Genetic Susceptibility to Alzheimer's Disease

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Chapter 1
Introduction

Chapter 1.1
General Introduction

Abstract

With the ageing of western society the contribution to morbidity of diseases of the elderly, such as dementia, will increase exponentially. Thorough preventative and curative strategies are needed to constrain the increasing prevalence of these disabling diseases. Better understanding of the pathogenesis of disease will enable development of therapy, prevention and the identification of high-risk groups in the population. Here, we review the genetic epidemiology of Alzheimer's disease, the most common cause of dementia in the western world. The search for genetic risk factors, though far from completed, has been of major importance for understanding the pathogenesis of Alzheimer's disease. Although effective therapy is still awaited, these findings have led to new avenues for the development of drugs.

Introduction

Although in the past century major progress has been made in unravelling the genetics of Alzheimer's disease (AD), many questions remain to be answered. The debates about its pathogenesis, diagnosis, therapy and prevention have not been settled yet. It is clear that Alzheimer's disease is a complex multifactorial disorder. A great number of possible genetic risk factors have been investigated, but for most of these no clear association has been found.[1] The search for genetic risk factors has yielded three genes (amyloid precursor protein,[2-5] presenilin-1,[6-10] and presenilin-2 genes [11, 12]) in which mutations were found which result in rare autosomal dominant forms of AD. One susceptibility gene (apolipoprotein E gene) has been identified which is a risk factor in the general population.

In this paper the prevalence and risk factors for AD are reviewed. The emphasis will be on the genes known to be involved in AD, their role in understanding the development of the disease, and their implications on diagnosis and clinical counselling.

Clinical epidemiological aspects of Alzheimer's disease

Diagnosis and prognosis

Alzheimer's disease (AD) is the most common cause of dementia in the western world. The disease is clinically characterised by insidious onset and slow progression of cognitive decline. Most frequently, loss of short-term memory and impaired imprinting of new information are the presenting symptoms of AD. During the course of the disease, symptoms may further include disturbance of speech, poor judgement, personality change and deterioration of visuospatial skills, with preserved level of consciousness. Patients gradually lose the ability to be self-supportive, and eventually they will become bedridden. Death usually occurs due to complications of immobility and malnutrition.[13] The average duration of Alzheimer's disease is 8-10 years, although at old age survival may be shorter. Available therapeutic strategies (cholinesterase inhibitors) are not curative, but may halt the process of decline for half a year to two years in the early stages of the disease in a small number of patients.[14]

The diagnosis is based on clinical examination and neuropsychological testing. These should yield no clues for systemic or other brain diseases capable of causing dementia, such as vascular dementia and subcortical dementia's

(NINCDS-ADRDA).[15] Although the precision of the diagnosis has improved considerably with improvement of neuropsychological tests and neuroimaging, during life only a probable diagnosis can be made, with an accuracy of 80-90%. The definite diagnosis of AD is always based on histopathological findings in the brain[16]: neuritic plaques, neurofibrillary tangles and loss of neurons in hippocampus and cerebral cortex.

The neuritic plaques are composed of aggregations of beta amyloid, which are surrounded by dystrophic dendrites, microglia and astrocytes. These plaques are located preferentially in limbic and association cortices of the brain, areas important for memory and cognition.

Neurofibrillary tangles consist of intraneuronal aggregations of hyperphosphorylated tau. Tau is a protein that is normally present in adult human brain, where it exerts its function through stabilising microtubules, which are essential for cell shape and support and intraneuronal transport. Hyperphosphorylated tau destabilises the microtubule network within neurons. Due to subsequent neuronal dysfunction and deficits in neurotransmitters, normal brain function is impaired.[17]

Prevalence of AD

The number of patients affected with AD (prevalence of disease) is remarkably stable in western society. The major determinant of the prevalence of disease is age. Less than 1% of the people aged 70 years or younger is affected with AD. But with each five years increase in age the prevalence of AD doubles, until by age 90 years up to 30% is affected.[18] A large European follow-up study has shown that, especially at older age, women are more often affected than men.[19, 20]

Although AD is considered to be a disease of the elderly, there are patients in whom first symptoms of AD may be present as early as at age 35 years. Frequently, a distinction is made between "early-onset Alzheimer's disease" (EOAD) and "late-onset Alzheimer's disease" (LOAD). The distinction is arbitrary, since clinical and pathological features are extremely similar in both groups. Age criteria for EOAD vary widely, but usually, when the age at onset of the disease is before 65 years of age, a patient will be diagnosed with EOAD.[1]

Given its strong association with age, AD will be an increasing health care problem in the next decades. With the aging of western society, the number of patients is expected to increase exponentially. By the year 2025, over 22 million patients with dementia are expected around the world.[21]

Risk Factors

Alzheimer's disease has a complex etiology. Research in the past century has focussed on many putative environmental factors that may either increase or decrease the risk of AD. These included age, smoking, maternal age at birth, head trauma, depression, thyroid disease, ant-inflammatory drugs, estrogen replacement therapy, alcohol, occupational exposure, aluminum, education and diet.[22] Findings on these risk factors have been inconsistent. Only increasing age and genetic predisposition are consistently correlated with the disease.[1]

Perhaps most interesting from an epidemiological perspective is the finding that studies on vascular risk factors such as hypertension, diabetes mellitus, atherosclerosis, and high cholesterol have yielded promising results, showing an up to two times increased risk for AD.[23-32] The mechanism through which these vascular factors are associated with AD remains to be elucidated. It has been argued that these factors may be a primary cause of AD pathology.[33] An alternative explanation may be that vascular pathology is not a primary cause of AD, but rather that it accelerates the primary neurodegenerative process.

The findings of the relationship between vascular pathology and AD are in line with cross-cultural observations by Hendrie et al.[34] They recently published results on differences in age-standardised annual incidence rates of AD in an industrialised versus a non-industrialised country. A possible explanation for the decreased rate in the non-industrialised country is the lower prevalence of cardiovascular disease in the non-industrialised population. However, also differences in genetic make-up between populations may partly explain these findings. As discussed in the next chapter, genetic susceptibility is, in addition to increased age, the most important determinant of AD.

Genetics of AD

Familial aggregation

As opposed to the difficulties encountered in finding environmental risk factors for AD, the genetic component of the disease has long been evident. Epidemiological studies have clearly shown that AD aggregates within families.[35] First degree relatives of AD patients have a 3.5-times increased risk of developing AD. The relative risk increases with a decrease in the age at onset of the affected proband. In relatives of patients with an onset before age 70 years the risk of having AD is increased over 4 times.[35] Concordance rates of up to 80% have been found in monozygotic twins. In dizygotic twins concordance rates were 35%.[36]

In few families an autosomal dominant pattern of inheritance can be recognised. A segregation analysis suggested an autosomal dominant model in less than 1% of 198 families with EOAD.[37] In LOAD, it is difficult to make a distinction between a dominant, recessive or additive model of inheritance.[38] In the majority of patients the aetiology appears to fit a multifactorial model in which multiple genes and environmental factors interact.[1]

Genes involved in AD

Research on genetic determinants initially focussed on families with an autosomal dominant pattern of inheritance. The first dominant mutation was found in the gene encoding the Amyloid Precursor Protein (*APP*) on chromosome 21.[2-5] Up until now, 32 families are known around the world with EOAD due to a dominant *APP* mutation (http://molgen-www.uia.ac.be/ADMutations/).

Besides *APP*, two homologous genes were identified, presenilin-1 (*PSEN-1*) at chromosome 14 and presenilin-2 (*PSEN-2*) at chromosome 1q31-q42, which account for families segregating AD as an autosomal dominant trait as well. So far, more than 80 mutations of the *PSEN-1* gene have been identified [6-10] (http://molgen-www.uia.ac.be/ADMutations/). Six mutations in *PSEN-2* are described [11, 12] (http://molgen-www.uia.ac.be/ADMutations/).

Frequency estimates of these mutations in EOAD patients are highly variable, ranging from less than 1% to 50%.[39-41] Differences might be due to more or less stringent diagnostic criteria in the population under study (e.g. probable versus autopsy confirmed AD), different maximum age when considering early onset, and selection of study populations. A study population derived from a highly specialised neurological centre is more likely to have an overrepresentation of cases with high familial aggregation. However, in a population based sample[41] *APP* mutations were found in only 0.5% of all EOAD patients and accounted for only 0.005% of AD in the general population. Although mutations in *PSEN-1* are more common, they still only accounted for 6.5% of all EOAD patients (i.e. 0.065% of AD in the general population). Mutations in *PSEN-2* were seen in less than 1% of all EOAD patients, and less than 0.01% of the general population. Together, dominant mutations in *APP*, *PSEN-1* and *PSEN-2* occurred in only 0.075% of AD patients at population level.[41, 42] Although these genes have a minor impact in the general population, for the individual carrier risk is extremely high. Almost all carriers of these mutations express the disease. As EOAD is rare, risk estimates for carriers of these mutations approximate infinity.

In addition to the three autosomal dominant genes, a fourth gene (Apolipoprotein E, *APOE*) was identified which is localised on chromosome 19

and has three common alleles coding for three different isoforms of the protein. The allele frequencies of this gene (*APOE*) are 0.08 for *APOE ε2*, 0.77 for *APOE ε3* and 0.15 for *APOE ε4* in populations of European ancestry.[42] *APOE ε4* is strongly associated with LOAD[43, 44] and EOAD.[45] Subjects homozygous for *APOE ε4* have an almost 15 times increased risk of developing AD, but 50% will not develop the disease.[46] Subjects with only one *APOE ε4* allele have a moderately increased risk (around 3 times).[47] Although risks are moderately increased for *APOE ε4* for the individual carrier, due to the fact that the allele is common *APOE ε4* may explain 17% of the occurrence of AD in the general population.[42] Homozygosity for *APOE ε4* contributes less than 2% to AD because of low prevalence of this genotype (0.0225). It is suggested that *APOE ε4* regulates when, rather than if, the disease occurs.[47, 48] Due to a relatively earlier onset of LOAD in those homozygous for *APOE ε4*, the influence of competing morbidity and mortality will be less, thereby enhancing the association between *APOE ε4* homozygosity and LOAD.

Genes and the pathogenesis of AD

The discovery of mutations in the genes involved in AD has been of great importance for the understanding of the biological mechanisms underlying AD. All causal mutations affect the normal metabolism of beta amyloid, suggesting that beta amyloid constitutes a central event in the pathogenesis of AD. Beta amyloid, or Aβ, is a peptide present under physiological circumstances in healthy subjects. Due to a mutation in any of the known AD genes, the equilibrium between production and clearance of Aβ gets disturbed, resulting in accumulation of Aβ in the brain. Amyloid fibrils are formed and subsequently deposited into plaques. At present, the most likely hypothesis is that at first diffuse plaques are formed. These plaques can also be seen in healthy subjects. In AD patients several of these plaques may evolve into "mature" neuritic plaques containing fibrillar aggregates, damaged neurons and activated glial cells in a cascade of pathological processes, eventually leading to profuse neuronal loss.[49-57]

APP is a transmembrane protein that is widely expressed on cell surface. Its functional properties are not clearly defined, but range from repair of vascular injury to mediation of growth and adhesion of neural and non-neural cells.[53] Recently it has been suggested that APP has a function in regulation of nuclear transcription.[58] APP is cleaved into Aβ. The different mutations that have been found so far in the gene coding for APP are located at or near cleavage sites.[40] By abnormal cleavage of APP larger amounts of a longer version of Aβ are produced, called Aβ42.[59-62] This longer version is more amyloidogenic and

therefore aggregates more easily into plaques.[63] There is increasing evidence that Aβ42 may play a crucial role in the pathogenesis of AD.

The function of the presenilin proteins is unclear, but it has been shown that mutations in *PSEN* also lead to altered APP processing. Aβ42 levels are raised in brain, plasma and fibroblasts[64] of carriers of a *PSEN* mutation. Furthermore, in experiments with *PSEN* transgenic mice and transfected cells higher Aβ42 levels are found as well.[65-68]

In sporadic LOAD the pattern of Aβ accumulation is less evident. It has been suggested that accumulation is rather the result of impaired clearance of Aβ than of increased synthesis.[57] In carriers of the *APOE ε4* allele, the predominant genetic risk factor in sporadic AD, APOE has increased affinity for Aβ and Aβ-aggregates. Although the precise mechanism is not yet elucidated, *APOE ε4* is suggested to facilitate Aβ-aggregation or to inhibit the elimination of the fibrillar aggregates.[44, 69, 70]

Although a body of genetic evidence supports the Aβ cascade hypothesis and although it is the most comprehensive theory so far, the debates on this hypothesis have not been settled yet.[50, 57, 71, 72] Disagreement ranges from details within the Aβ hypothesis (e.g. that not the total amount of Aβ is important but rather the relative proportion of Aβ42) to the reverse hypothesis that Aβ accumulation is a compensatory mechanism to ageing.[73] A finding difficult to explain has been that amyloid deposition does not seem to correlate very well with cognitive decline.[50] However, recent findings may have settled this argument by showing that Aβ plasma levels are elevated early in the course of the disease and are strongly related to cognitive decline, a finding in favour of the Aβ cascade hypothesis.[74, 75]

While the Aβ hypothesis is being refined other pathogenic models are considered as plausible. These include hypotheses on the involvement of tau,[76] on neuroplasticity, [77] ageing,[72] oxidative stress,[78] impaired cerebromicrovascular perfusion,[79] inflammation[80] and lipid homeostasis.[70]

Genetic counselling and risk prediction

The identification of genes involved in AD has been a major breakthrough. Yet there is ongoing debate on their use in clinical counselling.

Given the fact that *APP*, *PSEN-1* and *PSEN-2* mutations have a virtually complete penetrance and that these mutations are not found in healthy age-matched subjects, one might argue that these mutations are useful for risk prediction and genetic counselling. But the known mutations are only present in a minority of cases. Although mutations can be found frequently in patient populations from highly specialised centres due to selection bias,[81] these

mutations are rare in the general population. Thus, for risk prediction and counselling, the absence of a known mutation should not be conclusive. Even if the underlying mutation is known in a family with an autosomal dominant form of AD, there is a strong argument against screening relatives at risk, because curative therapy and prevention are not yet at hand. An argument in favour of screening relatives at risk and those that already have dementia without a definite diagnosis might be to take away incertitude and allow for future plans, but screening should always be preceded by thorough counselling, taking into account ethical considerations. Because of their low frequency, the mutations are not useful as a diagnostic tool for patients with early onset symptoms of dementia.[39]

Also the use of *APOE* is limited in clinical practice. *APOE ε4* increases the susceptibility to AD, but the increase in risk is modest, especially in the heterozygous carriers of the *APOE ε4* allele. As only 50% of the AD patients carry *APOE ε4* and a substantial number of patients with other dementias show similar frequencies, *APOE ε4* is not suitable for diagnostic purposes.[82] Even for subjects homozygous for the *APOE ε4* allele there is still a 50% chance not to develop the disease.[46] Thus despite the fact that *APOE* is a more important determinant of AD in the general population than *APP*, *PSEN-1* and *PSEN-2*, *APOE* is not suitable for risk prediction and counselling.

Considerations

The discovery of the dominant mutations in the *APP*, *PSEN-1* and *PSEN-2* genes in the families with autosomal dominant EOAD explains only a minor proportion of the disease in the general population. The clinical use is limited and awaits therapy and prevention. Nevertheless, genetic research has delivered a significant contribution to understanding the pathogenesis of the disease, even in sporadic patients. Genetic evidence is pointing towards a central role for amyloid metabolism in the aetiology of AD. Due to the genetic evidence towards Aβ research focussing on Aβ has expanded impressively. Recently, promising evidence has been found on vaccination strategies with Aβ in mice, decreasing formation of Aβ[83] and enhancing cognition in mouse models.[84-86] Verification of these findings in man, combined with useful biomarkers, will be of tremendous importance in prevention of this disabling disease. Recently, trials in humans were halted because of major side effects.

Meanwhile, other genetic and environmental factors should still be considered to fill the lacunae in our knowledge of AD. Better understanding of

the neuropathological mechanisms underlying AD, whether based on genetic findings or on results of other kinds of scientific research, will without a doubt aid future therapeutic and preventive strategies.

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Chapter 1.2
Scope of the Thesis

Studies exploring the genetic susceptibility to late-onset Alzheimer's disease have been faced with difficulties. After initial success (as reviewed in chapter 1.1), follow up by genome wide searches has provided evidence for additional loci encompassing susceptibility genes for Alzheimer's disease, but subsequent candidate gene studies have only given inconsistent results.

Tools are needed to reduce the heterogeneity of late-onset Alzheimer's disease. In this thesis we have employed several genetic epidemiological strategies in an attempt to further elucidate the genetic susceptibility to AD. Most of the work presented in this thesis is performed within the framework of the research program Genetic Research in Isolated Populations (GRIP). Genetic make up is likely to be more homogeneous in such a population than in outbred populations. Further, availability of complete genealogical data will allow more efficient and unbiased genetic studies. We believe this will facilitate the detection of possible susceptibility genes.

In chapter 2 two studies of familial aggregation are presented. In chapter 2.1 we study the familial clustering of various forms of dementia in the GRIP study, the contribution of known mutations to the occurrence of dementia in GRIP, and two previously reported candidate regions and candidate genes. Chapter 2.2 describes heritability of various cognitive traits, such as memory and executive control, in healthy middle aged and elderly individuals living in the GRIP area. In both studies we explore the role of vascular disease on familial aggregation of dementia or cognition, to gain more insight in the association between vascular disease and dementia. This question is further addressed in the first of the three genetic studies as presented in chapter 3. Chapter 3.1 describes the study of the vascular candidate gene encoding Angiotensin Converting Enzyme (*ACE*) in association to Alzheimer's disease in the Rotterdam Study. Chapters 3.2 and 3.3 describe two genome wide searches, the first performed in a consanguineous pedigree and the second in a large series of patients ascertained in the GRIP study. Finally, chapter 4 offers a synthesis of all work described in this thesis. The main findings will be discussed in a broader perspective of previous studies on Alzheimer's disease.

Chapter 2
Familial Aggregation

Chapter 2.1

Familial clustering and genetic risk for dementia in a genetically isolated Dutch population

Abstract

Despite advances in elucidating the genetic epidemiology of Alzheimer's disease (AD) and frontotemporal dementia, the etiology for most patients with dementia remains unclear. We examined the genetic epidemiology of dementia in a recent genetically isolated Dutch population, founded around 1750. The series of 191 patients ascertained comprised 122 probable AD patients with late onset and 17 with early onset, and 22 with possible AD. It further included ten patients with vascular dementia, 9 with Lewy body dementia and 6 with frontotemporal dementia. All patients, except those with vascular dementia, were more closely related than healthy individuals from the same area. Clustering was strongest for patients with early-onset AD or Lewy body dementia. Although 14% of late-onset AD patients had evidence of autosomal dominant disease, consanguinity was found in 3 late-onset AD patients, suggesting a recessive or polygenic model underlying the trait. We found no clustering of vascular dementia, implying a difference in genetic risk for late-onset AD and vascular dementia. Mutations in known genes could not explain the occurrence of dementia, but the population attributable proportion of APOE ε4 was high (45%) due to a high frequency of APOE ε4 carriers. Earlier identified regions on chromosomes 10 and 12, nor the effect of the A2M I/D polymorphism on AD could be confirmed in our study. We did find evidence for association between the A2M D-allele and Lewy body dementia.

Our data showed a strong familial clustering of various forms of dementia in this isolated Dutch population. A high percentage of late-onset AD could be explained by APOE ε4 but still, 55% of its origin is unknown.

Introduction

Dementia is known to aggregate in families.[1, 2] First-degree relatives of patients with Alzheimer's disease (AD) have a 3.5 times increased risk to develop AD. The risk of disease for relatives decreases with increasing age at onset of disease of the proband. Despite substantial evidence for familial aggregation, for late-onset AD the mode of inheritance is not clear.[2] Although the evidence is strongest for autosomal dominant and multifactorial segregation, there is recent evidence for recessive inheritance.[3] For other dementia subtypes, findings on familial clustering are less consistent. Several families have been reported in which frontotemporal or Lewy body dementia segregated as an autosomal dominant trait.[4-7] Little is known about the familial aggregation of vascular dementia, the most common form of dementia following AD.

Various genes are involved in the familial forms of dementia. Three major genes were identified which are involved in early-onset AD, i.e. amyloid precursor protein gene (*APP*),[8] presenilin 1 (*PSEN-1*),[9-13] or presenilin 2 (*PSEN-2*).[14, 15] Although these genes may be important in some families with early onset of the disease, in the general population mutations are rare, explaining less than 0.1% of all AD patients.[16] For sporadic late-onset AD, the most common genetic risk factor is the E4 allele of the apolipoprotein E gene (*APOE ε4*),[17] explaining up to 20% of all patients. Individuals homozygous for *APOE ε4* are estimated to have a 6 to 15 times increased risk for AD while carrying one allele is associated with a 1.5 fold increased risk.[16, 18] Numerous other loci were suggested to be associated with late-onset AD, but often the associations found were not replicated in other studies. However, there is consistent evidence for AD loci on chromosome 10, including the insulin degrading enzyme gene (*IDE*) and α-T catenin[19-24] and on chromosome 12 including alpha-2 macrogobulin (*A2M*).[25-27] Various genes are involved in other forms of dementia. Among those is the microtubule associated protein tau gene (*MAPT*), in which mutations are predominantly involved in frontotemporal dementia.[7]

Here, we aimed to study familial aggregation of dementia in a population-based study in a recent genetically isolated population in the South West of the Netherlands. This approach offered several advantages. Not only could we use genealogical data available through municipal and church records, rather than family history, we were also able to verify the diagnoses in all patients. Further, we could obtain DNA from all patients in this study. This allowed examination to which extent familial aggregation could be explained by known genes involved in various forms of dementia.

Material and Methods

Study population

The study is based in a genetically isolated population in the South West of the Netherlands (GRIP population). The population was founded in the middle of the 18th century, when a group of approximately 150 people settled in the area. The descendants of these founders lived in relative isolation until the middle of the 20th century. From the middle of the 19th century the population started to expand considerably, from 700 inhabitants in 1848 to more than 20,000 inhabitants by the end of the 20th century.

Patients

In cooperation with local general practitioners, neurologists and nursing home physicians, we asked patients with any dementia syndrome and their relatives to participate in our study. Written informed consent was obtained. The Medical Ethical Committee of the Erasmus MC Rotterdam, The Netherlands approved the study protocol. All ascertained patients (n=191) were examined by one of two research physicians to re-evaluate the clinical diagnosis. Examination consisted of neurological examination and brief neuropsychological testing. A standard interview was performed with close relatives concerning presenting symptoms, disease course, medical, social and family history. The degree of dementia was classified using the Clinical Dementia Rating Scale (CDR). In addition we reviewed all available medical records, neuropsychological test results, and hard copies of CT or MRI scans to establish a diagnosis according to NINCDS-ADRDA criteria [28]. Two neurologists (WAvG, JCvS) independently assessed all available information and made a clinical diagnosis. In case of discrepancy, the final diagnosis was established in a consensus meeting.

Genealogy

Genealogical data comprising name, date and place of birth of parents and grandparents was collected at a home interview with a close relative. This genealogical information was extended up to 22 generations using municipal registers and data from a large genealogy database that holds genealogical information on approximately 60,000 individuals from the GRIP region. Genealogical relationships between patients were expressed as the kinship coefficient (K).

Family and medical history

We obtained information on family history of dementia by means of interviews with first-degree relatives. Family history was defined positive if at least one first-degree relative had dementia. When at least 3 relatives in two generations were affected, independent of the number of first-degree relatives, we classified the disease as an autosomal dominant form of dementia.

History of cardiovascular disease was defined based on report of hypertension or treatment for hypertension, stroke or transient ischemic attack, myocardial infarction, angina pectoris, atrial fibrillation, and/or hypercholesterolemia in heteroanamnesis or medical records. History of diabetes mellitus was defined based on report of diabetes mellitus, or when patients used antidiabetic medication, or when increased fasting glucose levels or HbA1C were mentioned in the medical records.

