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Genetic Components of Late-Onset Alzheimer's Disease with Special Emphasis on ApoE, *IL-6, CYP46, SERPINA3* and *PPARγ*

Doctoral dissertation

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ABSTRACT

Alzheimer's disease (AD) is a multifactorial, progressive neurodegenerative disease the incidence of which increases strongly with age. AD is responsible for the most common form of dementia. Neuropathologically it is characterized by the accumulation of beta-amyloid and the formation of intracellular neurofibrillary tangles. AD is subdivided into early, EOAD and the more common late onset AD (disease onset ≥65 years), LOAD forms. The aetiology of LOAD is still largely unknown. It is widely accepted that genetic and non-genetic factors (e.g. lipid and glucose metabolism, inflammation) play crucial roles in predisposing the disease susceptibility.

Despite the extensive research to date, only four genes have been proven to either play a direct role in AD pathogenesis (genes for amyloid precursor protein, *APP*; presenilin-1, *PSEN-1*; presenilin-2, *PSEN-2* leading to rare autosomal dominant EOAD) or to significantly increase the disease susceptibility (i.e. apolipoprotein E gene, *APOE* ε4 allele in both EOAD and LOAD). Thus, the only established risk gene for LOAD is *APOE*. Several lines of evidence suggest the existence of additional susceptibility candidate genes for LOAD risk, but these still await identification. If we are to develop new strategies to prevent and treat AD, we need to understand the background (i.e. predisposing factors like risk genes) of the disease.

The present study focused on searching for novel risk genes for LOAD but also we explored the contribution of apolipoprotein E phenotype (ApoE) to the progression of LOAD in a Finnish population based sample. Furthermore, survival of the study population was examined in relation to AD, ApoE phenotype and gender.

We found that the ApoE ε4 phenotype did not influence the survival once AD had become manifested. However, in subjects with AD, ApoE ε4 non-carrier men had an increased risk of mortality compared to ApoE ε4 non-carrier women. Also in the whole study population, the risk of death was significantly increased in men.

On the basis of the previous pathophysiological studies we selected and studied genes encoding IL-6, cholesterol 24-hydroxylase (Cyp46), α-1-antichymotrypsin (ACT), and peroxisome proliferator-activated receptor γ (PPARγ), i.e. genes coding for enzymes involved in lipid or glucose metabolism and inflammation. Thus, these are potential biological candidate genes for LOAD risk.

The *IL-6* -174 G allele is over-represented in Finnish ApoE ε4 non-carrier LOAD patients. The *CYP46* gene dbSNP: 754203 T/C associated significantly with AD. Although dbSNP: 2146238 polymorphism failed to show a single allele association between cases and controls, we observed that the CG containing haplotype exhibites a modest odds ratio for the risk of AD. The analysis made between a nearby gene *SERPINA3*, which encodes ACT, and AD did not show any significant results. The genetic risk of AD was not significantly associated with the studied polymorphisms but the *PPARγ* Ala12- 478T genotype carriers were significantly younger at the onset of dementia than the other AD patients (p= 0.026).

These findings implicate *IL-6* and *CYP46* as possible risk genes for LOAD or, possibly they reflect the influence of a nearby gene in the AD risk. *PPARγ* does not seem to play major role in AD risk in Finnish LOAD patients.

To sum up, this data supports the theory that several susceptibility genes interact in the risk of AD or may modify the age of the disease onset. And, while ApoE is a significant genetic risk factor for the disease also in the aged population, its role in the disease progression is not as evident.

National Library of medicine classification: WT 155, QZ 50

Medical Subject Headings: Alzheimer disease/genetics; genes; apolipoproteins E; interleukin-6; cholesterol 24-hydroxylase; alpha 1-antichymotrypsin; PPAR gamma; aged; men; women; Finland

To Timo, Konsta and Sofia

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Kuopio, April 2006

Anne Koivisto

ABBREVIATIONS

Aβ	β-amyloid peptide
ACT/ *SERPINA3* (*ACT*)	α-1-antichymotrypsin protein/gene (alternative gene abbreviation)
AD	Alzheimer's disease
ApoE*/ APOE*	Apolipoprotein E protein/ gene
APP/ *APP*	Amyloid precursor protein/ gene
BSRT	Buschke Selective Reminding Test
CAA	Cerebral amyloid angiopathy
cM	centiMorgan
COX	Cyclo-oxygenase
CSF	Cerebrospinal fluid
Cyp46/ *CYP46 (CYP46A1)*	Cholesterol 24-hydroxylase protein/ gene (gene according to HUGO nomenclature)
EOFAD	Early -onset familiar Alzheimer's disease
EAOD	Early -onset Alzheimer's disease
IDE/ *IDE*	insulin-degrading enzyme /gene
IL1/ *IL1*	Interleukin-1 protein/gene
IL-6/ *IL-6 (IL6)*	Interleukin-6 protein/ gene (gene according to HUGO nomenclature)
LOAD	Late -onset Alzheimer's disease
LRP/ *LRP*	low density lipoprotein receptor-related protein /gene
MMSE	Mini-Mental State Examination
NFT	neurofibrillary tangle

NINCDS/ADRDA	The National Institute of Neurological Communicative Disorders and Stroke and the Alzheimer disease and Related Disorders Association
NSAID	nonsteroidal anti-inflammatory drug
OR	Odds Ratio
PPARγ/ *PPARγ* (*PPARG)*	peroxisome proliferator-activated receptor γ protein/ gene (gene according to HUGO nomenclature)
PSEN1/ *PSEN-1*	presenilin-1 protein/ gene
PSEN2/ *PSEN-2*	presenilin-2 protein/ gene
SNP	Single nucleotide polymorphism
TMT	Trail Making Test
VFT	Verbal Fluency Tests
VRT	Visual Reproduction Test

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by the Roman numbers **I-IV**

I Koivisto AM, Lempiäinen P, Koivisto K, Helkala E-L, Mykkänen L, Kuusisto J, Kervinen K, Kesäniemi YA, Laakso M, Soininen H. Apolipoprotein E phenotype alone does not influence survival in Alzheimer's disease: a population based longitudinal study. Neuroepidemiology. 2000;19:327-32.

II Koivisto AM, Helisalmi S, Pihlajamäki J, Moilanen L, Kuusisto J, Laakso M, Hiltunen M, Koivisto K, Hänninen T, Helkala E-L, Kervinen K, Kesäniemi YA, Soininen H. Interleukin-6 promoter polymorphism and late-onset Alzheimer's disease in the Finnish population. J. Neurogenet. 2005;19:155-161.

III Helisalmi S, Vepsäläinen S, Koivisto AM, Mannermaa A, Iivonen S, Hiltunen M, Kiviniemi V, Soininen H. Association study of CYP46 intron 2 polymorphism in Finnish Alzheimer's disease samples and a global scale summary. J. Neurol. Neurosurg. Psychiatry. 2006;77:421-422.

IV Koivisto AM, Helisalmi S, Pihlajamäki J, Hiltunen M, Koivisto K, Moilanen L, Kuusisto J Helkala E-L, Hänninen T, , Kervinen K, Kesäniemi YA, Laakso M, Soininen H. Association Analysis for the Peroxisome Proliferator-Activated Receptor Gamma Polymorphisms and Late Onset Alzheimer's Disease in Finnish population. Submitted.

(This thesis includes also analyses of other polymorphisms of *CYP46* gene and *SERPINA3* genes, not previously published)

CONTENTS

1 INTRODUCTION

Alzheimer's disease (AD) is a multifactorial, progressive neurodegenerative condition responsible for the most common form of dementia. Independent living becomes progressively more difficult for AD patients because their cognitive impairment interferes with basic activities of daily living. AD frequency increases strongly with age, from less than 1% in people aged 65 to 69 years to over 20% in those who are 90 years or older. AD is the most important cause of dementia (Lobo et al., 2000). As the population ages, AD is causing increasing public health and financial concerns because of its progressive and devastating effects on affected individuals as well as the substantial caregiver burden and the pressure on health care budgets (Winblad et al., 1996). If nothing is done to delay the disease onset, it has been suggested that the number of AD patients will quadruple within the next 50 years (Brookmeyer et al., 1998). Unless effective prevention or treatment is found for the common cause of dementia, the magnitude of the problem will continue to increase. However before one can develop of the effective strategies for AD therapy, it is necessary to understand the mechanisms of the pathogenesis, disease risk, onset and progression, including the genetic predisposition.

The predisposing factors of AD, with the exception of ageing (Fratiglioni et al., 2000), are still largely unknown, although much scientific effort has been expended over the years. While our understanding of pathophysiology of the AD remains fragmentary, it is widely accepted that genetic, environmental and life style factors play a crucial role in predisposing an individual to the disease (Bertram and Tanzi, 2004a). The involvement of vascular risk factors such as high midlife blood pressure, cholesterol, glucose intolerance and insulin resistance (Launer et al., 1999; Breteler, 2000; Kivipelto et al., 2001b) as well as the inflammatory response (McGeer and McGeer, 1998) in the aetiology of AD have received considerable attention. The contribution of the genetic factors in late-onset AD (LOAD) risk has been suggested to be very high, perhaps as much as 70 % (Bergem et al., 1997; Gatz et al., 1997). The difficulties to identify complex disease risk genes and the many failures to replicate studies are reflected in the fact that of the almost 100 candidate genes have been analyzed to date (Bertram and Tanzi, 2004a) and only four have been proven to either play a direct role in AD pathogenesis (genes for amyloid precursor protein, *APP*; presenilin-1, *PSEN-1*; presenilin-2, *PSEN-2*) (Goate et al., 1991; Levy-Lahad et al., 1995; Sherrington et al., 1995; Rogaeva et al., 1998) or to significantly increase disease susceptibility in almost every study population analyzed worldwide (i.e. apolipoprotein E gene, *APOE*, in chromosome 19) (Pericak-Vance et al., 1991; Poirier et al., 1993; Strittmatter et al., 1993; Dai et al., 1994; Kuusisto et al.,

1994). The mutations in *APP*, *PSEN-1* and *PSEN-2* lead to a rare autosomal dominant form of the AD, which usually appears before age of 65 years. However, the majority of AD patients are sporadic with onset age usually over 65 years (LOAD).

The presence of *APOE* ε4 allele increases the risk of early- and late-onset AD, sporadic and also familial forms of AD (Corder et al., 1993; Saunders et al., 1993; Chartier-Harlin et al., 1994; van Duijn et al., 1994). The *APOE* ε4 carriers have an earlier onset of the disease compared to non-carriers (Corder et al., 1993; Poirier et al., 1993; Tsai et al., 1994) but the results from those studies which have examined the influence of *ε4* allele in the progression of AD are controversial (Corder et al., 1995; Frisoni et al., 1995; Stern et al., 1997; Craft et al., 1998). Part of the LOAD cases can be explained by the presence of the *APOE ε4* allele (Slooter et al., 1998; Daw et al., 1999). While the magnitude of the effect of the *APOE ε4* allele as a risk factor for LOAD is age (Slooter et al., 1998) and ethnicity dependent (Corbo and Scacchi, 1999), the importance of the other genetic and environmental risk factors may increase in aged populations while that of the *ε4* allele decreases (Slooter et al., 1998). Further, although almost none of the 100 genes that have been tested for association with AD have yielded consistent results, several lines of evidence do point to the existence of additional susceptibility risk genes for LOAD risk, not yet identified (Daw et al., 1999; Bertram and Tanzi, 2004a).

Regions on at least nine other chromosomes in addition to chromosome 19 (probably representing *APOE*) have emerged in full genome screens, showing evidence for genetic linkage or association with *P*-values = 0.01 (Myers et al., 2002; Blacker et al., 2003) with the strongest evidence for linkage on chromosome 10 (Myers et al., 2002). Similar findings have been obtained for several additional genes, which are involved in AD-related pathophysiological cascades, such as the low density lipoprotein receptor-related protein (*LRP*) (Wavrant-DeVrieze et al., 1999), cystatin-C (*CST3*) (Finckh et al., 2000), cathepsin-D (*CTSD*) (Papassotiropoulos et al., 2000), bleomycin hydrolase (*BLMH*) (Montoya et al., 1998), interleukin-1 (*IL1*) (Grimaldi et al., 2000), interleukin-6 (*IL-6,* according to HUGO Nomenclature *IL6*) (Bagli et al., 2000; Arosio et al., 2004; Capurso et al., 2004) and neprilysin (*NEP*) (Helisalmi et al., 2004). Some genes, like the gene for insulin-degrading enzyme (*IDE*, located on chromosome 10q23.3 close to a region of linkage for LOAD) represent both positional and biologic candidates for LOAD susceptibility (Bian et al., 2004). Polymorphisms of these and of numerous other genes have been suggested as potential susceptibility factors for LOAD, and also possibly modifying the onset of the disease.

Novel AD genes will not only provide valuable clues for the development of novel therapeutic approaches, but will also allow the development of new genetic risk profiling strategies that will become an essential prerequisite for early prediction and prevention of this devastating disorder. The Finnish population is ideal for the study of genetic factors of diseases since a small number of founders brought a random assortment of disease genes to Finland. As a result of national and regional isolation, little further mixing of genes has occured. Therefore, the population has remained, so far, genetically homogenous, but also enriched with rare genetic mutations (Norio, 2003a; Norio, 2003b).

The present study is part of a larger project focusing on searching for novel risk genes for LOAD in the Finnish population. The studied candidate genes encode cholesterol 24-hydroxylase (Cyp46, referred to in the literature also as Cyp46a1), IL-6 and peroxisome proliferator-activated receptor γ (PPARγ) each of these enzymes acting in lipid metabolism (Papassotiropoulos et al., 2001; Raffai and Weisgraber, 2003; Cock et al., 2004), or in glucose metabolism and inflammation (Combs et al., 2000; Papassotiropoulos et al., 2001; Cock et al., 2004) thus they represent biological candidate genes for AD risk. The gene encoding α-1-antichymotrypsin (ACT), previously reported to associate with AD (Kamboh et al., 1998), was examined due to its proximity of cholesterol 24-hydroxylase gene (*CYP46,* according to HUGO nomenclature *CYP46A1*). Furthermore, our purpose was to study the contribution of apolipoprotein phenotype (ApoE), which also modulates lipoprotein transport and metabolism (Mahley, 1988; Mahley and Rall, 2000), in the progression of AD and in survival of the study population.

2. REVIEW OF THE LITERATURE

2.1. Alzheimer's disease (AD)

2.1.1. Epidemiology of dementia and AD

Dementing illnesses increase in the populations with advancing age. In a European collaborative study published in 2000, the prevalence of dementia in the elderly was 6.4 %, but it ranged from 0.8% in the group age 65 to 69 years and almost one third at age 90 years or older (Lobo et al., 2000). Currently in Finland, approximately 110 000 individuals suffer from mild to moderate dementia. As the population ages, the proportion of the demented subjects will continue to increase.

AD is the most common form of age-related dementia with onset during middle age but more commonly in the later years of life. The lifetime risk of AD varies with age, sex, and life expectancy (Seshadri et al., 1997). The incidence rate of AD increases from approximately 1% annually among people aged 65 to 70 years to approximately 6% to 8 % for people over age 85 (Mayeux, 2003a). The lifetime risk of AD varies from 12% to 19% for women over the age of 65 years and from 6% to 10% for men (Seshadri et al., 1997).

The duration of AD varies considerably with values ranging from 2 to 16 years after disease onset. The mean survival time for patients with AD was 3 to 7 years in two recently published studies (Wolfson et al., 2001; Fitzpatrick et al., 2005). For people over the age of 65, AD represents the eighth leading cause of death (Hoyert and Rosenberg, 1997).

In European populations, the overall prevalence of AD was 4.4 % (Lobo et al., 2000). The prevalence is higher among Afro-Americans and Hispanics compared with Caucasians living in the United States (Perkins et al., 1997; Gurland et al., 1999), but not among Africans in their native countries (Ogunniyi et al., 2000). Both the incidence and duration rates of illness influence the prevalence rate. Thus the prevalence of subjects surviving with clinically diagnosed AD varies with age. Before age 65 the disease is rare (<0.5% of this age group), but it increases by age 85 and older. Among the population over 85 years, between 13% to over 30% have this disease, depending on which the stage of disease severity is examined i.e. from mild to severe forms (Rocca et al., 1991; Skoog et al., 1993; Ott et al., 1995; Hy and Keller, 2000; Lobo et al., 2000; Polvikoski et al., 2001). Over half of all demented patients were diagnosed as having AD (Hy and Keller, 2000; Lobo et al., 2000).

2.1.2. Neuropathology and pathogenesis of AD

The characteristic histopathologic changes of AD at autopsy include neuronal loss, neurofibrillary tangles (NFTs) and neuritic plaques. Progressive neuronal dysfunction and loss of neurons in specific regions of the brain are associated with the clinical expression of the disease (Di Patre et al., 1999). Total neuron losses vary from 40% to 50% in the hippocampus (Mann et al., 1985), up to 79% in the nucleus basalis (Whitehouse et al., 1982) and from 15% to 58% in the cortex this being more severe in the temporal than in the parietal area (Hansen et al., 1988). Nerve cell loss in specific nuclei is accompanied by neurochemical deficiencies, and the combination of neuronal loss and neurotransmitter deficits leads to the appearance of the dementia syndrome. The destructive aspects involve the neurochemical deficits that disturb cell-to-cell communications, abnormal synthesis and accumulation of cytoskeletal proteins (e.g., tau) and beta-amyloid (Aβ), loss of synapses, decreased amount of dendrites, damage through oxidative metabolism and cell death (Di Patre et al., 1999).

The microtubule-associated protein, tau, appears to be critical for normal neuronal activity. The best established cause of dysfunctional tau in AD is the abnormal hyperphosphorylation of tau. This not only results in the loss of tau effect and stabilizing microtubules but also the modified form may have a toxic function and promote disruption of microtubules. The affected neurons react to the toxic tau by synthesizing new normal tau and by packaging the abnormally hyperphosphorylated tau into neurofibrillary tangles, twisted ribbons and straight filaments. Gradually and progressively the affected neurons degenerate (Iqbal et al., 2005).

The accumulation of non-soluble Aβ (Aβ42) is to date widely accepted as a central pathogenic event and hallmark of pathological diagnosis in AD (Hardy and Selkoe, 2002). All mutations known to cause AD (mutations in *APP, PSEN1* and *PSEN2*) increase the production of Aβ peptide (Hardy and Selkoe, 2002; Parihar and Hemnani, 2004). This protein is derived from amyloid precursor protein (APP) and, according to many studies when aggregated in a beta-pleated sheet configuration is neurotoxic (Hardy and Selkoe, 2002; Parihar and Hemnani, 2004). Aβ forms the core of neuritic plaques surrounded by dystrophic neurites, astrocytes and microglia. Unstructured amyloid consisting of diffuse plaques is also seen in AD brain, but they are detected also in normal brain. Diffuse plaques are not surrounded by neurites. In addition to extracellular form, β-amyloid is seen intracellulary in neurons (Selkoe, 2000). Amyloid plaques represent a side or final product in AD pathogenesis but the toxicity of Aβ and other amyloidogenic proteins is not mediated the through insoluble amyloid fibrils but it is the soluble oligomeric species which appear to be more

damaging. These soluble species include Aβ-derived diffusible ligands (ADDLs having globular structures) and protofibrils (strings of the globular structures) (De Felice et al., 2004).

Pathological alterations other than NFTs and plaques have also been shown to develop in AD brains and to parallel the severity of the clinical picture. At least three additional pathophysiological processes have been suggested to play a key role in the pathogenesis of AD: loss of synapses, inflammatory mechanisms and cerebral amyloid angiopathy (CAA). Loss of synapses in AD frontal cortex has been well documented by synaptophysin immunoreactivity and ultrastructural analysis and this has been shown to correlate with the degree of cognitive deficit (Cummings et al., 1998). Plaques are closely associated with a locally induced, non-immune mediated, chronic immune process. The role of inflammatory molecules in the pathological process is not fully understood, but numerous studies indicate that these molecules may be involved in the proposed Aβ-driven pathogenic cascade (van Gool et al., 2003). The concept that Aβ peptide is able to induce a local inflammatory response has received strong support by the finding that fibrillar Aβ binds to the complement factor C1 and activates the classical complement pathway in an antibody independent fashion (Rogers et al., 1992).The activated complement products may have a crucial role in the recruitment and activation of microglia (Eikelenboom and Veerhuis, 1996). Activated microglia produce numerous pro-inflammatory cytokines such as interleukin-1 and -6 (IL1 and IL-6) and tumour necrosis factor α (Griffin et al., 1989; Dickson et al., 1993). These cytokines may in turn induce further dysregulation of APP and local production of complement proteins and acute phase proteins (van Gool et al., 2003) as well as exacerbating plaque pathology and enhancing the hyperphosphorylation of tau and the subsequent development of neurofibrillary tangles (Kitazawa et al., 2004). On the other hand, microglial cell density in the white matter has been shown to be remarkably higher than that in the neocortex, suggesting that inflammatory responses involving the white matter may be more prominent than those in the gray matter and obviously this is not directly related to the development of the pathological features considered to be most characteristic of AD (Di Patre et al., 1999).

