Alzheimer’s disease

A genetic epidemiologic approach
The work presented in this thesis was conducted at the Genetic Epidemiologic unit, Departments of Epidemiology & Biostatistics and Clinical Genetics, of the Erasmus Medical Centre Rotterdam in close collaboration with the Flanders Interuniversity Institute for Biotechnology (V.I.B.), University of Antwerp (U.A.), Department of Biochemistry, Antwerpen, Belgium. Financial support for this study came from the Netherlands Organisation for Scientific Research (NWO), the NESTOR stimulation programme for geriatric research in the Netherlands (Ministry of Health, Welfare and Sports and Ministry of Education), the Netherlands Health Research and Development Council (ZON), the municipality of Rotterdam, the Fund for Scientific Research Flanders (Belgium; FWO-F), and DWTC Interuniversity Attractionpoles (IUAP).

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Alzheimer’s Disease

A genetic epidemiologic approach

De ziekte van Alzheimer

Een genetisch-epidemiologische aanpak

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op gezag van de Rector Magnificus Prof.dr.ir. J.H. van Bemmel en volgens besluit van het College voor Promoties.

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Papers and manuscripts based on the studies presented in this thesis

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Chapter 1

General introduction
Dementia is a frequent neurodegenerative disorder in the elderly with devastating consequences for both patients and caregivers. The most frequent cause of dementia in the elderly is Alzheimer’s disease (AD). AD is clinically characterised by an insidious onset of decline in memory and problems in at least one other area of cognition. It is a gradually progressive disease which will ultimately lead to a state of complete dependency. Because the disease mainly presents at old age, the prevalence of dementia and the health care costs associated with it will increase dramatically in the near future. In the Netherlands the estimated number of cases will increase to 250,000 by the year 2020.

To develop a rational therapeutic or preventive strategy for AD further insight in the pathophysiology is essential. Many studies have shown a strong familial clustering of AD which initiated the search for genes involved in the aetiology of AD in the early 80’s. The first AD gene, the amyloid precursor protein gene (APP), was identified not until 1991. Subsequently mutations in the presenilin-1 (PSEN1) and 2 (PSEN2) genes were identified as causes of AD. Mutations in these genes lead to an autosomal-dominant segregation pattern of the disease with a penetrance approximating 100%. However, mutations in these 3 genes are rare and only occur in families with early-onset AD (age at onset before 65 years). The E4 allele of the apolipoprotein E gene (APOE) only moderately increases the risk of developing both early and late-onset AD. However, because the E4 allele is frequent in the general population the impact on the occurrence of AD is larger than the impact of the other 3 AD genes. Despite these recent developments in the genetic epidemiology of AD it is clear that research on the genetics of AD is far from completed. Only a minor proportion of the occurrence of AD can be attributed to the 4 known AD genes.

The aim of this thesis was to identify new susceptibility genes for AD. For this purpose, two strategies were followed. First, a population-based approach using the candidate gene study was performed. A candidate gene is a gene suspected to be involved in the aetiology of a disease. This suspicion may rise because the gene product (the protein) is involved in the pathophysiology of the disease, or because the gene is homologues to another gene which is known to be involved in the aetiology of the disease. The probability of being involved in the disease will increase if the candidate gene is located in a region identified in a genomic screen. For the analysis of the candidate genes a case-control series of early-onset and a case-control series of
Chapter 1

late-onset AD patients were used. Secondly, a family-based approach was used to localise new AD susceptibility genes. Family-based studies are suitable for genomic searches. In a genomic search polymorphic markers equally distributed across the genome are genotyped. Classically these data are analysed with a linkage approach in which the co-segregation of the genetic markers with the disease in a family is analysed. Studies using this approach have been successful in identifying APP, PSEN1, and APOE. A drawback of this approach is that large families with multiple affected relatives are needed. In case of a late-onset disease such as AD these are hard to find. Furthermore, the mode of inheritance of the disease has to be defined which is only possible in the minority of AD families. Another method to analyse a genomic search is by association in which the frequency of marker alleles are compared between series of cases and controls. When family data are available in this case-control approach it is possible to combine linkage and association.

