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GENETIC ASSOCIATION ANALYSIS OF OVERLAPPING BIOLOGICAL PATHWAYS IN CARDIOVASCULAR AND ALZHEIMER DISEASE

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“A scientist in his laboratory is not only a technician: he is also a child placed before a natural phenomena which impress him like a fairy tale” Marie Curie

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ABSTRACT

To gain insight into the importance of the genome for diseases, sequencing and genotyping efforts aim to identify the consequences of genetic variation on both a functional and population level. The task involves the fine-resolution mapping of biologically significant genes and regions discerned by linkage analysis.

This thesis focuses on genetic variation in two candidate genes, Angiotensin-I Converting Enzyme (*ACE)* and ATP-Binding Cassette A1 (*ABCA1)* that are shown to potentially modify Alzheimer disease (AD) risk and related quantitative traits. AD is a disabling neurodegenerative disorder characterized by progressive memory loss that affects an increasing part of the aging population. Mutations in the Amyloid Precursor Protein (A*PP),* Presenilin 1 *(PSEN1),* and Presenilin 2 (*PSEN2)* have been described to cause the early-onset familial form of AD. However, the discovery of genes involved in sporadic late-onset AD has proven to be more difficult. Apolipoprotein-E (*APOE)* which mediates lipid and cholesterol metabolism is the only presently recognized susceptibility gene for sporadic AD.

The Angiotensin-I Converting Enzyme modulates not only blood pressure homeostasis but also the clearance of amyloid-β (Aβ), the pathogenic hallmark of AD, making *ACE* an intriguing biological candidate for both AD and cardiovascular disease. Significant effects for markers in the promoter and 3'- regions were found upon AD risk and disease age-of-onset, consistent with the presence of allelic heterogeneity in this genomic region. A unique differential relationship between genotypes for AD and obesity/ myocardial infarction was explored. The emerging pattern is consistent with the biological role of the ACE protein, but highlights the difficulties of analyzing pleiotropic genes. Computational analysis suggested functionally important promoter and splice variants that may be contributing to trait variability.

ATP-Binding Cassette A1 facilitates cholesterol transport and regulates APOE levels in cells. The gene lies in proximity to an AD linkage peak on chromosome 9q, making *ABCA1* both a biological and positional candidate. In four independent European populations, significant differences in genotype frequencies were found between cases and controls indicated by effects on disease risk. Correlations between quantitative traits related to disease progression complemented the data. To substantiate findings, cholesterol and metabolic traits were examined in a large cardiovascular disease population whereby significant association was determined only among smokers. The data highlight the importance of considering environmental factors that can modify genotype-phenotype relationships.

Applying association analysis across many traits using large replicating samples brings us closer to elucidating patterns of individual variations in genes that contribute to human diseases.

Key words: genetic variation, association, ACE, ABCA1, Alzheimer disease, pleiotropy, cardiovascular disease

ORIGINAL PUBLICATIONS

This thesis is based on the following papers:

I. Patrik G. Kehoe, **Hagit Katzov**, Lars Feuk, Anna Bennet, Boo Johansson, Björn Wiman, Ulf de Faire, Nigel J. Cairns, Gordon K. Wilcock, Anthony J. Brookes, Kaj Blennow , Jonathan A. Prince. (2003). Haplotypes Extending Across *ACE* are Associated with Alzheimer's Disease. *Hum Mol Genet.* 12: 859-867.

II. Patrik G. Kehoe, **Hagit Katzov**, Niels Andreasen, Margaret Gatz, Grodon K. Wilcock, Nigel J. Cairns, Juni Palmgren, Nancy L. Pedersen, Anthony J. Brookes, Kaj Blennow, Jonathan A. Prince. (2004). Genetic Variants of *ACE* Contribute to Age-at-Onset in Alzheimer's Disease. *Hum Genet.* 114: 478-483.

III. **Hagit Katzov,** Anna M. Bennet, Patrik G. Kehoe, Björn Wiman, Margaret Gatz, Kaj Blennow, Boris Lenhard, Nancy L. Pedersen, Ulf de Faire, Jonathan A. Prince. (2004). A Cladistic Model of *ACE* Sequence Variation: Implications for Myocardial Infarction, Alzheimer's Disease, and Obesity. *Hum Mol Genet.* 13: 2647-2657.

IV. **Hagit Katzov,** Katy Chalmers, Juni Palmgren, Niels Andreasen, Boo Johansson, Nigel J. Cairns**,** Margaret Gatz, Gordon K. Wilcock, Seth Love, Nancy L. Pedersen, Anthony J. Brookes, Kaj Blennow, Patrik G. Kehoe, Jonathan A. Prince. (2004). Genetic Variants of *ABCA1* Modify Alzheimer Disease Risk and Quantitative Traits Related to β-amyloid Metabolism. *Hum Mutat.* 23: 358-367.

V. **Hagit Katzov,** Anna M. Bennet, Kina Höglund, Björn Wiman, Dieter Lütjohann, Anthony J. Brookes, Neils Andreasen, Kaj Blennow, Ulf De Faire, Jonathan A. Prince. (2005). Quantitative Trait Loci in *ABCA1* Modify CSF-Amyloid-$\beta_{1\text{-}42}$ and Plasma Apolipoprotein Levels. *J Hum Genet.* 51: 171-179.

RELATED PUBLICATIONS

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ABBREVIATIONS

AAO	Age-at-Onset
ACE	Angiotensin-I Converting Enzyme
Aβ	Amyloid-beta
ABCA1	ATP-Binding Cassette, subfamily A, member 1
ACAT1	Acetyl-CoA acetyltransferase 1
AD	Alzheimer Disease
APO –A1,B,E	Apolipoprotein A1
APOB	Apolipoprotein B
APOE	Apolipoprotein E
APP	Amyloid beta A4 Precursor Protein
bp	Base Pair
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
CD/CV	Common Disease/Common Variant
CD/MV	Common Disease/Multiple Variant
cM	Centimorgans
CSF	Cerebrospinal Fluid
cSNP	Coding Single Nucleotide Polymorphism
CYP46	Cytochrome P450, Family 46, Subfamily A, Polypeptide 1
DASH	Dynamic Allele-Specific Hybridization
DNA	Deoxyribonucleic Acid
EM	Expectation Maximization
ENCODE	Encyclopedia of DNA Elements
EOAD	Early-Onset Alzheimer Disease
ESE	Exon Splice Enhancer
ESS	Exon Splice Silencer
HapMap	Haplotype Map
HDL	High Density Lipoprotein
HWE	Hardy Weinberg Equilibrium
I/D	Insertion/ Deletion
kb	Kilobase
LD	Linkage Disequilibrium
LDL	Low Density Lipoprotein
LINE	Long Interspersed Elements
LOAD	Late-Onset Alzheimer Disease
LOD	logarithm of the odds
MCI	Mild Cognitive Impairment
MI	Myocardial Infarction
MMSE	Mini Mental State Examination
MRI	Magnetic Resonance Imaging
NINCDS-ADRDA	National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association
NFT	Neurofibrillary Tangle
OR	Odds ratio
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography

PESN-1	Presenilin 1
PESN-2	Presenilin 2
rSNP	Regulatory SNP
SHEEP	Stockholm Heart Epidemiology Program
SINE	Short Interspersed Elements
SNP	Single Nucleotide Polymorphism
SR	Serine Arginine Rich
TDT	Transmission Disequilibrium Test
TC	Total Cholesterol
TM	Transmembrane
UK	United Kingdom
UTR	Untranslated Regions
UV	Ultraviolet
VNTR	Variable Number of Tandem Repeats
24-OHC	24-hydroxycholesterol

1 PREAMBLE

The study of genetic epidemiology encompasses statistics, population genetics, epidemiology and human genetics to understand the role of genes and environmental factors in complex traits. The goal of this field is to identify specific genes involved in determining disease risk or determining the underlying traits related to disease. Once the genes are identified, characterisation of the loci at both the individual and population level is undertaken.

