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Genetic Studies of Alzheimer's Disease

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2008

ISSN 1651-6206
ISBN 978-91-554-7346-4
urn:nbn:se:uu:diva-9397

Dissertation presented at Uppsala University to be publicly examined in the Rudbeck hall, Rudbeck laboratory, Dag Hammarskjölds väg 20, Uppsala, Friday, December 12, 2008 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract
Blom, E. 2008. Genetic Studies of Alzheimer's Disease. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 401. 47 pp. Uppsala. ISBN 978-91-554-7346-4.

Patients with Alzheimer's disease (AD) often have a family history of the disease, implicating genetics as a major risk factor. Three genes are currently known to cause familial early-onset AD (<65 years): the amyloid precursor protein (*APP*) and the presenilins (*PSEN1* and *PSEN2*). For the much more common late-onset disease (>65 years), only the *APOE* gene has repeatedly been associated to AD, where the *ε4* allele increases disease risk and decreases age at onset. As *APOE ε4* only explains part of the total estimated disease risk, more genes are expected to contribute to AD.

This thesis has focused on the study of genetic risk factors involved in AD. In the first study, we conducted a linkage analysis of six chromosomes previously implicated in AD in a collection of affected relative pairs from Sweden, the UK and the USA. An earlier described linkage peak on chromosome 10q21 could not be replicated in the current sample, while significant linkage was demonstrated to chromosome 19q13 where the *APOE* gene is located. The linkage to 19q13 was further analyzed in the second study, demonstrating no significant evidence of genes other than *APOE* contributing to this peak.

In the third study, the prevalence of *APP* duplications, a recently reported cause of early-onset AD, was investigated. No *APP* duplications were identified in 141 Swedish and Finnish early-onset AD patients, implying that this is not a common disease mechanism in the Scandinavian population.

In the fourth study, genes with altered mRNA levels in the brain of a transgenic AD mouse model (tgAPP-ArcSwe) were identified using microarray analysis. Differentially expressed genes were further analyzed in AD brain. Two genes from the Wnt signaling pathway, *TCF7L2* and *MYC*, had significantly increased mRNA levels in both transgenic mice and in AD brains, implicating cell differentiation and possibly neurogenesis in AD.

Keywords: Alzheimer's disease, Linkage, Affected sib-pairs, APOE, APP duplication, 10q21, 19q13, Wnt signaling

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ISSN 1651-6206
ISBN 978-91-554-7346-4
urn:nbn:se:uu:diva-9397 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-9397)

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List of papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals.

I **Blom ES**, Arepalli S, Hamshere ML, Adighibe O, Goate A, Williams J, Lannfelt L, Hardy J, Wavrant-De Vrièze F and Glaser A. "Further analysis of previously implicated linkage regions for Alzheimer's disease in affected relative pairs". *Manuscript*

II **Blom ES**, Holmans P, Arepalli S, Adighibe O, Hamshere ML, Gatz M, Pedersen NL, Bergem ALM, Owen MJ, Hollingworth P, Goate A, Williams J, Lannfelt L, Hardy J, Wavrant-De Vrièze F and Glaser A. "Does *APOE* explain the linkage of Alzheimer's disease to chromosome 19q13?" Am J Med Genet B Neuropsychiatr Genet. 2008 Sep 5;147B(6):778-83

III **Blom ES***, Viswanathan J*, Kilander L, Helisalmi S, Soininen H, Lannfelt L, Ingelsson M, Glaser A*, Hiltunen M*. "Low prevalence of *APP* duplications in Swedish and Finnish Alzheimer's disease patients". Eur J Hum Genet. 2008 Feb;16(2):171-5

IV **Blom ES***, Wang Y*, Skoglund L, Hansson A, Ubaldi M, Lourdusamy A, Sommer W, Hyman BT, Heilig M, Lannfelt L, Nilsson LNG, Ingelsson M. "Increased mRNA levels of *TCF7L2* and *MYC* of the Wnt pathway in tgAPP-ArcSwe mice and Alzheimer's disease brain". *Manuscript*.

*These authors have contributed equally to the work

Cover image: Metaphase chromosomes from a human male cell (www.genome.gov). Chromosomes studied in the present thesis are highlighted (chromosome 8 – Paper IV, chromosome 10 – Paper I and IV, chromosome 19 – Paper I and II, and chromosome 21 – Paper III)

Contents

Abbreviations

Aβ	Amyloid-β
AD	Alzheimer's disease
APOE	Apolipoprotein E (gene)
APOE	Apolipoprotein E (protein)
APP	Amyloid precursor protein
ARP	Affected relative pair
bp	Base pair
CAA	Cerebral amyloid angiopathy
cDNA	Complementary DNA
cM	Centimorgan
CNV	Copy number variation
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
et al.	*et alia*, and others
IBD	Identical by descent
IBS	Identical by state
LD	Linkage disequilibrium
LOD	Logarithm of odds
MCI	Mild cognitive impairment
MLS	Multi-point LOD score
mRNA	Messenger ribonucleic acid
NIMH	National Institute of Mental Health
NMDA	N-methyl D-aspartate
OR	Odds ratio
PCR	Polymerase chain reaction
PSEN1	Presenilin 1
PSEN2	Presenilin 2
qPCR	Quantitative PCR
SNP	Single nucleotide polymorphism
TgAPP-ArcSwe	Mouse transgenic for the Arctic (E693G, Aβ E22G) and Swedish (APP K670N/M671L) APP mutations
Zlr	Z-score of a likelihood ratio

Introduction

Alzheimer's disease

History

One hundred years ago, the German physician Alois Alzheimer published the first paper describing a patient with what has become known as Alzheimer's disease (AD) [1]. Dr. Alzheimer saw his patient, Auguste Dieter, for the first time in 1901 when she was 51 years old. She presented with rapidly deteriorating memory, disorientation and confusion; symptoms that did not fit any known diagnosis. After her death, Dr. Alzheimer performed an autopsy and found shrinkage of the brain and two types of protein deposits, plaques and tangles, today considered hallmarks of AD (Figure 1).

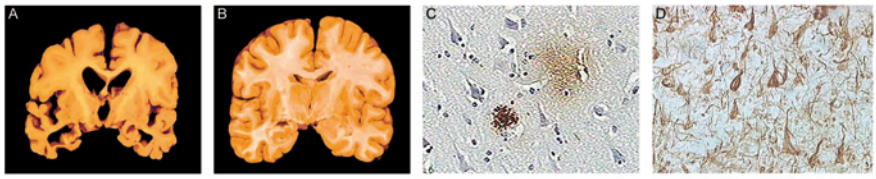

Figure 1. At autopsy, the AD brain is characterized by a reduced volume (A) as compared to normal brain (B). At higher magnification, amyloid plaques (C) and neurofibrillary tangles (D) are found in the AD brain. *Picture credit: Hannu Kalimo (A+B) and Martin Ingelsson (C+D)*

Neuropathology

Deposition of extracellular amyloid plaques is probably an early pathologic event, preceding the clinical symptoms of AD [2, 3]. Plaques consist mainly of aggregates of the peptide β-amyloid (Aβ) [4], which is formed through enzymatic cleavage of the amyloid precursor protein (APP). Three enzymes, α-, β- and γ-secretase, cleave APP at different positions (Figure 2). Cleavage by α-secretase within the Aβ sequence in combination with γ-secretase cleavage, result in the release of αAPPs, p3 and the APP intracellular domain. Conversely, when β- and γ-secretase cleave APP, Aβ is released together with βAPPs and the APP intracellular domain [5]. By cleavage at different positions, γ-secretase produces Aβ-peptides of varying lengths, where Aβ42

is more amyloidogenic than Aβ40. However, both peptides aggregate to form soluble protofibrils, which in turn form insoluble fibrils and plaques [6].