Amyloid can be deposited in the walls of blood vessels, more often arteries and arterioles, of the central nervous system. This phenomenon is known as CAA, which is an additional pathological process of AD. The other major clinicopathological manifestations of CAA include also cerebral hemorrhage and ischemic lesions. (Revesz et al., 2003). In addition to CAA, profound changes in cerebral microvessels and cerebral infarctions, independently of amyloid deposition, have often

been found in AD subjects. There are findings to indicate that almost 35% of AD subjects exhibit evidence of cerebral infarction at autopsy (Kalaria, 2003).

In addition, neuropathological overlaps have been reported between many neurodegenerative disorders. As many cases of other neurodegenerative diseases exhibit associated AD pathology, AD patients may also have pathological changes typical to other neurodegenerative diseases e.g. presence of lewy bodies or pathological changes in substantia nigra (Armstrong et al., 2005).

Pathological markers of AD develop in vulnerable brain regions in an uneven manner and at a variable rate. Indeed, cross-sectional studies indicate that the spreading of pathological alterations in AD does not appear in different brain regions at the same time but tends to begin in certain areas (namely, transentorhinal and entorhinal cortex and hippocampal formation), subsequently spreading to other areas (to the limbic areas of brain and finally to the neocortex) as the disease advances (Braak and Braak, 1995). Progression of cognitive impairment and manifestation of AD may not exclusively correlate with increasing plaque and NFT densities or with numerical changes of other pathological changes but, may also depend on the affected specific brain regions and the related brain changes. Involvement of subcortical nuclei (such as cholinergic forebrain nuclei) are crucial to memory functions and this contributes significantly to the cognitive impairment (Cummings et al., 1998).

Therefore, based on the available data, it is evident that AD, especially LOAD, may result from several parallel pathological changes that vary from patient to patient in intensity and distribution (Cummings et al., 1998). While the initiating factors and other antecedent events leading to the neurodegeneration in AD need to be clarified, it is widely accepted that many genes, most of them not yet identified, play an essential role in predisposing to disease onset and in modifying the course of the disease in conjunction with several non-genetic factors (Papassotiropoulos et al., 2001; Bertram and Tanzi, 2004a).

2.1.3. Aetiologies, predisposing and protecting factors of AD

The aetiology of AD, especially in the case of LOAD, is heterogenetic and multifactorial. Several genetic and non-genetic factors determine the risk for the development of AD and modify onset age and the course of the disease (**Table 1**). The real contribution, importance and interactions of many of these factors remain still unclear and are the topics of intense research (Bertram and Tanzi, 2004a).

Table 1. The factors involved in modifying AD risk.

	Hypotheses	
Power of evidence	**Predisposing factors**	**Protective factors**
Strong	• Clinical condition: Advanced age, Down's syndrome • Familial aggregation: First degree relatives with AD • Genetic factors: *APP, PSEN1, PSEN2, APOE ε4*	• Genetic factors: *APOE ε2*
Moderate	• Clinical condition: High midlife blood pressure • Female gender • Sosiodemographic factors: Low formal education, poor support during early years of life, lack of social and intellectual leisure-time activities	• Sociodemographic factors: higher educational level, intellectual leisure-time activities
Limited/insufficient	• Other genetic factors • Clinical condition: Cardio- and cerebrovascular diseases, other related vascular risk factors like high midlife cholesterol, high blood pressure or cholesterol in advanced age, diabetes, glucose intolerance, hyperinsulinemia, obesity. Depression. Previous severe or repeated head trauma. Lack of folate/B12-vitamin • Sociodemographic/ life style factors: low socioeconomic status, lack of physical activity, smoking, heavy intake of alcohol	• sociodemographic /life style factors: social activity, physical leisure activities, moderate alcohol consumption • Medication: History of estrogen replacement therapy in postmenopausal women, NSAIDs, lipid lowering agents, blood pressure therapy, vitamins

(Kivipelto 2002b; Mayeux, 2003a; Gorelick, 2004) and updated using summary of the Swedish Council on Technology Assesment in Health Care (SBU) report, 2006. Available in www.sbu.se/content0/document/Demens_sammanfattning.pdf).

2.1.4. Clinical features

AD preferentially affects individuals above 65 years. Clinically AD is characterised by an insidious onset and a progressive decline of memory and other cognitive functions interfering with daily functions at home and impairing occupational as well as social life. The deterioration of the cognitive functions is often followed by behavioural and mental disturbances. Motor difficulties appear in the late course of the disease. The disease progresses from a mildthrough a moderate to a severe phase with mounting severity of symptoms with the patient and need of, finally requiring continuous, assistance and care prior to death. The symptoms and severity of AD correlate with the neuropathological progress of the disease. The individual prognosis of AD varies but the expected survival time after manifestation of the disease is ten years, varying from two to 16 years (Reisberg et al., 1999).

2.1.5. Diagnosis of AD

Dementia is defined as "a loss of intellectual abilities of sufficient severity to interfere with social or occupational functioning" (APA, 1987). The specific diagnosis of AD is generally clinical and based of the symptoms and findings typical to AD. No specific biological markers are in use in routine AD diagnosis. The most widely used clinical criteria in the diagnosis of dementia and AD are defined in the Diagnostic and Statistical Manual of Mental Disorders, 3rd revised edition (DSM III-R) (APA, 1987) or 4th edition (DSM IV) (APA, 1990) and the criteria proposed by the National Institute of Neurological Communicative Disorders and Stroke and the Alzheimer disease and Related Disorders Association (NINCDS/ADRDA) (McKhann et al., 1984). According to NINCDS/ADRDA criteria (McKhann et al., 1984) the diagnosis of AD is classified into three categories- possible, probable and definite AD. The criterion for definite AD requires a histopathological confirmation of clinically probable AD. The criteria for neuropathological diagnosis of AD are defined by the Neuropathology Task Force of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Mirra et al., 1991). In clinical practice also the International Classification of Diseases, 10th revision criteria (ICD-10) (WHO, 1993) are used.

The patient has probable AD (McKhann et al., 1984) when the presence of dementia has been ascertained by a questionnaire and confirmed by typical findings in neuropsychological tests (like disturbances in memory, typically in episodic memory, in executive functions, and in language), plus that there is a gradual onset and progression of the symptoms. Other disorders which could possibly cause memory disturbances need to be absent. The dementia interferes with the patients

activities of daily living, and it induces altered patterns of behaviour. Regularly used laboratory analyses (blood and CSF tests) are normal. In the early phase of the disease, brain computed tomography is often normal but in magnetic resonance imaging, hippocampus atrophy can be seen. In serial observations, progressive brain atrophy appears. Electroencephalography is normal or some minor non-specific changes can be found. The patient has possible AD when he/she has also another brain disorder or systemic illness that is sufficient to cause dementia but is not considered to be the origin of the memory disease or the presentation of dementia varies compared to typical AD.

2.2. Subtypes of Alzheimer's disease

Previously the term Alzheimer's disease was used in the case of early onset (onset age <65 years) dementia of AD type. The late-onset type (onset age ≥ 65 years) of AD was called senile dementia. The typical brain pathology (e.g. NFT, plaques) of the disease are similar in EAOD and LOAD (Raskind et al., 1995) but in recent reports, certain differences have also been noted (e.g. the extent of atrophy in different parts of brain) (Frisoni et al., 2005). Furthermore, according to several studies, EOAD and LOAD patients have exhibited a different phenotype in several studies (Raskind et al., 1995; Devi et al., 2004; Hori et al., 2005) and, inheritance of AD shows an age-related dichotomy (Tanzi, 1999). Rare but highly penetrant mutations of *PSEN1* on chromosome 14, *PSEN2* on chromosome 1, and the amyloid protein precursor (*APP*) on chromosome 21 transmitted in an autosomal dominant manner have so far been found to account EOFAD. Instead, common polymorphisms like the APOE on chromosome 19 and alpha2-macroglobulin (*A2M*) on chromosome 1, with low penetrance appear to have their greatest effect as risk factors and/or genetic modifiers on the more frequent disease form LOAD (Tanzi, 1999).

Familial and sporadic AD was previously distinguished only by family history of other affected relatives. During the past decade genetic studies have increased our knowledge on AD aetiology and today molecular genetics provides an alternative approach to identify familial AD patients with defined genetic abnormalities. Nowadays, according to the genetic point of view, AD may be categorized into three forms: autosomal dominant familial AD, familial AD without clear Mendelian inheritance (familial aggregation) and the sporadic AD without familial aggregation (Papassotiropoulos et al., 2001). Only a minority (<5%) of all AD cases can be explained by the presence of known genetic factors (*APP*, *PSEN1* and *PSEN2*) responsible for the autosomal dominant, early onset familial form AD (Rocchi et al., 2003). However, 10 % of all AD cases are estimated to be autosomal dominant familial cases. The familial aggregation of AD may be due to both genetic and environmental risk factors in families without any clear mode of inheritance,

representing 30 % of all AD cases (Farrer et al., 1989). Sixty percent of all AD cases have been estimated to be without familial aggregation and they are called "sporadic AD" (Papassotiropoulos et al., 2001). Some clustering in families with longevity may be due to chance alone. Sporadic AD often manifests close to the terminal part of the life span (Tanzi, 1999). However, some contribution of genetics factors (e.g. modifying the age of onset or disease risk like *APOE*) in LOAD has been suggested to be higher, even 75 % of the disease risk (Gatz et al., 1997).

2.3. A strategy to identify and study candidate risk genes in complex diseases

Complex and heterogenetic diseases like coronary heart disease, arterial hypertension, diabetes and LOAD have no simple mode of inheritance. Furthermore, mutations and polymorphisms in multiple genes involved with these diseases interact with each other and also with non-genetic factors associated with disease risk. The genetic analyses of common and complex diseases have proved to be challenging. To date, almost 100 candidate risk genes have been analyzed for AD and only four (*APP, PSEN, PSEN2* and *APOE*) have been proved to associate with AD in several studies. Only one of these genes (*APOE*) has also associated with LOAD (Bertram and Tanzi, 2004a). In conventional family-based linkage analyses, these difficulties are related, in addition to the complex inheritance and heterogeneity, to the limited number of affected members in different generations available for genetic and phenotype analyses, the presence of phenocopies and to the possibility that carriers of the disease causing mutations or polymorphisms die before the appearance of the symptoms of late-onset disease. Therefore, alternative methods like linkage disequilibrium and association-based population and case-control studies have been used for identifying novel genes for LOAD and other complex diseases. However also in these studies, several factors such as sampling differences and stratification of the study population, make it difficult to identify and consistently replicate analyses of susceptibility in LOAD-related genes or other complex disease genes. Nevertheless, the LOAD risk caused by these susceptibility genes and consequently the importance in public health may be considerable due to their relatively common frequency in the population (Bertram and Tanzi, 2004a).

2.3.1 Candidate risk gene

Candidate genes are chosen as "positional candidates" or as "biological candidates" for the disease. "Positional candidate genes" are chosen from chromosomal regions implicated by genetic linkage or association. These candidate genes may also be "biological candidates" in the case when they have a positive association with the disease pathobiology. When the chosen candidate genes are purely

"biological candidates" implicated by pathobiological studies of disease under study then the chosen genes exhibit no evidence of genetic linkage or association.

2.3.2. Linkage studies

A common strategy to localize highly penetrant genes is linkage analysis. The co-segregation of specific genetic markers and disease phenotype is examined in extended families with several affected members. Linkage is derived from the consept of the distances between the two specific genetic loci. When different loci are situated in different chromosomes or are distant from each other in the same chromosome (and recombinations are possible), they are transmitted in meiosis independently of the other loci to the offspring. The specific loci are in linkage when they are located in the same choromosome, close enough that recombinations are rare between them. These close genetic alleles are transmitted to the next generation in a combination called the haplotype. The recombination fraction is a measure of genetic distance (1% recombination= 1 centiMorgan (cM)≈ 1 million nucleotides as a physical distance). The analysis of genetic linkage between two loci determines whether and how much the recombination fraction is smaller than 50%. For linked loci, it is less than 50% (likelihood that two loci are truly inherited in linkage, not inherited by coincidence). The origin of this strategy is that every subject carries two alleles at each gene locus transmitting with a probability of 50% one of two alleles from each locus to their offspring. In the estimation of likelihood of the true linkage, the most frequently used method is the logarithm of odds (lod –score method). Linkage is considered significant when the lod score reaches a value of 3 or more.

The strategy of linkage analysis and subsequent positional cloning has been very successful in monogenic disorders and in some rare variants of complex diseases like in EOFAD. This analytic approach has led to the identification of all three known EOAD genes on chromosomes 21 (*APP*), 14 (*PSEN1*) and 1 (*PSEN2*). However, the practical problems described above in complex and heterogenic diseases reduces the feasibility of using linkage studies in LOAD. It has been suggested that in complex and heterogenetic diseases, the loci associated with the disease under study may be identified more readily by studies of association and linkage disequilibrium (Risch and Merikangas, 1996).

2.3.3. Linkage disequilibrium

In linkage disequilibrium or allelic association, certain alleles located in close genetic loci are inherited together more often than expected according to the mathematical models. No recombinations, not even through several generations, are observed if these certain alleles are located close enough (Strachan and Read, 2004).

2.3.4. Association studies

Based on the pathophysiological studies related to the theory of the disease aetiology (e.g. in AD genes involved with Aβ, as well as other AD related pathways like inflammation, lipid metabolism etc.) or/and experimental findings (e.g. linkage studies), genes involved in disease pathogenesis are chosen as candidate genes in association studies. Markers or possible risk alleles e.g. single nucleotide polymorphisms, SNPs of these genes, which ideally alter gene function, are expected to be associated with the risk for the development of the disease. This hypothesis can be tested by cocducting an assessment of the frequency of the allelic or genotype variations in a sample of AD patients and control subjects in population or family-based or in classical clinical case-control studies. Evidence that the *APOE* ε4 allele was as a risk factor for AD was derived from numerous association studies while linkage analyses failed to replicate that finding. Furthermore, the replicability of the association studies in independent study populations and the possibility to study the haplotype structure of the gene increases the power of this method.

Homogeneity in the study population is crucial in the selection of the cases and controls for association studies. Heterogeneity, as well as stratification of the study population may skew the results. Concern about population stratification, control selection and prevalent case lead to biases and, therefore false positive results as well as controversial results, but these can be minimized by careful study design and by appropriate statistical analysis (Edland, 2004; Bertram and Tanzi, 2004a)

2.4. Genetics of Alzheimer's disease

Table 2. The established Alzheimer's genes and their functions.

Gene	Protein	Chromosomal Location	Associations	Model of Inheritance	Mutations/ N[b]	Affected families/N[b]	Onset age/ years (range)	Pathogenic relevance
APP	Amyloid precursor protein	21q21.2	EOFAD:+ LOAD: -	Autosomal-dominant	25	71	52 (35-60)	↑Aβ aggregation (↑$Aβ_{42/40}$-ratio)
PSEN1	presenilin 1	14q24.3	EOFAD:++ LOAD:+/-	Autosomal-dominant	155	315	44 (24-60)	↑Aβ aggregation (↑$Aβ_{42/40}$-ratio)
PSEN2	presenilin 2	1q31-42	EOFAD:+ LOAD:-	Autosomal - dominant	10	18	57 (56-71)	↑Aβ aggregation (↑$Aβ_{42/40}$-ratio)
APOE	apolipoprotein E, ε4 allele	19q13.32	EAOD:modifier[a] LOAD:modifier	complex	n.a.	n.a.	modifies onset age	↑Aβ aggregation ↓Aβ clearance

N= number; EOFAD = early -onset familial Alzheimer's disease; EOAD = early -onset Alzheimer's disease; LOAD = late -onset Alzheimer's disease; + = positive associations in several studies; -.= mostly negative association findings; +/-: both positive and negative association findings; n.a.= not applicable; modifier = modifies disease risk, age at onset and cognitive performance after disease onset (Marra et al., 2004); Aβ = beta- amyloid; ↑ = increase; ↓= decrease

The table is modified according to publications by Tanzi and Bertram (Tanzi and Bertram, 2005), Bertram and Tanzi (Bertram and Tanzi, 2004a) and, [a] (Marra et al., 2004) and updated on 6th Mar 2006 using [b] The Alzheimer's Disease Mutation Database (URL: http://www.molgen.ua.ac.be/ADmutations/).

2.4.1. Causative genes of early -onset AD

Less than half of the EOFAD cases are explained by the initial well known mutations found in APP, *PSEN1* and *PSEN* 2 genes (**Table 2**). All but one of the *PSEN1* mutations are 100% penetrant and are sufficient (but not necessary) to cause AD (Tanzi, 1999). (**Table 2**).

2.4.1.1. *APP*

Originally chromosome 21 had been selected for AD research from studies into the Down's syndrome. These subjects exhibit neuropathological changes typical of AD (Mann et al., 1985; Wisniewski et al., 1985). The first linkage between the gene encoding APP (**Table 2**) and familial AD was noted in 1987 (Goldgaber et al., 1987; Robakis et al., 1987; Tanzi et al., 1987). In EOAD families a common point mutation of *APP* gene in codon 717 leading to change of valine to isoleucine ("London mutation") was first described in 1991 (Goate et al., 1991). Currently, a total of 18 mutations, e.g. “Swedish” (double mutation at codons 670 and 671) and “Arctic” (at codon 693) mutations (Mullan et al., 1992; Nilsberth et al., 2001) have been identified in several families (**Table 2**). In all, 14 % of all EOAD families carry these known APP gene mutations (http://www.molgen.ua.ac.be/ADmutations). Almost all mutations are located on exon 17 (89%) and the remainder on exon 16 and consequently they are close to or within the Aβ peptide coding domain. Also genetic variations in the regulatory *APP* gene promoter region have been evaluated without any clear-cut findings (Wavrant-DeVrieze et al., 1999; Athan et al., 2002).

The *APP* 717 mutations produce more than doubling of the amounts of the insoluble forms of Aβ peptide (Aβ42-43), which rapidly aggregates to form amyloid depositions, initiating the formation of neuritic and senile plaques (Jarrett et al., 1993; Suzuki et al., 1994) though it is believed that, some mutations might be able to alter metabolism of APP (De Jonghe et al., 2001; Nilsberth et al., 2001).

The *APP* gene includes 18 exons and encodes for at least eight different isoforms of APP. The exon 15 is presented always in APP isoforms expressed in neurons. APP is a transmembrane protein with a long extracellular N-terminal domain and a short intracellular C-terminal domain (Hardy, 1997) **(Figure 1).** It undergoes at least two proteolytic cleavages (the possible cleavage pathways involve α-secretase, β- secretase or γ- secretase or combinations of these enzymes) in all cells. The membrane associated α-secretase cleaves APP within the Aβ- domain, leading to soluble and non-amyloidogenic formation of Aβ-peptide (Aβ40). The cofunction of β- secretase and γ- secretase is

critical since this leads to the release of Aβ-peptides with 42 or 43 amino acids (Aβ42- Aβ43), which are more fibrillogenic and possibly neurotoxic (Koo and Squazzo, 1994). The biological function of APP still remains poorly understood. In vitro studies suggest that secreted APP may stimulate cell proliferation and adhesion as well as support nerve growth factor-induced neurite outgrowth of certain cells (Milward et al 1992, Saitoh et al 1989).

