

Thesis for the degree of doctor of medicine

Susceptibility genes in conformational diseases

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Till pappa

5'-GATTAATTTATTAATAANTCTTAACATGCTCGTTAAATTAATATGTAATGATTGGTTAAGATTAATTGGAAGTTGAACGTTAAATTTAAGCTTTGTTGACTTAAGCTGGTTAAGTCGTTAA-3'

A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These papers are either already published or are manuscripts at various stages (accepted for publication, submitted or in manuscript).

ABSTRACT

Conformational diseases are characterized by protein misfolding and aggregation in the affected tissue. The aim of this thesis was to find genetic support for mechanisms in common for three prevalent conformational diseases: Alzheimer's disease (AD), Parkinson's disease (PD) and cataract.

The influence of genetic variability in candidate genes hypothesized to be involved in protein aggregation was investigated for association with risk of the sporadic forms of AD, PD and cataract. Furthermore, analysis of association with age at onset (AAO) of disease, and, for AD, association with mini-mental state examination (MMSE) scores and levels of the cerebrospinal fluid (CSF) biomarkers: A β ₄₂ (the 42 amino acid form of amyloid β), T-tau (total tau, i.e. all isoforms of tau) and P-tau₁₈₁ (hyperphosphorylated tau protein as measured by phosphorylation on amino acid 181) was carried out.

The kinesin protein is important for maintaining cell shape and function, especially in elongated cells such as neurons and lens cells. Previous molecular and genetic studies support impaired kinesin-mediated transport as a potential contributor in AD, PD and cataract. We analysed the contribution of variation in the kinesin light chain 1 gene (*KLC1*) encoding the kinesin light chain protein 1 protein (KLC1), initially by using a single nucleotide polymorphism (SNP) approach (paper I and II) and later in a haplotype study (paper III). Altogether, with the possible exception for cataract, the results of these papers do not support genetic influence of *KLC1* on risk of disease.

Oxidative stress is a contributing factor to aging and degenerative diseases. The proteins Nrf2 (nuclear factor (erythroid-derived 2)-like 2) and Keap1 (Kelch-like ECH-associated protein 1), constitute the two main regulators of the induced cellular oxidative stress defense called the phase II response. In paper IV and V we investigated their respective genes *NFE2L2* (Nuclear factor (erythroid-derived 2)-like 2) and *KEAP1* (Kelch-like ECH-associated protein 1) as possible susceptibility genes in AD, PD and cataract. We found that variation in one *NFE2L2* haplotype

window, which is in LD with functional promoter polymorphisms in the same gene, was associated with risk of PD in two independent European case-control materials (paper IV). In AD and cataract, variation in the same haplotype window was associated with AAO of the diseases (paper V). No association of *KEAP1* with any of the studied diseases was found.

The major finding of this thesis was the identification of *NFE2L2* as a potential susceptibility gene in PD adding genetic support to current indications that Nrf2 may have an important function in the cellular defense against PD.

Keywords: Conformational disease, Alzheimer's disease, Parkinson's disease, cataract, protein aggregation, cellular transport, oxidative stress, susceptibility genes, SNP, haplotype

LIST OF PUBLICATIONS

The thesis is based on the following papers which will be referred to by their roman numerals:

- I. **Malin E. Andersson***, Annica Sjölander, Niels Andreasen, Lennart Minthon, Oskar Hansson, Nenad Bogdanovic, Christina Jern, Katarina Jood, Anders Wallin, Kaj Blennow, Henrik Zetterberg. *Kinesin gene variability affects tau phosphorylation in early Alzheimer's disease*. The International Journal of Molecular Medicine. 2007, 20: 233-239.
- II. **Malin E. Andersson***, Madeleine Zetterberg, Gunnar Tasa, Mona Seibt Palmér, Erkki Juronen, Pait Teesalu, Kaj Blennow, Henrik Zetterberg. *Variability in the kinesin light chain 1 gene may influence risk of age-related cataract*. Molecular Vision. 2007, 13: 993-996.
- III. **Malin von Otter**, Sara Landgren, Staffan Nilsson, Caroline Lundvall, Lennart Minthon, Nenad Bogdanovic, Niels Andreasen, Deborah R. Gustafson, Ingmar Skoog, Anders Wallin, Anna Håkansson, Hans Nissbrandt, Madeleine Zetterberg, Gunnar Tasa, Kaj Blennow, Henrik Zetterberg. *Kinesin light chain 1 gene haplotypes in three conformational diseases*. Accepted for publication in NeuroMolecular Medicine, Oct 2009.
- IV. **Malin von Otter[§]**, Sara Landgren[§], Staffan Nilsson, Dragana Celojevic, Petra Bergström, Anna Håkansson, Hans Nissbrandt, Marek Drozdziak, Monika Bialecka, Mateusz Kurzawski, Kaj Blennow, Michael Nilsson, Ola Hammarsten, Henrik Zetterberg. *Association of Nrf2-encoding NFE2L2 haplotypes with Parkinson's disease*. Submitted manuscript, June 2009.
- V. **Malin von Otter**, Sara Landgren, Staffan Nilsson, Madeleine Zetterberg, Dragana Celojevic, Petra Bergström, Lennart Minthon, Nenad Bogdanovic, Niels Andreasen, Deborah R. Gustafson, Ingmar Skoog, Anders Wallin, Gunnar Tasa, Kaj Blennow, Michael Nilsson, Ola Hammarsten, Henrik Zetterberg. *Nrf2-encoding NFE2L2 haplotypes influence disease progress but not risk in Alzheimer's disease and age-related cataract*. Submitted manuscript, Sept 2009.

*Papers published before July 2009 were published in the name Malin E. Andersson

[§]These authors contributed equally to the work

LIST OF ABBREVIATIONS

A	Adenine
AAO	Age at onset
AD	Alzheimer's disease
APP	Amyloid precursor protein
APOE	Apolipoprotein E (gene)
APOE- ϵ 4	Apolipoprotein E ϵ 4 (allele)
APOJ	Apolipoprotein J (gene)
ARE	Antioxidant responsive element
ATP	Adenosine-5'-triphosphate
A β	Amyloid β
A β ₄₂	The 42 amino acid form of amyloid β
C	Cytosine
CEU	HapMap population consisting of Utah residents with ancestry from northern and western Europe
CLU	Clusterin (gene)
CNV	Copy number variation
CSF	Cerebrospinal fluid
DASH	Dynamic allele-specific hybridization
DNA	Deoxyribonucleic acid
EM	Expectation-maximization
EPHA2	EPH receptor A2 (gene)
G	Guanine
GBA	Glucosidase, beta (gene)
GST	Glutathione-S-transferase
GWA	Genome-wide association
HO-1	Heme oxygenase 1
HTT	Huntingtin (gene)
HWD	Hardy-Weinberg disequilibrium
HWE	Hardy-Weinberg equilibrium
kbp	Kilo base pair
Keap1	Kelch-like ECH-associated protein 1 (protein)
KEAP1	Kelch-like ECH-associated protein 1 (gene)
KHC	Kinesin heavy chains (proteins)
KLC	Kinesin light chains (proteins)

KLC1	Kinesin light chain 1 (protein)
<i>KLC1</i>	Kinesin light chain 1 (gene, human)
<i>Klc1</i>	Kinesin light chain 1 (gene, mouse)
LD	Linkage disequilibrium
<i>LRRK2</i>	Leucine-rich repeat kinase 2 (gene)
MCI	Mild cognitive impairment
MMSE	Mini-mental state examination
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acids
<i>NFE2L2</i>	Nuclear factor (erythroid-derived 2)-like 2 (gene)
Nrf2	Nuclear factor (erythroid-derived 2)-like 2 (protein)
NQO1	NAD(P)H:quinine oxidoreductase-1
OR	Odds ratio
PCR	Polymerase chain reaction
PD	Parkinson's disease
<i>PICALM</i>	Phosphatidylinositol binding clathrin assembly protein (gene)
P-tau	Phosphorylated tau
P-tau ₁₈₁	Hyperphosphorylated tau protein as measured by phosphorylation on amino acid 181
ROS	Reactive oxygen species
ROX	Carboxy-X-rhodamine
RR	Risk ratio
<i>SNCA</i>	α -synuclein (gene)
SNP	Single nucleotide polymorphism
T	Thymine
TMAD	TaqMan allelic discrimination
<i>TNK1</i>	Tyrosine kinase, non-receptor, 1 (gene)
T-tau	Total tau, i.e. all isoforms of tau
UGT	Uridine diphosphate glucuronosyltransferase
UV	Ultraviolet light
α S	α -synuclein (protein)
γ -GCS	γ -glutamylcysteinyl-synthetase

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INTRODUCTION

GENETICS

Definition of genetics

Genetics is the study of heredity and the variation of inherited characteristics, i.e. the underlying mechanisms for different personalities, traits, diseases etc. and how these are transferred from one generation to the next [1].

History of DNA and genes

Selective breeding as a way of improving cattle and plants has been used since prehistoric times. However, it was not until after the revolutionary work of Charles Darwin and Gregor Mendel in the mid nineteenth century, that the era of modern genetic research began and the term heredity was created and became a biological concept [2]. Darwin's "theory of natural selection" [3], evolved around his belief that existing species arose through modified descent from previous species. He did not, however, present a genetic basis for his theory [1]. Mendel, on the other hand, showed in his experiments with the garden pea, *Pisum sativum*, that different properties are inherited in units independently of each other [4].