1.1 HISTORICAL NOTE

The 20th century marked several scientific milestones that revolutionized human genetic research. The key to understanding how genetic information is replicated came in 1953, with Rosalind Franklin's images of DNA X-ray diffraction and James Watson's and Francis Crick's, publication of the structure of the DNA double helix[1]. This scientific discovery marked the beginning of developments of new techniques to unravel the genome. In 1961, French biologists Franois Jacob and Jacques Monod, together with Andre Lwoff isolated messenger RNA (mRNA) and presented a hypothesis on how genes are turned on and off with the lac-operon model for regulation of gene expression[2]. The 1970's brought together Fred Sanger and colleagues in Cambridge to develop the Sanger dideoxy sequencing method to 'read' the DNA double strand by whole genome shotgun sequencing[3]. Methods ranging from pyrosequencing[4] and chip-level sequencing[5] are all based on improving sequencing speed, read length, and base-call precision of the Sanger method, and have made whole genome sequencing of organisms from parasites such as trypanosomatids to mammals such as mice, dogs, and cows feasible[6]. The 70's also headlined Paul Berg's contribution to genetic engineering by devising a method to cut DNA sequences applied in recombinant DNA technology[7]. Another scientific breakthrough that allowed genetics to advance to the genome level was the development of a technique to amplify large amounts of DNA by polymerase chain reaction (PCR) in the 1980s by Kary Mullis[8]. PCR has become a commonly used method in molecular biology to detect DNA sequences. Many more scientific contributions bring us closer to understanding our genetic code. An industrial and academic effort that began in the 1980's made way for the publication of the reference sequence of the human genome in 2003[9-12]. The sequence revealed a genetic code made up of $3x10^9$ base pairs. This finding was only the beginning of possibilities to map genes to common diseases. Valuable resources such as the HapMap - ENCODE consortiums initiated mapping of genomic sequence variation to identify all functional elements in the human genome[10,13,14].

2 INTRODUCTION

Discovering the genetic components to common diseases has changed dramatically. The 'simple' Mendelian genetic view of diseases caused by single genes has turned into an awareness of complex interplays between interacting genes and environmental factors influencing diseases. The developments of tools and improvement of technologies has been a catalyst to discovering and understanding our genetic imprints. What does it all mean? How will genome projects benefit our society? Will we find treatments for complex diseases such as Alzheimer disease? Are only a few of the questions that we are now faced with while deciphering information from genome sequences. 'What does it all mean?' It means we have a code that determines appearance and behaviour, and our uniqueness is based on small differences in the code that are also responsible for making us more susceptible to diseases. These variations between individuals that amount to higher disease risk are what geneticist aim to identify in genetic association studies. The genetic markers of variation implicate genes and biological pathways involved in disease progression.

'How will genome projects benefit our society?' There are undoubtedly health benefits from research that provides information on genes, proteins and pathways that are involved in diseases and could be of potential drug targets. Disease markers can also be used in risk assessments. However, there are many ethical and social implications to the use of genetic information in regards to confidentiality and disclosures, genetic testing and discrimination. The prospect of personalized medicine based on genetic variation between individuals is not far fetched. 'Will we find treatments and cures for complex diseases such as Alzheimer disease?' There are many challenges to be made in our understanding of disease etiologies to be able find 'The treatment' or 'The cure', but advances in genomics do bring us a
step closer to this goal.

This thesis presents an investigation of variations in the genes encoding Angiotensin-I Converting Enzyme (ACE) and ATP-Binding Cassette A1 (ABCA1) in Alzhzeimer Disease (AD) and cardiovascular disease (CVD) patients with emphasis on shared molecular pathways.

3 GENETIC VARIATION

The first publications from the human genome project debated on the implications of findings on society and on future genetic research. Genetic research took a turn in terms of developments of bioinformatic tools to analyze the vast data and genetic variation was at a spotlight heralded as "the spice of life"[15]. The genetic basis of individual differences is encoded in the DNA sequence. There are only 6,000,000 million base pairs (bp) (around 0.1%) that are different between two individuals. These differences add to the diversity of our species. It is important to point out that large amounts of genetic information is common and shared not only between and within human populations but also between humans and other species[15].

Now that the era of genomics is in full blast, information and data is shared and explored including the study of genetic variation involved in complex disease. The identification of patterns and characterization of genetic variation in human populations is applied in the study human evolution by comparing sequences within and between species, in genetic studies to search for susceptibility genes in complex genetic diseases, and in the identification of regulatory elements in the genome[13].

3.1 EVOLUTIONARY FORCES

In the late 1800's, Charles Darwin's theories of evolution brought to light the notion that natural selection is required for the variation observed in all species[16]. It was only after Gregor Mendel's work demonstrating that units (or genes) passed on the hereditary information to the next generation, that natural selection was thought to act on these units[17]. It took several decades for scientist to discover that changes or mutations in genes give rise to genetic variation, and it was thus argued that evolution is dependent on these mutations.

Genetic variation however is determined by evolutionary forces. The degree of variation in the genome is dependent on demographics events such as changes in population size and migrations, as well as on chromosomal events such as recombination (crossing-over of alleles during meiosis) and gene conversion (unequal crossing-over)[18]. These chromosomal events also influence mutation rates, though new mutations can arise from environmental exposures to carcinogens, toxins and even to UV light[19]. Most mutations are neutral. If a mutation is deleterious it is removed from the gene pool by negative selection. On the other hand, positive selection will act to increase the frequency of an advantageous mutation in the population[20].

The random chance that a mutation will pass on to the next generation is known as genetic drift. Genetic drift is dependent on the effective population size (N_e), defined by the number of individuals contributing to the genetic pool in each generation[18]. It takes $4N_e$ generations for alleles to be fixed in the population. Thus, genetic drift is stronger in small effective populations than in large effective populations because it will take more generations for alleles to be fixed in the larger population[21]. The effects of genetic drift are most apparent in population bottlenecks whereby population size is dramatically reduced because of changes in the environment and in founder populations where a small group separates from the larger group and migrates to a new region[21,22]. An example of a founder population is the Ashkenazi Jewish population. Genetic diseases such as breast cancer caused by mutations in *BRCA1* and *BRCA2*, Tay-Sachs, Niemann-Pick and Gaucher disease are more prevalent in the Ashkenazi Jewish population and it is evident that alleles that were once rare and disease causing have increased in frequency in this founder population[23-25].