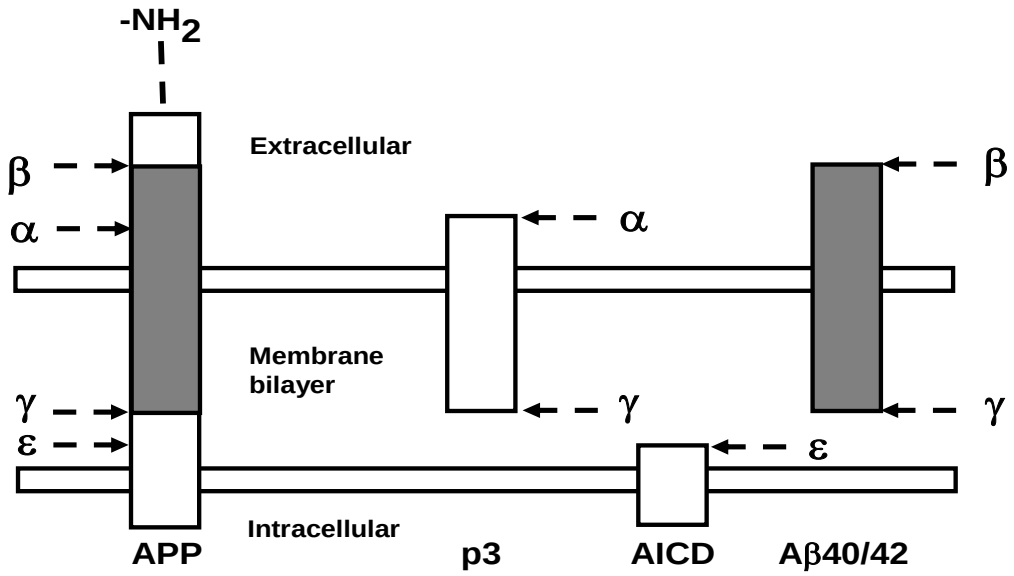

Figure 1. Domain structures of APP and secretase (α, β, γ and ε) cleavage sites in APP. Depending on the cleavage site of γ-secretase, either 40 or 42 Aβ-peptide is produced. Non-amyloidogenic peptides (P3 and βAPP Intracellular domain, AICD) are also produced.

2.4.1.2 Presenilins

A linkage between AD and a locus on the long arm of the chromosome 14 (Van Broeckhoven et al., 1992) was detected in 1992 and three years later Sherrington et al identified and isolated *PSEN1* (**Table 2**) by a positional cloning strategy (Sherrington et al., 1995). The *PSEN2* gene was found based on its homology to the *PSEN1* and it was localised to the long arm of chromosome 1 (**Table 2**). Both genes consist of 13 exons. To date there are 152 mutations found on these genes in almost 300 different EOAD families, most of them carrying the *PSEN1* mutations (**Table 2**) (http://www.molgen.ua.ac.be/ADmutations). Over 70 % of all known *PSEN1* mutations are located on exons 5-8, with the others on exons 4, 10-12 and five mutations on introns 4, 8 or 9. The most pathogenic mutations of *PSEN1* are mostly missense substitutions, two are nucleotide insertions

(one of them is not causative for AD but does cause frontotemporal dementia), three trinucleotide deletions, one hexanucleotide deletion and two splicing defects. The ten pathogenic missense mutations found in the *PSEN2* exist on exons 4, 5 7 and 12 (http://www.molgen.ua.ac.be/ADmutations). While several *PSEN* mutations have been associated EOFAD, an intronic *PSEN1* mutation has been claimed to be in association of sporadic LOAD, but this hypothesis is not supported by other studies (Kehoe et al., 1996; Nishiwaki et al., 1997; Scott et al., 1997).

The mutant *PSEN1* causes increased production of Aβ peptide in cell lines (Citron et al., 1997). *PSEN1* encodes a membrane protein, presenilin 1 (PSEN1), which influences γ-secretase activity which in turn is necessary for cleavage of Aβ from APP (Haass and De Strooper, 1999). The mutant PSEN1 has proven to increase also intracellular levels of the fibrillogenic Aβ42 (in endoplasmic reticulum and in Golgi compartments) (Sudoh et al., 1998).

The molecular mechanisms underlying the pathogenic effect of presenilins are not fully understood. Recent findings suggest that four membrane proteins (presenilin, nicastrin, aph-1 and pen-2) contain the limiting components of γ-secretase and when expressed together they form the active enzyme complex which catalyses the intramembrane proteolysis of Notch and APP protein and other substrates (Kimberly et al., 2003).

2.4.2 Apolipoprotein E

ApoE is a polymorphic protein synthesized in several organs but mainly by liver and brain in neurons and astrocytes. It is synthesized also by macrophages and monocytes. ApoE is crucial in lipid transport since it is a constituent of several classes of plasma lipoproteins as well as being the ligand that mediates the uptake of lipoprotein particles into cells via the low density lípoprotein receptor (LDLR) and LRP (Mahley, 1988). Furthermore, it is involved in the mobilization and redistribution of cholesterol during neuronal growth and after injury (Mahley and Rall, 2000) but is crucial in many other functions such as nerve regeneration, immunoregulation and activation of several lipolytic enzymes (Mahley and Rall, 2000).

ApoE contains 299 amino-acid residues, the amino terminal domain (residues 1-191) is a stable globular structure containing the receptor binding site. The carboxy-terminal domain (residues 216-299) is helical, less stable and this region contains the lipoprotein binding sites (Weisgraber, 1994). Utermann and colleagues have first described a polymorphism of ApoE in human serum (Utermann

et al., 1979). ApoE has three major isoforms, ApoE ε2, ApoE ε3 and ApoE ε4. A single locus with three alleles of APOE gene is responsible for this pattern. The three ApoE isoforms differ from each other at two sites, at residues 112 and 158 (**Figure 2**).

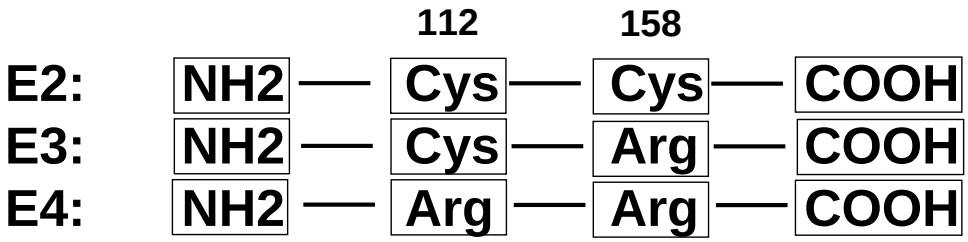

Figure 2. Apolipoprotein polymorphisms (modified according to Rocchi et al., 2003)

The prevalence of the *APOE* allele differs depending of the ethnic background and also geographical location. While the *APOE* ε2 allele is the rarest isoform with variations between 0.00 in Mayans and 0.13 and 0.14 respectively in New Guinea and China, the *APOE* ε3 allele is the commonest (range 0.49 in Sudan and 0.91 in Mayans) in all populations. The prevalence frequencies of *APOE* ε4 allele differ among populations (range 0.05-0.37, respectively, for Taiwanese and Papua New Guineans) (Gerdes et al., 1992; Siest et al., 1995). In Finland, *APOE* ε2 allele prevalence is lowest in children, becoming more common in the very elderly i.e. individuals with this allele live longer than their counterparts with the other alleles (**Table 3**) (Louhija et al., 1994). Furthermore, the ε4 allele frequency is higher in women than in men (Payami et al., 1996). In Finland, ApoE isoform frequencies appear to be rather similar in different parts of the country (Ehnholm et al., 1986; Lehtimaki et al., 1990; Lehtovirta et al., 1995; Kivipelto et al., 2002a).

Table 3. The prevalence of ApoE alleles in Finland (Louhija et al., 1994).

APOE	**3-18 years** N = 1577	**20-55 years** N = 615	**Centenarians** N=179
ε2	0.04	0.04	0.07
ε3	0.77	0.73	0.84
ε4	0.19	0.23	0.08

It has been proposed that the ApoE isoforms may posses distinct binding properties to Aβ peptide (Strittmatter et al., 1993) and tau-protein (Strittmatter et al., 1994) and that this might be the mechanism by which ApoE mediates its action. In particular, the ApoE ε4 isoform binds to Aβ faster than the ApoE ε3 isoform. ApoE ε4 in combination with Aβ forms monofibrils which deposit as dense structures of Aβ (Sanan et al., 1994). ApoE ε4 does not bind to tau in vitro whereas ApoE ε2 and ApoE ε3 do bind tau (Strittmatter et al., 1994). It has been speculated that interaction between tau protein and ApoE ε3 could protect against tau phosphorylation and NFT formation (Strittmatter et al., 1994). However, the exact mechanisms by which the ApoE isoforms can mediate their effects in the neuropathogenesis are still poorly understood. It is possible that ApoE ε4 mediates the risk for AD via its effects on cardiovascular disease, e.g. by increasing a carrier's risk for suffering hypercholesterolemia (Poirier, 2000).

In 1991 the chromosomal region of *APOE* on chromosome 19 was indicated in linkage analyses of AD families (Olaisen et al., 1982). Four years later, Lusis and colleagues succeeded in identifying the locus on the long arm of chromosome 19 (**Table 3**) (Lusis et al., 1986). Later, when Aβ had been shown to be bound to ApoE (Strittmatter et al., 1993), numerous researchers demonstrated an association of APOE ε4 allele with late –onset and sporadic forms of AD (Strittmatter et al., 1993; Farrer et al., 1997; Saunders et al., 2003). The association with the EOFAD is weak but some researchers have reported that the *APOE* ε4 allele may influence the age of onset in EOFAD cases carrying certain *PSEN1* or *APP* mutations (Levy-Lahad et al., 1995; Nacmias et al., 1995).

The presence of the ε4 allele is neither necessary nor sufficient to cause the disease. It act as more a genetic risk modifier in an interaction or co-operation with other genetic and/or environmental factors. The *APOE* ε4 allele modifies the age of onset and the presence of the ε4 allele is associated with AD risk. Its importance as a risk factor for the disease development is dose, age and ethnicity dependent (Corder et al., 1993; Farrer et al., 1997). Corder et al. showed that the risk for AD increases from 20 to 90% and the mean age of the LOAD decreases from 84 to 68 years with increasing numbers of ε4 alleles (Corder et al., 1993). While the impact of the *APOE* ε4 allele as a risk factor for disease development is evident in people from 40 to 90 years, it becomes weaker after the age of 75 years (Slooter et al., 1998). Generally the increased relative risk (as odds ratio, OR) is three fold for heterozygous and even 15 fold for the homozygous carriers of ε4 allele compared to ε3 homozygotes. The highest risk estimates have been found in Japanese population being 33- fold for ε4 homozygous subjects (Farrer et al., 1997) and lowest in Hispanics (only a two

fold risk) (Tang et al., 1996). Interestingly, in some African populations, *APOE* ε4 has not been associated with AD (Osuntokun et al., 1995). It is still uncertain whether these results are due to lower life expectancy or involvement of other environmental factors in African populations (Tang et al., 1996; Stewart et al., 2001).

The reports concerning the impact of *APOE* ε4 allele on the mortality in the general population and in AD patients have been contradictorary (Corder et al., 1995; Growdon et al., 1996; Stern et al., 1997; Craft et al., 1998; Slooter et al., 1999a; Fillenbaum et al., 2002; Lane et al., 2003). While Stern et al reported a slower deterioration and decreased risk of mortality in AD patients carrying ε4 allele compared to subjects without any ε4 alleles (Stern et al., 1997) other studies have indicated that *APOE* ε4 homozygosity was associated with increased deterioration of cognitive function (Craft et al., 1998) and mortality (Corder et al., 1995; Olichney et al., 1997; Tilvis et al., 1998; Dal Forno et al., 2002). However, some studies have failed to find any clear association between ApoE status, disease progression or mortality rate (Growdon et al., 1996; Slooter et al., 1999b; Fillenbaum et al., 2002). The mortality risk related to the *APOE* genotype appears to be age dependent, being less important or non-significant in the older age groups in European (people over 65 years) and Afro-American (people over 75 years) populations (Lane et al., 2003; Ewbank, 2004). The expected life span is generally lower in men than in women. With respect to AD, similar results have been found i.e., longer survival in woman after the disease diagnosis has been reported independently of ApoE status (Corder et al., 1995) whereas in other studies the presence of *APOE* ε4 associated with poor survival in men but not in women with AD (Dal Forno et al., 2002) but there are reports that neither *APOE* genotype nor gender have any influence on the progression of the disease (Growdon et al., 1996).

2.4.3 Candidate susceptibility genes for LOAD

2.4.3.1. Positional canditate genes

By the year 2005, almost 100 candidate AD genes had been analyzed but mostly without convincing evidence to claim an association with the disease risk (Bertram and Tanzi, 2004a). As discussed before, in complex diseases like in LOAD, there are several difficulties in identifying and replicating those genetic factors which cause only moderate or small effecs. Nevertheless, on the basis of full genome screens (used either linkage or association method), there are a number of positional candidate genes that have been linked with AD at a P-value ≤ 0.01 or a lod score ≥ 1.4 by at least two independent groups (Bertram and Tanzi, 2004a). Some of them are also biological

candidate genes for AD. In addition to the locus for *APOE* in chromosome 19q13, these studies have pointed to linkage with eight other different chromosomes (**Table 4**) with the most promising loci being located on chromosomes 9,10 and 12.

Table 4. Linkage or association regions for positional canditate genes according to published full genome screens modified according to Bertram and Tanzi (Bertram and Tanzi, 2004a)

Chromosome	Study method	References
1p36	linkage association	Myers et al 2002 Hiltunen et al 2001
4q35	linkage linkage	Li et al., 2002 Blacker et al 2003
5p13-15	linkage linkage linkage association	Myers et al 2002 Pericak-Vance et al 2000 Blacker et al 2003 Hiltunen et al 2001
6p21	linkage linkage association	Kehoe et al., 1999 Blacker et al 2003 Hiltunen et al 2001
6q15	linkage linkage	Pericak-Vance et al 1997 Myers et al 2002
9p21	linkage linkage	Pericak-Vance et al 2000 Myers et al 2002
9q22	linkage linkage association	Kehoe et al 1999 Blacker et al 2003 Bertram et al., 2005
10q21-22	linkage linkage	Myers et al 2002 Blacker et al 2003
10q24-25	linkage linkage	Li et al 2002 Blacker et al 2003
12p11	linkage linkage	Pericak-Vance et al 1997 Myers et al 2002
19q13	linkage linkage linkage linkage association	Pericak-Vance et al 2000 Kehoe et al 1999 Li et al 2002 Blacker et al 2003 Zubenko et al 1998
Xp21	linkage linkage	Kehoe et al 1999 Blacker et al 2003
Xp21-26	linkage association	Kehoe et al 1999 Zubenko et al., 1998

All these reported findings have a *P-value≤ 0.01* or two/multi point lod score ≥ 1.4 at least in two independent study.

Today, two genes on chromosome 9 have been associated to AD, either to disease risk or to onset age of AD. A Japanese group found in a case-control study, a significant association to AD with a polymorphism of the gene encoding for the very low density lipoprotein receptor (*VLDR-R*) in subjects carrying at least one *APOE* ε4 allele (Okuizumi et al., 1995). It is located in the neighbourhood of the signal peak observed in the linkage analyses (9p21) (Pericak-Vance et al., 2000). This *VLDR-R* polymorphism association has been replicated by another Japanese group and association has been seen also in Caucasian Europeans (or in Caucasians originating from Europe), but not commonly not in the patients from other ethnic populations (Okuizumi et al., 1995; Pritchard et al., 1996; Helbecque et al., 1998; Yamanaka et al., 1998). Further studies are needed to clarify whether *VLDR-R* is responsible for the linkage seen on chr 9p21. The gene encoding ubiquilin 1 (UBQLN1) is one of candidate genes for AD located near to a linkage peak on chromosome 9q22. Recent findings suggest that genetic variants in *UBQLN1* gene significantly increase the risk of AD (Bertram et al., 2005) Furthermore, another gene (ATP-binding cassette transporter A1, *ABCA1*) on chromosome 9 has been associated with onset age in AD and central nervous system cholesterol homeostasis in both a single allele (9q31.3) and haplotype association analyses, but this finding has not been confirmed in other studies (Wollmer et al., 2003; Katzov et al., 2004; Li et al., 2004b). A heavy cellular cholesterol load promotes Aβ formation and the ATP-binding cassette transporter A1 (ABCA1) mediates cholesterol efflux from cells. Genetic variability in ABCA1 may influence cholesterol metabolism in the central nervous system (CNS) and, thus, impact on the development of AD (Wollmer et al., 2003).

Recent findings from full genome screens and other studies indicate strong linkage of 10q with LOAD. Regions of interest derived from linkage analyses are 10q21-22 and 10q24-25 (Ertekin-Taner et al., 2000; Myers et al., 2000). It remains unclear whether these two linkage peaks represent an association to one or two underlying loci. Numerous genes have been mapped on these cromosomal regions. Most interesting, since it is also a plausible biological candidate for AD, is the *IDE* located on 10q23-25 (Bertram and Tanzi, 2004b). In addition to degrading insulin, IDE has a central role in the degradation and clearance of Aβ secreted by microglial cells and neurons (Vekrellis et al., 2000). Hippocampal IDE mRNA levels are lower on average in subjects with an *APOE* ε4 allele. This suggests that the genetic risk conferred by the *APOE* ε4 allele may be mediated in part by this allele's effect on IDE activity toward Aβ. It has been claimed that for the subjects not carrying ε4 allele, other factors which influence IDE may be relevant. One possible factor might be insulin which is a competitive inhibitor of IDE activity to Aβ (Edland, 2004). To date, several studies have succeeded in finding linkage between the genetic area consisting of *IDE*

and LOAD (Bertram et al., 2000b; Prince et al., 2003; Ertekin-Taner et al., 2004; Lee et al., 2004) but also opposite results have been published from a Japanese study (Sakai et al., 2004). The linkage of urokinase-type plasminogen activator gene (*PLAU*) mapped to chromosome 10q22.2 with LOAD has been reported though not consistently (Finckh et al., 2003; Bertram and Tanzi, 2004b; Papassotiropoulos et al., 2005). Urokinase-type plasminogen activator (uPA) converts plasminogen to plasmin. It modulates the cleavage of the APP and can degrade secreted and aggregated Aβ (Finckh et al., 2003).

LOAD candidate gene loci were described on chromosome 12p in 1997 and 1998 by three different study groups (Pericak-Vance et al., 1997; Blacker et al., 1998; Liao et al., 1998). While Pericak-Vance et al reported linkage with AD and certain loci in chr 12 (Pericak-Vance et al., 1997) Blacker et al (Blacker et al., 1998) focused on a pentadeletion/insertion polymorphism of the alpha-2-macroglobulin (α2M) gene (*A2M*) located in the 5'splice site of exon 18 and finding an association with familial LOAD. The exon 24 of the *A2M* contains a known second polymorphism which evokes an amino acid substitution (GTC→ATC, p.Val1000Ile). Liao et al (Liao et al., 1998) detected an increased risk for AD in carriers of the homozygous genotype coding Val instead of Ile. These findings have been confirmed by two other groups (Gibson et al., 2000; Wang et al., 2001).

The gene for LRP is located on chromosome 12, 50 cM distant from *A2M*. Several observations point to a role for this gene and the coded protein in the pathogenesis of AD. LRP is the main ApoE receptor expressed in neurons (Rebeck et al., 1993), mediating neurite outgrowth in an ApoE isoform-dependent manner (Holtzman et al., 1995). It is responsible for the endocytosis of secreted APP (Kounnas et al., 1995) and is detected in senile plaques, dystrophic neuritis and reactive astrocytes in AD brain (Rebeck et al., 1995).Two different *LRP* gene polymorphisms have been reported in association with AD (Kang et al., 1997; Lendon et al., 1997; Kolsch et al., 2003) but these reports have not been confirmed (McIlroy et al., 2001; Causevic et al., 2003). The other polymorphism is a silent point mutation in exon 3 of *LRP* gene, may reflect actually the linkage disequilibrium between this polymorphism and a functional variant of the *LRP* gene (Kang et al., 1997). Another chromosome 12 associated gene, a biallelic polymorphism (G>A) in the 3' untranslated region of the transcription factor LBP-1c/CP2/LSF has been implicated in AD susceptibility (Lambert et al., 2000). This gene is located in the neighbourhood of the *LRP* gene and it regulates the expression of several genes such as *α2M* and *IL1*. Further research on *LRP* and *LBP-1c/CP2/LSF* polymorphisms is needed to evaluate their effects on the risk of AD.