At the end of the nineteenth century, work on cell division by Walter Flemming led to the discovery of the chromosomes, but it was not until the beginning of the twentieth century with the rediscovery of Mendel's work, that one realized that the chromosomes were the actual units of inheritance [5]. Later, MacLeod and McCarty proved the nucleic acid, deoxyribonucleic acid (DNA) to be the bearer of the inherited information [6], through the nucleotides: adenine (A), cytosine (C), guanine (G) and thymine (T), that constitutes the four letter alphabet of DNA. Watson and Crick then went on to untangle the ladder-like, double helix structure of DNA [7].

By the end of the twentieth century, key methodology such as DNA sequencing [8] and polymerase chain reaction (PCR) [9] had evolved

sufficiently to enable genome-wide searches for disease associated loci using linkage methodology [10]. Soon the quest of sequencing the complete human genome began. The “Human Genome Project” aimed to decode the base pairs of the human genome, to discover all human genes and make the sequences accessible for further biological research [11]. The first draft of the human genome was published in 2001 [12] and the project culminated with the publication of the human genome in 2003 (presented in special issues: *Science*, 2003, vol. 300, no. 5617; and *Nature*, 2003, vol. 422, no 6934).

Genetic variation

Although the human genome is very similar between individuals there is room for genetic variations that makes all individuals unique. The most common form of variation is called single nucleotide polymorphism (SNP), i.e. the presence of two (or in rare cases three or four) different nucleotides (alleles) at a single base position of our DNA. SNPs are more common in non-coding than in gene-coding areas of the genome. If a variation is located in a position that is important for how well a gene is expressed or for the structure of the encoded protein, it has the potential of influencing risk of disease. If a gene does influence risk of disease it is referred to as a susceptibility gene for this disease.

A sequence of SNPs alleles on the same parental chromosome is called a haplotype. If two SNPs are inherited independently probability theory states that the haplotype frequency equals the product of the two separate SNP allele frequencies. However, if the SNPs are located close to each other on the chromosome, they will not be inherited independently (they are linked), and this equality may not longer hold, the SNPs are said to be in linkage disequilibrium (LD). The fact that SNPs are in LD with each other can be utilized when searching for unknown disease alleles in a candidate gene.

The HapMap project is an extension of the human genome project and aims at mapping the common patterns of genetic variation in the human genome by sequencing the genome of multiple individuals in varying populations. By making these variations publicly available this project aids researchers in their search for genetic components of diseases by making the design of genetic haplotype association studies possible. This initiative will eventually lead to important knowledge of disease mechanisms and possibly new treatments [13].

In addition to SNPs there are other types of variations in our genome that are all involved in risk of disease. Examples are: insertions/deletions (indels), repeat polymorphisms, structural duplications/deletions (copy number variations (CNV)), novel mutations, and epigenetic modifications (information other than the DNA sequence which is inherited during cell division), however these have not been studied in this thesis.

Benefits of genetic research

Genetic research has made an important contribution in understanding the underlying molecular mechanisms of, in particular monogenic diseases. Huntingtons disease is a good example of how the identification of the causing huntingtin (*HTT*) gene [14, 15] has stimulated research on causative molecular mechanisms [16]. Identification of genes contributing to genetically complex diseases, for which multiple genes and environmental factors affect the risk, has proved much more difficult. However, these diseases are much more common than monogenic diseases and are costly to society, motivating genetics as a tool for understanding these mechanisms [17]. Furthermore, genetics as a tool to support clinical diagnoses and therapeutic decision-making is also of great importance.

CONFORMATIONAL DISEASES

The term “conformational diseases” was introduced by Carrell and Lomas in 1997 [18] in an attempt to group diseases having similar underlying protein misfolding mechanisms, despite their clinically diverse characteristics. Conformational diseases were defined as:

“...a disease that arises when a constituent protein undergoes a change in size or fluctuation in shape, with resultant self-association and tissue deposition [...] with the limitation that at least some of the affected protein has to be correctly folded and released in its normal form upon production...”

This means that the conformational change cannot be a result of a genetic defect causing failure during the protein synthesis [18].

The misfolded proteins induce an unfavorable stress response that is disadvantageous for cell survival. Tissues with low cell turnover are especially vulnerable to this kind of intracellular disturbance since dying

cells cannot be replaced and cell death within the affected organ eventually will cause organ dysfunction. Conformational diseases therefore typically do not have a clinically recognizable onset until late in life [19].

This thesis focuses on the three conformational diseases; Alzheimer's disease (AD), Parkinson's disease (PD) and cataract, and two of the proposed mechanisms underlying peptide/protein misfolding and aggregation in these diseases; impaired kinesin-mediated transport and oxidative stress.

Alzheimer's disease

AD was first described by Alois Alzheimer in 1907 [20]. It is a slowly progressive disease clinically recognized by memory impairment and cognitive decline and is the most common disease that causes dementia. During the earlier stages, when memory dysfunction is present but the diagnostic criteria for AD with dementia are not yet fulfilled, the diagnosis mild cognitive impairment (MCI) can be made [21]. Many of these cases have incipient AD and will eventually develop AD with dementia.

Histologically, AD is characterized by neuronal loss, senile plaques consisting of aggregates of the amyloid β ($A\beta$) peptide, and of neurofibrillary tangles consisting of phosphorylated tau (P-tau) protein (figure 1, page 16). The neuronal loss primarily occurs in the medial temporal lobes that are involved in memory processing. Later, other cortical areas of the brain are also involved and the cognitive ability to coordinate thoughts and put things together in a context is affected [22]. The level of $A\beta_{42}$ (the 42 amino acid form of amyloid β), T-tau (total tau, i.e. all isoforms of tau), and P-tau₁₈₁ (hyperphosphorylated tau protein as measured by phosphorylation on amino acid 181) in the cerebrospinal fluid (CSF) can be used to reflect the AD pathology in patients and a combination of the three constitutes a good biomarker for AD [23]. Mini-mental state examination (MMSE) [24] is a widely used simple measure of the cognitive performance in the patient.

The mechanism behind sporadic AD remains unclear. Amyloid precursor protein (APP) is under healthy conditions processed into a number of resulting peptides, $A\beta$ being one of them. The functionality of this processing and the resulting peptides seem to be dependent on a precise balance between the produced peptides [25]. Although controversial, the

leading hypothesis of AD has long been the amyloid cascade hypothesis [26] postulating that it is the excessive production and subsequent deposition of $A\beta_{42}$ into senile plaques that leads to AD pathology. However, recent research indicates that it is the initial steps of aggregation, involving $A\beta_{42}$ oligomers rather than the mature plaques that are the toxic components responsible for neuronal death and eventually disease [27, 28].

There are two forms of AD. A very rare form, familial AD, caused by dominant mutations in genes coding for proteins involved in $A\beta$ -generating metabolism of the APP [29-32] (table 1, pages 18-19) and has an early age at onset (AAO). The vast majority of AD cases are sporadic and symptoms usually do not appear until after the age of 70. The genetic component of the sporadic form has been estimated as being up to 80% [33], and the apolipoprotein E $\epsilon 4$ (*APOE- $\epsilon 4$*) allele of the only established susceptibility gene, apolipoprotein E (*APOE*) [34-36] has been estimated to account for up to 50% of the genetic risk [37]. Lately, completions of a number of genome-wide association (GWA) studies have added enormous amounts of data to the search for new susceptibility genes for sporadic AD. Recently, results from two GWA studies, each including more than 10 000 individuals counting both discovery and replication case-control materials, indicated the presence of additional susceptibility genes. Besides the obvious associations of *APOE*, both studies independently reported replicated association of *CLU* (clusterin) also known as *APOJ* (apolipoprotein J) [38, 39]. Since the effect size of susceptibility genes other than *APOE* are small a vast number of individuals are needed to gain enough power to identify them [40]. The database AlzGene has been created in an attempt to facilitate identification of new susceptibility genes with less effect size. In this database all published AD gene association studies, i.e. GWA-studies, as well as candidate gene-studies, are registered and increased power of the association analyses is gained through continuous meta-analysis of the registered data [41]. To date AlzGene rates 35 high priority genes as a result of meta analysis from 1236 studies of 598 genes and 2335 polymorphisms and include the results of twelve original GWA-studies [38, 39, 42-51]. The most promising susceptibility genes after *APOE* today are: *CLU*, *PICALM* (phosphatidylinositol binding clathrin assembly protein) and *TNK1* (tyrosine kinase, non-receptor, 1). The top ten genes in AlzGene as of Sept 24th 2009 are given in table 1 on pages 18-19 [41]. Non-genetic risk of sporadic AD, other than old age, includes vascular disease, diabetes, low physical activity and degree of education [22].

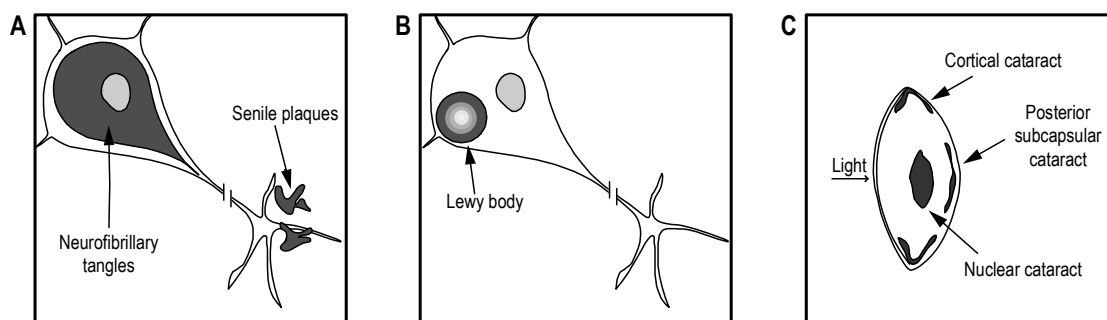


Figure 1: Schematic picture of the protein aggregates found in the studied diseases. A) Neuron with characteristic aggregates of Alzheimer's disease: intracellular neurofibrillary tangles and extracellular senile plaques. B) Neuron with a Parkinson's disease characteristic Lewy body. C) Lens with opacities at the location specific for each cataract subtype.

