

**Alzheimer's Disease: effect of Tau-related genes on the  
pathology, neurochemistry and risk of disease**

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*“A theory is something nobody believes, except the person who made it.  
An experiment is something everybody believes, except the person who made it”*  
Albert Einstein (1879-1955)

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## ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia in the elderly. It is a genetically heterogeneous disease characterized by progressive cognitive decline and memory impairment. In the rare early-onset familial form of the disease (FAD), causative mutations have been found in three different genes, the amyloid precursor protein (*APP*) gene and the presenilins 1 and 2 (*PSEN-1 and -2*). The predominant late-onset form of AD (LOAD) or sporadic AD is a genetically complex disorder probably involving a combination of genetic factors together with environmental influences. To date, the best established genetic risk factor identified for this complex form of AD is the *APOE*  $\epsilon$ 4 allele. However not all AD cases have the *APOE*  $\epsilon$ 4 allele, thus several susceptibility genes remain to be found.

One of the characteristics of AD is the intraneuronal accumulation of neurofibrillary tangles (NFTs). NFTs are composed of a hyperphosphorylated form of the microtubule-associated protein tau. When tau is in its hyperphosphorylated form, the role of maintain neuronal morphology by promoting the assembly and stabilization of microtubules is impaired. Since tau pathology is a central and important event in the AD pathogenesis this thesis has focused on studying genes that are directly or indirectly related to tau and investigate their potential as candidate genes for AD risk. Our aim was also to study how variants of these genes may affect the pathology and neurochemistry in AD.

In the initial investigation, a polymorphism in the cell division cycle (*CDC2*) gene was identified. Cdc2 belongs to the cyclin-dependent kinase (cdk) family and is a protein kinase involved in cell cycle regulation and in neuronal differentiation. In AD brain, cdc2 is expressed in neurons and is involved in the abnormal phosphorylation of tau. The polymorphism was tested for association with sporadic AD. A significant association between the *CDC2* II genotype and AD was found, and also an increased frequency of the *CDC2* I allele in both AD and frontotemporal dementia (FTD).

In the following study, patients with AD, FTD and Parkinson's disease (PD) were genotyped for a polymorphism in the Saitohin (*STH*) gene, a previously described gene located in on of the introns of the human *TAU* gene. Numerous polymorphisms span the human tau gene and are in complete linkage disequilibrium with each other yielding two separate haplotypes, termed H1 and H2. A single nucleotide polymorphism (SNP) in the *STH* gene has been suggested to be involved in sporadic AD and is in complete linkage disequilibrium with the *TAU* haplotype H1. Patients with AD, FTD and PD and control subjects were genotyped for the *STH* polymorphism and/or the *TAU* haplotype. Genotype data were tested for their association to the AD biomarkers total-tau, phospho-tau and  $A\beta_{1-42}$  in the cerebrospinal fluid (CSF) and to neuropathological scores of senile plaques (SPs) and NFTs in frontal cortex and in hippocampus in patients with autopsy-confirmed AD. The *STH* Q7R polymorphism and the *TAU* haplotype were in complete linkage disequilibrium in all patients (AD and FTD) and control subjects investigated for both genes. There were no significant differences in genotype or allele distributions in AD, FTD or PD patients compared to control subjects. Neither *TAU* haplotype nor *STH* influenced CSF biomarkers or neuropathological scores significantly.

In the next study, we followed up the findings from paper I and investigated the possible effects of the *CDC2* polymorphism on CSF biomarkers and neuropathological scores in AD patients. *CDC2* genotypes were evaluated for their association to CSF protein levels of total-tau, phospho-tau and  $A\beta_{1-42}$  and to amount of SPs and NFTs. The

*CDC2* I allele was associated with a gene dose-dependent increase of CSF total-tau levels and the homozygous *CDC2* II genotype was significantly more frequent among AD patients compared to control subjects.

In conclusion, the results from paper I suggest a link between the *CDC2* gene and AD. This is further supported by the findings from paper III, where we could provide evidence for an involvement of *CDC2* in the pathogenesis of AD. We found no evidence that could support a major pathogenic role of *STH* and *TAU* haplotype in AD, FTD or PD in paper II.

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers:

**I Johansson A**, Hampel H, Faltraco F, Buerger K, Minthon L, Bogdanovic N, Sjogren M, Zetterberg H, Forsell L, Lilius L, Wahlund LO, Rymo L, Prince JA, and Blennow K. "Increased frequency of a new polymorphism in the cell division cycle 2 (*CDC2*) gene in patients with Alzheimer's disease and frontotemporal dementia" *Neuroscience Letters* 2003 April 340 (1) 69-73.

**II Johansson A**, Zetterberg H, Håkansson A, Nissbrandt H and Blennow K. "TAU haplotype and Saitohin Q7R gene polymorphism do not influence cerebrospinal fluid levels of tau and  $\beta$ -amyloid<sub>1-42</sub>, in Alzheimer's disease and frontotemporal dementia" *Neurodegenerative Diseases* 2005 2 (1) 28-35.

**III Johansson A**, Zetterberg H, Hampel H, Buerger K, Prince J, Minthon L, Wahlund LO and Blennow K. "Genetic association of *CDC2* with CSF tau in Alzheimer's disease" *Dementia and Geriatric Cognitive Disorders* 2005 20 (6) 367-374.

**IV Sjölander A**, Andersson M, Zetterberg H, Minthon L, Bogdanovic N and Blennow K. "The *CDK5* gene and effect on CSF biomarkers and neuropathology in Alzheimer's disease" *Manuscript* 2007.

## RELATED PUBLICATIONS

**Johansson A**, Katzov H, Zetterberg H, Feuk L, Johansson B, Bogdanovic N, Andreasen N, Lenhard B, Brookes AJ, Pedersen NL, Blennow K and Prince JA. "Variants of *CYP46A1* may interact with age and *APOE* to influence CSF- A $\beta$  levels in Alzheimer's disease" *Human Genetics* 2004 May 114 (6) 581-587.

Andersson ME, **Sjölander A**, Andreasen N, Minthon L, Hansson O, Bogdanovic N, Jern C, Jood K, Wallin A, Blennow K and Zetterberg H. "Kinesin gene variability may affect tau phosphorylation in early Alzheimer's disease" *International Journal of Molecular Medicine* 2007 August 20 (2) 233-239.

**Sjölander A**, Minthon L, Bogdanovic N, Wallin A, Zetterberg H and Blennow K. "The *PPAR-a* gene in Alzheimer's disease: Lack of replication of earlier association" *Neurobiology of Aging* 2007 September, *in press*.

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## LIST OF ABBREVIATIONS

A $\beta$	Amyloid-beta
AD	Alzheimer's disease
ApoE	Apolipoprotein E protein
APOE	Apolipoprotein E gene
APP	Amyloid precursor protein
CDC2	Cell division cycle 2
CSF	Cerebrospinal fluid
CT	Computerised tomography
CYP46	Cholesterol 24-hydroxylase
DASH	Dynamic Allele-Specific Hybridization
DNA	Deoxyribonucleic acid
ELSA	Enzyme-linked immunosorbent assay
FAD	Familial Alzheimer's disease
FRET	Fluorescence resonance energy transfer
FTD	Frontotemporal dementia
LD	Linkage disequilibrium
LOAD	Late onset Alzheimer's disease
MRI	Magnetic resonance imaging
MMSE	Mini mental state examination
NFT	Neurofibrillary tangles
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association
PSEN1	Presenilin 1
PSEN2	Presenilin 2
RFLP	Restriction fragment length polymorphism
SNP	Single nucleotide polymorphism
SP	Senile plaque
STH	Saitohin
T <sub>m</sub>	Melting temperature

## INTRODUCTION

### 1.1 Genetic variation

The genetic material differ approximately 1 % between human individuals [1]. Differences in the DNA sequence (genotype) can result in a physical change (phenotype), but at most no dramatic change is seen. During the last century much research has been done to try to explain how genetic differences affect our phenotype, and especially the variation in complex traits and diseases.

#### *Single nucleotide polymorphisms*

Changes in the DNA sequence are called mutations, which can occur through natural internal processes like replication, recombination, gene conversion, but also through environmental factors like radiation and toxins. A polymorphism can be defined as a mutation that is more frequent than 1 % in a population, but another definition is when a DNA sequence variation gives rise to a disease or trait it is referred to as a mutation, otherwise it is termed a polymorphism. Different types of polymorphisms exist such as insertions, deletions, substitutions or repetitive elements.

The most abundant form of human genetic variation is single-nucleotide polymorphism (SNP), which only involve changes of a single base. The most frequent consequences of a SNP in an exon are a silent mutation or a missense mutation; the first does not change the resulting protein sequence and the latter leads to an amino acid change. One common application of SNPs is the use in association studies, that look for a statistically significant association between SNP alleles and phenotypes (usually diseases), in order to find candidate causative genes [2, 3]. Because of this, large databases of SNPs have been developed.

### 1.2. Genetic studies

#### *Mapping the genome*

Different maps are used to navigate the genome. One way to map the genome is the use of genetic markers, which are polymorphisms at specific locations on the chromosomes. These sequences usually do not affect the phenotype, but can be used to determine relative distances between the marker and a gene or other genetic characteristic.

In physical maps the actual DNA sequence on each chromosome is shown, in which distance is measured by nucleotides [4]. Another way to map the genome is by genetic maps and the distance between two genetic markers is measured in the unit centiMorgan (cM). This unit is a measure of the probability that a recombination will occur between the two markers. One cM is equivalent to 1 % recombination.

There are two copies of each chromosome and these have important genetic similarities and contain identical gene sites (loci). Some genes segregate as if they were linked together. Such genes are part of the same chromosome and are transmitted as a single unit and show linkage in genetic crosses. Alternative forms of the same gene can occur and are referred to as alleles. In a population of members of the same species, many different alleles of the same gene can exist. A specific combination of alleles along a chromosome is called a haplotype. During meiosis exchange of chromosome segments, called recombination, may take place and results in creation of new haplotypes. The use of haplotypes in genetic disease research has increased over the past years. Traditional

SNP association studies of one or a few candidate genes have limitations since only a small portion of the sequence variation is examined in each patient. The recently completed International HapMap project is a tool to evaluate how common genetic variations influence common diseases. The project aimed to describe haplotypes in four population samples in order to identify a minimum set of SNPs, so called “tagging SNPs”, sufficient to represent all common variants in our genome [5-8].