2.4.3.2 Biological candidate genes

The most interesting positional candidate genes for AD are involved, at least theoretically, in AD related pathophysiological cascades are those that are also biological susceptibility genes. Involvement of the immune system in the pathogenesis of AD has been discussed. Specific type T lymphocytes and reactive microglia as well as several markers of inflammation are evident in AD brain (Rogers et al., 1996; Tarkowski et al., 2003). The protective effect of NSAIDs for AD has been demonstrated in several epidemiological studies (Stewart et al., 1997; McGeer and McGeer, 1999). Furthermore, the involvement of vascular risk factors such as high midlife blood pressure, cholesterol, glucose intolerance and insulin resistance (Kuusisto et al., 1997; Breteler, 2000; Launer et al., 2000; Kivipelto et al., 2001a) in the etiology of AD has received considerable attention. These findings together with theory that different gene variations might influence the expression of the gene and consequently to the risk of AD have highlighted a group of the purely biological AD candidate genes (without linkage evidence) such as cystatin-C (Finckh et al., 2000; Goddard et al., 2004), cathepsin D (Kenessey et al., 1997; Sadik et al., 1999) and bleomycin hydralase (Namba et al., 1999) as well as the genes investigated in this study: *IL-6*, cholesterol 24S-hydroxylase (*CYP46*), α1-antichymotrypsin gene (*SERPINA3,* also the abbreviation *ACT* is used widely) and the peroxisome proliferator-activated receptor γ (*PPARγ,* according to HUGO nomenclature *PPARG*). The selection of these candidate genes on the basis of several published articles is presented in **Table 5** and the genes investigated in this study are described in detail. Furthermore there are several studied AD susceptibility genes like *IL1*, which are involved in inflammatory mechanisms, but according to large a recent recent large meta-analysis it was more associated with EOAD rather than LOAD (Rainero et al., 2004).

Table 5. Biological candidate susceptibility genes for LOAD (Papassotiropoulos et al., 2001; Prince et al., 2001; Rocchi et al., 2003)

Gene	Chromosomal localization	Studied polymorphisms	Association in AD risk	Protein	Expression in CNS	Pathophysiological relevance	References
ACE	17q23 int16	Deletion/Insertion (D/I)	+/- meta-anal:+	Angiotensin converting enzyme	-Cerebral blood vessels-endothelium, -neurons-supraoptic and paraventricular hypothalamic nuclei and basal forebrain and midbrain	Unclear Theoretical: links through vascular disease -blood pressure regulation? inflammatory cascade?	Elkins et al., 2004 Tian et al., 2004 Camelo et al., 2004 Kehoe et al 2004 totally over 30 studies
BLMH	17q11.1-11.2	A1450G	+/-	Bleomycin hydralase (cysteine protease)	Astrocytes	A candidate for the β-secretase secretion of Aβ-peptide	Montoya et al., 1998 Farrer et al., 1998 Namba et al., 1999 Malherbe et al., 2000
BChE-K	3q26.1	G1615GA	+/- or protection?	Butyrylcholinesterase K-variant	-Neurons -NFT -senile plaques	Hypothetical:Wild type *BChE*-participation in the transformation of Aβ to a neurotoxic dense form? *BChE*-K- protects from increased Aβ formation by having reduced enzymatic activity?Or increases risk?	Lehmann et al., 1997 Hiltunen et al., 1998 Wiebusch et al., 1999 Tilley et al., 1999 Alvarez-Arcaya et al., 2000 Mattila et al., 2000

Table 5. continued

Gene	Chromosomal localization	Studied polymorphisms	Ass in AD risk	Protein	Expression in CNS	pathophysiological relevance	References
CatD	11p15	exon 2 C>T	+/-	Cathepsin D (intracellular protease)	-Neurons containing NFT - endosomes of pyramidal neurons	Cleaves APP into amyloidogenic components, degrades tau protein secretase?	Touitou et al., 1994 Cataldo et al., 1997 Papassotiropoulos et al., 1999a Sadik et al., 1999 Callahan et al., 1999 Ingegni et al., 2003
CST3	20	5'flanking region G157C exon1 G73GA (p.Ala73Thr)	-73G/G age-dependent +/-	Cystatin C (amyloidogenic protein)	Neurons activated glia mainly in temporal cortex (hippocampus, entorhinal cortex) (cerebral bood vessels , NFT)	Extracellular inhibitor of cysteine proteases secreted by monocytes/ macrophages. Altered secretion of cystatin C, reduced rate of cystatin C synthesis	Finckh et al., 2000 Beyer et al., 2001 Deng et al., 2001 Goddard et al., 2004
NEP	3q25.1–q25.2	several polymorphic sites	Haplotype analysis + SNP:s +/-	Neprilycin (Aß degrading enzyme zinc dependent metallopeptidase)	Pyramidal neurons, smooth muscle cells of blood vessels	Aß degrading	Carpentier et al., 2002 Clarimon et al., 2003 Lilius et al., 2003 Helisalmi et al., 2004

Table 5. continued

Gene	Chromosomal localization	Studied polymorphisms	Association in AD risk	Protein	Expression in CNS	Pathophysiological relevance	References
NOS3	7q35	A298T (p.Glu298Asp) at least 4 other polymorphic sites	- Glu298Glu +/- others –not studied	Endothelial nitric oxide synthase	Cell membrane, cell cytosol, high concentrations in hippocampal pyramidal neurons	increased nitric oxide (NO) production by microglial cells, astocytes and brain endothelium> NO in neuronal death stimulation of oxidative stress by NO	Dahiyat et al., 1999 Kunugi et al., 2000
5-HTT	17q11.1-q12	promoter region deletion/insertion VNTR polymorphisms in 2. intron	D/I short variant +/- VNTR- not studied	Serotonin transporter	Serotonergic neurons?	Involved in serotonergic neurotransmitter system short variant- reduced transcriptional activity	Meltzer et al., 1998 Mundo et al., 2000 Hu et al., 2000 Kunugi et al., 2000 Hranilovic et al., 2004
TGF-β1	19q13.1-13.3	G800A C509T exon 5 missense mutation at codon 263	-800 – -263 – -509 +/-	Transforming growth factor -β1 (immunosuppressive cytokine)	Astrocytes	-Aβ accumulation -Aβ clearance by activated microglia	Lindholm et al., 1992 Luedecking et al., 2000 Araria-Goumidi et al., 2002 Lesne et al., 2003

Each gene written by using abbreviation, the full name of the gene is the same as the names of the protein. p. = protein, SNP = single nucleotide polymorphism, Aβ = β-amyloid, NFT = neurofibrillary tangle, CNS = central nervous system, + = associated with AD risk, - = association studied without significant findings, +/- = controversial findings in association between studied polymorphism and AD

IL-6 is a multifunctional inflammatory cytokine to involved in the. acute inflammatory response as ell as in the modulation of specific immune functions and B- and T-cell differentiation, aging, and bone metabolism (Papassotiropoulos et al., 2001). Furthermore, IL-6 may stimulate astrocytes to produce nerve growth factor (NGF) production and act by itself as neurotrophic factor in synergy with NGF. It mediates also neuronal degeneration (Papassotiropoulos et al., 2001; Tarkowski et al., 2003). It has several synonyms such as interferon-β2, hepatocyte stimulatory factor and many others in the literature (Papassotiropoulos et al., 2001).

Elevated IL-6 immunoreactivity has been observed in the amyloid plaques in AD patients' brain but it was claimed also to be in the plaques of nondemented elderly persons. In AD patients the majority of IL-6 – positive plaques were diffuse (71%) while immunoreactivity was absent in compact plaques. This suggests that IL-6 induction may be an early event in the AD neurodegenerative cascade (Huell et al., 1995). Several epidemiologic studies have shown that onset is delayed and prevalence of AD is reduced by 40 - 50% in persons using antiinflammatory drugs (McGeer and McGeer, 1999). Also, in transgenic mouse models, elevated CNS levels of IL-6 resulted in a chronic-progressive neurological disorder and a cognitive decline (Campbell et al., 1997).

The IL-6 has a specific receptor complex through which its functional effects are mediated. The complex consists of a membrane-anchored signal transducing glycoprotein gp130 (IL-6ST) and membrane-anchored ligand binding glycoprotein gp80 (IL-6R). Moreover, soluble forms of the ligand binding glycoprotein gp80 (sIL-6R) and the signal transducing glycoprotein gp130 (IL-6ST) binds to and are involved in IL-6 signaling. In contrast, an antagonistic mechanism has been proposed for gp130, which blocks IL-6 function (Papassotiropoulos et al., 2001).

The accumulation and aggregation of the Aß in the brain are crucial factors contributing to AD. Consequently, preventing the generation of Aß is an important preventive and therapeutic strategy. Recent work on the metabolism of Aß has identified several cellular proteins and proteases that collectively promote or prevent the generation of Aß. In addition, accumulating in vitro and in vivo evidence points to a role of cholesterol in modulating the cellular processing of Aß (Sparks et al., 1994) and there is clinical data to support its role in AD risk (Jarvik et al., 1995; Kivipelto et al., 2001b). A major portion of brain cholesterol is thought to be converted to soluble 24(S)-hydroxycholesterol which can diffuse across the blood brain barrier (BBB) entering into the cerebral circulation (Raffai and Weisgraber, 2003). Bodowitz and Klein (Bodovitz and Klein, 1996)

showed that plasma membrane cholesterol levels modulate APP processing via the α-secretase pathway in vitro. A Cholesterol-extraction agent or statin treated neuronal and non -neuronal cell lines exhibited increased α-secretase activity and the release of the soluble APP splicing products with decreased β-secretase activity.

Cyp46 plays a key role in the hydroxylation of brain cholesterol and regulates the elimination of brain cholesterol into plasma across the blood brain barrier (Bodovitz and Klein, 1996; Lund et al., 1999; Raffai and Weisgraber, 2003). It belongs to the sub -family of the cytochrome P450 enxymes and in humans it is highly expressed in the brain messenger RNA. In mouse brain, it has been found especially in neurons of the hippocampus, cerebral cortex and dentate gyrus, the areas vulnerable in AD pathogenesis. Therefore, functional alterations of Cyp 46 could modulate cholesterol concentrations in neurons and associated changes in APP processing and in Aβ production (Papassotiropoulos et al., 2003).

One of proteins involved in inflammatory reactions to be associated with AD brain lesions is α1-antichymotrypsin (ACT), an acute phase proteinase inhibitor of the serpin family(Abraham, 2001). ACT is one of the components of senile plaques where it binds Aβ with high affinity (Abraham et al., 1990) and pathological expression of ACT occurs in astrocytes in areas that develop amyloid lesions (Abraham, 2001). ACT synthesis is induced in cultured human astrocytes by IL1. IL1 is a lymphokine, the expression of which is up-regulated in microglial cells from affected areas of AD brain (Das and Potter, 1995). Furthermore, ACT and IL1 expression by microglia and astrocytes respectively showed a regional different distribution of expression occuring spontaneously on cortex (Das and Potter, 1995). Furthermore, AD patients with the *APOE* ε4 allele exhibited increased levels of ACT and an increased number of diffuse microglia positive cells (Cheng et al., 2002). In summary, in amyloid deposits ACT is found only in association with Aβ. ACT may have a role in the processing of β-APP or it stabilizes Aβ deposits. ACT levels have been significantly higher in AD patients compared to controls (Harigaya et al., 1995), their levels increasing in advanced stages of the disease (Matsubara et al., 1990).

Some “classical” and widely used NSAIDs such as indomethacin and ibuprofen are able to activite the PPARγ and this has been reported to inhibit the expression of a wide range of proinflammatory genes (Landreth and Heneka, 2001). This observation is supported by a re-analysis of the above mentioned epidemiological studies in which the reduced risk of AD correlated to the usage of this subset (e.g. ibuprofen and indomethacin) of Aβ lowering NSAIDs and, also the results from in vitro

and experimental animal studies showing the decreased Aβ production independently of COX-inhibition after treatment with unselective NSAIDs (Weggen et al., 2001; van Gool et al., 2003).

PPARs have ligand and DNA binding domains. They belong to the nuclear receptor super family of DNA-binding transcription factors whose transcriptional regulatory actions are activated after ligand binding. Activated PPARs form a heterodimeric complex with another nuclear receptor, the retinoic X receptor, and bind to certain PPAR response elements in the promoters of specific target genes and thus they are able to regulate their expression, either inducing or suppressing the transcriptional activity of the target gene (Plutzky, 2003) (**Figure 3**). There are three PPAR isoforms termed PPAR α, β, and γ. They are differentially expressed. The natural ligands for PPARs are fatty acids and lipid metabolites. Furthermore, each PPAR isoform has its own specific ligands (Combs et al., 2000).

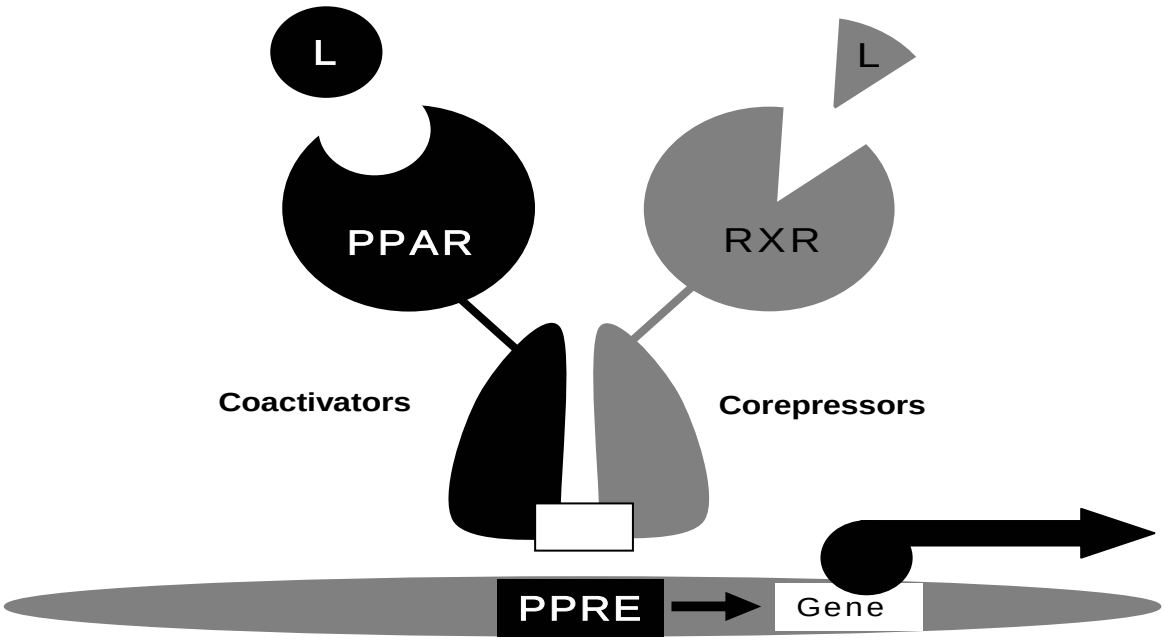

Figure 3. PPAR as transcription factor. PPARs control gene expression through a heterodimeric complex with retinoid X nuclear receptor (RXR). The activation of these receptors are controlled by binding of specific ligands (L). Furthermore, the transcriptional response is determined by the association or release of specific coactivators and corepressors. The formed complex binds to PPAR response elements (PPRE) in the promoter regions of target genes to modulate their expression (modified according to Pluzky, 2003).

PPARγ is expressed in fibroblasts, adipose cells, lymphocytes including B cells, in the major cellular constituents of vessel wall (endothelial cell, vascular smooth muscle cell, monocytes and macrophages), and in atherosclerotic lesions. PPARγ regulates adipogenesis and insulin sensitivity as well as lipid metabolism target genes, e.g. apolipoprotein A-1 and lipoprotein lipase which are a key mediators in metabolic syndromes such as diabetes and obesity. Recent studies suggest that some PPARγ mutations found in humans may result in severe insulin resistance and hypertension (Plutzky, 2003).

There is evidence that PPARγ also regulates the expression of other genes involved in a variety of other physiological functions (Willson et al., 2000; Landreth and Heneka, 2001; Willson et al., 2001) such as the regulation in lipid and carbohydrate metabolism (Willson et al., 2000; Willson et al., 2001). It is also implicated in atherosclerosis (Tontonoz et al., 1998).

The PPAR γ isoform has recently been found to be expressed in Aβ stimulated monocytes and macrophages. The principal function of PPARγ in these inflammatory cells is to suppress the expression of the proinflammatory cytokines IL1β, TNFα, IL-6 and other proinflammatory products. Simultaneously, decreased secretion of these products blocks astrocyte proliferation (Combs et al., 2000). In cell cultures, the J class prostaglandin PGJ2 (Kliewer and Willson, 1998) and, interestingly, also NSAIDs (Combs et al., 2000) and thiazolidinedione class antidiabetic drugs (Combs et al., 2000) functioning as PPARγ agonists can inhibit before mentioned Aβ stimulated secretion of proinflammatory products preventing microglial (mouse cells) and monocyte (human cells) mediated neurotoxicity (Combs et al., 2000) Furthermore, incubation of microglia with troglitazone inhibited the Aβ-mediated COX-2 immunoreactivity.

Furthermore, PPARγ may influence directly the neuroinflammatory responses seen in brain (Landreth and Heneka, 2001). Recently, Sastre et al. demonstrated that PPARγ reduction potentiated β-site amyloid precursor protein (APP) cleaving enzyme (BACE1) mRNA levels by increasing BACE1 gene promoter activity (Sastre et al., 2006). This indicates that an increase of PPARγ activity could inhibit the activity of BACE1 They also found and localized a PPARγ responsive element (PPRE) in the BACE1 gene promoter (Sastre et al., 2006). In conjunction with previous findings, this indicates that PPARγ may play a critical role in regulating the inflammatory responses of microglia and monocytes related to beta-amyloid i.e. PPARγ is involved in AD pathogenensis via its ability to interfere with inflammation (Combs et al., 2000; Sastre et al., 2006).

2.4.3.3. Interleukin-6 (*IL-6*) polymorphism

The gene coding for IL-6 found in the short arm of chromosome 7 (7p21) contains five exons and four introns. Several transcription factors such as nuclear factor IL-6 or activator protein-1 (AP-1) mediate the activation of the *IL-6* promoter while steroids result in inhibition of the activity of the *IL-6* promoter.

Two of several polymorphic sites have been used for genetic association studies: A biallelic G174C promoter polymorphism (according to Nomenclature Guidelines IL6 c.174G>C) and a multiallelic variable number of tandem repeats (VNTR; having AT repeats) polymorphism in the 3' flanking region of *IL-6*. The associations with several conditions and diseases have been studied. At least two independent studies have revealed an association between G174C polymorphism and bone mineral density, plasma IL-6 levels (also in AD patients) and the regulation of IL-6 transcription (Fishman et al., 1998; Papassotiropoulos et al., 2001; Capurso et al., 2004). Interestingly, considering the possible pathophysiological mechanisms involved in AD, a positive association with this polymorphism and lipid metabolism, atherosclerosis as well as with type II diabetes has been detected. The VNTR polymorphism is not as well confirmed in association with other diseases but there is at least one positive finding relating this polymorphism to other diseases (Papassotiropoulos et al., 2001).

The results from studies defining the AD risk associated with the *IL-6* single nucleotide polymorphisms are highly controversial (Bagli et al., 2000; Bhojak et al., 2000; Pola et al., 2002; Shibata et al., 2002; Faltraco et al., 2003; Licastro et al., 2003; Arosio et al., 2004; Capurso et al., 2004; Depboylu et al., 2004; Nishimura et al., 2004; Zhang et al., 2004). Most of them have investigated G174C promoter region polymorphism in association with the LOAD risk. Licastro et al postulated that the C- allele of the *IL-6* promoter region polymorphism increases the disease risk (Licastro et al., 2003; Capurso et al., 2004). Furthermore, Licastro et al found an increased level of IL-6 in the blood and brain in those subjects with the CC genotype. In contrast, other studies have suggested that it is the G allele (Shibata et al., 2002) or GG genotype (Pola et al., 2002) which associate with increased AD risk. to further to confuse the situation, Faltraco et al reported decreased disease risk in individuals with the C- allele (Faltraco et al., 2003). Finally, six other studies failed to find any association between this *IL-6* promoter region polymorphism and AD

(Bagli et al., 2000; Bhojak et al., 2000; Capurso et al., 2004; Depboylu et al., 2004; Nishimura et al., 2004; Zhang et al., 2004).