Parkinson's disease

PD is the second most common neurodegenerative disease after AD [52]. It is a movement disorder characterized by bradykinesia, tremor and postural instability [53], which was first described by James Parkinson in 1817 [54]. Histologically PD is characterized by neurons containing Lewy bodies that consist of aggregates of α -synuclein (α S) (figure 1), and by loss of the dopaminergic nigrostriatal neurons that are important for movement coordination. Eventually other transmitter systems are also affected which may lead to cognitive problems in the later stages of the disease [52]. The underlying mechanism for sporadic PD remains unresolved. Much focus has been on α S and similar to A β in AD, the current view is that oligomers of α S contribute to the neurotoxicity [55]

The genetic component of PD was considered negligible until about fifteen years ago. Since then multiple genes have been identified and shown to cause familial PD inherited in Mendelian manners (table 1, pages 18-19) [56-61]. Heredity in sporadic PD is less understood. Although familial aggregation of the disease is well recognized [62] twin studies have failed to prove a genetic component [63] or the found genetic components have been restricted to patients younger than 50 years of AAO [64]. It has even been suggested that instead of searching for pure susceptibility genes for sporadic PD, a search for genes affecting AAO might be more effective [65]. Recently, the first GWA-study using this approach was completed [66]. PDGene is the PD equivalent of the AlzGene database [67]. It currently lists 23 high priority genes as a result of meta analysis of 795 studies of 527 genes and 2259 polymorphisms and includes results from four original GWA-studies [68-71]. The most promising susceptibility genes today are: *GBA* (glucosidase, beta),

LRRK2 (leucine-rich repeat kinase 2) and *SNCA* (α -synuclein). The top ten genes in PDGene as of Sept 24th 2009 are given in table 1 on pages 18-19 [67]. The relatively low impact of genetics in sporadic PD suggests that environmental factors have a greater influence. Risk factors other than old age include low physical activity, obesity, exposure to pesticides and toxins, e.g. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Interestingly, smoking reduces the risk of PD [53].

Cataract

Cataract is one of the leading causes of visual impairment in the world. It is caused by lens opacities (figure 1), i.e. light scattering aggregates of crystallin proteins that reduce optical clarity [72]. There are three forms of crystallin: α -, β - and γ -crystallin. The biochemical nature of these proteins allows them to arrange themselves in crystal-like transparent structures which are fundamental to the clear properties of the lens [73-75]. In cataract, these structures are disrupted by various posttranslational modifications that change the interaction between crystallins and promote their aggregation [76].

As in AD and PD there are familial and sporadic forms of cataract. The familial forms are inherited in a Mendelian manner often with a debut within the first years of life. Mutations in a relatively large number of genes (table 1, pages 18-19) are associated with these forms of cataract [77]. The sporadic cataracts usually develop late in life and are often referred to as age-related cataract. Age-related cataract can be sub grouped into: cortical, posterior subcapsular, nuclear and mixed cataract, according to the positioning of the opacities in the lens (figure 1) [72]. Twin studies have shown that genetic as well as environmental factors influence risk of age-related cataract [78, 79]. However, the search for susceptibility genes is still in an early phase [80] and there are to date no established susceptibility genes for cataract. Recently, *EPHA2* (EPH receptor A2) was associated with cataract in two case-control materials [81, 82]. *EPHA2* is located at a loci previously associated with cataract [83] and is thus representing this developing field's most promising susceptibility gene for age-related cataract so far (table 1, pages 18-19). Examples of recognized environmental risk factors are: ultraviolet (UV) light exposure, smoking, diabetes and hypertension [72].

Table 1: Genes associated with Alzheimer's disease, Parkinson's disease and cataract

Disease	Gene	Protein
Alzheimer's disease		
Familial¹	<i>APP</i>	Amyloid beta precursor protein
	<i>PSEN1</i>	Presenilin 1
	<i>PSEN2</i>	Presenilin 2
Sporadic²	1. <i>APOE³</i>	1. Apolipoprotein E
	2. <i>CLU</i>	2. Clusterin (Apolipoprotein J)
	3. <i>PICALM</i>	3. Phosphatidylinositol binding clathrin assembly protein
	4. <i>TNK1</i>	4. Tyrosine kinase, non-receptor, 1
	5. <i>ACE</i>	5. Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1
	6. <i>TFAM</i>	6. Transcription factor A, mitochondrial
	7. <i>CST3</i>	7. Cystatin C
	8. <i>IL1B</i>	8. Interleukin 1, beta
	9. <i>CR1</i>	9. Complement component (3b/4b) receptor 1
	10. <i>hCG2039140</i>	10. Unknown
Parkinson's disease		
Familial⁴	<i>SNCA (PARK1/PARK4)</i>	α -synuclein
	<i>PRKN (PARK2)</i>	Parkin
	<i>PINK1 (PARK6)</i>	PTEN induced putative kinase 1
	<i>DJ-1 (PARK7)</i>	DJ-1 protein
	<i>LRRK2 (PARK8)</i>	Leucine-rich repeat kinase 2 (Dardarin)
Sporadic⁵	1. <i>GBA</i>	1. Glucocerebrosidase
	2. <i>LRRK2</i>	2. Leucine-rich repeat kinase 2 (Dardarin)
	3. <i>SNCA</i>	3. α -synuclein
	4. <i>USP24</i>	4. Ubiquitin specific protease 24
	5. <i>MAPT/STH</i>	5. Microtubule-associated protein tau/saitohin
	6. <i>BDNF</i>	6. Brain-derived neurotrophic factor
	7. <i>MAOB</i>	7. Amine oxidase (flavin-containing)
	8. <i>PDXK</i>	8. Pyridoxal (pyridoxine, vitamin B6) kinase
	9. <i>SLC6A3</i>	9. Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3
	10. <i>DRD2</i>	10. Dopamine receptor D2

Disease	Gene	Protein
Cataract Familial⁶	<i>BFSP1</i>	Beaded filament structural protein 1 (Filensin)
	<i>BFSP2</i>	Beaded filament structural protein 2 (Phakinin)
	<i>CHMP4B</i>	Chromatin modifying protein 4B
	<i>CRYAA</i>	Crystallin α A
	<i>CRYAB</i>	Crystallin α B
	<i>CRYBA1/A3</i>	Crystallin β A1
	<i>CRYBA4</i>	Crystallin β A4
	<i>CRYBB1</i>	Crystallin β B1
	<i>CRYBB2</i>	Crystallin β B2
	<i>CRYBB3</i>	Crystallin β B3
	<i>CRYGC</i>	Crystallin γ C
	<i>CRYGD</i>	Crystallin γ D
	<i>CRYGS</i>	Crystallin γ S
	<i>EYA1</i>	Eyes absent homolog 1 (Drosophila)
	<i>FOXE3</i>	Forkhead box E3
	<i>GCNT2</i>	Glucosaminyl (N-acetyl) transferase 2
	<i>GJA3</i>	Gap junction protein α 3 (Connexin 46)
	<i>GJA8</i>	Gap junction protein α 8(Connexin 50)
	<i>HSF4</i>	Heat shock transcription factor 4
	<i>LIM2</i>	Lens intrinsic membrane protein 2
<i>MAF</i>	V-maf musculoaponeurotic fibrosarcoma oncogene homolog	
<i>MIP</i>	Major intrinsic protein of lens fibre (Aquaporin 0)	
<i>NHS</i>	Nance-Horan syndrome protein	
<i>PITX3</i>	Paired-like homeodomain transcription factor 3	
<i>VSX2</i>	Visual system homeobox 2	
Sporadic⁷	<i>EPHA2⁸</i>	Ephrin receptor EphA2 precursor

¹[29-32]. ²Top ten susceptibility genes for sporadic AD in the AlzGene database, as of the 24 Sept 2009 [41]. ³*APOE* is as of today the only established susceptibility gene for sporadic AD. ⁴[56-61]. ⁵Top ten susceptibility genes for sporadic PD in the PDGene database, as of the 24 Sept 2009 [67]. ⁶[77]. ⁷There are no established susceptibility genes for age-related cataract, this list is assembled upon PubMed search for promising susceptibility genes using the search terms "age-related cataract AND gene" or "Case-control AND cataract". ⁸[81, 82].

PROTEIN AGGREGATION

Protein aggregation is normally described in a four-step process (figure 2):

1. Protein synthesis
2. Change in conformation
3. Formation of oligomers
4. Maturation of protein aggregate

Since it is becoming more and more evident that oligomers rather than mature aggregates are the toxic species during protein aggregation [84] and that mature aggregates may not be the cause but an end-point in a disease process, research on conformational diseases is now focused on early conformational changes and how they may initiate oligomer formation [19]. Both impaired cellular transport and dysfunctional oxidative stress response have been suggested to initiate and enhance protein misfolding [85, 86], hence representing mechanisms in common for conformational diseases.