Laboratory analysis

Blood was drawn from patients, spouses and offspring, or siblings. DNA was extracted from peripheral leucocytes according to a standard protocol.[29]
Given the tight relations between patients in this study, we limited mutation screening in *APP*, *PSEN-1* and *PSEN-2* to a representative subset of 80 patients. Basically, for each nuclear family one of the patients was selected. Exons 16 and 17 of *APP* and coding exons 3 to 12 of *PSEN-1* and *PSEN-2* were analysed for mutations. Mutations were analysed in 283 control individuals randomly selected from the general Dutch population (Netherlands and Flanders-Belgium) by pyrosequencing. Patients diagnosed with frontotemporal dementia who had a positive family history for dementia were screened for mutations in *MAPT*, using protocols described previously, sequencing all exons of *MAPT* except exon 6 and 8.[30]

We genotyped two markers flanking *APOE* (D19S420, D19S902; Applied Biosystems) for linkage analysis. *APOE* genotyping was successfully performed in 190 patients and a control set consisting of 156 spouses, spouses of siblings and spouses of patients with diabetes mellitus or Parkinson's disease originating from the same area, following previously described protocols.[31, 32] For linkage studies, a sample of 73 closely related patients was genotyped for nine short tandem repeat (STR) markers on chromosome 12 (D12S99, D12S336, D12S310, D12S1617, D12S345, D12S85, D12S368 and D12S83 from the Applied Biosystems version 2 MD-10 marker set) spanning approximately 60 cM and covering regions that showed evidence for linkage to AD in other studies.[25-27] On chromosome 10 seven STR markers (D10S196, D10S1652, D10S537, D10S1686, D10S185, D10S192 and D10S597) were genotyped, spanning an approximately 65 cM region that

showed evidence for linkage to AD in previous studies.[20-22] Polymerase chain reactions (PCR) were performed according to the manufacturer. PCR products were pooled and subsequently analysed on an ABI377 or ABI3100 automated sequencer (Applied Biosystems). Based on the results obtained in the linkage analysis, patients and 120 controls were genotyped for the *A2M* insertion-deletion (I/D) polymorphism,[33] and three STR markers on chromosome 10 (D10S192, D10S538 and D10S1686) surrounding the location of *IDE*.

Statistical analysis

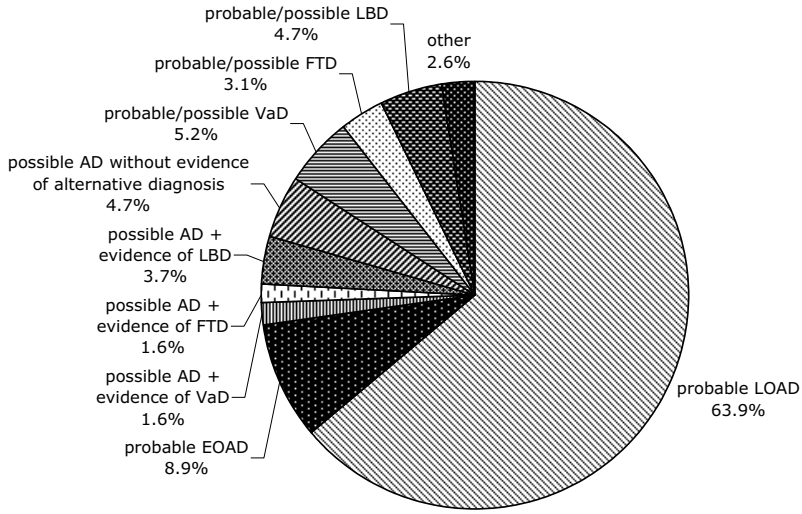
We performed general descriptive statistics using χ^2 statistics for dichotomous variables and a general linear model for continuous variables. The markers on chromosome 10 and 12 were analysed by means of Dislamb,[34] with disease allele frequency set to 0.01. Hardy Weinberg Equilibrium was tested using the GENEPOP-package (Raymond M. & Rousset F., 1995. GENEPOP version 3.1c). We calculated pair wise kinship coefficients (K) using PEDIG software (Boichard D., http://dga.jouy.inra.fr/sgqa/diffusions.htm) based on a pedigree of the total population, consisting of 56693 subjects. Distributions of K were compared between dementia subtypes using χ^2 statistics. Average kinship coefficients of patients were compared to that of their cognitively healthy spouses.

Results

Patients

We ascertained 191 patients (73.6% women, 26.4% men) with dementia in the isolated Dutch population (see figure 1). Of those, 122 (63.9%) had clinically probable late-onset AD and 17 (8.9%) had probable AD with an onset before 65 years (early-onset AD). In 13 patients, the clinical picture was compatible with AD but included symptoms suggestive of Lewy body dementia (n=7), frontotemporal dementia (n=3) or vascular dementia (n=3). We therefore classified those patients as having possible AD. The diagnostic group of possible AD further included 9 patients for whom available clinical data were not sufficient to fulfill criteria for probable AD. Ten patients were diagnosed with probable vascular dementia (5.2%), 9 patients with Lewy body dementia (4.7%), 6 patients with frontotemporal dementia (3.1%) and 5 (2.6%) patients had an unspecified type of dementia.

Figure 1. Frequencies of dementia subtypes



LOAD=Late-onset Alzheimer's disease, EOAD=Early-onset Alzheimer's disease, VaD =vascular dementia, FTD=frontotemporal dementia, LBD=Lewy body dementia, Other=possible Korsakov syndrome, possible amyloid angiopathy, or unspecified dementia.

Table 1. Characteristics of the patients with dementia

			Sex	Age at exam	Age at onset	Duration	Family History†	Family History†	DM *	CVD **
			%female	years	years	years	%positive	%autosomal dominant	%	%
Probable AD	LOAD	*(122)*	78.7	82.2 (5.3)	75.0 (5.3)	7.2 (3.9)	63.6	14.3	19.3	59.7
	EOAD	*(17)*	76.5	70.9 (5.8)	60.1 (5.0)	10.4 (3.9)	76.5	29.4	0	31.3
Possible AD	LOAD	*(18)*	77.8	83.2 (4.8)	77.8 (5.9)	5.4 (2.9)	77.8	38.9	16.7	61.1
	EOAD	*(4)*	75.0	67.0 (6.9)	61.0 (3.6)	6.0 (3.9)	75.0	0	25.0	50.0
LBD *(9)*			33.3	83.8 (8.1)	77.1 (6.1)	6.7 (2.6)	66.7	25.0	12.5	62.5
VaD *(10)*			50.0	79.0 (5.7)	69.6 (6.8)	9.4 (5.2)	20.0	0	55.6	88.9
FTD *(6)*			83.3	76.2 (10.6)	70.3 (10.5)	5.8 (4.8)	50.0	0	0	33.3
Other *(5)*			40.0	66.0 (10.9)	60.2 (13.7)	9.0 (4.6)	25.0	0	0	50.0

Values presented are % or mean (sd)
(†) positive family history: at least 1 affected first degree relative; autosomal dominant: at least 3 affected relatives in 2 generations.
(*) report of diabetes mellitus type II, anto diabetic treatment, or report of increased fasting glucose of HbAIC.
(**) report of / treatment for hypertension, stroke, transient ischemic attack, myocardial infarction, angina pectoris, arrhytmia, hypercholesterolemia, hyperlipidemia.
LOAD=late-onset, EOAD=early-onset AD, LBD=Lewy body dementia, VaD=vascular dementia, FTD=frontotemporal dementia

The general characteristics for all groups are summarized in Table 1. The patients with late-onset AD had a mean onset age of 75 (SD 5.3) years, with a mean duration of 7.2 (SD 3.9) years. At examination most patients had progressed into severe stages of dementia (median clinical dementia rating scale 3). More than half of all probable late-onset AD patients (64%) had at least one first degree relative with dementia, based on reported family history. The family history was compatible with that of an autosomal dominant disease in 14% of probable late-onset AD patients and in 39% of possible late-onset AD patients. The percentage of early-onset AD patients with at least one affected first degree relative was 77% while 29% of the families showed evidence for an autosomal dominant pattern of inheritance. Overall, familial aggregation of Lewy body dementia resembled that of late-onset AD, the percentage of patients with an apparent autosomal dominant form being higher for Lewy body dementia. For vascular and frontotemporal dementia we did not identify patients with a family history suggestive of autosomal dominant disease. Of note is that only 20% of the patients with vascular dementia had a positive family history of dementia.

Diabetes mellitus was present in 19% of the late-onset AD patients and a history of cardiovascular disease was found in 60%. Compared to patients with late-onset AD, vascular co-morbidity was lower in patients with early-onset AD and frontotemporal dementia, but not in those with Lewy body dementia.

Genealogy

In figure 2, a simplified pedigree is shown connecting 134 dementia patients to a common ancestor within 11 generations. Only the shortest connections are shown, while the multiple relationships existing between patients are omitted for ease of interpretation. To take into account these multiple relationships, we calculated pair wise kinship coefficients (K) for pairs of patients in separate diagnosis groups. Overall, patients were more closely related to each other than a group of cognitively healthy spouses from the same sampling area. When considering the sib pairs with late-onset AD (n=4), K was larger than 0.25 in three of the late-onset AD sib pairs (K=0.257; 0.262 and 0.271) suggesting consanguinity.

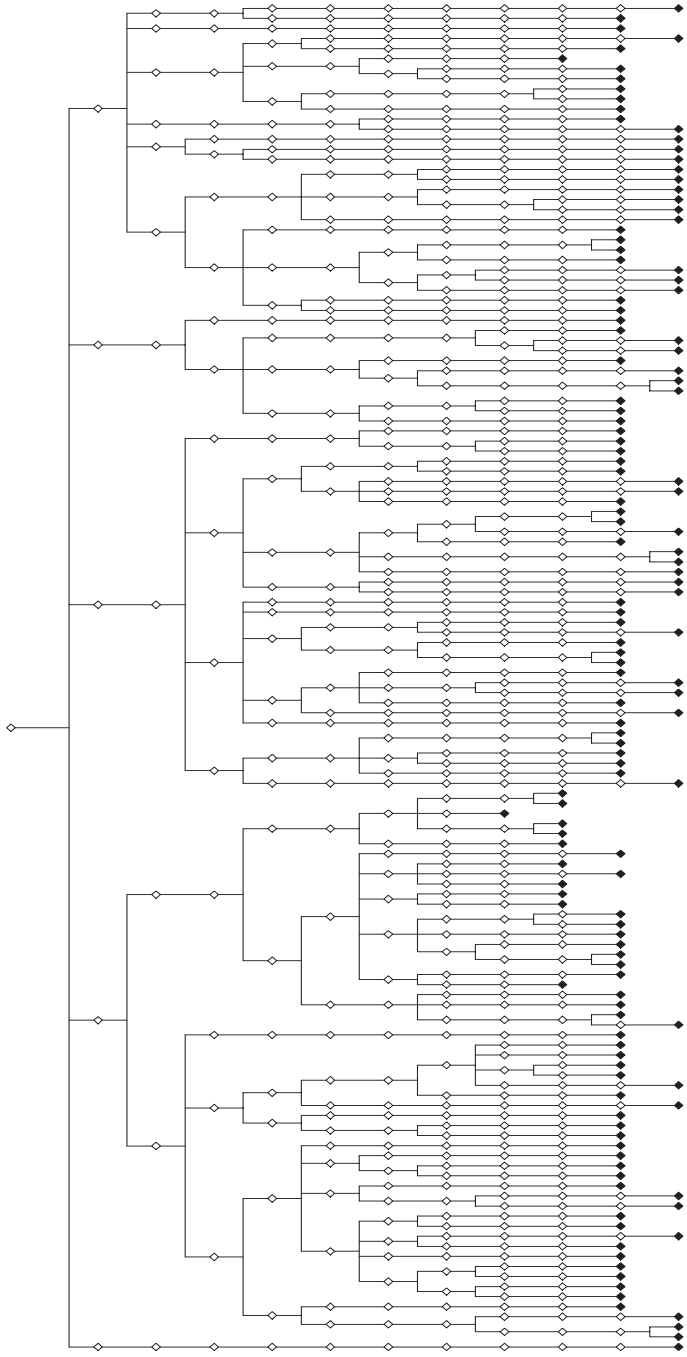


Figure 2. Interrelation of 134 patients with dementia
Phenotypic information is only shown for the last generation.
Only the shortest connection to a common ancestor is shown.

In table 2, proportions of pairs are shown by K. If K is zero, at least one of the pair of patients descends from another population. For early-onset AD patients, the proportion of pairs with K=0 was smallest (15%), differing significantly from that of late-onset AD (26%; p=0.01). Seventy percent of the pairs of patients with frontotemporal dementia had a kinship coefficient equal to zero. However, sample size for this group was small.

Table 2. Proportions of pairs in each kinship coefficient (K) category shown by diagnosis

K	LOAD	EOAD	LBD	FTD	VaD	Spouse*
$1 - ½^{3}$	0.001 (7)	0	0	0	0	0
$½^{3} - ½^{6}$	0.02 (131)	0.009 (1)	0.047 (1)	0	0	0.006 (25)
$½^{6} - ½^{9}$	0.08 (554)	0.14 (15)	0.09 (2)	0	0.03 (1)	0.03 (111)
$½^{9} - ½^{12}$	0.13 (918)	0.34 (36)	0.28 (6)	0.20 (2)	0.17 (6)	0.02 (60)
$½^{12} - ½^{15}$	0.23 (1646)	0.12 (13)	0	0.10 (1)	0.19 (7)	0.01 (55)
$< ½^{15}$	0.28 (2016)	0.23 (24)	0.28 (6)	0	0.39 (14)	0.03 (121)
0	0.26 (1868)	0.15 (16)	0.28 (6)	0.70 (7)	0.22 (8)	0.90 (3544)

Values are proportions of pairs per diagnosis, with absolute numbers between brackets LOAD=late-onset AD, EOAD=early-onset AD, LBD=Lewy body dementia, VaD=vascular dementia, FTD=frontotemporal dementia *Spouses of cases with LOAD

A kinship coefficient of $(½)^{9}$ or larger was found in 15% of the early-onset AD pairs, being very similar to the 14% of patients with Lewy body dementia in this category. The proportion of $K>(½)^{9}$ was slightly lower for late-onset AD pairs (10%), although the difference in distribution of K was only borderline statistically significant between late-onset and early-onset AD pairs (p=0.06). Kinship for Lewy body dementia pairs did not differ significantly from those with late-onset AD (p>0.1). When comparing subgroups of patients with late-onset AD based on presence or absence of cardiovascular disease, the proportion with $K>(½)^{9}$ was significantly higher for late-onset AD patients without cardiovascular disease than for those with cardiovascular disease (14% vs. 8%; p<0.00001). Patients with vascular dementia were even more distantly related; for only 3% K was larger than $(½)^{9}$. This was statistically significantly different from early-onset AD-pairs (p<0.05). The percentage of vascular dementia patients (3%) with $K > (½)^{9}$ was very similar to that seen in spouses of late-onset AD patients (3%).

Only for patients with late-onset AD, K was found to be significantly increased compared to their cognitively healthy matched spouses (p<0.0001).

For none of the other forms of dementia, K differed significantly from the K of their controls. However, given the fact that K of early-onset AD patients, as well as K of patients with Lewy body dementia, was higher than that of late-onset AD patients, this is most likely explained by the small number of subjects.

APP, PSEN-1 and PSEN-2

Since patients were closely related, we selected a sample consisting of 80 possible or probable late-onset AD patients as a representative group to screen for autosomal dominant mutations in *APP*, *PSEN-1* and *PSEN-2*. No mutations were found in *APP* or *PSEN-1*. In three probable late-onset AD patients we identified a single base change that predicted a missense mutation in *PSEN-2* (G34S, R62H and R71W). The variations did not segregate with disease in the families of these three patients. We detected the R71W variation in one of 283 healthy controls (566 chromosomes) as well (RR 3.56, 95%CI 0.22-57.72;p>0.1). We did not observe G34S and R62H in controls, but we did identify a R62C variation in one of the controls. Since *PSEN-2* includes a substantial number of polymorphisms that do not affect functionality [35], we tested if these missense mutations were associated with an increased secretion of $A\beta_{42}$ proportional to $A\beta_{40}$.[36, 37] No evidence was obtained that the amino acid changes altered the $A\beta_{42}/A\beta_{40}$ ratio (Theuns et al, unpublished data).

Chromosome 10 and 12

The chromosomes 10 and 12 STR markers did not show significant association with late-onset AD, except D12S336 (p-value 0.03). Since D12S336 is located close to *A2M*, we genotyped the I/D polymorphism in this gene in the entire sample. The genotype and allele proportions did not deviate significantly from Hardy Weinberg equilibrium. The frequency of the *A2M* genotypes did not differ significantly between patients with either probable or possible late-onset AD, and controls (table 3) making it unlikely that *A2M* explained the association between D12S336 and late-onset AD. Two (22.2%) of the patients with Lewy body dementia were homozygous for the D-allele, one was heterozygous and 6 were homozygous for the I-allele. This distribution differed significantly from controls, although the number of patients was very small compared to the control sample (Fisher’s exact p-value=0.009).

Table 3. Genotype distribution of *A2M* insertion/deletion (I/D) polymorphism in patients and controls

	I/I	I/D	D/D
Controls	73 (60.8%)	45 (37.5%)	2 (1.8%)
Probable LOAD	83 (71.6%)	30 (25.9%)	3 (2.6%)
Possible LOAD	12 (66.7%)	5 (27.8%)	1 (5.6%)

Values are presented as absolute numbers, with percentages between brackets
LOAD=late-onset AD

APOE

Linkage analysis yielded non-significant LOD scores for both flanking markers of *APOE* (LOD score 0.21; p=0.16 (D19S420) and 0.35; p=0.10 (D19S902)). *APOE* genotype frequencies were in Hardy Weinberg Equilibrium proportions. The frequency of carrying at least one allele *APOE ε4* is shown in figure 3. Of the late-onset AD patients 53.9% were heterozygous for APOE ε4 and 10.5% were homozygous for *APOE ε4*, compared to 30.1% heterozygous for *APOE ε4* and 2.6% homozygous in controls. Odds ratios were 3.34 (95%CI 2.14-5.36) for heterozygous and 7.72 (95%CI 2.53-23.56) for homozygous individuals. The population attributable proportion was 45%.

Of the 16 early-onset AD patients for whom *APOE* genotyping was available, 8 were heterozygous and 5 were homozygous for the ε4 allele. None of the nine patients with Lewy body dementia were homozygous, and 5 were heterozygous for *APOE ε4*. Of the 10 patients with vascular dementia, only one was heterozygous for *APOE ε4* and none were homozygous. Four of the 6 patients with frontotemporal dementia were heterozygous, and none were homozygous for *APOE ε4*.

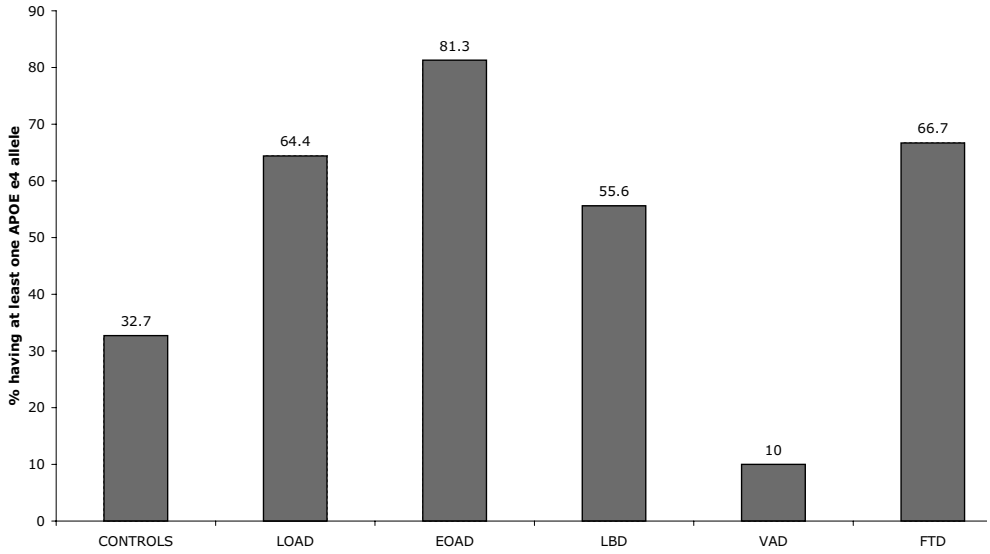


Figure 3. Frequency of *APOE ε4* carriership in dementia-patients and controls LOAD=Late-onset Alzheimer's disease, EOAD=Early-onset Alzheimer's disease, VaD =vascular dementia, FTD=frontotemporal dementia, LBD=Lewy body dementia

MAPT

We screened *MAPT* in 6 patients with frontotemporal dementia. None of the patients carried a known mutation. We found no evidence for haplotype association. In one patient we found a rare polymorphism in the intron following exon 9 (IVS9 +40 C/T). No DNA was available from affected relatives to test segregation of the polymorphism with affection status. This polymorphism was not found in 384 Dutch control chromosomes tested.[30] Three different splice prediction programs (NetGene2, Splice Site Prediction by Neural Networks and SpliceSiteFinder) predicted no change in splicing due to the +40 C/T base change, making it unlikely that this mutation explains the disease.

Discussion

In this paper we described the occurrence of dementia in a recent genetically isolated Dutch population. As expected, late-onset AD was the most common type of dementia (122 out of 191 patients), and its clinical picture resembled that of AD in the general Dutch population, including a high percentage of cardiovascular disease and diabetes mellitus.[38] We observed strong familial aggregation of dementia in this genetically isolated population. Clustering was strongest in patients with early-onset AD and Lewy body dementia. Although a relatively large portion of Lewy body dementia patients descended from different populations, the pairs that were related were as closely related as early-onset AD pairs. Vascular dementia showed weakest evidence for familial clustering. Of interest, in three out of four sib pairs with late-onset AD, kinship was larger than 0.25, indicating consanguinity in this patient group. This finding suggests a recessive mutation in a (small) subset of patients. Until now, no mutations causing autosomal recessive AD are known, but in an inbred Israeli-Arab community evidence for recessive inheritance of disease has also been found.[3]

Overall, we observed that patients were more closely related than a group of cognitively healthy spouses from the same sampling area. One might argue that the observed difference is based on the sex difference between patients and spouses, as patients in our study were predominantly female. However, we did not find significant evidence for a difference between men and women in patients, or between men and women in controls, arguing against a bias because of sex difference.

Having access to a database holding the genealogy of almost 60 000 individuals from the genetically isolated population allowed a thorough investigation of the clustering of dementia in seemingly unrelated patients. A finding of interest was the strong familial clustering in late-onset AD. In 14% of the patients the family history was compatible with autosomal dominant disease. A high percentage of patients with possible AD also had a family history compatible with autosomal dominant disease. Possibly, they shared a genetic risk factor conferring a distinct phenotype.

Epidemiological studies suggested that cardiovascular disease is a risk factor for late-onset AD.[38, 39] Indeed, patients diagnosed with late-onset AD in this present study had a high frequency of cardiovascular disease and diabetes mellitus. This raises the question whether similar genes are involved in the risk of vascular dementia and late-onset AD. Although only few patients had

vascular dementia in our study, we found no evidence for familial clustering of vascular dementia. This suggested that genes play only a minor role in vascular dementia, and that there is a difference in genetic etiology for late-onset AD and vascular dementia. Patients with late-onset AD who did not have a history of cardiovascular disease showed a significantly higher degree of familial aggregation than those with cardiovascular disease. This might indicate a stronger genetic factor in those without cardiovascular disease. But still, patients with late-onset AD and cardiovascular disease have a higher kinship than patients with vascular dementia, indicating separate genetic etiologies.