Papassotiropoulos et al found in 1999 for the first time an association between *IL-6* VNTR polymorphism and LOAD risk, claiming a protective effect for the VNTR C allele. The C allele carriers had delayed disease onset and their risk of AD was reduced (Papassotiropoulos et al., 1999b). This association has been confirmed later by Licastro et al (Licastro et al., 2003) who reported also increased blood IL-6 levels in the VNTR polymorphism DD genotype carriers (Licastro et al., 2003) whereas Shibata did not find any association with this polymorphism and AD (Shibata et al., 2002). The reasons for these highly conflicting results have been discussed. Capurso et al reviewed the German and Italian studies and concluded that geographical background might explain some of the discrepant findings on *IL-6* polymorphism in AD. They noted that in these European populations, the frequency of G allele increases as one moves towards the southern parts of the continent (Capurso et al., 2004).

Interestingly, the only published haplotype analysis showed a strong linkage disequilibrium between *IL-6*vntr and *IL-6*prom, demonstrating an association between the *IL-6*vntr C allele and *IL-6*prom C allele combination and reduced AD risk (Bagli et al., 2000).

2.4.3.4. Cholesterol 24 -hydroxylase (*CYP46*) polymorphism

The Cyp46 is encoded by the cholesterol 24-hydroxylase gene (*CYP46*) on chromosome 14q32.1 (Raffai and Weisgraber, 2003), in the neighbourhood of the *SERPIN A3* (also called *ACT*, encoding α1-antichymotrypsin which is a serine proteinase inhibitor, an acute phase protein). The encoding area of *CYP46* consists of 15 exons (Papassotiropoulos et al., 2003).

Some groups have reported significant associations with different *CYP46* polymorphisms (Kolsch et al., 2002; Papassotiropoulos et al., 2003; Borroni et al., 2004; Combarros et al., 2004; Johansson et al., 2004; Wang et al., 2004). By using an intronic SNP(Reverse rs754203 predicts a T>C substitution in intron two according to their report, but according to the genomic sequence of the *CYP46* gene, http://genome.ucsc.edu, designation of the forward rs754203 SNP is A>G) Papassotiropoulos et al reported an association between T- homozygosity and increased Aβ load, increased CSF levels of Aβ and phosphorylated tau protein (Papassotiropoulos et al., 2003). Furthermore, this *CYP46* polymorphism was significantly associated with increased LOAD risk in subjects having two T-alleles compared to C-allele carriers in two independent populations (OR

2.16). The presence of the *APOE* ε4 allele synergistically with the TT genotype increased the LOAD risk to an OR of 9.6 which was a two fold greater risk than that attributable to the ε4 allele alone (Papassotiropoulos et al., 2003). Previously Kölch et al (Kolsch et al., 2002) had found another SNP in intron 3 (rs4900442: C>T substitution) and shown that the C-allele at this locus is more frequent among LOAD patients. Moreover, CC genotype carriers had a significantly higher 24S-hydroxycholesterol/cholesterol ratio and total cholesterol of CSF compared to the T-carriers. the association of Intron 2 polymorphism with AD risk has been reported by later at least three independent groups (Borroni et al., 2004; Combarros et al., 2004; Wang et al., 2004). However, two of them reported that the increased AD risk was associated with the C-allele (Forward: G-allele). Borroni et al found a 2.8 fold AD risk for C-carriers; those subjects having both *CYP46* allele and *APOE* ε4 allele had an almost 18 fold increased risk to develop AD demonstrating their synergistic effect on AD risk while the risk attributable to the *APOE* ε4 allele was 4.1 fold (Borroni et al., 2004). Combarros et al claimed that intron 2 *CYP46* C/C (corrected G/G) genotype predisposed to the development of AD, with the association being independent of the *APOE* genotype (Combarros et al., 2004). Meanwhile, Johansson et al could not confirm the previous findings related to AD risk and intron 2 polymorphism, but noted that AA (CC in Papassotitopoulos study) homozygous LOAD patients carrying *APOE* ε4 allele had elevated Aβ concentrations in CSF compared to G-carriers in these stratified groups (Johansson et al., 2004). Their findings are in contrast with the findings reported by Papassotiropoulos (Papassotiropoulos et al., 2003; Johansson et al., 2004). Other workers have not found any association between intron 2 polymorphism and AD risk (Desai et al., 2002; Ingelsson et al., 2004). Furthermore, Ingelsson et al (Ingelsson et al., 2004) did not find increase in the brain levels of Aβ40, Aβ42 or in the levels of amyloid plaques or NFT.

2.4.3.5. *SERPINA3*

The gene encoding α-1-antichymotrypsin (*SERPINA3,* abbreviation *ACT* used also widely) is located ~5.5 cM 3' to the *CYP46* gene on chromosome 14q32.1. The gene harbours several polymorphic sites which are associated with either an increased or decreased risk to AD as well as the age of onset or plasma ACT concentration depending on the SNP (Kamboh et al., 1995; Meng et al., 2000; Wang et al., 2002).

2.4.3.6. The peroxisome proliferator-activated receptor γ (*PPARγ*) polymorphism

PPARγ gene is located on chromosome 3p25 (Beamer et al., 1997) including nine exons (A1, A2, B and exons1-6) spanning about 100 kb. Alternate transcription start sites and splicing generate four

PPARγ1, γ2, γ3 and γ4 mRNA isoforms (Meirhaeghe and Amouyel, 2004). *PPARγ*1, γ3 and γ4 encode the same protein product (Meirhaeghe and Amouyel, 2004). PPARγ1 is encoded by seven exons. The 5'-untranslated sequence of PPARγ1 is encoded by exons A1 and A2. Instead, the 5'-untranslated sequence of PPARγ2 and 28 amino acids containing a unique N- terminal, are encoded by exon B which is located between exons A1 and A2. The other six exons are shared at least by both PPARγ1 and PPARγ2 (Fajas et al., 1997) (**Figure 4**). PPARγ2 and PPARγ3 are predominantly expressed in adipose tissue whereas PPARγ1 is expressed in a wide variety of tissues. The expression pattern of PPARγ4 remains to be clarified.

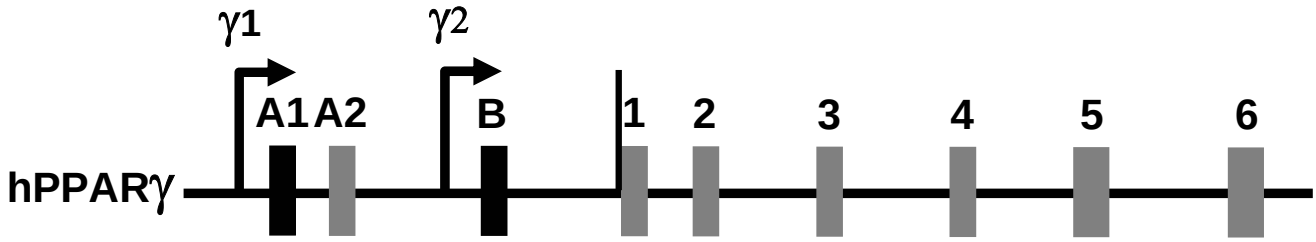

Figure 4. Organization of human *PPARγ* genes. Exons are represented by black and gray rectangles and introns by lines. Exons 1-6 are common for both *PPARγ1* and *PPARγ2*. Exons A1 and A2 of *PPARγ1* and Exon B (existing between A and B) of *PPARγ2* are represented separately (modified according to Fajas et al., 1997).

Rare mutations of *PPARγ* have been described in a few individuals, leading to a complex phenotype called "PPARγ ligand resistence (PLR) syndrome" with partial lipodystrophy, severe insulin resistance, type 2 diabetes, dyslipidemia, hypertension, and hepatic steatosis (Meirhaeghe and Amouyel, 2004). Common polymorphisms of *PPARγ* are quite frequent and they appear to have a significant impact on general health (Meirhaeghe and Amouyel, 2004). Most studies are related to the exon B specific Pro12Ala polymorphism (the c.12C>G variant predicting the substitution of Ala for Pro in position 12 of exon B) but some studies have also focussed on the silent single nucleotide polymorphism C478T (according to Nomenclatuere Guidelines c.478C>T) and C1431T (according to Nomenclatuere Guidelines c.1431C>T) in exon 6.

The *PPARγ* Pro12 Ala substitution was identified in 1997 by Yen and colleagues (Yen et al., 1997) after which numerous studies have been published on the impact of the Pro12Ala polymorphism and various diseases. The frequency of the rare Ala allele varies depending on ethnicity being lowest in Africans and Asians (1-3%) and highest (20%) in some Caucasian populations being 13% in the Finnish population (Valve et al., 1999). While the Ala12 allele has been associated most frequently with protection against type 2 diabetes and myocardial infarction, improved insulin sensitivity and lower BMI also conflicting results exist as well as reports from studies which did not find any association between Pro12Ala allele and these diseases (Deeb et al., 1998; Tontonoz et al., 1998; Valve et al., 1999; Meirhaeghe and Amouyel, 2004). Furthermore, in vitro studies have detected differences in binding affinity and transactivation of *PPARγ* Pro12Ala variants (Deeb et al., 1998)**.** The Ala isoform displayed a two-fold lower affinity for its PPAR response element (PPRE) and reduced ability to transactivate responsive promoters (Deeb et al., 1998)**.** The decreased activity of the *PPARγ* in 12Ala allele carriers has been confirmed in subsequent in vitro and in vivo studies (Koch et al., 1999; Masugi et al., 2000; Stumvoll et al., 2001; Schneider et al., 2002).

In 1998 Meirhaege et al published an association study of silent exon 6 1431T allele with leptin levels (Meirhaeghe et al., 1998). They reported that in obese T allele carriers, for a given leptin level, the BMI was lower than expected, assuming that the relationship between BMI and plasma leptin levels was related to the studied polymorphism (Meirhaeghe et al., 1998). Valve et al have shown that 478T homozygous obese women have greatly increased body fat-mass compared to C-carrier obese women (Valve et al., 1999). Since these polymorphisms are “silent”, the protein they code does not change. One has to consider whether these single nucleotide polymorphisms (SNP) reflect another real disease predisposing factor nearby or do they really have some impact on the functions, e.g., on the stability of the coded protein. Theoretically, *PPARγ* gene polymorphism might influence the expression, activity or ligand binding affinity of the PPARγ protein and consequently interfere with the PPARγ mediated reactions in brain and inthis way modify the AD risk (Kliewer and Willson, 1998; Combs et al., 2000). In the only reported study, no significant findings were noted between Pro12Ala *PPARγ* polymorphism and AD risk (Sauder et al., 2005). However, interestingly, that study revealed that AD patients carrying the Ala allele had higher plasma 24S- hydroxycholesterol/ cholesterol ratios compared to homozygote Pro- allele carriers (Sauder et al., 2005). The influence of the other variations in the *PPARγ* gene on the AD risk, disease onset or course remains to be clarified.

3. AIMS OF THE STUDY

This study is part of an ongoing project intending to search for novel risk genes for AD in the relatively homogenous Finnish population. Despite the well established effects of the *APP, PSEN1*, *PSEN2* and *APOE* genes in EOAD and LOAD, it is evident that additional susceptibility genes are involved in the underlying disease process. Based on the results from pathophysiological studies, four biological candidate susceptibility genes were selected for association studies in order to identify novel gene loci involved in AD, especially with LOAD. Furthermore the survival of the AD patients compared to controls and the effect of *APOE* and gender to survival in AD patients was examined

The specific aims of the study were:

1) To determine whether the survival is influenced by the ApoE ε4 phenotype, the presence of AD or gender in the aged Finnish population. Additionally, to investigate whether there are any gender and ApoE ε4 phenotype related differences in the survival in LOAD patients **(Study I).**

2) To investigate the previously described *IL-6* association with LOAD risk in our population based sample of aged Eastern Finnish subjects **(Study II).**

3) To test the hypothesis whether the *CYP46* gene associates with AD in age matched late-onset AD subjects and controls. Furthermore, due to the chromosomal proximity of *CYP46* and *SERPINA3* genes, the SERPINA3 gene was also examined in relation to the AD risk **(Study III)**.

4) To examine whether the PPARγ is involved in the risk or age of onset of LOAD in Finnish population **(Study IV).**

4. SUBJECTS AND METHODS

4.1. Subjects

Studies I, II and IV were carried out in a population based sample of aged subjects (living in Kuopio, Finland) in the Departments of Neurology and Medicine, University of Kuopio and Kuopio University Hospital. Study III was carried out in a sample of aged Eastern Finnish patients and age matced controls and conducted by the Department of Neurology in University of Kuopio and Kuopio University Hospital (study III). All of these experiments and analyses used for population based studies I, II and IV were carried out between 1986 and 2003. Study III was performed in 2004, comparing cases and controls and is part of the larger genetic study. The studies were approved by the Ethics Committee of the University of Kuopio and Kuopio University Hospital. All participants provided informed consent. In those cases when the patient was incompetent, the caregiver signed the consent form.

The study included four different samples (three of them based on the same original study population) of subjects. More women than men participated in these studies but the frequency of females was equal in both AD and control groups (**Table 6**). Generally the AD patients were slightly older and in studies I, II and IV less educated and had lower Mini-Mental State examination (MMSE) scores compared to the controls (all $P \leq 0.02$). The ApoE ε4 (in study IV *APOE* ε4) allele was significantly more common in AD patients ($p<0.001$). Demographic data concerning the AD patients and control subjects used in these studies are indicated in **Table 6.**

Table 6. Demographic data of the AD patients and controls.

	Study I		Study II		Study III		Study IV	
	AD	**CO**	**AD**	**CO**	**AD**	**CO**	**AD**	**CO**
Number of subjects	48	1251	65	542	422	469	125	461
Gender M/F	16/32	455/796	18/47	194/348	293/129	284/185	32/93	170/291
Age years (range)	70±3* (65-74)	69±3 (65-74)	78 ± 3 * (72-82)	76±3 (72-83)	72±7* (43-90)	70±5 (60-87)	77±3 (72-82)	76±3* (72-83)
Education years (range)	5±2* (0-10)	7±4 (0-21)	5±2 * (0-12)	7±4 (0-21)	-	-	6±3 (0-20)	7±4 (0-21)
ApoE4+ N (percentage)	27** (56 %)	393 (32 %)	37** (56 %)	151[1] (28 %)	311** (74 %)	129 (28 %)	69** (55%)	113[1] (25%)
Gluc mmol/L	-	-	6.8±2.6*	6.1±1.7	-		6.5±2.2*	6.1±1.8
Ins mmol/L	-	-	19.6±13.2*	15.3±8.1	-		18.4±11.8*	15.4±8.0-
TC mmol/L	-	-	6.4±1.1	6.6±1.3	-		6.5±1.2	6.6±1.3
HDL-C mmol/L	-	-	1.3±0.3	1.3±0.3	-		1.3±0.3	1.3±0.3
LDL-C mmol/L	-		4.2±1.1	4.5±1.1	-		4.3±1.1	4.5±1.2
Trigly mmol/L	-	-	1.9±1.0	1.8±0.9	-		2.0±1.1	1.8±0.8
SBP mmHg	-	-	162±25*	155±23	-		160±23*	154±23
DBP mmHg	-	-	81±10	81±10			81±10	82±10

The results are given as number of subjects (N) or as means ± standard deviation. Study I Age at the first clinical examination. Study II and IV age at the last cognitive examination in 1994. Study III Age at the cognitive screening for controls and age of onset for AD subjects. AD = Alzheimer's disease patients; CO = controls; M/F= male/ female; ApoE4+= number and (percentage) of apolipoprotein E ε4 allele carriers, one or two alleles; Gluc= glucose;Ins= insulin; TC= total cholesterol; HDL-C= high – density lipoprotein cholesterol; LDL-C= low –density lipoprotein cholesterol; Trigly= triglycerides; SBP = systolic blood pressure; DBP= diastolic blood pressure; All the laboratory values measured in 1986-88 at first study visit after 12 hours of fasting.[1] ApoE status for three controls were missing in studies II and IV.* $P \leq 0.02$ in univariate tests (t-test and Mann Whitney), AD patients compared to controls. ** $P < 0.001$ in Pearson χ^2 test, AD patient compared to controls.

Studies I, II and IV

Table 7. Crucial interventions and formation of the study population during follow-up.

Year	Intervention	Number of invited or evaluated subjects	AD subjects	Controls
1986	Invitation letter	1910	-	-
1986-88	First clinical examination, laboratory tests	1299	-	-
1990-91	First neurological examination, DNA sample	980 examined and 319 evaluated using medical records	48[1]	1251[2]
1993-94	Third neurological examination	632	65 [3]	542[4]
1995	Checking of survival	1299	48[1]	1251[2]
1997	Checking of survival	48	48[1]	
2003	Checking dementia diagnoses	1299	125[3]	461[4]

[1]Number of identified AD subjects in 1990-91 and used as cases in the study I. [2]Number of controls for the study I. [3]Number of identified AD subjects since 1986 who gave also a DNA sample. [4]Number of eligible non-demented controls with DNA sample for the study II and IV.

The subjects in studies I, II and IV had originally participated in a larger follow-up study launched in 1986. At that time, a cohort of 1910 subjects, aged 65-74 years, was randomly drawn from the register of eastern Finnish city of Kuopio and sent a letter inviting them to participate in the study. A total of 1299 of them responded to the postal questionnaire and participated in the first clinical evaluation. They represented totally 25 % of all subjects in that age group living in this area. First the evaluation of risk factors and prevalence of cardiovascular disorders was carried out in 1986-1988 in the Department of Medicine, University of Kuopio (Mykkanen et al., 1990; Kuusisto et al., 1997). The values (blood pressure, fasting glucose and insulin, total cholesterol) measured at that visit were used for the adjustment in the logistic regression analyses of the current studies. The next follow-up took place average 3.5 years after the original examination including also the blood

sample for DNA analyses and the screening of cognitive performance for detecting dementia and memory disorders (demographic interview, clinical examination, six neuropsychological tests including MMSE). From the 1192 subjects still living and eligible (then 69 to 78 years old) ultimately 980 (82 % of the original sample) participated in this first dementia screening (Koivisto et al., 1992; Helkala et al., 1995; Kuusisto et al., 1997). The second similar cognitive follow-up evaluation was carried out in 1992 and the third in 1993-94 (Helkala et al., 1995) approximately seven years after the first clinical examination and three to four years after the first cognitive screening. A total of 632 (71 %, then 72-82 years old) of the 890 eligible subjects were completely evaluated at this last follow up evaluation by 1994 (Helkala et al., 1995). The clinical and cognitive condition of all of the study subjects was followed up from available medical records and the settled dementia diagnoses from the National Research and Development Centre for Welfare and Health statistics up to 2003. The survival of the whole study group was followed up to 1995, and that of the subjects with AD in 1997 and 2003. In addition, the medical records were evaluated from a total of 319 study subjects, who did not participate in the first cognitive screening phase. The crucial interventions and formation of the study population during follow up are shown also in **table 7**.

The subjects with suspected memory disturbances in the screening evaluations underwent more detailed differential diagnostic examinations including several neuropsychological tests, clinical neurological, laboratory, radiological and neurophysiological examinations (Koivisto et al., 1992; Helkala et al., 1995). Dementia was diagnosed according to DSM-III-R criteria (APA, 1987) and AD according to the NINCDS-ADRDA criteria (McKhann et al., 1984). Controls neither showed any deterioration in repeated cognitive tests nor developed any kind of cognitive disorder in later life. The subjects with types of cognitive disturbances other than AD, missing genetic information or insufficiently studied cognitive performance were excluded from the study.

The cognitive evaluation of the study group as well as the diagnosis of dementia and follow up of the subjects with dementia were carried out by the Brain Research Unit (former Memory Research Clinic), Department of Neurology and Neuroscience, University of Kuopio and in the Department of Neurology, Kuopio University Hospital (Koivisto et al., 1992; Helkala et al., 1995).

For the study I survival analyses, all of the subjects originally participated in the first clinical examination were analysed. Grouping to the AD patients and controls was made according to first cognitive evaluation. Forty eight subjects were diagnosed to have AD at that time. Further survival analyses and evaluation of daily ability were examined in that group (48 AD subjects).

