Thesis for doctoral degree
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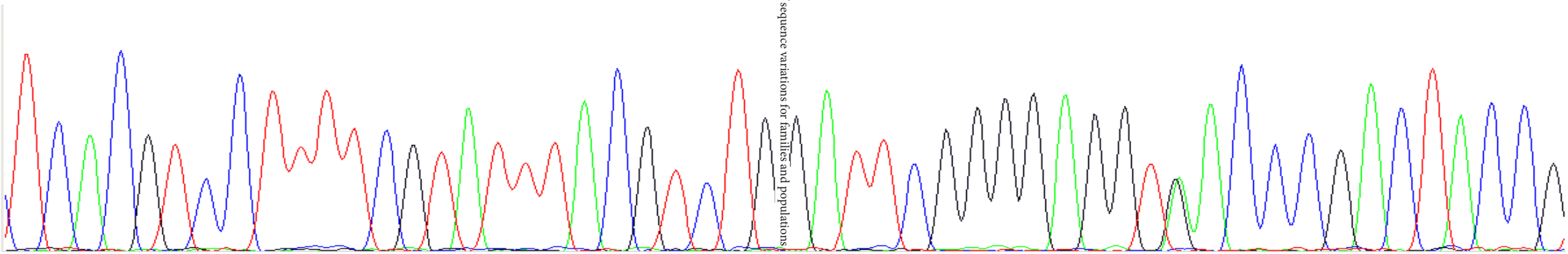
Genetics in Dementia

Impact of sequence variations for families and populations



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Genetics in Dementia: Impact of sequence variations for families and populations

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To my family

ABSTRACT

Even though the human genome sequences are remarkably similar, there is room for genetic variability that makes every human unique. The most common form of sequence variation in the genome is an exchange of a single nucleotide. The effect of sequence variations on the phenotype can be considered to be a continuum, from common polymorphisms with none or relatively mild effects, to severe and dramatic effects for mutations.

Dementia is a devastating disorder, which severely affects cognitive functions and eventually leads to death. Dementia is, not always but most of the time, caused by neurodegeneration, as in the case of Alzheimer disease (AD) and Frontotemporal dementia (FTD). The genetics in dementia is complex. The majority of cases are caused by a combination of variations in many genes, polymorphisms, in addition to an increased vulnerability due to exposure to harmful environmental factors during the life course. The effects of these variations are studied in populations, where both genetic and environmental factors can be assessed. In a few percent of dementia cases, the disease is caused by mutations in single genes. By identifying mutations in the affected families, important features about the disease etiology can be revealed.

In this thesis, variations in four genes were studied. **Study I** focuses on the impact of the widely confirmed genetic risk factor for dementia, ***APOE***, on mortality in a prospective community-based study, The Kungsholmen Project. The risk for mortality was increased for ε4 carriers, and decreased for ε2 carriers. The increased mortality was mainly explained by dementia. In addition, a gender specific effect was observed. In **Study II**, a G35fsX19 mutation was identified in the ***GRN*** gene, causing FTD in a Swedish family. Members in this family developed dementia with behavioral disturbances and progressive aphasia with an age at onset around 55 years. At autopsy, neurodegeneration and immunoreactivity for TDP-43 was observed. The G35fsX19 mutation resulted in a frameshift and was predicted to create a premature stop codon. Functional analyses of mRNA showed about 50% less expression of *GRN*. The mutated mRNA was not detected by cDNA sequencing, suggesting it was degraded by nonsense mediated mRNA decay. In **Study III**, an I143T mutation was identified in ***PSEN1*** in a Swedish family with early onset AD. The onset age was around 36 years. The mutation carriers were severely affected by cognitive deficits, in addition to neurological symptoms such as myoclonia. Neuropathologically, they were severely affected by Alzheimer pathology. Since one of the pathological hallmarks of AD, the amyloid plaques, consists of the Aβ peptide, the distribution of Aβ species in the postmortem brain of mutation carriers was investigated. Aβ42 was abundantly present both in plaques and vessels while Aβ40 was mainly present in vessels. Interestingly, we found Aβ43, which has rarely been studied in AD, to be present in all investigated brain regions, emphasizing a role for Aβ43 in the disease etiology. In **Study IV**, the ***FTO*** gene, which has been known for its involvement in body weight, was shown to influence the risk for dementia and AD for the first time, in persons 75+ years derived from the Kungsholmen project. The AA-genotype of the *FTO* rs9939609 polymorphism increased the risk about 50% compared to TT-carriers. This effect could not be explained by vascular risk factors measured at baseline, such as diabetes, high BMI, CVD or physical activity. Interaction between *FTO* and *APOE* was found, and together the two risk alleles increased the dementia risk almost three times. This finding supports a role for metabolic dysregulation in the dementia etiology.

To conclude, four genomic sequence variations were investigated for their impact on neurodegenerative diseases and mortality. Two mutations were identified, in *GRN* and *PSEN1* in two families suffering from FTD and AD, respectively. The *APOE* gene was found to increase mortality whereas *FTO*, the obesity-associated gene, was for the first time shown to increase the risk of dementia and AD in the old Kungsholmen population.

SAMMANFATTNING

Människans genom är väldigt lika individer emellan, men det finns rum för variationer som gör varje människa unik. Den vanligaste typen av variation i den genomiska sekvensen är ett utbyte av bara en nukleotid. Effekterna av variationer i DNA sekvensen kan vara omätbara eller milda för det som kallas polymorfier, till drastiska och sjukdomsframkallande för mutationer.

Demens är en förödande sjukdom, som i hög grad påverkar den drabbades kognitiva funktioner och som slutligen leder till döden. Demens orsakas, ofta men inte alltid, av neurodegeneration, vilket är fallet vid Alzheimers sjukdom och frontallobsdemens. Det genetiska bidraget till utvecklandet av demenssjukdomar är komplext. Oftast orsakas sjukdomen av en kombination av variationer i många gener, polymorfier, tillsammans med en ökad sårbarhet för sjukdomen som ackumuleras under livets gång på grund av att individen utsätts för olika miljöfaktorer som har en negativ påverkan på individen. Polymorfier studeras främst i populationer, där både genetiska variationer och miljöfaktorer kan studeras. I några få procent av alla som drabbas av demens beror sjukdomen på mutationer i endast en gen, som ger familjära former av demens med tidig debut. Identifiering av mutationer i dessa familjer kan leda till ökade kunskaper om sjukdomsorsakande mekanismer.

I den här avhandlingen har variationer i fyra gener studerats. I **Studie I** undersöktes den enda kända genetiska risk faktorn för demens, ***APOE***, och dess påverkan på dödligheten i en prospektiv populationsbaserad studie, Kungsholmsprojektet. Risken för dödlighet var större bland de som bar på ε4 allelen, och mindre för de som bar på ε2 allelen. Den ökade dödligheten kunde till största delen förklaras av den ökade förekomsten av demens. Dessutom kunde en könsspecifik effekt observeras. I **Studie II** identifierades en G35fsX19 mutation i ***GRN*** genen som orsakar frontallobsdemens i en svensk familj. Medlemmar i denna familj utvecklade demens med beteendestörningar och progressiv afasi vid ca 55 års ålder. Vid obduktion kunde neurodegeneration och immunoreaktivitet för TDP-43 påvisas. Mutationen ger upphov till en förskjutning i sekvensen som därför bildar ett för tidigt stop-kodon. Funktionella analyser visade att mutationsbärare hade ca 50 % lägre uttryck av *GRN* och vid sekvensering av cDNA kunde bara referens sekvensen detekteras, vilket sannolikt beror på ”nonsense mediated mRNA decay”. I **Studie III** påvisades en I143T mutation i ***PSEN1***, i en svensk familj med Alzheimers sjukdom. Debutåldern var ca 36 år. De drabbades av kognitiva störningar, och dessutom av neurologiska symptom så som myoklonier. Vid neuropatologisk undersökning observerades omfattande förändringar som är vanliga vid Alzheimers sjukdom. Eftersom ett kännetecken för Alzheimers sjukdom är amyloida plack, som består av Aβ-peptiden, undersöktes distributionen av olika varianter av Aβ-peptider i hjärnan hos de drabbade. Aβ42 förekom rikligt både i plack och i kärl, medan Aβ40 endast observerades i kärl. Aβ43, en längre och sällan studerad variant of Aβ-peptiden, observerades i alla undersökta regioner, vilket antyder att Aβ43 spelar en viktig roll i sjukdomen. I **Studie IV**, visades för första gången att ***FTO***, en gen som tidigare varit känd som en ”överviktsgen” ökar risken för demens och Alzheimers sjukdom. AA-bärare av *FTO* rs9939609 polymorfin hade ca 50 % högre risk jämfört med TT-bärare. Denna effekt kunde inte förklaras av vaskulära riskfaktorer så som diabetes, högt BMI, CVD och fysisk aktivitet. En statistisk interaktion mellan *APOE* och *FTO* observerades, där bärare av de båda riskgenotyperna hade nästan tre gånger högre risk. Detta fynd styrker betydelsen av metabola störningar för utvecklandet av demens.

Sammanfattningsvis, variationer i fyra gener studerades med avseende på deras betydelse för neurodegenerativa sjukdomar samt dödlighet. Två mutationer identifierades i *GRN* och *PSEN1* i två familjer med frontallobsdemens respektive Alzheimers sjukdom. *APOE* identifierades som en gen som ökar dödligheten i den äldre populationen på Kungshsholmen, medans *FTO*, påvisades för första gången som en risk faktor för demens och Alzheimers sjukdom.

LIST OF PUBLICATIONS

I. **Lina Rosvall***, Debora Rizzuto*, Hui-Xin Wang, Bengt Winblad, Caroline Graff, and Laura Fratiglioni. ***APOE*-related mortality: Effect of dementia, cardiovascular disease and gender.** *Neurobiology of Aging.* 2009, 30:1545-1551.

II. Huei-Hsin Chiang, **Lina Rosvall**, Jesper Brohede, Karin Axelman, Behnosh F Björk, Inger Nennesmo, Tiina Robins and Caroline Graff. **Progranulin mutation causes frontotemporal dementia in the Swedish Karolinska family.** *Alzheimer's and Dementia.* 2008, 4:414-420.

III. **Lina Keller***, Hedvig Welander*, Huei-Hsin Chiang, Lars O. Tjernberg, Inger Nennesmo, Åsa K. Wallin and Caroline Graff. **The *PSEN1* I143T mutation in a Swedish family with Alzheimer disease: Clinical report and quantification of Aβ in different brain regions.** *Manuscript.*

IV. **Lina Keller**, Weili Xu, Hui-Xin Wang, Bengt Winblad, Laura Fratiglioni and Caroline Graff. **The obesity related gene, *FTO*, increases the risk for Alzheimer disease in a prospective cohort study.** *Manuscript.*

*These authors contributed equally to the work

#Studies accepted for publication before September 2009 were published in the name Lina Rosvall. Thereafter in the name Lina Keller.

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid beta
ADL	Activities of daily living
APOE	Apolipoprotein E
AD	Alzheimer disease
APOC1	Apolipoprotein C1
APOJ	Apolipoprotein J
APP	Amyloid precursor protein
ATC	Anatomical Therapeutic chemical
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
CHMP2B	Charged multivesicular body protein 2b
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
DSM-III-R	Diagnostic and Statistical Manual of Mental Disorders, revised Third Edition criteria
EOAD	Early onset Alzheimer disease
FTD	Frontotemporal dementia
FTLD	Frontotemporal lobar degeneration
FTO	Fat mass and obesity associated gene
FUS	Fused in sarcoma
GRN	Granulin
GWAS	Genome-wide association study
ICD	International classification of disease
IHCD	Ischemic heart and cerebrovascular disease
LD	Linkage disequilibrium
LOAD	Late onset Alzheimer disease
MALDI-TOF	Matrix absorbed laser desorption ionisation-time of flight
MAPT	Microtubule associated protein tau
MMSE	Mini-mental state examination
NCI	Neuronal cytoplasmic inclusions
NII	Neuronal intranuclear inclusions
NMD	Nonsense mediated decay
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
PSEN1	Presenilin 1
PSEN2	Presenilin 2
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
TDP-43	TAR DNA-binding protein 43
TLS	Translated in liposarcoma
TOMM40	Translocase of outer mitochondrial membrane
TMEM106B	Transmembrane protein 106B
T2DM	Type 2 diabetes mellitus
VCP	Valocin-containing protein
Ub	Ubiquitin

INTRODUCTION

Through the increasing knowledge of the human genome, obtained by advancement in the technologies for DNA sequencing and genotyping, it has become feasible to study specific genetic variants in relation to different diseases and traits in an efficient way. For a substantial part of common diseases, such as dementia, the mode of inheritance is complex, as rare forms of the disease are inherited in a monogenic manner and common forms are determined by multiple genetic and environmental components. Studying these diseases using both genetic and epidemiological methods gives the possibility to explore both the underlying pathogenesis at the molecular and biochemical level, and the possible risk factors that can be both genetic and environmental factors as well as the interactions between them.