3.2 POLYMORPHISMS

Polymorphisms are common genetic variations that have been widely used as genetic markers. The variable tandem number repeat polymorphims (VNTRs) or the minisatellites were the first to be identified followed by the discovery of simple tandem repeats (di- tri- or tetra units) polymorphisms also known as microsatellites, commonly used in DNA fingerprinting[26,27]. Other types of polymorphisms are the insertion/ deletion (I/D) polymorphisms distinguished by presence or absence of DNA bases. All these types of polymorphisms have been used to study the genetic nature of diseases[28]. An example of insertional elements in the genome are the short interspersed elements (SINEs) such as *Alu* repeats[29,30], and the long interspersed elements (LINEs), both used as molecular markers. The Angiotensin-I Converting Enzyme contains a 250bp *Alu* I/D polymorphism on intron 16 which has been widely used as a genetic marker for several complex diseases[31-34].

The development of several high-throughput technologies to detect single nucleotide polymorphisms (SNPs) have led the field to focus on SNPs as genetic markers. With the improvements of genotyping techniques other types of structural variations such as duplications, copy number variants[35] and complex rearrangements are used to illuminate on the cause of complex disorders such as schizophrenia, autism and dyslexia[36].

3.2.1 Single Nucleotide Polymorphisms

On average, the genomes of two individuals are 99.9% identical[37,38]. Two chromosomes randomly selected from the human population have one single nucleotide difference every 1000 base pair[37,39]. When the least common allele has a frequency of more than 1% in the population it is referred to as a SNP. Single nucleotide polymorphsims are the most common type of variation in the human genome[40]. These polymorphsims are biallelic and can either be transitions (purine-purine A⇔G or pyrimidine-pyrimidine C⇔T) or transversions (purine-pyrimidine or pyrimidine-purine) substitutions[41] (Figure 1).

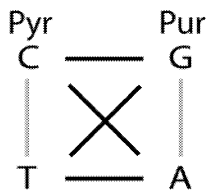


Fig. 1 - Transitions vs. Transversions

There are CG rich regions throughout our genome, known as CpG islands. The Cs of most CpG di-nucleotides are methylated whereby methyl-C tends to mutate to T[42], and in actuality 70% of all SNPs are C⇔T transitions[43]. Sequencing data indicate the existence of 10 million SNPs in the human genome[40,42]. More than 5 million of these SNPs are validated in NCBI's dbSNP database (www.ncbi.nlm.nih.gov/SNP)[44]. Other public databases with SNP annotations include: HGVbase (The European consortium; http://hgvbase.cgb.ki.se/)[45], Japan SNP database (JSNP; http://snp.ims.u-tokyo.ac.jp/)[46], the SNP consortium (TSC http://cshl.org/) and Seattle SNPs (http://pga.mbt.washington.edu/). As SNPs are abundant and genetically stable, they provide an excellent resource as genetic markers. Many studies including the work presented in the present investigation focus on identifying SNPs involved in complex disease.

3.2.2 SNP Functional Classification

The classification of SNPs is dependent on their genomic location.

Coding SNPs

Coding SNPs (cSNP) are located in exons and may be either synonymous or non-synonymous[47]. Synonymous SNPs are silent mutations that do not alter the amino acid sequence of the protein. Although, in some cases synonymous SNPs can affect alternative splicing by disrupting binding sites of proteins such as the serine/arginine-rich (SR) proteins that bind to exon splice enhancers (ESEs) and silencers (ESS)[48].

Non-synonymous cSNPs cause a change in the amino acid structure of the protein. They are prioritized as genetic markers because a change in the amino acid structure may impact on protein folding, as well as on interaction sites, solubility and stability of proteins[47,49] .

Regulatory SNPs

Most SNPs are located in the non-coding region of the genome. The majority of these SNPs have no known function; however some of these intronic SNPs may play a regulatory role in modulating gene expression[50,51]. These SNPs are termed regulatory SNPs (rSNPs). Regulatory SNPs located in the promoter region may affect transcription factor binding sites and rSNPs located in the 5'UTR and 3'UTR (untranslated regions) may also affect protein binding sites by changing sequence motifs[52]. Regulatory SNPs at exon-intron junctions or at ESE/ESS splice sites may cause exon skipping and are known as splice variants[51].

3.2.3 Prediction Tools

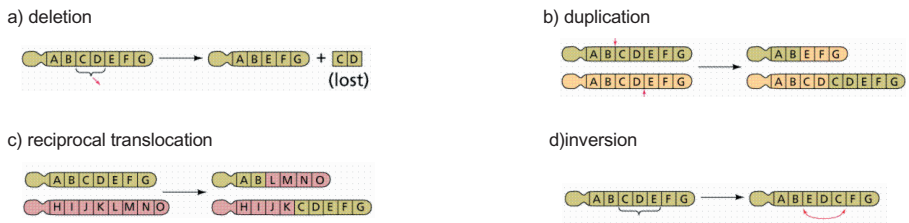
There are several tools that can be used to predict the effects on proteins caused by SNPs. Polyphen is one of the tools used in the thesis work to predict the possible impact of non-synonymous cSNPs on the structure and function of proteins (http://www.bork.embl-heidelberg.de/PolyPhen/) [53]. The damaging effects of synonymous cSNPs on splicing via ESE sequence motifs can be predicted with RescueESE (http://genes.mit.edu/burgelab/rescue-ese/)[54] and ESE finder (http://rulai.cshl.edu/tools/ESE/)[55]. The ESEfinder predicts whether SNPs have an effect on ESE binding sites for specific SR proteins. RAVEN (http://mordor.cgb.ki.se/cgi-bin/CONSNP/a) is one other tool that can be used to predict the effect of SNPs on transcription factor binding sites.

4 GENETIC STRATEGIES FOR GENE MAPPING

4.1 STRATEGY I - LINKAGE AND POSITIONAL CLONING

The goal for research in genetics is to understand gene function and regulation. Groundbreaking discoveries in the 'hunt' for disease genes have identified the genetic causes to many human diseases. More than 2000 of these are described in public databases such as The Online Mendelian Inheritance in Man (OMIM (www.ncbi.nlm.nih.gov/omim/)[56], and Human Gene Mutation Database (HGMD http://www.hgmd.cf.ac.uk/)[57]. Most monogenic diseases are inherited in either a recessive, dominant, codominant or X-linked manner and follow Mendel's laws of independent assortment and segregation[58,59]. Mendelian disorders are rare and are caused by different genetic aberrations such as deletions, duplications, inversions, and translocations of chromosomes in single genes (Figure 2). Mutations in disease causing genes have been traditionally discovered using linkage and positional cloning strategies[60]. The approach entails the determination of the chromosomal region by linkage followed by sub-cloning to identify the genes, and sequencing to identify the mutations[61]. Examples of genes identified by positional cloning and linkage are *CFTR, BRCA1,* and Huntingtin that cause Cystic Fibrosis, breast cancer, and Huntington's disease, respectively[62-64].