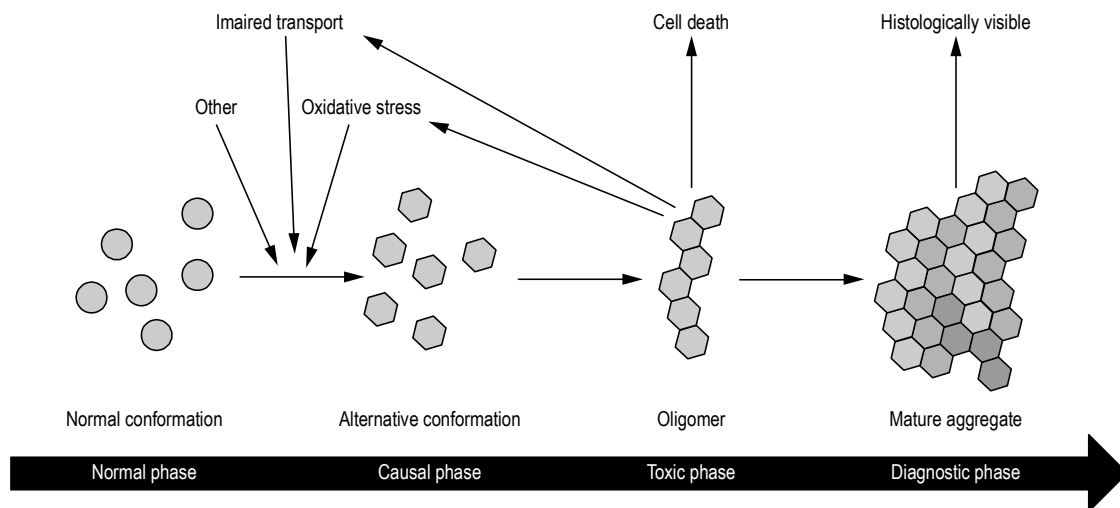


Figure 2: Schematic picture of protein aggregation in conformational diseases. The protein adapts normal conformation during synthesis. In the causal phase oxidative stress, transport disruptions or other factors induce changes in protein conformation which can lead to oligomerization and aggregation of the protein. The common belief today is that it is the oligomers that are the toxic species causing cell death during aggregation. The mature aggregates that are histologically characteristic for disease are considered end-products that might even be the result of a defensive mechanism in the tissue.

KINESIN TRANSPORT

An intact cellular skeleton and functional intracellular transport are essential for maintaining cell shape and function [87]. Especially elongated cells such as neurons and lens cells, in which long distance transport is needed, are vulnerable to transport disruptions [88-90].

Kinesin

Conventional kinesin (referred to as kinesin in this thesis) is the most abundant transport protein in most tissues [91]. It was discovered in 1985 through studies on fast axonal transport and represented a new kind of motor protein with properties different from the previously identified dynein and myosin transport proteins [92, 93].

Kinesin belongs to the kinesin superfamily of proteins which is the largest family of transport proteins [91]. It is an adenosine-5'-triphosphate (ATP)-dependent protein [92, 94] that travels in a plus-ended manner along the microtubule [95] transporting membrane-bound organelles and vesicles [96] from the cell nucleus to the cell membrane, or as in the case of neurons from the cell body to the synapse.

Kinesin is composed of two heavy chains (KHC) which can form tetramers with two light chains (KLC) [97-99]. The amino-terminal KHC contain the microtubule-binding and ATP-utilizing domains that perform the movement along the microtubules [100, 101]. Cargo-binding is mediated by the carboxy-terminal domain of KHC [102, 103] and by the KLC that further increase the cargo-binding specificity of kinesin [104] (figure 3, page 22).

Kinesin and conformational diseases

AD, PD and cataract can all be linked to impaired kinesin-mediated transport:

Alzheimer's disease. Some years ago "the axonal transport dysfunction hypothesis" in AD was proposed on the basis of results showing that APP functions as a cargo receptor for kinesin and that the proteolytic machinery, β - and γ -secretase, needed for production of $A\beta_{42}$ from APP is present in kinesin-transported vesicles (figure 3, page 22) [105-108]. Furthermore, gene knock-out kinesin light chain 1 (*Klc1*) mice showed enhanced $A\beta_{42}$ production in axonal swellings that were

histologically similar to early stage AD phenotypes in humans [109], and genetic variation in the kinesin light chain 1 gene (*KLC1*) had been associated with risk of AD [110]. Recently, tau abnormalities were also observed in the knock-out *Klc1* mice [111].

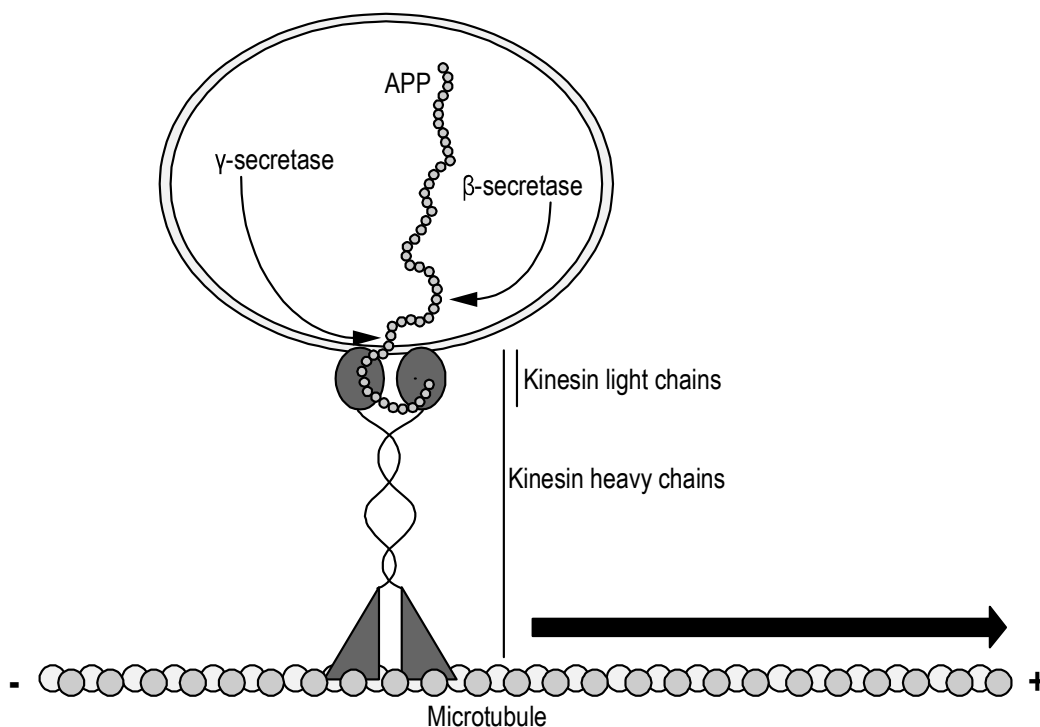


Figure 3: Schematic picture of kinesin transport describing its putative role in AD. The kinesin protein is composed of two heavy chains and two light chains. The heavy chains bind to the microtubule and are responsible for the anterograde movement. They are also involved in binding to the transported cargoes. The light chains bind to the heavy chains and increase the specificity of the cargo interaction. Behind the axonal transport dysfunction hypothesis in AD are, among others, the findings that APP can act as a cargo receptor for kinesin light chain and that the constituents of β - and γ - secretase are transported in vesicles carried by kinesin.

Parkinson's disease. Axonal pathology has been observed in the early stages of PD pathology [112]. More specific indication of kinesin importance for PD is that α S is transported by kinesin [113] and that the PD-inducing toxin MPTP causes kinesin transport disruption [114].

Cataract. Associations between cataract and kinesin transport are less specific. Kinesin has been shown to be important for lens growth and maintenance [90]. APP and APP-like proteins have been co-localized with kinesin in the lens. Also, similarities between the microtubule network and cargo-trafficking between neurons and lens fibre cells suggest that transport dysfunction may cause pathology both in the central nervous system and the lens [88, 90].

OXIDATIVE STRESS

Production of reactive oxygen species (ROS) is an inevitable effect of oxygen utilization in cell metabolism. Elevated levels of ROS lead to oxidative stress which is toxic to the cell and is a recognized contributor to aging and age-related diseases [115, 116]. Posttranslational oxidation of proteins has been suggested to be potential seeding sites for protein aggregation, and thus represents a potential mechanism in common for conformational diseases [86]. Cellular defence mechanisms that neutralize ROS have evolved over time, and one of the most important systems is the so-called phase II oxidative stress response [117].

The phase II oxidative stress response

Nrf2 (nuclear factor (erythroid-derived 2)-like 2) and its repressor Keap1 (Kelch-like ECH-associated protein 1) are the main regulators of the phase II system [117]. Under normal conditions, Nrf2 is located in the cytoplasm where it is bound to Keap1 [118, 119]. Keap1 marks Nrf2 for proteasomal degradation through ubiquitination and thereby keeps Nrf2 at basal levels [120-123]. As a response to oxidative stress or other electrophilic compounds the ubiquitination by Keap1 is disrupted and newly produced Nrf2 can enter the nucleus [124]. Here, Nrf2 forms heterodimers with small Maf proteins that bind to the antioxidant responsive element (ARE), a common motif in the promoters of the phase II genes, and induces expression of the antioxidant phase II enzymes [125]. The classic phase II enzymes: γ -glutamylcysteinyl-synthetase (γ -GCS), Glutathione-S-transferase (GST), NAD(P)H:quinine oxidoreductase-1 (NQO1), uridine diphosphate glucuronosyltransferase (UGT) and Heme oxygenase 1 (HO-1) detoxify ROS through conjugation reactions. In addition to these, Nrf2 drives expression of two transporter proteins that regulate the intracellular level of antioxidant glutathione [117, 126] (figure 4, page 24).