In the analysis of genetic risk factors already known in dementia, we detected 3 missense mutations in *PSEN-2* in three patients with probable late-onset AD, and an intronic polymorphism in *MAPT* in a patient with frontotemporal dementia. Although the sequence variations in *PSEN-2* did not segregate with affection status in relatives, and although they did not affect the $A\beta_{42}/A\beta_{40}$ ratio in an in vitro assay, and although one of the missense mutations in *PSEN-2* (R71W) was detected in a control, we cannot fully exclude the relevance of these missense mutations for AD. New missense mutations continue to be reported more often in patients than in controls, but their frequency is so low that statistical evidence for association can hardly be shown. To exclude functionality of the *MAPT* polymorphism we relied on splice prediction programs, since no affected relatives were available for further study of the segregation of this polymorphism with the clinical phenotype. Definitive evidence arguing against functionality should come from in vitro studies, but up until now polymorphisms located this far outside the functional loop have not been shown to affect splicing.[40]

Despite increasing evidence for a locus on chromosome 10 in other studies[20-24] we found no association between markers in this chromosomal region and late-onset AD in the isolated population. Inherent to the genetic structure of an isolated population, genetic factors that do have considerable impact on disease in an outbred population might not be present or only with undetectable frequencies in a genetically isolated population. As did others, we did find some evidence for association with AD on chromosome 12,[25-27] close to the location of *A2M*. Evidence was weak however, and the I/D polymorphism in *A2M* could not explain this association, which is consistent with other studies that have been negative for *A2M*.[41] Some studies reported a possible association between Lewy body dementia and the region on chromosome 12 and the *A2M* D-allele.[42, 43] Correspondingly, we did find evidence for excess homozygosity of the *A2M* D-allele in patients with Lewy body dementia. Since the deletion in *A2M* appears not to have biological consequences,[44] this polymorphism might be located close

to a true disease locus elsewhere on chromosome 12. Another candidate gene on chromosome 12 is low-density lipoprotein receptor-related protein (*LRP*),[45,46] but *LRP* is located at a distance of 50 cM from the marker suggestive of linkage on chromosome 12. Further, none of the markers close to *LRP* conferred evidence for linkage. We therefore excluded *LRP*.

The frequency of *APOE ε4* was slightly higher, both in late-onset AD cases and controls from the isolated population, compared to the general Caucasian population. Although the odds ratios associated with *APOE ε4* were similar to those seen in Caucasian populations, the risk of AD in the isolated population attributable to *APOE ε4* is higher (45%) due to the high frequency of the allele.[18]

We found a high degree of relationship between patients in this isolated population, especially in early-onset AD and Lewy body dementia. More importantly, also patients with late-onset AD showed strong evidence for familial aggregation as opposed to vascular dementia. Consanguinity in several patients suggested an underlying recessive mutation. Mutations in known genes causing autosomal dominant disease were unlikely to explain dementia in this population. We did find evidence for an association between Lewy body disease and *A2M*. The frequency of *APOE ε4* was high, resulting in an attributable risk of 45% for AD. This suggested that this allele is important as a determinant and/or a modifier of disease in the isolate. Still, a large portion of AD remains unexplained, asking for further research on the genes involved in this type of dementia.

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Chapter 2.2
Vascular risk genes do not contribute to the genetic variance of cognitive functioning later in life

Abstract

Impaired cognitive function in later life may result from pathology related to Alzheimer's disease, but also from vascular pathology. We studied to what extent vascular risk explains the heritability of cognitive functioning in 500 individuals aged 50 years or over, who are related in one extended pedigree in a genetically isolated population, as featured in the Erasmus Rucphen Family (ERF) study.

Heritability of various measures of cognition was estimated using variance components modeling (SOLAR). Univariate analyses were performed using a model with and without vascular disease, bivariate analyses included both a cognitive and a vascular trait, such as blood pressure, intima media thickness of the carotid arteries, serum glucose or lipids.

Heritability estimates for immediate and delayed recall, recognition, semantic fluency and the Stroop test were significant, with estimates from 0.19 to 0.38. Except for immediate recall and the Stroop test, we observed no significant effects of vascular factors on average cognitive functioning. Heritability estimates did not change significantly when adjusted for vascular disease, and we found no genetic correlation between cognitive functions and vascular traits.

This suggests that in this population vascular disease is not strongly associated with cognitive dysfunction, and in those with vascular disease, vascular risk genes do not contribute to the genetic variation in cognitive function at later age.

Introduction

There is strong evidence that genes influence cognitive functioning in late adult life. Twin studies have reported high heritability estimates (i.e. the proportion of total variance, or inter-individual variation, of a trait that is attributable to genes).[1-4] Heritability of general cognitive function, or intelligence, is estimated to be as high as 0.80.[3] Memory, which can be assessed by immediate and delayed recall of a word list-learning test, has a heritability of approximately 0.40.[2, 3] However, estimates vary with the mean age of the study population and the study design used. Heritability estimates for executive control functions, such as complex attention and concentration, cognitive flexibility and execution of action plans, range from 0.34 to 0.68.[5]

In concert with this strong genetic influence, education and occupational attainment also affect cognitive functioning at later age.[6] Furthermore, cardiovascular health status has been related to cognitive performance, specifically to executive control function.[7] Diabetes mellitus and impaired glucose tolerance, hypertension, atherosclerosis and white matter lesions on brain MRI have all been, albeit sometimes modestly, associated with cognitive impairment.[8-13]

Given the association between vascular risk factors and cognition, genetic influences involved in vascular disease might also account for variation in cognitive function. We studied heritability of cognitive function in a single extended pedigree including 500 individuals aged 50 years or older, and investigated to what extent vascular pathology contributes to the genetic and environmental variance of cognitive function.

Methods

Study population

The present study is embedded in the Erasmus Rucphen Family study (ERF), a large family-based cohort study, which aims to identify susceptibility genes for complex disorders by studying quantitative traits. The cohort study is set in a recent genetically isolated population in the South West of the Netherlands, with a computerized genealogy database available up to approximately the year 1600. Eligibility for participation in the cohort was based on genealogical background. Twenty couples living in this community in the 19th century were selected, provided they had given birth to at least 6 children. By means

of baptizing and municipality records, all their living descendants in three generations were traced and invited to participate. Thus, subjects were not selected with respect to phenotypes of interest. Participation involved a 3-hour visit to the research center and filling out of an extensive home questionnaire. During the visit blood was drawn for DNA and clinical chemistry, and participants underwent an extensive examination, which included an interview by a physician, anthropometric measurements, cardiovascular assessment, an eye examination and cognitive function tests. All participants gave informed consent, and the Medical Ethical Committee of the Erasmus MC approved the scientific protocol of the ERF study.

As our aim was to study the heritability of cognitive functions at older age, we limited the analyses to participants aged 50 years or older. Participants with clinically evident dementia (n=5), and participants with a history of cerebrovascular accidents (n=35) were excluded from the analyses as their condition interfered with performance on cognitive tests. Patients with other neurodegenerative conditions or severe psychiatric disorders did not participate in this part of the study. Phenotypic information was available for a total of 500 individuals who were all related in one extended pedigree.

Assessment and definitions of cognitive phenotypes

Neuropsychological tests were administered by three trained assistants in a standardized test environment. A 50-minute test battery was designed, including 4 cognitive function tests. The tests were selected based on the following criteria: they should be applicable over a wide age range and have different levels of complexity to overcome floor or ceiling effects. Furthermore, a validated version for the Dutch language should to be available, and the tests should not exceed the time available within the setting of the ERF study. The selection of tests included a 15-word test[14] (after Rey's Auditory Verbal Learning Test [15]), a semantic word fluency test,[16] the color word card of the Stroop Color Word test,[17] and part B of the Trail making test.[18]

The three latter subtests each generated a single score for further analysis. The score on the semantic word fluency test was defined as the sum of the number of correctly named items in two categories (animals and professions) in two 1-minute trials. The score on the Stroop Color Word test was defined as seconds to completion of the color word card of this test; the Trail making test was scored by seconds to completion of card B. Individuals with illiteracy were excluded from Trail making and Stroop tests, individuals with color blindness were excluded from the Stroop test.

From the 15-word test we derived 3 scores for further analyses, i.e.

immediate recall, delayed recall and recognition. We defined immediate recall as the total number of correctly recalled words on the 5 learning trials of the 15-word test, delayed recall was defined as the number of correctly recalled words of the 15-word test after 20 minutes, and recognition was defined as the sum of the number of correctly recognized words and correctly rejected words of the 15-word test.

To adjust for intelligence and education, we estimated intelligence with the Dutch Adult Reading Test [19] and asked the participants for their highest level of education and occupational attainment (according to the Dutch standard for classification of occupation, Office of Population Statistics Netherlands 1992).

Assessment and definitions of vascular risk

Hypertension was defined as mean systolic ≥ 160 mmHg or diastolic tension ≥ 100 mmHg or use of anti-hypertensive medication. Blood pressure was measured twice with an automated device (OMRON 711, automatic IS; Vernon Hills Illinois, USA) at the right upper arm in sitting position. Intima media thickness (IMT) was measured by ultrasonography with a 7.5-MHz linear array transducer (ATL Ultra-Mark IV; Advanced Technological Laboratories, Bethell, Washington, USA) of the left and right common carotid artery. The maximum carotid IMT was determined as the mean of the maximum IMT of near and far wall of both common carotid arteries. Diabetes mellitus was defined based on use of insulin or oral glucose lowering medication or fasting serum glucose levels ≥ 7 mmol/l. Dyslipidemia was defined based on use of statins, or fasting lipid spectrum abnormalities in serum. Serum triglyceride levels were considered abnormal when higher than 4.0 mmol/l. Serum fasting cholesterol was considered abnormal when ≥ 9.0 mmol/l, or when ≥ 6.5 mmol/l if cholesterol/HDL ratio ≥ 5.0, or when cholesterol/HDL ratio ≥ 8.0. Blood chemistry was performed on a spectrophotometric Chemistry analyser (Synchron LX20; Beckman, Fullerton, California, USA).

The role of vascular disease on cognition was studied both with quantitative and discrete traits. Quantitative traits were as mentioned above, discrete vascular traits were defined based on presence or absence of hypertension, diabetes mellitus or dyslipidemia, or on IMT, dichotomized at the 75th percentile. A composite score expressing vascular risk profile was categorized as follows: none of the above vascular risk factors, one of the above, or two or more.

Statistical analysis

Inbreeding coefficients were calculated using PEDIG software (Boichard D., http://dga.jouy.inra.fr/sgqa/diffusions.htm) based on a database of the total population, holding genealogical data of 56693 subjects. General descriptive statistics were performed using χ^2 statistics and univariate analysis of variance in SPSS for Windows, version 11.0. General linear models were used to test phenotypic association between various cognitive traits and discrete vascular traits in SPSS for Windows, version 11.0. For the heritability analyses, covariates were included based on a significant effect on the trait under study in a stepwise procedure, or based on biological plausibility. All covariates significant at the 0.10 level were retained to maintain covariates with possible important effects in the final model. When unstandardized residuals were not normally distributed (according to the one sample Kolmogorov-Smirnov test), traits were transformed by taking the natural logarithm (Trail making, Stroop test) or biquadratic transformation (recognition). The final models included the following covariates: age, sex, level of inbreeding, estimated intelligence, highest education, occupational attainment, history of depression or use of anti depressants, and alcohol use.

We used a variance component approach to estimate heritability of each phenotype, using maximum likelihood methods for univariate models (SOLAR 2.1.2. software package).[20] Univariate analyses were performed on model I, including all final covariates, and model II, including all final covariates and the parameter summarizing vascular risk profile. We used the likelihood ratio test to compare the likelihood of model II with that of model I (χ^2 statistic at 1 degree of freedom).

Bivariate models were maximized to obtain genetic (ρ_G) and environmental (ρ_E) correlations between a cognitive trait and a quantitative vascular trait. Bivariate analyses were additionally adjusted for medication use if relevant.

Results

Phenotypic information was available for a sample of 500 individuals who were all connected in one extended pedigree. The pedigree included 21 parent offspring pairs, 235 sibling pairs, 8 half-sibling pairs, 206 avuncular pairs, 1038 first cousin pairs, and 20 half-cousin pairs. A considerable proportion of participants was inbred, i.e. their parents were consanguineous (table 1). The mean inbreeding coefficient indicates that subjects' parents were, on average, second cousins once removed.

Table 1. General characteristics of the study population

	Men (n=211)	**Women (n=289)**
Age, years (SD)	62.5 (7.6)	61.2 (7.7)
Inbreeding		
Inbreeding (%)	66.5	72.6
Mean inbreeding coefficient	0.0082	0.0098
Level of education**		
Elementary school (%)	51.4	47.7
Vocational technical training (%)	28.6	34.1
Higher education (%)	20	18.2
Elementary or low occupation (%)	80.9	93.6
Depression (%)**	9.1	19.6
Alcohol use (%)**	66.5	34.1
Consumption in drinkers, u/w (SD) **	10.5 (11.7)	6.0 (6.3)
Benzodiazepine use (%)**	11.8	23.9
Anti-depressant use (%)	6.6	10.7
Hypertension (%)	71.1	63.7
Diabetes Mellitus (%)*	17.5	10.7
Hyperlipidemia (%)	41.7	39.1
Intima Media Thickness**	1.02 (0.1)	0.93 (0.1)

SD is standard deviation, u/w = units (glasses) of alcohol per week
*)0.05>p>0.01, **) 0.01>p>0.001

In this population, women reported depression or use of benzodiazepines significantly more often than men, whereas men tended to have higher alcohol consumption and a more adverse vascular risk profile (table 1). Hypertension, dyslipidemia and diabetes mellitus were all more prevalent in men, though only for diabetes mellitus the prevalence was significantly higher. Of note, half of the men and women only finished 6 to 8 years of elementary education, and almost 80% achieved only an elementary or low occupation. The low level of education and occupation was usual for this region in the first half of the previous century.

In table 2, performance on the different cognitive function tests is shown for men and women separately, adjusted for age and education. The 15-word test, addressing immediate and delayed recall and recognition, was done significantly better by women; men did better at the semantic fluency test. The difference between men and women in scores on the Stroop test and Trail making were not significant.

Table 2. Mean test performance in men and women

Test	Men (n=211)	Women (n=289)
Immediate recall *	29.1 (0.5)	32.3 (0.5)
Delayed recall *	5.5 (0.2)	6.4 (0.2)
Recognition *	26.1 (0.2)	26.9 (0.2)
Semantic fluency *	33.0 (0.6)	30.4 (0.5)
Stroop Color Word	127.1 (2.6)	124.7 (2.1)
Trail making B	138.9 (4.4)	145.4 (3.7)

Values are estimated marginal means (standard error), given at mean of covariates age and education. Immediate recall is the sum of the number of correct words in 5 trials; delayed recall is the number of words correctly recalled after 20 minutes; recognition is the number of words correctly recognized or rejected; semantic fluency is the number of correctly named items in two 1-minute trials; color word interference is seconds to completion of card III; Trail making B is seconds to completion.
* $p < 0.01$

Table 3. Relation between vascular disease and cognitive function
Values are estimated marginal means given with standard error at mean of covariates age, sex and education.
*) $p \leq 0.05$, as compared to reference of no disease

Table 3. Relation between vascular disease and cognitive function

	Immediate Recall	Delayed Recall	Recognition	Semantic Fluency	Stroop Color Word	Trail making B
Hypertension						
Absent (165)	31.7 (0.6)	6.1 (0.2)	26.7 (0.2)	32.1 (0.6)	122.8 (2.9)	148.2 (4.9)
Present (326)	30.5 (0.4)	6.0 (0.1)	26.5 (0.1)	31.2 (0.4)	127.2 (2.0)	139.9 (3.5)
Diabetes Mellitus						
Absent (428)	31.0 (0.4)	6.0 (0.1)	26.6 (0.1)	31.5 (0.4)	125.2 (1.8)	142.7 (3.0)
Present (63)	30.3 (0.9)	5.9 (0.3)	26.4 (0.3)	32.0 (1.0)	128.9 (4.6)	143.3 (7.9)
Dyslipidemia						
Absent (293)	31.0 (0.4)	6.0 (0.1)	26.5 (0.2)	31.3 (0.5)	124.6 (2.1)	142.5 (3.6)
Present (198)	30.7 (0.5)	6.0 (0.2)	26.7 (0.2)	31.8 (0.6)	127.3 (2.6)	143.1 (4.4)
Intima Media Thickness						
< 75th percentile (351)	31.0 (0.4)	6.0 (0.1)	26.6 (0.1)	31.4 (0.4)	122.7 (2.0)	143.7 (3.3)
>75th percentile (120)	30.6 (0.8)	5.9 (0.2)	26.3 (0.3)	30.5 (0.8)	133.7 (3.7)*	140.6 (6.2)
Vascular composite score						
0 (89)	32.8 (0.8)	6.2 (0.3)	26.9 (0.3)	31.6 (0.9)	120.1 (3.9)	144.6 (6.4)
1 (166)	30.3 (0.6)*	5.9 (0.2)	26.4 (0.2)	31.3 (0.6)	123.1 (2.8)	141.1 (4.7)
2 or more (186)	30.6 (0.6)*	5.9 (0.2)	26.7 (0.2)	31.0 (0.6)	129.7 (2.7)*	143.9 (4.6)

The relationship between vascular disease and performance on the various cognitive tests is presented in table 3. Despite slightly lower mean performance on most tests for participants with hypertension, diabetes mellitus, dyslipidemia, high IMT or a combination of these conditions, the association between vascular pathology and cognitive test performance was only significant when combining the effect of all vascular factors into the composite score, with the exception of high IMT which was associated with worse performance on the Stroop Color Word test. The composite score was significantly related to the Stroop Color Word test as well as to immediate recall.

To assess whether vascular risk genes might contribute to the genetic variation in cognitive functioning, we studied heritability of cognitive functions both in a univariate model with and without vascular pathology (Model II and I, respectively; Table 4). Heritability of immediate recall was higher than of delayed recall (h^2 = 0.38 and 0.19, respectively), but for both traits the contribution of genes was significant. The heritability for recognition was 0.30 ($p<0.01$). Only the heritability of immediate recall showed a slight decrease in model II compared to model I, suggesting that genes involved in vascular disease might explain part of the genetic variance of this trait. However, the difference was minor, and the two models did not differ significantly.

Genetic factors further explained a significant proportion of the variance of semantic fluency (h^2 = 0.27) and performance on the Stroop Color Word test (h^2 = 0.36), whereas the genetic variance of the Trail making test was non-significant. The genetic variance of the Stroop test decreased slightly when adding the vascular risk parameter to the model, but the difference between Model II and Model I was not significant. None of the other heritability estimates changed in Model II.

The proportion of variance explained by the covariates entered in Model I ranged from 0.15 to 0.40, with highest proportions for semantic fluency, the Stroop test and Trail making B (Table 4). Covariates contributing most to the total variance were age, intelligence and education. Inbreeding had a negligible effect.

For those traits for which a significant heritability estimate was obtained, we followed up with bivariate genetic analysis of the correlation of vascular factors and cognition measurements. Maximization of bivariate models, including both a cognitive and a vascular trait, showed that there was no significant genetic correlation between cognitive traits and any of the vascular traits studied (Table 5). Moreover, environmental correlations were not significant either.

Table 4. Heritability of cognitive function

Trait	h^2		Model II vs. I		Proportion of variance due to all covariates
	Model I	*Model II*	χ^2	p-value $_{(1\ df)}$	
Immediate recall	0.38 (0.13)**	0.37 (0.14)**	0.68	ns	0.26
Delayed recall	0.19 (0.12)*	0.19 (0.12)*	0.13	ns	0.15
Recognition	0.30 (0.12)*	0.30 (0.12)*	0.40	ns	0.18
Semantic fluency	0.27 (0.15)*	0.27 (0.15)*	0.37	ns	0.36
Stroop Color Word	0.36 (0.13)**	0.34 (0.13)**	1.02	ns	0.35
Trail making B	0.15 (0.19)	0.15 (0.19)	0.82	ns	0.40

h^2= heritability; heritability estimates are presented as proportions with standard error
Model I includes age, sex, inbreeding, level of education, intelligence estimate, alcohol use and depression
Model II consists of all covariates in Model I, plus a vascular risk composite score (no vascular disease, one vascular condition, or two or more vascular conditions)
χ^2 = chi squared test statistic when comparing Model II to Model I
* $0.05 > p \geq 0.001$, ** $p < 0.001$, ns = non-significant

Table 5. Genetic and environmental correlation between cognitive and vascular traits

	Mean*	**Immediate Recall**		**Delayed Recall**		**Recognition**		**Semantic Fluency**		**Stroop Color Word**	
		ρ_G	ρ_E	ρ_G	ρ_E	ρ_G	ρ_E	ρ_G	ρ_E	ρ_G	ρ_E
SBP (mm Hg)	150.6 (1.0)	-0.41 (0.30)	0.14 (0.17)	-0.05 (0.32)	-0.01 (0.15)	0.04 (0.25)	-0.03 (0.15)	0.01 (0.36)	0.04 (0.16)	0.26 (0.26)	-0.08 (0.16)
DBP (mm Hg)	82.1 (0.5)	-0.29 (0.45)	0.03 (0.16)	-0.16 (0.40)	-0.01 (0.15)	-0.03 (0.27)	-0.08 (0.14)	-0.01 (0.45)	0.05 (0.15)	0.87 (0.49)	-0.23 (0.14)
Glucose (mmol/l)	5.0 (0.05)	0.25 (0.31)	-0.20 (0.16)	0.40 (0.31)	-0.27 (0.16)	0.17 (0.27)	-0.12 (0.15)	0.46 (0.37)	-0.27 (0.17)	0.01 (0.31)	0.05 (0.16)
IMT (mm)	0.99 (0.01)	-0.46 (0.27)	0.29 (0.22)	-0.47 (0.26)	-0.01 (0.13)	-0.31 (0.18)	0.37 (0.25)	-0.24 (0.27)	0.07 (0.26)	0.39 (0.27)	-0.10 (0.20)
Cholesterol (mmol/l)	5.8 (0.05)	-0.10 (0.27)	0.12 (0.18)	-0.22 (0.30)	0.11 (0.15)	-0.30 (0.26)	0.17 (0.16)	0.35 (0.37)	-0.14 (0.17)	0.21 (0.25)	-0.16 (0.17)
HDL (mmol/l)	1.3 (0.02)	0.22 (0.19)	-0.24 (0.28)	-0.26 (0.28)	0.13 (0.19)	-0.22 (0.23)	0.21 (0.20	-0.08 (0.23)	0.16 (0.23)	-0.05 (0.20)	-0.05 (0.22)
Chl/HDL ratio	4.8 (0.06)	-0.25 (0.26)	0.19 (0.17)	0.24 (0.34)	-0.01 (0.13)	0.15 (0.24)	-0.09 (0.15)	0.66 (0.35)	-0.29 (0.15)	0.10 (0.25)	-0.03 (0.16)

All values are presented with their standard error
ρ_G = genetic correlation, ρ_E = environmental correlation, SBP = systolic blood pressure, DBP = diastolic blood pressure, IMT = Intima Media Thickness, HDL = high-density lipoprotein, Chl/HDL ratio= cholesterol/HDL ratio
* adjusted for age and sex

Discussion

In this large family based cohort study we estimated heritability of cognitive functions, and the role of vascular disease. Heritability estimates for immediate and delayed recall, recognition, semantic fluency and the Stroop Color Word test were significant, with estimates ranging from 0.19 to 0.38. No significant heritability was observed for performance on the Trail making test. Except for immediate recall (15-word test) and the Stroop test for color word interference, we did not observe significant effects of vascular risk factors, such as hypertension, diabetes mellitus and dyslipidemia, on average cognitive functioning. Heritability estimates did not change significantly when considering vascular disease, and we found no genetic correlation between cognitive functions and vascular traits. This suggests that in the ERF study population vascular disease is not strongly associated with cognitive dysfunction, and in those with vascular disease, vascular risk genes do not contribute to the genetic variation in cognitive function at later adult life.

Due to the difference in study design between the various twin studies on cognitive function and this family based cohort design, comparisons of heritability estimates should be made with caution. However, heritability estimates observed for immediate recall and Stroop color word interference were close to what has been reported before.[2, 3, 5] We found no significant heritability for performance on the Trail making test, as opposed to others.[5] Although the Trail making test covers a different aspect of executive functioning than the Stroop test or verbal fluency, the absence of significant heritability might have a different explanation. Perhaps the socio culturally determined low level of education in this population impedes correct completion of the Trail making test. Despite the low self-reported frequency of illiteracy, 10% of the participants were unable to complete part B of the test in a timely manner due to poor knowledge of the alphabet, which is reflected in large standard errors around the mean score at this test.

In our heritability analyses we did not model shared environment. Shared environment is of lesser importance for cognitive function later in life, as is shown in studies of twins reared apart or together.[2, 3] Further, because we studied correlations in all possible pairs of individuals in this large family based cohort instead of in nuclear families, the confounding effects of shared environment on heritability estimates are reduced. In a genetically isolated population with consanguinity in over 2/3 of the study population, dominance variance, which is the genetic variance due to dominance of one allele over

the other at a single locus, needs careful consideration.[21] However, the lack of effect of inbreeding on the cognitive tests argues against a strong effect of dominance-variance components.