Numerous recombinational events can occur for each chromosome. The rate of recombinational events is generally proportional to the distance between two loci on the chromosome. However, there is no absolute correlation between genetic and physical distance and it varies between different regions of the genome. It is therefore complicated to have a correct conversion factor between genetic and physical maps, but a common estimate is that 1 cM is equivalent to approximately 1 mega-bases (Mb) of the DNA sequence. Alleles of neighboring loci tend to be inherited together and this association of alleles in the human genome is called linkage disequilibrium (LD). This means that for two markers a specific combination of alleles is more frequent in a population than would be expected by chance.

When studying diseases, physical maps, genetic maps and linkage disequilibrium (LD) can together be used to navigate the genome to find interesting parts [4], and a central issue in human genetics is the use of LD in mapping genes involved in different diseases.

#### *Disease research*

A disease that does not follow the basic Mendelian laws of inheritance is referred to as a complex disease [9]. Many complex diseases, like Alzheimer’s disease, are not caused by a defect in a single gene. It may be an interaction between multiple genes, environmental factors and/or a combination of several gene products. Many of the complex diseases have a higher age of onset compared to most monogenic diseases. This may mirror an accumulation of environmental factors or of long-term gene interaction effects. Few complex diseases in humans have been completely understood, probably there is a mixture of genetic and allelic heterogeneity. There are two common methods to find human disease genes. The previous knowledge about the disease decides which method to use. In the functional cloning method knowledge about the function or structure of the protein is used to isolate the related gene. The method positional cloning requires no previous biological information of the disease. This method has been used for simple diseases. The gene encoding presenilin (PSEN) involved in AD has been identified through positional cloning [11, 12]. In the first step of this method the chromosomal region likely harboring the disease gene must be identified, followed by isolation of the gene. Linkage studies and association studies are two tools for these strategies, where neutral polymorphisms (genetic markers) are used to find the mutations that cause the disease. Combinations of linkage and association methods have been shown to be a good way to map genetic risk factors of complex disease. Initial mapping by linkage studies often result in a large region of <10 Mb on the chromosome. If these linkage studies are followed by association studies of the candidate genes in these regions, size of the assumed region can be reduced. Risk alleles for late-onset Alzheimer’s disease have been isolated according to this approach [13].

The aim of a linkage study is to see how genes are transmitted in a pedigree. Information from numerous families can expose certain regions of the genome that is

transmitted together with the disease in the pedigree. Linkage studies have been shown to be a good way to explore monogenic diseases. One of the main differences between linkage studies and association studies is that the latter are not normally based on family materials. For many complex diseases, families can be difficult to find or recruit and therefore an alternative approach is to perform population-based association analysis. In this approach a population with large groups of not related patients and control subjects are selected. To maximize the chances for success, it is important to “match” the two groups as much as possible in regard to age, sex, ethnic background and any environmental factors, so the only known difference between the two groups is the diagnosis.

During the last years, large efforts have been made to complete the sequencing of the human genome. This together with the fact that the majority of all SNPs now have been cataloging in databases and the development of more effective SNP genotyping technologies have made a non-hypothesis driven genome-wide association methodology more easily viable. A minimum of 300,000 SNPs throughout the genome are necessary to perform a genome-wide association study [14, 15].

#### *Problems in genetic studies*

When setting up an association study one important decision to make is choose of genetic marker. Genetic markers located in or around genes that are suspected to be involved in the disease are selected. These so-called candidate genes are said to be biological candidates if previous knowledge suggests that a certain protein or biological pathway is important for the development of the disease. Then a hypothesis can be set up; are mutations in the gene causative for the disease? Alternatively, candidate genes can be positional candidates because of their locations on the chromosomes. Selection of SNPs for association studies can be done so those SNPs that are most likely to directly influence the disease are included [16].

A strong correlation does not automatically mean that the studied allele directly influences the disease phenotype. There are number of reasons why an association can be found when there is no real involvement of the studied genetic marker in the disease. One alternative explanation could be that the chosen genetic marker happens to be on the same haplotype as the pathogenic one. In this scenario, the linkage disequilibrium (LD) between the markers is responsible for the strong association.

Errors can also explain association signals, which can stem from improper selection of cases and controls. Another likely problem is the random chance that the chosen sample not is representative for the large population. Two types of errors can occur. Firstly, false positive results can be obtained, which is referred to as Type I errors. On the other hand, a false negative finding can be obtained, called Type II errors [17]. These types of errors can be reduced by increasing the numbers of cases and controls studied, but also increasing the stringency of the statistical method, alone or in combination with increased sample size. There is a direct relationship between the number of individuals included in the study and the statistical measure of power. With power means: “the probability to find a correlation if it truly exist”. Power should be between 80 and 95 % to find a statistical significant association of a variant genotype that doubled the risk for disease.

Another problem with genetic studies could be the phenomenon of gene interaction, when effects of one or more genes in determining the occurrence of a disease

are modified by the presence or absence of another gene or genes. Expression of one of the genes can mask the expression of the other gene or genes. In other cases, the involved genes may influence each other in a complementary or cooperative way.

#### **1.4. Alzheimer's disease**

In 1906 a Bavarian psychiatrist named Alois Alzheimer was the first to define the clinicopathological syndrome Alzheimer's disease (AD). He described a 51-year old woman, who had developed memory deficits and progressive loss of cognitive abilities. Her memory became increasingly impaired and she died within five years after the onset of illness. Alois Alzheimer observed severe atrophy of the brain and the characteristic neuropathological changes consisting of neurofibrillary tangles (NFTs) and senile plaques (SPs) [18, 19]. These hallmarks are still used to confirm the diagnosis. AD usually begins with episodic memory impairment and encompasses language, visuospatial and behavioral dysfunction. Progression of the disease leads to degeneration and death of more nerve cells with subsequent cognitive and behavioral changes. The ability to recognize faces and to communicate is completely lost in the final stages. Patients lose bowel and bladder control, and eventually need constant care. This stage of complete dependency may last for years before the patient dies. The average length of time from diagnosis to death is 4 to 8 years, although it can take 20 years or more for the disease to run its course.

##### *Epidemiology*

AD is the most common form of dementia in the elderly and is responsible for more than 50 % of all dementias. AD represents the fourth largest cause of death in the developed world [20]. One of the risk factors for developing dementia is high age. After an age of 60 years has been reached, the incidence is doubled every 5 years [21].

##### *Diagnosis*

AD is usually classified according to its age of onset. When the disease occurs before 65 years of age it is called early-onset ("presenile") AD, while late-onset ("senile") AD occurs in subjects over 65 years of age. The most common way to clinically diagnose AD is based on the criteria of the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV-TR) and National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association (NINCDS-ADRDA) Work Group [22].

Based upon neurological examination, neuropsychological tests and brain imaging techniques like magnetic resonance imaging (MRI) and computerised tomography (CT), the accuracy level of a clinical diagnosis can be "probable" or "possible" AD. A diagnosis of "probable" AD indicates that all other disorders that may be causing dementia have been ruled out and that symptoms are most likely be the result of AD. A diagnosis of "possible" AD indicates the presence of another disorder that may be affecting the known progression of AD, so that the process is somewhat different than what is normally seen. In this case, however, AD is still considered the primary cause of dementia symptoms. A common way to determine the cognitive status is by the Mini Mental State Examination (MMSE) [23]. Questions are asked in a specific order and scored immediately. The maximum MMSE score is 30, and subdivisions have been created to determine the severity of AD: mild AD (21-26), moderate AD (12-20) and severe AD (<12).

A diagnosis of “definite” AD requires post-mortem detection of SPs and NFTs and can be made only at the time of autopsy. This is the generally accepted way to diagnose AD with certainty [22]. Unfortunately, there is a large overlap in neuropathology between AD and other forms of dementia [24], and an important number of non-demented individuals have been post-mortem diagnosed with AD due to their amounts of SPs and NFTs [25].

According to the NINCDS-ADRDA recommendations, cerebrospinal fluid (CSF) examination should be used to exclude non-AD dementia. Total-tau, phospho-tau and  $A\beta_{1-42}$ , are three markers that are widely used to reflect the central pathogenic processes in the brain. In AD, both total-tau and phospho-tau are elevated in CSF compared with levels in healthy control subjects. For  $A\beta_{1-42}$ , it is typical that the CSF levels are decreased. This is however not specific for AD since decreased levels of  $A\beta_{1-42}$  and increased levels of total-tau and phospho-tau are found in other forms of dementias [26]. The best way is to combine these three CSF biomarkers in the diagnostic procedure. A combination of these can predict AD in a follow-up for 4-6 years with a sensitivity and specificity of >90% and >85% respectively [27].

Recently a revision of the above mentioned diagnostic criteria has been proposed [28]. Since the understanding of AD is improved due to greater scientific knowledge this working group suggests a new way to diagnose AD by a combination of old and new criteria [28]. These proposed criteria aim to define the clinical, biochemical, structural, and metabolic presence of AD to establish a positive diagnosis [28].

### *Neuropathology*

The characteristic hallmarks for AD are intracellular accumulation of NFTs, extracellular deposits of SPs and degeneration of neurons and synapses. These neuropathological changes also occur in non-demented elderly individuals, but to a lesser extent than in AD patients [29, 30].

NFTs consist of insoluble twisted fibers that are found inside the neurons. NFTs is composed of a hyperphosphorylated form of the microtubule-associated protein tau [31]. When tau is in its hyperphosphorylated form, the role of maintain neuronal morphology by promoting the assembly and stabilization of microtubules is impaired [32].

Two types of plaques occur that can be morphologically distinguished from each other, termed senile (or neuritic) and diffuse plaques. Senile plaques are spherical lesions that contain fibrillized  $A\beta$ , a 39-43 amino acid peptide derived from proteolytical processing of the amyloid precursor protein (APP), and are surrounded by dystrophic neurites reactive glia. Diffuse plaques contain amyloid deposits but in contrast to senile plaques there are few or no amyloid fibrils.