Fig. 2 ñ Common chromosomal aberrations. a) deletion b) duplication c) reciprocal translocation d) inversion Modified from Purves et. al.[65]



4.1.1 Linkage analysis

Linkage analysis detects the pattern of transmission of alleles in a pedigree. Linkage studies often use widely spaced microsatellites (5-10cM)[66,67] or SNP panels to generate linkage peaks of susceptibility loci[68-71]. Recombination fraction (θ) is a measure of linkage defined by the proportion of cross-overs between two alleles (or loci) during meiosis. Recombination fraction can be used as a measure of relative physical distance between two loci measured in centiMorgans (cM) also known as the genetic map distance[72]. According to Mendel's law of independent assortment, the recombination fraction will be 50% if the alleles are located on different chromosomes. Two loci are considered linked if a recombination event occurs between them with a probability of less than 50%[73,74]. Linkage is measured by logarithm of the odds (LOD) scores which calculate the likelihood that two markers are linked divided by the likelihood that they are not linked[75]. Large positive scores are evidence of linkage[76] and LOD score of 3 (p=0.001) has been regarded as significant evidence for linkage[73].

In AD research, linkage analysis has identified chromosomes 1, 2, 5, 6, 9, 10,12, 13, 14,15,19, 21 to be linked to the disease[77]. These linkage studies provide broad regions where susceptibility genes reside. Fine resolution mapping of the region are further required to identify specific gene that confer disease risk.

4.2 STRATEGY II - ASSOCIATION

The majority of complex diseases are not caused by single gene, they do not follow Mendelian laws and they are influenced by a variety of genetic and environmental factors. These complex diseases remain largely unresolved on a genetic level[78,79]. To identify the genetic components of complex diseases a candidate gene or genes in a pathway are chosen for fine mapping based on their biological relevance or based on previously defined linkage regions[80,81]. Single nucleotide polymorphisms are then selected and prioritized according to predicted function to identify alleles which are either increased or decreased in frequency in a population of patients versus controls[82-86]. This type of study design is referred to as a case-control association study. It is assumed that the frequency distribution of alleles amongst patients and healthy individuals in the study can provide an estimate of the distribution of alleles in the population[84,87]. Odds ratio (OR) measures are used to compare the odds of patients exposed to the risk factor (in this case the risk alleles) compared to the odds that healthy individuals are not exposed to the risk alleles[87-90] (Box 1).

Box 1 - Odds Ratios

Exposure	Disease: Yes	Disease: No
Yes	a	b
No	c	d

$$OR = \frac{ad}{cd}$$

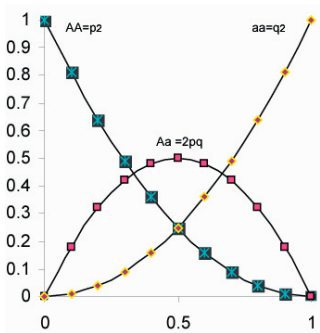
OR>1 – positive association
OR<1 – negative association

Association studies assume Hardy Weinberg Equilibrium (HWE) (Figure 3) to test the null hypothesis (H_0) of no association[87]. There are several advantages of case-control association studies compared to linkage analysis which include the simple and efficient study design to refine small genomic regions and the large number of samples that can be readily ascertained, especially for late-onset diseases such as AD[91] (Box 2).

Fig. 3 - HardyñWeinberg Equilibrium

$$p^2 + 2pq + q^2 = 1$$

Alleles are the different forms of a gene. The set of alleles at a specific locus (chromosomal position) are termed genotypes which represent the genetic make-up of the individual. By applying HWE allele frequencies can be used to predict genotype frequencies. The genotype frequencies for a locus with alleles A and a, and with allele frequencies *p* and *q* respectively, will be: AA homozygote = p^2; Aa heterozygote = 2*pq* and aa homozygote = q^2. There are several assumptions in HWE which include a large, random mating population that is not subjected evolutionary forces of mutation, migration and natural selection[92].



Box 2 – Linkage studies vs. Case-control association studies[91]

Linkage Studies
Use extended families or certain types of study cases and unrelated controls
Use genetic markers situated throughout genome.
Linkage of genotype for a genetic marker to disease may be unique to the particular family

Association studies
Use cases and appropriately matched unrelated controls
Determine genotype for polymorphism in candidate gene of biological relevance to disease
Association of a genotype or phenotype with disease is a statistical finding

4.2.1 Linkage Disequilibrium

Linkage disequilibrium is the non-random association of alleles often termed allelic association. In principal, closely linked alleles tend to be inherited together. This means that a specific combination of alleles may occur more often than would be expected by chance alone[93]. At first, LD can be mistaken with linkage which measures the co-segregation of alleles in a pedigree, though LD is a measure of the co-segregation of alleles in a population[94-96] (Figure 4).

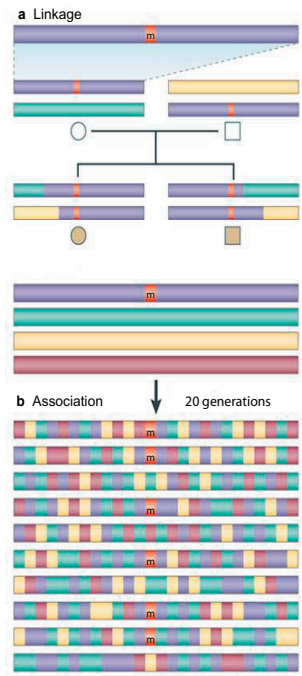


Fig.4 ñ a) Linkage vs. b) Association
m - mutation[81]

4.2.2 Patterns of Diversity

Linkage disequilibrium is influenced by the same factors that influence genetic variation, that is by recombination and gene conversion events[97] in the population history, as well as by demographic events such as isolation, migration, admixture and population bottlenecks[38,98-100]. The distribution of genetic markers such as *Alu*'s and SNPs are used to understand patterns of diversity in different populations.

It is estimated that 80-90% of human SNPs are shared between populations at different frequencies[38,101,102] and that variation between populations is comparatively new. The shared markers between populations indicate mutation events from the past 100,000-200,000. These data provide evidence for the 'out of Africa hypothesis'[103-105], that is the dispersal of *Homo erectus* from Africa to different geographic regions at that time[106].

4.2.3 Indirect Association

Linkage disequilibrium mapping has become a widely used tool for genetic disease mapping and population studies[107-112]. When measuring LD between genetic markers such as SNPs, the presence of one marker makes it possible to predict other markers on a different locus, depending on the strength of LD. The power of linkage disequilibrium to detect allelic associations with disease is limited by the amount of allelic heterogeneity in the region[99,113-115], implying the contribution of several alleles to the disease phenotype. Another limitation to this method is that a lack of association with a SNP does not necessarily rule out the functional effects of other SNPs that are in LD[40,111,116]. Information on LD parameters between SNPs in five different populations (Nigerians, Japanese, Chinese, Western and Northern Europeans) is available from the HapMap project (www.hapmap.org)[117] and currently tested for viability in disease association studies[118-122].

4.2.4 Measures of Linkage Disequilibrium

There are several methods to calculate LD but the most commonly used are measures based on Lewontin's D and r^2. Both methods calculate the pairwise distributions between the allele frequencies of two bi-allelic markers[123-125].

$$D = P_{AB} - P_A \times P_B$$

D is known as the linkage disequilibrium coefficient and ranges from -0.25−0.25. It measures the difference between the observed frequency of a two-locus haplotype (discussed below); P_{AB} for alleles *A* and *B*, and the expected frequency if the alleles were independent ($P_A \times P_B$).