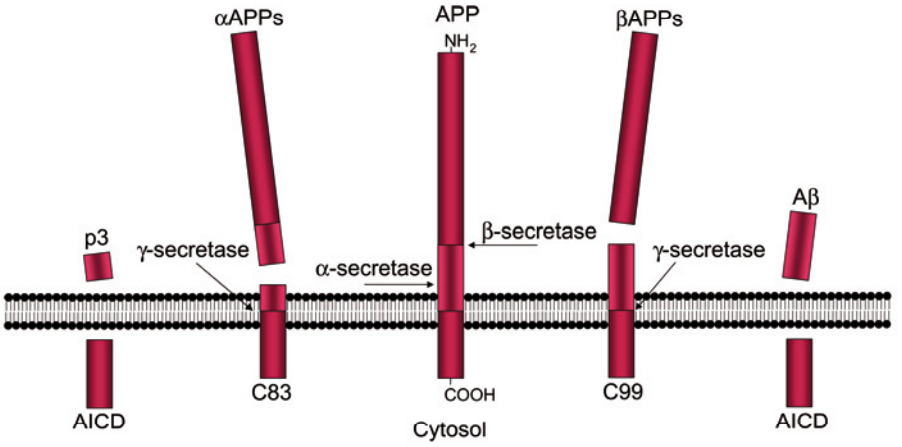

Figure 2. APP is cleaved by α- and γ-secretases to release non-amyloidogenic products, and by β- and γ-secretases to release the amyloidogenic Aβ.

The role of plaques in AD has been debated. The initial version of the amyloid cascade hypothesis stated that aggregation and amyloid deposition of the Aβ peptide causes AD [7]. As cognitive decline is not well correlated to the amount of amyloid deposits in the brain [8] this theory has received criticism. Levels of soluble Aβ may be better correlated to both synaptic density and cognition [9] and there is growing evidence that they might be the principal neurotoxic species in AD, whereas insoluble fibrils are relatively inert or even protective [10]. Therefore, the main focus of the theory today is Aβ aggregation rather than plaques, in the pathogenesis of AD [11].

Neurofibrillary tangles consist mostly of aggregated hyperphosphorylated tau protein. The normal function of tau is to bind tubulin and thereby stabilize microtubules in neuronal axons, allowing nutrients and neurotransmitters to be transported along the axons between the cell body and the synapses. In AD, hyperphosphorylated tau detaches from the microtubules and aggregates into paired helical filaments and neurofibrillary tangles [12, 13].

In studies on a transgenic mouse model for AD, tangles appear to be an event secondary to Aβ plaques, where Aβ might induce or aggravate the aggregation of tau [14].

Risk factors

The major risk factor for AD is age. Between 65 and 74 years of age, 1.6% of the US population is affected by AD, while 43% is affected above the age of 85 years [15, 16]. As the population is aging, the number of people af-

fected by AD will also increase, adding burden to already strained health budgets. Considering the exponential increase in incidence of AD after retirement age, it is plausible that everyone would develop AD if we only lived long enough. However, there are also studies showing a decrease in incidence in the very high ages, possibly due to the depletion of susceptible individuals, suggesting that some individuals are invulnerable to AD [17].

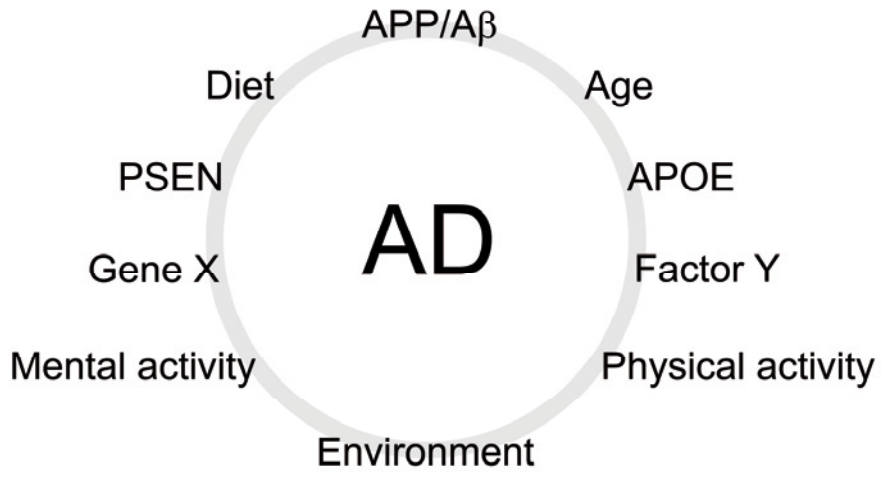

Figure 3. Factors that may influence the development of AD

Another major risk factor for AD is genetics, where first-degree relatives of a person with AD have a greater risk of developing the disease than those without a family history of AD. Twin studies have estimated heritability for AD to be as high as 79% [18]. In addition, estimations of concordance have demonstrated that if one twin develops AD, the other twin will also develop disease in 59% of the monozygotic twin pairs, whereas this only occurs in 32% of same-sex dizygotic twins and in 24% of opposite-sex dizygotic twin pairs. As twins are assumed to share not only genes but also the environment during a critical period for brain development, this demonstrates a high genetic component to AD [19].

AD and cardiovascular disease have many risk factors in common, such as high blood pressure, hypercholesterolemia and diabetes [20, 21]. Accordingly, long-term use of statins, a drug used to lower cholesterol levels, has been shown to reduce the risk of developing AD [22]. There have also been studies demonstrating that people who eat fish at least once a week, and thereby have a high intake of omega three fatty acids, have a decreased risk of developing AD [23].

Moreover, there are studies showing that individuals with a higher level of education have a lower risk of developing AD than those with less education. Theories about cognitive reserve state that cognitive failure is not no-

ticeable until it reaches a certain threshold. Therefore, for an individual with a greater cognitive reserve, the threshold of disease may be reached later and onset of symptoms will be delayed. This protective effect may also apply to people who stay socially, mentally and physically active throughout their lives [24]. Also, studies have demonstrated that transgenic mouse models of Aβ deposition, which are exposed to an enriched environment with e.g. running wheels, colored tunnels and toys display reduced Aβ levels and amyloid deposits in the brain [25].

Symptoms, diagnosis and treatment

The disease process normally starts in the hippocampus and the medial temporal cortex, explaining early symptoms such as failing short-term memory. As the disease progresses through the cerebral cortex, more functional areas of the brain are affected, causing difficulties with activities of daily living, language impairment and sometimes personality changes. In the final stages, patients are often withdrawn and eventually become bedridden.

The average disease duration of AD is dependent on the patient's age at diagnosis, ranging from eight years if diagnosis is made at 65, to three years for patients diagnosed at 90, ranging from one to twelve years [26]. Death is usually not due to the disease itself, but rather to a secondary infection such as pneumonia or urinary tract infections.

There are many possible causes for memory loss and cognitive deterioration. AD is therefore diagnosed through a combination of medical history, clinical evaluation including cognitive testing such as the mini mental state examination, and changes in levels of different biomarkers such as Aβ42, tau and phosphorylated tau in cerebrospinal fluid (CSF). These proteins can be measured for different neuronal dysfunctions, where elevated levels of tau and phosphorylated tau, along with a decrease in Aβ42 imply AD with high sensitivity and specificity [27]. Also, brain imaging techniques such as magnetic resonance imaging, computed tomography and positron emission tomography aid in reaching a diagnosis. However, a definite diagnosis of AD can only be made *post mortem* using the same pathological criteria as was observed by Alois Alzheimer; cerebral atrophy and the presence of plaques and tangles in the hippocampus and cortex (Figure 1).

Today there is no cure for AD and the existing treatments only temporarily slow the progression by modifying neurotransmitter signaling in the brain. There are four registered drugs for AD in Sweden; three cholinesterase inhibitors and one N-methyl D-aspartate (NMDA) antagonist. The cholinesterase inhibitors increase the concentration of acetylcholine at the synapses and provide some improvement of memory and cognition in mild to moderate AD patients. However, they only work for some patients, and only for a limited time period. The NMDA antagonist reduces negative effects of excessive amounts of the neurotransmitter glutamate, and is used for moderate to severe

AD patients. Also, chronic presymptomatic use of certain non-steroidal anti-inflammatory drugs, e.g. ibuprofen, has been shown to reduce the risk of AD in patients with rheumatoid arthritis [28]. A lot of effort is now put into developing new treatments for AD, where immunotherapy is a strong candidate [29]. There are two immunotherapeutic approaches. In passive immunization, premade antibodies against Aβ are administered to the patient, whereas in active immunization, the patient's own immune response is raised against Aβ to produce a long-lasting antibody response.