Despite a high prevalence of vascular disease in this population, phenotypic association between vascular disease and cognitive function could only be shown for immediate recall and color word interference. Vascular damage is believed to lead to impairment of frontally mediated executive control functions due to interruption of frontal subcortical neuronal circuits.[7] Of the measures of executive function we studied, only color word interference was significantly associated with vascular risk. Differences between verbal fluency and other measures of executive control have been reported before.[5] Verbal fluency is influenced by executive functions, but also involves short-term memory and verbal abilities. Similarly, the Trail making test part B not only reflects executive control, but also psychomotor speed. We excluded 35 participants with a cerebrovascular accident from this study, to avoid incomplete and unreliable test results. Any consequent bias is likely to be small, given the high remaining prevalence of vascular risk in this study population.

In addition to the modest phenotypic association, we did not find any noticeable effect of vascular disease on the heritability estimates of cognition, and there was no significant evidence for genetic correlation between vascular quantitative traits and the various cognitive functions. So, not only average performance on various cognitive functions, but also inter-individual variation in cognitive functioning at later adult life was independent of vascular disease. This suggests that, at least in this population, cognitive dysfunction is not under influence of vascular susceptibility genes.

Our findings are in agreement with a study investigating heritability of executive function and white matter hyperintensities on brain MRI. White matter hyperintensities are considered to be a sign of vascular damage to the brain. Only 8% of the total genetic variance of executive function was explained by white matter hyperintensities.[22]

We observed significant heritability of measures of memory and executive function in people aged 50 years or over, related in a single extended pedigree. Our findings dispute a primary causal role for vascular risk genes in cognitive dysfunction. In this population, the genetically determined inter-individual variation in cognitive functioning is most likely attributable to genes more directly involved in Alzheimer-related pathology.

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Chapter 3
Genetic Studies

Chapter 3.1

Angiotensin Converting Enzyme gene is associated with atrophy of medial lobe structures on brain MRI and Alzheimer disease

Abstract

Despite biological support for a role of Angiotensin converting enzyme (ACE) in Alzheimer's disease (AD), studies assessing the ACE I/D polymorphism in AD are conflicting. We re-evaluated this association in the Rotterdam Study, a population-based cohort study. The mechanism of association was further explored by adjusting for vascular factors, and by analysing atrophy, white matter lesions and infarcts on MRI in non-demented individuals. Genotypes were available for 6488 participants. During average follow-up of 6 years 250 subjects developed AD. MRI data were available for 494 non-demented participants.

Homozygosity for the I-allele conferred a slightly increased risk of AD compared to carrying a D-allele (RR 1.12 (95%CI 0.99-1.25)). This increase was only significant in women, and independent of vascular factors (RR 1.39 (95%CI 1.14-1.69)). Non-demented women with the II genotype had smaller hippocampal and amygdalar volumes. Vascular pathology was not significantly associated with ACE.

This suggests a modest but significant increase in risk of AD and early AD pathology in women homozygous for the ACE I-allele independent of vascular factors.

Introduction

Angiotensin converting enzyme gene (*ACE*) is studied as a candidate gene for Alzheimer's disease (AD) because ACE plays an important role in different pathways that may lead to AD, such as the cardiovascular and the amyloid β pathway. [26] ACE is part of the renin-angiotensin system that modulates vascular homeostasis. [36] Subjects with a deletion of a 287 base pair repeat at intron 16 of ACE (D-allele) have higher plasma ACE levels than those with an insertion of this repeat (I-allele). [38] The D-allele has been associated with cardiovascular disease and mortality. [18 29] Given growing evidence for common determinants of AD and cardiovascular disease this increased risk may have implications for AD as well. [6 20]

Apart from its vascular properties, the *ACE* gene may be involved more directly in AD given the altered presence of ACE and other renin-angiotensin system components in brains with AD pathology. [3 25 41] The local brain renin-angiotensin system was found to exert a direct function on memory performance. Angiotensin II and its metabolite angiotensin IV can excite neurons in the hippocampus and amygdala,[43] which are structures important for memory and cognition. Furthermore, ACE may lower amyloid β levels by promoting its degradation [21 26] reinforcing the hypothesis of a role of ACE in the pathogenesis of AD.

Notwithstanding the evidence for a role of ACE in AD pathogenesis, studies assessing the relation between the I/D polymorphism and risk of AD have yielded conflicting results.[1 24 33 34 44 37 45 8 32] The inconsistencies might be due to the relatively small risk attributable to the *ACE* gene, but also to differences in methodology. Populations under study were small, and little attention was paid to the role of vascular risk factors, opening the opportunity for spurious findings. A recent meta-analysis [13] found carriers of the I-allele to be at increased risk of AD, but concluded that the pathophysiologic implications of this association still are not fully understood, and that large studies are needed to replicate this finding.

Our aim was to re-evaluate the association between the I/D polymorphism and AD in a large population based cohort study. To explore the underlying pathophysiological mechanisms, we studied the effects of cardiovascular risk factors on this association. In addition we studied the association between the I/D polymorphism and structural brain abnormalities on MRI in individuals who were clinically free from dementia. These brain abnormalities included white matter hyperintensities, infarcts and hippocampal and amygdalar atrophy.

White matter hyperintensities and infarcts are presumed to reflect vascular damage to the brain [35] whereas hippocampal and amygdalar atrophy are putative in vivo markers of Alzheimer neuropathology. [16]

Methods

Study population

The study was performed within the Rotterdam Study, a population-based cohort study in the Netherlands that investigates the prevalence, incidence and determinants of chronic diseases in the elderly.[19] The medical ethics committee of Erasmus Medical Centre Rotterdam, the Netherlands approved the study protocol. Baseline examinations, including a detailed interview, physical examination, and blood sampling, were conducted between 1990 and 1993. Of the total cohort of 7983 participants, 6869 subjects were genotyped for the *ACE* I/D polymorphism.

Participants with prevalent dementia (n=381) at baseline examination were excluded. The presence of dementia was assessed by means of a stringent protocol. First, participants underwent a combined mini mental state examination (MMSE) [14] and geriatric mental state (GMS) [9] schedule. If participants were screen positive (MMSE<26 or GMS>=1) they were subsequently examined with the Cambridge Mental Disorders of the Elderly Examination (CAMDEX).[40] When CAMDEX score was lower than 80, participants were examined by a neurologist and neuropsychologist and, if possible, underwent neuroimaging. Dementia was diagnosed according to DSM-IIIR criteria and AD and vascular dementia were diagnosed according to international criteria.[31 39]

The cohort of 6488 participants, with *ACE* genotype and without dementia at baseline, was followed for incident dementia. To screen for dementia, during follow-up examinations in 1993-1994 and 1997-1999 a diagnostic procedure equal to the baseline scheme was used. In addition, information from general practitioners and the Regional Institute for Outpatient Mental Health Care was continuously reviewed until December 31, 1999. From 1995 to 1996, as part of the Rotterdam Scan Study, we obtained brain MRI scans in 494 non-demented participants.[5]

MRI measurements

Standard T1, T2 and proton-density weighted MR sequences of the brain were made in a 1.5 Tesla MR unit (VISION MR, Siemens, Erlangen, Germany). We added a custom-made double contrast 3D MRI sequence (half-Fourier acquisition single-shot turbo spin echo) [17] to the protocol for volumetric assessments of hippocampus and amygdala. Based on the 3D MRI sequence, we reconstructed a series of coronal brain slices (contiguous 1.5-mm slices) aligned to be perpendicular to the long axis of the hippocampus. The procedure of segmenting hippocampus and amygdala has been described in detail.[17] Briefly, we manually traced the boundaries of hippocampi and amygdalae on each slice by means of a mouse-driven pointer to obtain surface areas (mm^2). The summed surface areas were multiplied with slice thickness to yield estimates of left and right hippocampal and amygdalar volume (ml). Total hippocampal and amygdalar volumes were calculated by summing left and right volumes. As a proxy for head size, we measured the intracranial cross-sectional area on a reformatted middle sagittal MRI slice.[17] To correct for head size difference across individuals [7 30] we first divided the uncorrected volumes by the subject's calculated head size area, then we multiplied this ratio by average head size area (men and women separately) to yield normalized volumes. White matter lesions were considered present if visible as hyperintense on proton density and T2 weighted images, without prominent hypointensity on T1 weighted scans. We separately scored periventricular and subcortical white matter lesions, using a method described in detail elsewhere.[12] Briefly, we summed three region specific grades (lesions adjacent to the frontal horns, lateral walls, and occipital horns of the lateral ventricle) to get a total periventricular white matter lesions grade (range 0-9). We counted subcortical white matter lesions in three size categories based on their maximal diameter (< 3 mm, 3-10 mm, >10 mm). A total volume was approximated by assuming that these subcortical lesions were spherical with a fixed diameter (volume range 0-29.5 ml). We defined infarcts as focal hyperintensities on T2 weighted images, 3 mm in size or larger. Lesions in the white matter also had to have corresponding prominent hypointensities on T1 weighted images, in order to distinguish them from cerebral white matter lesions. We defined lacunar infarcts as focal hyperintensities on T2-weighted images 3 to 20 mm in size and located in the subcortical white matter or basal ganglia. A single trained physician, blinded to history of stroke and transient ischemic attack, rated infarcts.

Vascular factors

As vascular factors may be effect modifiers in the association between the *ACE I/D* polymorphism and AD, we controlled our analyses on AD for blood pressure, intima media thickness of, and plaques in the carotid arteries. Blood pressure was measured at baseline in sitting position at the right upper arm using a random zero sphygmomanometer. The average of two sequential measurements was used. Hypertension was defined as a diastolic blood pressure of 100 mm Hg or higher and/or a systolic blood pressure of 160 mm Hg or higher and/or use of antihypertensive medication. Intima media thickness of the right and left carotid artery and atherosclerotic plaques in the carotid arteries were assessed by means of ultrasound, using a procedure described previously.[4] Three or more carotid plaques were considered abnormal.[20]

DNA study

II, ID and DD genotypes were detected using a PCR technique according to Lindpaintner et al. with some modifications.[28] The amplification yielded a 319-bp amplicon for the D-allele and a 597-bp amplicon for the I-allele. Preferential amplification of the D-allele in heterozygous subjects causes misclassification of ID genotypes into DD genotypes in 4-5%. To avoid this we performed a second independent PCR with a primer pair that recognizes an insertion-specific sequence.[28] To optimize the second PCR, 10% DMSO, 0.35 units AmpliTaq Gold DNA polymerase and GeneAmp PCR Gold buffer (Applied Biosystems) was added to the PCR mix. This reaction yielded a 335-bp amplicon only if the I-allele was present. In post-PCR analyses, 10 µl of PCR product was loaded on 3% Agarose gel. Fragments were visualized using Ethidium Bromide staining and UV trans-illumination. Two independent investigators read pictures from each gel. All ambiguous samples were analyzed a second time.

Data analysis

Hardy-Weinberg Equilibrium of the *ACE* I/D polymorphism was tested in controls and cases. Univariate analysis of variance and χ^2-statistics were performed to compare baseline characteristics of the total study population stratified by genotype, using SPSS for windows software package version 10.0. To assess the influence of the I/D polymorphism on AD, we estimated hazard ratios using Cox proportional hazard models. For the time-to-event or Alzheimer-free survival analysis we specified age as the underlying time variable instead of follow-up time, taking into account delayed entry (left truncation) by using the counting process notation of S-Plus V.6.0.[2] Participants with incident dementia other than AD were censored at the time of diagnosis.

The analyses were performed both crude and adjusted for sex and possible cardiovascular effect modifiers (systolic and diastolic blood pressure, intima media thickness, carotid atherosclerosis (3 or more plaques)). We used multivariate linear regression to compare mean hippocampal and amygdalar volumes and white matter lesion severity in non-demented individuals across genotypes, adjusted for age and sex. We assessed the relation between *ACE* genotypes and infarcts using multiple logistic regression, adjusted for age and sex. As a report on the relation between *ACE* genotype and dementia suggested sex-specific effects,[10] we repeated all analyses in men and women separately.

Results

In table 1, baseline characteristics for subjects of the Rotterdam Study and the MRI subset are shown by genotype. Genotype frequencies in controls (p=0.4) and cases (p=0.1) were in Hardy-Weinberg Equilibrium proportions and did not change significantly in different age strata. Age and sex distribution were similar across genotypes. Hypertension was more frequent (p=0.07) and intima media thickness increased (p=0.06) in carriers of the D-allele in the total sample.

During 37890 person-years of follow-up (mean follow-up 5.8 years), 250 participants (71.2% women) developed AD. The mean age at diagnosis was 82.0 (SD 7.3) years. In only 3.6% of the incident AD cases an MRI had been obtained for the Rotterdam Scan Study.

In table 2 hazard ratios for AD are presented by genotype in the total sample and for men and women separately. Results are given both crude and adjusted for sex (in the total sample), systolic and diastolic blood pressure, intima media thickness and plaques in the carotid arteries. Carriers of the II genotype had a modestly increased hazard for AD compared to subjects with a D-allele. When we studied men and women separately, we found that the relative risk for women with the II genotype was increased to 1.24 (95%CI 1.08-1.41; p=0.002). Since hazard rate curves were similar for homozygous and heterozygous D-allele carriers these data were pooled. The relative risk for women homozygous for the I-allele increased to 1.39 (95%CI 1.14-1.69; p=0.001). In men, no evidence for an association between *ACE* and AD was seen.

After baseline, 50 participants developed vascular dementia. We found no evidence for association of *ACE* and risk for vascular dementia (data not shown).

Given the sex-specific association between *ACE* and AD, we analysed the data of the MRI sample accordingly. Figure 1 and 2 show mean volumes of hippocampus and amygdala by genotype in non-demented subjects, stratified by sex. Women with the II genotype had significantly smaller hippocampal and amygdalar volumes than women with the DD genotype (difference in hippocampal volumes 0.35 ml (95%CI 0.07-0.64), in amygdalar volume 0.28 ml (95%CI 0.01-0.48)). In men we found no evidence for a similar association between II genotype and hippocampal or amygdalar volumes. Instead an opposite effect was present, significant only for hippocampal volumes (p-trend=0.04).

Finally we studied the association between *ACE* and vascular pathology on MRI, as shown in table 3. Of all infarcts (n=139) 83% were lacunar. We found no significant association between *ACE* and infarcts, subcortical or periventricular white matter hyperintensities overall or in sex strata.

Table 1. Baseline characteristics by *ACE* genotype in the total cohort and in the subset with MRI measurements

	Total cohort (n=6488)			MRI subset (n=494)		
	DD	*ID*	*II*	*DD*	*ID*	*II*
Frequency, % (n)	27.8 (1806)	50.3 (3264)	21.9 (1418)	28.3 (140)	49.6 (245)	22.1 (109)
Mean age, years	68.9 ± 8.8	69.1 ± 8.6	68.4 ± 8.7	69.5 ± 8.2	68.4 ± 7.9	68.4 ± 7.7
Sex, % men	40.2	40.8	41.5	57.1	48.9	50.5
Hypertension, %	34.4	35.1	31.2	41.2	36.7	38.7
Systolic blood pressure, mm Hg	140.1 ± 22.0	139.2 ± 22.6	138.1 ± 21.7	137.3 ± 20.1	135.1 ± 20.1	138.9 ± 19.8
Diastolic blood pressure, mm Hg	74.0 ± 11.0	73.6 ± 11.6	73.6 ± 11.6	73.4 ± 10.4	73.2 ± 10.9	72.0 ± 10.8
Carotid plaques ≥ 3, %	22.7	22.7	20.3	16.5	17.1	22.7
Carotid IMT[A], mm	0.80 ± 0.16	0.80 ± 0.16	0.79 ± 0.15	0.77 ± 0.14	0.76 ± 0.13	0.76 ± 0.14

Values are percentages or unadjusted means ± standard deviation. [A]IMT=intima media thickness

Table 2. Relative risk of AD by *ACE* genotype, overall and stratified by sex

	ACE genotype		
	DD	*ID*	*II*
Model I[A]			
Overall	1.00 (ref)	0.96 (0.83-1.11)	1.08 (0.99-1.20)
Men	1.00 (ref)	1.10 (0.83-1.46)	1.03 (0.85-1.25)
Women	1.00 (ref)	0.89 (0.75-1.07)	1.12 (1.00-1.25)*
Model II[B]			
Overall	1.00 (ref)	1.00 (0.84-1.20)	1.12 (0.99-1.25)
Men	1.00 (ref)	1.16 (0.86-1.57)	0.87 (0.68-1.12)
Women	1.00 (ref)	0.92 (0.74-1.14)	1.24 (1.08-1.41)**

Values are hazard ratios with 95% confidence intervals

[A] Model I: crude, and adjusted for sex in the total sample

[B] Model II: additionally adjusted for systolic and diastolic blood pressure (continuous), intima media thickness (continuous) and carotid atherosclerosis (dichotomized at 3 plaques)

* p=0.05; II compared to DD. ** p=0.002; II compared to DD

Figure 1.
Mean volumes of the hippocampus by genotype overall and stratified by sex

Volumes (standard error), adjusted for age and when applicable sex.

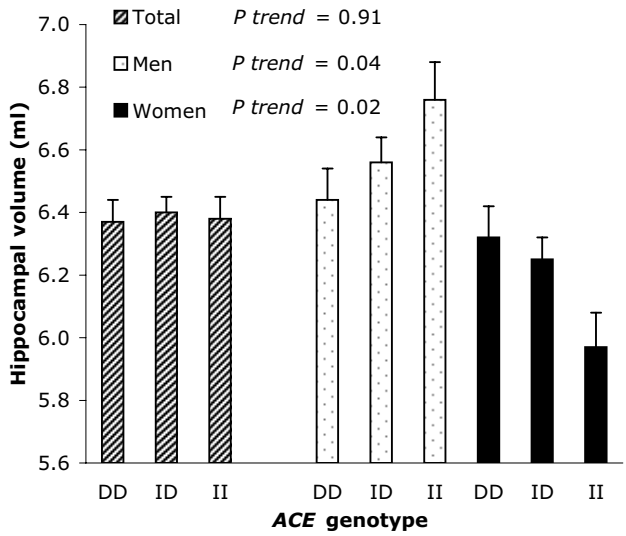

Figure 1

Figure 2.
Mean volumes of the amygdala by genotype overall and stratified by sex

Volumes (standard error), adjusted for age and when applicable sex

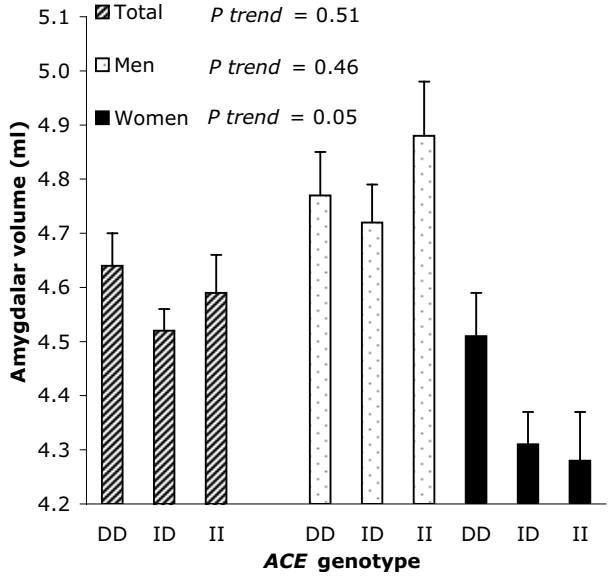

Figure 2

Table 3. *ACE* polymorphism in relation to vascular brain abnormalities on MRI

	ACE genotype			*P* for trend
	DD	*ID*	*II*	
Overall				
White matter lesions				
Periventrical[A]	2.7 ± 0.2	2.7 ± 0.2	2.8 ± 0.2	0.83
Subcortical[B]	1.6 ± 0.3	1.8 ± 0.2	1.9 ± 0.3	0.37
Infarcts, n (%)	35 (24.8)	73 (29.4)	31 (29.2)	0.60
OR (95%CI)	1.00 (ref)	1.39 (0.85-2.30)	1.49 (0.83-2.70)	0.33
Men				
White matter lesions				
Periventricular	2.8 ± 0.2	2.8 ± 0.2	2.4 ± 0.3	0.25
Subcortical	1.7 ± 0.4	1.6 ± 0.3	1.7 ± 0.3	0.98
Infarcts, n (%)	17 (21.5)	39 (32.0)	15 (27.3)	0.27
OR (95%CI)	1.00 (ref)	1.82 (0.91-3.63)	1.63 (0.71-3.77)	0.23
Women				
White matter lesions				
Periventricular	2.6 ± 0.3	2.6 ± 0.3	3.2 ± 0.3	0.14
Subcortical	1.4 ± 0.4	2.0 ± 0.3	2.1 ± 0.4	0.22
Infarcts, n (%)	18 (29.0)	34 (27.0)	16 (31.4)	0.84
OR (95%CI)[C]	1.00 (ref)	1.03 (0.50-2.12)	1.33 (0.57-3.09)	0.75

[A] Values are age and sex adjusted mean grade ± standard error
[B] Values are age and sex adjusted mean volumes ± standard error
[C] Values are odds ratios with 95% confidence interval

Discussion

In this cohort of people aged 55 years and over, we found that women homozygous for the I-allele of the *ACE* gene had a small increased risk to develop AD. This finding was supported by the fact that the I-allele was associated with early AD-related markers on MRI, i.e. smaller hippocampal and amygdalar volumes in women without dementia. Although the MRI scans were made in a sample of non-demented elderly, smaller volumes of medial temporal lobe structures predict the development of clinical AD.[22] Moreover, atrophy on MRI is strongly correlated to the extent of neurofibrillary tangles, even in non-demented elderly.[16] The fact that the I-allele was associated with both the risk of clinical Alzheimer's disease and, in an independent sample, with MRI markers indicative of AD pathology, decreases the possibility of a chance finding. No significant association was observed between the *ACE* gene and vascular lesions on MRI, neither did adjustment for cardiovascular risk factors change the association between *ACE* and AD.

In three meta-analyses studying the effect of the *ACE* I/D polymorphism on the risk of AD, the I-allele was associated with risk for AD, [34 23 13] but none of these meta-analyses was stratified by sex. The association of the I-allele with AD in women has been reported before in one study.[10] However, not in all studies sex-stratified analyses were or could be performed due to small sample size. To our knowledge one study found an increased risk of cognitive impairment associated with the D-allele in men only, although confidence intervals of the risk estimates were rather wide.[1] In line with this we found that men homozygous for the D-allele had smaller hippocampal volumes than men carrying the I-allele. This trend was not confirmed in the analyses of amygdala or AD. Although the results in men carrying the DD genotype may be chance findings we cannot exclude a potential opposite effect in men and women. The number of events in men was small, which may reduce power to detect an association.

Since the D-allele has been associated with myocardial infarction, a cause of death earlier in life, one could hypothesize that (I-allele carrying) survivors develop AD at later age. The association would then be biased by competitive mortality. In the Rotterdam Study we found no statistically significant difference in genotype distribution in different age strata, arguing against a major survival bias.

Since AD has a complex etiology in which numerous interactions between environmental and genetic factors are likely to play a role, confounding effects

of gene-environment interactions should be anticipated when performing candidate gene studies. However, environmental risk factors other than age and sex are rarely taken into consideration in candidate gene studies. Availability of data on vascular risk factors in the Rotterdam Study allowed us to explore to what extent the vascular properties of ACE were affecting the association between *ACE* and AD. When we controlled the analyses for vascular risk factors, we found an association between *ACE* and AD independent of vascular risk factors. This is compatible with our finding of the I-allele being the risk allele for AD, since the D-allele is generally considered to be the risk allele for cardiovascular disease. In addition, vascular brain abnormalities on MRI were not associated with *ACE*. We also assessed the relation between *ACE* and vascular dementia and found no evidence for association. Again this suggests the relation between *ACE* and AD is not mediated by vascular pathology.

One of the interesting mechanisms through which *ACE* may be involved in AD is that the I-allele is associated with a higher amyloid β load in the brain.[26] ACE has the ability to degrade amyloid β in vitro.[21] This suggests that the aging brain may benefit from high levels of ACE, which will lower the total amyloid β load. Subjects with the II genotype have the lowest levels of ACE in plasma,[38] and most likely also in the brain, which diminishes the capability to degrade amyloid β. This could contribute to accumulation of amyloid β seen in AD.