The monogenic autosomal dominant forms of the disease often give rise to early disease onset for the mutation carriers. The risk of getting the disease is 50% for first degree relatives. Diseases with a monogenic dominant form of inheritance are most efficiently studied in families. Linkage analysis to find the chromosomal location containing the causative mutation and screening for mutations in genes known to cause disease are two common strategies to study the genetics in these families. Once the causative mutation is found, the effect can be studied using different molecular and biochemical techniques, requiring tissue/blood samples from both affected and healthy individuals. For dementia and Alzheimer disease (AD), the monogenic forms of disease account at most for a few percent of all cases. Anyhow, by studying the effect of different mutations, knowledge about the underlying mechanisms is gained and the etiology of the disease can be explored and understood more in detail.

When the cause of disease does not follow any clear pattern of inheritance (although there may be familial clustering) and is influenced by both environmental factors and many genetic factors, the disease etiology is referred to as complex. In the dementia field, the sporadic forms with late age at onset are complex, accounting for approximately 95% of all cases. The effect size of each contributing gene is small and the power to detect such an effect requires large groups of patients and controls. When one or more polymorphic genetic markers that possibly contribute to the disease risk are examined, an association can be detected if the marker is causative or in strong

linkage disequilibrium (LD) with a causative genetic variant. Moreover, even though genetic risk factors are not modifiable, the knowledge about their contribution to disease reveals important information about the underlying mechanisms and might also improve the identification of individuals at higher risk for that specific disease.

HISTORY OF GENETICS

The revolutionary work of Gregor Mendel and Charles Darwin in the nineteenth century gave rise to the era of modern genetics and the concept of heredity. Darwin presented the theory of natural selection that is of high impact for the understanding of the nature of genetic variations. A genetic variation originates from a nucleotide exchange in single germ cells and, unless it is detrimental for the species, it will be passed on to the next generation. The variant might have a beneficial effect in terms of survival, and will thereby rapidly increase in the population. Mendel, on the other hand, showed that properties and traits are inherited independently of each other and follow particular laws. The concept of dominant and recessive inheritance was assessed. By the work of Walter Flemming, the knowledge about chromosomes as carriers of the material for inheritance was gained. The next important contribution to the field was published in 1944 when MacLeod and McCarty showed that deoxyribonucleic acids (DNA) contain four different nucleotides: adenine (A), cytosine (C), guanine (G) and thymine (T), and that these are not randomly distributed throughout the chromosomes and possess important biological information that is transmitted to the next generation (Avery et al., 1944). Watson and Crick discovered the double helical structure of DNA, containing complementary nucleotides in a ladder like shape. The finding was published in *Nature* in 1953 (Watson and Crick, 1953a; Watson and Crick, 1953b). They were awarded the Nobel Prize for their work on DNA in 1962. The knowledge of the structure opened up new possibilities for the development of methods to study DNA. The polymerase chain reaction (PCR) technique and DNA sequencing are today widely used both in research and in clinical practice (Mullis and Faloona, 1987; Sanger et al., 1977). Kary Mullis was awarded the Nobel Prize in chemistry in 1993 for his invention of the PCR method. The Human Genome Project aimed to sequence the human genome in order to discover all human genes and make the sequence accessible for research. An important milestone in this achievement was the integration of the genetic and physical map. A first draft of the human genome sequence was published in 2001 (Lander et al., 2001; Venter et al., 2001). A high quality version of the human genome sequence was made publicly available in 2003.

GENETIC EPIDEMIOLOGY

The concept of genetic epidemiology has been established in the last decades. It has been defined as "A science that deals with the etiology and control of disease in groups of relatives, and with inherited causes of disease in populations" (Morton and Chung, 1978). A number of various types of investigations can be used in genetic epidemiology, for examle linkage analysis to study familial aggregation and case/control studies to study genetic contribution to disease in populations. The most common way to study small genetic contributions is by using a case/control sample set. However, when the studied disease is relatively common in the population, the use of a prospective population-based sample set is enabled and will provide more reliable risk estimates. The genetic variant can be used as a variable in classical statistical models, e.g., logistic regression- and Cox regression models. Both the case/control design and the prospective population-based design have their advantages and disadvantages, and are often used as complements to each other. The case/control studies are more efficient (lower cost, quicker to complete and require fewer individuals) but are, for several reasons, prone to selection bias, e.g., because the outcome is measured before the exposure. The cohort study is often prospective, measuring the outcome subsequent to the exposure and yields true incidence rates and relative risks. However, these studies are prone to attrition bias (loss of participants during the study period) and sensitive to changes in methods over time (e.g., laboratory measurements). Due to the small effect sizes by every gene that contributes to the disease, a major challenge has been to collect sufficiently large samples to reach an acceptable study power to avoid false negative results.

GENETIC VARIATIONS

Even though our genomes are tremendously alike, there is room for genetic variability that makes every individual unique. The genome consists of approximately 10^9 base pairs. Genetic variations can be found with about a thousand base pairs in between. The most common variation in the genome is *single nucleotide polymorphisms* (SNP), meaning that a nucleotide differs at one specific position when comparing two different chromosomes. The different nucleotides are referred to as different alleles. Most of our genome is "gene-deserts" without any information that is converted to proteins, and most of the variability occurs in these regions. However, some of the variations are located in protein coding regions. These variations may alter the expression or the

function of the proteins, and they are more likely to influence the individual's risk of different diseases than the non-coding variations. Sometimes the change of a nucleotide results in an amino acid change in the protein chain, which further increases the likelihood that the variation has a pathogenic nature. Genetic variations that are pathogenic are often referred to as mutations, while a polymorphism has two or more common alleles in the population that are often phenotypically indistinguishable. The discrimination between a polymorphism and a mutation is not always evident. However, the effect of genetic variations on the phenotype can be considered to be a continuum, from common polymorphisms with no or relatively mild effects, to severe and dramatic effects for mutations.

In this thesis I will refer to the studied genetic variations in Papers I and IV as SNPs and those in Papers II and III as mutations, which is the most commonly used terminology for these types of variations.

Capital italic letters are used for human genes (e.g., *PSEN1*). Capital letters are used for human proteins (PSEN1). Lower case letters are used as symbols for genes (*Psen1*) and proteins (Psen1) in other species.

CHARACTERISTICS OF DEMENTIA

The concept of the dementia syndrome is wide and includes numerous clinical characteristics, simplified as a global deterioration of mental functioning, making the person with dementia dependent on others for their activities of daily living. The word dementia is often associated with memory impairment, which has also been essential for the diagnosis in the past. However, due to increased knowledge about the clinical features, the concept of "neurocognitive disorders" was recently proposed to replace the term "dementia" by the DSM-5 Neurocognitive Disorders Work Group, American Psychiatric Association, to avoid the confusion that memory impairment is required for the diagnosis and to increase the flexibility to include other cognitive deficits in the concept. The development of cognitive deficits can occur for different reasons, for example brain hemorrhage, Multiple Sclerosis and long-term alcohol abuse. However, it is most commonly caused by neurodegeneration. There are several different types of neurodegenerative disorders. In this thesis two of them are explored in detail, namely AD and frontotemporal dementia (FTD).

Alzheimer disease

AD is a devastating disorder during which patients suffer from dramatic loss in cognitive functions, such as memory loss, delusions and disorientation in time and space. The disease is slowly progressive and often begins with, for example, impaired memory and disorientation. Some patients describe an inability to find their way home, even though the way was previously well known by the patients. As the disease progresses the patients become more and more dependent on care and have problems with carrying out functions of daily living. Relatives often bear witness to a complete personality change of the patient, in addition to a high care burden. Out of the about 24 million people in the world with dementia, at least 50% of them are classified as having AD (Qiu et al., 2007). The numbers are increasing as the population becomes older, since age is the most pronounced risk factor. Among the oldest individuals (90 years or above), the prevalence is about 50% (Graff, 2005). AD was first described, and named, by Alois Alzheimer who published his observations on a female patient that suffered from a progressive presenile dementia in 1907 (Alzheimer, 1907). At autopsy, abnormal changes in the brain of the patient in terms of amyloid plaques and neurofibrillary tangles were observed. These changes are today widely recognized as the two pathological hallmarks of AD. The amyloid plaques mainly consist of aggregates of the amyloid β (Aβ) peptide, and appear sometimes with a distinct core and sometimes without (diffuse plaques and cotton-wool like plaques). The core of the extracellular amyloid plaques are about 15-20 μm in size (Söderberg et al., 2006). The diffuse and, the rarely observed, cotton-wool like plaques are extensively larger. The amount of amyloid plaques differs between cases, but are present both in early onset AD (EOAD) cases and late onset AD (LOAD) cases (and sometimes even in old healthy individuals (Polvikoski et al., 1995)). The tangles are twisted fibers inside of nerve cells consisting of the phosphorylated microtubule associated tau protein. It is well documented that the phosphorylation of tau contributes to degeneration of neurons by inhibiting binding of the protein to the microtubule, and thereby reduces its stability (reviewed in Lebouvier et al., 2009). Except for these abnormalities, AD-brains are also characterized by a decreased volume due to atrophy, neuronal and synaptic loss, signs of inflammation and oxidative stress. The diagnosis is definitive once a neuropathological examination of the postmortem brain verifies the presence of accumulated extracellular plaques and intracellular neurofibrillary tangles (Mirra et al., 1991), as well as atrophy. Among the many theories proposed to explain the pathogenesis of AD, “the amyloid cascade hypothesis” is the most prominent (Hardy

and Higgins, 1992). It states that the amyloid β (Aβ) peptide, cleaved from the amyloid precursor protein by secretases, is responsible for the neurodegenerative processes in the brain (see *PSEN1*-section for details).

Frontotemporal dementia

FTD is the second most common form of neurodegenerative dementia after AD (McKhann et al., 2001). The age at onset is generally below 65 years, and lower than the age at onset of AD (Rademakers and Hutton, 2007). FTD is not mainly characterized by loss of memory, but instead of abnormalities of personality, behavior and/or language. Signs of Parkinsonism are sometimes observed and the prevalence of motor neuron disease, such as amyotrophic lateral sclerosis, is higher in FTD patients than in the general population. Progressive non-fluent aphasia and semantic dementia are part of the FTD spectrum, sometimes without any memory deficits. Neuropathologically, FTD cases can be divided into immunohistochemically defined subgroups. Frontotemporal lobar degeneration (FTLD) is used as an umbrella term for these subgroups. One group of FTD patients show neuronal immunoreactivity for the microtubule associated protein tau (FTLD-tau), while another group is negative for tau, but instead immunoreactive for ubiquitin and in the majority of cases also for TAR DNA-binding protein 43 (FTLD-TDP) (Mackenzie et al., 2010; McKhann et al., 2001). It was recently shown that a small group of patients (about 10%) that were not immunoreactive for tau or TDP-43 were instead immunoreactive for a protein called FUS (fused in sarcoma, also known as translated in liposarcoma, *TLS*) coded by a gene on chromosome 16p11 (Neumann et al., 2009a; Neumann et al., 2009b). Macroscopically, lesions are most commonly found in frontal and temporal cortex, in addition to the dentate gyrus of hippocampus, in all subgroups of FTLD that cannot be clinically distinguished. In FTLD-tau patients, neurofibrillary tangles are abundantly observed. The FTLD-TDP patients are characterized by TDP-43 positive dendrites and axons as well as neuronal cytoplasmic inclusions (NCI) consisting of hyperphosphorylated, ubiquinated and c-terminally fragmented TDP-43 (Arai et al., 2006; Neumann et al., 2006). Patients that are immunoreactive for FUS have neuronal intermediate filament inclusion and glial cytoplasmic inclusions (Neumann et al., 2009a; Neumann et al., 2009b).