Genetics

Only a few percent of all AD cases are early-onset (before 65 years of age). Of these, approximately 40% are sporadic and 60% have a familial dominantly inherited AD (Figure 4) with mutations in any of three genes: *APP* on chromosome 21, presenilin 1 (*PSEN1*) on chromosome 14 and presenilin 2 (*PSEN2*) on chromosome 1. However, these three genes do not explain all cases of familial AD.

The majority of AD cases are late-onset (after the age of 65 years), for which approximately 75% are sporadic (Figure 4), probably caused by a genetic predisposition in combination with environmental factors. The remaining 25% of late-onset cases have a family history of AD (Figure 4) where several genes have been implicated [30]. So far only the apolipoprotein E gene (*APOE*) has repeatedly been associated to late-onset AD [31, 32]. However, *APOE* is neither sufficient nor required for the development of AD, as not all AD cases have an *APOE ε4* allele and not all individuals with an *APOE ε4* allele will develop AD. Therefore, additional, yet unidentified disease genes are likely to exist.

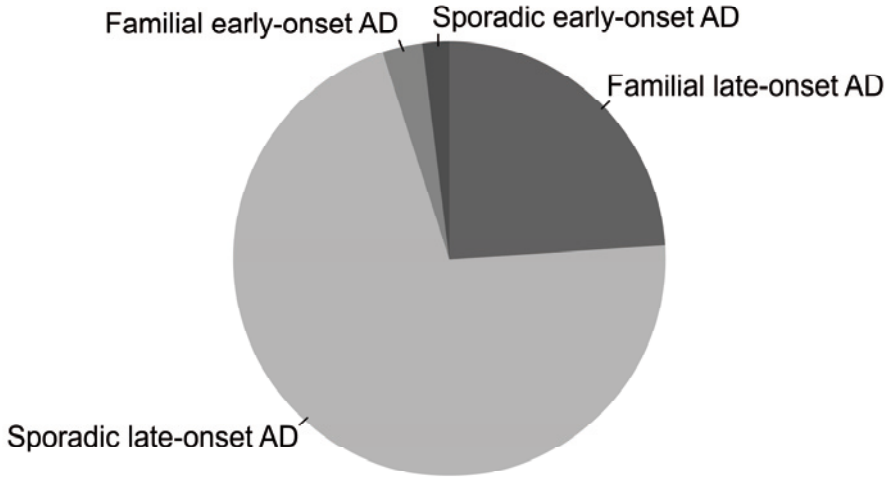

Figure 4. Approximate prevalence of subtypes of AD [33]

APP

In 1984, Glenner and Wong purified and sequenced the protein, which later became known as Aβ, from cerebrovascular amyloidosis in AD patients [4]. It was later demonstrated that Aβ was derived from APP and the *APP* gene was mapped to chromosome 21 in 1987 [34]. Some years later, in 1991, the first mutation in *APP* causing familial AD was found [35]. Mutations within the *APP* sequence are all in exons 16 or 17, where the sequence for Aβ is located. Mutations affecting the γ-secretase cleavage site alter the processing of APP into more Aβ42, whereas mutations affecting the β-secretase cleavage site lead to the production of more total Aβ (Figure 2). There are also mutations located within the Aβ sequence that increase the aggregation rate of Aβ. For an updated list of AD mutations, see alzforum.org/res/com/mut.

Patients with Down's syndrome (trisomy 21) usually develop AD-like neuropathology already in early middle age [36]. Once the *APP* gene was mapped to chromosome 21, the co-occurrence of Down's syndrome and early-onset AD could be explained by a gene dose effect due to the extra copy of the *APP* gene. Recently, two groups reported that duplications of *APP*, in the absence of trisomy 21, can cause familial early-onset AD with cerebral amyloid angiopathy (CAA) [37, 38]. There are also reports that polymorphisms in the promoter of the *APP* gene affecting expression levels are associated with AD [39]. These findings demonstrate that an increased expression of *APP* is enough to cause AD even when the sequence of *APP* is not altered.

The APP protein consists of multiple structural and functional domains. It has been proposed to function as a cell surface receptor, in cell adhesion and in synaptic plasticity [40]. Homozygous APP knock-out mice are viable and fertile, but are smaller and have decreased locomotor activity [41].

PSEN1/PSEN2

In 1995, genome-wide linkage analysis led to the identification of a novel gene for early-onset AD on chromosome 14 [42]. Shortly thereafter a highly homologous gene on chromosome 1 was discovered [43]. In view of the early onset of dementia in the mutation carriers and their considerable homology, the two genes were named presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*).

Together with nicastrin, presenilin enhancer 2 (PSENEN), and anterior pharynx defective 1 (APH1), PSEN constitute the γ-secretase protein complex [44]. This enzymatic complex is not only involved in the cleavage of APP, but also in regulated intramembrane proteolysis of several other cell surface receptors, e.g. Notch [45]. Many *PSEN* mutations increase the production of Aβ42, but this might be at the expense of Aβ40 cleavage, leading to a reduction in Aβ40 levels. This in combination with neurodegeneration and impairment of hippocampal memory in mice with a conditional knock-

out of both *PSEN1* and *PSEN2* [46] have brought forward the suggestion that *PSEN* mutations cause AD through a loss of function mechanism rather than a gain of toxic function [47].

APOE

The *ε4* allele of *APOE* on chromosome 19 was the first genetic risk factor to be identified for late-onset AD [31, 32]. Three different alleles, *ε2, ε3* and *ε4,* encode the isoforms of APOE that differ by cysteine/arginine substitutions at positions 112 and 158. These base pair substitutions result in changes in the relative affinity of the APOE protein for receptors and lipoproteins. The *ε2* allele is the most unusual of the three and is considered somewhat protective for AD [48]. The most common variant, *ε3*, is neutral for AD risk, whereas *ε4* decreases the age at onset in carriers in a dose dependent manner [31]. In addition, polymorphisms within the promoter may influence the expression of *APOE* and thereby disease risk [49].

Table I. Frequencies of *APOE* genotypes (%) (Adapted from Bird 2008 [33])

***APOE* genotype**	**General population**	**AD patients**
ε2/ε2	1.3	0
ε2/ε3	12.5	3.4
ε2/ε4	4.9	4.3
ε3/ε3	59.9	38.2
ε3/ε4	20.7	41.2
ε4/ ε4	0.7	12.9

In the brain, APOE is synthesized by astrocytes and microglia, whereas APOE in the periphery is mostly synthesized in the liver. It is a lipid transporter in CSF and plasma and the primary protein component of lipoproteins in the central nervous system. Through the interaction with cell surface lipoprotein receptors it is involved in cholesterol homeostasis, where APOE4 is associated with increased levels and APOE2 with decreased levels of cholesterol in plasma, as compared to APOE3 [50]. APOE also binds Aβ, where the binding of E4 is more rapid than E3 [51], leading to an enhanced fibril formation [52]. Moreover, APOE3 has been demonstrated to inhibit the neurotoxic effect of Aβ, while APOE4 does not [53]. APOE4 also has a reduced efficiency in promoting the degradation of soluble Aβ by microglia, as compared to APOE2 and APOE3 [54].

Other genes

A large number of additional genes have been suggested to be associated with AD. However, most lack confirmation in independent studies or their replications have been inconsistent. Due to the complexity of AD pathogenesis where probably several genes contribute small individual effects in com-

bination with environmental risk factors, susceptibility loci are often difficult to replicate, especially in small individual samples. Nonetheless, replication remains a critical step in the validation of genetic studies. There have been many attempts at finding and replicating susceptibility genes for AD, resulting in data for over 500 candidate genes [30]. To help interpret these studies, the AlzGene database presents meta-analyses of all published association studies of AD, resulting in combined odds ratios (OR) for the different genes (alzforum.org/res/com/gen/alzgene) [55]. The top five results at AlzGene (as of October 2008) are listed in Table II.