We found only women with the I-allele were at increased risk for AD, a finding more difficult to explain. Postmenopausal women on oestrogen hormone replacement therapy have lower ACE levels than those without hormone replacement therapy.[42] Likewise serum ACE is lower in pregnant than in non-pregnant women.[27] Oestrogen attenuates tissue ACE activity by down regulating ACE mRNA in rats.[15] In contrast androgens do not influence ACE levels.[11] This might explain why premenopausal women with high circulating oestrogen levels have lower ACE levels than men. Hypothetically, women homozygous for the I-allele have the lowest ACE levels throughout their lives. After menopause, ACE levels will increase but this may also be less pronounced in women homozygous for the I-allele. Given the possible beneficial effect of ACE on amyloid β degradation, women with the II genotype will then be at highest risk of AD. Moreover, ACE is involved in formation of angiotensin II and angiotensin IV, which can excite hippocampal neurons, leading to improved memory.[43] In women homozygous for the I-allele, this stimulatory role of the brain renin-angiotensin system on memory performance will be most impaired.

Not only studies of *ACE* and AD, but also of *ACE* and cardiovascular disease have yielded conflicting results, with large population heterogeneity[29]. This might reflect that the I/D polymorphism is in close linkage disequilibrium with

another truly causal polymorphism in *ACE*. Indeed, recently a haplotype study showed evidence for association with AD at a marker located in the promoter region of *ACE.*[23] The I/D polymorphism we studied was found to be in linkage disequilibrium with this marker, implying the I/D polymorphism may be a valid proxy in our population, but findings may be stronger when the precise causal variation has been established.

Our results suggest that women homozygous for the I-allele of the *ACE* I/D polymorphism are at increased risk of AD compared to women with the D-allele. This finding was supported by the presence of smaller amygdalar and hippocampal volumes on MRI in clinically non-demented women carrying the I-allele. Future research should focus on the role of *ACE* in the specific pathogenesis of Alzheimer's disease.

Acknowledgements

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Chapter 3.2
Evidence of linkage of Alzheimer's disease to chromosome 3 in five inbred families

Abstract

Late-onset Alzheimer's disease (AD) is a complex genetic disease without a single mode of inheritance. We studied AD in 5 multiply affected inbred families from a recently founded genetically isolated Dutch population, suggesting a recessive mutation underlying the disease. We performed homozygosity mapping on the data of a genome screen with 420 markers (average spacing 10 cM) in 10 descendants of these families, who could be connected to a common ancestor in 8 generations, and who were affected with probable late-onset AD. Homozygosity mapping revealed a large region with evidence of linkage on chromosome 3 (maximum LOD score 3.1 at 3q13, and 3.4 at 3q26). After fine mapping, the region (initially spanning 90 cM) could be narrowed down to two peaks, one of which extended over 19 cM, with a maximum LOD score of 3.9 at 3q22. The physical size of this peak is 9 Mb, and includes 90 genes. Further fine mapping is needed. The second peak at 3q26 could be narrowed down to a 5.8 cM interval (physical size 3.87 Mb, encompassing 13 genes) after testing additional markers, with a maximum multipoint LOD score of 3.2. This region contained the candidate gene butyrylcholinesterase (BCHE)*. Sequencing of coding exons of* BCHE *revealed no variants that could explain the occurrence of disease. In an association analysis in a series of 153 patients and 75 controls from the isolate we observed significant evidence of association with markers D3S1282 and D3S1565. Our findings suggest a new locus on chromosome 3, which plays a role in late-onset AD.*

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder, progressively affecting memory and other areas of cognition until a state of dependency, through an as yet not fully understood etiology. Mendelian genes involved in AD typically show (co-) dominant segregation of disease in families. Mutations in the amyloid precursor protein gene (*APP*), presenilin-1 (*PSEN-1)* and presenilin-2 (*PSEN-2*) are dominant mutations leading to early-onset AD with an age dependent penetrance.[1] Apolipoprotein E gene (*APOE*) has a co-dominant effect, with an over twofold risk increase for homozygous *ε4* carriers when compared to heterozygous carriers.[2] Genome screens show convincing evidence for additional AD loci on chromosomes 9, 10 and 12.[4] Whereas in families with early-onset AD the disease most often segregates as a dominant trait, for late-onset AD findings of segregation analyses are compatible with dominant, recessive and polygenic models.[3] Recently, a locus for AD was reported on chromosome 9 that appears to encompass a recessive mutation in a genetically isolated population.[5] Evidence increases that deleterious recessive alleles influence susceptibility to complex late-onset disease.[6] The presence of inbreeding, one of the major determinants of recessive traits, may facilitate the detection of recessive mutations in complex late-onset diseases.

We found evidence of inbreeding in five families multiply affected with AD in a genetically isolated Dutch population. Here we report the findings on a genome-wide search aiming to localize a possible recessive mutation using homozygosity mapping.

Methods

Subjects

The study was conducted in a genetically isolated Dutch population, within the framework of the research program Genetic Research in Isolated Populations (GRIP)).[7] The population was founded around 1750, by approximately150 people. The medical ethical committee of the Erasmus MC Rotterdam approved the study protocol. Two neurologists diagnosed all patients ascertained for the GRIP study independently, according to NINCDS ADRDA criteria.[8] Genealogical data were available up to 22 generations. We ascertained 191 patients, of whom 161 were diagnosed with probable or possible AD.[7] For the present genome screen, we identified 6 patients who were offspring of 5 consanguineous unions between 3rd cousins or more closely related partners. These families were again related distantly, within 8 generations. Table 1 presents the general characteristics of the patients. The probands (sib pair A.1 and A.2, and B.1, C.1, D.1 and E.1; upper case letters represent the five families) were all diagnosed with probable late-onset AD. All living affected relatives (n = 6) were ascertained. Of those, two second-degree relatives (B.2 and D.2) and two first cousins once removed (B.3 and B.4), all diagnosed with probable AD, were included in the statistical analyses of the genome screen. The other two second-degree relatives (C.2 and C.3) with possible AD and clinical features suggestive of Lewy body dementia, were excluded from the statistical analyses, but included in the haplotype analysis. The age at onset of dementia in the patients ranged from 66 to 85 years, with a mean of 73.3 years (Table 1).

Table 1. General description of the patients

Family	Patient	Relationship to the proband	Sex	Age onset	Inbreeding Coefficient	Diagnosis*
A	A.1	Proband	F	74	1.38E-02	Probable AD
	A.2	Proband	F	71	1.38E-02	Probable AD
B	B.1	Proband	F	74	2.79E-02	Probable AD
	B.2	Avuncular	F	79	1.33E-02	Probable AD
	B.3	1st cousin once removed	F	68	1.37E-02	Probable AD
	B.4	1st cousin once removed	F	75	1.37E-02	Probable AD
C	C.1	Proband	F	66	2.97E-02	Probable AD
	C.2	Avuncular	M	76	1.62E-03	AD or LBD
	C.3	Avuncular	M	72	1.62E-03	AD or LBD
D	D.1	Proband	F	69	1.65E-02	Probable AD
	D.2	Avuncular	F	85	9.09E-03	Probable AD
E	E.1	Proband	F	71	2.84E-02	Probable AD

* AD = Alzheimer's disease, LBD = Lewy body dementia

Genotyping

DNA was extracted from peripheral leucocytes following a standard protocol.[9] Mutations in *APP*, *PSEN-1* and *PSEN-2* were excluded previously, and all probands carried the *APOE ε3/4* genotype.[7] A genome-wide search was conducted using 420 micro-satellite markers (ABI Prism Linkage Mapping Set MD-10 Version 2 (Applied Biosystems, Foster City, CA, USA)). Polymerase chain reactions (PCR) were performed according to the manufacturer. PCR products were pooled and analyzed on ABI377 or ABI3100 automated sequencers (Applied Biosystems). Ten additional markers (D3S2406, D3S3556, D3S3695, D3S3606, D3S1549, D3S3626, D3S1275, D3S1607, D3S3668, D3S1282) were used for fine mapping of candidate regions on chromosome 3.

Statistical analysis

Pair wise kinship coefficients and inbreeding coefficients were calculated using PEDIG software (Boichard D., http://dga.jouy.inra.fr/sgqa/diffusions.htm). Genotype errors were checked using Pedcheck[10] and Merlin[11] software. In the linkage analysis we used a recessive model with disease allele frequency 0.01 and penetrances of 0.01, 0.01 and 0.99, allowing us to detect both recessive susceptibility alleles and rare dominant alleles. LOD scores were calculated with MAPMAKER/HOMOZ software.[12] Allele frequencies were based on 150 chromosomes of spouses of patients, originating from the GRIP region. Haplotypes were constructed for the 12 patients from genetic data of their close relatives. Candidate alleles were tested for association in the full patient and control series excluding those patients contributing to linkage, using Chi-square statistics and logistic regression adjusted for sex, in SPSS for windows 11.0.

Mutation analysis

Mutation analysis of the gene encoding butyrylcholinesterase (*BCHE*; OMIM 177400) was performed by direct sequencing of PCR amplicons of the coding exons. The gene sequence was obtained from the UCSC database (http://genome.ucsc.edu/). PCR reactions were performed in 50µl, containing 10x Invitrogen PCR buffer, 1.5mM $MgCl_2$, 20µM of each dNTP, 10 pM forward primer and 10pM reverse primer, 1.25 units of Taq Polymerase (Invitrogen) and 50 ng/µl genomic DNA. For exons fragments 1.2 and 3.2, we used 2mM $MgCl_2$. For exon fragment 1.2 we used DMSO 6%. PCR cycle conditions were 10 minutes at 94°C, 35 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at temperatures available in Table 2, and 90 seconds extension at 72°C. Final extension was set at 72°C for 5 minutes.

Primers, annealing temperatures and size of the amplified fragments were as shown in Table 2. Direct sequencing of both strands was performed using Big Dye Terminator V 3.0 chemistry (Applied Biosystems). Fragments were loaded on an ABI 3100 automated sequencer and analyzed with DNA Sequencing Analysis V 3.7 and SeqScape V 2.1 packages (Applied Biosystems).

Table 2. Primers, annealing temperatures and exon fragment sizes of *BCHE*

Exon Fragment	Forward Primer 5′	Reverse Primer 5′	Annealing T°	Size bp
1.1	CAATAAAGTATAATATGCTATAT	GCTTTTTGAATCGAAGTCTAC	55	~ 400
1.2	CTATGCACAGCCACCTCTTGG	CATGTTCCCTGGAGCCTCAG	60	~ 400
1.3	CAAACTGGAACATCATCTTTAC	CAGTCAATTTAGCTAAGTTCAAC	55	~ 400
1.4	CAGGAGCAGCTTCAGTTAGC	GAAGCCAGGAGCACCATAG	55	~ 400
1.5	CCTATGGGACTCCTTTGTCAG	GAACTTCTTGGTGAACTCCAAG	55	~ 400
1.6	CATTACACAGACTGGGTAGATG	CAGAGACCAAGCAAAGCTAAG	55	~ 400
2	GTTCACATACGTTTCATATCATC	GTGCCTTGGAGAGTATACTTC	54	~ 400
3.1	CTGTGTAGTTAGAGAAAATGGC	GCACATAATTAACTGTAGAAC	55	~ 450
3.2	GCCAGAAGGATAATATTGATTC	CAATTCTTATTTTAATTTAGGAAA	54	~ 250

Results

As shown in Figure 1, multiple inbreeding loops were present in all probands. Their affected relatives, not depicted in Figure 1, each were the offspring of a consanguineous marriage as well, as can be concluded from the inbreeding coefficients presented in Table 1. The closest connection to a common ancestor of all 12 patients was within 8 generations, but multiple complex connections between the patients were present. The kinship coefficients between the probands are presented in Table 3. Kinship was highest between probands C.1 and E.1, whereas kinship between the patients from family A and the other probands was lowest, suggesting a distinction between this sib pair and the other patients.

We performed a genome search with 420 markers in the six probands and their four close relatives with AD. Homozygosity mapping revealed 3 peaks with a LOD score over 1.5. On chromosome 3, two peaks reached a LOD score over 1.5, extending over a large region of 90 cM. One peak had a maximum multipoint LOD score of 3.1 at 3q13, and a second peak reached a maximum multipoint LOD score of 3.4 at 3q26 (D3S1279 to D3S1565). On chromosome 9 a maximum multipoint LOD score of 1.6 was reached at 9q34.

Table 3. Kinship coefficients between probands

Family	**A.1**	**B.1**	**C.1**	**D.1**	**E.1**
A.2	2.58E-01	1.53E-02	1.06E-02	1.03E-02	1.50E-02
B.1		*	2.10E-02	2.36E-02	2.00E-02
C.1			*	1.73E-02	3.00E-02
D.1				*	2.60E-02
E.1					*

* No sibling

We further investigated the region on chromosome 3 with ten extra markers (D3S2406, D3S3556, D3S3695, D3S3606, D3S1549, D3S3626, D3S1275, D3S1607, D3S3668 and D3S1282). The first peak, initially spanning ~ 50 cM, could be narrowed down to a 19 cM region with a maximum LOD score of 3.9 in the region between D3S1292 and D3S1549 (Figure 2). Haplotypes are shown in Figure 3a. Patient B.1 is homozygous for a haplotype over a large region, spanning 25 cM. Her relative, B.2, is the only one who is heterozygous for part of this haplotype, but many of the other patients are homozygous for one or

Figure 1.

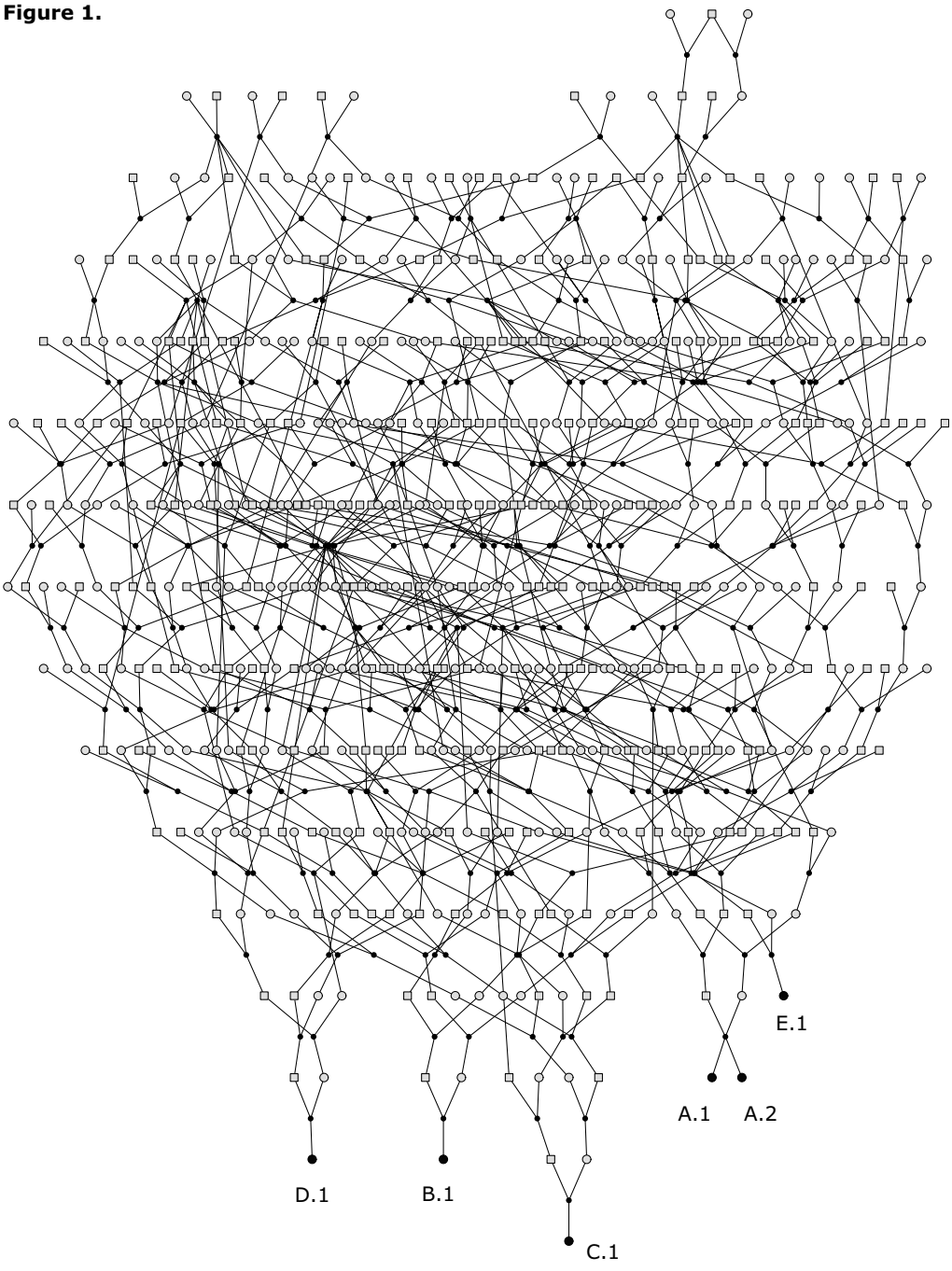

Figure 1. Pedigree of all probands and their relationships to common ancestors
Grey solid squares indicate men, grey solid circles indicate women, and black solid circles indicate probands. Black dots indicate meioses. Affection status is only shown for the probands.

two alleles at this haplotype (e.g. A.1, A.2, B.2, and C.1 for allele 1, and B.3 and B.4 for allele 2 at marker D3S3606; C.3, D.1 and D.2 for allele 3 at marker D3S1292, for which several others are heterozygous; nuclear family B and C.1 for allele 1 at marker D3S1569). Another haplotype (from D3S1267 to D3S1292) is shared by C.2 and C.3, D.1 and E.1. An association analysis in an independent series of 153 probable and possible AD patients and 75 spouses originating from the study population, however, did not reveal association of any of the candidate alleles (as based upon the haplotypes in Figure 3a) with AD.

For the second peak (3q26), a maximum LOD score of 3.2 was obtained after fine mapping; see Figure 2. Most of the evidence for linkage was derived from nuclear family A, for which the Lod score was 2.2 in the region between markers D3S3668 and D3S1614. Haplotypes are shown in figure 3b. The two siblings A.1 and A.2 were homozygous for a haplotype of 10.6 cM (D3S3668 to D3S1565). Patient C.1 was heterozygous for this haplotype, excluding D3S1565. The patients with possible AD and possible Lewy body dementia (C.2 and C.3) shared this haplotype with their relative with probable AD (C.1). They further shared a distinct haplotype, for which C.2 was partly homozygous. This haplotype could also be found in their relative with probable AD (C.1).

For this second region on chromosome 3 we conducted an association analysis of haplotype-based candidate alleles as well, again using the independent series of 153 probable and possible AD patients and 75 spouses originating from the study population. None of the alleles of D3S1607, D3S3668 and D3S1614, as observed in the haplotypes depicted in Figure 2, were significantly associated with AD. We did find evidence for association with disease status for marker D3S1282 (allele 1, as observed in nuclear families B, C, D and E) and D3S1565 (allele 1, as observed in nuclear family B, C.3, nuclear family D and E). For D3S1282, the sex adjusted odds ratio (OR) was 2.4 (95% CI 1.0-5.8; p = 0.05) for individuals homozygous for allele 1. Heterozygous individuals were at increased risk (OR 1.7, 95% CI 0.9-3.2), albeit non-significant. For marker D3S1565, OR was 1.8 (95% CI 1.0-3.4; p = 0.06) for individuals heterozygous for allele 1, and 2.1 (95% CI 0.9-5.0; p= 0.09) for those homozygous for allele 1.

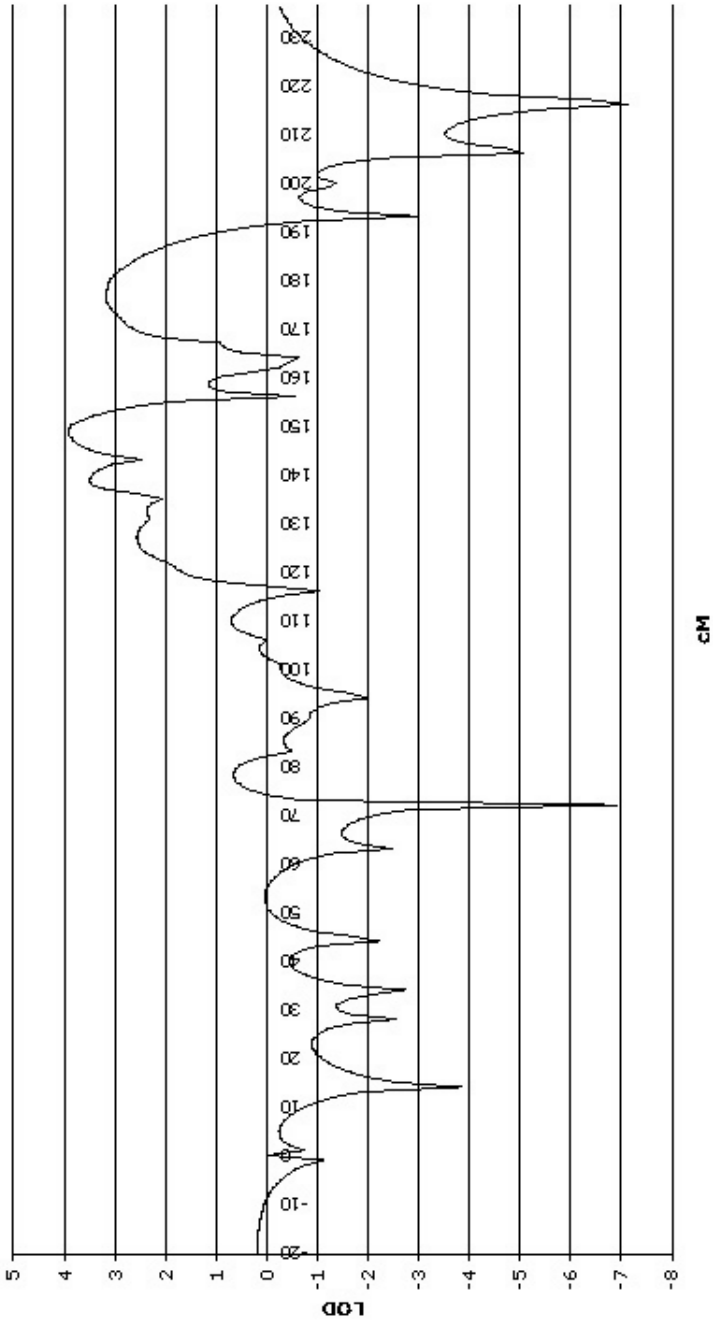

Figure 2.
Multipoint LOD score graph of chromosome 3 after fine-typing
The region from 97 cM to 186 cM is additionally typed with 10 extra markers.

Figure 3a

Marker	cM	Family A				Family B								Family C						Family D				Family E	
		A.1		A.2		B.1		B.2		B.3		B.4		C.1		C.2		C.3		D.1		D.2		E.1	
D3S1267	139.12	3	5	3	5	1	1	1	3	1	3	1	3	3	8	8	4	5	4	4	4	6	4	4	7
D3S3606	143.94	1	1	1	1	1	1	1	1	2	2	2	2	1	1	1	4	3	4	4	2	4	1	4	2
D3S1292	146.6	3	1	3	1	7	7	7	4	7	4	7	4	11	6	6	3	3	3	3	3	3	3	3	5
D3S1549	151.49	3	4	3	4	1	1	0	1	6	5	6	5	5	6	6	5	0	5	4	5	4	0	3	3
D3S1569	158.38	2	4	2	4	1	1	1	1	1	1	1	1	1	1	1	2	1	2	4	4	2	1	1	5

Figure 3b

D3S1279	169.6	5	4	5	4	1	8	1	8	1	8	1	8	4	6	6	3	1	3	6	5	5	5	7	1
D3S1607	172.27	5	3	5	3	11	5	11	5	11	5	11	5	3	6	6	4	5	4	6	7	3	5	6	1
D3S1275	172.28	4	3	4	3	2	5	2	5	2	5	2	5	3	7	7	3	3	3	7	5	6	6	7	2
D3S3668	175.47	3	3	3	3	2	1	2	1	2	1	2	1	3	1	1	3	5	3	4	3	2	3	1	2
D3S1614	177.75	2	2	2	2	3	4	3	4	3	4	3	4	2	2	2	2	3	2	5	5	6	3	2	4
D3S1282	180.8	2	2	2	2	1	1	5	1	5	1	5	1	2	1	1	1	3	1	1	1	4	1	1	1
D3S1565	186.04	4	4	4	4	3	1	1	1	1	1	1	1	3	3	3	3	1	3	1	1	2	1	1	1

Figure 3a and 3b.
Haplotypes at the first part of the region (3q21.3-3q22.3) in patients (3a)
Haplotypes at the second part of the region (3q26) in patients (3b)

Given the small difference in OR between heterozygous and homozygous individuals, the 2 groups were pooled, leading to an OR of 1.9 (95% CI 1.0-3.3; p = 0.03).