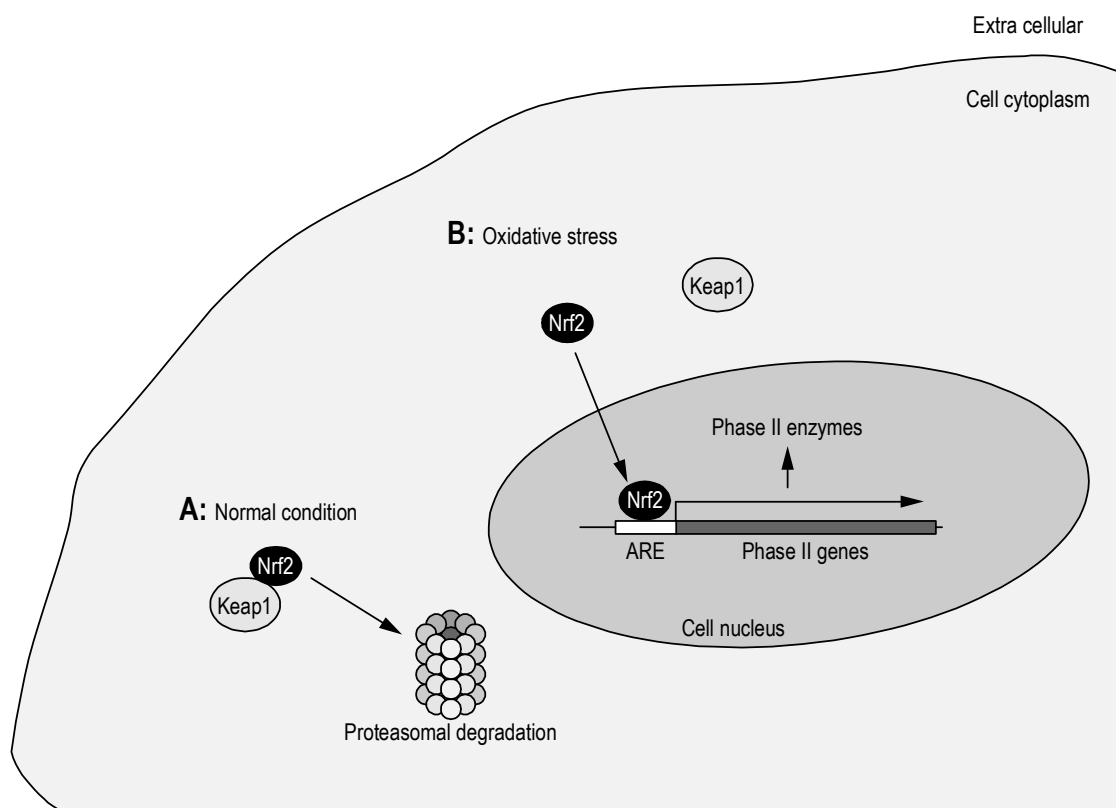


Figure 4. The phase II oxidative stress response: During normal conditions (A) the transcription factor Nrf2 is localised to the cytoplasm and is kept at basal levels through binding to its repressor Keap1 that mark Nrf2 for proteasomal degradation. As a response to oxidative stress (B) Nrf2 is detached from Keap1 and newly synthesised Nrf2 can enter the nucleus and induce expression of the Phase II genes which will result in production of antioxidant Phase II enzymes.

The phase II oxidative stress response and conformational diseases

AD, PD and cataract can all be linked to aberrations in the phase II oxidative stress response:

Alzheimer's disease: Growing evidence suggests that oxidative stress from multiple sources contributes to the pathogenic process of AD. In addition, A β seems to enhance this stress in a feed-forward manner by inducing ROS while ROS stimulates A β production [127]. In AD patients, the Nrf2 activation is reduced in the hippocampal areas [128]. Induction of Nrf2 seems to protect against A β cytotoxicity *in vitro* [129, 130], and induced expression of human Nrf2 in this region improved spatial learning in a transgenic mouse model of AD [131]. Altogether, these results indicate an important role of the phase II oxidative stress response in AD pathogenesis.

Parkinson's disease: Multiple observations point towards involvement of oxidative stress in PD [132]. Lately, evidence of the importance of the phase II oxidative stress response in PD has gained increasing attention. First, DJ-1, encoded by one of the familial PD genes (table 1, pages 18-19), stabilizes Nrf2 during its activation by preventing its interaction with Keap1 [133]. Further, Nrf2 activation is induced in the substantia nigra of PD patients [128]. Finally, Nrf2 induction protects from dopaminergic neuronal cell death caused by oxidative stress *in vitro* [134] and from MPTP-induced dopaminergic cell death in a transgenic PD mouse model [135].

Cataract: The lens is avascular and relies on diffusion of oxygen through the aqueous and vitreous humors. Since the lens is constantly exposed to UV-radiation and the lens fibres remain throughout life, oxidatively damaged proteins may accumulate in this tissue [136, 137]. There are no studies directly addressing the influence of Nrf2-induced ROS defence in the lens or during cataractogenesis. However, reduced activities of anti-oxidant GST and γ -GCS enzymes and polymorphisms in the genes encoding these have been associated with risk of cataract [138-141].

THE STUDIED GENES

All gene data in the following sections were assembled from the Entrez Gene Database (October 3rd 2009) at the NCBI home page (<http://www.ncbi.nlm.nih.gov>), and from the HapMap Genome Browser (Phase 1 & 2 - full dataset of CEU (Utah residents with ancestry from northern and western Europe)) at the International Haplotype Mapping Project web site (www.hapmap.org).

KLC1

KLC1 (also known as *KNS2*; Entrez gene ID: 3831) was identified and cloned in 1993 [142] and is located on chromosome fourteen. It is a 72 kilo base pair (kbp) long gene that includes eighteen exons. Alternative splicing occurs downstream of exon thirteen and gives rise to at least three messenger ribonucleic acids (mRNA) and protein isoforms. Much more extensive splicing, suggested to be important for the kinesin light chain 1 (*KLC1*) protein cargo-binding specificity, has been proposed [143]. However, most of these forms have not been investigated in detail. *KLC1* is covered by one haplotype block that includes 47 SNPs with a

minor allele frequency of >5% in the CEU population. dbSNP report one coding amino acid changing (non-synonymous) SNP with a frequency >5% in a European population.

NFE2L2

NFE2L2 (nuclear factor (erythroid-derived 2)-like 2; Entrez gene ID: 4780) was cloned and characterized in 1994 [144] and is located on chromosome two. It is a 34 kbp long gene including six exons that after alternative splicing give rise to three mRNAs and protein isoforms that differ at the amino-terminus. *NFE2L2* is covered by one haplotype block that includes 24 SNPs with a minor allele frequency of >5% in the CEU population. dbSNP does not report any coding SNPs with a frequency >5% in a European population. However, there are three known functional SNPs in the promoter of *NFE2L2* [145, 146].

KEAP1

KEAP1 (kelch-like ECH-associated protein 1; Entrez gene ID: 9817) was first identified in 1999 as a gene encoding a direct binding partner to Nrf2 [119] and is located on chromosome nineteen. It is a seventeen kbp long gene including seven exons that after alternative splicing give rise to two mRNAs both encoding identical proteins. *KEAP1* is covered by two haplotype blocks that include nine SNPs with a minor allele frequency of >5% in the CEU population. dbSNP reports two coding but amino acid consistent (synonymous) SNPs with a frequency >5% in a European population.

METHODOLOGICAL CONSIDERATIONS

GENOTYPING

Polymerase chain reaction

PCR was developed during the early 1980s by Mullis et al. [9] and is a method used for selective amplification of DNA. It is probably the most important method in modern genetic research, since genotyping methods require an initial amplification of the template DNA. The method is based on the principle of DNA replication. Two oligonucleotides (primers), enclosing the DNA sequence of interest, are hybridized to the template DNA. During the PCR the primers are used as initiation sites for the DNA polymerase which travels along the template DNA extending the primer into a complementary copy of the DNA sequence using free nucleotides. Exponential production of DNA copies is managed by using the thermo stable DNA polymerase, *Taq polymerase*, in a three step thermocycling reaction of which the produced copies from one cycle act as template DNA in the next.

The PCR thermocycle:

1. 95 °C: De-hybridization of the template DNA
2. 50-60 °C: Primer-hybridization to the template DNA
3. 72 °C: Replication by *Taq polymerase*

Being the first step in all genotyping experiments the importance of a pure PCR product is crucial. Though straight forward in theory, producing a perfect PCR product can be challenging and a vast number of optimization possibilities, including variation of reagent and primer concentrations, annealing temperatures, *Taq polymerase* as well as repeated PCRs, have been developed for this purpose. In this thesis, PCR represent the base of all experimental methods used.

Sequencing

DNA sequencing is used to determine the exact order of nucleotides in a DNA sequence. The automated sequencing methods commonly used are based on the method developed by Sanger et al. in 1977 [8]. Today, however, DNA sequencing is preceded by amplification of the DNA fragment of interest by ordinary PCR. The amplified fragment is then used as the template DNA in the following sequencing reaction. In this reaction, just one primer is used to create DNA fragments, starting from one end only. In addition to the ordinary free nucleotides, the sequencing reaction contains a small amount of fluorescent termination nucleotides (dideoxynucleotides), each carrying a nucleotide-specific dye. When a termination nucleotide is incorporated by the *Taq polymerase*, the elongation is terminated resulting in a DNA fragment with a fluorescing molecule in its 3'-end. Since the incorporation of termination nucleotides is random, the DNA fragments produced will be of different sizes. Separation of the fragments using capillary electrophoresis then sorts the fragments by length and the order of the nucleotides in the sequence can be monitored by a laser as they reach the end of the capillary.