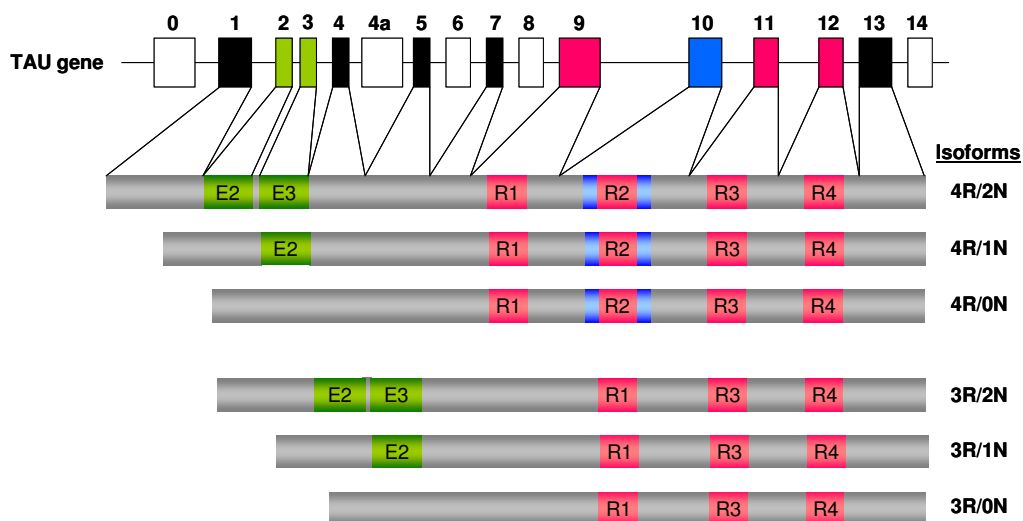
There is an overall shrinkage of brain tissue as AD progresses. In addition, the ventricles are noticeably enlarged. In the early stages of AD, short-term memory begins to decline when the neurons in the hippocampus, which is part of the limbic system, degenerate. As AD spreads through the cerebral cortex judgment declines, emotional outbursts may occur and language is impaired.

### *Tau*

Microtubules and their associated proteins (MAPs) are key cytoskeletal elements in neuronal cells, and transport nutrients and other important substances from one part of the nerve cell to another. Tau, MAP1 (A/B) and MAP2 are the main neuronal MAPs

found in normal mature neuron and are present in different compartments of the neuron. MAP2 is found in the somatodendritic part, whereas tau is localized in the cell body and axons. MAP 1B have been found to be abundant in the newborn brain and decreases with development, in contrast to MAP 1A which increased with development [33].

The function of tau is to promote microtubule (MT) assembly and stabilization [34, 35]. Tau is a phosphoprotein and interacts with microtubule via specific binding domains in its C-terminus [32]. The human *TAU* gene is located on chromosome 17q21 and contains 16 exons (E), including E0, which is a part of the promoter (Fig. 3). Alternative mRNA splicing of E2, E3, and E10 produces six tau isoforms [36-38], which differ by the presence of either three (3R) or four (4R) carboxy-terminal tandem repeats of 31-32 amino acids (Fig. 3). In neurodegenerative disorders like AD and frontotemporal dementia (FTD), tau protein becomes abnormally hyperphosphorylated and is accumulated as intraneuronal tangles of paired helical filaments (PHF) [39]. Hyperphosphorylation causes detachment from MT and thereby tau loses its ability to MT assembly. Hyperphosphorylation is believed to be an early event that leads from soluble to insoluble and filamentous tau protein [40]. However, it is unclear whether this is sufficient for assembly into filaments. The accumulation of the abnormally hyperphosphorylated tau is associated with neurofibrillary degeneration and dementia.



**Figure 3.** Genomic structure of the human *TAU* gene, located in chromosome 17q21. *TAU* contains 16 exons and by alternative splicing six isoforms are produced. The isoforms by the presence of either three (3R) or four (4R) carboxy-terminal tandem repeats of 31-32 amino acids, and are referred to the designations seen in the figure.

The findings of mutations in *TAU* gene and their association with the inherited frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) disease has established that abnormalities in tau protein can lead to neurodegeneration and dementia [41-43]. The phosphorylation state of tau is regulated by the balance between protein kinases and protein phosphatases. Tau is a substrate for numerous protein kinases

[44] and is phosphorylated at over 30 serine/threonine residues in AD [45, 46]. Among these kinases both cell division cycle (*cdc2*) and cyclin dependent kinase 5 (*cdk5*) have been implicated in the tau hyperphosphorylation process [47].

In addition to kinase and phosphatase activities, tau phosphorylation is also substrate regulated. Certain mutations in TAU gene give rise to a more favorable substrate for hyperphosphorylation than wild-type tau [48].

### *Biomarkers in CSF*

Biochemical changes in the brain are reflected in the cerebrospinal fluid (CSF), since the liquid is in direct contact with the extracellular space of the brain. CSF biomarkers should reflect the central pathogenic processes in the brain. Total-tau, phospho-tau and  $A\beta_{1-42}$  are three biomarkers for AD that have been evaluated in numerous studies.

CSF total-tau is increased in AD patients, which has been shown in several studies [49]. Enzyme-linked immunosorbent assay (ELISA) has been used to develop methods to measure total-tau in CSF [50]. Levels of total-tau probably reflect the extent of neuronal damage and degeneration.

Hyperphosphorylation of tau is found during neuronal development and in several neurodegenerative disorders [32, 51]. Approximately 30 phosphorylation sites have been found on the tau protein [32]. Whether some of them are specific for AD is not clear. In this thesis an ELISA method for quantification of tau phosphorylated at threonine 181 has been used [52]. The levels of phospho-tau in CSF should reflect the phosphorylation state of tau. Elevated levels of phospho-tau in CSF is not simply a measurement of neuronal degradation, since these levels are normal or just mildly increased in Creutzfeldt Jacob's disease (CJD) [53].

Immunoassays specific for detection of the 42 amino acid variant of  $A\beta$  have been developed, since this is the predominating form found in diffuse plaques [54, 55]. Decreased CSF levels of  $A\beta_{1-42}$  in AD patients have been found in several studies [56]. The reduced levels might be caused by deposition of  $A\beta_{1-42}$  into plaques, since strong correlations between low levels of  $A\beta_{1-42}$  in ventricular CSF and number of plaque have been found [57]. It has also been shown that in vivo brain amyloid load well correlates with CSF levels of  $A\beta_{1-42}$  [58]. Via positron emission tomography imaging of the amyloid-binding agent, Pittsburgh Compound-B (PIB) the in vivo brain amyloid load was determined and then compared with  $A\beta_{1-42}$  levels in CSF [58]. It was evident that subjects with positive PIB binding had the lowest CSF  $A\beta_{1-42}$  level, and those with negative PIB binding had the highest CSF  $A\beta_{1-42}$  level [58].

{“The environment is everything that isn't me”  
Albert Einstein}

### *Genetics*

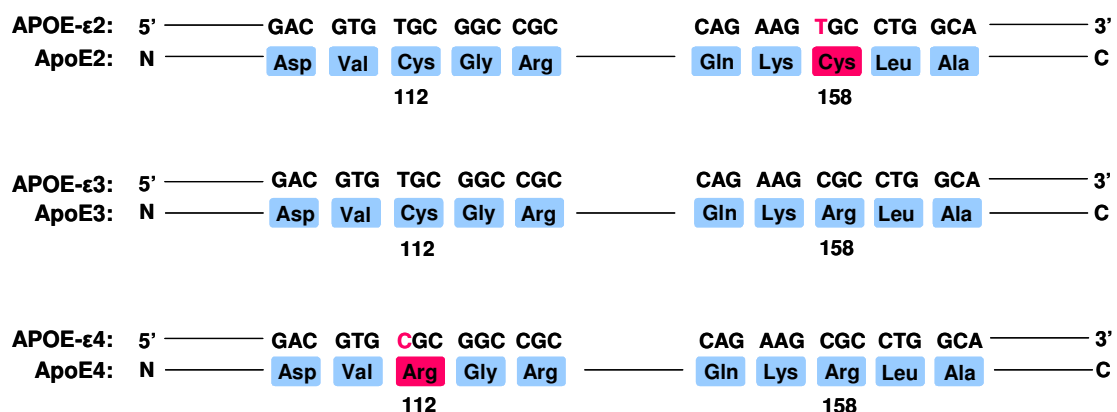
Many complex diseases, like AD, are not caused by a defect in a single gene, rather they arise from the interaction of multiple genes and environmental factors. AD is a genetically heterogeneous disorder, which can be divided into an early-onset familial form (FAD) and a late-onset sporadic form (LOAD). FAD has an autosomal dominant inheritance, and to date causative mutations have been found in three different genes, the amyloid precursor protein (*APP*) and the presenilins 1 and 2 (*PSEN-1* and *-2*). In the mid 1980s it was shown that individuals with Down's syndrome develop the clinical and neuropathological features of AD [59, 60]. These data pointed to the involvement of



chromosome 21 in AD, since individuals with Down's syndrome have an extra copy of chromosome 21. The first genetic linkage was discovered between a locus on chromosome 21q and FAD [61], and additionally the gene encoding *APP* was mapped to chromosome 21 [62, 63]. The first specific genetic cause of AD to be identified was the occurrence of mutations in the *APP* gene [64]. Direct nucleotide sequencing of exon 17 of the *APP* gene led to the discovery of several missense mutations in families with FAD. The mechanism by which the *APP* mutations are causative for AD has been suggested to be an altered metabolism of APP, since the mutations are located very close to the sites of secretase cleavage flanking the A $\beta$  sequence [65-67]. The Swedish mutation *APP* 670/671 produces elevated levels of the soluble A $\beta$  peptide [68, 69], and the *APP* 717 mutation produces more than two-fold of the longer and more insoluble form of the A $\beta$  peptide [65].

Since most FAD families do not have mutations in the *APP* gene, it was expected that other AD loci might exist. In 1992 a locus on the long arm of chromosome 14 was detected by linkage analysis. A few years later the *PSEN1* gene was identified and isolated by positional cloning strategy [12, 70]. A second gene was found based on its homology to *PSEN1* and mapped to 1q31-q42 [11]. Mutations in these two genes (*PSEN1* and *PSEN2*) are believed to cause up to 80 % of the familial early-onset AD cases. There is strong evidence showing that *PSEN1* has  $\gamma$ -secretase activity or at least is a cofactor for  $\gamma$ -secretase, the enzyme involved in the metabolism of APP and Notch [71-73]. To date there are more than 120 different mutations identified in the *PSEN1* gene, while only eight missense mutations have been identified in *PSEN2* (<http://molgen-www.uia.ac.be/ADmutations>).