Alternative measures of LD are r^2 and $|D'|$. Both measures range from 0−1 and defined by:

$$|D'| = \frac{D}{D_{\max}}$$

$|D'| = 1$ (complete LD) occurs when two markers have not been separated by recombination. $|D'|$ is independent of allele frequencies and relates to the recombination rates between markers[126]. $|D'|$ measures do tend to overestimate LD in small samples [97,125,127]; $D_{\max}$ - maximum possible value of D when all double heterozygotes are either AB/ab or Ab/aB.

$$r^2 = \frac{D^2}{P_A \times P_a \times P_B \times P_b}$$

The correlation coefficient $r^2 = 1$ (complete LD) measures statistical associations between markers depending on the allele frequencies. The r^2 value is correlated to the χ^2 distribution, and it is also inversely proportional to the sample size required to find the same association with a different marker[93,115].

4.2.5 Haplotypes and Linkage Disequilibrium

Alleles on the same chromosome that are in linkage disequilibrium with each other form a haplotype. In accordance to LD patterns, new haplotypes are created by recombination and mutation events in the population history[97,127]. Across the human genome there are regions of strong LD (where alleles are tightly linked) and regions of weak LD[128-131]. In regions of strong LD, where almost no recombination takes place, only a few common haplotypes are found. Thus the genome can be divided into haplotype blocks that are separated by recombination 'hot-spots'[14,128]. These haplotype blocks (block-like patterns of LD) can extend up to 100kb[132-134] and can be identified or 'tagged' by only a small number of SNPs[129]. Informative SNPs that can capture haplotype diversity and knowledge of gene architecture makes the prospect of whole genome-wide association attainable[116,135].

4.2.6 Haplotype Analysis

To test for association with disease risk and quantitative trait models, haplotype frequencies are first estimated using various prediction tools. The programs use algorithms to infer haplotypes from observed (phased) and unobserved (unphased) haplotypes from the genotype data. Inferring haplotypes from double heterozygote genotypes cause problems of phase uncertaintly[136]. For example, for a single marker locus with alleles *A* and *a*, the haplotype for the single marker is either A or a and the diplotype (multilocus genotypes) heterozygotes will be *Aa* and *aA*. These diplotypes are indiscernible from one another[137].

Haplotypes can be deduced by molecular haplotyping methods; however rarely used because current techniques such as creating somatic cell hybrid methods[138] and allele-specific polymerase chain reaction[139] are inefficient for large distances and technically challenging[128,140].

4.2.7 Haplotype Prediction Tools

The haplotype inference programs used in the thesis work are those of Haplotyper (http://www.people.fas.harvard.edu/~junliu/Haplo/docMain.htm)[141], PHASE (http://www.stat.washington.edu/stephens/software.html)[142,143] and Arlequin (http://lgb.unige.ch/arlequin/)[144]. Arlequin is based on the expectation maximization (EM) algorithm which produces an estimate of the maximum likelihood of haplotype frequencies[145]. PHASE and Haplotyper are both Bayesian methods with prior assumptions as a guide for unobserved haplotypes[146].

4.2.8 Clades

Clades are defined by mutations in regions represented by haplotypes. They are constructed from evolutionary relationship of the haplotypes[147,148]. The haplotype trees or clades are estimated using phylogenetic inference algorithms in candidate gene region where little or no recombination has taken place[147,149-151]. Accordingly, it is assumed that haplotypes (branches) of the haplotype tree (clades) will exhibit similar associations[148,152,153]. For example, in European populations the *ACE* locus is defined by two major clades (clade A and clade B) that explain 36% of the variation in ACE activity. A third clade (clade C) was formed by a recombination event between clades A and B [154-157].

4.2.9 Issues in Study Design

Stratification and Admixture

There are several issues that have been debated because of inconsistencies among the results from association studies. These issues include population choice and the power to detect associations[158,159]. Inappropriate study design may cause both false positive (type I error) and false negative (type II error) associations[81,135]. Subpopulations in the study base due to admixture and stratification are caused when cases and controls are poorly matched with respect to age, gender, and ethnic background[160-162]. To control for stratification and type I error in a study the threshold of statistical significance can be increased. This approach is used in genomic control whereby loci unrelated to the disease are evaluated in both cases and controls[163]. Another method to control for stratification is by group- matched study designs such as the transmission disequilibrium test (TDT) where matching is by nuclear families[164,165].

Admixture mapping takes advantage of the proportion of known genetic markers in the subpopulation to measure the degree of admixture in a population[160,161,166,167]. Several markers that are far apart (not to be in LD) are typed and tested for deviation from HWE at each locus. Admixture is indicated by population associations amongst pairs of loci and by differences in disease risk between loci[162,161,168]. This method does require a map of polymorphic markers or admixture panels that differentiate between the founding populations.[160,166].

Power

In association analysis, power is the probability that the test statistic indicates that the observed marker is close to the disease locus[169]. Power is also related to type I error rate which can be controlled by setting thresholds for the test statistic. For example, specifying power of 95% to detect an association at 5% type I error rate[170]. The power to detect association depends on a number factors including sample size, effect size, (defined by the extent to which a factor influences the outcome), the frequency of alleles, and the strength of LD[96,171].

Genotype errors may cause a loss in power[169,172,173] and can be avoided by using both positive and negative controls or by replicating the results with different genotyping methods[174,175]. Genotyping a number of genes and polymorphisms in the same population of cases and controls increases the chance of false associations (Type I error) due to multiple testing[90]. The Bonferroni correction (multiplying the p-value with the number of tests performed) can be applied; however, the Bonferroni correction is conservative and may even lead to loss of real associations(Type II error)[176].

4.3 DISEASE HYPOTHESES

The common disease/common variant (CD/CV) hypothesis proposes that the genetic risk for common diseases will often be caused by high frequency (<1%) disease alleles found in the population[146]. Thus, the disease is common because the alleles influencing the disease are common. This hypothesis assumes that the detrimental effect of each disease allele is relatively low. Although, the effects of susceptibility alleles may not be strong enough to cause the disease they may influence disease traits and biological pathways[177,178]. An extension of the CD/CV hypothesis is the common variant multiple disease (CV/MD) hypothesis[179]. The CV/MD hypothesis proposes that common alleles which contribute to disease may act on several outcomes due to gene-gene and gene-environment interactions[180,181]. The model emphasizes the overlap in etiological factors among related disorders. This hypothesis closely relates to genetic pleiotropy, defined by a mutation in a single gene that produces effects on several phenotypes[182], and the studies presented in this thesis represent the pleiotropic nature of both *ACE* and *ABCA1*.

5 ALZHEIMER DISEASE

Alzheimer disease brain pathology was first described in 1907 by the German physician, Dr. Alois Alzheimer[183]. A century later, AD is one of the major diseases causing dementia, afflicting 15 million people worldwide[184,185]. The disease is characterized by a progressive loss of memory and decline in cognitive function. Histopathologic features seen in the brain of AD patients are the presence of senile plaques with amyloid-β (Aβ) accumulation and neurofibrillary tangles (NFTs)[186,187]. Considerable neuronal damage and loss of synapses are also found in AD brain pathology[188]. AD is an age-dependent disorder, with prevalence rates of 1% for age group 60-64 to 40% in the older than 90 age group[185]. While age is an important known risk factor, AD is multi-factorial with a strong genetic component[189].