GENETICS IN DEMENTIA

Genetics in Alzheimer disease

The pathogenesis of AD is thought to be multi-factorial, with both environmental and genetic influences. The majority of all AD cases do not show any obvious pattern of inheritance. However, the contribution of genetic factors has to be considered in most cases. For the most common form, LOAD, the strongest and most replicated genetic risk factor is located on chromosome 19q13, a region that harbors a cluster of genes that might be involved in the disease, i.e., apolipoprotein ε (*APOE*), apolipoprotein C1 (*APOC1*) and translocase of outer mitochondrial membrane 40 (*TOMM40*). The ε4 allele of *APOE*, which both reduces the age at disease onset and increases the risk of developing the disease is the most well studied (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993). The *TOMM40* gene was recently shown to contain a polymorphic poly-T variant (rs10525423) that was suggested to increased the precision of age at onset estimations in *APOE* ε3 carriers. Phylogenetic analyses showed that those carrying ε3 allele of *APOE* in combination with a long poly-T variant of *TOMM40* had an earlier onset of the disease, compared with those carrying ε3 in combination with the shorter *TOMM40* variant (Roses et al., 2009). Even though the *APOE* ε4 allele most likely has an effect on the development and the onset age of AD, the surrounding genes might have important contributions that should not be neglected. Except for the genes on chromosome 19q13, several genes have been reported to contribute to the risk of AD. Since those genes have small effects, large sample sizes are needed to gain enough power to detect them. In the summer of 2009, two large genome-wide association studies (GWAS) were published identifying three novel risk genes for AD (Harold et al., 2009; Lambert et al., 2009). The clusterin gene (*CLU*), also called *APOJ*, on chromosome 8 was identified in both studies. Due to its functional similarities to *APOE* it was already in 1996 investigated for an association to AD (Tycko et al., 1996) but the study had limited power to detect the association. The phosphatidylinositol binding clathrin assembly protein (*PICALM*) on chromosome 11

and the complement component receptor 1 (*CR1*) on chromosome 1 reached whole genome-wide association significance in one of the two studies. To increase the power to detect genes with small effects, data from all association studies, both GWAS and candidate gene studies of susceptibility genes of AD, have been assembled in the ALZgene database (http://www.alzgene.org), where meta analyses are performed systematically and continuously as new data appear (Bertram et al., 2007). Today, the database includes 1355 studies of 660 genes and 2836 polymorphisms. The genes with the strongest evidence for association to AD are presented as top-candidates and except for the already mentioned genes the sortilin-related receptor 1, *SORL1* and a region on chromosome 14, an association detected by GWAS to which no specific gene has been assigned, should be mentioned as likely to have an effect on the disease risk.

AD susceptibility genes – top hits
APOE
CLUSTERIN
PICALM
SORL1
Chr 14q32.13

A few percent of all AD cases have an early onset with autosomal dominant inheritance. Mutations in three genes (Amyloid Precursor Protein; *APP* [OMIM 104760], Presenilin 1; *PSEN1* [OMIM 104311] and Presenilin 2; *PSEN2* [OMIM 600759]) have been shown to be disease causative. Mutations in the *PSEN1* gene have been reported to account for up to 55% of all EOAD cases (Janssen et al., 2003; Lleo et al., 2002). Mutations in the *APP* gene and the *PSEN2* gene also cause EOAD, but less frequently than the *PSEN1* gene. However, the frequency of mutations seems to differ depending on the population origin. In Sweden, the frequency seems to be very low, compared with other European countries. From Sweden two mutations in APP: APP_{swe} (Mullan et al., 1992) and APP_{arc} (Nilsberth et al., 2001) and three in *PSEN1*: Met146Val (Clark, 1995), His163Tyr (Clark, 1995) and Arg269His (Forsell et al., 1998) have been described. In total, 102 unrelated Swedish cases have been screened for mutations in *PSEN1* and *APP*, but no additional pathogenic mutations have been identified (Clark, 1995; Forsell et al., 1997; Forsell et al., 1998). Moreover, in a recent report, a Swedish sample set of 77 AD cases was screened for *APP* duplications, since it is believed that increased levels of the Aβ-peptide is a cause of AD. No duplications were identified, indicating that also the frequency of *APP* duplications is lower in Sweden compared with other European countries (Blom et al., 2008). The AD/FTD database is continuously updating the list of genetic variations found in cases and families with AD and other neurodegenerative diseases

(http://www.molgen.ua.ac.be/ADMutations). Linkage analyses have identified regions suggested to harbor disease causing genes on chromosomes 6, 9, 10 and 12 (reviewed in Kamboh, 2004). In a Swedish sample set of 109 families, the known *APOE* locus on chromosome 19 was confirmed, but no additional region was detected (Sillen et al., 2006; Sillen et al., 2008)

Genetics in Frontotemporal dementia

A substantial part of all cases with FTD have a positive family history and a strong genetic influence can be suspected to the disease etiology. Mutations have been found in a few genes and two of them, in which mutations causing FTD most commonly have been found, are located in a narrow region on chromosome 17. The finding of two genes located very close to each other, in which mutations cause a very similar phenotype, is extremely rare, and as far as we know today this has to be considered as a coincidence. At a consensus conference in 1996, it was found that 13 kindreds with FTD were linked to chromosome 17q21 and the searching for a causative gene was initiated (Foster et al., 1997). Many of these cases showed immunoreactivity for a protein coded by a gene located in the chromosomal region, the *MAPT* gene. Two years later, mutations in this gene were reported to be disease causative in a proportion of these families (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). *MAPT* mutations explained the disease in nine of the initial 13 families. Today, more than 40 different *MAPT* mutations have been shown to be causative of FTD associated with taupathy (http://www.Molgen. ua.ac.be/FTDMutations), and these mutations seem to explain about 5-10% of all FTD cases. However, many cases were not immunoreactive for tau, and no mutations in *MAPT* could be found in the remaining four families linked to chromosome 17. Further neuropathological research revealed that these cases were instead immunoreactive for ubiquitin and the search for the disease causative mutations in these families continued. Since the linage peak on chromosome 17q21 was still present, all possible defects of the tau gene were excluded: mutations, deletions and duplications, genomic rearrangements, abnormal mRNA and abnormal protein isoforms (Cruts et al., 2006). After this careful investigation of tau, the search for a new gene causing the same clinical features in a very narrow chromosomal region began. The region was narrowed down to 6.3 megabases (Mb) in a Dutch family (Cruts et al., 2006) harboring 165 genes. In 2006 the first mutation in the granulin gene, *GRN*, located only 1.7 Mb centromeric to *MAPT*, was reported (Baker et al., 2006; Cruts et al., 2006). Mutations in *GRN* were shortly thereafter shown to be present in three of the

four remaining FTD families, leaving just our Swedish Karolinska family to study (Rademakers and Hutton, 2007). Also in this last family we were able to identify a disease causing mutation in *GRN*, as described in Paper II (Chiang et al., 2008). Nowadays, we know that mutations in *GRN* are as common as the *MAPT* mutations and account for about 5-10% of all FTD cases (Rohrer et al., 2009). From a clinical point of view, carriers of mutations in *MAPT* and *GRN* cannot be distinguished. However, carriers of mutations in *MAPT* have been observed with behavioral changes and semantic deficits more often than *GRN* mutation carriers that more often suffer from non-fluent progressive aphasia (Pickering-Brown et al., 2008) but these observations need to be confirmed.

Except for mutations in *MAPT* and *GRN*, mutations in three other genes have been found to cause FTD: the charged multivesicular body protein 2B gne (*CHMP2B*) on chromosome 3 (Skibinski et al., 2005), the valocin-containing protein gene (*VCP*) on chromosome 9 (Watts et al., 2004), and the fused in sarcoma gene (*FUS*) on chromosome 16 that was described a few weeks ago for the first time in a FTD patient (Van Langenhove et al., 2010).

In February 2010 the first GWAS on FTD was published (Van Deerlin et al., 2010), including individuals with TDP-43 immunoreactive inclusions, both with and without mutations in *GRN*. The *TMEM106B* gene, coding an uncharacterized transmembrane protein, on chromosome 7, was identified as a susceptibility gene for FTD, primarily in those carrying mutations in *GRN*. For a summarized and simplified illustration of the genetic- and neuropathological subgroups of FTD, see Figure 1. Note, however, that not all individuals that are immunoreactive for TDP-43 are *GRN* mutation carriers. In addition, both the TDP43+ and FUS+ group of patients are immunoreactive for Ub, as well as those with mutations in the *CHMP2B* gene.

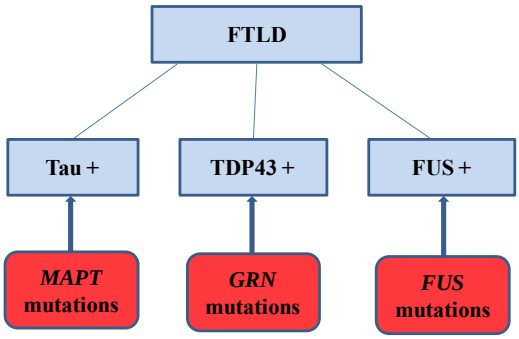

Figure 1. A simplified picture of the neuropathological features found in FTD patients with mutations in different genes.

RISK AND PROTECTIVE FACTORS

In order to deal with the development of prevention strategies, epidemiological research focuses on the identification of both risk and protective factors. The period in life in which a person is exposed to risk and protective factors has gained some attention, as many researchers today advocate that the sum of risk and protective factors during the life course results in the final risk for dementia. In addition, some factors might influence the risk during a specific period of life, but not in another. Moreover, risk and protective factors may act differently in different ethnic groups and for example, in a gender specific manner. The evidence for factors with a suggested role in the etiology of dementia is mainly derived from longitudinal studies. The evidence has been summarized by 26 experts in a report from 2008, published by the Swedish Council on Technology Assessment in Health Care (SBU, 2008). The present literature was systematically reviewed and each risk and protective factor was scored according to internal validity and causal criteria. The evaluation was done separately for dementia and AD. Except for the role of **age** and the above mentioned ***APOE* ε4 allele**, factors that received highest quality grades (at least "moderate scientific evidence") are listed below:

Familial aggregation: First degree family members to a person with AD were summarized to have an increased risk for the disease. The evidence in the literature was limited for a role of familial aggregation in other dementias. This association might be due to both genetic and environmental factors.

Low education: Education was shown to be an important factor which can modulate the risk for dementia. This association may be explained by the fact that lower educated individuals are more prone to adopt an unhealthy lifestyle. However, it might also be due to an increased brain reserve, related to higher education which may contribute to a reduced risk of dementia or at least postpone the dementia onset (Fratiglioni and Wang, 2007).

High midlife blood pressure: Elevated blood pressure in midlife was suggested to increase the risk for both dementia and AD. However, in late life even low diastolic blood pressure seems to be predictive of dementia and AD, as has been shown in the Kungsholmen Project (Qiu et al., 2003).

Antihypertensive drugs: The risk for dementia, especially vascular dementia, can be reduced by using antihypertensive drugs in subjects with high blood pressure. The evidence for an effect on other types of dementia is limited.

Diabetes: The association between diabetes and dementia is supported by a moderately strong evidence, but the results are inconsistent for AD.

Leisure activity: Frequent engagement in leisure activities is inversely related to dementia and AD. A substantial number of studies have explored the association between leisure activities and risk of dementia and AD. These activities can be divided in three groups depending on the activity type and their social, physical and mental components. Among them, physical activity is the most studied activity. In spite of the differences in study design and type of activities studied, most of the reports on the topic have suggested a protective effect of leisure activities against dementia (Fratiglioni and Wang, 2007). The contribution of each type of activity to the protective effect is not known, but the most favorable situation is linked to activities that include all three components (Karp et al., 2006), as for example dancing.

Body Mass Index (BMI): The evidence for a role of BMI on dementia risk was at the time for the SBU report insufficient. However, the evidence has accumulated in the past two years (Fitzpatrick et al., 2009; Hassing et al., 2009; Whitmer et al., 2008). A higher BMI at midlife seem to increase the risk for dementia and AD in late life, whereas an accelerated decline in BMI the years preceding the dementia onset has been observed (Gustafson, 2006). Overweight and obesity are closely related to, e.g., hypertension, hypercholesterolemia and diabetes, which might influence the BMI-mediated dementia risk.

Except for the above mentioned risk and protective factors for which the evidence received a high quality score, there are other factors that need to be further investigated. The prevalence of dementia is higher in women than in men, especially among the oldest old. This **gender** difference seems to be confined to AD. Other risk factors are related to vascular damage and vascular diseases, i.e., **heart- and cerebrovascular disease** and **hyperlipidemia**. The risk for dementia is modulated also by our social and physical context. Some environmental factors have been extensively studied in relation to dementia development such as **socioeconomic status, occupational exposure, cigarette smoking** and **alcohol consumption.**

THE STUDIED GENES

APOE – a risk factor for AD and mortality

Apolipoprotein E, APOE, was discovered several decades ago, and the knowledge about *APOE* as a risk factor for AD has been known since the early 1990s and has consistently been confirmed in many studies since then (Corder et al., 1995; Corder et al., 1993; Poirier et al., 1993; Strittmatter et al., 1993). A lot of investigations have tried to understand the possible mechanism underlying the association between *APOE* and AD, but the current knowledge is still limited. The protein is 299 amino acids long, consists of one lipid binding site and one receptor binding site and exists in three common variants, the ε2, ε3 and ε4 isoforms. The major differences between the three isoforms are the amino acids at residues 112 and 158 (Figure 2), having a tremendous effect on the protein structure and the biological mechanisms in which APOE is involved (Strittmatter and Bova Hill, 2002). APOE is a protein that binds to and transports lipids. APOE is a major transporter of lipids in the central nervous system. Neuropathologically, carriers of the ε4 allele have been shown to have a higher degree of deposition of Aβ than those without the ε4 allele, even in the absence of cognitive deficits (Polvikoski et al., 1995).