Sequencing is a very important method for biomedical research. It is indispensable for the mapping of new genomes and allows for robust generation of reliable results when other genotyping methods cannot be used. In this thesis sequencing has been used mainly for two purposes; to verify the Dynamic allele-specific hybridization (DASH) assays in paper I and II, and in paper IV to genotype SNPs located in the promoter region of *NFE2L2*, for which TaqMan allelic discrimination (TMAD) assays could not be developed.

Dynamic allele-specific hybridization

DASH is a melting point-based genotyping method in which induced fluorescence resonance energy transfer is used to determine the genotype of a specific SNP [147, 148]. The target DNA is amplified using a biotin-bound primer. The PCR product is bound to a streptavidin-coated surface. An acceptor dye, e.g. carboxy-X-rhodamine (ROX), containing probe complementary to the normal SNP allele of the target DNA is allowed to hybridize with the product in the presence of the donor dye SYBR Green. SYBR Green binds to double-stranded DNA, and as long as the probe remains hybridized to the target DNA the SYBR Green will cause the acceptor dye to fluoresce. During the experiment the temperature is increased and when the melting point of the probe is reached the

SYBR green can no longer feed the donor dye sufficient energy for it to fluoresce. This can be monitored as a drop in fluorescence as measured by a laser. Since the melting point depends on the match between the probe and the target DNA, a mismatch caused by a variant allele in the target DNA will result in a drop in fluorescence at a lower temperature compared with a perfectly matched probe and the genotype can be determined through the melting point profile in each reaction.

Although less time consuming than sequencing, the DASH method still requires a significant amount of laboratory work including post-PCR handling which is undesirable due to the risk of cross contamination between samples. Furthermore, the amount of work during genotype scoring for the method is not optimal since every sample has to be manually controlled. Today the DASH method has been replaced by other methods, e.g. TMAD, which is suitable for small scale genotyping studies. In this thesis the DASH method was used for genotyping in paper I and II.

TaqMan allelic discrimination

TMAD is a PCR-based genotyping method in which cleavage of sequence specific probes carrying a reporter dye and a quencher dye are used to genotype SNPs [149]. Two probes, one complementary to the normal SNP allele and one to the variant SNP allele, each carrying an allele-specific fluorescing dye and a quencher molecule, are added to the PCR. During DNA amplification, if the probe is perfectly hybridized to the DNA, the 5'-nuclease activity of the *Taq polymerase* will cleave the probe resulting in the detachment of the quencher from the fluorescing dye and an allele-specific increase of fluorescence. If the probe is hybridized with a mismatch on the other hand, the probe is knocked off the target DNA in its intact state and the quencher keeps absorbing the fluorescence from the dye. Plotting the allele-specific fluorescence intensity against each other allows for scoring of SNP genotypes.

TMAD represents an adequate genotyping method for smaller candidate gene studies in which a number of SNPs are analysed. The method is relatively fast, samples can be scored plate-wise (i.e. 96 or 384 samples at a time), and no post-PCR handling is required. In this thesis TMAD has been used for genotyping in papers III-V.

GENETIC STATISTICS

Statistical significance and power

Statistics is used as a tool to provide evidence of associations. In genetic association studies the null hypothesis is that there is no association between the genotype and the disease. If this null hypothesis can be refuted at a specific significance level (often set to 0.05), this is considered as a support of the genes involvement in the disease. If a hypothesis is refuted when there is no true association it is called a type I error. If the hypothesis cannot be refuted if there is a true association it is called a type II error. Power is a measure of how well a study can identify a true association, i.e. a high power means that the risk of a type II error is low. The power is dependent on the significance level used, the effect size of the studied relationship, the sample size and the quality of the data.

Correction for multiple testing

In genetics it is common to test several genetic markers in multiple models in relation to a specific outcome, e.g. disease risk. The risk of at least one type I error grows with the number of statistical analyses performed and correction for multiple testing has become an important issue in genetic research. Several methods have been developed addressing this problem [150]. In this thesis the following methods have been used as specified in the individual papers:

Bonferroni correction. This is the simplest of the correction methods in which a global significance level is kept, and the corrected p-value is retrieved through multiplication of all p-values by the number of total tests performed [151, 152]. However, this method is conservative when analysing several SNPs that are in tight LD with each other since these tests are not really independent of each other [150].

Permutation tests. A better approach to correction for multiple testing when tightly linked SNPs are analysed is permutation analysis. This method is based on random shuffling of the relation between an individual's phenotype data and genotype data, recalculating all the n statistics and their p-values for each shuffle and thus achieving an empirical null hypothesis distribution of the smallest among the n p-values. The reshuffling is repeated many times, e.g. 10 000 and the corrected p-value for a SNP or haplotype is estimated as the propor-

tion of the simulated p-values that are smaller than the originally observed p-value [150].

Replication. This is the ultimate way of discrimination between true and false associations [150]. Replication requires that an association is observed in at least two independent populations, and is today required by most genetic journals for publication of new associations.

Risk estimation

Risk estimations are normally presented with either risk ratio (RR) or odds ratio (OR). They are both measurements of risk for an outcome (e.g. a disease), if exposed versus not exposed to a risk factor (e.g. a susceptibility gene). RR is used when individuals are recruited randomly from the population or when sampling from carriers and non-carriers of the risk factors respectively. However, when studying diseases this method is not usually feasible for practical reasons, i.e. the numbers of individuals that have or develop the disease are too few resulting in problem with power in the study. OR on the other hand can be calculated in studies designed to balance the number of individuals according to the outcome, e.g. the diagnosis, and is therefore commonly used in case-control studies in medical research. Both RR and OR are interpreted by the outcome in relation to the number 1 since a ratio =1 means that the genotype does not affect risk of the disease. Therefore, RR or OR >1 indicates increased risk, whereas RR or OR <1 indicates a decreased risk of the disease. In this thesis we have solely used OR for the measurement of disease risk.

Hardy-Weinberg equilibrium

In a randomly mating population that is not under any evolutionary pressure the relationship between allele and genotype frequencies will remain stable and is said to be in Hardy-Weinberg equilibrium (HWE) [153, 154], this balance can be easily assessed with χ^2 -statistics [155]. Analysis of HWE can be a helpful, considered by some even necessary, to detect genotyping errors, thereby avoiding false associations [156, 157]. However, it is important to remember that Hardy-Weinberg disequilibrium (HWD) also can be a result of evolutionary drift caused by true association with disease. In this thesis HWE was controlled before association analyses were performed in order to detect genotyping or data handling errors. In the presence of significant HWE, all genotyping data and the following data handling was thoroughly

controlled for genotyping or human errors. If no such errors were found, the SNP was kept in the analysis.

Haplotype phasing

When several SNPs are genotyped it is not possible to resolve which combination of alleles from adjacent SNPs that have the same parental origin (the phase). Hence, in the case of multiple heterogeneous genotypes, it is not possible to determine haplotypes with certainty using SNP genotype data. The Expectation-Maximization (EM)-algorithm is a method for estimating haplotype frequencies [158]. This algorithm is an iterative procedure that from an initial assumption of haplotype frequencies (often the product of the constituting SNP alleles) adjusts the frequencies into new frequencies that are more likely given the observed data. The iteration goes on until the likelihood no longer increases. However, the initial condition of the calculation can affect the result. Therefore, a larger sample group, as well as performing the calculation multiple times with different random initiation sites, is crucial for a reliable result. In this thesis, we used the haplotype approach and the EM-algorithm in papers III-V.

STUDY DESIGNS AND ANALYSIS APPROACHES

All papers in this thesis are based on a candidate gene approach, i.e. genes have been selected according to previously published data that indicate that variation in these genes potentially could influence the risk of the studied disease.

SNP analysis

In paper I and II single SNP analyses were performed on the basis of previous association of the studied SNP with disease. Single SNP studies were relatively common in candidate gene studies up until a few years ago and were often performed on SNPs with no known functional effect. This was considered of interest since a SNP can be truly associated with risk of disease if it is in LD with a functional SNP that is the actual cause of the observed association. However, the likelihood of finding casual SNPs using a single SNP candidate gene approach is small compared with the risk of an observed association being a type I error. Therefore, the only single SNP studies considered to be of interest today are those

of single SNPs with a recognised functional effect, i.e. non-synonymous SNPs or SNPs with a known effect on gene expression or gene splicing.

Haplotype analysis

In paper III to V we have used a haplotype design followed by sliding window analysis of the data in order to investigate genetic variation of the whole genes in a more explorative manner than in the first two studies. Haplotype analysis is more powerful than single SNP analysis when exploring data to identify new susceptibility genes. This is because of the fact that discrimination between all haplotype alleles rather than between single SNP alleles of the gene is more specific when untangling which haplotype an unknown functional SNP or haplotype is located on. Sliding window analysis, i.e. analysis of consecutive SNPs and/or haplotypes of various sizes, is powerful for identification of the haplotype window that best represents the optimal association with the studied parameter (figure 5, page 34). This approach is mainly used for candidate gene studies and for further analysis of genetic areas that has been identified as potential susceptibility loci in GWA studies.