However, the predominant sporadic form of AD is a genetically complex disorder probably involving a range of genetic factors combined with environmental influences. In the vast majority of sporadic AD there is no recognizable pattern of classical Mendelian inheritance, although epidemiological, family and twin studies suggest a significant proportion of sporadic AD being attributable to genetic factors [74].



**Figure 1.** The positions of the three allelic forms existing in the *APOE* gene, designated  $\epsilon$ 2,  $\epsilon$ 3 and  $\epsilon$ 4. Under the nucleotide sequence their corresponding isoforms of the ApoE protein are shown: ApoE2, ApoE3 and ApoE4. These isoforms differ in amino acid sequence at two sites, residue 112 and residue 158.

The best established susceptibility gene is the apolipoprotein E gene (*APOE*), located on chromosome 19q13.2, which has been shown to modify both the risk and age at onset of sporadic AD [75, 76]. ApoE is a plasma glycoprotein synthesized mainly by the liver, by neurons and astrocytes in the brain, and also by other cell types including macrophages and monocytes [77]. In the brain apoE is involved in the mobilization and redistribution of cholesterol during neuronal growth and after injury [78]. It is also involved in many other functions in human beings, like nerve regeneration, immunoregulation and activation of several lipolytic enzymes [79, 80]. The *APOE* gene exists in three allelic forms designated  $\epsilon 2$ ,  $\epsilon 3$  and  $\epsilon 4$ , which give rise to three isoforms of the apoE protein: apoE2, apoE3 and apoE4. These isoforms differ in amino acid sequence at two sites, residue 112 and residue 158 (Fig. 1) [81]. One study has shown an association between the *APOE*  $\epsilon 4$  allele and sporadic AD in a dose-dependent manner [82]. Taken together, the *APOE*  $\epsilon 4$  allele, only accounts for a fraction of all sporadic AD cases, thus several susceptibility genes remain to be found. Other potential susceptibility genes for AD have been suggested, but the allele frequencies have often been only marginally different from controls, and the associations have not always been confirmed in subsequent studies.

#### *APP and A $\beta$*

The major constituent of extracellular plaques is the 39-43 amino acid residue A $\beta$  [83, 84]. The presence of A $\beta$  deposits in AD brains depends not only on the rate of synthesis of the peptide, but also on its aggregation and degradation. A $\beta$  is derived from the proteolytic processing of APP by two proteases, referred to as  $\beta$ - and  $\gamma$ -secretases. APP occurs in three alternatively spliced forms with 695-, 751- and 770-residue polypeptides.

Cleavage by  $\beta$ -secretase generates an approximately 100 kDa soluble N-terminal fragment and a 12 kDa C-terminal fragment of APP called C99.  $\beta$ -secretase can also cleave APP at another site nearby yielding C89, a C-terminal fragment of 89 amino acids (Fig. 2) [85]. The C99 fragment can be further cleaved by  $\gamma$ -secretase to yield two major variants of A $\beta$ , the 40 amino acid long peptide A $\beta_{(1-40)}$  and the 42 amino acid long variant A $\beta_{(1-42)}$ . In the major processing pathway,  $\alpha$ -secretase cut APP in the middle of the A $\beta$  region, which prevents the formation of the A $\beta$  peptide (Fig. 2). The  $\alpha$ -secretase cleavage products are an N-terminal soluble fragment ( $s\alpha$ APP) and a membrane-bound C-terminal fragment (C83) [86]. The resulting C83 can subsequently be cleaved by  $\gamma$ -secretase to yield p3<sub>40</sub> and p3<sub>42</sub> (Fig. 2).

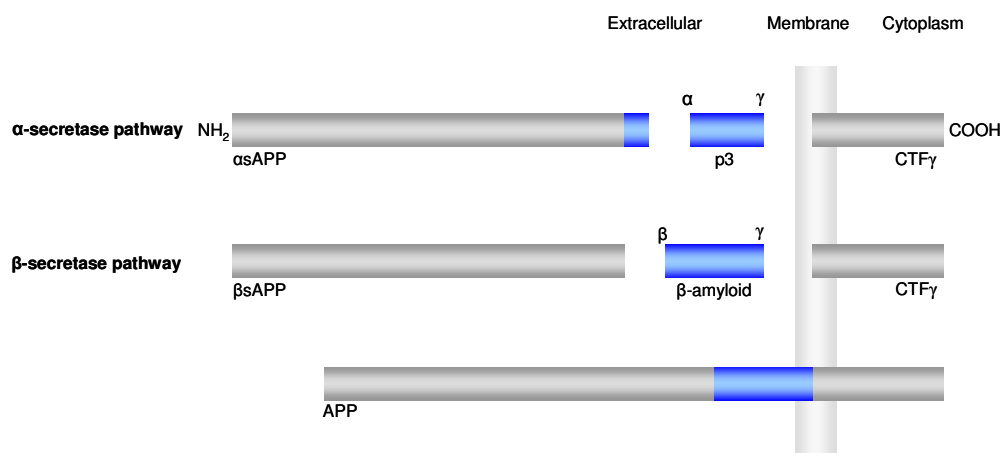
Once A $\beta$  is generated, some portion of monomeric A $\beta$  species can undergo oligomerization to form oligomers, which can subsequently aggregate into fibrils. Extensive amyloid plaque formation in brains of AD patients presumably arises as a result of an imbalance between production and clearance of A $\beta$ .

#### *$\alpha$ -, $\beta$ - and $\gamma$ -secretase*

Several independent studies lead to identification of the  $\beta$ -site APP cleaving enzyme (BACE) [85, 87-89]. The cloned aspartyl protease, BACE, is a 501 amino acid transmembrane protein and shows all the properties described for the  $\beta$ -secretase. Overexpression of BACE yield increased  $\beta$ -secretase cleavage products and presence of an antisense inhibitor to BACE reduce these cleavage products [85, 88]. BACE is expressed in all tissues and at high levels in all brain regions. Furthermore, BACE cleaves APP at the known  $\beta$ -secretase cleavage sites, Asp 1 ( $\beta$ -site) and Glu 11 ( $\beta'$ -site) [85].

To date, the proteins presenilin, nicastrin, aph-1 and pen-2 have been shown to be parts of the  $\gamma$ -secretase complex [90]. PSEN1 and PSEN2 are transmembrane proteins that undergo constitutive endoproteolysis in many cell types and in the brain, and thus exist in major part as stable heterodimers composed of the N-terminal and the C-terminal fragment [91, 92]. PSEN1 plays a required role in the  $\gamma$ -secretase cleavage of APP [93] and has been suggested to contain the active site of  $\gamma$ -secretase [94, 95]. Mutation of either Asp 257 or Asp 385 in PSEN1 was found to inhibit A $\beta$  production and to accumulate the  $\gamma$ -secretase substrates C83 and C99 [96].

Cleavage at the  $\alpha$ -secretase site in APP prevents the formation of full length A $\beta$ , and is referred to as the non-amyloidogenic pathway. Recent studies have proposed some proteases to be responsible for the  $\alpha$ -secretase activity. These proteases belong to a family of disintegrin and metalloproteases, ADAM 9, 10 and 17 [97], and overexpression of ADAM 10 in cells, leads to elevated  $\alpha$ -secretase activity. In addition, a member of the ADAM family was also found to regulate the similar extracellular  $\alpha$ -secretase-like cleavage of the Notch protein [98]. In vitro studies showed that the activity responsible for this cleavage is due to ADAM 17 or tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-converting enzyme (TACE).



**Figure 2.** Proteolytic cleavage of APP. In the  $\beta$ -secretase pathway, APP is first cleaved by  $\beta$ -secretase, generating  $s\beta$ APP and membrane-bound C-terminal fragments (CTFs): CTF $\beta$  (or CTF $\beta'$  depending on the cleavage site). The following  $\gamma$ -secretase cleavage produces A $\beta$  and CTF  $\gamma$ . In the  $\alpha$ -secretase pathway, APP is first cleaved by  $\alpha$ -secretase, generating  $sa$ APP and membrane-bound CTF $\alpha$ . The following  $\gamma$ -secretase cleavage produces p3 peptide and CTF  $\gamma$ .

### 1.5. Frontotemporal dementia and Parkinson's disease

Frontotemporal dementia (FTD) is a progressive neurodegenerative syndrome with diverse clinical presentations. FTD is the third most common form of the presenile dementias [99], and occurs on average in patients about a decade earlier than the onset of AD [100, 101]. In the majority of cases onset occurs between the ages of 45 and 60 [102].

The clinical diagnosis is often made on the basis of a detailed cognitive and behavioural assessment and is supported by cerebral imaging showing involvement of the frontal lobes and/or the anterior part of the temporal lobes, and normal EEG. FTD patients have in common a good day-to-day episodic memory and no spatial disorientation, which distinguish them from AD [103, 104]. Disorders that share abundant filamentous tau pathology and brain degeneration in the absence of extracellular amyloid deposits are referred to as tauopathies [105]. Progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) and Pick's disease (PiD) are three such disorders, and they have been classified as disorders belonging to a group of diseases known as frontotemporal dementia (FTD).

PiD was first described by Arnold Pick in 1892. Argyrophilic inclusions (Pick bodies) and swollen cells (Pick cells) were found in frontal and temporal brain regions. The plaques and tangles typical for AD brains are not found in PiD. The form of tau that accumulates in many neurodegenerative disorders, including PiD, is hyperphosphorylated. Isoforms of human tau include either three or four consecutive repeat motifs of the microtubule binding region. The three-repeat tau is the predominant form found in brains of these patients [106]. Because of the difficulty of establishing a diagnosis of PiD in a living individual, FTD is the preferred designation for cases meeting the clinical criteria for PiD. Several distinct pathological conditions have been associated with FTD, but it has not been possible to map anyone of these conditions to a particular clinical presentation. It has been suggested that the most common condition underlying FTD is frontal lobe degeneration of the non-Alzheimer-type [107], also referred to as dementia lacking distinctive histopathology (DLDH) [108].