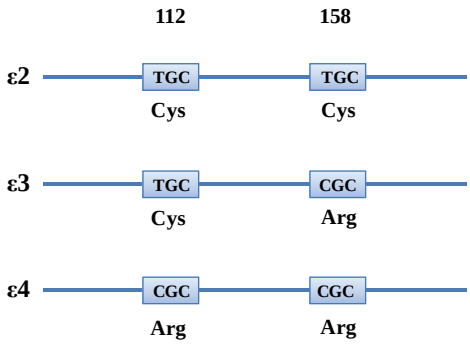

Figure 2. The common variants of APOE differ at residues 112 and 158, harboring either an arginine or a cystein encoded by exon 4 of the *APOE* gene.

The ε4 allele has been suggested to be the ancestral allele (Finch and Sapolsky, 1999; Mahley and Rall, 1999). One argument for this is the fact that all the great apes carry a

ε4-like allele, harboring an arginine in residue 112. In addition, it is unlikely that a thymine (T) to cytosine (C) transition has occurred in a GpG rich region like this one, which would be required for the substitution from ε3 to ε4. In regions like this, methylation of C, and subsequently spontaneous deamination into a T, is the favored mechanism for substitutions. The frequencies of the *APOE* alleles differ in different ethnic groups and are relatively high in the northern European countries (Gerdes et al., 1992). In Sweden, about 20-25% of all individuals carry one ε4 allele (Hannelius et al., 2005), and more than 50% carry one ε3 allele. The ε2 allele is relatively uncommon, with a frequency less than 10%. The ε4 allele seems to increase the risk for AD in all ethnic groups, with a possible exception for individuals of African origin (Farrer et al., 1997). The risk exerted by the ε4 allele seems to interact with age, and is attenuated among the oldest old (Farrer et al., 1997).

Except for its role in AD etiology, the *APOE* gene has also been thoroughly studied for its role on all cause mortality and reduced lifespan. The majority of studies have shown a lower frequency of the ε4 allele and a higher frequency of the ε2 allele in aged populations (Lewis and Brunner, 2004). Apart from its role in AD, the *APOE* gene has also been associated with cardiovascular diseases (CVD) (Stengard et al., 1996; Stengard et al., 1995; Utermann et al., 1984; Wilson et al., 1994; Vogt et al., 1997) and might therefore lead to an earlier death for affected individuals. However, it should be kept in mind that the ε4 allele is not sufficient or necessary to cause disease or an early death. Among the participants in the Kungsholmen Project with genotypes available, 29 were carriers of two ε4 alleles and 11 of them were non-demented. Five of the ε4/ε4 homozygote participants were relatively well educated females, who survived to the age of 90!

***GRN* – a role in neurodegeneration**

Progranulin (GRN) is a protein that can be processed into smaller granulins or epithelins. The protein is coded by the granulin gene (*GRN*). The granulins were identified in the early 1990s and have mostly been recognized for their role as growth factor peptides. However, new functions have repeatedly been identified for GRN, for example, regulation of cell division and an important role in cancer have been consistently shown, in addition to functions in wound repair and inflammation, reviewed by He and Bateman (He and Bateman, 2003). With the discovery of pathogenic mutations in *GRN* causing neurodegenerative disease, it became clear that

GRN also increases neuronal survival. *GRN* is located on chromosome 17q21 and contains 13 exons, of which the initial exon, the first bases in the second exon and the last part of exon 13 are non-coding regions (Figure 3). At present, 68 mutations in the *GRN* gene have been detected to cause neurodegeneration (http://www.molgen.ua.ac.be/FTDMutations). Several types of mutations have been found to cause FTD. Most of them seem to result in a premature stop codon that subsequently results in degradation of the mutated mRNA by nonsense mediated decay (NMD). NMD is known as an mRNA surveillance mechanism to prevent the translation of truncated proteins that might have deleterious effects for the organism. Mutations that activate the NMD machinery can result in null alleles, i.e., complete absence of the gene product. The presence of such a mutation can be detected by analyses on the protein level, as it is decreased by about 50%. Neuropathologically, carriers of mutations in *GRN* are negative for tau, and instead immunoreactive for ubiquitin and TDP-43 (FTLD-TDP). Atrophy is most pronounced in the frontal lobes, but also observed in the temporal lobes. Interestingly, the temporal atrophy is often asymmetric, with a more extensive atrophy on the left side than the right, which was also described in the family with GRN mutation described in Paper II (Basun et al., 1997).

Figure 3. The genomic structure of *GRN*. Grey color indicates untranslated regions, while the coding regions are indicated in blue.

***PSEN1*– the amyloid cascade hypothesis**

Based on the knowledge that the amyloid plaques in AD brains mainly consist of the Aβ peptide (Masters et al., 1985), a cleavage product of APP, in addition to the fact that Trisomy 21 patients that harbor three copies of the *APP* gene accumulate amyloid plaques and develop AD, the amyloid cascade hypothesis was proposed by Hardy and Higgins in 1992 (Hardy and Higgins, 1992). They suggested that the deposition of insoluble Aβ is the initial cause of AD pathology, followed by the formation of neurofibrillary tangles, cell loss and vascular damage. In an updated version of the amyloid cascade hypothesis, the soluble Aβ oligomers rather than the final amyloid deposits are in focus for the toxicity of Aβ (Hardy and Selkoe, 2002). The first pathogenic mutation in *APP* was described in 1991, a few years after the region on

chromosome 21 had shown linkage to AD (Goate et al., 1991; Tanzi et al., 1987). At the time for the proposal of the amyloid cascade hypothesis, three mutations in the *APP* gene had been discovered to cause hereditary forms of AD. In 1995, the first pathogenic mutations in *PSEN1* on chromosome 14 were described (Sherrington et al., 1995). The finding that also the *PSEN1* (and *PSEN2*) is involved in the processing of APP gave further support for the theory of the amyloid cascade that has been in focus for research since then. The processing of APP can be divided in two pathways: the non-amyloidogenic pathway and the amyloidogenic pathway, differing in terms of protease cleavage and the resulting cleavage products. APP can be either cleaved by the α-secretase, which precludes the production of the toxic Aβ since it cleaves the peptide within the Aβ region, or by the β secretase and subsequently by the γ-secretase, producing the Aβ-peptide. The PSEN1 protein is part of the γ-secretase complex, responsible for the second cleavage of APP in the amyloidogenic pathway influencing the production of the Aβ-peptide that exists in different lengths. The name "amyloid" refers to the ability of a protein to form β-sheets and to aggregate into insoluble depositions. The longer forms of the Aβ-peptide are more amyloidogenic and more prone to aggregate. The mechanism by which mutations in *PSEN1* cause AD is not fully understood. An increase in the $A\beta_{42}/A\beta_{40}$ ratio has been observed for the majority of *PSEN1* mutations, which also seems to correlate with age at onset (Kumar-Singh et al., 2006). At first glance, this might look like a gain of a toxic function since accumulation of toxic Aβ is increased in the AD brain, in line with the amyloid cascade hypothesis. However, when looking more closely at the nature and function of *PSEN1* mutations, some critical points against this theory have been identified. The increase in the $A\beta_{42}/A\beta_{40}$ ratio has most often been attributed to a decrease in the $A\beta_{40}$ and total inactivation of Psen1 prevents the formation of Aβ in cells from mice (reviewed in De Strooper, 2007). The fact that the *PSEN1* mutations are distributed all over the gene, and not specifically close to the active site, makes it more reasonable to believe that the mutations cause a general instability of the protein structure and therefore decreases its function (Figure 4).

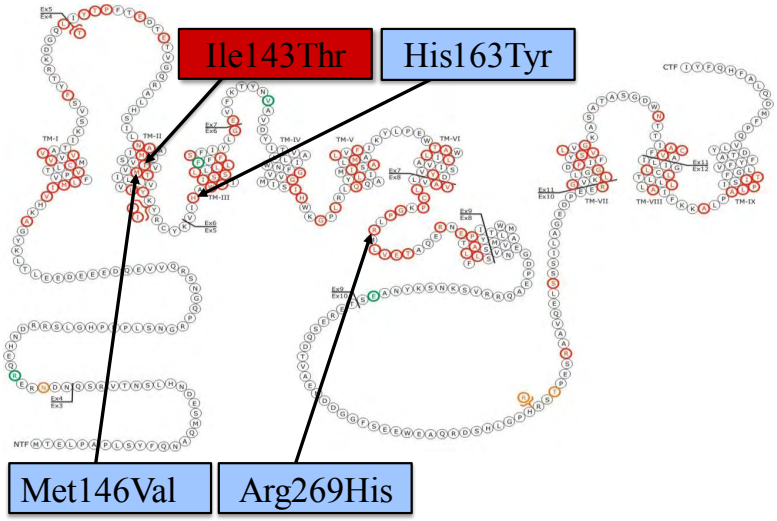

Figure 4. The distribution of the mutations in *PSEN1*. The location of the four mutations detected in Swedish families are indicated with boxes/arrows. The red box indicates the mutation described in Paper III.

***FTO* - fat mass and obesity associated gene**

Researchers in the obesity field had for a long time been struggling to find genes with importance for body weight. In 2006, the *FTO* gene was found to be associated with increased Body Mass Index (BMI) in both childhood and midlife in a GWAS for type 2 diabetes mellitus (T2DM) (Frayling et al., 2007). In that study, the *FTO* association to increased risk for T2DM was abolished after adjustment for BMI. The association between variants in *FTO* and obesity/T2DM has been consistently replicated in several GWAS (Burton et al., 2007; Hinney et al., 2007; Loos et al., 2008; Meyre et al., 2009; Scuteri et al., 2007; Thorleifsson et al., 2009). *FTO* is a large gene (~400kb) located in chromosome 16q12.2 and codes for a protein suggested to be involved in the repair system of both single stranded DNA and RNA, by oxidative demethylation of 3-methylthymine and 3-methyluracil (Jia et al., 2008). The *FTO* gene was cloned by Peters et al. from a region that was shown to be deleted in a mouse model with fused toes (Peters et al., 1999). They named it "fatso" due to its large size. The associated SNPs in the above mentioned studies are located in a conserved region in the first intron of the gene. The gene is widely expressed in the human body in various tissues. Of special interest is the abundant expression in the brain, especially in the hypothalamus and the pituitary gland, areas of importance for body weight regulation

and feeding behavior (Dina et al., 2007). From studies on mice, with a similar expression pattern as in humans, expression of the gene has been observed predominantly in neurons, and could not be observed in astrocytes or glia cells (Fredriksson et al., 2008). As expected for a protein with a role in the DNA/RNA repair system, the protein was mainly found in the cell nucleus (Gerken et al., 2007). How the protein acts to increase body weight is not known. Physical activity has been suggested, by epidemiological studies, to counteract the effect of *FTO* on BMI (Andreasen et al., 2008; Rampersaud et al., 2008) but this finding was not supported by another study (Jonsson et al., 2009). The effect of *FTO* on body weight might also involve the processing of insulin. The brain is highly responsive to insulin, and the effect of insulin on cortical activity has been shown to be dependent on body weight. Tschritter et al. found that cerebrocortical activity was increased by insulin in lean humans, while this effect was absent in obese humans (Tschritter et al., 2006). *FTO* may exert its role on obesity through lowering the insulin response in the cortical area, since carriers of the risk allele had a low response to insulin similar to obese humans, and this effect was independent of BMI (Tschritter et al., 2007).

Moreover, a recent study shed light on a possible involvement of leptin and its downstream signaling pathway since an increased expression of Fto in the arcuate nucleus of the hypothalamus in rats elevated the mRNA expression of Stat3, a transcription factor with importance for the leptin receptor signaling (Tung et al., 2010). Since leptin is produced by fat cells and adipose tissue, it is well established that the levels of leptin correlate with body weight in humans. In line with that observation, carriers of the obesity associated allele of the *FTO* gene have been shown to have higher levels of circulating leptin (Andreasen et al., 2008; Do et al., 2008; Welsh et al., 2010). The gene has not been investigated in relation to dementia or AD before.

THESIS OBJECTIVES

The overall aim of this thesis was to identify genetic variations with importance for the development of dementia and to investigate their consequences both in families and in populations.

Paper I: To investigate the role of *APOE* on mortality in an old Swedish population that was followed in 18 years from age 75+. This study specifically took into account the effect of dementia, cardiovascular disease and gender.

Paper II: To identify the pathogenic mutation that caused a dominantly inherited frontotemporal dementia in a Swedish family and to describe its clinical, neuropathological and biochemical consequences.