In study II 65 AD subjects were identified by 1994 and for the study IV a total of 125 AD subjects by 2003. The probable AD diagnosis was made as described above and confirmed after follow-up of the clinical course of the disease. All the control subjects had participated in the last cognitive screening without any signs of cognitive deterioration. The subjects who did not participate in the last cognitive evaluation or did not undergo the more detailed diagnostic examinations in the case of suspected memory deficit, subjects with types of dementia other than AD, subjects with a suspicion of some disease interfering with the cognitive performance (e.g. depressed subjects) or the subjects with missing the crucial genetic information were excluded from the analyses of these studies. Furthermore, for study IV, we checked that control subjects had not been iued with any dementia diagnosis by 2003.

Study III

In study III, all the subjects were examined in Department of Neurology, Kuopio University Hospital and University of Kuopio. To confirm the homogeneity of the study group, the community based voluntary, unrelated control subjects were derived from the same restricted area of northern Savo as the AD patients. That area was settled in the late 16^{th} and early 17^{th} century (Soininen, 1981; Pirinen, 1982). All the participants were interviewed for demographic information, medical history, current medication, living habits and subjective assessment about memory disturbances or depression during the past year also for this study. Family data of dementia was obtained from the medical records, interviewing the study subject or by interviewing the next of kin (in the case of individuals with dementia). In addition to the interview, all the controls underwent at least one to three cognitive screening tests (Wechsler, 1945; Benton, 1974; Folstein et al., 1975).The interview and cognitive screening tests were carried out by trained personnel (nurse, psychologist or doctor) during a single visit. Standardization of the interview and tests was accomplished with the help of an instruction manual. The blood sample for DNA genotyping was taken at the same visit.

Controls exhibited no signs of dementia during the interview and neuropsychological testing at the time of the cognitive evaluation.

All AD subjects underwent a comprehensive clinical evaluation (including clinical and neurological examination, neuropsychological testing, imaging study of the brain and laboratory tests) based on which the clinical diagnosis of possible or probable AD was made according to the NINCDS-ADRDA criteria (McKhann et al., 1984; APA, 1987).

These 891 study subjects who participated in study III included 354 LOAD cases (84% of all AD cases) and 68 EOAD cases (16% of all AD cases with onset age 65 years or below). Sixty three of EOAD subjects were screened for known AD mutations in *APP, PSEN-1* or *PSEN-2* genes. Although no such mutations were found, it is still possible that some of these patients may carry rare variants in these genes. Approximately 40% of AD patients displayed a positive family history of AD (at least another first-degree relative with AD type dementia), but there was inconclusive evidence of autosomal dominant transmission. If no reliable family history was available, patients were termed as sporadic cases.

4.2. Methods

4.2.1. Clinical and laboratory examinations

The clinical examination was similar to the comprehensive neurological examination regularly performed in clinical practice, including medical history, physical examination directed especially towards cardio- and cerebrovascular problems, detailed neurological examination and assessment of mental state, especially depression and cognitive functions.

Blood pressure was measured in the supine position with a mercury sphygmomanometer after 5 minutes rest. Two readings were taken with a 1.5 minutes break. The latter value was used in the statistical analyses.

Blood samples were taken in the morning after 12 hours fasting. Venous blood samples for glucose and insulin determinations were taken into chilled tubes. Plasma glucose was determined by the glucose oxidase method (Glucose Auto & Stat HGA-1120 analyzer, Daiichi, Kyoto, Japan). Plasma insulin was determined from samples stored at -70ºC by a commercial double-antibody solid-phase radioimmunoassay (Phadeseph Insulin RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden) (Hales and Randle, 1963). Commercial enzymatic methods and control sera were used in the determination and standardizing the measurements of total cholesterol (Monotest, Boehringer Mannheim, Mannheim, Germany; Seronorm and Seronorm Lipid, Nycomed, Oslo, Norway) (Siedel et al., 1983).

4.2.2. Neuropsychological tests

Neuropsychological screening tests were carried out by psychologist during a single visit. Standardization of the tests was accomplished with the help of an instructional manual.

Neuropsychological evaluation for studies I, II and IV included six neuropsychological tests (Reitain, 1958; Borkowski et al., 1967; Buschke and Fuld, 1974; Folstein et al., 1975; Russel, 1975; Butters et al., 1987) which were repeated at the follow-up visits.The MMSE total score adjusted for education (cut off scores were 23, 24 or 25) was used for clinical phase examinations. Also, those subjects scoring one or more standard deviations below the mean on at least three of the above specified subitems of four other screening tests were considered to be potentially demented.

In Study III, most subjects underwent three cognitive screening tests (Wechsler, 1945; Benton, 1974; Folstein et al., 1975) with cut off points in MMSE >25, in Benton visual Retention test >6 and in Paired Associate learning subtest >13 without history of memory impairment in the interview. These tests are described in detail previously (Hanninen et al., 1997). Fifty one controls were screened only by MMSE with a higher cut off point (27 points or more, no signs of impaired delayed recall of words) and interview (no subjective memory complaints or signs of memory impairment during the interview) (Hiltunen et al., 2001).

MMSE includes a selection of short items testing different aspects of cognitive function like orientation, repetition and recall of words, attention, language and constructional ability. The score used was the sum of the scores of all items (Folstein et al., 1975).

In the Buschke Selective Reminding Test (BSRT) the subject has to recall as many as possible of the ten words that have just been read out by the examiner. The scores obtained are the total number of words and the words which were recalled in consecutive trials without being repeated (i.e. words in long term memory) (Buschke and Fuld, 1974).

Visual Reproduction Test (VRT) includes three subtrials in which the person must reproduce increasingly complex geometric, nonrepresentative features from memory immediately after seeing them and after 30 minutes of unrelated testing. In order to assess any visuoperceptual dysfunction, the subject is asked to copy the figures after the recall. The scores are given for the number of components present in the original drawings. The maximum score in each of three subitems is 21 (Russel, 1975).

Verbal Fluency tests (VFTs) includes two parts. First the subjects are given 60 seconds to produce as many words as possible beginning with each of the letters P, A and S (VFT-letters). For the VFT category, the subject is asked to generate the names of as many animal names as possible within 60 seconds. The performance was scored by counting the total number of correct words produced for each letter or category. Study I analysed the results from VFT-L (Borkowski et al., 1967; Butters et al., 1987).

Study I used Trail Making Test (TMT) part A where the subjects must draw a line to connect consecutively numbered circles. The scores are the time required to complete the trial. The time limit is 150 seconds (Reitain, 1958).

In the Benton Visual Retention test, form C, administration A (BVRT), the subject copies from memory ten different figures after their presentation for ten seconds. The scores are awarded for each correctly reproduced figure (Benton, 1974).

In Paired Associate Learning subtest (PAL) of the Wechsler memory Scale (WMS), the examiner reads ten pairs of words. The subject is afterwards required to recall the latter word of each pair when the first one is presented as a cue. This procedure is repeated three times. The total score was calculated according to the specific manual for this test (Wechsler, 1945).

4.2.3. Determination of apolipoprotein E phenotype (studies I, II and IV)

The ApoE phenotype was determined from venous serum samples with isoelectric focusing of heparin/Mg^{2+} -precipitated lipoproteins and immunoblotting techniques using commercial antibodies as described in detail (Ehnholm et al., 1986; Menzel and Utermann, 1986).

4.2.4. Gene analyses

For studies II-IV, genomic DNA was extracted from peripheral blood leukocytes using the standard phenol/chloroform extraction method (Vandenplas et al., 1984).

Table 8. Primers used in the PCR and SNaPshot analyses.

Study	Gene	Target Location	Used Primers
Study II:	*IL-6*		
		Promoter	F: 5'-TGACTTCAGCTTTACTCTTTGT-3'
			r: 5'-CTGATTGGAAACCTTATTAAG-3'
Study III:	*CYP46*		
		5' to exon 6	F: 5'-ATTGCTCAAGGACAGGCAGT-3'
			R: 5'-GCTGTTCCCTTGCTCTTGTC-3'
		SNaPshot	5'-T_{40}GGCCACAGTGACCAACAGAG-3
		Intron 2	F: 5'-AAT GCA TGC TAC CAA AAG AG-3'
			R: 5'-AAT CAT TTG ATT CCC AGG AC-3'
		SnaPshot	5'-T_{31}CAACAGGGCAGAGCCTTGCCCCC-3'
	SERPINA3		
		Intronic dbSNP:4934	F: 5'-CAG AGT TGA GAA TGG AGA-3'
			R: 5'-TTC TCC TGG GTC AGA TTC-3'
		SnaPshot	5'-T_{38}AGAGAATGTTACCTCTCCTG-3'
	APOE		F: 5'-GCACGGCTGTCCAAGGAGCTGCAGGC-3'
			R. 5'-GGCGCTCGCGGATGGCGCTGAG-3'
Study IV:			
	PPARγ	Exon B	F: 5'-GACAAAATATCAGTGTGAATTACAGC-3'
			R: 5'-CCCAATAGCCGTATCTGGAAGG-3'
		Exon 6	F: 5'-CCGCCCAGGTTTGCTGAATGTG-3'
			R: 5'-CAGTGGCTGAGGACTCTCTG-3'

***IL-6* (study II) and *PPARγ* (study IV)**

The *PPARγ* exon B and exon 6 as well as the *IL-6* promoter were analyzed by PCR with the primers shown in **Table 8.** The *PPARγ* variants and the IL-6 C-174G polymorphism were detected by single strand conformation polymorphism analysis. The screening of the studied polymorphisms in the *PPARγ* and *IL-6* has been described previously (Laakso et al., 1994; Valve et al., 1999).

***CYP46, SERPINA3* and *APOE* (Study III)**

For *CYP 46* and *SERPINA3* gene analyses the studied SNPs were validated from the National Center for Biotechnology Information (NCBI) data library. Two intronic SNPs investigated in *CYP46* gene were dbSNP:754203 T-to-C substitution in intron 2, and >14kb distant in an intronic dbSNP:2146238 G-to-T substitution 5' to exon 6. In the *SERPINA3* gene, an intronic dbSNP:4934 A-to-G substitution was determined. Assays for the SNPs were genotyped in one multiplex polymerase chain reaction (PCR) to perform a single SNaPshot reaction. Forward and reverse amplification primers for each SNP were used as presented in **Table 8**. A product of each PCR-amplification was used as a template in an ABI PRISM® SNaPshot™ Multiplex assay (Applied Biosystems) and specific primers **(Table 8**) were used in a SNaPshot reaction with the length of tail described: SNaPshot multiplex reactions were performed according to the manufacturer's instructions and samples were analysed with ABI 3100 Genetic Analyzer and GeneMapper software (Applied Biosystems).

For recognition of the *APOE* allele forms ε2, ε3 and ε4, PCR and H*ha*I (New England Biolabs) digestion of the PCR products were carried out with primers **(Table 8)** and conditions as previously published (Tsukamoto et al., 1993). The heterozygote *APOE* ε2/4 sample was used as control in each run.

These gene tests were analyzed by two researchers independently.

4.2.5. Statistical analyses

The distributions of genotypes in studies II-IV, both in all and in stratified groups, were found to be in Hardy-Weinberg equilibrium (Genepop web version of 3.4). Statistical significance was established at $P<0.05$.

Study I

The study population was divided first into two groups identified on the basis of the examinations in 1991: subjects with AD and control subjects and subsequently for the presence of ApoE ε4 phenotype (ApoE ε4 carriers/ ApoE ε4 non-carriers) and gender.

Data analysis was conducted with the SPSS /PC+ programs (6.1.). A multivariate Cox regression model (Cox, 1972) was used to investigate the association of risk factors with allcause mortality. Otherwise the $\chi 2$ -test or analysis of t-test was used in the assessment of differences between the studied groups.

Study II-IV

In the risk analyses, AD patients were compared to non-demented control subjects in the total study group and also stratified according to the presence of the ApoE ε4 phenotype (genotype in study III) or gender (study IV). In study IV, to estimate the difference in the age of AD onset, the AD patients were subdivided according to their genotypes. The relative risk of AD or difference in age of onset was estimated using two-tailed Pearson χ^2, Fischer exact or Mann Whitney tests, and adjusted logistic regression analysis (SPSS program, version 10.1) Adjustment was made with ApoE, age, gender, education and cardiovascular risk factors (systolic blood pressure, serum total cholesterol, fasting blood glucose) previously described to be in association with the risk of AD. Power determinations were performed using Query Advisor Release 3.0 software (http://www.statsol.ie/nquery/review3.htm). Estimation of haplotype frequencies for study III was performed with Arlequin version 2.0 (http://lgp.unige.ch/arlequin). Haplotype frequencies were compared for case and control subjects using the RxC-program employing the metropolis algorithm to obtain unbiased estimates for exact p-values with standard errors.

5. RESULTS

For all these studies ApoE ε4 (or *APOE* ε4 for study III) allele and allele carrier frequencies were analyzed and compared between AD patients and control subjects. In every study presented in this thesis, as expected, the proportion of ApoE ε4 allele and this allele carriers were significantly higher among AD patients ($p<0.001$) compared to non-demented subjects **(Table 6)**. The numbers of ApoE ε4 carriers and demographics of the study populations are presented in the "Subjects" part of this thesis **in Table 6**.

5.1. Survival of AD patients in relation to ApoE phenotype (Study I)

In all 4% of the total study group were diagnosed as having AD at the time of the first cognitive screening in 1990-91. Eight of them had already an AD diagnosis with early manifestations of the disease and the other 40 were diagnosed at the study visit. The distributions of the genders and ApoE ε 4 phenotype carriers are shown in **tables 6 and 9**.

5.1.1. Survival of the whole study population

During the seven years' follow-up time of the whole study group, 12 (25.0%) of 48 AD patients and 280 (22.4%) of 1251 controls died. This difference in allcause mortality as well as in stroke or cardiovascular mortality was not statistically significant between the groups **(Table 9).** AD patients were slighty older than controls (70 vs. 69 years) but this did not influence mortality. With respect to the analysed risk factors, the ApoE phenotype (ApoE ε4 non-carriers vs. ApoE ε4 carriers) did not influence survival, nor did the presence of AD, whereas the risk of mortality was significantly higher among men than among women (131/ 30% of 471 men and 151/ 18% of 828 women died, $p<0.001$) **(Table 10)**. There was no significant difference in age between men and women at baseline (mean age of both groups was 69 years).

Table 9. Characteristics and mortality of study subjects after seven years of follow-up (Study I).

	All	Subjects with Alzheimer's disease	Controls
Number of subjects	1299	48	1251
Men/Women	471/828	16/32	455/796
ApoE4+	420 (30.9)	27 (56.3)	393 (31.5)*
All Cause mortality	292 (22.5)	12 (25.0)	280 (22.4)
Stroke mortality	35 (2.7)	3 (6.3)	32 (2.6)
CHD mortality	119 (9.2)	3 (6.3)	116 (9.3)

The $\chi 2$ -test *$p<0.001$; Values are numbers (percentages); ApoE+= one or two ApoE ε4 allele; CHD= coronary heart disease

Table 10. Univariate Cox regression analysis on risk factors (Sex, ApoE ε4, AD) of mortality during seven years' follow-up in aged subjects (65-74 years in baseline).

	HR	95% CI	p value
Sex (Men/ Women)	0.56	0.44 - 0.70	<0.001
ApoE4 (0/1)	0.95	0.74 - 1.22	0.692
Alzheimer's disease (no/yes)	1.18	0.61 - 1.93	0.784

HR= Hazard Ratio; 95% CI= 95 % ConfidenceI; ApoE 0/1= Subjects not carrying ApoE ε4 allele (marked as 0) compared to subjects carrying at least one ApoE ε4 allele (marked as 1).

5.1.2. Survival of the AD patients in relation to gender and ApoE phenotype

The subjects with AD (N=48) were followed up for a further two years. Twenty six (54%) of them were still alive and 22 (46%) had died by 1997. Of those still alive, 11 had severe, six moderate, six mild and two still exhibited early dementia, data from one of those 26 still alive was missing. Fifteen subjects with AD were living at home (eight of them alone and six without any major need of help, data on one was missing) and 11 were in a nursing home or hospital. At the cognitive screening visit in 1990-91, the survivors (AD patients living in 1997) and the non-survivors (died AD patients by 1997) had the same age (mean age, range in years: 70±2 (65-74) in survivors vs. 71±3 (65-74) in non-survivors) and duration of dementia from thr onset of symptoms (mean duration, range in years: 2.9±4.6 (0-15) in survivors vs. 3.6±3.7 (0-12) in non-survivors), but in baseline cognitive testing, those patients deceased by 1997 had had lower scores in MMSE (mean MMSE points, range: 22±3 (17-28) in survivors vs. 19±5 (9-26) in non-survivors, $p=0.01$) and the verbal memory in BSRT (mean points, range: 21±5 (8-30) in survivors vs. 17±4 (7-24) in non-survivors, $p<0.01$), but not in Visual Reproduction Test (VRT) assessing visual memory, TMT or Verbal Fluency Test.

Of all AD patients, 52% of subjects with the ApoE ε4 phenotype and 38% of subjects without ApoE ε4 had died by 1997 **(Table 11)**. At cognitive screening the ApoE subgroups did not differ in age or performance in cognitive tests. Although mortality was higher in Apo ε4 carrier AD subjects than in ApoE ε4 non-carrier patients, the difference was not significant ($p = 0.31$). However, mortality in the ApoE ε4 non-carriers males (80% had died) was significantly higher than that of the women (20% had died)**(Table 11).**

Table 11. Mortality of the patients with Alzheimer's disease according to the ApoE phenotype and gender in 1997.

	ApoE4-			ApoE4+		
	All	**Men***	**Women**	**All**	**Men**	**Women**
Survivors	13	1	12	13	5	8
Non-survivors	8	5	3	14	5	9
Total number of subjects	21	6	15	27	10	17

ApoE4- = subjects with apolipoprotein ε 2/2, 2/3 or 3/3 phenotype; ApoE4+ = subjects with apolipoprotein ε 2/4, 3/4 or 4/4 phenotype. $\chi 2$ -test *$p<0.01$ between genders in ApoE4- group;Values are numbers of subjects.

5.2. Interleukin-6 polymorphism and risk of AD (study II)

The frequency of finding the *IL-6* -174 G allele was significantly higher (p= 0.03) among ApoE ε4 non-carrier AD subjects, but not in the total study group or the ApoE ε4 carriers **(Table 12**). The CG and GG genotypes in ApoE ε4 non-carrier AD patients were also more common than in ApoE ε4 non-carrier controls but not statisticaly significantly **(Table 12)**. When we combined the 174-CG or GG genotypes, we found a borderline risk for AD of the G allele carriers compared to the subjects with CC genotype in a univariate test (OR 3.4, 95%CI 1.0-11.4; p= 0.04) but after adjustment, this finding did not reach significance (OR 3.4, 95%CI 1.0-12.0; p= 0.06). In the total study group or the ApoE ε4 carriers, no significant differences were observed for allelic or genotype distributions of IL-6 -174 G/C promoter polymorphism (**Table 12**).

No significant additive influence on the risk of AD was found between ApoE ε4 and *IL-6* -174 G allele carriers (**Table 13**).

Table 12. *IL-6 -174 G/C* promoter polymorphism genotype and allele distributions in AD cases and controls.

Group		n	Genotypes				Alleles		
			CC	***CG***	***GG***	***OR(95%CI); P*** [1]	***C***	***G***	***OR(95%CI); P*** [2]
All	AD	65	0.23	0.49	0.28		0.48	0.52	
	CO	542	0.27	0.48	0.25	1.3 (0.8-1.9); 0.27	0.51	0.49	1.2 (0.8-1.7); 0.44
ApoE4-	AD	29	0.10	0.52	0.38		0.36	0.64	
	CO	388	0.28	0.46	0.26	1.7 (0.9-2.9); 0.08	0.51	0.49	**1.8 (1.1-3.2); 0.03**
ApoE4+	AD	36	0.33	0.47	0.20		0.57	0.43	
	CO	151	0.26	0.53	0.21	0.9 (0.5-1.7); 0.68	0.52	0.48	0.8 (0.5-1.4); 0.48

n= Number of Alzheimer's disease patients (AD) and controls (CO);

ApoE4- = Apolipoprotein E ε 2/2, 2/3 or 3/3 phenotype;ApoE4+ = Apolipoprotein E ε 2/4, 3/4 or 4/4 phenotype; The ApoE status was missing in three control subjects; Only uncorrected *P*-value used.

[1] Adjusted binary logistic regression analysis was performed in three groups (all, ApoE4- and apoE4+). ApoE**,** age, sex, education and cardiovascular risk factors (blood pressure, total cholesterol and fasting insulin values) previously associated with AD as covariates were used in the regression model.