5.1 CLINICAL ASPECTS

Post mortem neuropathology based on Braak[190] provides the only method for a definite AD diagnosis. To make appropriate diagnosis several health outcome measures are used in combination with established criteria such as NINCDS-ADRDA and CERAD, for diagnosing the different stages of the disease[191,192]. Diagnosis is divided into possible, probable and definite AD depending on the progression of disease in patients. A commonly used tool by clinicians to assess cognitive impairment is the MMSE, a score based questionnaire (maximum score = 30; a score < 23 indicates cognitive impairment) that tests six areas of cognitive function: orientation, registration, attention, calculation, recall, and language[193]. In addition, neuroimaging techniques such as PET and MRI are used to diagnose AD. Functional imaging techniques reveal the changes in metabolism, while structural imaging detect atrophy and blood flow changes in the brain[194].

5.2 AMYLOID PLAQUES AND NEUROFIBRILLARY TANGLES

Amyloid-β and NFTs are considered to be the hallmarks of AD[195-197]. The main constituents of amyloid plaques are the 40–42-residues of the amyloid protein (Figure 5), whilst NFTs are composed of the microtubule-associated phosphoprotein tau. The deposition of Aβ and NFTs in cerebrospinal fluid (CSF) are used as biomarkers to detect the early stages of AD, termed mild cognitive impairment (MCI)[198,199]. CSF-tau levels are significantly higher in AD patients compared to healthy individuals[200]. On the other hand, CSF-Aβ levels are lower in patients compared to controls[201-203]. Amyloid-β load measured by immunohistochemical staining of autopsy brains is also used to assess the deposition of Aβ in the different brain regions. The amyloid cascade hypothesis has been confirmed in many studies and proposes that the irregular clearance and degradation Aβ initiates a cascade of neurodegenerative changes that eventually lead to AD pathology[186,195].

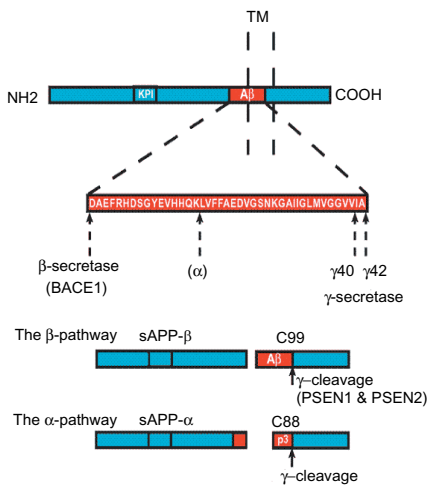


Fig. 5 - APP processing
APP is a type I glycoprotein. Aβ (red) is produced by the β-pathway, where APP is cleaved at the N-terminus (β-cleavage) of amyoid and then in the transmembrane domain (γ-cleavage), at either position 40 or 42. In most cases APP is cleaved at the α-position without Aβ formation. C88, C99 represent the C-terminal fragments produced after cleavage, (numbers are based on the number of amino acids)[204] ;TM ñ transmembrane

5.3 ALZHEIMER'S AND CARDIOVASCULAR DISEASE

Vascular risk factors influence AD as indicated by several epidemiological studies showing strong relationships between AD and CVD[205,206]. Hypertension, history of stroke, hypercholesterolemia and diabetes are all associated with increased risk of AD[205,207-211]. While a number of different biological pathways are implicated in AD, the data point to perturbed cholesterol and lipid metabolism as being central to the disorder[212,213]. The principal evidence for this is the association of APOE, the primary cholesterol carrier protein in the brain, with AD and Aβ deposition[214-218]. APOE-ε4 genotype (discussed below) is correlated with plasma LDL cholesterol levels, which contributes to atherosclerosis[219]. Atherosclerosis in itself is an additional AD risk factor[220]. Moreover, NFTs similar to those in AD have been reported in patients with Niemann-Pick type C, a disorder characterizes by elevated levels of free cholesterol (FC)[221]. Findings from animal models and in-vitro studies indicate that cholesterol is involved in APP processing whereby a reduction in cholesterol causes a reduction in Aβ production[222-226] . The role of cholesterol in Aβ production is still unknown but it appears that cholesterol, as an integral component of cell membranes may affect the clustering of APP secretases in lipid rafts[227,228].

5.4 BRAIN CHOLESTEROL

Studies indicate that cholesterol is synthesized locally in the brain[229], making the brain one of the organs richest in cholesterol[230]. Cells maintain a constant flow of cholesterol. The redistribution of cholesterol in the brain involves the formation of complexes with apolipoproteins - APOA, APOB and APOE[230]. In these complexes cholesterol is also transported to neurons for membrane and synapse formation[231-234]. Removal of brain cholesterol occurs by the conversion of FC to 24-hydroxycholesterol (24-OHC) which can then pass through the blood brain barrier[235]. This reaction is mediated by the enzyme 24S-hydroxylase (CYP46), a brain specific enzyme. Studies have shown that AD patients have increased levels of 24-OHC in plasma and CSF[235] making 24-OHC a potential AD biomarker[236]. In the cell, cholesterol is stored as either FC or in an esterified form. The conversion of FC to cholesteryl esters is mediated by acyltransferase 1 (ACAT1). Reports have indicated that changes in ACAT1 levels influence Aβ production[237,238]. Cholesterol homeostasis in the brain is maintained by a balance of transport, storage and clearance and a disruption of this balance can lead to neurodegeneration (Figure 6).

5.5 STATINS

Cholesterol reducing drugs such as Statins have been suggested as a treatment for AD patients[239-243]. Statins cross the blood brain barrier and inhibit the enzyme HMG-CoA reductase, which catalyzes the formation of melovonate, the rate limiting step in cholesterol formation[230]. Clinical trials have been initiated to assess the effect of Statin use on both cholesterol and Aβ concentration in the blood[243,244].

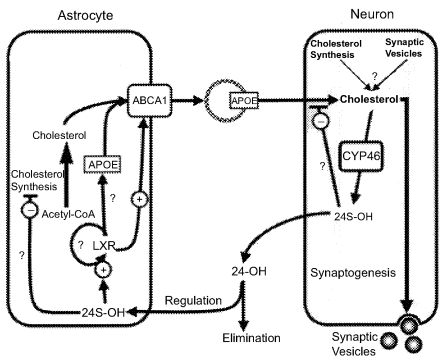


Fig.6 - Cholesterol homeostasis in the central nervous system. LXR- liver-X receptors (important proteins in the regulation of cholesterol homeostasis)[229]

5.6 GENETICS OF ALZHEIMER DISEASE

5.6.1 Early-Onset Alzheimer disease

Mutations in the Amyloid Precursor Protein (*APP),* Presenilin 1 (*PSEN1),* and Presenilin 2 (*PSEN2)* are responsible for the early-onset (EOAD) familial form of AD with autosomal dominant inheritance[245-248]. EOAD accounts for 2-5% of all AD cases with an age-at-onset (AAO) before the age of 65[249]. Six missense (non-synonymous) mutations in *APP* located on chromosome 21 have been identified to cause AD and are responsible for 5-10% of all published EOAD[245,247,250]. A recent study showed that duplication of *APP* with a frequency of 8% in the studied population may be involved in the etiology of disease in a dose dependent manner[251]. Both *PSEN1* and *PSEN2* are active sites of the γ-secretase complex involved in APP processing[252] (Figure 5). At least 50 different mutations have been found in *PSEN1* located on chromosome 14[253,254] and account for 30-50% of familial EOAD[255], whilst mutations in *PSEN2*, a gene homologous to *PSEN-1* located on chromosome 1 are quite rare[256,257].