Paper III: To identify the pathogenic mutation that caused a dominantly inherited early onset Alzheimer disease in a Swedish family and to describe its clinical, neuropathological and biochemical consequences. Specific aims were to investigate the regional distribution of different Aβ species.

Paper IV: To identify and explore a possible role of the "fat mass and obesity associated" gene, *FTO*, on the risk for Alzheimer disease and dementia in an old Swedish population.

METHODOLOGICAL CONSIDERATIONS

CANDIDATE GENE STUDIES

A common way to study the contribution of genes to the development and progression of complex diseases has been to genotype polymorphisms in candidate genes and test them for association to the disease. These genes might be interesting candidates due to a possible functional role in the disease, or because of their location in the genome identified by previous linkage studies in affected families. Due to the advancement in the molecular techniques, it is now possible to perform genome-wide analyses in a more unbiased and hypothesis free manner, by genotyping several hundreds of thousands of SNPs at once. This design has become more popular as the cost for genotyping decreases. Anyhow, the coverage of the genome is still not complete, and a bias is present in terms of the best covered genes (Li et al., 2008). In addition to that, the large amount of data gathered from a GWAS is a major computational challenge and inconvenient for many researchers. Therefore, the candidate gene studies have not been completely replaced by GWAS. If we look ahead, the technology will be further expanded and include the concept of "whole genome deep sequencing" and also structural variants and copy number variations will be analyzed in a genome-wide manner. Until now, 13 GWAS on AD have been performed some of which have used overlapping populations. Most of them were performed with the highly appreciated intention to make the data publicly available for researchers to download and to investigate their gene of interest. These databases constitute an excellent source for replication of findings from candidate gene studies, as exemplified in Paper IV.

METHODS TO ASSESS GENETIC VARIATIONS

DNA quality

The first step to assess genetic variations in DNA samples is usually amplification of the DNA sequence of interest by using a polymerase chain reaction (**PCR**). This technique is based on thermal cycling and DNA replication to obtain many copies of a short fragment, harboring the variation of interest. Since this is the initial step to determine a variation, optimization of its conditions is crucial. However, except for an optimized PCR, also the quality of DNA is crucial for this initial step. A human DNA sample can be extracted from a number of different sources, most commonly a blood sample but also from for example buccal cells, brain tissue as well as other tissues.

Different protocols for preparation of the different kind of samples are available, but the final purity and concentration of the DNA differs. It is a complex task to collect samples for DNA preparation. To investigate segregation of disease causing mutations in families, DNA samples from more than one generation are needed. In Paper II, all samples were prepared from blood samples obtained during the clinical investigation. However, in Paper III, the mutation was detected in DNA extracted from a blood sample from case III:1 and confirmed in an independent sample extracted from brain tissue. Moreover, DNA from case II:2 was extracted from formalin fixed paraffin embedded brain tissue that had been stored for more than 20 years, and thus were to a high degree degraded. It was certainly a demanding challenge that finally succeeded.

In Papers I and IV, blood samples were obtained during the baseline examination of the Kungsholmen Project and extracted using a method previously described (Higuchi et al., 1989). The samples had been stored for a long period of time but could efficiently be amplified using PCR, and were analyzed for the desirable SNPs after a number of optimization steps.

The appropriate way to analyze a DNA sample depends on several components. First of all, the research question has to be the initial guide. In Papers II and III, the question was whether mutations could be found in specific genes in a limited number of individuals. For that reason, the exact order of the nucleotides in these particular genes had to be determined by **DNA Sequencing**. In Papers I and IV, we were instead interested in specific polymorphisms in a rather large sample set. For that purpose, we found it appropriate to use the **MALDI-TOF** (Matrix Adsorbed Laser Desorption-Ionisation-Time Of Flight) analysis on a Sequenom MassARRAY™ platform.

DNA sequencing

After the initial PCR that amplifies the region of interest, a sequencing reaction takes place. In this reaction, the amplified DNA is used as a template and either a forward or a reverse primer is used in parallel reactions to create sequences in either direction. The sequencing reaction contains a small amount of modified nucleotides, provided with a nucleotide specific fluorescent termination dye. Each time these nucleotides are randomly incorporated, which terminates the elongation step, DNA fragments of different lengths and with different fluorescent dyes in the 3´end are created. The sequences can thereafter be visualized using capillary electrophoresis. Both in Papers

II and III, all exons in respective genes were sequenced in both the forward and the reverse directions using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) and thereafter visualized on an ABI 3100 genetic analyzer using the software ABI PRISM®.

Genotyping

With approximately 1,200 samples and about 30 SNPs, including the *FTO* rs9939609, to analyze, we took advantage of the Mutation Analysis Facility, MAF, at the Karolinska Institutet. Genotyping was performed using MALDI-TOF analysis on the Sequenom MassARRAY™ platform. In brief, the DNA is amplified using primer pairs designed to amplify the desired SNP loci. Thereafter, a single base extension reaction with a primer adjacent to the SNP-site is performed with mass modified dideoxynucleotides. The resulting allele specific extension products are differentiated by their respective mass, which are analyzed by MassARRAY MALDI-TOF mass spectrometry. Calling of the alleles was performed using the software MassARRAY Typer 3 by cluster analysis.

THE KUNGSHOLMEN PROJECT

Both Studies I and IV are based on data gathered from the Kungsholmen Project, which is a community-based longitudinal study on aging and dementia carried out in a central area of Stockholm, Sweden. The initial population included all registered inhabitants who were living in the Kungsholmen district of Stockholm and were aged 75 years and above in October 1987 (Fratiglioni et al., 1992b; Fratiglioni et al., 1997). Of the 2,368 individuals invited to participate in the study, 181 had died before the first examination, 69 moved out of the area and 308 refused to participate in the study, leaving 1810 individuals to be examined (76.4%). Baseline examination and four follow-ups with approximately three-year intervals were completed before the data collection was terminated in the year 2000. All participants underwent a structured interview, medical examination, and psychological assessment. At baseline all participants went through a Mini-Mental State Examination (MMSE) to screen for possible dementia cases. Those with an MMSE<24 were suspected to have dementia. In order to assess the final dementia diagnoses, an extensive medical investigation was carried out on the cognitively impaired subjects and in a similar number of individuals with normal MMSE scores (Phase II). Of the 1,810 participants examined at baseline, 110 dropped out in phase II

and the final non-demented cohort consisted of 1,473 individuals. Up to 1998, 440 incident dementia cases were detected. The age ranged from 75 to 101, with a mean age of 81.7 years. At this day, less than 50 individuals are still alive. Even though this study population has been thoroughly studied in many different perspectives, the research concerning genetic contribution to disease has focused on the *APOE* gene that was assessed at baseline. However, old but still useful DNA samples from 1,259 of the participating individuals were available for genotyping. The study populations used in Studies I and IV are described in Figure 5.

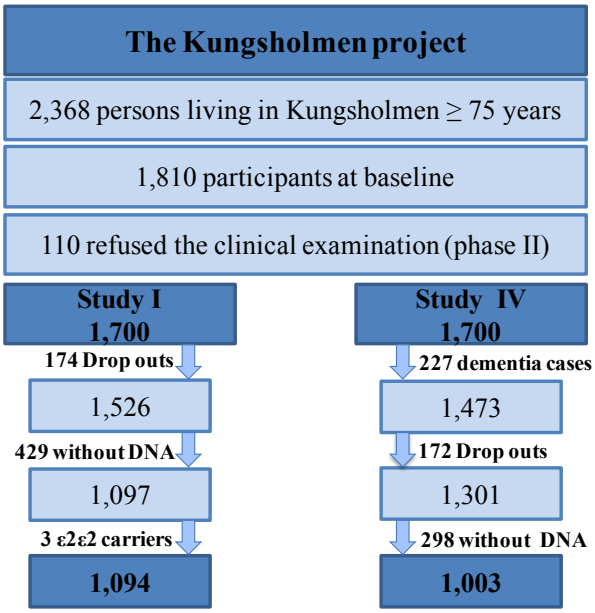

Figure 5. The study population of studies I and IV, derived from the Kungsholmen Project.

EPIDEMIOLOGICAL AND BIOSTATISTICAL CONCEPTS

Epidemiological studies aim to address our understanding of risk and protective factors for diseases at a population-based level. To do that, a wide variety of statistical analyses have to be performed using suitable statistical software. For the analyses performed in Papers I and IV, STATA 9 has been used. Comparison of demographic characteristics was done using χ^2 for categorical variables and ANOVA for continuous variables. In Paper IV, logistic regression models were used to estimate the odds ratios and corresponding confidence intervals for the different vascular risk factors measured at baseline.

Cox proportional hazard models

A popular way to assess relative risks is to use Cox proportional hazard models. The main results both in Studies I and IV were obtained by using different Cox models. By using these kinds of statistical models, we aimed to assess the effect of several variables on the time until an event occurs, the hazard ratios (death in Study I and development of dementia/AD in Study IV). The hazard ratio is an approximation of the relative risk, which is a ratio of the probability for an event to occur between the group that is exposed and the non-exposed group. When the time to an event is available, the Cox regression model is preferred to use, instead of the logistic regression model, as it uses the information about time and censoring to give a more precise estimation of the risk. However, even though this is a robust model that can be used for a wide variety of data sets, the proportional hazard assumption has to be fulfilled. To fulfill the assumption, the hazard function for two groups at any time point has to be proportional. The most straightforward way to check this is to plot the survival curves for the compared groups. Crossed survival curves might violate the hazard assumption. The assumption of proportional hazards was tested in both papers both for each independent variable, and globally for the models.

In Paper I, the outcome; death, and the covariates; dementia and IHCD, were strongly associated with age. For that reason age was used as the time-scale in the Cox regression models, instead of using time in the study, that is more commonly used (Commenges et al., 1998). As each individual came into the study at different ages, from 75 years and above, delayed entry was used in the models (Cleves, 2004). To get a precise estimate of the influence of the covariates on the *APOE*-related mortality, IHCD and dementia were used as time dependent covariates in the meaning that the exact age for the occurrence of the different diseases for each individual were specified in the models. In Paper IV, the analyses were repeated using age as the time scale. The results were similar to those reported in the manuscript; hence, the adjustment for age at baseline was considered as sufficient to obtain accurate estimates of the relative risks.

Interaction

Working both within the genetic and epidemiological fields, interaction appears as a confusing concept, which has also been implied by others (Ahlbom and Alfredsson, 2005). In epidemiology, the simplest case of interaction describes a situation when the effect of the exposure on the outcome differs depending on whether another

exposure/characteristic is present or not. This kind of interaction is often assessed by the introduction of an interaction term in the regression model. To reach significance, the interaction term has to depart from the multiplicative effect, which is a rather stringent requirement. Some researchers state that this requirement is too ambiguous, and advocate using the p-value of 0.1 as the limit for significance for interaction. This kind of interaction is also referred to as statistical interaction. On the other hand, in a biological sense, the term interaction is used to describe the situation when the effect of two exposures on the outcome is bigger than just adding the effect of each exposure. This kind of interaction is also referred to as a biological interaction, and can be evaluated as the departure from the additive effects. Both in Papers I and IV, the interactions were assessed as departure from the multiplicative effects.

Outcome measurements

Death: Information about death was obtained from death certificates. As the study population was old and followed for a relatively long time, the proportion of individuals that died was high. In Study I, 95 individuals in the study population were still alive in 2005, as was defined as the end of the study.

Dementia: In Papers I and IV, using data gathered from the Kungsholmen Project, dementia was diagnosed according to the *Diagnostic and Statistical Manual of Mental Disorders*, revised Third Edition criteria *(DSM-III-R*) (Association, 1987) by using data obtained at the clinical examinations at baseline and at three follow-up occasions. Diagnoses were assessed by following a rigorous three-step procedure where two physicians independently made preliminary diagnoses, and a third opinion was obtained in case of disagreement. In Paper I, dementia was used as a covariate to assess its influence on the *APOE*-related mortality. All types of dementia cases were used in the analyses. Also, both prevalent and incident dementias were included in the analyses, since separate analyses gained similar results. Prevalent cases included all subjects who received a diagnosis of dementia at baseline, while incident cases included all subjects who developed dementia during the nine years of follow-up. In Paper IV, only incident dementia cases were included, as we sought to estimate the RR of the disease. Dementia and AD were used separately as the main outcomes. By using all dementia cases, some power is gained due to increased numbers in the analyses. However, it can be speculated that the group of AD cases has a more homogenous etiology, possibly with a greater genetic influence, compared with dementia in general,

making it relevant to use both in separate analyses. Postmortem examinations were not conducted in the project. Despite a rigorous examination procedure, it should be remembered that the possibility to separate different types of dementia is limited without a postmortem examination. In the initial diagnostic procedure, about 75% of the dementia cases were believed to have AD. In a later project, aimed to re-evaluate the diagnoses in the Kungsholmen Project, it was found that more than half of the AD-cases actually had some vascular component (Aguero-Torres et al., 2006).