In chapter 2 the current knowledge on the genetic epidemiology of AD is reviewed. Population-based studies on several candidate genes for AD are described in chapter 3 case-control studies on polymorphisms in PSEN1, the tau gene (MAPT), the cystatin C gene (CST3), and the APOE promoter are presented. In chapter 4 family-based studies are described. First, the complex clinical picture of a family with a known mutation in APP was described in detail. As a novel strategy for future genomic screens, a genetically isolated population in the Netherlands is presented. Finally, two chromosomal regions identified in previous genomic screens are tested in this isolated population. In chapter 5 methodological issues regarding genetic case-control are discussed together with the main findings in this thesis and clinical relevance.
Chapter 2

Genetic epidemiology of Alzheimer’s disease

2.1 Introduction
2.2 Alzheimer’s disease diagnosis and subtypes
2.3 Epidemiology of Alzheimer’s disease
2.4 Familial aggregation
2.5 Genes involved in Alzheimer’s disease
2.6 Efforts to identify new AD genes
Chapter 2.1

Introduction

Dementia is a major health problem in the elderly. It is a syndrome characterised by impairment in intellectual functioning resulting in a distressing condition both for the patient and caregiver. Alzheimer's disease (AD) is the most common cause of dementia in western society. AD is clinically characterised by an insidious onset of decline in memory and problems in at least one other area of cognition. Additional characteristics are a gradually progressive course, a preserved level of consciousness, and absence of other conditions able to cause these symptoms. The pathological hallmark in brains of AD patients are extracellular plaques composed mainly of the amyloid-β peptide and intracellular neurofibrillary tangles containing hyperphosphorylated tau protein.⁹

In chapter 2 a review of the current knowledge on the genetic epidemiology of AD is given. First the diagnostic issues in AD will be discussed briefly. Secondly, the epidemiology of AD and the familial aggregation are discussed. Thirdly, known AD genes are described together with their estimated contribution to the occurrence of the disease in the general population. Finally, efforts to identify novel genes involved in AD are discussed in light of the developments in molecular biological research.
Chapter 2.2

Alzheimer’s disease diagnosis and subtypes

Although improved considerably, the clinical and pathological diagnosis of AD is still ambiguous. At the neuropathological level none of the characteristics, including the amyloid plaques and tau pathology, are unique as they can also be found in non demented subjects or patients with other neurodegenerative disorders.

The second most frequent cause of dementia is vascular dementia 10. Although both AD and vascular dementia have widely accepted criteria 11, 12, it is sometimes difficult to distinguish these subtypes clinically and even pathologically. Typically, vascular dementia is more acute in onset and has a stepwise progression, contrasting the gradually slow progression in AD. However, a considerable number of patients have features of both AD and vascular dementia. Subjects developing a slowly progressive cognitive decline after a vascular event, or subjects in whom the gradually cognitive decline is complicated by cerebrovascular disease, are difficult to classify exclusively as AD or vascular dementia. The situation is even more complicated by the fact that vascular pathology and its determinants may be risk factors for AD 13.

Another disease showing substantial overlap with AD is Parkinson’s disease. About 33 percent of patients with Parkinson’s disease suffer from dementia while AD patients often develop Parkinsonian symptoms 14. Further, the familial clustering of both diseases points to the overlap in clinic, pathology, and aetiology 15. At the level of pathology the distinction from idiopathic Parkinson’s disease is clear with cortical plaques and tangles in AD and Lewy bodies in the substantia nigra in Parkinson’s disease. However, there is ongoing debate on the existence of a distinct form of dementia, which is pathologically characterised by Lewy bodies but clinically presents as an AD like disorder 14.

In addition to problems in the differential diagnosis, genetic epidemiological research is further hampered by the fact that AD itself is a heterogeneous disorder. A subgroup of AD, which is often distinguished in clinical as well as research settings, is the early-onset form of AD. There is no agreement on the cut-off point to define early-onset. Although age 65 years is classically used, others prefer 50 or 70 years as a cut-off. Whatever cut-off is used, no consistent differences in clinical or
pathological features between patients with early and late-onset AD have been found. Another frequently used subdivision is autosomal-dominant, familial, and sporadic AD. The autosomal-dominant form of AD is rare, probably accounting for less than 5% in early and late-onset AD. The familial cases, i.e. cases with at least 1 affected first-degree relative, may explain about 30% of early-onset patients and about 20% of the late-onset. Thus, for both early and late onset AD, sporadic cases are most common. There are no clear differences in clinical presentation and pathological changes in the brain between dominant, familial, and sporadic AD, other than that autosomal-dominant and familial AD more often present at an earlier age than the sporadic form.
Chapter 2.3

