ApoE genotype in DS cases has not revealed an increased frequency of the ε4 allele (Saunders et al., 1993a; Hardy et al., 1994; Pickering-Brown, 1994; Van Gool et al., 1995). Our results confirmed the lack of association with the ApoE ε4 allele. It is possible that AD pathology is induced in DS regardless of the ApoE genotype or AD pathology seems to be induced more slowly in the presence of the ApoE ε2 (Hardy et al., 1994).

The ApoE ε4 allele frequency was 0.44 in AD patients compared to controls 0.17 in our study. In two other studies in the same area, the ApoE ε4 allele frequencies were 0.45 and 0.36 in AD patients compared to controls 0.11 and 0.17, respectively (Kuusisto et al., 1994; Lehtovirta et al., 1995). Thus the ε4 allele frequencies were similar to ours in both studies.

6.6 Analysis of acetylcholinesterase enzyme activity

ChE and AChE activities in the CSF were observed to be higher in AD patients (early stage of the disease) with ApoE ε4 allele compared to controls and to be highest in AD patients with two ApoE ε4 allele. Furthermore, this finding was independent of patients's age, disease duration or severity of dementia. Previously, decreased AChE activity in CSF has been described in AD patients in a severe stage of the disease (Reinikainen et al., 1988; Soininen et al., 1981). Whether the high enzyme activities in AD patients carrying the ApoE ε4 allele is due to increased release or decreased degradation of these enzymes remains to be studied.

Two post-mortem studies have shown that the degree of the deficit of acetylcholine containing neurons in AD brains is related to the number of ApoE ε4 alleles (Poirier et al., 1994; Soininen et al., 1995). AD patients carrying the ApoE ε4 allele have a more severe cholinergic deficit in the hippocampus and temporal cortex (Poirier et al., 1994) and in the frontal cortex (Soininen et al., 1995).

Our finding may have interesting implications with respect to therapeutic approaches using ChE inhibitors in AD patients. Poirier et al. (1995) have reported that AD patients carrying ApoE ε4 allele differ in their response to treatment with tetrahydroaminoacridine (tacrine), being at a greater risk for loss of their acetylcholine synthetic capacities and therefore less capable of responding to the drug (Poirier et al. 1995). This supports the concept that AD patients carrying the ApoE ε4 allele have a more intense cholinergic dysfunction.

6.7 Alpha-1-antichymotrypsin polymorphism

Selection of the control population is of importance in studies of polymorphism. It is possible that differences may arise if patients and control subjects are not derived from the same population (Kamboh 1997a; Haines 1997).We collected our AD patients and control subjects from the same geographical area to avoid allele heterogeneity in the studied groups. It was possible that differences between our results and previously reported positive findings (Kamboh et al., 1995) might be lack of power in our study due to its small sample size, especially in controls. The number of AD patients was two times higher than the control group, which could affect the informativeness of results in cases of bi-allelic polymorphism, and also uneven distribution of ApoE genotypes in AD patients and controls. Another problem may be in the method of selection of AD patients and controls. We used only sporadic probable AD patients. It is also possible, that the differencies in studies may be due to heterogeneity between populations in the ACT gene. Furthermore, in our study, the number of of females was much higher than males in both AD and in control group. Kamboh and coworkers (1997b) have reported that ACT polymorphism can be gender-specific in the general population. In Caucasian and blacks Nigerian women, the frequency of the ApoE ε4 allele was significantly lower in ACT/AA phenotype than the corresponding frequency in men (Kamboh et al., 1997).

6.8 The PS-1 intron 8 polymorphism

The same subjects were used as in the study of ACT. In addition to considering the role of ACT, Wragg and coworkers (1996) sought possible causes for the linkage disequilibrium between AD patients and controls. They did sequence analysis of the open reading frame of ten AD cases with genotype 11 and failed to reveal coding changes in the PS-1 gene. Thus, it was possible that any relevant variability in the PS-1 gene might be in the promoter or somewhere else in the non-coding regions (Wragg et al., 1996). In another study, we sequenced exons 4-8 and 11 of the PS-1 gene to screen for mutations, but the sequenced part of the open-reading frame in familial AD samples with allele 1 failed to reveal any changes. Thus, our results in these exons of the PS-1 gene provided support for the concept that there was no disequilibrium in intron 8 polymorphism.

7. SUMMARY AND CONCLUSIONS

The purpose of this study was to delineate the molecular genetic basis of AD in eastern Finland. Both familial and sporadic early-onset and late-onset AD cases were studied. At present three different gene regions of FAD (APP, PS-1, PS-2) and the major risk factor (ApoE) are known, but the frequency of these disease causing mutations in Finnish population is not known.

The major results and conclusions are:

1. We screened for APP mutations at codons 665, 670/671 and 717 (Val to Ile). In accordance with the low frequency of APP gene mutations reported all over the world, we did not find any of these mutations.

2. No linkage was found in 15 FAD pedigrees to chromosome 14 or 1. The main problem was the high age at onset in affected individuals and possibility of heterogeneity in our AD families, which reduces the power to detect linkage. Instead, we found a mutation at codon 318 of exon 9 in the PS-1 gene in one early-onset FAD pedigree.

3. The frequency of the ApoE ε4 allele was increased in AD compared to controls.

4. AD patients with two ApoE ε4 alleles had higher ChE and AChE activities in their CSF than controls and AD patients with 1 or no ApoE ε4 allele. This finding was independent of the patient's age, disease duration or severity of dementia. This may indicate altered cholinergic metabolism in ApoE ε4 carriers which in turn may have implications for therapeutic approaches in AD, such as the response of the patients to cholinesterase inhibitors.

5. ACT or the PS-1 intron 8 polymorphism did not increase the risk of developing AD, when these risk factors segregate with or without ApoE ε4 allele. This result confirmed the findings of many other studies, that these factors do not modify the risk of suffering AD.

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