Table II. Top five genes with association to AD, according to AlzGene (Adapted from Bertram *et al.* 2007 [55])

Position	Gene	Gene name	OR	Location	Linkage	Link to AD
1	*APOE*	Apolipoprotein E	3.68	19q13	Yes	Affects Aβ aggregation [52] and clearance [54]
2	*CHRNB2*	Cholinergic receptor, nicotinic, beta 2	0.67	1q21	Near	Reduced cholinergic signaling in AD brain [56]
3	*GAB2*	GRB2-associated binding protein 2	0.84	11q13	No	Suppresses GSK3-dependent phosphorylation of tau [57]
4	*CH25H*	Cholesterol 25-hydroxylase	1.44	10q23	No	Lipid metabolism, up-regulated in AD brain [58]
5	*SORL1*	Sortilin-related receptor	0.9	11q24	No	Affects trafficking and processing of APP [59]

Linked chromosomal regions

Several whole genome linkage scans have been conducted for AD [60-64]. Three of the studies, Pericak-Vance *et al.* 2000, Myers *et al.* 2002 and Blacker *et al.* 2003, are all based on the National Institute of Mental Health (NIMH) sample collection [65]. Pericak-Vance *et al.* included 413 ARPs from NIMH and also 326 other American ARPs, all from families with a mean age at onset >60 years of age. Myers *et al.* included 80 American ARPs and 94 ARPs from the UK, in addition to 277 ARPs from the NIMH, all with age at onset >65. Blacker *et al.* did not count ARPs, but included 437 families with 994 affected individuals from the NIMH, where 117 of the families included members with age at onset <65 years of age.

Chromosome 9

On chromosome 9 there seem to be two linked loci, 9p22-21 and 9q22, and both loci are reported from the three overlapping NIMH studies [61-63]. For 9p21, cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*), which is involved in cell cycle regulation, was recently suggested [66]. For 9q22, death-

associated protein kinase 1 (*DAPK1*), which is involved in the apoptotic cascade [67], has been suggested.

Chromosome 10

Several linkage studies have identified chromosome 10 as a good candidate for a new AD gene. Myers *et al.* initially identified a locus at 10q21 with a multi-point LOD score (MLS) of 3.8 [68]. Also high levels of Aβ42 in plasma have been linked to 10q21 [69]. Blacker *et al.* found the highest linkage at 10q22, 10 cM distal the peak found by Myers *et al.,* with an MLS of 1.8 in their total sample. They also found a locus on 10q24 in their late subsample, displaying a two-point LOD score of 1.9.

At 10q21 several genes have been implicated in AD, e.g. transcription factor A, mitochondrial (*TFAM*), where impaired mitochondrial function and apoptosis in AD links *TFAM* [70]. Calcium homeostasis modulator 1 (*CALHM1*) is located at 10q24 and encodes a Ca^{2+} channel, which has also been demonstrated to regulate Aβ levels [71]. As stated above, *CH25H* is located between these linked regions at 10q23. There have also been a number of studies on the insulin degrading enzyme (*IDE*) at 10q23, an enzyme known to degrade Aβ [72]. However, meta-analysis of published studies only produced an OR of 0.98, demonstrating inconsistencies in study results.

Table III. Maximum MLSs from original genome wide linkage studies on Caucasian AD samples [60-64]

Chromosomal region	Pericak-Vance *et al.* 1998	Pericak-Vance *et al.* 2000	Myers *et al.* 2002	Blacker *et al.* 2003	Sillén *et al.* 2008
9p22-21	-	4.3[b]	1.8	1.3[e]	-
9q22	-	-	1.8[c]	2.9	-
10q21-22	-	-	3.9	1.8[e]	-
10q24-26	-	-	-	1.9[a]	-
12p13-11	3.2[a]	-	1.4[d]	-	-
19q13	-	5.7	1.3	7.7[f]	5.3[c]

[a]Two-point LOD score [60, 61]
[b]Autopsy confirmed subsample [63]
[c]APOE4+ subsample [62, 64]
[d]APOE- subsample [62]
[e]Total sample [61]
[f]Early/mixed subsample [61]

Chromosome 12

At 12p11-13 there seems to be a locus with linkage that is stronger in *APOE ε4* negative families [60, 62, 63]. Alpha-2-macroglobulin (A2M) received attention due to its ability to reduce aggregation and fibril formation of Aβ [73] and to influence the clearance of Aβ across the blood-brain barrier [74]. However, as with *IDE*, genetic studies have demonstrated inconsistent results.

Chromosome 19

A majority of linkage studies report linkage to 19q13, which is where *APOE* is located. *APOE* is by far the strongest risk gene with an OR of 3.68. Apolipoprotein C-I (*APOC1*) is also located under this peak, with an OR of 2.07. However, *APOC1* maps ~5 kb distal to, and is in linkage disequilibrium (LD) with *APOE*, which could explain the association of *APOC1* to AD. Also, in paper II of this thesis, we have demonstrated that *APOE* is exclusively responsible for the 19q13 peak in included cohorts [75].

Transgenic mouse models

Genetic findings in families with autosomal dominant early-onset AD have provided insights into the pathogenesis of AD. By introducing these genes and mutations into animals, models for the disease can be created. These transgenic animals have contributed greatly to elucidating disease mechanisms and they are also important for testing new drugs and treatments. The most widely used transgenic mouse model for AD research is the Tg2576 mouse. It expresses human APP with mutations causing familial AD (APP K670N/M671L) and displays some of the characteristics of AD pathology with cerebral Aβ deposition, plaques, and cognitive deficits [76]. Another mouse model, the triple transgenic mouse carrying human mutated APP, PSEN1 and tau, display both Aβ plaques and tangles [77]

Materials and methods

The following section is an overview of materials and methods used in this thesis. For a more detailed description, please see the included papers and manuscripts.

The human genome

The era of modern genetics started in 1865, when Gregor Mendel presented his famous work on the inheritance of traits in pea plants [78]. His work was forgotten until the early 1900's, but was followed by many other important discoveries. In 1953, Watson and Crick presented the structure of deoxyribonucleic acid (DNA) [79] and in 1986, Kary Mullis and co-authors published the Nobel prize winning method polymerase chain reaction (PCR) [80]. In a PCR, a single stretch of DNA can be amplified to several million copies within hours. PCR is today used in many, if not most, genetic methods where amplification is necessary for detection.

With the sequencing of the human genome, the field of human genetics took a great leap [81-83]. The human genome consists of approximately 3 billion base pairs (bp) divided into 23 chromosome pairs (see cover image), where one chromosome is inherited from the mother and the other from the father. Today, there are 20,067 known protein coding genes and 1,461 novel protein-coding genes (October 2008, ensembl.org/Homo_sapiens). However, it has been estimated that there might be as many as a million different protein molecules produced during the lifetime of a human. Through alternative splicing and post-translational modifications, in combination with differential expression of genes in different tissues and at different stages of development, the variation is increased multifold. Nonetheless, the extent of the genome encoding for proteins is only a small fraction of the total genome, covering only a few percent of the sequence [81]. Today, more and more attention is being directed to the non-coding regions, since they are believed to contain regulatory domains and other biologically important regions.

Genetic variation

Compared to the non-human hominid apes, humans are very similar in their genomic sequences, reflecting a founder effect from the recent African ori-

gin of our species. At approximately one in every 250 bp across the human genome, the sequence differs in what is known as a single nucleotide polymorphism (SNP) [84] (Figure 5a). Other small sized genetic polymorphisms are microsatellites, which usually consist of di-, tri- or tetranucleotide repeats of varying lengths (Figure 5b). SNPs usually occur through errors in DNA repair or in DNA copying in the gametes, and microsatellites by slippage of the DNA polymerase during DNA replication [85].

Figure 5. (A) The upper panel exemplifies a SNP with alleles T or C in the forward strand. (B) The lower panel exemplifies a tetra repeat microsatellite marker with alleles 20, 24 and $(GATA)_n$ bp.

Copy number variation (CNV) is another form of genetic variation, which, considering the total number of nucleotides affected, causes more sequence variation than SNPs. CNVs are segments of the genome larger than 1000 bp that are deleted, inserted or duplicated when comparing individuals [86]. These events affect gene expression in different ways, e.g. through the duplication of an entire gene leading to increased expression, or through the deletion of a repressor element, affecting the regulation of gene expression [87].