Since dementia is a slowly progressive disease, the exact time for an individual's conversion from healthy to demented is difficult to determine. In Papers I and IV, the individuals were examined every three years and the time for dementia onset was assumed as being halfway between two examinations, or between examination and death if the dementia diagnosis was recorded in the national inpatient registry, where hospital discharge diagnoses since 1969 are registered.

Exposure assessments

Baseline demographic characteristics: Data on age, sex and education was collected from participants at baseline using standardized protocols (Fratiglioni et al., 1992a; Fratiglioni et al., 1997). Age was used a continuous variable in the analyses. The education level was measured by the maximum years of formal schooling. Subjects were divided into two categories according to their highest education level (low education ≤ 8 years and high education > 8 years).

IHCD (Ischemic heart and cerebrovascular disease): Diseases with a documented association to *APOE* were included in our variable for IHCD that was assessed at baseline and during the nine years of follow-up by using the computerized Stockholm Inpatient Registry System, where hospital discharge diagnoses from 1969 up to 1998 were registered. It should be noticed that some diseases that are usually included in the definition of CVD were not included in the IHCD-variable (heart failure for other reasons than ischemia) because these could not be found as *APOE* related diseases in the literature. To eliminate confusion we used the term IHCD instead of CVD. Data from the two sources were combined to create one variable for IHCD defined as the presence of any of the following diseases classified according to the International Classification of Disease (ICD, eight, ninth and tenth revision; WHO, 1969): ischemic

heart disease (ICD8-9: 410-414, ICD10: I22-25) or cerebrovascular disease (ICD8-9: 430-438, ICD10: I60-69**).**

BMI: Weight and height were measured at the baseline examination in light clothes and without shoes. BMI was calculated as weight (kg) divided by height (meters) squared. BMI ≥30 was classified as high BMI, as the definition used by WHO.

Physical activity: Information about physical inactivity was obtained from an open question during a personal interview at baseline concerning leisure activities. Each activity was scored based on their physical, mental and social components as described in (Karp et al., 2006). Those that did not report any activity with a physical component were classified as physically inactive. To investigate whether the level of physical activity was a matter of physical independence, activities of daily living (ADL) measured with ADL-Katz index were taken into account.

Cardiovascular disease: Information was obtained from the inpatient register and included the following diagnoses: ischemic heart disease (ICD-8 and 9 codes 410–414), heart failure, left ventricular failure and other myocardial insufficiencies (ICD-8 codes 427 and 428; ICD-9 code 428.x), atrial fibrillation (ICD-8 code 427.9; ICD-9 code 427) and stroke (ICD-8 and 9 codes 430–438).

Diabetes: Subjects were classified as diabetic if the ICD-8 and 9 code 250 was recorded in the inpatient registry, if the participant used anti-diabetic drugs, or if the blood glucose level (in samples taken without any instructions to the participants regarding fasting) was ≥11.0 mmol/l at the baseline examination (Xu et al., 2004). Medical drugs were coded according to the Anatomical Therapeutic Chemical (ATC) classification system (Nordic Council on Medicines). Anti-diabetic drugs included all oral and injected glucose-lowering drugs (ATC code A10). Information on medical drug use for the two weeks prior to the baseline interview was collected from the subjects.

Pre-diabetes: Diabetes-free subjects with random blood glucose level of ≥7.8 but lower than 11.0 mmol/l (Xu et al., 2007; Xu et al., 2009) was defined as pre-diabetic.

Generalizability

Participants in the Kungsholmen Project were all living in the Kungsholmen district in Stockholm. Inhabitants in this area had some specific characteristics that should be considered before results from the study are generalized to other populations. The mean age at death was relatively high as well as the proportion of women. They were relatively well educated and differed in their marital status from other urban areas of Sweden. These features might have influenced some of the information used in Studies I and IV. As they became old, it can be speculated that they had adopted a healthier lifestyle compared to inhabitants in other areas by for example, having a healthier diet and a more physically active way of life. Both *APOE* and *FTO* seem to be involved in lipid metabolism and affect vascular risk factors, and a healthy lifestyle seems important to overcome the harmful effect of these genes. As a result of that, a healthy lifestyle might have led to an underestimation of the effect of these genes in this population. However, the results may be generalized to other urban areas in the Western countries.

ETHICAL CONSIDERATIONS

STUDIES I AND IV – THE KUNGSHOLMEN PROJECT

The invited participants were sent a personal letter, which explained the nature of the project and the importance of their participation. However, it was clearly stated that the participation was voluntary. At the first visit, the aim of the project was presented, as well as the fact that all obtained information would be kept confidential. Thereafter, informed consents were obtained from each subject. If a participant was suspected to be cognitively impaired, a proxy gave consent. The examinations and interviews were interrupted in case the participant expressed any hesitation to continue. All phases of the project received approval from the Ethics Committee at the Karolinska Institutet in Stockholm.

- Baseline: Dnr. 87:234
- First follow up: Dnr. 90:251
- Second follow up: Dnr. 94:122
- Third follow up: Dnr. 99:308
- Death certificates: Dnr. 99:025
- Inpatient register data: Dnr. 01:020

STUDIES II AND III – CLINICAL IMPLICATIONS

These studies were approved by the Ethics Committee at the Karolinska Institutet in Stockholm.

The ethical considerations in Studies II and III are of a different nature than those in Studies I and IV, since they are carried out on an individual/family level, in contrast to the population-based studies.

- Sample collection - Alzheimer disease: Dnr. 485/02
- Genetic research - Alzheimer disease: Dnr. 484/02
- Genetic research - other diagnoses (FTD): Dnr. 2007/1212-32
- Biochemical research: Dnr. 2007/661-32

As the knowledge about genetic contributions to diseases increases, the demand for and the debate concerning genetic testing are growing. Genetic tests have been used for diagnostics in recent decades to determine the cause of symptoms in an already diseased patient. However, the use of genetic tests in a predictive manner before disease onset of a dominant inherited disease is much more complex both in its ethical and procedural issues. When genetic tests are desired, they should be done with assistance from a genetic counselor. With lack of prevention strategies for the disease, like AD and FTD, the gain of knowing the mutation status should be carefully considered. Additionally, the penetrance of these mutations is not always 100%, as in the case of the *GRN* mutation in Paper II, which further complicates the application of genetic tests. The age limit for taking a decision about these tests is today 18 years, in Sweden. Different options are available to handle the increased risk in the coming generation. By using prenatal genetic tests and Pre-implantation Genetic Diagnosis (PGD) parents can be helped to improve their chance of giving birth to a child without the mutation. PGD is a genetic test of the embryo before pregnancy occurs, while prenatal genetic tests are performed during pregnancy. So far, PGD has not been used in Sweden for individuals affected by a mutation causing EOAD, but the technique is available and has been reported for early onset dominant AD (Verlinsky et al., 2002).

The definitive diagnosis of AD requires a post mortem examination of the brain. In Paper III, the diagnosis of AD was definitive and set using both the clinical examinations (NINCDS-ADRDA, The National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer Disease and Related Disorders Association)(McKhann et al., 1984) and postmortem autopsy confirmation using both Braak (Bancher et al., 1993) and CERAD criteria (Mirra et al., 1991). In addition, the knowledge about a disease causing mutation in the family further increases the certainty of the diagnosis. However, this information was not available some generations ago. Due to similarities of the involuntary and myoclonic movements in the family carrying the *PSEN1* mutation, one family member was misdiagnosed with Huntington disease. Moreover, the dominant mode of inheritance in this family was at that time most well explored among patients with Huntington disease and gave further support for the faulty diagnosis. Some of the family members were tested for the incorrect disease, Huntington disease. Today, genetic testing is not performed before mutation is confirmed in a diseased family member. Since the identification of the true pathogenic mutation, the family members can now be offered genetic testing again.

The susceptibility genes only contribute to the disease risk and are not generally predictive of disease. When genetic testing is used for complex diseases, such as LOAD, the gene is not necessary or sufficient in order to get the disease. For AD, the *APOE* gene is the only susceptibility gene that has been in debate for clinical use. Sometimes, *APOE* is tested for confirmation of the AD diagnosis. *APOE* is a multifaceted gene, which might also influence the levels of cholesterol and triglycerides, and the risk for cardiovascular diseases and even the mortality risk. In addition, the risk allele is relatively common in the population. The *APOE* gene is not recommended to be used for predictive genetic testing.

RESULTS AND DISCUSSION

In this section of the thesis the main results from Papers I-IV are presented, and discussed. For a detailed description see the respective paper.

STUDY I

APOE-related mortality: Effect of dementia, cardiovascular disease and gender.

As mentioned earlier, the *APOE* gene has been associated with both AD and cardiovascular diseases with a potential effect to shorten the life of affected individuals. By observing the allele frequencies in populations of different ages, it seems that the frequency of the ε4 allele decreases with age, in favor of the ε2 allele. The majority of these studies have been performed using a cross sectional design and the results might be dependent of cohort effects. We took advantage of the longitudinal cohort study, The Kungsholmen Project, to assess the *APOE*-related mortality risk. It should be mentioned that the frequency of the ε4 allele in the study population, that was 75+ years, was similar to the reported frequencies for younger populations in Sweden. Figure 6 shows the allele frequencies at baseline, to the end of the study.

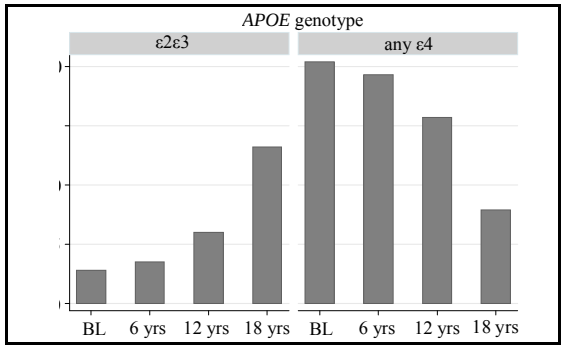

Figure 6. Proportion of individuals with the ε2ε3 genotype and one or two ε4 alleles (any ε4) in the study population at baseline and among survivors at 6, 12 and 18 years of follow-up (BL=baseline).

Survival curves for carriers of the ε2ε3 genotype and any ε4 were different from those with ε3ε3 ($p<0.001$). The longer survival for carriers of the ε2ε3 genotype were more

pronounced in very old age (>90). After basic adjustment (gender and education), individuals with at least one ε4 allele were at a significantly greater risk of dying (RR=1.22; 95% CI: 1.07-1.41) compared to persons with the ε3ε3 genotype, whereas individuals with the ε2ε3 genotype were at a significantly lower risk of dying (RR=0.72; 95% CI: 0.59-0.88).

The next step in the analysis was to investigate if this difference in survival could be explained by the *APOE*-related diseases. Diseases with a documented association to *APOE* genotypes were included in the analyses, including both prevalent and incident cases of **dementia** and **IHCD (Ischemic heart and cerebrovascular disease)** that was assessed at baseline and during the nine years of follow-up (Table 1).

Table 1. Relative risks (RR) and 95% confidence intervals (CI) of mortality for the entire study population, and stratified by gender, in relation to *APOE* genotype derived from different Cox regression models.

		Basic-Adjusted	Basic-Adjusted + IHCD	Basic-Adjusted+ Dementia
Study population				
	ε2ε3	**0.72 (0.59-0.88)**	**0.77 (0.63-0.94)**	**0.76 (0.62-0.92)**
	ε3ε3	1.00 (Ref.)	1.00 (Ref.)	1.00 (Ref.)
	Any ε4	**1.22 (1.07-1.41)**	**1.24 (1.08-1.43)**	1.08 (0.94 -1.25)
Male				
	ε2ε3	1.07 (0.73-1.57)	1.17 (0.79-1.71)	1.11 (0.76-1.62)
	ε3ε3	1.00 (Ref.)	1.00 (Ref.)	1.00 (Ref.)
	Any ε4	**1.48 (1.11-1.98)**	**1.72 (1.27-2.32)**	1.29 (0.96-1.74)
Female				
	ε2ε3	**0.63 (0.50-0.79)**	**0.67 (0.53-0.85)**	**0.66 (0.52-0.83)**
	ε3ε3	1.00 (Ref.)	1.00 (Ref.)	1.00 (Ref.)
	Any ε4	1.13 (0.96-1.33)	1.11 (0.95-1.31)	1.00 (0.85-1.18)

Basic-Adjusted = Adjustment for gender (in unstratified analysis) and education.