All haplotypes in the second region (physical size 3.87 Mb, encompassing13 genes; Figure 3b) contain the gene *BCHE*. We sequenced *BCHE* in the probands and one healthy age-matched spouse. All sequenced individuals carried a known T insertion (non-coding) in exon-fragment 3.1 (rs3836432; NCBI), and a known G/A change in exon-fragment 3.2. The control and all patients except D.1 were homozygous for the A-allele, D.1 was heterozygous G/A (frequency A: 0.321 (rs3495; NCBI)). We detected no novel sequence variants. None of the sequenced probands were carrier of the K variant of *BCHE* (*BCHE-K;* ala539thr) that has previously been associated with AD.[13]

Discussion

We have found evidence of linkage of late-onset AD to chromosome 3 in five inbred families. After fine mapping of the candidate region, significant LOD scores of 3.9 (3q22) and 3.2 (3q26) were calculated under a recessive model. Family A contributed most to the LOD score that was obtained at 3q26, whereas the other families contributed most to the LOD score of 3.9 at 3q22. Homozygosity mapping can be susceptible to false positive findings, but with our genealogical data we have shown the presence of similar haplotypes in patients related in at least 7 generations. In addition, markers at the second peak showed association with AD in an independent sample from the same population, making a false positive finding unlikely.

No recessive mutations are established to increase risk for AD, but in an inbred Israeli-Arab community, evidence for a recessive locus for AD on chromosome 9p has also been found.[5] These findings imply there may be families in which the disease can be explained by recessive mutations. It is difficult to ascertain these families, as the disease often appears to be sporadic, and inbreeding loops can only be determined when extensive genealogical data are available. The chances of detecting such families are higher in genetically isolated populations.

Our haplotype analyses do not support the view that a single mutation exists at this region in the GRIP population. The presence of different haplotypes suggests that different mutations may have occurred at different locations in the same gene. When considering the second peak (3q26), patient C.1 appears to be compound heterozygous for 2 haplotypes, which may be compatible with multiple recessive mutations. Patients B.1, C.2, D.1 and E.1 are homozygous for allele 1 of D3S1282, and patients B.2, B.3, B.4, D.1 and E.1 are homozygous for allele 1 of D3S1565, which is still compatible with a recessive effect. However, our association analysis showed that individuals heterozygous for allele 1 of D3S1565 are also at increased risk, with a risk estimate similar to that in homozygous individuals. The odds ratios observed suggest a susceptibility gene with an increased risk for both hetero- and homozygous individuals. Nevertheless, the presence of such a risk-increasing variant does not preclude a recessive mutation in the same gene. In other neurological disorders various mutations in the same gene have been found to cause disease, e.g. Parkin mutations in Parkinson's disease. [14] The presence of a somewhat distinct phenotype (i.e. features of Lewy body dementia) in two of the heterozygous patients might be compatible with this situation.

Although the first part of the chromosome 3 region (3q21.3-3q22.3) conferred the highest LOD score, the size of the region (9 Mb, containing 90 genes) precludes meaningful haplotype analysis. Further refinement is needed in order to localize a possible susceptibility gene. Of interest, the region does show overlap with a locus detected previously in familial AD.[15] Fronto temporal dementia also has been observed to map to chromosome 3, but to a locus upstream of our peak.[16]

Direct sequencing of the coding regions of *BCHE*, a candidate gene in the second region, gave no evidence of a causative mutation, despite plausibility for a role of *BCHE* in AD, [17] and the positive association studies of *BCHE*-K variant and AD.[13] None of the sequenced patients were carriers of *BCHE-K*, excluding this association as an explanation. We cannot exclude a disease-associated mutation in the non-coding regions. Another candidate gene in this region is *Neuroserpin* (OMIM 602445), but it has only been associated with very early onset autosomal dominant dementia with a distinct phenotype.[18]

In this study of inbred families in a genetically isolated population we detected evidence for linkage of AD to chromosome 3. The first part of the region still needs refinement, but shows overlap with a region previously described to be linked to dementia. The mutation underlying the second peak is likely to be recessive given the pedigree structure, evidence of homozygosity by descent and compound heterozygosity, but mutations with a co-dominant effect may have occurred. Most of the evidence at this peak is derived from a single family. However, our association analysis in the full cohort suggests the gene is involved in a larger number of patients, at least in the isolated population studied.

Acknowledgement

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Chapter 3.3

A genome wide search for susceptibility genes for Alzheimer's disease in a genetically isolated Dutch population

Abstract

Given the genetic heterogeneity of late-onset Alzheimer's disease (AD), studies in more homogeneous, genetically isolated populations are advantageous in localizing susceptibility loci using an association-based approach. This report describes the results of a genome wide association study to search for susceptibility loci for AD in a recently founded, genetically isolated Dutch population. A series of 156 patients with Alzheimer's disease and 75 cognitively healthy spouses were genotyped with 420 micro-satellite markers (average spacing 10-12 cM). Single marker association analyses were performed using Dislamb and Clump software. We detected association in 6 regions (D1S2797, D10S196, D15S127, D17S1857 and D17S798, and D19S210) at a significance level of $p < 0.01$. The association with D15S127 has not been reported before. All other loci have previously been implicated in AD. The locus on chromosome 1 seemed to confer risk especially in those patients without an APOE ε4 allele. D19S210 was only associated with increased risk for AD in APOE ε4 negative individuals, suggesting a locus other than APOE on chromosome 19.

The identification of both known loci and a new locus on chromosome 15 shows the applicability of genetically isolated study populations in the search for susceptibility loci for AD. The structure of our genetically isolated population will enable further investigation of these loci.

Introduction

Alzheimer's disease (AD) demonstrates strong familial clustering.[1] To date, four genes have been identified in the etiology of AD. Mutations in three of these (coding for amyloid precursor protein (*APP),*[2] presenilin-1 (*PSEN-1)* [3] and presenilin-2 (*PSEN-2)* [4]) cause early-onset autosomal dominant AD. The fourth gene, encoding apolipoprotein E (*APOE)* [5, 6] is implicated in the common form of AD. The *ε4* allele of *APOE* is associated with increased susceptibility to both early- and late-onset forms of AD, but *APOE ε4* is not sufficient to cause AD. Together, *APP*, *PSEN-1* and *PSEN-2* and *APOE* explain only about 20% of all patients with AD.[7] There is compelling evidence that more genes are involved in AD.[8] Linkage to several chromosomal regions is reported with considerable consistency in different studies on AD, such as regions on chromosome 9, [9-14] chromosome 10 [15, 16,17,18] and chromosome 12. [19, 20 21] On chromosome 10, several genes have been associated with AD, amongst which are the genes encoding insulin degrading enzyme (*IDE*),[22] choline acetyltransferase (*CHAT*),[23] plasminogen activator urinary (*PLAU),[24]* and alpha-T-catenin (*VR22*).[25] Similarly, on chromosome 12 several genes have been put forth as candidate genes, such as the genes encoding alpha-2-macroglobulin (*A2M*)[26] and low-density lipoprotein receptor-related protein (*LRP*).[27]

Most studies conducted to date have been based on classical linkage analysis. This method has its limitations in late-onset disease. When genotype relative risks of common variants are low, large samples of families are required to obtain significant results. An alternative approach is to conduct an association study. The rationale behind association is linkage disequilibrium (LD) between a marker and a disease locus. In an outbred population, the extent of LD around a disease locus is limited, requiring a dense set of markers to detect association. There is increasing interest in the use of genetically homogeneous study populations for genome-wide association studies. Not only is the allelic heterogeneity of the disease decreased, but in recently founded isolated populations, patients can be expected to share relatively large haplotypes flanking a mutation, thereby facilitating its detection with a coarse set of markers.[28] Full genome screens for susceptibility to AD have been performed in a genetically homogeneous Finnish population, founded during the 16th and 17th century, [29] and in an inbred Arab community. [13] Both identified several loci previously reported (1p36;[29] 9p21 and 10q21-22[13]), and a locus on chromosome 2p23-24[13, 29] which had not been detected before.

We performed a genome screen for AD in a recently founded, genetically isolated population in the Netherlands that is studied as part of our Genetic Research in Isolated Populations (GRIP) program.[30] The population was founded in the middle of the 18th century, leading to strong LD.[31] In GRIP, LD extends up to 20 cM, allowing for a genome-wide search with a coarse marker set.

Methods

Study population

The study is based in a genetically isolated community in the southwest of the Netherlands, as part of the GRIP program. The descendants of 150 founders of the GRIP population lived in relative isolation until the middle of the 20th century. From the middle of the 19th century the population expanded substantially, from 700 inhabitants in 1848 to more than 20,000 inhabitants by the end of the 20th century. The Medical Ethics Committee of the Erasmus MC Rotterdam, The Netherlands approved the study protocol.

Patients

Patients were traced through local general practitioners, neurologists and nursing home physicians. All patients suspected of or diagnosed with dementia were seen by a research physician. Of all ascertained patients (n=191), 156 were diagnosed with AD according to the criteria of NINCDS-ADRDA.[32] The diagnosis was based on a neurological examination, brief neuropsychological testing, a standard interview with close relatives concerning presenting symptoms, disease course, medical, social and family history, and review of all available medical records, neuropsychological test results, and hard copies of CT or MRI scans of the patients prior to participation in this study.[30] Genealogical information, collected during the proxy interview, was extended up to 22 generations using municipal registers and data from a large genealogy database that holds genealogical information on approximately 60,000 individuals from the GRIP region.

Laboratory analysis

Blood was drawn from patients, spouses and offspring, and siblings. In total, 75 cognitively healthy spouses were included as control subjects. The inclusion of first-degree relatives allowed for segregation checks and the construction of haplotypes. Genomic DNA was isolated from peripheral blood according to a standard protocol.[33] Mutations in known genes (*APP*, *PSEN-1* and *PSEN-2*) were excluded, and *APOE* was genotyped previously.[30] A genome wide scan

was performed using 420 micro-satellite markers from the ABI Prism Linkage Mapping Set MD-10 Version 2 (Applied Biosystems, Foster City, CA, USA) in two different genotyping experiments. Polymerase chain reactions (PCR) were performed according to the manufacturer's protocol. PCR products were pooled and subsequently analyzed on an ABI377 or ABI3100 automated sequencer (Applied Biosystems). The Marshfield sex-averaged genetic map was used to determine the order of the markers.

Statistical analyses

Because a small proportion of the genotypic data was obtained in a different genotyping experiment (*see Chapter 3.2*), the data of the two screens were pooled using a statistical pooling method (POOL_STR 1.1; Aulchenko YS et al, Ann Hum Genet, *in press*). Association analysis was performed using Dislamb and Clump software, and included all AD patients and controls from the same region. Dislamb is a likelihood based program testing for association at a single locus, under the assumption that one allele will be over-represented on chromosomes that carry the disease mutation.[34] Allele frequencies are estimated for patients and controls, and a likelihood ratio test is performed yielding a chi-square statistic and corresponding p-value. The parameter lambda (λ) signifies the proportion of patients in whom the disease is associated with the ancestral allele.

The Clump program allows multiple alleles at marker loci to be in linkage disequilibrium with a disease locus, as might occur in complex diseases in which multiple founders have brought numerous mutations into a population. In addition, the program allows for early recombinations or mutations of highly polymorphic micro-satellite markers. Significance is tested using a Monte Carlo approach on four 2 by N tables. The first 2 by N table contains the data as observed; the second contains the original data, but alleles with low frequencies are clumped together; the third is a 2 by 2 table of a non-rare allele versus all other alleles; and the fourth is a 2 by 2 table clumping the columns of the original table together to maximize the chi-squared value.[35]

Markers were investigated further when the evidence for association reached a p-value of $p<0.05$. To assess if observed associations were dependent or independent of *APOE ε4*, we calculated the odds ratios (OR) with 95% confidence intervals for associated markers in two strata defined by *APOE ε4*. To test for interaction, a case-only analysis was performed. The rationale of this test is that unlinked genes will not segregate independently in the presence of interaction.

Results

A genome wide scan was conducted on 156 AD patients originating from the GRIP region and 75 age-matched controls from the same region (Table 1). A relatively high proportion of probable and possible AD patients were *APOE ε4* carrier. Probable AD patients were more closely related to each other than possible AD patients. In total, 19 markers at 15 chromosomal regions showed a significant difference in allele frequency distribution between patients and controls, either in the analysis based on Dislamb or Clump, with p-values ≤ 0.05 (Table 2).

Table 1. General descriptives

		Age at onset/inclusion*	Female	*APOE ε4* +	Kinship > (½)[9]
AD	Probable (136)	73.0	0.78	0.64	0.10
	Possible (20)	74.8	0.77	0.77	0.04
Control	(75)	74.2	0.45	0.32	0.03

* mean age at onset for patients, age at inclusion for controls

The strongest evidence for association (p ≤ 0.01) was obtained at 6 markers (D1S2797, D10S196, D15S127, D17S1857 and D17S798, and D19S210). Three of these (D10S196, D15S127 and D19S210) were found to be associated with AD in both the Dislamb and Clump analyses. This also holds for a marker on chromomsome 4 (D4S1539), albeit at lower level of significance. Based on both Dislamb and Clump analyses and significance levels, evidence was strongest for D19S210, with $p_{dislamb}$ = 0.001 and p_{clump} = 0.003. Allele 2 was associated to AD in both analyses, with a frequency of 0.22 in patients and 0.08 in controls. This marker is 16 cM distant from D19S571 (p_{clump} = 0.085), which has previously been linked to AD. This linkage was attributed to *APOE*.[15]

Strong evidence for association was also obtained on chromosome 15, where we detected association with marker D15S127 in both the Dislamb and Clump analyses ($p_{dislamb}$ = 0.03 and p_{clump} = 0.004). This marker has not been implicated in AD before. Association to a locus approximately 30 cM distant of the locus detected here was found in a subset of patients with very-late age at onset (≥ 79 years) previously.[12] This could not be observed in our study, as the mean age at onset of the patients contributing to the association on chromosome 15 was 71.6 years. The odds ratio for the alleles associated at D15S994 was highest of all markers (OR 4.4, 95% CI 1.7-11.1; p = 0.0009 (see table 3)).

Table 2. Association in AD patients versus controls

		Dislamb					Clump			
Marker	cM	Allele	Patient (312)*	Control (150)*	p	λ	Alleles	Patient (312)	Control (150)	p**
D1S234	55.1	-	-	-	-	-	2,3,5,7	0.61	0.42	0.019
D1S2797	75.7	-	-	-	-	-	1,2,4,6,8,9	0.98	0.79	0.005
D1S2878	177.9	-	-	-	-	-	1,2,4,5,7,9	0.85	0.68	0.024
D2S142	161.3	-	-	-	-	-	2,3,4	0.82	0.70	0.037
D2S335	175.9	-	-	-	-	-	1,2,3,4,8	0.62	0.45	*0.054*
D2S126	221.1	-	-	-	-	-	1,2,3,4,5	0.91	0.62	0.017
D2S125	260.6	-	-	-	-	-	1,2,4,5	0.79	0.65	0.044
D4S1539	176.2	2	0.41	0.28	0.018	0.17	2,4	0.46	0.31	0.020
D6S264	179.1	-	-	-	-	-	1,2,6	0.34	0.18	0.035
D8S550	21.33	-	-	-	-	-	3,4	0.56	0.40	0.015
D9S285	29.5	-	-	-	-	-	2,6,7,8,9	0.82	0.67	0.022
D9S171	42.7	-	-	-	-	-	1,3,4,5,9	0.77	0.59	0.010
D10S547	29.1	3	0.27	0.16	0.043	0.13	-			-
D10S196	70.2	2	0.33	0.20	0.016	0.16	2,4	0.36	0.20	0.008
D13S159	79.5	-	-	-	-	-	3,4,5,8,10,12	0.42	0.30	*0.089*
D13S173	93.5	-	-	-	-	-	1,4,5,6	0.82	0.68	0.048
D15S994	40.3	-	-	-	-	-	1,6,7	0.16	0.04	0.030
D15S127	86.8	11	0.21	0.09	0.030	0.12	1,3,4,6,8,11-13	0.70	0.45	0.004
D15S130	100.6	-	-	-	-	-	2,3,4,6,7	0.89	0.78	0.07
D17S1857	43.0	-	-	-	-	-	1,2,3,4,5,6,8	0.95	0.84	0.006
D17S798	53.4	-	-	-	-	-	1,3,4,5,6,7	0.78	0.59	0.009
D19S571	84.1	-	-	-	-	-	2,4,5	0.52	0.38	*0.085*
D19S210	100.0	2	0.22	0.08	0.001	0.14	2,6	0.23	0.08	0.003

* The maximum number of chromosomes is indicated for patients and controls, but due to genotype failures these numbers may vary per marker

** All markers at p-values < 0.05 are shown, and 3 markers with p < 0.1 that were located within 20 cM distance of a positive marker (p-values for these markers are in italics)

Table 3. The odds ratio of AD for markers showing overall association, stratified by *APOE ε4*

Marker	cM	Associated	All				*APOE ε4+*				*APOE ε4-*			
		alleles*	Case †	Control ‡	OR	p	Case†	Control ‡	OR	p	Case †	Control ‡	OR	p
D1S234	55.1	2,3,5,7	0.61	0.42	2.1 (1.4-3.2)	0.0007	0.60	0.65	0.8 (0.3-1.9)	0.6	0.66	0.31	4.2 (2.0-8.8)	0.0001
D1S2797	75.7	1,2,4,6,8,9	0.98	0.79	3.2 (1.8-5.9)	0.0001	0.89	0.88	1.1 (0.3-4.0)	0.9	0.97	0.85	4.0 (1.0-16.8)	0.04
D1S2878	177.9	1,2,4,5,7,9	0.85	0.68	2.5 (1.5-4.3)	0.0004	0.83	0.68	2.3 (0.9-6.2)	0.08	0.88	0.69	3.5 (1.4-8.5)	0.005
D9S285	29.5	2,6-9	0.82	0.67	2.3 (1.5-3.5)	0.0001	0.84	0.70	2.4 (1.0-5.9)	0.06	0.78	0.67	1.8 (0.9-3.7)	0.1
D9S171	42.7	1,3-5,9	0.77	0.59	2.3 (1.4-3.5)	0.0004	0.80	0.61	2.5 (1.1-5.8)	0.02	0.79	0.63	2.2 (1.1-4.5)	0.03
D10S547	29.2	3	0.27	0.16	2.0 (1.1-3.5)	0.013	0.25	0.18	1.5 (0.5-4.1)	0.5	0.32	0.17	2.2 (1.0-5.1)	0.05
D10S196	70.2	2	0.33	0.20	2.0 (1.1-3.4)	0.007	0.34	0.28	1.3 (0.5-3.3)	0.6	0.32	0.20	1.3 (0.6-2.8)	0.5
D15S994	40.3	1,6,7	0.16	0.04	4.4 (1.7-11.1)	0.0009	0.18	0.04	5.5 (0.7-41.7)	0.07	0.18	0.04	5.8 (1.3-26.2)	0.01
D15S127	86.8	11	0.21	0.09	2.6 (1.3-5.2)	0.007	0.16	0.08	2.0 (0.5-9.1)	0.3	0.27	0.09	3.5 (1.2-9.8)	0.01
D15S130	100.6	2-4,6,7	0.89	0.78	2.2 (1.3-3.8)	0.002	0.87	0.80	1.6 (0.6-4.8)	0.4	0.90	0.78	2.5 (1.0-6.2)	0.03
D17S1857	43.0	1-6,8	0.95	0.84	3.7 (1.8-7.3)	0.0001	0.97	0.88	3.5 (0.8-14.4)	0.06	0.96	0.86	3.7 (1.0-13.3)	0.03
D17S798	53.4	1,3-7	0.78	0.59	2.3 (1.5-3.5)	0.0001	0.87	0.95	0.3 (0.02-2.4)	0.2	0.72	0.58	2.0 (1.0-3.9)	0.05
D19S571	84.1	2,4,5	0.52	0.38	1.8 (1.2-2.7)	0.009	0.56	0.40	1.9 (0.8-4.5)	0.1	0.46	0.38	1.4 (0.7-2.7)	0.4
D19S210	100.0	2	0.22	0.08	3.2 (1.6-6.4)	0.0005	0.22	0.14	1.7 (0.6-5.2)	0.3	0.19	0.07	3.0 (1.0-9.4)	0.04

* Alleles are selected for odds ratio calculation based on their association in the whole series in Dislamb (for a single allele) or Clump (> 1 allele) analyses. In case of association in both analyses, only the odds ratio for the allele associated in Dislamb is given, to provide the most conservative estimate.
Values shown are proportions (of patients, controls), odds ratios with 95% confidence intervals and p-values.
† The maximum number of chromosomes is n = 312 for all patients, n = 210 for *APOE ε4+* patients, n = 102 for *APOE ε4-* patients. ‡ The maximum number of chromosomes is n = 150 for all controls, n = 28 for *APOE ε4+* controls, n = 60 for *APOE ε4-* controls.

The region on chromosome 10 encompassing *IDE* [16-18] was negative in the overall analysis of the whole patient-control series, but we did find association with marker D10S196 adjacent to the candidate gene *CHAT*, both with Dislamb (allele 2; p = 0.016, λ = 0.16) and with Clump (alleles 2 and 4; p = 0.008). At two chromosomes (1 and 17), a significant association (p < 0.01) was only obtained in the Clump analysis. Two adjacent markers on chromosome 17 (D17S1857 and D17S798) showed strong association with AD based on the odds ratio and p-values (see table 3). D17S798 is ~13 Mb upstream from *MAPT,* encoding tau. The association is based on over 75% of cases, making it unlikely that as yet unrecognized features of fronto-temporal dementia explain this association, since this is a rare trait. To further test if the association at these markers was indeed driven by fronto-temporal dementia, we analysed all markers on chromosome 17 in a sample of 10 possible and probable FTD-patients from the GRIP area.[30] There was no significant evidence of association at D17S1857 ($p_{dislamb}$ = 0.08) and at D17S798, which is located closer to *MAPT* ($p_{dislamb}$ = 0.5). On chromosome 1, two regions showed association, of which the 1p32 region (D1S234, D1S2797) provided the strongest evidence. Of interest, D1S2878 in the second region is located close to *NCSTN*, a candidate gene encoding for nicastrin, which is a component of the presenilin containing γ-secretase complex implicated in APP processing.[36, 37]

Two markers on chromosome 9 giving evidence for association at p < 0.05 (D9S285 and D9S171; p_{clump}= 0.02 and p_{clump}= 0.01) overlapped with regions observed in previous studies on AD.[9-14] The markers giving evidence for association on chromosome 2 did not overlap with loci observed in the other screens performed in isolated populations.[13, 29] No flanking markers of known genes *APP*, *PSEN-1* and *PSEN-2* showed evidence of association.

When we evaluated the effect of *APOE ε4* (table 3), the 1p32 region association was much stronger in the *APOE ε4* negative stratum than in the *APOE ε4* positive stratum. Most other markers showed similar associations in both strata; D10S547, D15S127 and D19S210 appeared significantly associated only in *APOE ε4* negative patients yet we cannot exclude an association in *APOE ε4* carriers given the sample size. No markers showed significant interaction with *APOE ε4* in a case-only analysis. Further, we conducted a haplotype analysis. Allele 1 at marker D9S171 and allele 3 at D9S161 together constituted a haplotype that occurred at a frequency of 11.4% in patients and 4.5% in controls, giving an odds ratio of 2.7 (95% CI 1.5-4.7; p = 0.0004). For other regions, no single haplotypes could be reconstructed.