Gene duplications have been stated to be the single most important factor in evolution [88] where an extra copy of a gene alleviates selective constraints and allows for new proteins to evolve. It has been estimated that genomic rearrangements occur in the human genome at a rate two to four times greater than that of point mutations [89]. Positive selection has been demonstrated for copy numbers of the salivary amylase gene encoding an enzyme that degrades starch, where a high-starch diet in agricultural societies have resulted in more copies than the low-starch diet of rainforest hunter-gatherers [90].

The distribution of CNVs across the genome is highly non-uniform, probably due to chromosomal 'hot spots', where rearrangements occur more frequently. Furthermore, certain functional classes of proteins are more often found to be duplicated than others. For example, proteins involved in environmental response are more often found to be duplicated than proteins vital

for development [91]. CNVs are usually formed through non-allelic homologous recombination during meiosis, where repetitive sequences in the genome are used as substrates for homologous recombination, resulting in the deletion or duplication of the intervening segment [92]. Also, an uneven recombination between aligned chromosomes during meiosis can produce one chromosome with a duplicated segment and another with a deleted segment.

Most of the variation present in the human genome is silent, but some occur in coding regions where they influence phenotypic differences between humans through changes in the amino acid sequence of proteins or the regulation of gene expression. Most of these variants have no connection to disease; however, some variants mediate an increased susceptibility to, or directly cause a disease. There are monogenic diseases caused by a mutation in a single gene, or complex diseases, for which polymorphisms in many genes with small individual effects are believed to interact together with environmental risk factors to cause disease.

Genetic mapping

Genetic mapping is based on recombination. During meiosis, the process of cell divisions resulting in egg and sperm cells, the maternal and paternal chromosomes align and recombination of chromosomal segments occurs, creating a new combination of chromosomal segments in the off-spring. The closer together two loci are on a chromosome, the fewer recombinations will occur between them and the more often they will be inherited together. The genetic distance separating two loci is measured in centimorgans (cM), where 1 cM corresponds to a 1% probability of recombination occurring between two loci. Due to recombination 'hot spots' and 'cold spots', the genetic distance is not the same as the physical distance between loci, but 1 cM roughly corresponds to 1 Mbp in humans.

Materials

For linkage studies of human disease, families with several members affected by the disease are most often used. Large and extended multigenerational families with a clear inheritance pattern are highly valuable. However, such families are difficult to find, especially for diseases with a late onset, where the previous generation is no longer available for DNA sampling at disease onset of the present generation, and the next generation is still unknown for possible disease development. Therefore, smaller families, affected sib-pairs or, when including cousins or half-sibs, affected relative pairs (ARPs) are frequently used.

To gain sufficient statistical power, a large number of ARPs are needed. However, using several unrelated families in genetic mapping of complex diseases can introduce confounding factors such as selection bias, when samples are not representative for the population, or population stratification, when study samples with different demographic background might differ naturally in allele frequencies without association to disease status. Also, a gene contributing to disease might be neither sufficient nor necessary to cause disease, and different genes might cause similar disease phenotypes.

Genetic markers distributed over the entire genome or over entire chromosomes are used to trace a disease locus in genetic mapping. Any polymorphic genetic character that shows a Mendelian inheritance pattern and that can easily be detected can be used as a genetic marker. A marker should be sufficiently polymorphic so that a randomly selected individual has a good possibility of being heterozygous. Both SNPs and microsatellites are inherited and fairly easy to genotype, whereby they are frequently used as markers in genetic mapping. SNPs usually only have two alleles and are therefore less informative (Figure 5a), but as they exist in abundance and are today easy to genotype, high throughput compensates for low information contents. Microsatellites have numerous alleles and are highly informative (Figure 5b), but occur less frequently across the genome and are somewhat more difficult to genotype.

SNPs can be genotyped by sequencing or by different kinds of arrays. On the array, nucleic acid targets of the different SNP variants are immobilized, and by detecting which spot the sample hybridizes to, the genotype can be elucidated. Microsatellites can be genotyped by amplification in a PCR where one of the primers is labeled with a fluorophore, and subsequent detection of the length of the amplicon, i.e. the microsatellite allele, through capillary electrophoresis.

Linkage studies

The aim of a linkage study is to find chromosomal regions where genetic polymorphisms contributing to disease are located. Two loci, e.g. a marker and a disease locus, are linked when they are inherited together more often than expected by random segregation. The closer on a chromosome two loci are located, the fewer recombinations will occur between the two and the more often they will be inherited together during meiosis. Marker alleles that are inherited together more often than expected are said to be in LD and a block of co-segregating alleles are called a haplotype.

It is assumed that family members who share the same inherited disease will also share the markers close to the disease loci or in genomic regions relevant for disease development, whereas unaffecteds will not. Therefore, by evaluating the number of alleles shared in a region, a disease locus can be identified. Alleles that are shared due to a common parental inheritance are

identical by descent (IBD). However, markers can have the same alleles even though they are not of the same parental origin and are then identical by state (IBS).

The probability of linkage between two loci can be calculated as the logarithm of odds (LOD), as a Zlr (Z-score of a likelihood ratio) or as a p-value [93]. A LOD score is the $\log_{10}$ of the ratio of the likelihoods that the loci are linked rather than not linked. A Zlr is the number of standard deviations by which the sharing exceeds the expected. The p-value is the probability of finding a linkage as high as the one observed by chance. It can either be a pointwise p-value, which reflects the probability of observing allele sharing as high as the one observed at a specific locus, or a genome-wide p-value, which reflects the probability of observing allele sharing as high as the one observed somewhere in an entire genome scan. When using affected sib-pairs to study linkage of complex traits, linkage can be considered suggestive if the LOD score is above 2.2 and significant if the LOD reaches 3.6 or higher. The corresponding numbers when using a Zlr are 3.2 and 4.1, and for pointwise p-values 7.4×10^{-4} and 2.2×10^{-5}, respectively [93]. However, factors such as marker density and number of included sib-pairs affect linkage results, whereby a simulation of the statistical power in the current study setup can be worth conducting [94].

When marker positions on the chromosomes are known, multipoint analysis where several adjacent markers are co-analyzed relative to the disease locus, can be used. This increases the information in the analysis and enhances power as compared to a two-point analysis, where each marker is analyzed independently, relative to the disease locus. For analysis of a Mendelian disease where mode of inheritance, gene frequencies and penetrance are known, parametric analysis can be used. However, for a complex disease, model-free non-parametric linkage is more useful, where sharing of alleles between affected individuals is analyzed.

Gene expression

In order to survive, a cell has to respond quickly to changes within and in the surrounding environment. This requires an alteration of the levels of existing proteins, or for new proteins to be made. The central dogma of molecular biology states that DNA is transcribed into mRNA (messenger ribonucleic acid), which is in turn translated into proteins. By measuring levels of mRNA, the level of expression of genes can be estimated. A complicating factor is that mRNA levels are influenced by both transcription and degradation of mRNA. Thus, changes in mRNA levels may not strictly reflect the transcriptional regulation of individual genes. However, mRNA is a very unstable molecule and is normally readily degraded in order to facilitate quick changes in gene expression.

The instability of the mRNA molecule requires certain precautions when using post-mortem brain tissue to study events in the living brain. Post-mortem time, handling and storage of the samples are crucial; however, studies have shown that degradation of transcripts in the same brain correlates, maintaining the relation to reference genes [95]. However, care must still be taken as it has been demonstrated that a subset of mRNA transcripts are reduced after an extended post-mortem interval of 48 h, which could especially affect microarray studies that include a broad spectrum of genes [96]. Also factors such as gender, age at death and brain pH can affect mRNA integrity [97].

Microarrays

Gene expression can be measured either at a transcriptome level, i.e. all mRNA transcripts present at a given moment, or on an individual gene level, measuring one transcript at a time. A microarray is used to measure the transcriptome, and is, simplified, a small glass slide with thousands of tiny spots on it. There are several approaches as to the details, but on the microarrays used in paper IV of this thesis, each spot contains a short section of a gene used as a probe, immobilized to the glass. By hybridizing fluorescently labeled cRNA or cDNA (complementary RNA/DNA, reverse transcribed from a cDNA/mRNA template, respectively) from the tissue of interest to the probes and measuring fluorescence at the different spots, the expression of all genes in the tissue can be estimated [98]. Through comparisons of the gene expression between e.g. cases and controls, genes that are affected in the diseased tissue can be identified.