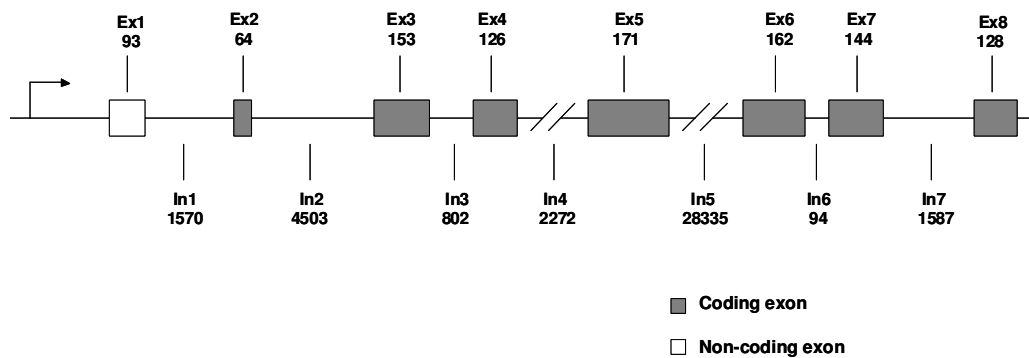
The group of syndromes known as frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) consists of autosomal dominantly inherited neurodegenerative diseases with diverse, but overlapping, clinical and neuropathological features [109]. Genetic linkage of this disease to chromosome 17q21-22 has been shown [110]. Clinically, they are characterized by FTD and parkinsonism [109]. Missense mutations have been identified in the *TAU* gene in FTDP-17 families [42, 111], which are predicted to affect the microtubule-binding properties of the tau protein.

Parkinson's disease (PD) is a common progressive neurodegenerative disorder and the most common cause of parkinsonism. Parkinsonism is also a feature of other disorders, such as progressive supranuclear palsy (PSP), pathologically characterized by the presence of neurofibrillary tangles [112]. Genes involved in the rare Mendelian forms of PD have been identified [113], but in most sporadic late-onset cases of PD the genetic component involved is unclear and thus other susceptibility genes may contribute to the disease process. Evidence has implicated the *TAU* gene in the pathogenesis of PD and linkage to the *TAU* locus on chromosome 17q has been reported in late-onset cases [114].

## 1.6. Cell division cycle

In 2000, three different papers reported linkage of AD to two different chromosome 10 markers. Two studies found linkage to the marker D10S1225, one using sibling pairs with late-onset AD [115], the other using plasma-A $\beta$ 42 as a surrogate marker [116]. The third study used genetic markers mapping near the insulin-degrading enzyme (*IDE*) gene in multiplex AD families, finding an association between AD and the marker D10S583 [117]. Subsequent studies have identified several SNPs in the *IDE* gene. Significant associations to AD have been found [118, 119], as well as negative findings [120]. Taken

together, these findings support the presence of an important susceptibility gene for AD on the long arm of chromosome 10. The cell division cycle (*CDC2* or *p34*) gene (Fig. 4) is located on chromosome 10q21.1 [121], approximately 2 Mbp from the marker D10S1225 linked to AD. Cdc2 is a 34 kDa protein kinase involved in regulation of the cell cycle [122] and in neuronal differentiation [123]. In AD brain, cdc2 is expressed in neurons [124], and catalytically active cdc2 is present in NFTs bearing neurons [124, 125]. Cdc2 has also been shown to be involved in the abnormal phosphorylation of tau in AD [126]. Furthermore, cdc2 has been shown to phosphorylate APP at Thr668 [127], and also the A $\beta$  peptide at Serine 26 [128]. The *CDC2* gene may therefore be a candidate susceptibility gene for AD.



**Figure 4.** Schematic overview of the *CDC2* gene. Coding exons are shown in light color, whereas non-coding exons are represented in dark color.

### 1.7. TAU haplotype and Saitohin

Numerous polymorphisms spanning the human *TAU* gene are in complete linkage disequilibrium with each other and have been defined as two separate haplotypes, termed H1 and H2 [129]. The H1 haplotype has been associated with AD [130, 131], FTD [132] and progressive supranuclear palsy (PSP) [129], the latter is another neurodegenerative disorder characterized by NFTs.

Previous studies have described Saitohin (*STH*) [133], a gene located in the intron 9 of the human *TAU* gene, a critical region for alternative splicing of exon 10. Inclusion of exon 10 in *TAU* mRNA results in a protein with four repeated microtubuli binding domains (4R), compared to exclusion of exon 10, which give rise to three microtubuli binding domains (3R). *STH* and *TAU* have similar expression pattern in human tissues and brain areas, and to date the biological function of *STH* is unknown. A SNP (A  $\rightarrow$  G) in the *STH* gene was identified, and results in a amino acid change from glutamine (Q) to arginine (R) in position seven (Q7R) [133]. In a case-control study it was found that the RR genotype and the R allele were over represented in sporadic AD.

## EXPERIMENTAL THEORIES

### 2.1. Polymerase chain reaction

The polymerase chain reaction (PCR) is a widely used technique for the selective amplification of particular DNA sequences, such as individual genes or gene fragments. PCR is quick, sensitive and robust and is particularly useful when dealing with small amounts of DNA, or where rapid and high-throughput screening is required.

PCR is based on the DNA polymerization reaction and amplifies DNA that is flanked by known sequences. The known sequences correspond to two synthetic oligonucleotide primers which are used to initiate the reaction. These flank the region to be amplified and face inwards, on opposite DNA strands, so that synthesis occurs across the central region. The products of the DNA synthesis reaction can themselves act as templates for DNA synthesis. Therefore, after one round of synthesis, the amount of template DNA is doubled. Doubling occurs in every cycle of the PCR leading to exponential amplification of the target. Each time DNA synthesis is carried out, a single stranded template is converted into a double-stranded product. Before further synthesis is possible, this product must be separated into single strands. Each PCR cycle therefore comprises three processes:

#### 1. Denaturation

The DNA is heated usually to 95 °C to render it single-stranded

#### 2. Primer annealing

The two primers bind the appropriate complementary strand. The temperature for this step varies depending on the GC content and the size of the primer and its homology to the target DNA, often 50-60 °C

#### 3. Primer extension

DNA polymerase extends the primer by its polymerase activity. This is done at a temperature optimal for the particular polymerase that is used. Taq polymerase is often used, a DNA polymerase from the thermophilic bacteria *Thermus aquaticus*, with an optimal temperature of 72 °C.

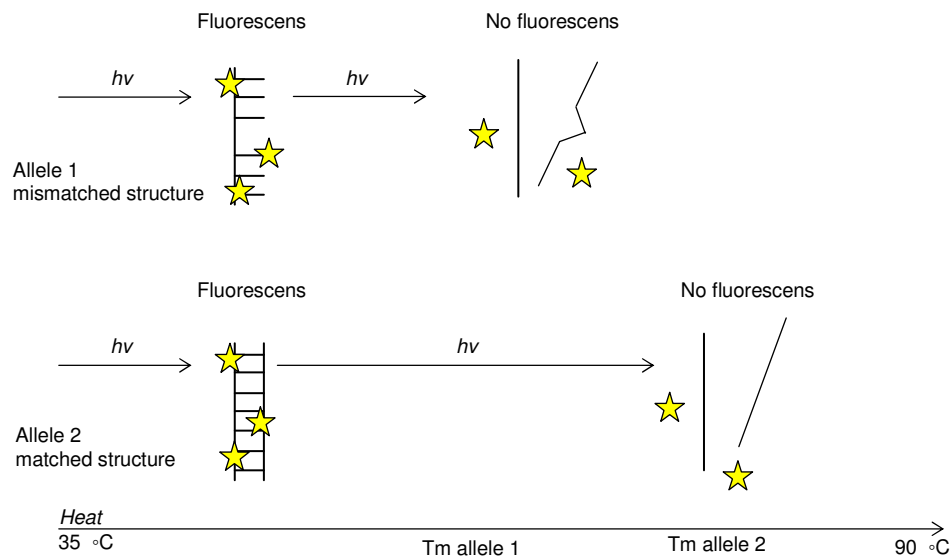
### 2.2. DNA sequence analysis

DNA sequencing is the process of determining the exact order of the bases A, T, C and G in a piece of DNA. The DNA to be sequenced is provided in single-stranded form. This acts as a template upon which a new DNA strand is synthesized. DNA synthesis requires a supply of the four nucleotides, the enzyme DNA polymerase and a primer. The nucleotides added to the growing DNA strand are complementary to those in the template strand.

Sequencing is achieved by including in each reaction a nucleotide analogue that cannot be extended and thus acts as a chain terminator. Four reactions are set up, each containing the same template and primer but a chain terminator specific for A, C, G or T. Because only a small amount of the chain terminator is included, incorporation into the new DNA strand is a random event. Each reaction therefore generates a collection of fragments, but every DNA strand will end at the same type of base (A, C, G or T).

These fragments are then analyzed on a fully automated capillary electrophoresis machine and detected by a laser to generate a string of nucleotides representing the DNA sequence of the starting template. We have used ABI PRISM 3100 Genetic Analyzer

(Perkin-Elmer, Waltham, USA), a 16 capillary electrophoresis instrument for automated DNA sequencing and DNA fragment analysis.



**Figure 6.** DASH method strategy. Denaturation of probe-target complex (DNA duplex) is monitored over a wide temperature range by measuring fluorescence while heating. As the duplex destabilizes due to heating it falls apart, so releasing the dye, which thus ceases fluorescing. This drop in fluorescence, which occurs earlier for mismatched structures than for matched duplexes

### 2.3. Dynamic allele-specific hybridization

#### Basic principles

Dynamic allele-specific hybridization (DASH) was created as a method to genotype SNPs as well as short insertion or deletion polymorphisms. An overview of the DASH procedure is shown in figure 6. A target sequence containing the variation is amplified by PCR in which one primer is biotinylated. The biotinylated product strand is bound to a streptavidin-coated microtiter plate well, and the non- biotinylated strand is rinsed away with alkali (NaOH). An oligonucleotide probe, specific for one allele, is hybridized to the target at low temperature and a duplex DNA region is formed. This can interact with a double strand-specific intercalating dye, in this thesis Sybr Green I.