To verify whether IHCD and dementia may partially or completely explain the association between the *APOE* genotype and mortality, we entered IHCD and dementia separately in different Cox regression models. The main results in table 1 show that the effect of *APOE* on mortality is modified by adjustment for dementia, but not at all affected by the adjustment for IHCD. The mortality risk was mainly increased for

males, while the protective effect for the ε2ε3 genotype was present only among females. The adjustment for dementia modified the mortality-related effect both for males and females. As supplementary information, it can be mentioned that once demented, there was no difference in survival between those carrying an ε4 allele and the ε3ε3 carriers (data not shown).

STUDY II

Progranulin mutation causes frontotemporal dementia in the Swedish Karolinska family.

Already in 1997 the clinical characteristics of a Swedish family with FTD were described (Basun et al., 1997). The family had shown linkage to chromosome 17, but no mutation in the *MAPT* gene could be identified (Froelich et al., 1997). About ten years later, six new family members were affected by the disease, with a mean age at onset of 54.8 years (range 50-58 years) and average disease duration of 8.2 years (range 4-13 years) and two were still alive (Figure 7).

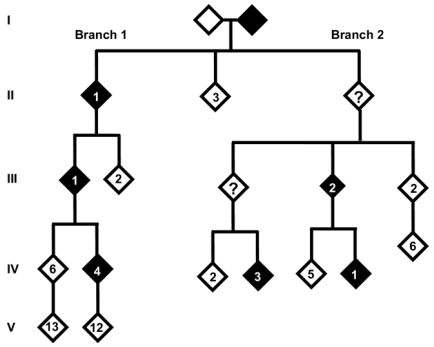

Figure 7. The pedigree of the Swedish Karolinska family with FTD.

The disease often started with personality changes, and the first cognitive symptoms were reported to be language problems. Accompanied with memory disturbances, progressive aphasia was a prominent symptom later in the disease course. Two new cases had been subjects for autopsy. Briefly, both showed neurodegeneration, vacuolization and gliosis in the superficial layers in cortical regions. Ubiquitin (ub) positive neuronal cytoplasmic inclusions (NCI), neuronal intranuclear inclusions (NII) and neurites in the superficial layers of cortex and in the cytoplasm of granular cells in

the hippocampus were detected in both cases. TDP-43 immunoreactivity was observed in similar pattern as the immunoreactivity for ub. When the *GRN* gene was shown to harbor disease-causing mutations in other families with FTD, we initiated a screening for *GRN* mutation in the family. In addition to several polymorphisms, we found a deletion in exon 2 that potentially could cause the disease. The g.102delC mutation (Figure 8) results in a frameshift and was predicted to create a premature stop codon at amino acid position 35 (p.G35fsX19).

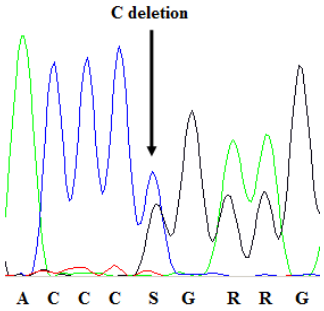

Figure 8. Elektropherogram showing the g102delC mutation (S=C↔G). The initial overlapping and differently colored peaks correspond to guanine (black) and cytosine (blue). Cytosine represents the normal allele that has been deleted on one of the two chromosomes.

This mutation has previously been reported (Gass et al., 2006). The g.102delC was identified in 13 out of 30 analyzed family members of whom seven were diagnosed with FTD. The remaining six mutation carriers did not show any symptoms of FTD of which four were below the mean age at onset for the whole family (53.3 years) and two were above the mean age at onset. The mutation was absent in four unaffected family members above the age of 70 years. The age-dependent cumulative mutation penetrance among the 13 mutation carriers is illustrated in Figure 9. This figure corresponds well with the documented penetrance of about 90% for 70 years old *GRN* mutation carriers (Gass et al., 2006). However, this mutation seems to give rise to a wide range of age of onset. Diseased members of this family had an age between 50 and 58 when symptoms debuted, but a substantially older age at onset (83 years) was previously reported for one case (Gass et al., 2006). *APOE* genotype was not detected as a modifier of the onset age in this family. On the other hand, the *TMEM106B* gene,

recently detected as a susceptibility gene for FTD with TDP-43 immunoreactive inclusions, as mentioned before, is a possible modifier of the onset age which has been assessed in this family (Personal communication C. Graff).

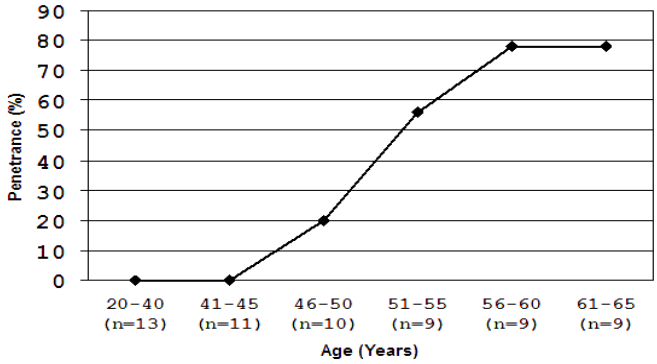

Figure 9. Cumulative penetrance curve for g.102delC mutation carriers in the Karolinska family. n= number of mutation carriers that have reached the age group.

Most of the reported *GRN* mutations result in a reduction of functional protein, likely by degradation of the mutated mRNA by nonsense mediated mRNA decay. As the detected deletion gives rise to a frameshift and a premature stop codon, degradation of the mRNA was suspected also in this case. The level of total RNA from fibroblasts from one affected mutation carrier was compared with the total RNA level from one healthy family member and from five control individuals by real time quantitative PCR. The analyses showed an approximately 50% reduction of the *GRN* transcript in the mutation carrier. Additionally, the cDNA was sequenced to analyze if any mRNA was transcribed from the mutated DNA, but only the strand lacking the mutation was detected.

The pathogenicity for the identified mutation was strongly supported by this study. A reduction of the transcript in a carrier of the mutation gives further support to the hypothesis that loss of functional GRN can result in FTD. Due to incomplete penetrance of the mutation and a wide variety of onset ages and clinical symptoms, the search for modifying genes and environmental factors has to continue, and the usefulness of the knowledge about this mutation in genetic testing has to be carefully considered.

STUDY III

The *PSEN1* I143T mutation in a Swedish family with Alzheimer disease: Clinical report and quantification of Aβ in different brain regions.

A Swedish family was severely affected by a pre-senile neurodegenerative dementia segregating in the family with an autosomal dominant inheritance pattern. The affected family members had a low age at onset (mean=35.6 years) and a short duration of disease (mean=6.8 years). The first clinical symptoms were reported to be related to cognition and typical for AD, such as memory deficits. In addition, these family members suffered from neurological symptoms such as coordination problems and myoclonic jerks. In the late stages of the disease also psychiatric symptoms appeared.

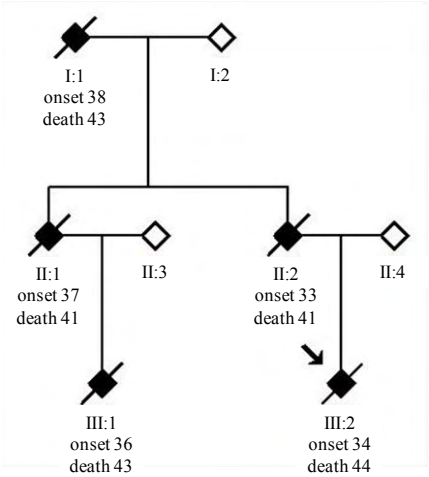

Figure 10. The pedigree of the Swedish family with EOAD.

To screen for a mutation in this family we initially discussed all the three genes known to cause EOAD (*PSEN1*, *PSEN2* and *APP*). As the clinical description of this family corresponds well to that observed for other families with mutations in *PSEN1*, especially the very early age at onset and the rapid progression, we found it reasonable to start to screen the *PSEN1* gene. In the fifth exon of *PSEN1*, a transition of a thymine to a cytosine was identified. The codon ATC, coding for an isoleucine at residue 143, was replaced with ACC, coding for a threonine. The I143T mutation is illustrated in the electropherogram below (Figure 11). The mutation was confirmed in an independent

sample, namely in DNA extracted from frozen brain tissue from the same individual. We were able to detect the mutation in three individuals in the family, in two generations from whom we had obtained DNA.

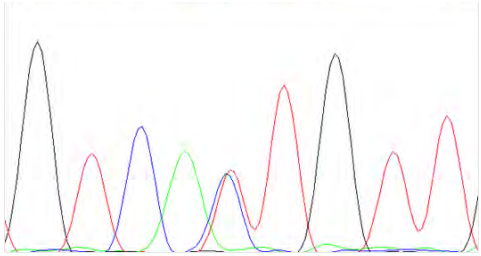

Figure 11. Electropherogram showing the I143T mutation (Y=T↔C). The two overlapping and differently colored peaks correspond to thymine (red) and cytosine (blue). Thymine represents the normal allele that has been mutated to a cytosine on one of the two chromosomes.

In the clinical-based work, it is favorable to exclude the presence of the mutation in a healthy family member, before any conclusion about its pathogenic nature is drawn. However, in this case, the mutation was described before in four single unrelated cases, in three siblings, and two families where segregation was proven (Arango et al., 2001; Cruts et al., 1995; Martin et al., 1991; Miravalle L, 2002; Raux et al., 2005; Rogaeva et al., 2001). According to the Alzheimer Disease & Frontotemporal Dementia Mutation Database the mean age at onset for this mutation was 32.5 years, and the mean age of death 41.2 years (duration 3-12 years), which is very similar to the Swedish family. The families, in which segregation was proven, originated from Belgium and Colombia, and a common ancestor has been excluded by haplotype analyses (Arango et al., 2001). Martin et al. first described the Belgian family clinically and neuropathologically in 1991 (Martin et al., 1991), and the molecular genetics were presented in 1995, by (Cruts et al., 1995). The clinical features of the Belgian family are similar to the Swedish family, with early signs of rapidly progressing memory loss, myoclonus and epilepsy. Furthermore, there was a clinical variability within both families. Therefore, we had no doubts that this was the disease causing mutation. Other mutations in the same codon have been reported. An African family with an isoleucine to methionine substitution has been reported with a mean age at onset of 50 years (Heckmann et al., 2004). A British family carries a substitution to phenylalanine, with age at onset of 55 years. Interestingly, incomplete penetrance was noted in that family (one healthy

mutation carrier was observed at age 68) (Palmer et al., 1999; Rossor et al., 1996). A third family, originating from France, carried a substitution to asparagine, with an age at onset of 45-50 years (Raux et al., 2005). Of all families with mutations in codon 143 in *PSEN1*, carriers of the I143T mutation seem to be the most severely affected, with the earliest onset age, and with the additional symptoms of myoclonus and epileptic seizures, which has not been reported for the other mutations in the same codon.

Three mutation carriers in the family were subject for autopsy. They were severely affected by AD pathology (Figure 12). Atrophy was observed, particularly in the frontal lobes. Numerous amyloid plaques were detected, sometimes with a distinct core, and the tau-pathology was widespread.

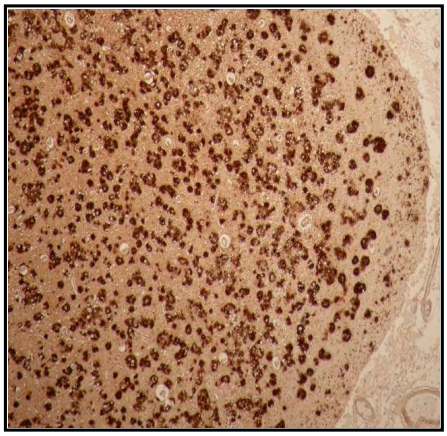

Figure 12. Parietal cortex from case II:2, stained with 4G8 antibody to visualize β-amyloid.

This specific mutation has been suggested to create different lengths of the Aβ peptide to a higher extent than other mutations, e.g., the $A\beta_{1-39}$ and the N-terminal truncated $A\beta_{11-42}$ (Kumar-Singh et al., 2006). A method to measure the concentration of different Aβ species, including a longer fragment consisting of 43 amino acids in brain tissue, was previously developed in our lab (Welander et al., 2009). It is reasonable to believe that longer, and more hydrophobic, fragments of the Aβ peptide are more prone to aggregate than Aβ42, hence; it can be speculated to be readily found in brains from severely affected AD cases. With the opportunity to include six brain regions from two

affected mutation carriers (frontal-, parietal-, temporal-, and occipital cortex, anterior hippocampus and cerebellum), we analyzed the quantity of Aβ40, Aβ42 and Aβ43 by high performance liquid chromatography (HPLC) coupled to an electrospray ionization (ESI) ion trap mass spectrometry (MS) both in plaque cores and in total amyloid preparations. Our findings illustrate that the plaque cores in the analyzed brain regions mainly consist of the Aβ42 peptide, as has been described before (Iwatsubo et al., 1994). Interestingly, we found the longer Aβ43 to be present in all brain regions, except in plaque cores from the cerebellum, where also the concentration of Aβ42 was relatively low. The levels of both Aβ42 and Aβ43 were highest in the occipital cortex (both in plaque cores and in the total amyloid preparations). Interestingly, Aβ40 was not at all detected in plaque cores in any of the regions and was only present in the occipital cortex and cerebellum in total amyloid preparations in case III:2.