Quantitative PCR

Gene expression of individual genes can be measured through e.g. quantitative PCR (qPCR) [99]. One of the available detection methods is the SYBR green chemistry. The SYBR green dye binds to double-stranded DNA, and is used to visualize the PCR product as it accumulates stepwise during a qPCR [100]. A camera detects the increasing fluorescence, and the cycle where enough double-stranded DNA has been produced for the fluorescence to reach a threshold value is called the threshold cycle (Ct). Less starting material requires more PCR cycles to reach the threshold, thereby resulting in a higher Ct value (Figure 6). A control gene, usually a housekeeping gene that has constant expression in all tissues, is used to normalize for unequal starting concentrations. By comparing the normalized Ct value of the gene of interest between tissue from e.g. cases and controls, relative gene expression can be calculated.

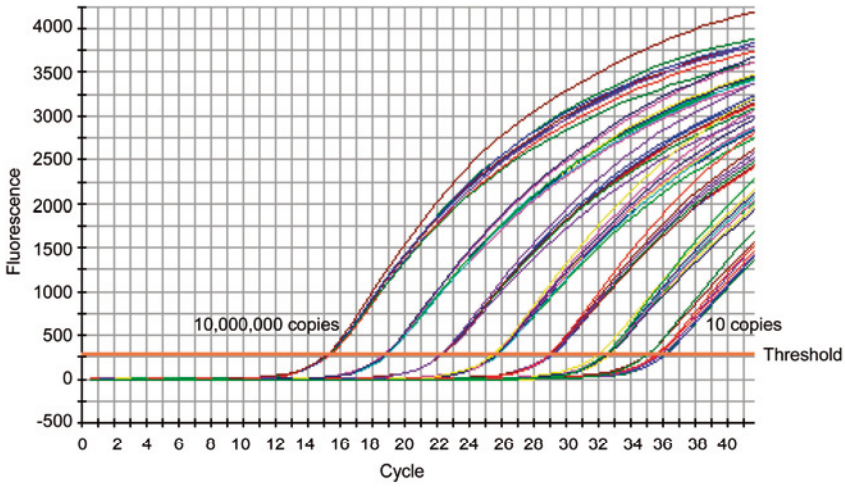

Figure 6. Results from a qPCR using a 10-fold dilution series, ranging from 10,000,000 to 10 copies/well. The amount of starting material is measured in Ct, the cycle where fluorescence reaches a threshold value.

Present investigations

Aim of the thesis

The overall aim of this thesis was to further elucidate the genetics of AD, which is a complex condition with several genetic and environmental factors contributing to disease. The specific aim of each paper was:

Paper I	To investigate previously implicated linkage regions in a collection of relative pairs affected by AD
Paper II	To investigate if the *APOE* gene is exclusively responsible for the linkage of AD to 19q13
Paper III	To estimate the prevalence of duplications of the *APP* gene in Swedish and Finnish patients with early-onset AD
Paper IV	To identify differentially expressed genes in young tgAPP-ArcSwe mice, and to further investigate the findings in AD brain

Paper I

Linkage analysis of previously implicated regions for AD

Background

Several whole genome linkage scans have been conducted in order to identify genetic factors involved in the development of AD, where regions on chromosomes 9, 10, 12, and 19 are the most replicated (Table III) [60-64]. In the present study, we have included ARPs from the study by Myers *et al.* 2002, using the whole UK sample whereas the USA sample was modified (171 affected individuals were added and 118 were removed due to ambiguous phenotypes). Also, 277 ARPs from the NIMH sample used in Myers *et al.* were not included in the present study. Instead, 116 ARPs from Sweden were included. Using this collection of ARPs, we analyzed previously implicated linkage regions for AD.

Results and discussion

The linkage analysis of chromosomes 1, 9, 10, 12, 19 and 21 to AD in the present study revealed a significant MLS at 19q13 of 3.0 in the total sample collection, increasing to 8.3 in the *APOE ε4+* subsample. Also, 10p15 demonstrated suggestive linkage with an MLS of 1.6 in the total sample.

As we have a large overlap with the samples used in Myers *et al.* where the highest linkage peak was on 10q21, it is somewhat surprising to not detect this linkage peak also in the present scan. Other linkage scans using the NIMH sample, e.g. the scan by Blacker *et al.* also found linkage to the region, 10 cM distal at 10q22. Further, the scan by Kehoe *et al.* used the NIMH samples included also in Myers *et al.*, and found linkage to the same region [101]. This suggests that the linkage found to this region results from the NIMH sample, which was not included in the present study. There are many factors influencing linkage, e.g. population heterogeneity, diagnostic differences or random statistical fluctuations [93].

The Swedish subsample is to some degree an overlap with the sample used by Sillén *et al.* [64]. This is also noticeable in the similar results, where chromosome 19 demonstrates the strongest linkage, with a strong influence of *APOE* [75], whereas the other analyzed chromosomes did not demonstrate significant linkage.

Studies have demonstrated linkage to the same chromosomes but to slightly different regions, e.g. 10q21-22 where Myers *et al.* found maximum linkage at 82 cM whereas the maximum linkage in Blacker *et al.* was at 92 cM. The question arises whether these peaks represent linkage to the same loci, or whether they are separate loci close to each other. A simulation study of variability in linkage positions for complex diseases revealed that vari-

ability can be substantial, up to tens of cM, and can be even larger in smaller sample cohorts [102]. Considering the generally low LODs reported in linkage studies for AD, the differences could also be due to random statistical fluctuations, where some of these peaks are false positives. Some peak variation might also be due to the presence of multiple genetic loci contributing with differing strength in different samples.

Paper II

Analysis of the contribution of APOE to the linkage peak at 19q13

Background

Linkage studies of complex diseases usually result in large chromosomal regions demonstrating linkage to the disease. The region borders might be better defined through fine mapping using additional markers, or the regions might be narrowed down through the addition of more samples with more informative recombinations between markers and the disease locus. However, linked regions might still contain hundreds of genes that could be responsible for the disease. Due to functional properties, some genes might seem to be better candidates than others, but sequencing or functional testing of hundreds (or even tens) of genes is not always realistic. It might also be that several neighboring genes within a linked region are associated to disease but are in LD, making it difficult to separate genetic influences.

Genome-wide linkage studies of AD often demonstrate linkage to chromosome 19q13, and as *APOE* is located in this region, it has been assumed that *APOE* is the only gene responsible for the peak. However, additional genes in the region have also been associated with AD, but due to LD they have been disregarded. Therefore we wanted to investigate whether the *APOE* gene is exclusively responsible for the linkage peak of AD to 19q13, or whether other genes in the region might contribute to the linkage.

Results and discussion

To investigate how *APOE* affects the 19q13 linkage peak and if *APOE* is the only gene contributing to the linkage, we used the sample cohort from paper I in a hypothesis test under the null hypothesis that *APOE* is the only cause of linkage. Zlr scores for the *APOE* locus were calculated using replicated data sets, and the proportion of replicated Zlrs being higher than the actual Zlr was used as a p-value to test the hypothesis. As the replicated Zlrs were calculated based on the allele frequencies of *APOE*, they can be considered a measure of the linkage contributed by *APOE*. Therefore, if the actual Zlr was comparable to the replicated Zlr, the null hypothesis would be true, i.e.

APOE would be considered exclusively responsible for linkage. On the other hand, if the actual Zlr was significantly higher than the replicated Zlr, it would suggest that linkage cannot be explained exclusively by *APOE* and the null hypothesis would be rejected. Our results showed a tendency of additional gene influence in the SWE subsample, but it was not significant ($p = 0.064$). In the combined sample however, the study demonstrated that *APOE* does explain the linkage peak on its own ($p = 0.18$).

The contribution of *APOE* to 19q13 has also been investigated in previous studies. For example, the study by Myers *et al*. compared the number of *APOE ε4* alleles in individuals sharing two markers IBD close to *APOE*, with individuals who did not share any alleles. This revealed a higher number of *ε4* alleles among sharers, demonstrating a high impact of *ε4* on the linkage at 19q13 [62].