Epidemiology of AD

The number of patients affected with AD (prevalence of disease) increases strongly with age. In figure 2.1, data on the prevalence of AD in 12 European studies have been pooled and re-analysed. Below age 70, less than 1% of the subjects in the general population suffer from AD. However, the prevalence increases exponentially with advancing age (see figure 2.1). By age 90, up to 30% of subjects are affected with AD. There are only minor differences in prevalence between men and women, with a slightly higher prevalence in women before age 90 years (see figure 2.1).

Figure 2.1 Age-specific prevalence of Alzheimer's disease from 6 pooled European studies for both sexes combined, men, and women.

Studies comparing geographical variation have yielded important clues for the aetiology of common disorders such as cardiovascular disease, cancer, and
osteooporosis. Since differences in prevalence of a chronic disorder as AD may be
determined for a large part by regional differences in mortality, it is preferred to
compare the number of newly diagnosed patients (incidence of disease). There is
considerable variation in incidence of AD between populations, in particular after age
75 years 2. However, there is no evidence for a geographical trend in incidence rates,
e.g. North-South trends in Europe that have been observed in cardiovascular disease
and cancer 2. The differences observed in AD are most likely due to methodological
problems, including a low number of subjects, non-response and competing mortality
in the elderly, and co-morbidity complicating the diagnosis of AD at old age.

There is some evidence for a difference between the relative proportion of AD and
vascular dementia between different populations 19. In Caucasian populations AD is
by far the most frequent type of dementia while in Asian populations vascular
dementia has been diagnosed in up to 60% of the dementia patients. Given the
similarity in incidence of AD in Asian and Caucasian populations, there may be a
higher frequency of genetic or environmental determinants for vascular dementia in
Asian populations. However, an alternative explanation may be that the differences in
subtypes of dementia are difficult to interpret due to the lack of biologic markers and
unique clinical features for these subtypes.

Putative risk factors for AD include thyroid disease, depression, vascular disease
and its risk factors, aluminium and head trauma 2. Potential protective factors include
hormone replacement therapy and anti-inflammatory medication 2. For most of these
factors the evidence is not convincing up to date as findings of studies have continued
to be contradicting 2. A major limitation of research of non-genetic factors to date is
the lack of longitudinal follow-up studies of AD 2. This type of study has proven to be
extremely valuable in cardiovascular and cancer research.

Perhaps the most important development in research of the epidemiology of AD
concerns the role of vascular factors. In recent years, there has been growing evidence
for a role of various vascular factors associated with a modest (up to 2-fold) increase
of AD. Vascular factors that have been implicated in the risk of AD include
hypertension, diabetes mellitus, atherosclerosis, estrogen replacement therapy, and
smoking 13, 20-29. Although few studies have addressed these factors in a long-term
follow-up approach, the hypothesis of vascular pathology modifying the risk of AD
offers a novel framework to study risk factors for and perhaps also prevention of AD.
The underlying biological model through which vascular factors may be involved in AD remains to be elucidated. These factors by themselves may have (small) additive effects. Together their effects may result in a load of vascular pathology, which influences the expression of AD. Indeed the risk of AD appears to be associated with the number of vascular (risk) factors. How the risk of AD is modified by vascular pathology is unclear. Vascular pathology may merely exaggerate already ongoing AD pathology in brain. The additional vascular pathology may compromise the brain further, leading to an earlier onset (and subsequently higher risk) of disease. However, it may be speculated that vascular pathology can be a primary cause of AD in a subset of patients.
Chapter 2.4

Familial aggregation

Several twin studies have been performed in the past. A study summarising the data of three twin-studies showed concordance rates in monozygotic twin pairs of 80% and 35% in dizygotic twins, resulting in a heritability estimate of 0.84. However, twin studies of AD are not straightforward in interpretation. A major problem is the AD diagnosis, which requires extensive clinical examinations. Differences may exist between medical centres in particular when twins were diagnosed in the past when the diagnosis was not yet standardised. Given the late and variable onset of AD, differences in onset age between twin pairs may lead to false concordant findings.