[2] Pearson χ^2 test used to analyse allelic data.

Table 13. Adjusted odds ratios for AD with ApoE ε4 non-carriers and IL-6 CC genotypes as a reference. Total study group (n=604).

ApoE4	IL-6 G	AD	Controls	OR(95% CI)*	*P*
-	-	3	109	1.0	
-	+	26	279	3.4(1.0-12.0)	**0.06**
+	-	12	39	14.8(2.9-74.8)	**< 0.01**
+	+	24	112	8.5(2.3-31.2)	**< 0.01**
		65	539		

AD=Alzheimer's disease; ApoE4 (ApoE 2/2, 2/3, 3/3 phenotypes= -; ApoE 2/4, 3/4, 4/4 phenotypes= +); IL-6 -174 G/C (174CC genotype= -; 174CG/GG genotypes= +);
ORs with 95% Confidence Interval (CI) for AD were calculated using binary logistic regression models and they were adjusted for age ,gender, education, systolic blood pressure, fasting total cholesterol and insulin; Statistical significance (*P*) was established at <0.05.

5.3. Cholesterol 24 -hydroxylase (*CYP46*) polymorphism and risk of AD (Study III)

The distribution of APOE ε2, ε3 and ε4 alleles in the AD and controls was 0.02/0.51/0.47 and 0.04/0.80/0.16, respectively. The APOE ε4 allele was significantly associated with AD (OR 4.8) as described in **table 6** and age at onset was approximately three years earlier for *APOE* ε4 carriers vs. ε4 non-carriers (71 vs. 74 years, p<0.001). In chi-square and logistic regression tests, the subjects were also split on the basis of *APOE* genotype into the *APOE ε4* carriers and non-carriers (**Table 14 and 15**).

No significant differences were observed for allelic or genotype frequencies of dbSNP: 2146238 between AD patients and controls. However, a strong pairwise LD (D'=0.99; p<0.001) between dbSNP:754203 and 2146238 was found in all samples (1782 alleles) or in AD case and control groups, separately.

The results show that a significant association between dbSNP:754203 CC genotype and AD was observed in all subjects and in subjects carrying *APOE ε4* (**Table 14**). The age and sex adjusted odds ratio for the risk of AD in the carriers of the dbSNP:754203 CC genotype was 2.13 (95% CI 1.25 to 3.62; p=0.005) and 3.58 (95% CI 1.21 to 10.56; p=0.021) compared with the CT/TT genotypes for all and *APOE ε4* carrier group, respectively. **Table 15** describes the combined effects

between the dbSNP:754203 CC and *APOE ε4* genotypes. We found an increased risk of AD (OR 3.34) in the subjects with both the dbSNP:754203 CC genotype and *APOE ε4* allele, taking as reference subjects those who had neither of these genotypes. Thus, we determined whether our results support an independent or synergistic model of these two genes. Our finding of the predicted OR 21.4 in those having both risk genes separately in the binary regression model (data not shown) compared to the OR in the subjects with the combined risk effect (3.34) suggested strongly to an independent model of action for these two genes.

For the meta-analysis of the dbSNP:754203, fifteen case-control studies, including subjects from this study, were pooled to achieve a total of 3307 cases and 3328 controls (**table 16**). Fixed effects meta-analysis was performed since the likelihood ratio test did not provide any significant evidence of between-study heterogeneity (χ^2 = 20.49, df =14; p=0.12). In the pooled analysis of different ethnic populations, a pooled effect of CC-genotype frequencies in AD cases was very similar to those found in controls (OR 1.16; 95% CI 0.97 to 1.38). We also estimated a pooled effect of C-allele frequencies in all populations with random effects meta-analysis, finding OR 0.95; 95% CI 0.83 to 1.10.

In haplotype analysis, haplotypes for dbSNP:754203 and 2146238 with the following frequencies CG, 31% in AD vs. 26% in controls; TG, 53% vs. 56%; TT, 16% vs. 18% were included into statistical tests. Haplotype analysis with 844 alleles in all AD cases and 938 alleles in all controls showed that CG haplotype was more common in AD cases than in controls (p<0.03).

The frequency of the *SERPINA3* dbSNP:4934 for A-allele was 54.8% in AD cases and 56% in controls. No association between *SERPINA3* and AD was found. In addition, non-significant LD (D'=0.001; p=0.97) between *CYP46* and *SERPINA3* markers was observed.

Table 14 *CYP46* genotype and allele distributions for dbSNP:754203

Group		n	Genotypes, n (%)			P[a]	Alleles, n (%)		P[b]
			CC	CT	TT		C	T	
All	**AD**	422	43 (10)	172 (41)	207 (49)	0.015 (0.030) *	258 (31)	586 (69)	0.040 (0.080) *
	CO	469	24 (5)	196 (42)	249 (53)		244 (26)	694 (74)	
APOE4-	**AD**	111	12 (11)	43 (39)	56 (50)	0.203	67 (30)	155 (70)	0.391
	CO	340	20 (6)	145 (43)	175 (51)		185 (27)	495 (73)	
APOE4+	**AD**	311	31 (10)	129 (41)	151 (49)	0.032 (0.062) *	191 (31)	431 (69)	0.019 (0.038) *
	CO	129	4 (3)	51 (40)	74 (57)		59 (23)	199 (77)	

[a]Fisher's exact test and [b]unbiased estimate of exact p value. *To compensate for multiple testing, we used the Bonferroni method and corrected for two tests (APOE4 status). APOE4- = Apolipoprotein E ε22/23/33 genotypes and APOE+ = Apolipoprotein E ε 24/34/44 genotypes. n= number of subjects. AD= Alzheimer's disease; CO=controls.

Table 15 Age and sex adjusted odds ratios for AD with *APOE ε4* non-carrier and *CYP46* dbSNP:754203 CT/TT genotypes as a reference

APOE4	*CYP46* CC	AD, n (%)	CO, n (%)	OR (95% CI) [a]	P[a]
-	-	99 (24)	320 (68)	1 (reference)	
-	+	12 (3)	20 (4)	2.03 (0.92-4.47)	0.81
+	-	280 (66)	125 (27)	2.89 (2.46-3.41)	<0.001
+	+	31 (7)	4 (1)	3.34 (2.26-5.03)	<0.001
		422	469		

[a]Odds ratios (ORs) were calculated using binary logistic regression model. *APOE4* (24/34/44 genotypes= +; 22/23/33 genotypes= -) and *CYP46* CC (CC genotype= +; CT/TT genotypes= -).

Table16. Meta-analysis of the dbSNP:754203 CC-genotype carriers.

Study (Population)	**AD (n/N)**	**Control (n/N)**	**OR (fixed) 95% CI**	**Weight %**
[15]Desai (USA)	45/434	41/401	1.02 (0.65-1.59)	16.33
[15]Desai (African American; USA)	1/54	2/61	0.56 (0.05-6.32)	0.79
[16]Ingelsson (USA) [#]	19/178	5/105	2.39 (0.86-6.60)	2.40
[17]Wang (China)	2/99	3/113	0.76 (0.12-4.62)	1.17
[11]Combarros (Spain)	27/321	10/315	**2.80 (1.33-5.89)**	3.95
[8]Papassotiropoulos (Greece, Italy)	5/107	6/76	0.57 (0.17-1.95)	2.86
[8]Papassotiropoulos (Switzerland)	7/94	19/172	0.65 (0.26-1.60)	5.31
[18]Borroni (Italy)	10/143	10/134	0.93 (0.38-2.32)	4.10
[19]Kabbara (France)	57/601	57/631	1.06 (0.72-1.55)	21.51
[13]Kölsch (Germany)	10/115	16/144	0.76 (0.33-1.75)	5.54
[20]Chalmers (UK) [#]	4/86	3/58	0.89 (0.19-4.15)	1.46
[14]Johansson (Sweden)	54/440	45/398	1.10 (0.72-1.67)	17.72
[14]Johansson (Sweden) [#]	5/93	11/102	0.47 (0.16-1.41)	4.24
[14]Johansson (Scotland) [¤]	9/120	11/149	1.02 (0.41-2.54)	3.88
Present study (Finland)	43/422	24/469	**2.10 (1.25-3.53)**	8.73
Total (95% CI)	3307	3328	1.16 (0.97-1.38)	100.0

OR and 95% CI of the genotype frequencies are shown. [#]Autopsy-confirmed sample; 54 AD cases confirmed (Ingelsson). [¤]Early-onset AD sample. n/N= Number of CC-genotype carriers/ Number of subjects. Weight % = Weighting proportion of each individual study (used when combining ORs). In the separate pooled analysis of USA and European populations, a pooled effect of CC-genotype frequencies was similar to those found in controls (OR 1.17; 95% CI 0.78 to 1.73 and OR 1.16; 95% CI 0.95 to 1.41, for USA and European, respectively).

5.4. The peroxisome proliferator-activated receptor γ polymorphism and AD (study IV)

The distributios of the ApoE alleles ε2/ ε3/ ε4 were significantly different ($p<0.001$) in subjects with AD (0.02/0.66/0.32) compared to controls (0.07/0.80/0.13).

The allele or genotype frequencies of *PPARγ* exon 6 C478T (C/T allele and CC/CT/TT genotype frequencies in AD patients vs. controls: 0.80/0.20 vs. 0.81/0.19 and 0.62/0.35/0.03 vs. 0.64/0.33/0.03) and exon B encoding for Pro12Ala (Pro/Ala allele and ProPro/ProAla/Ala/Ala genotype frequencies in AD patients vs. controls: 0.86/0.14 vs. 0.85/0.15 and 0.74/0.24/0.02 vs. 0.72/0.26/0.09) did not differ significantly between AD cases and controls in the total study group or in subgroups stratified by ApoE ε4 phenotype or gender. The additive influence of the combined *PPARγ* exon 6 C478T and Pro12Ala genotype for the risk of AD was also studied with 478CC and Pro12Pro as reference. No significant additive influence on the risk of AD was found in the total study group or in the stratified groups.

The influence of the *PPARγ* C478T or Pro12Ala genotypes on age of onset was studied in the total AD group. Interestingly, AD subjects carrying both *PPARγ* 478T and 12Ala allele, that is the less common of each allele, manifested clinical AD significantly earlier than the subjects without these alleles (75 years vs. 78 years, $p= 0.026$, **Table 17**). Furthermore, we studied whether the significance of 12Ala-478T carrier genotype on age of onset was influenced by confounding factors. The AD group was dichotomized according to the mean age of onset (77 years) and groups were compared using Pearson χ^2 tests and adjusted logistic regression models. This significance seemed to be independent from the adjusted variables (**Table 18**). The influence of the 12Ala variant or 478T allele carrier genotypes separately on the age of onset did not quite reach significance ($p= 0.057$ and $p=0.068$ respectively). Furthermore, the ApoE phenotype di not influence the age of onset in this aged AD population (ApoE ε4 carriers vs. non-carriers, age of onset in both groups was 77 years).

Table 17. The additive influence of combined PPARγ Pro12Ala and exon 6 C478T genotypes on age of onset in total AD group (n= 125) with Pro/Pro-C/C as reference.

Pro12Ala	C478T	N	Age of onset	P
Pro/Pro	C/C	71	77.6±5.3	1
Pro/Pro	C/T or T/T	22	77.3±4.6	0.597
Pro/Ala or Ala/Ala	C/C	7	77.4±3.9	0.957
Pro/Ala or Ala/Ala	C/T or T/T	25	75.4±4.7	**0.026***

Pro/Pro, Pro/Ala and Ala/Ala = PPARγ2 Pro12Ala variants; C/C, C/T and T/T= PPARγ exon 6 C478T genotypes; N= number of subjects carrying each genotype combination; Age of onset given as years ± SD; Mann Whitney test used in statistics.

Table 18. The effect of 12Ala and 478T carrier genotype on age of onset (onset ≤77 year, N=25 or onset >77 years, N=75) in AD patients with Pro/Pro-C/C carriers as reference . Pearson χ^2 test and adjusted binary logistic regression tests.

	Model 1		**Model 2**		**Model 3**	
	P	**OR(95%CI)**	**P**	**OR(95%CI)**	**P**	**OR(95%CI)**
Ala+ /T+	**0.018**	**3.4 (1.2-9.4)**	**0.022**	**3.5 (1.2-10.3)**	**0.014**	**4.0 (1.3-12.1)**
ApoE4+	-	-	0.753	0.9 (0.4-2.1)	0.764	0.9 (0.3-2.2)
Sex	-	-	0.547	0.7 (0.3-1.9)	0.396	0.7 (0.2-1.7)
Age at baseline	-	-	0.337	0.9 (0.8-1.1)	0.373	0.9 (0.8-1.1)
systolic BP	-	-			0.317	1.0 (1.0-1.0)
TC	-	-			0.870	1.0 (0.6-1.4)
Gluc					0.150	1.2 (0.9-1.6)

Model 1: Univariate test, Pearson χ^2 test used;

Model 2: Adjusted binary logistic regression. Adjustment made with ApoE ε4, gender and age at baseline.

Model 3: Adjusted binary logistic regression. Adjustment made with the variables used in Model 2 and also with the cardiovascular risk factors previously associated with AD (systolic blood pressure, fasting total cholesterol and fasting glucose);

Ala+/T+ = combined PPARγ 12Ala and exon6 478T carries; ApoE4+ = apolipoprotein E ε4 carriers; systolic BP= systolic blood pressure; TC = fasting total cholesterol; Gluc = fasting glucose; OR (95% CI) = Odds Ratio with 95% confidence interval.

6. DISCUSSION

6.1. Methodological aspects

6.1.1. Study populations and design

The strength of the present study is in the homogeneity of these Caucasian patients and control subjects. The Finns are generally considered as being genetically isolated. In our study, both study patients and controls were derived from a geographically restricted area in northern Savo or Kuopio city. Homogeneity of the study population is essential for the genetic association studies but due to this genetic isolation, the reported results are mainly validated in our population can not be generalized to other populations.

Men and women were represented equally in both AD and control groups. The patients were on average slightly older and less educated than the controls but this was taken into account when the results were adjusted in multivariate analyses to verify the findings.

Considering the possibility to find true differences between the compared groups, there was an adequate number of subjects in the whole study populations (in studies I-IV) but the number of subjects in AD group became finally quite limited in studies I, II and IV (48 subjects in study I, 65 subjects in study II and 125 subjects in study IV). When the subjects (Studies I, II and IV) were invited for the follow-up study we were only able to estimate how many of them would convert to the dementia during the next years. In 1990-91 the numbers of identified AD cases (3.7.%) out of all study of the subjects resembles the prevalence of AD in this age group (Ott et al., 1995). During several years follow-up time, naturally, some of the study subjects died and some others did not respond to the next study invitation (Helkala et al., 1995). Some of the patients were not willing to provide a DNA sample or were not otherwise eligible for these genetic studies.

In studies I, II and IV all the subjects were LOAD cases with comparable clinical phenotype and duration of the disease at the beginning of the follow-up. In study III, some of the study subjects (both controls and AD subjects) were under 65 years. Most of them were screened for the known EOFAD mutations without any findings, and none of the AD cases showed an autosomal dominant inheritance in their family. Thus, it is more likely that they share a similar genetic background as the LOAD subjects.

In 1997, the AD patients' need of maintenance in everyday life (for study I) was evaluated by interviewing the caregivers over the phone. This method provides more reliable picture of the patients' current situation than can be obtained by interviewing patients themselves. It is possible that selection of a face-to-face approach would have been even more informative. The diagnosis of AD was made using widely accepted criteria for dementia and AD. For studies II and IV, the AD subjects were accepted as cases if also the clinical course of the disease had followed the "classical" progression of this disease

Also controls, not only demented subjects, were interviewed to evaluate their ability of daily living and cognitively screened by at least one neuropsychological test, usually with several tests. For studies II and IV they were followed up for years and for study IV their cognitive survival was estimated by checking the dementia diagnoses in 2003. For that study, the controls had survived without any cognitive symptoms for the whole follow up period. The subjects named as controls in study I included also cases with other cognitive problems. Several of study I and II controls developed also clinical AD during the later years of their lives. Also, study IV, which is a classical case control study, we cannot exclude the possibility that some controls would have suffered AD after the study and this would obviously affect the results. However, this same problem is shared with most of the studies conducted around the world.

All the personnel who carried out the examinations and finally made the diagnoses were trained and qualified to identify subjects with cognitive disturbances. On the other hand, due to the several years follow-up, some of the personnel changed. This might have influenced some of the estimations during the examinations.

6.1.2. Cognitive screening tests

Widely used, and replicable neuropsychological screening tests were selected to examine different aspects of learning and different kinds of memory functions (e.g. MMSE- delayed word recall, orientation, VFT- long term semantic memory, VRT, BSRT- to ability to to learn and retain verbal or pictorial information) but also to estimate the subject's attentional state and ability to solve problems (e.g. MMSE, TMT). Education, age and gender were taken account in the cognitive performance which was noted during the interview considering the ability to take care of everyday life. All the subjects were capable of performing the tests at the beginning of the follow-up study or at the time of the cognitive screening in the study III.

The more tests, repetitively used after follow-up, to test different kinds of memory functions, the better we may identify the subjects with memory problems. Therefore, a rather wide selection of the neuropsychological tests were performed and in the most cases, repeated once or twice. Furthermore, an interview may reveal the deterioration of the cognitive functions compared to the level the subject had in the earlier phase of the life. A minority of the study IV controls performed only one neuropsychological test (MMSE). In view of the weakness of one test to recognize cognitive decline, we performed the interview and elevated the MMSE cut off point in these controls (MMSE ≥27 without history of the memory impairment by the interview). We postulate that demented subjects were excluded also by this method from controls, but we cannot exclude the possibility that some control subjects may have some milder cognitive disturbances.

6.1.3. Diagnosis of AD

The diagnostic procedure consisted of the interview of the study patients and also the caregivers and the wide range of differential diagnostic examinations used in clinical practice. These detailed examinations were carried out by experienced personnel. The diagnosis of AD was accepted if made according to the golbally used DSM-IIIR criteria for dementia (APA, 1987) and NINCDS-ADRDA criteria for AD (McKhann et al., 1984). Therefore, we are convinced that the diagnosis of clinical AD was made as well as possible. According to several studies, the accuracy of the clinical AD diagnosis is around 85-90%, the definite diagnosis of AD requires neuropathological confirmation of typical AD changes in brain. To obtain as accurate an AD diagnosis as possible for studies II and IV, the progression of the disease was one criterion to be fulfilled (WHO, 1993) with other characteristic symptoms and findings typical to this dementing disease.

6.1.4. Selection of susceptibility risk genes

All the studied candidate susceptibility genes for AD risk were selected for the analyses as biological candidate genes based on previous studies indicating that the proteins the genes encode have been implicated in AD pathophysiology and/or there is evidence of the functional changes in the specific genes caused by single polymorphisms. This is the other generally accepted method to select possible new genes for association studies as in the current study.

We concentrated on the genes which share at least theoretically the possibility to affect inflammatory mechanisms (*PPARγ, IL-6* and *SERPINA3*) (McGeer and McGeer, 1998; Landreth and Heneka, 2001; Papassotiropoulos et al., 2001; Cheng et al., 2002) and/or lipid metabolism and

atherosclerosis (*PPARγ, IL-6, CYP46* and *APOE*) (Lund et al., 1999; Papassotiropoulos et al., 2001; Plutzky, 2003; Raffai and Weisgraber, 2003). By the time the study was completed, the pathophysiological mechanisms (inflammation and lipid metabolism) in which these genes are involved have become an area of great interest in AD research and consequently the genes possibly regulating these reactions are the focus of much research activity. On the other hand, the impact of selected genes, like many other AD candidate susceptibility genes, seems to be quite limited and appear to be evident only in small subgroups of AD. The most interesting of the currently studied genes may be *PPARγ* which may regulate the expression of other genes and influence, in theory, directly the neuroinflammatory responses seen in brain (Landreth and Heneka, 2001; Sastre et al., 2006).

6.1.5. Molecular genetic analyses

The methods we used to study the genes (SNPs and haplotype) and ApoE phenotype are widely used and validated. ApoE phenotype was determined from venous samples at the time (at the beginning of the 1990's) when genetic analyses were not available yet. There is no absolute concordance between genotype and phenotype method. Analyses of the Framingham Offspring Study (Lahoz et al., 1996) have revealed that a discrepancy may occur in 3% of the cases. However, both methods resulted in similar ApoE allele frequencies and for example, no differences were observed regarding the average allelic effect on total cholesterol (Lahoz et al., 1996).