Much effort is put into the search for new genes contributing to AD, and due to repeated linkage results to certain chromosomes, much effort is directed to these regions. At chromosome 19q13, *APOE* is the strongest candidate gene, but there are also other genes in the region demonstrating association to AD [103, 104]. However, these genes are in strong LD with *APOE*, making it difficult to separate the respective effects. One of the genes is *APOC1* with an OR of 2.07, as compared to the OR of *APOE* of 3.68 [55]. *APOC1* and *APOE* are both apolipoproteins and their close localization on chromosome 19 indicates a common ancestry prior to gene duplication. They might therefore have similar functions, where both could affect Aβ and possibly AD [105]. The tendency for additional genetic contribution in the Swedish subsample could possibly be a small contributing effect from *APOC1*. However, our study did not show any statistical evidence for additional genes contributing to the linkage in the whole sample, demonstrating that the major genetic risk factor at 19q13 is *APOE*.

Paper III

Screen for APP duplications in Swedish and Finnish patients with early-onset AD

Background

Many neurodegenerative conditions display protein deposits in the brain, such as plaques of Aβ in AD and Lewy bodies of α-synuclein in Parkinson's disease. This suggests that the amount of protein, and the expression levels of the corresponding gene, may play a central role in disease pathogenesis. In this context it has long been recognized that most patients with Down's syndrome develop early-onset AD due to the extra copy of the *APP* gene, located on chromosome 21 [36]. Recently, two groups reported that duplica-

tions of *APP*, in the absence of trisomy 21, can cause familial early-onset Alzheimer's disease with CAA [37, 38].

Results and discussion

To investigate the incidence of *APP* duplications in a Scandinavian population, subjects with early-onset AD from Sweden and Finland were included. By qPCR using the SYBR green chemistry, the Swedish subjects were screened for gene dose.

As the qPCR method is dependent on high quality DNA and the samples included were collected during a number of years, our approach was first assessed by screening with glycogenin 2 (*GYG2*), a gene located on chromosome X. As women have two X chromosomes and men have only one, the observed gene dose could be correlated to a predictable level in the form of gender. As a result, eight samples were removed from the study, leaving 75 individuals from families with features of early-onset AD and 66 individuals with early-onset AD without a known familial inheritance pattern.

No cases of *APP* duplications were verified in the screen. This could be due to a genetic differences between the Swedish and Finnish populations studied by us, and the French and Dutch populations studied previously [38, 106]. However, considering the fairly short geographic distance between the populations, a mixing of populations is likely. The lack of *APP* duplications could possibly also be due to the unknown frequency of CAA in our early-onset AD cases. In both previous studies, *APP* duplications have been found in patients with early-onset AD and CAA [37, 38, 107], and CAA is also frequent in patients with Down's syndrome [108].

It has been questioned whether the occurrence of AD in Down's syndrome is due only to the duplication of *APP*, or whether additional genes on chromosome 21 might contribute. The finding of a duplication spanning only the *APP* gene in an AD patient provides evidence that APP is enough for AD development [38]. This is further emphasized by the finding that polymorphisms in the promoter of *APP* found in AD patients increases the expression levels of *APP* twofold [39].

The occurrence of several different duplication sizes surrounding the *APP* gene, rather than a common duplication size in all patients, demonstrate that these duplications are separate events and do not stem from a common ancestor. It also demonstrates that this region is a recombinational 'hot spot' where genomic rearrangements occur frequently at slightly different locations. A recent study on multiplications of the α-synuclein gene in Parkinson's disease demonstrated that two different mechanisms, both intraallelic duplication and interallelic recombination with unequal crossing over were responsible [109]. Both duplications and triplications have been identified for α-synuclein, where a triplication resulted in earlier disease and more severe symptoms [110-112]. No triplication of *APP* has yet been identified,

but considering the resemblance between the two diseases in other areas, the same scenario of more severe symptoms with additional copies of the gene is possible for AD. A recent case report of a patient presenting with early-onset AD due to a 10% mosaicism for trisomy 21 [113] demonstrated that even rather modest increases in *APP* dose can cause early-onset AD with approximately the same age at onset as in patients with full trisomy 21 [114]. Furthermore, a recent study on the allelic expression of *APP* in single cloned human B-lymphoblastoid cell lines demonstrated that the maternal and paternal alleles were randomly active, with monoallelic expression in some clones and biallelic expression in others [115]. A similar mechanism could explain why only some neurons in an AD brain are affected. If so, individuals with a more biallelic expression may be more prone to developing AD, while those with a more monoallelic expression are less prone. A large proportion of monoallelic expression might also be an explanation as to why *APP* was not found to be up-regulated in a study on gene expression in Down's syndrome brain [116].

Paper IV

Gene expression studies in a mouse model for AD and in human AD brain

Background

The creation of transgenic animal models has been instrumental to the increased understanding of AD pathogenesis. A transgenic mouse model with both the Swedish (APP K670N/M671L) and the Arctic (APP E693G, Aβ E22G) APP mutations (tgAPP-ArcSwe) has been developed in our group [117]. The tgAPP-ArcSwe develops strong intraneuronal Aβ aggregation already at one month of age, i.e. long before the extracellular senile plaque formation begins at five to six months. Data suggests that this intraneuronal Aβ is in fact soluble oligomers as opposed to the insoluble Aβ that is found in extracellular deposits [118]. As soluble oligomers have been suggested to be more toxic than insoluble fibrils found in plaques, we decided to investigate mRNA levels in tgAPP-ArcSwe mice at an age when they have soluble intraneuronal Aβ aggregates, but when plaques have not yet deposited. Since results found in a mouse model for a disease cannot be directly translated into the human situation, we also wanted to replicate the findings in human AD brain.

Results and discussion

In this study, we sought to identify biological processes that are involved in the pathology of soluble Aβ aggregates. Using Affymetrix microarrays we

have assessed mRNA levels in the brains of young tgAPP-ArcSwe mice and non-tg mice on an equal genetic background. This revealed three pathways with altered expression of several genes, of which the most affected was the Wnt signaling pathway. Genes from this pathway were also examined in human brain tissue using qPCR. Two genes, *TCF7L2* and *MYC*, were found to have significantly increased mRNA levels in both transgenic mice and in AD brains. These two genes are functionally related, where the transcription factor TCF7L2 regulates transcription of the *MYC* gene [119]. The Wnt pathway has previously been connected to AD in several ways, with regulation of APP cleavage by disheveled (DVL1) [120], stimulation of β-catenin degradation by PSEN1 [121], and the hyperphosphorylation of tau by glycogen synthase kinase 3 beta (GSK3B) [122] as examples.

The microarray results were attained in young tgAPP-ArcSwe mice with soluble intraneuronal Aβ aggregates but not yet plaques. Therefore, it would have been fitting to also investigate brain samples from humans with early signs of disease development, i.e. mild cognitive impairment (MCI). However, disease progression in humans is very disparate and not all MCI patients develop AD. Still, in a study of levels of tau and Aβ42 in CSF from MCI patients, incipient AD could be predicted with a sensitivity of 95% and a specificity of 83% [123]. Nonetheless, MCI patients rarely come to autopsy. Thus, we only had the possibility to analyze brains from patients with manifest AD.

The use of animal models for AD allows us to investigate disease pathogenesis in ways that would be impossible or considered unethical in patients. The mouse is a good model organism as it is small and has a fairly short life span. However, the short life span of a mouse is also a problem when studying a late-onset disease that develops during several decades in a human. Generalization between organisms is possible due to a common descent and conserved pathway structures. However, care must still be taken, especially when studying brain functions in a model with a less developed brain, compared to the human brain.

Concluding remarks and future perspectives

Genetic studies of Alzheimer's disease had great successes in the early 1990's, when all the presently known AD genes were identified; *APP* in 1991 [35], *APOE* in 1993 [31, 32] and the presenilins in 1995 [42, 43]. These genes all affect Aβ in one way or another, either affecting the production, the aggregation or the clearance of Aβ. Moreover, they have provided great insights into the pathogenesis of AD.