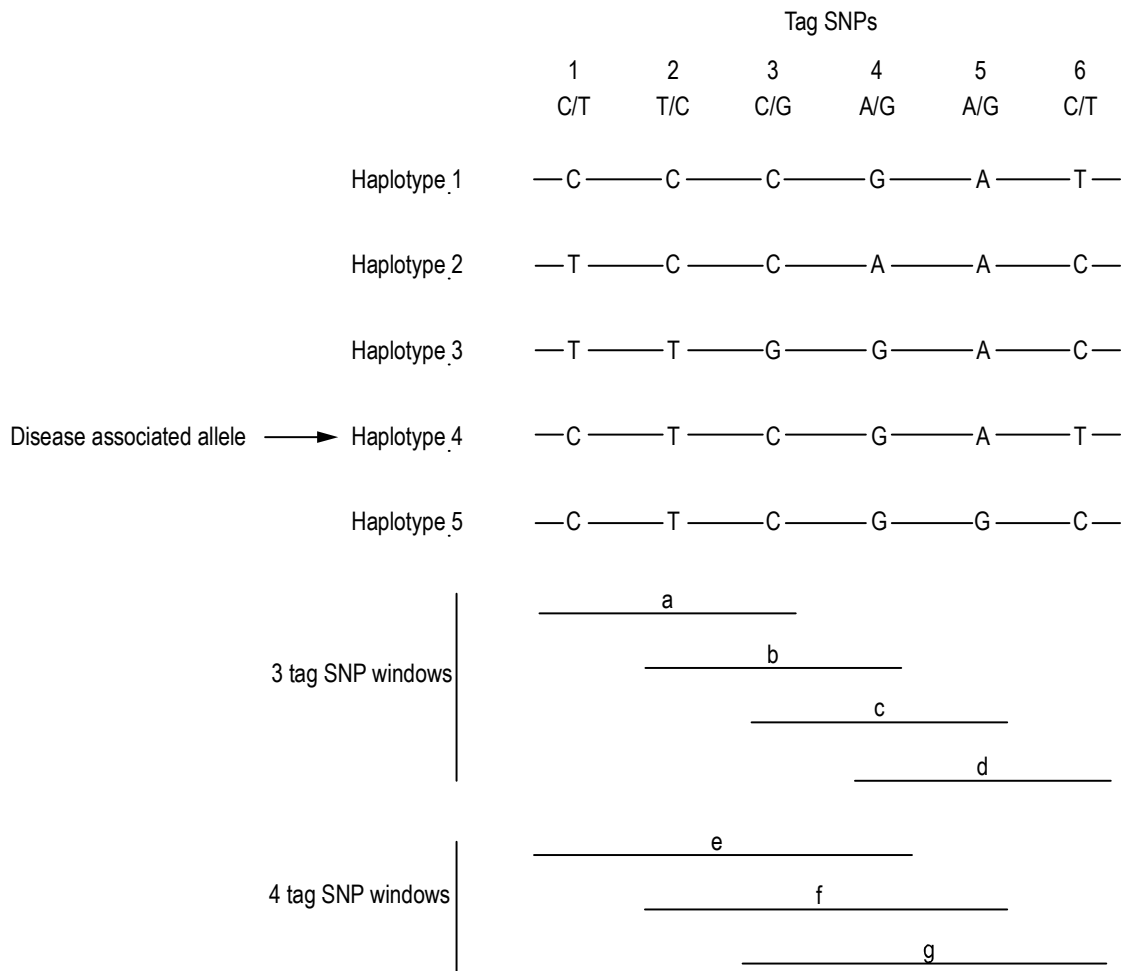


Figure 5: Schematic description of haplotype analysis and the sliding window approach. Haplotype analysis is more powerful than single SNP analysis when exploring data to identify new susceptibility genes. This is because of the fact that discrimination between all haplotypes alleles rather than between single SNP alleles of the gene is more specific when untangling which haplotype an unknown functional SNP or haplotype is located. In this example haplotype 4 is the disease-associated allele. Since none of tag SNPs 1-6 has the variant allele exclusively on haplotype 4, analysis of one SNP will always result in dilution of the group carrying the risk haplotype by individuals carrying the minor allele of the analysed tag SNP on another haplotype, which will result in a weaker association or no association at all. For example, if solely tag SNP 6 is genotyped, the T allele would probably be found associated with risk of the disease although not as strongly as if haplotypes were analysed since the group with genotype T would contain individuals both with haplotype 1 and 4, the latter of which is not disease-associated. By genotyping several tag SNPs and analyse them together in windows of different size in a sliding manner one can identify the haplotype window that have the strongest association with disease since a specific combination of SNP alleles will be present on the associated haplotype only, i.e. the group of individuals carrying the disease-associated haplotype will therefore not be diluted by individuals not carrying the associated haplotype. For example, the 4 tag SNP window (f) carrying the combination TCGA is exclusively present on the disease-carrying allele. This block will therefore show the strongest association with disease and it is likely that the disease-causing SNP or haplotype is part of or in LD with the identified haplotype.

AIMS

The overall aim of this thesis was:

To find genetic support for mechanisms in common for three prevalent conformational diseases: AD, PD and cataract, through investigation of common genetic variations of *KLC1*, *NFE2L2* and *KEAP1* with risk of the sporadic forms of these diseases.

The specific aims of the different studies were:

- I. To investigate the previously AD-associated *KLC1* SNP rs8702 for association with risk of AD and MCI, and/or association with cognitive measures and levels of CSF AD biomarkers in AD and MCI (paper I).
- II. To investigate the *KLC1* SNP rs8702, for association with risk of cataract (paper II).
- III. To perform a haplotype study of the common genetic variation of *KLC1* and investigate if SNPs and/or haplotypes are associated with risk of, or AAO in sporadic AD, PD or cataract, and in AD for association with cognitive measures and levels of CSF biomarkers (paper III).
- IV. To investigate common genetic variation in *NFE2L2* and *KEAP1* for association of SNPs and/or haplotypes with risk of and AAO in PD (paper IV).
- V. To investigate common genetic variation of *NFE2L2* and *KEAP1* for association of SNPs and/or haplotypes with risk of and AAO in AD and cataract, and in AD for association with cognitive measures and levels of CSF biomarkers (paper V).

RESULTS AND DISCUSSION

KLC1 AND CONFORMATIONAL DISEASES

It has been suggested that impaired cellular transport might be an early event in protein misfolding and hence in conformational diseases [85]. Several lines of evidence have indicated that transport mediated by kinesin could be involved in conformational diseases (see Introduction).

In **paper I**, we investigated the previously AD-associated *KLC1* SNP rs8702 [110] in an AD case-control material and in a followed-up MCI material. We could not replicate the association with AD in our material, but did find an association between the previously associated rs8702 minor allele and higher levels of P-tau₁₈₁ in patients with incipient AD, i.e. MCI patients that after follow-up developed AD. We also found that the rs8702 genotypes were not inherited equally between the different *APOE-ε4* genotypes in the AD patients. This imbalance was not found in a non-demented control group.

In **paper II**, we investigated if rs8702 was associated with risk of cataract and found that the rs8702 minor allele was associated with reduced risk of cataract.

Around the time of publication of papers I and II, two additional papers analysing the possible impact of rs8702 in leukoaraiosis and multiple sclerosis were published [159, 160]. The authors reported association of the rs8702 minor allele with increased risk of leukoaraiosis in hypertensive smokers and with reduced risk of multiple sclerosis. The inconsistent associations with increased and reduced risk of disease caused by the minor allele as observed in our studies and others could possibly be explained by the fact that the diseases are different in nature and that changed kinesin-mediated transport might affect the progress of these diseases differently.

The SNP rs8702 is located in exon fifteen of the gene. This exon is part of the 3' untranslated region of the *KLC1* isoform 1 transcript but is spliced away in the isoform 2 and 3 transcripts. Since alternative splicing occurs only after exon thirteen in *KLC1* [143], this location gives cause for

speculation that the rs8702 genotype could possibly affect alternative splicing of the gene [110, 159-162] that may give rise to varying vesicle-binding properties of the resulting KLC1 protein. It is more likely, however, that, if real, these observed associations are caused by another/other SNPs that are in linkage with rs8702. In order to investigate this hypothesis we decided upon a thorough evaluation of *KLC1* using a haplotype approach in multiple diseases: AD, PD and cataract.

In **paper III** we used the HapMap Genome Browser (Phase 1 & 2 - full dataset of CEU) to design a study covering the common genetic variation in the block that covers *KLC1*. Genotyping of six tag SNPs, including the one SNP (rs3212102) reported as non-synonymous in one of the three reported *KLC1* isoform transcripts, covered >80% of the common variation in the gene.

We found no indication of association of *KLC1* single SNPs or haplotypes with risk of PD. Associations were observed for risk of AD and cognitive measures in AD. However the associations were rather vague and somewhat contradictory since an allele associated with reduced risk of AD was also associated with reduced cognitive performance in the AD group. It is very possible that these associations are a result of type I errors rather than being true associations. In cataract, one additional SNP, other than rs8702 which was identified in paper II, was associated with risk of disease. Haplotype analysis did not add significantly to any of the associations observed in AD or cataract. Hence, the haplotype analysis did not support the hypothesis that the previously observed associations were caused by association to other SNPs or haplotypes in linkage with rs8702. The functional SNP rs3212102 was not associated with risk of disease or other disease parameters in any of the investigated diseases.

Since haplotype phasing requires a rather large number of individuals to successfully predict haplotype frequencies [158], the limited numbers in the different MCI subgroups made analysis of this material unreliable. Hence, the association of rs8702 with increased P-tau₁₈₁ levels in paper I could not be investigated using the haplotype approach.

Altogether, these results do not support *KLC1* as a major susceptibility gene for PD and AD. A genetic contribution of *KLC1* to the risk of cataract cannot be excluded but would need to be confirmed in other populations and through functional studies.

NFE2L2, *KEAP1* AND CONFORMATIONAL DISEASES

Oxidative stress and protein misfolding seem to be involved in a dysfunctional feed forward relationship that leads to cellular dysfunction and death. Several lines of evidence support a role of the phase II oxidative stress response in conformational diseases (see Introduction).