Table 4. New regions suggesting association after stratification by *APOE* strata

	Marker	cM	Dislamb					Clump				Odds Ratio*	
			Allele	Patient†	Control‡	p	λ	Alleles	Patient†	Control‡	p	(95%CI)	p(OR)**
APOE ε4+	D3S1580	207.7	-	-	-	-	-	2-4,6-9	0.65	0.74	0.002	0.7 (0.3-1.7)	0.4
	D4S405	56.9	-	-	-	-	-	1-4,6,8	0.82	0.80	0.01	1.1 (0.4-3.3)	0.8
	D5S416	28.8	-	-	-	-	-	1-3,5	0.95	0.71	0.002	7.2 (2.3-22.4)	0.0001
	D5S419	40.0	-	-	-	-	-	1,4,6,7	0.46	0.14	0.043	5.1 (1.7-15.1)	0.0015
	D5S426	52.0	-	-	-	-	-	1-8	1.00	0.89	0.005	∞	∞
	D5S407	64.7	-	-	-	-	-	2,8,9	0.42	0.07	0.046	9.3 (2.1-40.2)	0.0004
	D10S591	13.5	-	-	-	-	-	1,2,4,5	0.71	0.46	0.05	2.9 (1.3-6.5)	0.007
APOE ε4-	D1S2800	252.1	1	0.40	0.19	0.021	0.24	1,2	0.44	0.21	0.049	2.8 (1.3-6.4)	0.009
	D4S1575	132.1	-	-	-	-	-	1,3	0.63	0.39	0.017	2.6 (1.3-5.1)	0.006
	D12S345	53.1	-	-	-	-	-	1,3,6-8	0.85	0.60	0.039	3.8 (1.7-8.3)	0.0008

Values shown are proportions (patients, controls or λ) or p-values.

* The odds ratio is calculated over the allele or alleles associated in either Dislamb or Clump analyses.

** The p-value of the odds ratio is given by p(OR).

† The maximum number of chromosomes is n = 210 for *APOE ε4+* patients, n = 102 for *APOE ε4-* patients.

‡ The maximum number of chromosomes is n = 28 for *APOE ε4+* controls, n = 60 for *APOE ε4-* controls

In table 4 several chromosomal regions are presented that only gave evidence for association after stratification by *APOE ε4*. Although these results might very well be false positives due to over-stratification, a series of 4 markers on chromosome 5, spaced ~12 cM distance apart, shows a potential locus that is relevant in *APOE ε4* carriers. Also of interest is the association at D12S345, with an OR of 3.8 (95% CI 1.7-8.3; p = 0.0008) in *APOE ε4* negative individuals only. This marker is ~14 cM distant from *LRP*.[19-21] Studies have suggested phenotypic heterogeneity in association to chromosome 12. The phenotype Lewy body dementia was found to be associated with *A2M*.[38] We performed an association analysis of all markers on chromosome 12 in 16 patients with possible or probable Lewy body dementia from the GRIP region.[30] Of interest, the only marker on chromosome 12 showing significant association was D12S336, adjacent to *A2M*. In the Clump analysis 100% of the patients carried one of the 3 AD-associated alleles, as opposed to only 50% of controls (p_{clump} = 0.05).

Discussion

In this genome-wide search for susceptibility genes for AD in a genetically isolated Dutch population we identified 6 chromosomal regions with evidence of association to AD. The regions on chromosome 1p32, 9p21, 10 and 19p13.4 were reported to be associated to AD previously. In addition to these regions, we also detected association at chromosome 15, which might concern a new locus. Association to chromosome 15 has been reported before in a subset analysis in very-late-onset AD. However, association was found ~ 30 cM distant of the region detected in our study. [12] Also in the Finnish study of an isolated population some evidence existed of association on chromosome 15, but the locus at which LD was detected did not show an increased odds ratio in their analyses.[29] Thus the present study is the first to provide evidence for an AD locus on chromosome 15 in the initial analyses. Interestingly, both of the regions on chromosomes 1 and 9 are close to, or overlap with regions observed in other studies performed in genetically homogeneous populations. [13, 29] The region on chromosome 9 has also been found in numerous other studies [9-12, 14] several of which were conducted on parts of the same data set (NIMH-AD genetic consortium), but with different statistical approaches and different inclusion criteria. Replication of these regions in independent samples, such as the inbred Arab community or the GRIP population, presents a strong argument for the presence of an underlying disease gene. In the inbred Arab community, the chromosome 9 region appeared to encompass a recessive or additive mutation because of excess homozygosity. Only two patients were homozygous at the haplotype observed in the GRIP population, suggesting an additive rather than a recessive effect.

Despite a high prevalence of *APOE ε4* in the GRIP population, [30] the flanking markers of *APOE* did not show evidence of association ($p = 0.9$, $p = 0.8$ respectively). It has been suggested by others that linkage disequilibrium around *APOE* can only be detected at very short intervals around *APOE*.[29] We did observe suggestive association at D19S571, which has been linked to AD previously.[15] Given its relatively close location to the *APOE* locus (15 cM), association at this marker is ascribed to *APOE*. Marker D19S210 at a distance of 16 cM from D19S571, however, showed association only in individuals without *APOE ε4*. This suggests the possibility of another locus for AD on chromosome 19 in this population.

Interestingly, a number of markers showed a difference in allele frequency distributions depending on *APOE ε4* status. The 1p32 region especially, showed

strong association in those without *APOE ε4*. Association to 1p36 has been observed in the Finnish study, with no evidence for a modifying role of *APOE ε4.* [29] We also detected association at a second region on chromosome 1, near the location of *NCSTN*, the gene encoding Nicastrin. Previously, we found that this gene may be involved in familial early-onset AD, especially in those patients without *APOE ε4.*[39] *APOE ε4* negative late-onset patients were at increased risk at this locus in the present study as well, but the difference in odds ratio between *APOE ε4*+ and *APOE ε4*- individuals was not statistically significant. On chromosome 12 an association between AD and a marker (D12S345) located at ~14 cM distance of *LRP*, only became apparent after stratification by *APOE ε4* in those without APOE *ε4*. It is difficult to interpret this finding that occurred only after stratification, as stratified analyses are susceptible to false positive findings. This marker is located in a region implicated in AD by others,[19] and an association in *APOE ε4* negative individuals only has been reported before, making a true association more likely.[38]

In the present genome-wide search we found no evidence for association on chromosome 10 at the region including the candidate gene *IDE*. Most evidence on chromosome 10 pointed towards a region more closely located to *CHAT*, a candidate gene of the acetylcholinesterase pathway. Several studies have reported conflicting results on the role of *CHAT* in AD. [23, 40, 41] In our study we cannot exclude a role of *CHAT*.

Despite exclusion of patients with fronto-temporal dementia, we found evidence for association in the *MAPT* region on chromosome 17, which only appeared present in *APOE ε4*+ AD patients. Several studies have addressed the role of *MAPT* in AD. A *MAPT* mutation, Arg406Trp, is known to present clinically with early-onset AD.[42] However, although some studies have reported association of a *MAPT* polymorphism or haplotype in limited subsets of AD patients, e.g. defined by male gender, early-onset disease[43] or *APOE ε4* status,[44, 45] other studies have been negative.[46, 47] Similarly, associations with the Saitohin gene (*STH*), located in the intron 9 of *MAPT*, have been inconsistent.[48] An alternative explanation for the observed association in the present study is that the AD-patient series might have included patients who actually had fronto-temporal dementia. Our AD-patient series did include several patients with possible AD who were related to patients with probable AD. It is however unlikely that the observed association of *MAPT* is due to inclusion of these patients, as the association was based on a vast majority of cases. Further, when we performed the analysis in patients with fronto-temporal dementia we found no significant evidence of association. Although numbers are small, this is in accordance with previous findings in 6 of these

patients, in whom a mutation screening of *MAPT* had been negative.[30]

Chromosome 12 was screened in a series of 16 patients with probable or possible Lewy body dementia, on the basis of previous data suggesting involvement of a locus on this chromosome,[38] for which *A2M* was put forth as a candidate gene.[26] We found evidence of an association to D12S336 in this series of Lewy body dementia patients, which is a marker adjacent to *A2M*. Correspondingly, in a candidate gene study on *A2M* and Lewy body dementia we also found evidence of association.[30] This warrants further study of Lewy body dementia in this population.

Recently, we detected significant evidence of linkage to chromosome 7 in a Dutch family segregating AD as an autosomal dominant trait (*Rademakers et al, submitted*). At one of the markers contributing to linkage (D7S798) we observed some evidence of association, although non-significant (p_{clump} = 0.08), in the present genome wide survey. Further, on chromosome 3 we recently observed evidence for a recessive locus on chromosome 3, based on 8 patients originating from the GRIP region (*see Chapter 3.2*). This region did not appear in the present association-based analysis. The explanation most likely lies within the fact that in the previous analysis we restricted ourselves to a genome screen in families with evidence for a recessive form of disease, which gave us the specific allele to study further in the total patient series.

A genome-wide association analysis increases the likelihood of spurious findings due to multiple testing. For several regions that were reported previously, however, we found association with AD significant at $p < 0.01$, making false-positive findings less likely. We observed association at a new locus on chromosome 15, and found several regions (1, 9, 10, 19) that have been implicated in numerous other studies on AD, underscoring the likelihood of susceptibility genes for AD at these loci.

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Chapter 4
General Discussion

This thesis describes an investigation of genetic susceptibility to Alzheimer's disease (AD). Several favourable qualities for gene finding, such as a high heritability[1] and evidence for the existence of as yet undetermined AD-influencing genes,[2] do not outweigh the difficulties encountered when studying the genetics of a complex disorder such as late-onset Alzheimer's disease, for which pertinent genes have already been identified.[3-6]

Our approach was that of reductionism. On the one hand, we aimed to create a level of homogeneity in the tangle of pathways that lead to the end point disease. We used classical approaches set in the background of a study population with a genetically more homogeneous composition. On the other hand, we aimed to gain information by studying intermediate phenotypes, such as cognitive functions (memory, executive control) and structural brain abnormalities (atrophy of hippocampus and amygdala). Further, we put special emphasis on one of the many pathways thought to be involved in late-onset AD. Below, the main findings presented in the chapters of this thesis will be discussed briefly, and put into perspective of each other and of previous findings.

Familial aggregation in a genetically isolated population

Clustering of a disease in relatives is an indication that shared genetic background might contribute to this disease. For AD, twin studies[7] and population-based studies[1] have shown familial aggregation. Even though the results are difficult to interpret due to late and insidious onset of AD and diagnostic imprecision, a genetic background for AD has long been accepted. For other dementias clustering is less well studied. In Chapter 2.1 we studied familial aggregation of dementia in a recently founded community included in the research program Genetic Research in Isolated Populations (GRIP). To study familial aggregation in this community we had access to a large genealogical database holding information on over 60,000 inhabitants of this area. The use of this database in the visualization of relationships that patients themselves are unaware of, allowed a highly unbiased estimation of familial aggregation by calculation of pair wise kinship coefficients. Recall bias, that is likely to occur in studies that are relying on interviews about family history, is avoided in this approach.

We observed familial clustering of AD in this population, more so in early-onset than in late-onset AD. For vascular dementia, there was no evidence of familial clustering. For Lewy body dementia patients the degree of kinship was as high as for early-onset AD patients, suggesting a strong genetic background. The reported association to the I-allele of the alpha-2 macroglobulin (*A2M*)

I/D polymorphism (Chapter 2.1) might explain part of the familial clustering of Lewy body dementia, but it is mandatory to conduct further genetic studies, for which the GRIP study will provide a powerful setting.

Even though kinship for late-onset AD patients was lower than for early-onset AD patients, late-onset AD patients still had a higher degree of kinship than cognitively healthy spouse-controls originating from the same population. Thus, the observation of a higher kinship in AD patients provided us with evidence of a genetic background of AD in the GRIP region. Although the pattern of inheritance was not formally studied by segregation analysis, in many families dementia appeared to segregate in an autosomal dominant pattern of inheritance, with multiple affected patients over several generations. However, a high kinship in several sib pairs was indicative of inbreeding. This suggested an underlying recessive mutation in a selected group of patients.

There is an increasing recognition that recessive mutations influence susceptibility to complex late-onset disease,[8] but the possibility of recessive components in the susceptibility to late-onset Alzheimer's disease has gained little attention, even though early segregation studies were compatible with recessive forms of disease.[9] In this light, it is of interest that somatic (and germ line) mosaicism was recently described, suggesting a novel molecular mechanism for AD.[10]

Only few studies have addressed the possibility of recessive models in AD. A genealogical study of AD in an inbred population in Canada observed that late-onset AD patients carrying an *APOE ε4* allele were, on average, more inbred than controls, but this estimate was largely driven by a few patients, whose parents were related at the first cousin level, which makes these findings difficult to interpret.[11] More convincing evidence for the presence of recessive effects in AD comes from a study in an inbred Israeli-Arab community.[12] The prevalence of AD in this community is high, despite low frequencies of *APOE ε4*. In a genomic screen performed in this community excess homozygosity was present at one locus, suggesting a recessive mutation.

Evidently, part of the reason why recessive disease models for AD have been underexposed is the difficulty to identify individuals at increased risk for homozygosity in outbred populations. In outbred populations, "isolated" cases of late-onset AD could be due to homozygosity for a common deleterious allele. In this light, studying the genetics of Alzheimer's disease in a population such as the GRIP population is advantageous. Because extensive genealogy of the entire population is available, inbreeding loops can be characterized. Although inbreeding is not necessarily a phenomenon of isolated populations, most founder populations with a limited number of ancestors show evidence

of inbreeding.[13] Thus, recently founded populations may elucidate the genetic architecture of Alzheimer's disease to a greater extent than would be possible in outbred study populations. In addition to this advantageous feature of genetically isolated populations, and the clearly beneficial decrease in genetic variation due to founder effects,[14] another merit is the opportunity to perform association based genome-wide case control studies to map genes.

In a recently founded population such as the GRIP population, which is founded in the 18th century, less meiotic events will have occurred since a founder introduced the mutation in the gene pool. Therefore, haplotypes shared around mutations will be relatively large, facilitating the detection of the mutation.[13] Indeed, in the GRIP population, linkage disequilibrium extends over up to 20 cM around a locus.[15]

Genetic susceptibility for Alzheimer's disease in the GRIP study

In chapter 3.2 and 3.3 we described the results of a genome-wide search in patients from the GRIP region. Based on the abovementioned properties of genetically isolated populations, we followed two approaches. In chapter 3.2 we availed ourselves of the potential to identify recessive mutations that might go undetected in outbred populations, by studying the affected offspring of consanguineous marriages and assuming a recessive mode of inheritance in our statistical model.

We used a genetic epidemiological tool to search for excess homozygosity at marker loci on haplotypes in six inbred patients and their affected relatives. Homozygosity for a haplotype shared by patients would be indicative of a recessive mutation underlying the trait. [16] Homozygosity mapping has the advantage that relatively few affected individuals are required. Genetic heterogeneity is reduced because a pair of closely related affected individuals will probably share the same mutation. We found evidence of linkage under a recessive model at chromosome 3. After typing extra markers at this initially large (90 cM) region, the maximum LOD scores were 3.9 at 3q22, and 3.2 at 3q26. The region with the highest LOD score was still of considerable size after typing extra markers, and contained 90 genes. Further refinement of this region is warranted, especially since this region has been reported in familial AD before.[17] The second peak on chromosome 3 (3q26) could be narrowed down to a considerably smaller size, but analysis of the haplotypes revealed a situation less straightforward than would be expected. Inspection of the genealogy had already revealed a remotely related sib pair. They were both homozygous for an identical haplotype (and thereby contributed most to the LOD score) at the second peak on chromosome 3 (3q26), suggesting a recessive mutation.

Several other patients were heterozygous for this haplotype. This would argue against a recessive mutation. Among possible explanations is that this is a spurious finding. Homozygosity is known to be susceptible to false-positive results, one reason being the underestimation of the level of inbreeding.[18] Multiple problems are encountered in genealogical studies that might lead to bias in the estimation of the level of inbreeding. With our genealogical database we were able to accurately assess the level of inbreeding, but of course, non-paternity several generations ago will not be apparent in inheritance checks of people alive. However, we showed segregation of the haplotype over many generations, and we found association of markers at the locus with AD in an independent sample from the GRIP area, arguing against a false-positive finding. A more plausible explanation lies in compound heterozygosity. Given the presence of a second distinct haplotype in the heterozygous patients, several mutations might have occurred at the same gene, combinations of which may lead to expression of disease. A similar situation exists in the *parkin* gene, which is involved in early-onset Parkinson's disease.[19] Recessive mutations were thought to cause this disease, but patients were identified in whom two single heterozygous mutations at different locations in *parkin* were sufficient to cause disease.[19] Two of the compound heterozygous patients in our study showed clinical signs of Lewy body dementia, possibly reflecting different mutational effects.

Due to computational limits of the software to perform homozygosity mapping,[20] we could only include 10 patients for whom the number of meioses by which their parents were related did not exceed 20. A considerably greater number of patients showed a lesser degree of inbreeding. These patients may still yield valuable information. Alternatively, these patients could be studied in a classical linkage analysis, assuming a recessive model but ignoring the level of inbreeding. However, this approach would be less powerful than homozygosity mapping.

In chapter 3.3 an association-based genome screen is described in 156 AD patients and 75 controls from the GRIP community. We detected association with AD at six chromosomal regions. Several of these loci have previously been identified, except for a locus on chromosome 15, that was significant in both Dislamb and Clump analyses, and was associated with the largest odds ratio obtained in this study. Two other studies have reported loci on chromosome 15. But since one of these loci was observed only in a subset of very-late-onset AD and was located more than 30 cM distant from our locus,[21] and the other locus did not reach statistical significance,[22] we are the first to provide convincing

evidence for a locus in our initial analyses.

As other loci with evidence of association in our study (on chromosomes 1, 9, 10,19) have been observed before,[12, 21-26] this implies that results from this isolated population may very well be extended to the "general" population. Of course, our findings remain to be confirmed, preferably in the general population, but the GRIP population has already proven to provide information that can be applied in other populations as well. The role of mutations in *DJ-1*[27] in early-onset recessive parkinsonism for example, has since its discovery in GRIP been confirmed worldwide.[28, 29] Similarly, mutations in *SLC11A3* causing autosomal dominant hemochromatosis[30] have been identified outside the GRIP population as well.[31-33]

In a small series of patients with Lewy body dementia, for whom we had previously established strong familial clustering and association to *A2M* (Chapter 2.1), we now obtained association at a marker adjacent to *A2M*. This association has been reported before in other populations.[34, 35] Even though the association requires further investigation in GRIP, this again illustrates the likelihood of a more widespread implication of the observations done in the GRIP study. Furthermore, as the association of Lewy body dementia to both *A2M* and its adjacent marker is not likely to be coincidental, our findings underscore that GRIP offers a powerful setting for association studies, even in small patient series.

Of further interest in our genome screen was the association we observed at a marker > 30 cM distant from *APOE* on chromosome 19. A marker more closely located to *APOE* showed an association exclusively in *APOE ε4+* patients, probably reflecting *APOE*. This has been reported in other studies as well.[23, 36] The marker >30 cM distant from *APOE*, however, showed association only in *APOE ε4* negative patients. This is indicative of a distinct locus underlying the association on chromosome 19.

We obtained similar results at chromosome 19 in a linkage study of a multiply affected Dutch family (Family 3355) living in the eastern part of the country, including eight patients with a mean onset of 64 years (*Arias-Vasquez et al, manuscript in preparation*). A full genome screen was performed using 420 markers (spaced 10-12cM). In order to gain additional phenotypic information, we measured Amyloid beta 42 (Aβ42) levels, using an enzyme immunoassay (Innogenetics, Ghent, Belgium) in 15 non-affected relatives who were still at risk of AD. Aβ42, which plays a central role in the pathogenesis of AD (see Chapter 1.1), is increased in plasma of people who later on develop AD.[37] It has previously been used as an intermediate phenotype for AD, leading to the fine-mapping of the α-T-catenin gene to a quantitative trait locus on

chromosome 10.[38]

We performed a non-parametric linkage analysis, for which we defined our disease set in two ways. Our first analysis (Model I) included only AD patients, the second analysis (Model II) included AD patients plus 4 at-risk relatives with Aβ42 levels over 205 pg/mL. Model I yielded NPL scores of 1.92 at D19S902, and 1.92 at D19S571, which increased to 2.91 and 3.09 respectively in Model II. To bring to light if linkage was attributable to *APOE*, in a second phase we included *APOE* genotypes as a marker in the analyses. The NPL score at *APOE* was 0.37 in Model I. After inclusion of the *APOE* genotype, the NPL scores at D19S902 and D19S571 changed to 1.64 and 1.66 respectively in Model I and 1.95 and 1.94 in Model II. The marginal change in Model I as compared to Model II suggests that *APOE* cannot fully account for the linkage observed on chromosome 19 in patients, corresponding to what we observed in Chapter 3.3, but has a more pronounced effect on Aβ42 levels. The physiological relationship between *APOE* and Aβ42 is very complex. Several possible mechanisms by which apolipoprotein E might enhance Aβ deposition have been previously described. Apolipoprotein E might be internalised by neurons, inducing lysosomal accumulation of Aβ42 and amyloidogenic APP fragments. Another mechanism of action is extra cellular: apolipoprotein E-containing lipoproteins are shut in by Aβ42 deposits, mobilizing soluble Aβ peptides. Intra and extra cellular mechanisms may operate at different stages of the pathogenesis of AD, and suggest a chaperone-like function for the apolipoprotein E molecule.[39]

Of interest, in the analysis of family 3355 including only AD patients, an NPL score of 1.86 was obtained at 1p32 (D1S255), which lies in between two markers (D1S234 and D1S2797) that gave evidence of association in the study described in chapter 3.3. Moreover, on chromosome 7 an NPL score of 2.12 was obtained in Model I, at marker D7S2465 in family 3355. This marker lies 12 cM upstream of marker D7S798, suggestive of association in GRIP as discussed in chapter 3.3. Significant evidence of linkage was obtained at this region in another Dutch family with autosomal dominant AD (*Rademakers et al, submitted*).

The role of vascular genetic susceptibility

Cognition and Alzheimer's disease

The clinical endpoint AD is a synthesis of many, mostly undefined intermediate phenotypes with undefined effect sizes. These intermediate phenotypes, in turn, are not likely to be monogenic, but at least they will be more directly under influence of the genetic (and environmental) risk factors eventually affecting the clinical endpoint. Studying the genetics of an intermediate phenotype might thus contribute to the understanding of the etiology of complex diseases. Since AD is clinically characterized by decline in memory and other areas of cognition, neuropsychological measures of cognitive function may be suitable as intermediate phenotype for AD as well, with the caveat that they are under influence of many modifying factors, amongst which are intelligence, depression, and attention. Similar to genetic studies in clinical endpoints, intermediate phenotypes are only valuable in the study of genes, when they are shown to be heritable. Significant estimates of heritability of cognitive function have been observed in large twin cohorts.[40-46] Our study of heritability of cognitive function, as presented in chapter 2.2, differed from previous studies in two ways. First, our study population did not exist of twins, but included 500 healthy middle aged and elderly participants of the Erasmus Rucphen Family (ERF) study, who could all be connected to a common ancestor in the GRIP region. The heritability estimates we obtained based on a large variety of relative pairs were comparable to the estimates reported in twin studies. Second, we went one step beyond by studying vascular factors that might in turn be intermediate factors for cognitive functioning at later age, or give evidence of pleiotropic effects. This research is a first step in the exploration of the role of genetics of vascular pathology in AD.

The rationale to study the effect of vascular factors on cognition is based upon observations of increased memory impairment and impaired executive control in patients with vascular disease.[47-52,53] Further, increasing evidence associates vascular disease with AD,[54, 55] but the mechanism of association is as yet unclear. Numerous hypotheses exist to explain the mounting evidence of association. Vascular disease and AD can of course co-exist in elderly people in the absence of causation, but vascular disease could serve a direct causal function through decreased perfusion of the brain, thereby hampering the energy supply to brain tissue and subsequently causing neuronal death.[56] Cognitive impairment through vascular disruption of neuronal circuits may simply expose incipient AD at an earlier age, but vascular disease might also cause secondary acceleration of pre-existing Alzheimer-pathology through

ischaemia and free radical formation.[57] These observations have fuelled the debate about the distinction or similarity between AD and vascular dementia.

In our study of familial aggregation (Chapter 2.1) we compared pair wise kinship of patients with vascular dementia to patients with AD and concurrent vascular disease. We observed that AD patients with concurrent vascular disease had higher pair wise kinship coefficients than vascular dementia patients. This observation suggests that there is at least a distinction in etiology between vascular dementia and AD at the genetic level. The absence of familial aggregation of vascular dementia in GRIP raised the question if we could observe a contribution of vascular factors to the heritability of cognitive functioning at older age. We observed a relation between a vascular composite score and memory and executive function, but there was no evidence that vascular disease, or a vascular trait such as blood pressure, cholesterol, intima media thickness, or glucose levels, did affect the observed heritability estimates of cognition (see chapter 2.2). Genes underlying the variance in vascular traits are therefore not likely to also account for a large part of the variance in cognitive function later in life. This argues against both an intermediate effect of vascular risk genes on cognition, and against pleiotropic effects.