In support of twin research, epidemiological studies of risk in first-degree relatives also point to a strong familial clustering of the disease. However, also in these studies problems related to the diagnosis and age-related expression are encountered. In a re-analysis of 7 case-control studies, first-degree relatives of AD patients had a 3.5 fold increase in risk for developing AD. This relative risk decreased with increasing age at onset of the proband but even by age 80 years a statistically significant 2.6 fold increase in risk for first-degree relatives was observed. From these relative risks it can be calculated that at the population level up to 34% of the occurrence AD may be explained by familial factors. For clinical genetic practice, absolute risks for relatives of patients are relevant, which have been adjusted for censored observations given the age-dependent expression. The lifetime cumulative risk for first degree relatives of early and late-onset AD patients was found to be 39% compared to about 12% in first-degree relatives of controls.

With regard to the mode of inheritance, numerous extended families with multiple AD cases have been published. However, most families concern cases with an early-onset AD. The inheritance patterns in these families is predominantly autosomal-dominant. It is important to realise that these families are an atypical subgroup also within the early-onset AD group. Segregation analysis in 198 early-onset AD families indicated that patterns of familial clustering can be explained by transmission of a major autosomal dominant gene in less than 1% of the families.

Even in early-onset AD, the pattern of familial aggregation in the majority of the
Chapter 2.4

families fits a dominant gene with a multifactorial component (21%). In late onset AD the situation is technically more complex because of censoring of at risk individuals, small families, and incomplete family histories. In a segregation analysis comprising 400 late-onset families there was evidence for a single major gene with a population frequency of 1.5% 16. However, due to lack of power no distinction could be made between dominant, recessive, or additive models.

In conclusion, there is a substantial contribution of genetic factors to the aetiology of AD. However, heritability is difficult to assess in AD. Besides some early-onset AD families, no definite conclusion about the mode of inheritance can be drawn. In most cases, the disease appears to be of multifactorial origin with several genetic and environmental factors involved.
Chapter 2.5

Genes involved in Alzheimer’s disease

Autosomal-dominant mutations

Early genetic research focussed on early-onset families segregating AD in an autosomal-dominant pattern. Given the rare occurrence of the disease before age 65 (see figure 2.1) there is a high probability that there is a single gene underlying AD in these cases. Initial genetic research targeted chromosome 21 as a candidate chromosome because of the observation that trisomy 21 patients develop pathology identical to that observed in AD patients. Using linkage analysis, early-onset AD was found to be linked to the q11.2-q21.2 region on chromosome 21, which contains the gene encoding the Amyloid Precursor Protein (APP). Yet the first mutation in APP responsible for AD was not found earlier than 1991. Since then, eight different mutations have been found that cause autosomal-dominant forms of AD with an early onset. The APP gene consists of 18 exons but the mutations are all located in exon 16 and 17 that code for the βA4 amyloid, suggesting that either this is the functional region or mutations in the other regions are not compatible with life.

The most frequently described mutation is a valine to isoleucine change at codon 717. This mutation is found in 11 families while the others are described in one family only (AD mutation database: http://molgen-www.uia.ac.be/ADMutations/). APP mutations are not uniquely associated with AD. A mutation in codon 693 is associated with cerebral haemorrhages due to vascular depositions of amyloid, while one single mutation in codon 692 has been shown to present with either a cerebral haemorrhage or AD.

Given the limited impact of APP mutations in terms of families explained by mutations in this gene, several genome-wide searches were conducted. In 1992 findings of the first genomic screen of autosomal-dominant AD were published suggesting linkage to chromosome 14. Three independent groups confirmed these findings. The gene was isolated in 1995. This gene was identified in patients with early-onset AD and designated presenilin-1 (PSEN1). As APP, PSEN1 is a
Chapter 2.5

transmembrane protein, with 7 transmembrane domains and one hydrophilic loop. Mutations associated with AD are clustered in the transmembrane domains, especially domain 2, and the membrane associated region of the hydrophilic loop 44. Up to date, 56 mutations in 7 different exons of the PSEN1 gene have been found (AD mutation database: http://molgen-www.uia.ac.be/ADMutations/). The function of the presenilin protein is unclear, but mutations in the gene lead to altered APP processing in vivo, cell cultures, and transgenic animals 45 46 47.