5.6.2 Late-Onset Alzheimer Disease

The common sporadic form of AD is known as late-onset (LOAD) and represents 85-90% of all cases worldwide[184,185]. *APOE* which is involved in the transport of cholesterol and the metabolism of lipoprotein particles is the only confirmed susceptibility gene identified so far for LOAD[214,216]. The ε4 variant of *APOE* has been shown time and again to be more frequent in AD patients when compared to healthy individuals[214,216,258-263] and the associated risk may be both gender and age-dependent[264-267]. Susceptibility of the APOE-ε4 variant in AD exemplifies not only the CV/CD hypothesis but also the CV/MD hypothesis as a gene associated with several disorders including CVD, Parkinson's disease, Schizophrenia, and diabetes[268-271]. Even-though, APOE-ε4 is an established risk factor for AD, the exact role of APOE in the pathogenesis of disease is still unresolved[272] and not all individuals with the ε4 allele are afflicted with AD[214]. It is more than likely that other genes are involved in disease etiology.

6 CANDIDATE GENES

Genetic association studies in different ethnic populations have implicated a number of susceptibility genes in AD[273]. These findings include cholesterol related genes such as *ACAT1* and *CYP46* to be associated with AD risk and quantitative measures[274-280]. Though, replication attempts in independent samples have been generally unsuccessful[281-285], raising questions on genetic risk factors and association methods. Two candidate AD genes, *ACE* and *ABCA1* will be further explored in the present investigation in studies relating genetic variation to disease risk and measures of AD severity.

6.1 ANGIOTENSIN-I CONVERTING ENZYME

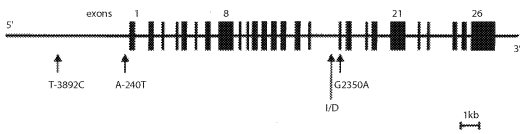


Fig. 7 – Schematic diagram of human *ACE* gene illustrating the location of 3 selected tag SNPs in the present investigation. Exons 1-26 are indicated with vertical bars[154].

Located on chromosome 17, *ACE* is very well characterized with its simple architecture and distinguishable haplotype structure[154-157] (Figure 7). Genetic variation in *ACE* has an effect on the activity of ACE plasma levels making it possible to correlate a measurable trait with a genotype [154,155,286-288]. Extensive evidence exists that variants of *ACE* contribute to CVD and AD[289,290]. The effect however, while heavily replicated appears to be quite weak, which has resulted in many researchers questioning the relevance of the gene. The majority of studies have also been conducted using a single *Alu* I/D polymorphism in the gene, leaving the question about potential genetic heterogeneity largely unanswered. Three studies corroborate on the biological candidacy of *ACE* in AD by describing the involvement of ACE in Aβ degradation[291-293]. These studies and the present investigations provide strong evidence that *ACE* is involved in disease etiology.

6.2 ATP-BINDING CASSETTE A1

The present investigation also explores *ABCA1*'s candidacy as a potential AD susceptibility gene. Previous reports have identified and tested both promoter and non-synonymous cSNPs in *ABCA1* for association, mostly with CVD risk and related quantitative traits[295-301], but also with AD[302]. As a positional candidate, *ABCA1* is located on chromosome 9q31.1 in proximity to previously identified AD linkage peaks[303]. The protein spans the membrane as an integral transmembrane protein (Figure 8). It is involved in the transportation of cholesterol across cell membranes to APOA1 in the plasma membrane, the rate limiting step in the formation of HDL particles[304,305]. Mutations in *ABCA1* cause Tangier disease, characterized by the inability of cells to clear out cholesterol, and by low HDL and APOA1 concentrations[306-308]. Interestingly reduced HDL and APOA1 have also been evident in AD patients[309,310]. ABCA1 is required for the regulation of APOE levels in the brain[311] and it appears that an increase in ABCA1 levels or function may cause a decrease in Aβ via increasing APOE lipidations[311-313] making *ABCA1* a plausible biological candidate for AD.

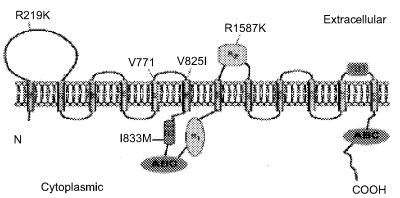


Fig. 8 – A topological view of ABCA1. The position of the amino acid substitution presented in the present investigation are shown; ABC - ATP-hydrolyzing domains; R – regulatory segments[294].

7 PRESENT INVESTIGATIONS

Aims

The studies presented in this thesis aim to explore candidate Alzheimer and cardiovascular disease susceptibility genes by employing SNP strategies and association models with emphasis on quantitative trait analysis and replication efforts. The ultimate goal of the work is to present the applicability of genetic variation research for use in the public health domain.

Paper I and II

To study the role of *ACE* gene polymorphisms in sporadic AD populations by examining single markers and haplotypes in relation to disease risk and age-at-onset.

Paper III

To extend studies on *ACE* variants by investigating the pleiotropic effects of *ACE* in CVD and AD patients and controls, by a systematic investigation of metabolic traits in both men women, by investigating disease associations, and by investigating potential regulatory markers.

Paper IV and V

To examine the link between cholesterol and AD, and the extent by which coding markers within *ABCA1* influence AD risk, as well as to examine measures of disease severity, and cholesterol traits in CVD and AD populations.

7.1 METHODS

7.1.1 Genotyping

To allow for the rapid assessment of SNP allele frequencies in large numbers of individuals, Dynamic allele specific hybridization (DASH) was employed as the main genotyping method. The reaction principal in DASH is based on the differences in the interaction of target alleles with a probe[314] (Figure 9).

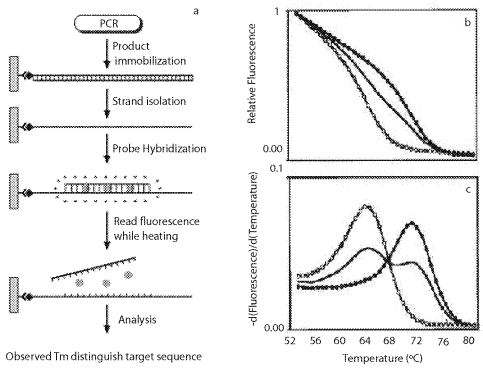


Fig. 9 – The DASH assay

a) DASH involves the design of short PCR primers that span the SNP position. One of the primers is biotinylated and immobilzed to a streptavidin microtiter coated plate or membrane. After the PCR reactions, the non- biotynilated strand of the PCR product is removed by an alakli rinse and an allele specific probe is annelaed to the bound target molecule along with a double stranded specific flourescent dye. This is followed by dynamic heating and real-time monitoring of the hybridization status. b) Denaturation of the probe-target duplex is plotted as a function of flouresence intensity over temperature. c) The negative derivative of the denaturation curves displays the peaks at the melting temperature of the probe-target duplexes which can then be assigned to either match (○), mismatch (•) or heterozygous genotypes (solid line)[315].