In paper IV and V we used the haplotype approach to investigate if common genetic variations in *NFE2L2* and *KEAP1* influence the risk of AD, PD or cataract. As in paper III we used the HapMap Genome Browser (Phase 1 & 2 - full dataset of CEU) to design a study covering the common genetic variation in the blocks covering *NFE2L2* and *KEAP1*. Eight SNPs in *NFE2L2* and three SNPs in *KEAP1* tagged for >80% of the common variation of the genes.

In **paper IV** the advantage of haplotype analysis over SNP analysis described in figure 5 on page 34 is exemplified. In agreement with a previous publication, including single SNP analysis of *NFE2L2* in a GWA study [163], none of the studied single SNPs were associated with risk of PD. Haplotype analyses on the other hand resulted in a replicable association of *NFE2L2* with risk of PD in two independent Caucasian case-control materials, and association with later AAO of PD in one of the materials. These associations could be linked to functional SNPs in the promoter of *NFE2L2*. Together with previous and subsequently published data [128, 133-135], the results support an important function of the phase II oxidative stress response in pathological mechanisms underlying PD. No associations were observed for single SNPs or haplotypes in *KEAP1* with risk of PD.

In **paper V** we found no associations of *NFE2L2* SNPs or haplotypes with risk of AD or cataract. However, we did find an association of the PD-associated *NFE2L2* haplotype with earlier AAO in AD and cataract. No associations were observed for single SNPs in *NFE2L2* or for single SNPs or haplotypes in *KEAP1* with risk of AD or cataract.

This study indicates that *NFE2L2* and *KEAP1* are not susceptibility genes for AD or cataract. However, the results suggest that genetic variation in the *NFE2L2* gene may affect how well cells are able to withstand oxidative stress in a way that influences the rate of an ongoing disease process. It is important to note, however, that the opposing results of this haplotype, resulting in decreased risk of PD while causing an earlier

onset of AD and cataract, do not support a shared functional effect of this haplotype in all three diseases.

CONCLUSION

This thesis aimed at studying common variation in candidate genes representing two of the proposed mechanisms underlying peptide/protein misfolding and aggregation in the sporadic forms of the three conformational diseases AD, PD and cataract. The *KLC1* gene represented impaired cellular transport and the *NFE2L2* and *KEAP1* genes represented oxidative stress.

A role of Nrf2 in diseases involving oxidative stress, such as conformational diseases, is already established (see Introduction). The major finding of this thesis was the identification of *NFE2L2* as a potential susceptibility gene in PD adding genetic support to current indications that Nrf2 may have an important function in cellular defence against PD.

Our results do not support the hypotheses that *KEAP1* and *KLC1* are important susceptibility genes in any of the studied diseases, even though an effect of *KLC1* on cataract cannot be excluded.

A BROADER PERSPECTIVE

The potential of today's high throughput genotyping methods is immense, provided that the resources and required disease materials are available. Not only is it possible to run GWA SNP analyses, but network analysis is also available to integrate these data with data from genome-wide gene expression analyses and epigenetic analyses in order to characterize the function of loci associated to genetically complex diseases [164]. Many frontline laboratories are currently using these methods to identify important susceptibility genes hoping to untangle the underlying mechanisms of common diseases. It is important to bear in mind, however, that the materials studied must be well-characterized. In Scandinavia and many other regions, there are highly organized registers of citizens making it possible to perform long-term follow-up studies and epidemiological studies in which multiple parameters, including biomarkers, can be repeatedly monitored and used in genetic analyses. Well-organized studies of this kind are also valuable as confirmatory populations for the GWA-studies in which potential susceptibility genes are identified.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Alzheimers sjukdom, Parkinsons sjukdom och grå starr är sjukdomar som de flesta människor stöter på någon gång i sin vardag, kanske för att en vän eller nära anhörig drabbas. Dessa sjukdomar är till synes mycket olika. Alzheimers sjukdom påverkar först och främst minnet och förmågan att ta hand om sig själv, medan Parkinsons sjukdom huvudsakligen påverkar motoriken. Gemensamt för Alzheimers och Parkinsons sjukdom är dock att de är obotliga och att man i dagsläget endast kan behandla dem med symptomlindrande mediciner. Grå starr är en ögonsjukdom som drabbar linsen och som gör individen blind om den lämnas obehandlad. Man kan dock operera grå starr och sätta in en artificiell lins vilket gör att gråstarrsorsakad blindhet är mycket ovanligt i I-länder.

Trots att dessa tre sjukdomar tar sig mycket olika uttryck är de inte så olika som det kan verka. De är alla konformationssjukdomar (eng. conformational diseases) vilket innebär att de involverar felaktigt veckade och hopklumpade proteiner i den drabbade vävnaden. Det har föreslagits många olika anledningar till hur denna hopklumpning startar. Forskare tror idag att det skulle kunna bero på att det blir stopp i proteintransporten i cellerna eller att kemiska reaktioner som inkluderar fria syreradikaler kan modifiera strukturen hos proteiner så att dessa lättare klumpar ihop sig.

Genetik har med framgång använts för att kartlägga hur ärftliga sjukdomar överförs till nästa generation men även för att förstå de processer som orsakar eller bidrar till utvecklandet av sjukdomar. Även om olika individers arvs massa (genom) är mycket lika varandra så finns det variationer (genotyper) som gör oss unika, och som kan påverka hur stor risk vi har att utveckla vissa sjukdomar. Den genetiska effekten på en sjukdom kan vara stor eller liten och benämns genotypens penetrans för sjukdomen. Om en specifik genotyp är det enda som krävs för att man ska utveckla en sjukdom är denna genotyps penetrans =100%. Om en genotyp endast resulterar i en ökad risk att bli sjuk så är genotypens penetrans låg. Om man hittar genetiska varianter med hög penetrans kan dessa användas i diagnosutredningar.

Genom att bestämma genotypen hos ett stort antal patienter och friska personer kan man statistiskt beräkna om de undersökta variationerna i de utvalda generna påverkar risken för en viss sjukdom, d.v.s. om det är statistiskt signifikant fler patienter än friska kontroller som har en sjukdomspåverkande variant av en den studerade genen. Man har därmed fått ett genetiskt stöd till den teori som man utgick ifrån när man planerade sin kandidatgenstudie och genen kan benämnas som riskgen (eng. susceptibility gene) för sjukdomen.

I denna avhandling har vi undersökt hur vanliga genetiska förändringar påverkar risken för Alzheimers sjukdom, Parkinsons sjukdom och grå starr. Vi har använt en så kallad kandidatgensapproach. Detta innebär att vi har utgått från tidigare publicerad vetenskaplig litteratur och bestämt oss för att undersöka ett fåtal gener där vi tror att genetiska variationer har särskilt stor sannolikhet att påverka risken för att utveckla dessa sjukdomar. Vi har studerat de former av Alzheimers sjukdom, Parkinsons sjukdom och grå starr som inte har en hög grad av ärftlighet. Därför förväntar vi oss inte att hitta gener som påverkar risken för sjukdom med mycket hög penetrans. Vi söker främst genetiskt stöd för de mekanismer vi valt att studera och att hitta genotyper som tillsammans med andra genotyper och omgivande faktorer till viss del kan påverka risken för sjukdom. Vi har undersökt följande gener:

KLC1. Denna gen kodar för ett protein (*KLC1*) som behövs för att transportera andra proteiner och organeller från cellkärnan till de yttre delarna i cellen. Denna gen är särskilt intressant eftersom nervceller och linsfiberceller är särskilt känsliga för transportförändringar då dessa celler är mycket utdragna i sin form och transport över stora avstånd därför krävs.

NFE2L2 och *KEAP1*. Dessa gener kodar för två proteiner (*Nrf2* och *Keap1*) som utgör regleringen av ett av våra cellers viktigaste försvar mot fria syreradikaler genom produktionen av kroppens egna antioxidanter.

Våra resultat tyder på att genotyper i *KLC1* inte påverkar risken för Alzheimers sjukdom och Parkinsons sjukdom, men att en effekt på risk för grå starr inte kan uteslutas. Fyndet i relation till grå starr måste dock upprepas i andra studier innan man kan se *KLC1* som en etablerad riskgen för denna sjukdom. Det största fyndet i denna avhandling är att genotyper i *NFE2L2* verkar ha betydelse för utvecklandet av Parkinsons sjukdom. Vi fann att en variant av *NFE2L2* påverkade risken för Parkin-

sons sjukdom i två oberoende patient-kontrollmaterial. Samma *NFE2L2* variant påverkade även vid vilken ålder patienterna utvecklade Parkinsons sjukdom, Alzheimers sjukdom och grå starr. Detta beror troligen på varianter som finns i den reglerande regionen framför genen (promotorn) som påverkar hur mycket protein som produceras och kan användas för att försvara cellen mot fria syreradikaler. Detta i sin tur är viktigt för hur länge cellerna kan stå emot andra riskfaktorer som ligger bakom de studerade sjukdomarna. Varianter i *KEAP1* verkar inte påverka risken för någon av de studerade sjukdomarna.

Sammantaget tyder våra resultat, tillsammans med övriga publicerade data inom området, på att genetisk variation i promotorregionen av *NFE2L2* kan vara viktiga för risken att få Parkinsons sjukdom, samt för hur snabbt en redan pågående sjukdom fortskrider. Det senare gäller även för Alzheimers sjukdom och grå starr. Genetisk variation i *KLC1* och *KEAP1* verkar inte vara viktigt för de studerade sjukdomarna, även om en effekt av *KLC1* på risk för grå starr inte kan uteslutas.

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