Upon excitation, the dye emits fluorescence proportional to the amount of double stranded DNA (probe-target duplex) present. The sample is then steadily heated through and beyond its melting temperature. In presence of a DNA duplex, the dye will fluoresce, emitting a detectable light signal that can be measured in real time. As the duplex destabilizes due to heating it falls apart, so releasing the dye, which thus ceases fluorescing (Fig. 6). This drop in fluorescence, which occurs earlier for mismatched structures than for matched duplexes, allows one to infer the SNP allele contained within the amplified DNA (Fig. 6). To make observed denaturation profiles more readily interpretable, the

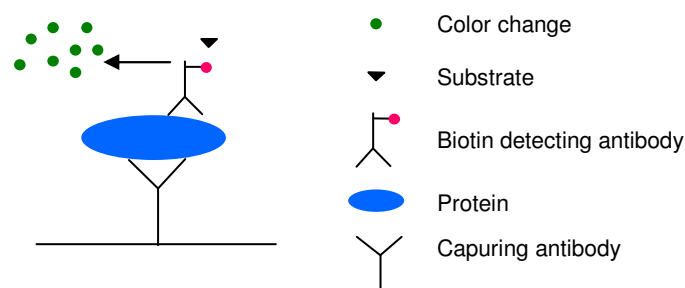
negative first derivative of the fluorescence versus time is plotted. Peaks that occur at low temperatures are scored as homozygous mismatches, peaks occurring at higher temperatures as homozygous matches and cases where both peaks are present are scored as heterozygous.

#### *Induced fluorescent resonance energy transfer (iFRET)*

Using only Sybr Green I for fluorescent detection has a lot of advantages. The dye is not expensive and produces a large amount of fluorescence, which can easily be detected. Since Sybr Green will bind to all DNA that is double stranded it can of course also bind to secondary structures, which occurs when base pairs form within a single strand of DNA.

An alternative strategy is to use fluorescence resonance energy transfer (FRET). FRET is a phenomenon that occurs between distinct pairs of fluorophores, a donor and an acceptor molecule. Instead of emitting light, the donor transfers the energy to an acceptor, which in turn can release light corresponding to its emission spectrum. For FRET to occur, the emission spectrum of the donor must overlap with the excitation spectrum of the acceptor. FRET occurs only when the donor and acceptor molecules are kept close together, as they are in a DNA duplex formation.

We have used a relatively new version of FRET, termed induced FRET (iFRET), which is ideally suited for melting curve analysis. Here is Sybr Green used as donor molecule for a FRET reaction between this intercalating dye and an acceptor attached to the allele-specific probe. The iFRET technology combines the high fluorescence as observed with Sybr Green detection, with the specificity for particular DNA duplex that is normally exclusively reserved for FRET detection. Thus it is possible to effectively filter out background fluorescence caused by the non-specific nature of Sybr Green I. We have used a common FRET acceptor molecule called 6-Rhodamine (ROX), which is able to harvest the energy from Sybr Green I with high effectiveness.



**Figure 7.** Schematic overview of the principle of the sandwich Enzyme-Linked Immunosorbent Assay (ELISA) technique.

## 2.4. Enzyme linked immunosorbent assay (ELISA)

Enzyme-Linked Immunosorbent Assay (ELISA) is a useful and powerful method in quantification of proteins in a solution, such as cerebrospinal fluid (CSF), serum, urine or culture supernatant.



In our studies, we have used a variant of this technique called sandwich ELISA. The basis of the method is the binding of the protein by an antibody (capturing antibody) that is linked to the surface of a plate well. Unbound protein is washed out and a second antibody (detecting antibody) specific for another epitope (part) is added. To the detecting antibody an enzyme is conjugated, in this case biotin. Finally the protein-antibody complex is detected by peroxidase-labeled streptavidin. After addition of substrate solution, positive samples will develop color and the intensity of color produced is proportional to the protein concentration originally present in the sample.

## **SPECIFIC AIMS**

One of the characteristics of AD is the intraneuronal accumulation of NFTs. NFTs are composed of the microtubule-associated protein tau. Thus, tau pathology is a central and important event in the AD pathogenesis and we decided to study genes that are directly or indirectly related to tau and investigate their potential as candidate genes for AD risk. Our aim was also to observe how variants of these genes influence quantitative AD biomarkers in CSF and neuropathological scores of SPs and NFTs in brain tissue.

### **3.1. Paper I**

The aim of this study was identify new polymorphic sites in the *CDC2* gene, which has been suggested to be involved in AD, and test for their possible association with increased risk for AD or FTD.

### **3.2. Paper II**

The study aimed to explore the possible relationship of the *STH* Q7R polymorphism and the *TAU* haplotype with AD, FTD and PD, and test genotype data for their influence on biomarkers in CSF and neuropathological scores of SPs and NFTs.

### **3.2. Paper III**

In this study we wanted to follow up the findings in paper I by investigating if and how the *CDC2* SNP influences pathogenic processes in AD. We measured levels of CSF biomarkers and determined SP-NFT scores and tested if they were associated with *CDC2* gene variants.

## MATERIAL AND METHODS

### 4.2. Clinical characterization of study participants

Participants in our studies have been patients with AD, FTD and PD, and control individuals. AD patients can be sub grouped in clinical and neuropathological diagnosed post-mortem populations. Patients in the clinical group were clinically diagnosed with probable late-onset AD according to the NINCDS-ADRDA criteria [22]. Clinical diagnoses included a medical history, physical, neurological and psychiatric examination, screening laboratory tests, ECG, X-ray of the chest, EEG, and computerized tomography (CT) of the brain. Mini-Mental State Examination (MMSE) was performed according to standard procedures [23]. No patient had a family history of autosomal dominant dementia. In clinically diagnosed AD patients CSF samples have been available for quantification of the AD biomarkers total-tau, phospho-tau and  $A\beta_{1-42}$ . In the clinical control group only individuals without history, symptoms or signs of psychiatric or neurological disease, malignant disease, or systemic disorders were included. Individuals with MMSE scores below 28 were not recruited to the control group.

In the post-mortem group AD patients were clinically diagnosed with probable late-onset AD according to the NINCDS-ADRDA criteria [22] and confirmed as definitive AD if the neuropathological diagnoses fulfilled the neuropathological CERAD criteria [147]. All AD brains had a histopathological score above five and revealed no infarcts or other changes that could account for the dementia. Neuropathological examinations of brain tissue have been available for the post-mortem populations. Included in the neuropathological control group were individuals who had died from cardiac disease or malignant disease. They had no history of dementia, psychiatric or neurological diseases and all brains had a histopathological score [148] of four or lower.

FTD patients were diagnosed according to the Lund-Manchester criteria [149] and no FTD patient had any of the known mutations in the *TAU* gene, as presented earlier [150].

PD patients were recruited at the Sahlgrenska University Hospital in Gothenburg and fulfilled the PDS Brain Bank criteria for idiopathic PD, except that presence of more than one relative with PD was not an exclusion criterion [151].

All diagnoses were set without knowledge of the results from biochemical and/or genetic analyses and vice versa.

### 4.2. Cerebrospinal fluid analyses

In both paper II and III, samples of CSF were obtained by lumbar puncture in the L3/L4 or L4/L5 interspace, under standardized conditions. A CSF volume of 12 ml was collected and gently mixed to avoid gradient effects. All samples were centrifuged to remove cell debris and stored in -80 °C. Further details of CSF collection can be found elsewhere [152].

#### *Sandwich ELISA for quantification of tau protein*

Quantification of tau protein in CSF was performed using the Innostest hTau Ag (Innogenetics, Gent, Belgium) [153]. This is a solid-phase enzyme immunoassay in which the wells of polystyrene microtiter plates are coated with an anti-human tau monoclonal antibody, AT120. CSF samples are incubated in these wells together with a pair of biotinylated tau-specific monoclonal antibodies, HT7 and BT2, which recognized

different epitopes. The biotin will then bind to peroxidase-conjugated streptavidin and produce a color reaction. The intensity of color produced is proportional to the tau concentration originally present in the sample.

#### *Sandwich ELISA for quantification of phosphorylated tau protein*

For quantitative determination of phosphorylated tau protein in CSF the Innostest phospho-Tau<sub>(181P)</sub> (Innogenetics, Ghent, Belgium) was used [52]. This is also solid-phase enzyme immunoassay in which the phosphorylated tau protein is captured by a first monoclonal antibody, HT7. CSF samples are added and incubated with a biotinylated antibody, AT270<sub>bio</sub>. This protein-antibody complex is then detected by a peroxidase-labeled streptavidin. Positive samples will develop a color proportional to the concentration of phosphorylated tau originally CSF sample.

#### *Sandwich ELISA for quantification of $\beta$ -amyloid<sub>(1-42)</sub>*

Cerebral fluid  $\beta$ -amyloid<sub>(1-42)</sub> level was determined using the Innostest  $\beta$ -amyloid<sub>(1-42)</sub> (Innogenetics, Ghent, Belgium) constructed to specifically measure  $\beta$ -amyloid<sub>(1-42)</sub> [154]. The monoclonal antibody 21F12, which is highly specific for the C-terminus, is used as capturing antibody. The biotinylated monoclonal antibody 3D6, specific for the N-terminus, is used as detector. The protein-antibody complex is then detected by a peroxidase-labeled streptavidin. This reaction results in a color change, which can be measured. The intensity of the color is proportional to the concentration of phosphorylated tau in the originally CSF sample.

### **4.3. Post-mortem brain analyses**

The post-mortem examination revealed no infarcts or other changes that could account for the dementia. Two areas of the right hemisphere, the frontal lobe and the anterior part of the hippocampal formation, were fixed in 10% buffered neutral formalin for 4-6 weeks, and thereafter embedded in paraffin blocks. Six  $\mu$ m-thick sections were stained with Bielschowsky staining. The absolute number of SPs and NFTs was counted in five randomly selected fields at magnification x 125, and a mean count of SPs and NFTs was obtained for each brain region, and rated on a four-step scale (0= no, 1= mild, 2= moderate and 3= severe) [148]. All AD brains had a histopathological score above five, mean  $\pm$  SD  $7.5 \pm 2.0$ .

The neuropathological control group consisted of patients who had died from cardiac disease or malignant disease. Their medical records revealed no history of dementia, or psychiatric or neurological diseases. The post-mortem examination revealed no macroscopic infarcts, and all brains had a histopathological score of four or lower, mean  $\pm$  SD  $1.3 \pm 1.4$ .

### **4.4. Genotype analysis**

Genomic DNA was extracted from whole blood samples or brain tissue using the GenoPrep<sup>TM</sup> DNA Blood kit or DNA MagAttract kit (Qiagen, Hilden, Germany) together with the GenoM-48 Robotic Workstation (GenoVision, Oslo, Norway). Approximately 100  $\mu$ l of whole blood or 10 mg of brain tissue was used for each extraction. The isolated genomic DNA was stored at -20 °C until it was used for gene analysis.