7.1.2 Population selection

In order to empower studies aiming to map genes underlying complex diseases like AD and CVD the selection of study populations is important. The study population consisted of several hundred well-defined late-onset AD patients and their age-matched controls from the Northern European population. These collections included samples from the Swedish Twin Registry (STR)[316] and other collections around Sweden and the UK. Unique to these samples are a wealth of phenotypic data which allowed for investigations into genetic factors which influence not only AD itself, but also many other traits related to Aβ metabolism and cognitive function.

To specifically explore questions about metabolic phenotypes, in parallel with the AD studies, 3,000 individuals from the Stockholm Heart Epidemiology Program (SHEEP) were examined. The SHEEP is a population based case-referent study evaluating risk factors in myocardial infarction (MI) and includes extensive measures of metabolic traits[317,318].

7.2 RESULTS

Paper I

A meta-analysis of published data on the commonly studied *Alu* I/D polymorphism in *ACE* indicated a significant association of the insertion allele with AD. Several SNPs in case-control samples from five independent European populations were genotyped and analyzed based upon single markers and haplotypes. Significant evidence of association was found for a promoter SNP and an intergenic SNP indicating genetic heterogeneity in the region. The

results suggested that alleles that are associated with AD are the same alleles that are associated with reduced circulating ACE levels. This implied a relationship whereby reduced ACE activity, either by modification of the protein or reduced expression levels, underlies an increase in disease susceptibility.

Paper II

A replication study provided further evidence implicating *ACE* in AD by testing age-at-onset (AAO) models. Three SNPs previously demonstrated to have maximum effects upon *ACE* plasma levels[288] were analyzed across 2861 individuals from three European populations. As in previous studies, independent effects were observed for both promoter and intergenic SNPs. The association of *ACE* with AD in case-control models was significant, whereby risk alleles appeared to reduce AAO regardless of *APOE*-ε4 carrier status and gender. These results complement existing data confirming ACE'*s* involvement in AD.

Paper III

A strategy examining haplotypes and haplotype combinations (clades) was employed in order to find unique effects of *ACE* in disease risk models of AD and MI, and on quantitative traits related to CVD. To explore the pleiotropic effects of *ACE*, a systematic analysis of metabolic phenotypes of samples from the SHEEP material was carried out. Effects were detected upon several traits and measures of obesity only in men, indicating gender specific effects. Population frequencies of genotypes changed with age adding to the emerging evidence linking *ACE* to longevity. Computation analysis of the regulatory effects of *ACE* variants predicted promoter and splice variants as potential functional markers. In addition, clade models were applied in both MI and AD case-control samples, which allowed for the refinement of the region that harbours pathogenic variation. The data provide evidence of the pleiotropic effects of *ACE* and the importance of testing for genetic heterogeneity.

Paper IV

Analysis of the potential role of *ABCA1* in AD was performed in early and late-onset AD cases and controls which included 1750 individuals from three European populations. Significant association was evident for three common cSNPs, previously associated with CVD[296,301]. Haplotype based association analysis of disease risk and quantitative traits of AD severity showed stronger effects in the early-onset samples. These analyses revealed inconsistencies between allele frequencies, suggesting varying degree of linkage disequilibrium and genetic heterogeneity in the region. Moreover, in AAO models, single marker tests indicated modest evidence of association; however no evidence for association was evident in haplotypes based analysis. Data indicated that variants of *ABCA1* do contribute to variable CSF-tau, CSF-Aβ42 protein levels, and brain Aβ load. The study implicates *ABCA1* in AD, though much work remains to solve the molecular mechanism by which ABCA1 affects the disease.

Paper V

Further support of *ABCA1*'s role in AD and lipid metabolism was demonstrated in a study analyzing the genetic association of common cSNPs of *ABCA1* with CSF- Aβ42 and apolipoprotein levels. Consistent with previous data, a common marker in *ABCA1* was significantly associated with CSF- Aβ42 in AD case-control samples. To define a link between AD and cholesterol the correlation between CSF-cholesterol and CSF-Aβ42 was determined in a

small sample set. In an effort to distinguish the relationship between cholesterol and Aβ42, an alternative approach examining lipid traits in the SHEEP material was employed. Non-synonymous cSNPs in *ABCA1* modulated APOB, LDL and total cholesterol (TC) levels. An independent effect of the markers indicated allelic heterogeneity in the region. Results showed that plasma APOB was elevated among smokers providing evidence that smoking and variants of *ABCA1* may be interacting to affect lipid profiles. The data provide an example of an environmental exposure that may modify a genotype-phenotype relationship and adds to the emerging evidence linking cholesterol to AD.

7.3 CONCLUSIONS AND PERSPECTIVES

The genetic association studies on *ACE* and *ABCA1* brought forward many impending issues in genetic disease associations.

For *ACE*, association findings were consistently replicated across well-defined study populations. The tight LD structure of the gene in European populations makes the identification of the 'real' disease variant difficult. It is more than likely that several alleles determine the ACE trait. Genes may have several different functions as demonstrated by the pleiotropic effects of ACE, whereby differences in ACE levels, determined by genetic variants had an effect on both AD and CVD. The studies on *ACE* did depict specific genetic markers that may be further studied in functional analysis to determine
their role in modulating gene expression or alternative splicing.

For *ABCA1*, findings in single marker analysis and haplotypes were only modestly replicated across samples. The differences between early and late-onset samples and differences in ethnic backgrounds between the samples, as well as the varying degree of LD between markers may have contributed to these modest findings. Non-synonymous cSNPs were prioritized and selected for in the study; however other promoter or downstream markers may also be contributing to trait variability. Findings between common variants in smokers and changes in apolipoprotein levels signify complex gene-environment interactions.

The fine-mapping studies of *ACE* and *ABCA1* provide evidence that the genes are implicated in disease. The studies model known gene architecture in association analysis by selecting SNPs that distinguished the haplotype structure of the genes. The strength of the studies lies in the analysis of quantitative traits related to disease progression added to disease risk findings.

'What does it all mean? How will genome projects benefit our society? Will we find treatments for complex diseases such as Alzheimer disease?' With respect to Alzheimer disease, the data illuminate on the relevance of genetic variation in *ACE* and *ABCA1* in disease and advance our understanding of the molecular basis of AD. With knowledge about pathways involved in disease such as cholesterol metabolism and data coming from clinical trials, the use of cholesterol reducing drugs may become commonly used for the treatment of AD. Our knowledge of the function of each of the ~25 ,000 protein encoded genes in the human genome increases. The challenge for the future will be to identify all DNA sequence variants that confer increase disease risk and to identify the network of gene- interactions in the context of environmental exposures.

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