The third gene harbouring dominant mutations was found based on its homology with PSEN1 (67% amino acid sequence homology concentrated in the transmembrane domains) 48. Two missense mutations in this second presenilin gene (PSEN2) on chromosome 1 (q31-q42) were identified in two families with early-onset AD 5. Until now only four mutations in PSEN2 are described of which three are localised transmembranic(AD-mutation-database:http://molgen-www.uia.ac.be/ADMutations/). The functional relationship of these mutations to AD remains unclear.

Genetic Susceptibility

In 1991, linkage to a region on chromosome 19 in families with late-onset AD was reported 49. Albeit that evidence was weak and based solely on an affected only analysis. The Apolipoprotein E gene (APOE) on chromosome 19 was tested as a candidate because of the presence of apolipoprotein E in senile plaques and its affinity to amyloid 50. The APOE gene has three common alleles (the wildtype APOE*3, APOE*2 and APOE*4) coding for three different isoforms of the protein 51. The frequency of APOE*4 was found to be significantly increased in familial AD patients 50. The number of studies that confirmed the association between APOE*4 and AD is overwhelming 7. In a meta-analysis of 40 studies conducted up to 1995 7, both early and late-onset AD, sporadic and familial forms, were associated with APOE*4. Early findings of a decreased frequency of APOE*2 in AD patients 52, 53 were not confirmed by others 54, 55. APOE*4 is not exclusively associated with AD. A number of studies also found association with vascular dementia 56, or Lewy body dementia 57, which makes this gene unsuitable for clinical diagnostics 58. Risk may differ across subgroups. There is some evidence that APOE interacts with other genetic and environmental risk factors. Vascular risk factors in generalised atherosclerosis and serum cholesterol levels may modulate the APOE*4 associated risk of AD 13, 59.
Other factors include head injury 60, herpes simplex virus 61, smoking 25, and estrogen use 29. However, identification of gene-gene and gene-environment interaction requires large population-based studies of which there are few available at present. This makes confirmation of the findings on gene-gene and gene-environment interaction difficult.

**Population attribution**

The frequency of major mutations depends for a large part on the ascertainment of families. Families ascertained for linkage studies 40 or genetic counselling 62, 63 have shown high mutation frequencies. When considering a population-based sample of families with an autosomal dominant form of early-onset AD, 18 percent was explained by mutations in known genes, of which PSEN1 mutations are most common 64. When considering all early-onset AD patients in a population-based sample, 0.5 percent of the patients carry APP mutations, 6 percent carry PSEN1 mutations, and 1 percent carry PSEN2 mutations 64, 65. Frequencies of these mutations in controls approach zero implying that the relative risk associated with these mutations is very high. The absence of asymptomatical carriers after age 70 years in families segregating APP or PSEN1 mutations suggest a high penetrance approaching 100 percent. From a clinical genetic perspective, this makes these genes useful for risk prediction and diagnosis of AD. Nevertheless, the mutations causing AD remain unknown in 82% of the families with early-onset AD, this concerns also extended pedigrees segregating AD.

In the general population early-onset AD is only a minor fraction of all AD (<1%) 10. Therefore, on the level of the general population the attribution of these mutations to the occurrence of disease is estimated to be very small. Together, the three dominant genes do not appear to explain more than 0.1 percent of the occurrence of all AD 6.

At the population level, APOE is a more important determinant of AD. Although the relative risk of AD is moderately (1.3 to 3.2 times) increased for carriers of one APOE*4 allele (APOE24, APOE34), the population attributable risk suggests that the APOE*4 allele may explain about 10 to 17 percent of the disease in the general population 6. The substantial contribution to the frequency of AD in the population is for a large part explained by the large number of subjects heterozygous for the
Chapter 2.5

APOE*4 allele (±25% in Caucasians). For homozygotes the situation is different. Despite the high risk for AD (6 to 15 times increased), the contribution to AD in the general population is limited (<2%) because of the very low frequency of this genotype. APOE is a susceptibility gene that increases risk but not always leads to AD. Given its modest increase in risk, its role in risk prediction in the clinical genetic practice and diagnosis in the neurological practice is limited.
Chapter 2.6

Efforts to identify new AD genes

It is clear that research on the genetics of AD is far from completed. Several strategies to identify novel genes have been followed including candidate gene approach and genome screening. None of these approaches yielded equivocal results so far.