### *Paper I and paper III*

The primer sequences were designed according to the *CDC2* genomic DNA sequence deposited in the NCBI database (accession no. 14739455 and D32223). PCR amplification was performed in a final volume of 50  $\mu$ l, containing approximately 40 ng of template DNA, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 1.25 unit of Taq DNA polymerase in 1 x PCR buffer and 0.4 pmol/ $\mu$ l of each primer. The thermal cycling profile was designed as follows: an initial 4 min denaturation at 95 °C, followed by 35 cycles of 95 °C for 1 min, 57 °C for 45 s and 72 °C for 1 min. A final extension step was 72 °C for 10 min.

DNA sequencing was performed using cycle sequencing with fluorescent dNTPs (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, USA), separation by capillary electrophoresis and detection by laser-induced fluorescence in an ABI 3100 genetic analyzer (Perkin-Elmer, Waltham, USA). The sequencing reactions were conducted in both the forward and reverse directions.

For genotyping of intron 6, DNA sequencing was performed using the primers 5'-GCACCATATTTGCTGAACTAG-3' and 5'-GTCCTGTAAAGATTCCACTTC-3' (Invitrogen, Carlsbad, USA), covering the region -80 bp to +57 bp of intron 6. The PCR amplification was performed as described earlier except that the annealing temperature was 53 °C. *APOE* genotyping was performed using minisequencing as described before [155]. Two SNPs, rs7412 and rs429358, were used to determine the presence of the  $\epsilon$ 2,  $\epsilon$ 3 and  $\epsilon$ 4 alleles.

### *Paper II*

PCR amplification of *STH* gene was performed with the forward primer 5'-CCCTGTAAACTCTGACCACAC-3' and the reverse primer 5'-ACAGGGAAGCTACTTCCCATG-3' (Invitrogen, Carlsbad, USA). The reaction was performed in a final volume of 50  $\mu$ l, containing approximately 40 ng of template DNA, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M deoxynucleotide triphosphates (dNTP), 1.25 unit of Taq DNA polymerase in 1 x PCR buffer (Roche, Basel, Switzerland) and 0.4 pmol/ $\mu$ l of each primer (Invitrogen, Carlsbad, USA). The PCR program consisted of an initial 4 min denaturation at 95 °C, followed by 35 cycles of 1 min at 95 °C, 45 s at 60 °C and 1 min at 72 °C. A final extension step of 10 min at 72 °C.

The DNA sequencing reactions were conducted in both forward and reverse direction using the same primers as above. DNA sequencing was performed using cycle sequencing with fluorescent dNTP (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, USA), and separated by capillary electrophoresis and laser-induced fluorescence in an ABI 3100 genetic analyzer (Perkin-Elmer, Waltham, USA).

The 238-bp *TAU* deletion was amplified as described earlier [129]. Primer sequences used were forward: 5'-GGAAGACGTTCTCACTGATCTG-3' and reverse: 5'-AGGAGTCTGGCTTCAGTCTCTC-3'. The reaction was performed in a volume of 50  $\mu$ l and contained MgCl<sub>2</sub>, dNTPs, unit of Taq polymerase (Roche, Basel, Switzerland). The PCR product was examined on an agarose gel (2%) for the presence of the intronic 238 bp deletion giving fragments of 484 or 246 base pairs. *APOE* genotyping was performed using minisequencing as described before [155]. Two SNPs, rs7412 and rs429358, were used to determine the presence of the  $\epsilon$ 2,  $\epsilon$ 3 and  $\epsilon$ 4 alleles.

## 4.5. Statistics

### *Paper I*

*CDC2* allele and genotype frequencies were compared using Pearson Chi squared test. Odds ratios were calculated with 95% confidence intervals. P-values lower than 0.05 was considered statistically significant. All statistical analyses were performed using the SYSTAT program version 10.2 (SYSTAT Software GmbH, Erkrath, Germany). Haplotype frequencies were inferred using the HAPLOTYPER program which implements a partition ligation/Gibbs sampling algorithm [158]. Linkage disequilibrium (LD) was estimated between pairs of diallelic loci using the measure of  $r^2$  ( $r^2 = (\pi_{AB} - \pi_A\pi_B)^2 / (\pi_A\pi_a\pi_B\pi_b)$ ), where  $\pi$  represents allele frequency and A, B, a, and b represent alleles at compared loci.

### *Paper II*

Genotype and allele distributions of both *STH* polymorphism and *TAU* haplotype were analyzed using Pearsons Chi squared test, since normal distribution was obtained for these data. Fisher exact test was used where appropriate. In the case of the effect parameters (total-tau, phospho-tau and  $A\beta_{1-42}$ ), normal distribution were not obtained and therefore Kruskal-Wallis one-way analysis non-parametric statistics was used. Odds ratios and 95% confidence intervals were calculated according to description by Altman [17]. Deviations from Hardy-Weinberg equilibrium were assessed using chi-square statistics. All analyses were performed using the SYSTAT program version 10.2 (SYSTAT Software GmbH, Erkrath, Germany). P-values lower than 0.05 was considered statistically significant.

### *Paper III*

Genotype and allele distributions for the *CDC2* Ex6+7I/D polymorphism were analyzed using the Pearson Chi squared test. Comparisons involving three or more groups were performed using analysis of variance (ANOVA) and since the CSF traits are slightly skewed we also performed the non-parametric Kruskal-Wallis analysis of ranks. Odds ratios and 95% confidence intervals were calculated according to description by Altman [17]. Comparing observed and expected genotype frequencies using a chi-square test assessed deviations from Hardy-Weinberg equilibrium. P-values lower than 0.05 was considered statistically significant. All analyses were performed using the SYSTAT program version 10.2 (SYSTAT Software GmbH, Erkrath, Germany).

## RESULTS

### 5.1. Paper I

We identified a polymorphism in the *CDC2* gene with a frequency above 5% at position +7 in the splice site after exon 6, and investigated if this polymorphism was associated with tauopathies (AD and FTD).

We found a significant association between the *CDC2* II genotype and AD, and also an increased Ex6+7I allele frequency in AD compared with control subjects. A similar association was found in the FTD group, although the difference did not reach statistical significance, probably due to the smaller sample size.

When the AD and FTD groups were combined to a tauopathy group, there was a clear overrepresentation of the *CDC2* II genotype in the tauopathy group, and a significant increased allele frequency compared with control subjects. We found no interaction with the *APOE*  $\epsilon$ 4 allele, suggesting that the *CDC2* I allele may act as a susceptibility gene for tauopathies, independently of the *APOE*  $\epsilon$ 4 allele.

We could show that the *CDC2* I allele is associated with tauopathies, including both AD and FTD. Alternatively, the *CDC2* I allele may be in linkage disequilibrium with a gene located close to the *CDC2* gene. Further studies are warranted to confirm our findings, as well as studies exploring the neurobiological effects of the *CDC2* I allele.

### 5.2. Paper II

Our findings with regards to genotype and allele frequencies in patients and control subjects showed no involvement of *TAU* haplotype and/or *STH* in AD, FTD or PD. The *STH* Q7R polymorphism and the *TAU* haplotype were in complete linkage disequilibrium in all patients (AD and FTD) and control subjects investigated for both genes. Neither *TAU* haplotype nor *STH* influenced CSF levels of total-tau, phospho-tau and  $A\beta_{1-42}$  in AD patients significantly. In AD patients with neuropathological scores of SPs and NFTs no associations with *TAU* haplotype and *STH* were found.

The PD patients were subdivided into early-onset PD (onset at or before 50 years) and late-onset (onset after 50 years) according to a commonly used stratification [159, 160]. This stratification did not contribute to changes of genotype or allele distributions. Since we had information from the participants about their family history of PD and AD, we included these parameters in the analysis. We noticed that 23.5 % of the PD patients had a family history of PD, compared to 8.2 % of the controls, which was statistically significant. Unfortunately, the numbers of PD patients and controls were too small to yield enough power for stratified investigations of risk gene effects based upon family history of PD and AD. Our results do not support a major pathogenic role of the *TAU* haplotype and *STH* in AD or in FTD.

### 5.3. Paper III

We studied the *CDC2* Ex6+7I/D gene variant in relation to quantitative measurements of total-tau, phospho-tau and  $A\beta_{1-42}$  proteins in CSF. We sought to systematically test for the potential impact of the *CDC2* Ex6+7I/D gene variant upon these AD related quantitative traits, as well as MMSE scores, age at onset (AAO), and a neuropathological scores of SPs and NFTs. Comparisons for these CSF traits between AD patients and controls have previously been performed demonstrating highly significant differences, whereby higher total-tau and phospho-tau levels and lower  $A\beta_{1-42}$  levels are evident in AD

patients compared to control subjects [161] and this was true for the present sample as well. To model these effects, we tested each trait in turn using ANOVA to explore for significant differences in genotypic means. For CSF traits, this initial screen was performed in combined patients and controls. For MMSE and for SP-NFT scores, this was only done in AD patients.

The *CDC2* I allele was associated with a gene dose-dependent increase of CSF total-tau levels and the homozygous *CDC2* II genotype was significantly more frequent among AD patients compared to control subjects.

Genotype distributions for the *CDC2* Ex6+7I/D conformed to Hardy Weinberg equilibrium in both AD patients and control subjects. The *CDC2* Ex6+7I/D genotype distribution differed significantly between AD patients and control subjects. Specifically, the homozygous *CDC2* II genotype and the *CDC2* I allele frequencies were significantly increased in AD. As expected the *APOE*  $\epsilon$ 4 genotype and allele frequencies were significantly increased in the AD and *APOE* status did not influence *CDC2* frequencies. Our results provide further evidence for an involvement of *cdc2* in the pathogenesis of AD.