6.1.6. Statistical analyses

We used the diagnosis of AD (study I survival analysis), alleles or genotypes and haplotypes of studied polymorphisms as primary categorical variables in these analyses. This is a common approach in epidemiological and genetic studies, providing a possibility to study odds ratios (risk of the disease caused by the studied factor) through application of univariate tests and logistic regression. All the used statistical tests throughout the study are in general use and easy to repeat. On the other hand, the possibility of false positive findings increases when multiple tests are used. In these cases, Bonferroni correction was used to correct the level of significance as e.g. in study III.

In genetic studies focussing on complex diseases such as AD, the signal obtained from association analyses is believed to be more prominent than that obtained from linkage studies. Moreover, identification of genes and single polymorphisms as well as statistical methods used in these kinds

of studies are easily carried out and replicated in the other study populations. While the number of studied genes and also the significant findings from these kinds of studies are high, the replicated studies achieving similar results are few and false positive findings may also occur. Also, the effect of the possible single gene on AD risk is low, perhaps being influenced by other genes and several other non-genetic factors. We tried to take this into account by adjusting in the logistic regression model for cardiovascular risk factors previously associated with AD risk (Kuusisto et al., 1997; Breteler, 2000; Kivipelto et al., 2001b), gender, age and education as well as with ApoE phenotype.

The adjusted factors were selected due to their association with AD in previous studies. All the study subjects were Caucasians, thus the adjustment with race was not appropriate. Age is the most important risk factor for LOAD (Ott et al., 1995) and the effect of some genes as AD risk factors may also be age dependent as in the case of *APOE* (Ott et al., 1995; Slooter et al., 1998. Protective effect of early life education for AD is well described {Fritsch, 2002 #145) Protective effect of early life education for AD is well described (Fritsch et al., 2002) and higher socioeconomic status including higher education affect general health and consequently survival (Roberge et al., 1995). Lifetime risk of dementia for women is twice as high as for men, which reflects not only the longer life span of women but also the higher dementia risk at very old age (Ott et al., 1998). Adjusted cardiovascular risk factors (high blood pressure, glucose and insulin metabolism and total cholesterol levels) used in the studies II and IV have been associated with increased LOAD risk in clinical studies (Kuusisto et al., 1997; Breteler, 2000; Kivipelto et al., 2001b). Based on the evidence that these cardiovascular factors may affect AD risk prior to the clinical onset of the disease (Kivipelto et al., 2001b) we used the values measured at the clinical baseline i.e., before the disease had become clearly manifested in these study subjects. Furthermore, *PPARγ* and *IL-6* polymorphisms or the proteins encoded by these genes are involved in the regulation of inflammation, lipid metabolism and atherosclerosis (Lund et al., 1999; Papassotiropoulos et al., 2001; Plutzky, 2003; Raffai and Weisgraber, 2003). These adjusted factors are a major research focus for AD risk in their own right and also, they may interact with the possible candidate genes for AD risk. Therefore, it was important to study the effect of these factors in relation to AD risk caused by the studied polymorphisms.

6.2. Apolipoprotein E, gender and late-onset AD- effect on survival

6.2.1. ApoE, gender and LOAD in relation to survival in the aged population

The frequencies of ApoE ε4 in both controls and AD subjects in studies I, II and IV were comparable to other previously published population based data from the same geographical area (Lehtovirta et al., 1995; Kivipelto et al., 2002a).

Middle-aged carriers of the ApoE ε4 phenotype are known to have an unfavourable lipid-profile and increased risk of cardiovascular diseases, and consequently increased risk of death (van Bockxmeer and Mamotte, 1992; Wilson et al., 1994; Wilson et al., 1996) but, in elderly subjects the results have been contradictory (Kuusisto et al., 1995; Olichney et al., 1997; Heijmans et al., 2002; Lane et al., 2003). This is most likely due to increased mortality risk (4.8 fold) in ApoE ε4 carriers compared to E3/3 carriers when they are younger, suggesting that the carriers of the ApoE ε4 have died before entry into these studies which are conducted in aged populations. This is supported by the reduced ε4 allele frequency in the aged compared with young cohorts (Talmud et al., 2004).

Cerebrovascular deaths have not been reported to be increased in the aged carriers of ApoE ε4 (Kuusisto et al., 1995; Olichney et al., 1997). In the case of intracerebral hemorrhage (ICH) Woo et al (Woo et al., 2002) reported that in a comparison of ApoE with ICH location, the ApoE ε2 and ε4 allele carriers had increased risk of lobar ICH.

In the present study, we did not find any significant difference between subjects with AD and non-demented subjects in their risk of succumbing to cardiovascular disease or stroke. Our results confirm the reports that the importance of the ApoE ε4 phenotype as a risk factor for cardiovascular and also stroke deaths decreases with age (Kuusisto et al., 1995; Olichney et al., 1997; Talmud et al., 2004). Although the ApoE ε4 phenotype did not seem to be an important risk factor for cardiovascular or cerebrovascular deaths in this aged population, mortality from these diseases does increase with ageing. For example, the risk of stroke among the elderly has been reported to double with each successive decade of life (Wolf et al., 1992).

Furthermore, we studied the influence of gender and AD on survival duration. In our study, in line with one other study (Corder et al., 1995), the survival of women was significantly longer than thet of men. The longer survival of women was independent of ApoE phenotype. Meanwhile, we were unable to show, in contrast to two other studies, that the presence of AD had any influence on survival in our aged study population (Olichney et al., 1997; Larson et al., 2004). Larson with his

colleagues from U.S. reported in 2004 (Larson et al., 2004) that in the subjects who received a diagnosis of AD (in both EOAD and LOAD subjects) the survival duration was shorter than predicted on the basis of the national population data. In contrast to our study, they studied also the survival of the EOAD patients and it seemed that especially within that group was the survival duration shortened. This may be one possible reason for the difference between the results from our study and those of Larson.

6.2.2. ApoE and gender in relation with survival in LOAD

While the effect of the ApoE ε4 allele in AD risk is clear, the effect in the course of the disease and survival of the AD patients is stil a matter of depate. For example, Tilvis et al reported (Tilvis et al., 1998) that the Apo E ε4 carrier phenotype correlated with increased mortality in AD patients studied in a population based sample while Slooter et al (Slooter et al., 1999b) did not find any relation between APOE ε4 allele and survival in AD. In the present study among all AD patients, we coud find no significant influence of ApoE ε4 carrier genotype on AD survival. Subsequently on, results consistent with our study and also with the Tilvis study have been published (Dal Forno et al., 2002; Fillenbaum et al., 2002). However after stratification of the AD population according to gender, ε4 negative men with AD had higher mortality compared to ε4 negative woman with AD. One has to bear in mind that there were only six ε4 negative men with AD and a false positive finding due to small sample size cannot be excluded. On the other hand, previous studies have suggested that the risk of death in men with AD is increased (Corder et al., 1995) and progression of AD is faster in Apo E ε4 non-carriers (Frisoni et al., 1995; Stern et al., 1997).

6.2.3. Neuropsychological tests and survival

We report that the progression of AD may be predicted by the results achieved in MMSE and BSR at the time of diagnosis. Larson et al, in line with our results, have reported that reduced survival correlated with severity of cognitive impairment (poorer results in MMSE and Blessed) at the time of diagnosis(Larson et al., 2004). This suggests that non-survivors were suffering from a more advanced form of the disease at entry. In the present study, the duration of AD did not explain the difference in survival nor did the ApoE phenotype. On the other hand, it has been observed that some subtypes of AD, for example Lewy body variant of AD, are characterised by faster cognitive decline and increased mortality (Drachman et al., 1990). It is also likely that many environmental and genetic factors contribute to the progression of AD. It would be necessary to conduct a more

detailed evaluation of clinical features, environmental factors and neuropathological and genetic data to confirm this suspicion.

6.3. Susceptibility genes and risk of AD

6.3.1. An allelic association of *IL-6* polymorphism in AD risk

Previously two SNPs (*IL-6* -174 G/C and VNTR polymorphisms) have been investigated in relation to AD risk in different populations with inconsistent results (Bagli et al., 2000; Bhojak et al., 2000; Papassotiropoulos et al., 2001; Shibata et al., 2002; Faltraco et al., 2003; Licastro et al., 2003; Arosio et al., 2004; Capurso et al., 2004; Depboylu et al., 2004; Nishimura et al., 2004). Capurso et al (Capurso et al., 2004) interpreted their study results and from estimations of other published data that geographical background could well have affected the results. This encouraged us to evaluate the role of the *IL-6* -174 G/C promoter polymorphism in relation to LOAD risk in our population based sample.

Our results are in line with the Shibata (Shibata et al., 2002) and the Pola (Pola et al., 2002) studies that ApoE ε4 non-carrier AD patients had significantly higher *IL-6*- 174G allele frequency with OR 1.8 compared to controls. Furthermore, in the same group, CG and GG genotypes were more common in AD patients compared to controls but, possibly due to the limited number of AD cases, this was not statistically significant. On the other hand, in allsubjects or in the ApoE ε4 carrier group, we did not find any allelic or genotype associations in agreement with previous reports from German (Bagli et al., 2000) and other Italian populations (Bagli et al., 2000; Capurso et al., 2004). Interestingly, the G allele frequency was lower in our population in both AD patients (0.52) and in controls (0.49) compared to the respective values for to German (0.60/0.58) or Italian (0.75/0.77) study populations (Capurso et al., 2004). This supports the previous observation made by Capurso about the increasing geographical distribution of the *IL-6*-174 G allele from northern to southern Europe as being a possible, at least a partial, explanation for the controversial results (Capurso et al., 2004).

The *IL-6*-174 G/C polymorphism, G allele as risk factor, is interesting in view of the findings by Fishman et al (Fishman et al., 1998) in their study evaluating in healthy controls and subjects with juvenile chronic arthritis. They reported (Fishman et al., 1998) that *IL-6*-174 C construct was associated with lower expression of *IL-6* gene and lower IL-6 plasma levels and, on the contrary, that the *IL-6-174 G* allele as a risk factor increased the expression of *IL-6* gene and IL-6 plasma

levels. They proposed that there is genetically determined difference in the degree of the IL-6 response to stressful stimuli between individuals and that the CC genotype of this polymorphism is a potentially protective genotype. Based on these findings, the *IL-6* genotype may be relevant also in other conditions in which IL-6 is implicated (Fishman et al., 1998).

There are studies which have evaluated the impact of the IL-6 in the pathophysiological reactions seen in AD. Elevated production of IL-6 has been linked to the early stages of amyloid deposition and plaque formation, leading to the neurodegenerative process seen in AD (Gruol and Nelson, 1997). Consistently, IL-6 and other cytokines can be stimulated in glial cells surrounding Aβ plaques in an animal model of AD (Mehlhorn et al., 2000) and in cultured cells (Toro et al., 2001). Recently, Luterman et al (Luterman et al., 2000) found higher IL-6 mRNA expression in both entorhinal cortex and superior gyrus of AD patients with an advanced stage of the disease compared to normal controls. This difference was not noted between the AD patients at an earlier stage of the disease and controls (Luterman et al., 2000). They suggested that the inflammatory cytokine response to the pathological effects of AD does not occur until the later stages of the disease (Luterman et al., 2000). The factors which are responsible for the overexpression of IL-6 are not clear but it appears that age is an important factor for IL-6 (Wei et al., 1992; Ye and Johnson, 2001).

Since ApoE is an established risk factor for AD (Corder et al., 1993; Saunders et al., 1993), the interaction with *APOE* genotype has been studied in relation to IL-6 polymorphisms and AD but there is no evidence for any possible synergistic effect (Arosio et al., 2004; Depboylu et al., 2004) even though Bhojak et al (Bhojak et al., 2000) did find in ApoE ε4 carriers a marginal association between GC/GG genotypes and increased AD risk compared to CC carriers. Here we could not detect any synergistic effect in the AD risk between ApoE ε4 allele and the *IL-6*-174 CG/GG genotype.

6.3.2. The *CYP 46* and *SERPINA3* polymorphisms and risk of AD

The present candidate gene based association study with the *CYP46* gene in a large series of AD and controls found evidence for an increased AD risk in the *CYP46* dbSNP:754203 CC genotype carriers in all subjects and *APOE ε4* carriers. Our data of an association between dbSNP:754203 CC genotype and AD is in line with a study in the Spanish population (Combarros et al., 2004). However, a significant risk in AD patients carrying CC genotype was only evident in these Spanish subjects with *APOE ε4* genotype. This discrepancy between results of these two different ethnic

populations may be due to differences in the *APOE* frequencies. The frequency of the *APOE* ε4 allele was 16% in our controls and only 6%, in the study of Bullido et al (Bullido et al., 1998) in their Spanish population. It seems also that dbSNP:754203 CC genotype may not possess an additive risk effect within *APOE ε4* carriers in our population and thus our data supports an independent risk factor mode of action for *APOE* and *CYP46* genes.

To test whether there exists LD in the *CYP46* gene, we screened another marker dbSNP:2146238. Our finding of considerable LD (D') between SNP pairs (dbSNP:754203 and 2146238) showed a robust intermarker association, which may be evidence of a haplotype block structure as also described by SNPbrowser™ software (Applied Biosystems). Although the dbSNP:2146238 polymorphism failed to show a single allele association between cases and controls, we observed that the CG containing haplotype exhibited a modest odds ratio of 1.25 for the risk of AD.

There are several published contradictory studies describing either no association or even an opposite effect of dbSNP:754203 T/C and AD. Although the results for a significant association of dbSNP:754203 T/C and AD may be population specific and related to AD via some unknown mechanism, the meta-analysis argues against the hypothesis that the dbSNP:754203 T/C plays a crucial role between the *CYP46* gene and AD. Since the relevant variation dbSNP:754203 T/C is intronic, it is not clear how it can influence the function of the enzyme coded by this gene (Kolsch et al., 2002; Johansson et al., 2004). The dbSNP:754203 T/C LD may reflect a pathogenic variation in the *CYP46* gene itself but it may also, be present in a nearby gene. A genetic association with AD has earlier been found in the dbSNP:4934 of the *SERPINA3* gene. However, our data from a single marker seems to exclude *SERPINA3* gene as a candidate gene for AD. The frequency of *SERPINA3* A-allele (previously AD risk related allelic form) was equivalent in AD patients and in controls. In addition, no significant LD between *CYP46* and *SERPINA3* polymorphic markers was observed, which most likely rules out any genetic association between the genes. It remains to be tested whether the dbSNP:754203 T/C may be in LD with functionally relevant variability elsewhere in the *CYP46* gene.

6.3.3. *PPARγ* polymorphisms in risk of AD and age of disease onset

While we could not find an association between studied *PPARγ* polymorphisms on AD risk in line with Sauder and colleagues (Sauder et al., 2005), our data does suggest that the carriers of both 12Ala and 478T allele have a lower age of onset (OR 3.4) compared to Pro12Pro/478 CC carriers. This modifying effect on age of LOAD onset seems to be independent of ApoE and other adjusted

variables. Interestingly, in the present study there were no significant differences in age of LOAD onset between ApoE ε4 carriers and non-carriers as described previously (Slooter et al., 1998).

These *PPARγ* loci have been previously described to be associated with increased body fat-mass (Deeb et al., 1998; Valve et al., 1999; Cock et al., 2004), insulin sensitivity and unfavourable lipid profile (Deeb et al., 1998; Cock et al., 2004) which are factors also associated with the risk of AD (Breteler, 2000). Therefore the 12Ala/478T carrier genotype rather than being be a direct risk factor, may represent a disease course modifying factor for developing AD. This theory finds support from the findings that the *PPARγ* variant carrying the polymorphic region coding Pro12Ala is expressed extensively in adipose tissue (Meirhaeghe and Amouyel, 2004). Furthermore, the chronic inflammatory reaction related to AD may be regulated by *PPARγ* agonists (Combs et al., 2000). On the other hand, our data suggested that the earlier age of AD onset related to the *PPARγ* Pro12Ala and C478T polymorphism was independent from the studied cardiovascular risk factors**.** The recent findings by Sartre et al. support the concept for a role for PPARγ as a modulator of the β-amyloid generation by inflammation (Sastre et al., 2006)**.** A direct functional impact on the activity of *PPARγ* is also possible in the modulation of the AD development as are also changes in the transactivation of the *PPARγ* gene in the case of the Pro12Ala polymorphism (Deeb et al., 1998). The possible functional mechanisms of the silent C478T polymorphism remain unknown.

To evaluate whether the allele number used in the analysis was sufficient to find a true association, a power analysis was also undertaken. According to the simulation analysis, on the basis of the Ala12Pro or C478T minor allele proportions described previously in this area population (Valve et al., 1999), approximately 600 alleles or 300 subjects, respectively would have been needed in each group to have a statistically significant p-value of 0.05 at 70% power and a moderate odds ratio (OR) of 1.5

6.3.4. Importance of the findings in AD risk and implications for the future

In the case of *IL-6* we showed that IL-6 prom 174 G- allele is over-represented in Finnish LOAD patients, at least in those LOAD patients not having any ApoE E4 alleles. Also, *CYP46* is a possible risk gene for Finnish AD patients. However, in the case of *CYP46*, the meta-analysis argues against the hypothesis that the dbSNP:754203 T-to-C plays a crucial role generally between the *CYP46* gene and AD. Our data seems to exclude *SERPINA3* gene as a candidate gene for AD but indicate that polymorphisms within the *PPARγ* gene in exon 6 C478T and in the region coding Pro12Ala can modify age of onset in LOAD independently of ApoE phenotype. While no direct genetic

influence on AD risk was found, this data in conjunction with a previous report (Sauder et al., 2005) suggests that the studied *PPARγ* polymorphisms do not play a role in AD risk in these European populations.

The genetic associations between PPARγ, CYP46 and IL-6 and AD would highlight the importance in the disease development of the immune functions and lipid metabolism of brain. The recently published data related to studied polymorphisms and the alteration of their function as well as the clarification of the functions of the encoded proteins in brain support the findings of the genetic association studies. Thus also indicates that the studied genes are pathophysiologically relevant susceptibility candidate genes for AD.

However, the results of these studies should be interpreted with some degree of caution. This caution applies in particular to the results from the ApoE stratified tests, owing to the smaller sample size in the study groups, and when the results are principally based on one intronic marker. These approaches increase the possibility of false positive findings.

Furthermore, there are several issues from a genetic point of view which are not yet clearly resolved. There are several polymorphic sites in the studied genes which need to be identified if possible and examined. In that way, one can determine which site has the strongest signal for AD, how they influence gene expression and what are the kinds of functions or the pathophysiological effects of the encoded proteins. The gene to gene as well as the gene to environmental interactions in relation to the studied genes are unknown. It would be interesting to determine, whether these polymorphic genes can influence the therapeutical response to drugs. Should it be the case that these genes are involved in AD, it is likely that the effect of one gene in the complex disease risk (i.e. AD risk) is quite small. However, in view of the previous reported associations with conditions related also with AD, *IL 6, CYP46* as well as *SERPINA3* and *PPARγ* remain interesting candidate susceptibility genes for dementia. Confirmation of the association between these genes and AD as well as investigating their functional role in the pathogenesis of AD will be challenging task for researchers in the future.To sum up, this data supports the theory that several susceptibility genes contribute to the risk of AD development.

CONCLUSIONS

1. The presence of ApoE ε4 phenotype or AD did not influence the risk of mortality in the elderly population while the risk of death was significantly increased in men compared to that of women. Once AD had manifested itself, the ApoE ε4 carrier phenotype did not influence the survival.

2. Our findings point to an influence of *IL-6* on the LOAD risk in ApoE ε4 non-carriers in the Finnish population and consequently, support the hypothesis for an involvement of *IL-6* in AD.

3. The *CYP46* is a possible risk gene for Finnish AD patients or it reflects the influence of a nearby gene which increases the AD risk. On the other hand, the meta-analysis with dbSNP:754203 T/C argues against this hypothesis. Furthermore, the evaluation of a nearby gene *SERPINA3* polymorphism did not detect any significant association between this gene and risk of AD.

4. No direct genetic influence on LOAD risk was for the polymorphisms in *PPARγ* and the AD risk. Instead, *PPARγ* seem to modify the age of onset in LOAD independently of ApoE phenotype.

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