In parallel to the quantitative study, immunohistochemistry was performed on consecutive brain sections, using antibodies directed against Aβ40, Aβ42 and Aβ43. Immunoreactivity towards both Aβ42 and Aβ43 was detected both in plaques and in vessels in all regions. In contrast, Aβ40 immunoreactivity was only detected in a few vessels.

In addition, the production of Aβ in cultured fibroblasts was investigated using a highly sensitive ELISA. Secreted and cellular Aβ was analysed from one *PSEN1* I143T mutation carrier, one carrier of the *APP*Swe mutation and in three healthy controls. The fibroblasts from the carrier of the *APP*Swe mutation had substantially higher production of secreted Aβ40 compared to the others as well as a noticeable higher level of Aβ42. The ratio between Aβ42 and Aβ40 in the secreted fraction was highest (Aβ42/Aβ40=0.50) for the *PSEN1* I143T carrier. Both cellular Aβ40 and Aβ42 were similar in all samples, with about five times higher level of Aβ40. Unfortunately, there was no commercial ELISA detecting Aβ43 available.

The Aβ43 peptide was recently shown to be present in sporadic AD cases, as well as AD caused by mutations, in similar concentrations as we found in the *PSEN1* I143T carriers (Welander et al., 2009). However, in Welander et al. 2009, only frontal and occipital cortex was available for analyses. Herein, we show that the longer Aβ43 peptide was present in all six analysed regions. Further analyses are needed to assess whether the widespread presence of the Aβ43 peptide is associated with the

aggressiveness of the disease, or the low onset age. It would also be of interest to elucidate the phenotypic correlates of the Aβ43 peptide in brain, for example, the levels of soluble Aβ43 in the brain of mutation carriers since soluble Aβ seems to correlate with cognition and synaptic plasticity, rather than the aggregated forms. To investigate whether Aβ43 is expressed in brains from cognitively healthy controls would be of interest to clarify its role in pathogenesis. Furthermore, to investigate the presence of Aβ43, and possibly the ratio between Aβ43/Aβ40 in CSF, would lead to a better understanding of its potential as a biomarker for AD.

The low frequency of reported *PSEN1* mutations in Sweden and the availability of brain tissue from affected individuals as well as clinical records from several affected family members and generations gave us a unique opportunity to study a causative mutation with clinical, biochemical and neuropathological approaches and to gain knowledge about the way in which it causes EOAD.

STUDY IV

The obesity related gene, *FTO*, increases the risk for Alzheimer disease in a prospective cohort study.

About three years ago the *FTO* gene was first suggested by a GWAS to be an obesity related gene. Since then, about 330 articles have been published about the *FTO* gene, and the effect on BMI has been consistently confirmed both in childhood and in midlife around the world. As this gene has a prominent effect on BMI in the younger ages, we were curious to find out if the effect of the gene could be detected also in the elderly population. In addition, as the gene also has been associated with several possible risk factors for dementia, i.e., diabetes, insulin resistance, leptin levels and lower physical activity we sought to investigate the relation between the *FTO* gene and dementia in the old population. Of the 1,003 subjects that were followed for nine years to detect incident dementia, 346 developed dementia and of them 261 were diagnosed with AD.

The genotype frequency was as follows: 314 (33.6%) carried the TT genotype; 460 (49.1%) carried the AT genotype and 162 (17.3%) carried the AA genotype. The frequencies did not diverge from the Hardy Weinberg Equilibrium (p=0.77) The A allele is the risk allele for high BMI in previous studies. Using data from the baseline

examination, the distribution of genotypes was significantly different between diabetic and non-diabetic participants, as well as for physically inactive and active participants, where AA carriers had a higher prevalence of diabetes and were estimated to be more physically inactive. Otherwise, no difference was observed for the baseline characteristics. In a logistic regression model, adjusted for age, gender, education and vascular risk factors, the OR for having a BMI≥30 was increased for the AT carriers, but not significantly increased for carriers of the AA genotype. However, it was relatively uncommon with a BMI≥30 in this old population, and only eight participants with the AA genotype had such high BMI and it is reasonable to believe that the non-significant result in this group is a matter of the power to detect the association.

The *FTO* AA genotype was found to increase the risk for AD with 58% (95% CI: 1.11-2.24) and for dementia with 48% (95% CI: 1.09-2.02), when adjusted for age, gender, education and *APOE*. Interaction between *FTO* and *APOE* on dementia risk was found, as shown in Table 2. The observation that the *FTO* AA-carriers appear to be less physically active than the TT-carriers, might be part of the explanation for the interaction between *FTO* and *APOE* as physical activity has been shown to be an important factor to overcome the effect of the ε4-allele on AD and dementia risk (Rovio et al., 2005). Another possible explanation for the interaction might be that AA-carriers have been shown to have a higher fat intake and an increased appetite compared to TT-carriers (Wardle et al., 2008). Considering the involvement of APOE in the metabolism of lipoproteins and triglycerides, increased fat intake might further exacerbate the deleterious effect for carriers of the ε4 allele, which less efficiently removes dietary lipids from the bloodstream. In the presence of both the *APOE* ε4 allele and the *FTO* AA genotype, the relative risk for dementia was increased to 2.73 (95% CI: 1.71-4.37). It should be noticed that even without *APOE* in the model, there was a significant effect of *FTO* on AD and dementia. However, a non-significant trend was observed in those without an *APOE* ε4 allele (RR_{AD}=1.30, 95% CI: 0.82-1.97 and $RR_{dementia}$=1.19, 95% CI: 0.82-1.74) in analyses stratified on the ε4 allele.

Table 2. Relative risks (RR) and 95% confidence intervals (CI) of developing AD and dementia during the nine years of follow-up with respect to *FTO* and *APOE* genotypes and their interaction. Model 1 is adjusted for age, gender, education and *APOE* in similarity to Model 2, which additionally illustrates the effect of the interaction between *FTO* and *APOE* due to introduction of the interaction term into the model.

	RR (95% CI)			
	AD		**Dementia**	
FTO genotypes	Model 1	Model 2	Model 1	Model 2
TT	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
AT	1.11(0.84-1.48)	1.01(0.74-1.37)	1.11(0.87-1.42)	1.00 (0.76 1.30)
AA	**1.58 (1.11-2.24)**	1.30 (0.85-1.99)	**1.48 (1.09-2.02)**	1.20 (0.82-1.74)
APOE ε4	**1.74 (1.33-2.26)**	**1.66 (1.26-2.19)**	**1.59 (1.26-2.01)**	1.17 (0.80-1.71)
*FTO*APOE*		1.35 (0.93-1.96)		**1.42 (1.02-1.98)**

All models are adjusted for age, gender and education.

As the *FTO* gene might increase the risk for different vascular risk factors, such as diabetes, high BMI, physical inactivity and CVD, these factors were taken into account in the Cox regression models to investigate a possible modification of the *FTO*-related dementia risk. As presented in Table 3, after adjustments for all factors simultaneously the effect of *FTO* on AD and dementia were still present (RR_{AD}: 1.47, 95% CI: 1.03-2.12 and $RR_{dementia}$: 1.38, 95% CI: 1.00-1.90), indicating that these factors do not explain the increased risk for dementia and AD. However, with increasing evidence for a role of midlife vascular risk factors in late life dementia, it would have been desirable to adjust for these factors measured earlier in life.

Table 3. Relative risks (RR) and 95% confidence intervals (CI) of developing AD and dementia during the nine years of follow-up.

	AD	**Dementia**
FTO genotypes		
TT	1.00 (ref)	1.00 (ref)
AT	1.15 (0.86-1.54)	1.11 (0.86-1.43)
AA	**1.47 (1.03-2.12)**	**1.38 (1.00-1.90)**
BMI≥30	1.00 (0.54-1.84)	0.88 (0.50-1.54)
Physical inactivity	**1.30 (1.00-1.70)**	**1.46 (1.16-1.83)**
Diabetes	1.28 (0.80-2.07)	1.45 (0.99-2.14)
CVD	0.78 (0.56-1.10)	**1.30 (1.01-1.69)**

All models are adjusted for age, gender, education and *APOE*.

As the effect of *FTO* on dementia was shown herein for the first time, we found it important to replicate our finding in an independent sample set. We took advantage of the publicly available GWAS data containing genotype information on the *FTO* rs9939609 from 859 AD cases and 552 controls (Reiman et al., 2007). In this dataset we were able to confirm our findings.

This is the first time a gene related to BMI is shown to increase the risk for dementia and AD. The effect of *FTO* on BMI has been shown with remarkable consistency. High BMI in midlife and increased risk for later development of dementia has been shown in several studies (Kivipelto et al., 2005; Rosengren et al., 2005; Whitmer et al., 2005; Whitmer et al., 2007; Whitmer et al., 2008), but the relationship is complex. Since it is not known whether the AA-carriers in this population had an increased BMI in midlife, it can only be speculated that their increased risk for dementia and AD in late life was mediated through BMI. On the other hand, since *FTO* is highly expressed in the brain and the FTO protein seems to act on, e.g., insulin processing in the CNS, it can be speculated that *FTO* might have a role on dementia risk that is independent of its effect on BMI.

CONCLUSIONS

Study I: The *APOE* ε4 allele increases the risk for mortality. The increased risk was mainly explained by dementia. The ε2 allele had a protective effect on mortality only for women, and the increased risk for carriers of the ε4 allele was present mainly in men, indicating a gender specific effect by *APOE* on mortality.

Study II: A g.102delC mutation in *GRN*, resulting in a premature stop codon at residue 35, causes dominantly inherited FTD in the Swedish Karolinska family. With segregation analyses, as well as functional analyses, and a neuropathological investigation we found evidence that strongly supported its pathogenic nature.

Study III: An I143T mutation in exon 5 of *PSEN1* causes early onset, dominantly inherited Alzheimer disease with myoclonia in a Swedish family. Mutation carriers were severely affected both clinically and neuropathologically. Of special interest, we found a longer Aβ peptide, consisting of 43 amino acids, to be present in all six investigated brain regions.

Study IV: The AA genotype of the rs9939609 polymorphism in *FTO* increases the risk for dementia, and in particular for Alzheimer disease. The increased risk could not be explained by vascular risk factors measured at baseline. Moreover, the effect of *FTO* on the risk for dementia was found to interact with *APOE*.

FUTURE PERSPECTIVES

Even in the relatively short period of time that this thesis has been under construction, the advances in the genetic field have been impressive. At a conference in the beginning of my PhD studies, some researcher mentioned that samples from more than 10,000 individuals have to be collected to yield sufficient power to detect susceptibility genes with effect sizes common in complex diseases like AD. To find new pathways with importance for the disease, it was stated that the analysis should be done in a hypothesis free manner, which required that the whole genome was covered. For many of us, it felt undoable. However, with huge collaborative and methodological efforts by researchers around the world, three novel genes that likely have a role in the etiology of AD were presented last summer.

The early discoveries of molecular mechanisms involved in the pathogenesis of dominantly inherited AD, pinpointing a role for Aβ, revolutionized the field and have been in focus for the majority of the research since then. In light of the strong association between *APOE* and AD, in addition to the lack of replication of many suggested risk genes, introduction of novel genes into the field has often been received with skepticism. The newly found risk genes for AD point out a role for lipid metabolism and inflammation in AD etiology. The effect on the research focus of these findings remains to be addressed.

To be able to cure or prevent dementia we have to face the challenging fact that all individuals have a risk for the disease that is built up by numerous factors, both risk and protective, both genetic and environmental, and they lead to an accumulated risk during the whole life course. In the pipeline for genetic research in the dementia field are the post-GWA analyses, dealing with epistasis between genetic factors, as well as between genetic and environmental factors. Combining the effect of all variants with nominal significance in the GWAS might explain a greater proportion of cases than the individual genes with stronger evidence for a causal effect, but these analyses remain to be carried out. The future avenue of research is to use meta-analyses of the GWAS together with pathway analyses on the novel genes and epidemiological studies to verify the findings in the general population and to explore interactions with environmental factors. This combined strategy might bring research towards the

identification of personalized risk profiling. To look ahead, the new generation sequencing techniques will not only cover the whole genome, but also simultaneously assess copy number variations and larger structural rearrangements. To interpret the results accurately, the genetic and epidemiological fields have to be unified in a sophisticated way. What we can only imaging today, will probably be doable in a few years.

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