## DISCUSSION

In the past years, several genetic association studies have been performed on AD [162]. To date, the  $\epsilon 4$  variant of the *APOE* gene is the only established risk gene for AD [75, 163]. This only account for a fraction of all sporadic AD cases, thus several susceptibility genes remain to be found and is therefore it is of great importance to study and try to find other genes that can influence the risk of AD. In paper I we found a significant association between the *CDC2* II genotype and AD, and also an increased *CDC2* I allele frequency in both AD and FTD. In the combined tauopathy (AD and FTD) group, there was a clear overrepresentation of the *CDC2* II genotype, and a significant increased allele frequency. The *CDC2* I allele, which was found to be associated with tauopathies, may have functional consequences, since it might influence splicing mechanisms. Intriguingly, intronic mutations located close to the splice site of exon 10 in the *TAU* gene have been shown to alter the splicing of exon 10 [31]. Accumulating evidence also suggest involving of the *cdc2* in the pathogenesis of AD. Intracellular accumulation of tau protein is a characteristic hallmark of both AD and FTD. *Cdc2* has been shown to be involved in the phosphorylation of tau in AD [126], and catalytically active *cdc2* is found in NFT-containing neurons in AD [124]. One possibility might be that the *CDC2* Ex6+7I/D polymorphism affects the phosphorylation of tau. We found no clear difference in the *CDC2* I allele frequency between patients that did, or did not possess the *APOE*  $\epsilon 4$  allele, suggesting that the *CDC2* I allele may act as a susceptibility gene for tauopathies which is independent from the *APOE*  $\epsilon 4$  allele.

Other pathogenic mechanisms may also link *cdc2* to AD. Reactivation of cell cycle events in postmitotic neurons may be linked to the pathogenesis of AD, including dysregulation of cell cycle proteins [164]. Inappropriate activation of the cell cycle protein *cdc2* might thus be linked to neurodegeneration in AD [122]. Further, *cdc2* phosphorylates both APP [127] and  $\beta$ -amyloid [128]. Thus, an alternative explanation may be that the *CDC2* Ex6+7I/D polymorphism affects the processing of APP and  $A\beta$ .

In paper II we genotyped cases with AD, FTD and PD and control subjects for the *TAU* haplotype and the *STH* Q7R polymorphism. Our goal was to clarify if the *TAU* haplotype and the *STH* gene constitute a risk for AD, FTD or PD. Since we have one group of individuals with measures of total-tau, phospho-tau and  $A\beta_{1-42}$  in CSF, and another group with neuropathological scores of SPs and NFTs, we could test if gene variants affected these traits. Most of previous studies have only compared genotype and allele frequencies between cases and control subjects.

Based on the molecular basis both AD and FTD are referred to as tauopathies and the pathogenic relationship between tauopathy and these neurodegenerative diseases is well established [105]. Mutations in the human *TAU* gene have been linked to FTD [42, 111], but no *TAU* mutations have been identified in AD patients. Beside the known mutations several polymorphic sites have been identified through the *TAU* gene and association studies have reported conflicting results [130, 131, 165-169]. Since series of these common polymorphisms in the *TAU* gene are in complete linkage disequilibrium, two separate haplotypes, H1 and H2, have been defined.

The haplotype H1 has been suggested to be associated with FTD [170, 171] and also with AD [130, 131]. Since the H1 allele is in complete linkage disequilibrium with *STH* Q allele, it is even more confusing when other results have shown that the R allele and the RR genotype of *STH* are significantly more frequent in patients with AD

compared with control subjects [133]. In their study 51 AD patients and 30 control subjects were included, and maybe the positive association of the R allele in *STH* to AD is due to a Type I error (e.g. too small sample size).

Most of the patients (AD and FTD) and control subjects were genotyped both for the *TAU* haplotype and the *STH* Q7R polymorphism and in all these the *TAU* haplotype H1 was in complete linkage with the Q allele in *STH*, and H2 with the R allele. This result is in agreement with previous studies [171-173].

Our findings with regards to genotype frequencies in cases and controls are in line with several previous studies where no involvements of *TAU* haplotype/*STH* have been seen neither in AD [165, 171, 174, 175] nor in FTD [176]. We have not been able to replicate any of the previous positive findings [130, 131, 133, 170, 171]. The strength in our study is that we have been able to investigate if *TAU* haplotype/*STH* influence either CSF levels of total-tau, phospho-tau and  $A\beta_{1-42}$  or amounts of SPs and NFTs in two different brain areas, the hippocampus and the frontal cortex. In spite of the fact that no associations were found of *TAU* haplotype/*STH* to AD, FTD or PD, we still wanted to see if CSF biomarkers or neuropathological scores were affected. This idea is supported by studies of the *IDE* gene, another gene suggested to be implicated in AD. When studying *IDE* polymorphisms, no associations of *IDE* genotypes to AD were found, but still levels of  $A\beta_{1-42}$  was dramatically influenced [119, 177].

Thus, we found no evidence that could support an involvement of *TAU* haplotype/*STH* in AD, FTD or PD. *TAU* haplotype/*STH* did not influence CSF levels of total-tau, phospho-tau and  $A\beta_{1-42}$  in AD significantly. In AD patients with neuropathological scores of SPs and NFTs no associations to *TAU* haplotype/*STH* were found. These results do not support a major pathogenic role of the *TAU* haplotype and *STH* in AD, FTD or in PD.

To explore possible effects of the *CDC2* Ex6+7I/D polymorphism, found in paper I, on CSF biomarkers we examined AD patients in which we measured CSF levels of total-tau, phospho-tau and  $A\beta_{1-42}$ . We found the *CDC2* polymorphism to be associated with elevated CSF total-tau protein levels in AD patients. The *CDC2* I allele influenced CSF total-tau protein levels in a dose-dependent manner, suggesting a biochemical effect and an involvement of *cdc2* in the pathogenesis of AD. Interestingly, the same tendency could be seen in controls. This was, however, not significant, probably due to less power since the sample size was smaller. The results from the *CDC2* genotyping of the AD patients and control subjects were in line with our previous results in paper I where the homozygous *CDC2* II genotype is significantly over represented in AD patients compared to control subjects. We note that *APOE*  $\epsilon 4$ , the strongest known genetic risk factor for AD, was more frequent in AD patients compared to control subjects in our material. When we explored potential interactions of age, gender, or *APOE* genotype, none of these covariates affect the results significantly. This suggests that the relationship between the *CDC2* gene and AD is independent of *APOE*  $\epsilon 4$  status. Although mutations in the *APP* gene are causative for familial AD, the role of  $\beta$ -amyloid in sporadic AD is unclear. The amyloid cascade hypothesis suggests that deposition of  $\beta$ -amyloid into SPs is the event causative for the formation of NFTs and neurodegeneration [178]. Tau pathology has been observed in absence of SPs in other neurodegenerative disorders, such as progressive supranuclear palsy (PSP), Pick's disease (PiD) and corticobasal degeneration (CBD). Alternatively, cell cycle events may precede the formation of SPs and NFTs found in AD. The general assumption is that adult neurons in the brain are postmitotic and

unable to re-enter mitosis and thereby lack their ability to divide, conversely, accumulating evidence shows that postmitotic neurons in AD are able to re-enter the cell cycle. Specific cell cycle components have been found to be activated in AD brain [125, 179, 180] and altered regulation of cdks and cyclins point towards cell cycle activation in postmitotic neurons in AD [180, 181]. Active cdc2/cyclin B complex is localized in NFT-bearing neurons [125] and this accumulation of active cdc2 can occur before the formation of PHF, and without involvement of  $\beta$ -amyloid [182]. Cdc2 has been suggested to be one of the kinases involved in AD pathology since it is able to phosphorylate tau protein at sites known to be phosphorylated in AD [182-184]. In the present study we detected a non-significant trend towards increased phospho-tau levels in patients with the *CDC2* II and ID genotypes. In our ELISA method we measure tau protein phosphorylated at residue 181, which is a relatively isolated phosphorylation site in the proline-rich region [153] and is preferentially phosphorylated by proline directed kinases [40]. Even though it is evident that cdc2 activity is increased in AD it needs to be determined whether cdc2 alter tau phosphorylation in a direct or indirect manner.

Additionally, cell cycle events have been linked to neuronal cell death [185]. Several studies support the hypothesis that cell cycle proteins are expressed in postmitotic neurons, thus force them to re-enter the cell cycle and by this means eventually cause neuronal death [185]. Critical check points of the cell cycle regulate the destiny of the cell, towards further progression through the cell cycle, growth arrest or induction of an apoptotic pathway. In terminally differentiated cells, like neurons, an apoptotic response might prevent an improper activation of the cell cycle [186]. Cdc2 activity is elevated in apoptotic cells and many of the sites on tau that are hyperphosphorylated during apoptosis are also found in AD brain [184, 187, 188]. Cdc2 can induce phosphorylation of the Bcl-2 associated death agonist (Bad) at Ser128, which in turn results in activation of Bad-mediated apoptosis [189, 190] and previous results have found elevated levels of Bad protein in AD brains [191]. Moreover, APP can be phosphorylated by cdc2, which results in altered  $\beta$ -amyloid production [127]. In turn,  $\beta$ - amyloid can stimulate proliferating, drive cultured neurons into the cell cycle and thereby maintain the proliferating signal [192, 193].

The results from paper III further supports the involvement of cdc2 in AD and is in line with the results from paper I. The *CDC2* I allele influence CSF total-tau protein levels in a dose-dependent manner where individuals with II and ID genotypes have elevated protein levels of CSF total-tau compared to DD individuals. Biological studies of the cdc2 protein are needed to investigate how activity and expression is affected by the *CDC2* polymorphism.

## CONCLUSIONS

In this thesis genes, directly or indirectly related to *TAU*, have been tested for their association with AD risk, and the effects of genetic variations in these genes on the biochemistry and pathology. The main findings are listed below.

### 7.1. Paper I

We could show that the *CDC2* I allele frequency was significantly increased in AD and FTD patients. The *CDC2* I allele may be in linkage disequilibrium with a gene located close to the *CDC2* gene and therefore further studies are warranted to confirm this finding, as well as studies exploring the neurobiological effects of *CDC2* I allele.

### 7.2. Paper II

Our results could not support an involvement of *TAU* haplotype and/or *STH* in AD, FTD or PD. Neither *TAU* haplotype nor *STH* influenced CSF levels of total-tau, phospho-tau and A $\beta$ <sub>1-42</sub> in AD significantly. In AD patients with neuropathological scores of SPs and NFTs no associations to *TAU* haplotype and *STH* were found. These results do not support a major pathogenic role of the *TAU* haplotype and *STH* in AD, FTD or in PD.

### 7.3. Paper III

When following up the findings from paper I we found the *CDC2* I allele to be associated with a gene dose-dependent increase of CSF total-tau levels in AD patients. The homozygous *CDC2* II genotype was significantly more frequent among AD patients compared to control subjects. The results from this paper provide further evidence for an involvement of *cdc2* in the pathogenesis of AD.

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