Since these first genetic discoveries, more than 500 genes have been tested for AD association, but they have often been disproved in other studies [30]. However, heritability for AD has been estimated to 60-80%, indicating that genetics is a major risk factor for AD [18]. Moreover, it has been estimated that there could be as many as seven additional risk loci affecting age at onset for AD [124]. Many AD patients do not have an *APOE ε4* allele and *APOE* accounts for less than a third of the estimated disease risk [125, 126], indicating a high probability of finding additional genes for AD.

AD is a monogenic disease in the few cases with familial early-onset AD, and a complex disease in most other cases. The study of monogenic diseases is fairly straightforward, as demonstrated by the early identification of *APP* and the *PSENs*. However, studying complex diseases is more difficult. Firstly, both genes and environmental factors contribute to complex diseases and both might cluster in families, where environmental factors can be confounding factors in genetic studies. The genes contributing to disease risk might be neither necessary nor sufficient for disease development, as is the case with *APOE*. Also, different genes or different mutations in the same gene, in combination with different environmental factors might produce similar disease phenotypes in different individuals. This emphasizes the need for either very well defined phenotypes or the use of isolated populations where cases may be assumed to be due to the same genetic polymorphism. The advantage of using well defined sample material has recently been demonstrated with the identification of the progranulin gene for frontotemporal dementia (FTD), which was identified in a pathological subtype of the disease defined by ubiquitin-positive inclusions in the brain [127, 128]. This finding further demonstrates that mutations in different genes give rise to different pathological subtypes in FTD, even though the subtypes appear identical in the clinic.

The methodology for complex genetic studies is rapidly evolving, with the advent of genome wide association studies using hundreds of thousands

of SNPs to search for genetic association in cases and controls. In 2007, genes or loci for a number of complex diseases were identified [129], e.g. for type 1 [130, 131] and type 2 diabetes [132-134] and heart disease [135-137]. Three genome wide association studies have been conducted on AD; however, they used relatively small sample sizes [103, 138, 139]. All three studies identified *APOE* or SNPs in LD with *APOE*, but the few additional loci presented in the original publications were all much smaller than *APOE*. As has been exemplified by more recent genome wide association studies, tens of thousands of patients and millions of SNPs increases the power of a study, so that also smaller genetic variants can be identified [140-144]. As AD is a common disease, this approach raises the hope of finding additional disease loci.

Alzheimer's disease is a devastating disease that gradually breaks down the personality and the individual; something the patient often is very aware is happening, at least initially. After the patient is in oblivion, the disease continues to disturb the next of kin who is no longer recognized or remembered, nor do they recognize the affected person. AD usually affects people after their retirement, when for many people it is finally time to enjoy life. It is not only a catastrophe for the individual and next of kin, the society is afflicted with huge costs for medical care and assisted living. Even though we have known of this disease for over one hundred years, there is still no effective cure. Therefore, genetic studies of AD are crucial, and the quest for new susceptibility genes or genes that can modify age at onset must go on in order to improve diagnosis, treatment and prevention of AD. A delay in age at onset of only a few years would decrease suffering and costs to a great extent [145]. Many hypothesis and theories about the disease pathology are now being investigated as new drugs and treatments, and some are currently also being tested in clinical trials. Hopefully, one of these will soon reach and aid suffering patients.

Acknowledgements

The work leading to this thesis was carried out in the group of Molecular Geriatrics, Department of Public Health and Caring Sciences, Uppsala University. I would like to express my sincere gratitude to all the people who have contributed to this thesis for your support and help in various ways. I would especially like to thank the following:

My main supervisor **Anna Glaser**, for your vast knowledge on genetic theory and helpful discussions on projects;

My co-supervisors **Martin Ingelsson** for your enthusiasm and over-flow of new project ideas, and **Lars Lannfelt** for letting me to do my PhD work in your group, and for providing nice co-workers in the group;

My co-authors for all your work resulting in this thesis, **Omanma Adighibe** and **Sampath Arepalli** for your help with the ARP project, and for nice lunches at NIA, **Mina Bergem**, **Margaret Gatz**, **Anna Glaser** for all our discussions on linkage and qPCR, **Alison Goate**, **Marian Hamshere** for our endless email conversations working out a pedfile for the ARPs, **Anita Hansson** for helping me ultra clean the lab before mRNA work at NIAAA, **John Hardy** for welcoming me to the NIA, **Markus Heilig** for welcoming me to the NIAAA, **Seppo Helisalmi**, **Mikko Hiltunen**, **Paul Hollingworth**, **Peter Holmans** for all your help understanding the mysteries of Sun *et al.*, **Bradley Hyman**, **Martin Ingelsson** for help with mRNA and qPCR work and for expression discussions, **Lena Kilander**, **Lars Lannfelt**, **Anbarasu Lourdusamy**, **Lars Nilsson** for doing the killing, **Michael Owen**, **Nancy Pedersen**, **Lena Skoglund** for helping me co-co-supervise, **Hilkka Soininen**, **Wolfgang Sommer** for helpful discussions on microarrays, **Massimo Ubaldi**, **Yijing Wang** for being a good master student, always wanting more work, **Fabienne Wavrant-De Vrièze** for introducing me to the not always so friendly robot and for teaching me how to make Excel dance, **Julie Williams**, and **Jayashree Viswanathan** for being crazy you;

All the patients who through their donations of blood and tissue have made the research presented in this thesis possible;

All the happy people at the Rudbeck Laboratory and especially in MolGer for fika and Friday beers and for making it funnier to go to work: **Anna G** for all stories about orientation and cake baking, **Anna L** for much appreciated kick-offs and activities, **Astrid** for your happiness, **Barbro** for chats at coffee breaks, **Charlotte** for fun in the office and at conferences, **Dag** for your never ending coffee enthusiasm, **Frida** for discussions at coffee and lunch, **Hille** for much appreciated kick-offs and activities, **Jocke** for your positivism, **Lena** for helping me keep the genetics flag high, **Martin** for scientific advice, **Lars** for providing papers impossible to find, **Mimmi** for always caring, **Ola** for Friday beers, **Paul** for trying your best to awaken my, then, non-existing beer taste buds, **RoseMarie** for all your laughs, **Sofia** for providing new blood to the group, **Sofie** for discussions at genetics meetings and at coffee, **Therese** for fun in Chicago and at work, **Tomas** for adding your Norrlandian humor to the group, **Vilmantas** for your immense knowledge in genetic statistics and computers, and **Xiao** for laughs; A sincere THANK YOU ALL for these years!

Former members of MolGer: **Anna B**, **Ann-Sofi, Fredrik, Jovanka, Pär G**, **Sara,** and **Yijing;**

Present and former members of the Uppsala University Medical faculty PhD-student council for all the fun pizza filled meetings and for support during my time as chairman, especially **Jessica**, **Per L**, **Sara,** and **Térèse**; also my fellow student companions in the faculty board, **Karin, Per Ö** and **Shima;** and finally **Ulf P** and **Britt** for teaching me so much about how the Medical faculty is run, we've had our discussions but in the end I think we all won;

Everyone at NIA for making my six months memorable and fruitful, especially **Janet** and **Sampath** for lunches; **Amanda, Behnosh** and **Leslie** for party nights; **Elisa** for beer quiz nights and for being the best roommate I could have wished for, and **Fabie** for all you've taught me;

Everyone at the Clinic of Geriatrics;

The administration of the Department of Public Health and Caring Sciences, especially **Marianne Carlsson** and **Karin Torbratt**;

All administrative and computer staff at Rudbeck laboratory for making the house run smoothly;

H/järngänget, **Carolina**, **Henrietta**, **Hille**, **Karin**, **Kristina,** and **Lina** for fun during the Biomedicine program and reunions over the years;

My second family, **Anna, Helena, Marcus, Robert**, and **Roger**, thanks for Åland and for all our fun dinners and outings;

Ann-Sofi for all our party nights and crazy trips (yes, it all works out in the end!);

And finally **Markus** and my **family** for all your love and support!

The following foundations are acknowledged for financial support: **Alzheimerfonden**, **Bertil Hållstens forskningsstiftelse**, **Demensfonden**, **Emma Petterssons testamente**, **Gun och Bertil Stohnes stiftelse**, **Hjärnfonden**, **Stiftelsen Goljes minne**, **Stiftelsen Ragnhild och Einar Lundströms minne**, and **Vetenskapsrådet**.

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