A study of a candidate gene

To further explore the genetics of vascular pathology in AD, we assessed the nature of association between AD and an often-studied candidate gene that is implicated in vascular physiology and disease as well (Chapter 3.1). We studied the candidate gene *ACE,* encoding angiotensin converting enzyme, in relation to Alzheimer's disease in a different, outbred study population (The Rotterdam Study). *ACE* was postulated as a candidate gene for AD mainly because of its central role in vascular homeostasis. Interestingly, whereas the D-allele in this gene is associated with vascular disease,[58] numerous independent studies[59-63] and two meta-analyses found the I-allele, instead of the alleged deleterious D-allele, to be more frequent in patients with AD.[64, 65] As discussed in chapter 3.1, there is sufficient reason to believe that ACE can exert a direct action on brain areas implicated in AD, for example through its role in Aβ metabolism[66]. To investigate the nature of the association between the I/D polymorphism in *ACE* and AD, we decided to consider vascular risk factors in our analyses, an approach that had not been followed before. We observed an association of the I-allele with increased risk for AD, in the absence of vascular risk factors. This suggests a pathogenic relation between ACE and AD that is not vascular in origin. This finding is in line with our observations in the studies on familial aggregation as described in chapters 2.1 and 2.2. Even though

vascular factors undeniably are associated with AD and cognitive impairment, our data consistently show that shared genes are not likely to fully explain this association.

The challenge of candidate gene studies

The risk estimate observed in our study on the *ACE* I/D polymorphism as well as in other studies was small (HR 1.12 (0.99-1.25) overall and HR 1.24 (1.08-1.41) for women only; see chapter 3.1). When studying a complex, most likely polygenic disorder, this is however to be expected. Ancient risk alleles like *APOE ε4* may have an important influence on disease liability, but more often high allelic diversity will reduce the contribution of the individual genes to the susceptibility to disease, especially for variants that are subject to natural selection, like *ACE*.[67] The challenge for a late-onset disorder like AD may be to find genes with such a small effect. Arguably, one of the reasons why association studies for AD have been so inconclusive is that small effects are often not significant, and are thus prone to false negative as well as to false positive outcomes. As for *ACE*, previous, predominantly small studies have indeed generated conflicting results. Meta-analyses exhibit ethnic heterogeneity in these studies,[64, 65, 68] possibly influenced by ethnic differences in patterns of LD with the true causal variant, or by differences in environmental background of diverse populations. Further, considerable selection pressure is likely to exist, as *ACE* is associated with fatal conditions like cardiac disease and renal failure and with longevity.[58, 69, 70] Consequent selection bias and confounding by unexplored risk factors will further increase the chance of spurious associations in cross-sectional studies. The Rotterdam Study provides a more favourable setting for candidate gene studies because of its large size and prospective nature. Moreover, extensive data on numerous phenotypes and risk factors are available to control for confounding factors. We adjusted our study of *ACE* for vascular risk factors, and showed an increased risk of AD in the absence of vascular disease, thereby showing that the *ACE* gene confers risk, at least partly, through a different pathway leading to AD.

Another strategy to avoid false acceptance or rejection of results is to obtain independent replication. We studied the association of the insertion/deletion polymorphism in *ACE* with intermediate phenotypes of AD and cognitive impairment on MRI in healthy participants of the Rotterdam Scan Study.[54] Again, vascular pathology such as silent infarcts and white matter hyperintensities showed no association, but early AD-related pathology (atrophy of brain structures such as hippocampus and amygdala[71]) did show an association.

Future prospects

Breakthroughs in the genetics of AD in the past have led to advances in the development of therapeutic strategies. Genetic research pointed out a central role of amyloid β in the cascade leading to dementia, which offered the clues to develop immunization against Alzheimer's disease.[72-75] Unfortunately, although animal studies were very promising, trials in humans have been halted because of serious side effects. Other (partly) available therapeutic interventions, such as the cholinesterase inhibitors, still offer no more than a stay of execution.[76] At present no well-documented preventive measures exist for this devastating disease, let alone a cure.

Although we should not be pessimistic for the future, genetic research still is indispensable to provide better understanding of the etiology of AD. New insights in molecular genetics, through the detection of novel genes and their specific mutations, may further elucidate how we have to intervene in the cascade leading to AD.

Despite an enormous effort worldwide, bearing a wealth of putative associations, many observations cannot be replicated.[77] Reasons like confounding and small relative risks have already been discussed in detail. In addition, it is of concern that most candidate gene studies only focus on a single variant, with little attention to the properties of the variant studied, such as the type of mutation or polymorphism, or allele frequency.[78] As can be learned from the emerging information on SNPs and their function in haplotype blocks, properties such as allele frequencies and background marker correlation are essential in the interpretation of study results and warrant more attention in candidate gene studies.[79]

In this light, much can be expected of large-scale SNP projects[80] and haplotype block mapping efforts,[81-83] that allow association studies in general populations as well. At highly dense spacing, SNPs can be used to detect small haplotypes around disease mutations. Still, the immense amount of SNPs required (estimates range from over 100.000 to even over 1.000.000 SNPs[84]), the unfavourable distribution of allele frequencies of these SNPs[85, 86] and the low relative risks associated with a disease genotype[84] will require large sample sizes to give sufficient power to detect mutations at genome-wide significance level. It is prudent to select SNPs based on their location,[87] in coding regions that alter or terminate amino acid sequence, disrupt splice sites or occur in promoter regions, or in regions highly conserved over species that are thus more likely implicated in disease.[88] The development of databases covering

information on physiological pathways to disease and all genes and proteins involved in these pathways, will further enable a careful design of interaction studies.[89-92]

One of the merits of genetic epidemiology has been and will be to provide clues for further therapeutic research. It is therefore our responsibility to watch against spurious associations. Sound study design, stringent significance criteria and independent replication should prevent false-positive results, but given the low relative risks of individual risk factors in polygenic disease, we should also put more emphasis on avoiding false-negative results. We should not hesitate to perform analyses in subgroups (if sample size allows), to test multiple intermediate phenotypes, to test haplotypes instead of single polymorphisms, and in particular we should pay more attention to epistatic effects and confounding by environmental factors.

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Chapter 5
Summary

Alzheimer's disease (AD) is the most common cause of dementia in the western world. The disease is clinically characterised by insidious onset and slow progression of cognitive decline. Old age and genetic predisposition are the most important risk factors for AD. With the ageing of western society, AD will create an increasing burden for health in the next decades. By the year 2025, over 22 million patients with dementia are expected around the world. In Chapter 1.1 a review of the epidemiology and genetics of Alzheimer's disease is given.

In chapter 2, two studies are described focussing on familial aggregation. Both studies were performed in a recently founded, genetically isolated community in the southwest of the Netherlands that was part of the research program Genetic Research in Isolated Populations (GRIP). We ascertained 191 patients with various forms of dementia. Availability of extensive genealogy of this community allowed accurate assessment of familial aggregation. In Chapter 2.1, familial aggregation is studied, not only of Alzheimer's disease, but also of other forms of dementia for which patterns of aggregation are less well known. In addition, various known genetic risk factors were studied to assess to what extent they could explain the occurrence of dementia in this population. We found a high degree of relationship between patients in this isolated population, especially in early-onset AD and Lewy body dementia. Patients with late-onset AD showed strong evidence of familial aggregation as opposed to vascular dementia. This implies a difference in genetic make up between Alzheimer's disease and vascular dementia. A considerable proportion of patients had a family history compatible with autosomal dominant disease, but consanguinity in parents of several AD patients suggested an underlying recessive mutation as well. Mutations in known genes causing autosomal dominant disease (*APP, PSEN-1, PSEN-2* and *MAPT*) were unlikely to explain dementia in this population. We did find evidence for a significant association between Lewy body disease and *A2M*. The frequency of *APOE ε4* was high, resulting in an attributable risk of 45% for AD. This suggested that this allele is important as a determinant and/or a modifier of disease in the isolate. Still, a large portion of AD remains unexplained, asking for further research on the genes involved in this type of dementia.

In chapter 2.2, a study on heritability of cognition is presented. This study was performed in the same community as described in chapter 2.1, but this time, the study population consisted of participants of the Erasmus Rucphen Family (ERF) study. Those participants were selected based on their genealogy, regardless of their health status. The first 500 participants who were aged 50 years or over were included in the analyses described in chapter 2.2. In the

ERF study, extensive phenotypic information is collected, not only on cognitive performance, but among others, also on vascular disease status. Using these data we addressed the question whether the association between vascular disease and poor cognitive function at later age might be caused by shared genes. Heritability estimates for immediate and delayed recall, recognition, semantic fluency and the Stroop test were significant, with estimates ranging from 0.19 to 0.38. Except for immediate recall and the Stroop test, we observed no significant effects of vascular factors on average cognitive functioning. Heritability estimates did not change significantly when adjusted for vascular disease, and we found no genetic correlation between cognitive functions and vascular traits. This suggests that, at least in the ERF study, in individuals with vascular disease, vascular risk genes do not contribute to the genetic variation in cognitive function at later age.

In chapter 3 the genetic studies are presented. The first part (Chapter 3.1) again focuses on the relationship between Alzheimer's disease and vascular disease, by studying the association between AD and a polymorphism in a candidate gene (Angiotensin Converting Enzyme gene (*ACE*) I/D polymorphism) that is also implicated in vascular disease. This study was performed in the Rotterdam Study, a large prospective population based study, which also offers access to extensive phenotypic information. The large sample size of the Rotterdam Study, its prospective nature and the availability of data on vascular risk factors allowed a different approach than had been followed before in numerous conflicting studies. In addition, we studied the association between the *ACE* I/D polymorphism and several brain abnormalities, either related to preclinical AD- or vascular pathology, on MRI data that were obtained through the Rotterdam Scan Study. We observed that homozygosity for the I-allele conferred a slightly increased risk of AD compared to carrying a D-allele (RR 1.12 (95% CI 0.99-1.25)). This increase was only significant in women, and independent of vascular factors (RR 1.39 (95%CI 1.14-1.69)). In accordance, non-demented women with the II genotype had smaller hippocampal and amygdalar volumes, which can be considered as a preclinical indication of increased risk for AD. Vascular brain abnormalities, such as white matter hyperintensities and infarcts, were not significantly associated with the *ACE* I/D polymorphism. This suggests a modest but significant increase in risk of AD and early AD pathology in women homozygous for the *ACE* I-allele independent of vascular factors.

In Chapters 3.2 and 3.3 two genomic screens are presented that were performed in the GRIP study. The first genomic screen is performed in five inbred families that could all be connected to a common ancestor. Following the

hypothesis that recessive mutations might play a role in late-onset neurological disease, and that such mutations can be more easily identified in inbred multiply affected families, we analyzed the genome screen using homozygosity mapping. After fine mapping of a large region on chromosome 3 with evidence of linkage under a recessive model, we obtained a LOD score of 3.2 on 3q26, and a LOD score of 3.9 on 3q22. The peak with the highest LOD score extended over a region with a physical size of 9 Mb, containing 90 genes. Further fine-typing is needed at this region. The second peak (3q26) was considerably smaller (3.87 Mb, including 13 genes). One of the genes in this region was Butyrylcholinesterase (*BCHE*). Sequencing of the coding exons revealed no mutations. An association analysis of markers at this peak revealed evidence of association in a larger, independent sample from the GRIP region, suggesting a new locus for AD at chromosome 3.

Chapter 3.3 describes the results of a genome screen in all AD patients of the GRIP study. Given the presence of strong linkage disequilibrium in GRIP, we analysed the data using an association-based approach. Single marker association analyses were performed using Dislamb and Clump software. We detected association in 6 regions (D1S2797, D10S196, D15S127, D17S1857 and D17S798, and D19S210) at a significance level of $p < 0.01$. We were the first to obtain evidence of association on chromosome 15 in our initial analysis. The locus on chromosome 1 seemed to confer risk especially in those patients without an *APOE ε4* allele. D19S210 was only associated with increased risk for AD in *APOE ε4* negative cases, suggesting a locus other than *APOE* on chromosome 19.

Finally, in chapter 4, all findings are put into perspective and future prospects are discussed.

De ziekte van Alzheimer (AD) is de meest frequent voorkomende vorm van dementie in de westerse wereld. De ziekte wordt klinisch gekarakteriseerd door een sluipend begin en gestage achteruitgang van cognitieve functies. Oudere leeftijd en genetische predispositie zijn de belangrijkste risicofactoren voor het krijgen van AD. Door de vergrijzing van de westerse samenleving zal AD in de komende decennia een toenemend gezondheidsprobleem vormen. Er wordt verwacht dat rond het jaar 2025 22 miljoen mensen ter wereld de ziekte van Alzheimer zullen hebben. In hoofdstuk 1.1 wordt een overzicht gegeven van de epidemiologie en van de genetica van de ziekte van Alzheimer.

Hoofdstuk 2 bevat twee studies die zich richtten op familiaire aggregatie, ofwel het clusteren van bepaalde eigenschappen of aandoeningen in families. Dit kan een indruk geven van een eventuele genetische achtergrond van dergelijke eigenschappen of aandoeningen. Beide studies zijn uitgevoerd in een recent ontstane, genetisch geisoleerde gemeenschap in het zuid westen van Nederland. Deze gemeenschap vormt de basis van het onderzoeksprogramma Genetic Research in Isolated Populations (Genetisch onderzoek in geisoleerde populaties; GRIP). De beschikbaarheid van uitvoerige genealogie maakt deze gemeenschap uitermate geschikt voor het betrouwbaar onderzoeken van familiaire aggregatie.

Aan de hand van 191 patienten met dementie bestudeerden we in hoofdstuk 2.1 de familiaire aggregatie van de ziekte van Alzheimer, maar ook van andere vormen van dementie. Voor andere vormen van dementie is daarover namelijk minder bekend. Naast familiaire aggregatie bestudeerden we de rol van reeds met dementie geassocieerde genen. Aan de hand van de genealogische gegevens vonden we dat patienten een hoge mate van onderlinge verwantschap hadden. Dit gold met name voor patienten met de vroege vorm van de ziekte van Alzheimer en patienten met een vorm van dementie die histologisch wordt gekenmerkt door Lewy lichaampjes (dementie met Lewy lichaampjes). Maar ook patienten met de late vorm van Alzheimer hadden nog altijd een hogere mate van verwantschap dan gezonde leeftijdsgenoten uit de zelfde gemeenschap. Bovendien toonden we aan dat patienten met de late vorm van Alzheimer een hogere mate van onderlinge verwantschap hadden dan patienten met vasculaire dementie. Deze bevinding is interessant in het licht van de lopende discussie over het al dan niet bestaande onderscheid tussen beide aandoeningen, en duidt op een verschil in genetische achtergrond. Een aanzienlijk aantal patienten had een familie anamnese die pastte bij een autosomaal dominante aandoening, maar bloedverwantschap in ouders van enkele patienten met de ziekte van Alzheimer suggereerde dat ook een

recessieve mutatie aanwezig zou kunnen zijn in de GRIP populatie. Mutaties in bekende genen die autosomaal dominante dementie kunnen veroorzaken (*APP, PSEN-1, PSEN-2, MAPT*) bleken niet verantwoordelijk voor het voorkomen van dementie in GRIP. Ook een associatie tussen de ziekte van Alzheimer en regio's op chromosoom 10 en 12, en twee daar gelocaliseerde kandidaatgenen (*IDE* en *A2M*), was in GRIP niet aantoonbaar. Wel vonden we significante associatie tussen dementie met Lewy lichaampjes en *A2M*. The frequentie van *APOE ε4* was hoog, waardoor het een attributief risico van 45% voor de ziekte van Alzheimer gaf. Dit suggereerde dat het een belangrijke determinant voor ziekte is in de GRIP gemeenschap. Toch kan het een groot deel van het voorkomen van de ziekte niet verklaren, waardoor verder onderzoek gewenst is.

In hoofdstuk 2.2 wordt een "heritability" of overervingsstudie beschreven. Een overervingsstudie kan worden beschouwd als een studie naar de familiaire aggregatie van een (kwantitatieve) eigenschap in plaats van, zoals beschreven in 2.1, een kwalitatieve uitkomst zoals ziekte. We bestudeerden de overerving van verschillende cognitieve functies in de GRIP populatie, zoals onmiddellijk en uitgesteld geheugen, aandacht, concentratievermogen, herkenning, mentale flexibiliteit en interferentieverschijnselen. In tegenstelling tot het vorige hoofdstuk onderzochten we dit keer niet de patienten van de GRIP gemeenschap, maar 500 (gezonde) inwoners van 50 jaar of ouder, die deelnamen aan de Erasmus Rucphen Familie (ERF) studie die in de GRIP gemeenscap wordt uitgevoerd. In de ERF studie wordt elke deelnemer uitvoerig medisch onderzocht door bijvoorbeeld echo-onderzoek van de halsvaten, bloeddruk meting, en chemisch bloedonderzoek. Hierdoor hadden we niet alleen beschikking over neuropsychologische testresultaten, maar ook over vasculaire risicofactoren. Door middel van deze gegevens konden we onderzoeken of de associatie tussen vasculaire aandoeningen en verminderd cognitief functioneren op oudere leeftijd veroorzaakt wordt door een gezamenlijke genetische achtergrond. Voordat we op deze vraag ingingen bewezen we eerst dat in ERF een aanzienlijk deel van de variatie in cognitief functioneren verklaard kon worden door overerving. Dit varieerde van 19% voor uitgesteld herinneren tot 38% voor het onmiddellijke geheugen, beide getest door de 15 woorden test. Ook het deel van de variatie van de Stroop test (voor kleur-woord interferentie) dat verklaard wordt door genetische factoren was aanzienlijk (36%).
Daarna testten we de associatie tussen de cognitieve functies en vasculaire risicofactoren. We toonden aan dat alleen op het onmiddellijke geheugen en de Stroop test slechter gescoord werd wanneer de deelnemer vasculair belast was. Bij de overervingsanalyses bleek vervolgens dat vasculaire aandoeningen zoals hoge bloeddruk, suikerziekte, atherosclerose en te hoog cholesterol

geen invloed hadden op de genetische variatie in cognitief functioneren. Het zelfde konden we ook aantonen als we bloeddruk, glucose, cholesterol etcetera als kwantitatieve uitkomsten bestudeerden in relatie tot de cognitieve eigenschappen. Dit pleitte tegen een gedeelde genetische achtergrond.

Hoofdstuk 3 bevat drie genetische studies. In het eerste deel (hoofdstuk 3.1) gingen we wederom dieper in op de relatie tussen de ziekte van Alzheimer en vasculaire risicofactoren. We onderzochten de associatie tussen AD en het insertie/deletie (I/D) polymorfisme in het kandidaatgen *ACE* (Angiotensin Converting Enzyme), dat ook betrokken is bij vasculaire aandoeningen. Deze studie voerden we uit binnen de Rotterdam studie, een groot prospectief populatie onderzoek, waarin ook uitgebreide medische informatie beschikbaar is over elke deelnemer. Hierdoor konden we een andere aanpak volgen dan in eerdere, conflicterende studies mogelijk was. Als eersten onderzochten we of de associatie tussen AD en het *ACE* gen bestaat ten gevolge van een intermediaire rol van vasculaire aandoeningen, of juist onafhankelijk daarvan is. Om onze bevindingen te bevestigen bestudeerden we ook de relatie tussen het *ACE* gen en verschillende afwijkingen die zichtbaar zijn op MRI van de hersenen. Hierin maakten we onderscheid tussen preklinische Alzheimer-gerelateerde afwijkingen, zoals kleine volumina van hippocampus of amygdala, of vasculaire pathologie, zoals witte stof afwijkingen en herseninfarcten. Homozygotie voor het I-allel bleek geassocieerd met een licht verhoogd risico om AD te krijgen (RR 1.12 (95% CI 0.99-1.25)). Het risico was alleen in vrouwen significant verhoogd, en we toonden aan dat de associatie onafhankelijk was van vasculaire factoren (RR 1.39 (95% CI 1.14-1.69)). Vrouwen zonder dementie die homozygoot waren voor het I-allel bleken bovendien significant kleinere hippocampus en amygdala volumina te hebben. Vasculaire pathologie op MRI was niet geassocieerd met het *ACE* I/D polymorfisme. Uit deze bevindingen blijkt een bescheiden maar significant verhoogd risico op het krijgen van dementie in vrouwen die homozygoot zijn voor het *ACE* I-allel, onafhankelijk van de invloed van vasculaire factoren.

In hoofdstuk 3.2 gingen we verder op onze eerdere bevinding van bloedverwantschap in ouders van enkele patienten met AD. We voerden een koppelings analyse uit op volledige genomische data van 10 patienten uit 5 families met bloedverwantschap, en vonden koppeling van een grote regio op chromosoom 3 met AD onder een recessief model. Nadat we nog 10 extra genetische markers in deze regio hadden getypeerd, bleek dat de regio uit twee pieken bestond. De eerste bereikte een maximale LOD score van 3.9 op 3q22, maar bestreek nog steeds een afstand van 9 Mb, en bevatte 90 genen. Het zou voorbarig zijn hieruit conclusies te trekken. Verdere verfijning van de regio

is nodig. De tweede piek bereikte een maximale LOD score van 3.2 op 3q26, en bestreek een kleinere regio van 3.87 Mb, waarin 13 genen te vinden zjin. Een van deze genen is butyrylcholinesterase (*BCHE*), een gen dat al eerder is geassocieerd met AD. We doorzochten de hele (exonische) DNA volgorde van dit gen in de patienten en een gezonde partner, maar vonden geen variaties in de DNA code die dementie zouden kunnen veroorzaken. Wel toonden we aan dat twee van de markers in de tweede regio geassocieerd waren met AD in een onafhankelijke groep patienten en controles uit de GRIP gemeenschap. Onze bevindingen duiden op een nieuw locus voor AD op chromosoom 3.

In het laatste hoofdstuk van de genetische studies (hoofdstuk 3.3) beschrijven we een associatie analyse uitgevoerd op de volledige genomische data van 156 patienten en 75 cognitief gezonde mensen uit de GRIP gemeenschap. In recent ontstane, genetisch geisoleerde gemeenschappen als de GRIP populatie zijn de genomische regio's waarover koppelings- (of 'linkage') disequilibrium tussen genetische markers en ziekte loci bestaat groter dan in de algemene populatie. Dit maakt een associatie analyse van volledige genomische data efficienter. Door middel van twee statistische programma's voor associatie analyse (Dislamb en Clump) vonden we aanwijzingen voor associatie ($p < 0.01$) in 6 genomische regio's (D1S2797, D10S196, D15S127, D17S1857 en D17S798, en D19S210). Wij waren de eersten die in initiele analyses aanwijzingen voor een locus voor AD op chromosoom 15 vonden. De regio op chromosoom 1 leek alleen in patienten zonder *APOE ε4* geassocieerd te zijn met AD, evenals de genetische marker op chromosoom 19 (D19S210), wat erop duidde dat een ander locus dan *APOE* op chromosoom 19 bestaat.

Ten slotte worden in hoofdstuk 4 alle bevindingen besproken, en in perspectief geplaatst. Vooruitzichten voor het voortzetten van genetisch epidemiologisch onderzoek naar de ziekte van Alzheimer worden gegeven.

...

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About the author

Kristel Sleegers was born June 23rd, 1975 in Geldrop, The Netherlands. After she graduated from the 'Lorentz Lyceum' (Gymnasium β) in Eindhoven in 1993, she commenced her medical training at Leiden University. During her medical training she took part in clinical research projects at the Departments of Psychiatry and of Neonatology at Leiden University Medical Center (LUMC), and she successfully completed a specialized academic training in Philosophy at Leiden University. She received the medical degree in 1999. She worked as a resident in Neurology and Emergency Medicine at the Holy Hospital, Vlaardingen from December 1999 until December 2000. In December 2000 she started the work described in this thesis at the Department of Epidemiology & Biostatistics of the Erasmus Medical Center, Rotterdam. In 2003 she obtained a Master of Science degree in Genetic Epidemiology at the Netherlands Institute for Health Sciences, Rotterdam. Since September 2004 she continues working in the field of Alzheimer research at the Department of Molecular Genetics, Flanders Interuniversity Institute for Biotechnology, at the University of Antwerp, Belgium.