Table 2.1 possible susceptibility genes for Alzheimer’s disease

<table>
<thead>
<tr>
<th>Candidate gene</th>
<th>Possible role</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>α1-antichymotrypsin</td>
<td>Binds to β amyloid</td>
<td>67, 68</td>
</tr>
<tr>
<td>VLDL receptor</td>
<td>Binds to APOE</td>
<td>69, 70</td>
</tr>
<tr>
<td>LDL receptor related protein (LRP)</td>
<td>APOE receptor in brain</td>
<td>71, 72</td>
</tr>
<tr>
<td>α2-Macroglobuline</td>
<td>Binds to β amyloid</td>
<td>73, 74</td>
</tr>
<tr>
<td>Angiotensin converting enzyme (ACE)</td>
<td>Gene involved in vascular pathology</td>
<td>75</td>
</tr>
<tr>
<td>α-Synuclein (NACP)</td>
<td>Component of plaques</td>
<td>76, 77</td>
</tr>
<tr>
<td>HLA</td>
<td>Inflammation</td>
<td>78, 79</td>
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<tr>
<td>Bleomycin hydrolase</td>
<td>APP processing</td>
<td>80, 81</td>
</tr>
<tr>
<td>Tau</td>
<td>Main protein in tangles</td>
<td>82, 83</td>
</tr>
<tr>
<td>Buterylcholinesterase</td>
<td>Associated with plaques and tangles</td>
<td>84, 85</td>
</tr>
<tr>
<td>Nitric Oxide synthase</td>
<td>Dilatation of small vessels</td>
<td>86</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Known AD genes</th>
<th>Possible role</th>
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<tr>
<td>PSEN1 intron 8</td>
<td>Alteration in known AD gene</td>
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<tr>
<td>PSEN1 promoter</td>
<td>PSEN expression</td>
<td>89</td>
</tr>
<tr>
<td>APOE promoter</td>
<td>APOE expression</td>
<td>90, 91</td>
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The candidate gene approach has been successful in identifying AD genes. However, these studies were based on strong evidence towards region of interest, e.g. observations of the occurrence of amyloid pathology in Down syndrome patients pointing to chromosome 21 (APP), linkage studies flagging chromosome 19 (APOE) or chromosome 14 (PSEN1), or homology to a known gene (PSEN2). Recent
candidate gene studies have been less successful despite the fact that for each of them there is a biological justification (see table 2.1). A large number of genes was studied but none of the studies of the candidate genes listed in Table 2.1 have been replicated consistently. Methodology in some studies may explain part of the inconsistencies. In a number of studies genotype frequencies in control samples were not in Hardy Weinberg equilibrium suggesting the presence of bias 92. This bias might have occurred during control ascertainment or at laboratory assessment. An important issue in case-control studies is the source population of controls. Especially hospital based controls are prone to selection bias. Inclusion of subjects with unknown pathology related to the genetic factor under study may result in false positive findings. Furthermore, errors in the genotype classification due to reading or genotyping mistakes may cause deviation from Hardy Weinberg equilibrium.

Although candidate gene studies have also been disappointing in other fields than AD, it is only fair to say that these studies in AD are far from their limits. Critical evaluation of studies in terms of possible false positive findings due to ascertainment and stratification has been limited both by investigators and referees, evidenced by findings based on control data not in Hardy Weinberg equilibrium. On the other hand, there is an issue of small studies underlying false negative results, which is often neglected. To overcome the problem of false negative results in small studies pooling valid studies in a meta-analysis may lead to a more intelligible interpretation of scientific evidence.

With regard to the candidate gene approach there are opportunities for progress in studies of the promoter areas of known genes (APOE, APP, PSEN1) 89-91 in that more detailed studies on the functionality of these polymorphisms is needed. There have been limited efforts to increase the validity of candidate gene studies by using other designs, for example the transmission disequilibrium test (TDT) or sibpair design, or by studying samples for the presence of population admixture, potentially a source of bias 93.

Successful candidate gene studies in AD up to date distinguish themselves from unsuccessful ones by the fact that they were supported by positive results in linkage analysis. Unfortunately genome screening for new AD genes has not yielded consistent results. Several searches have been conducted, targeting the most common late-onset form of AD. Different approaches have been followed including the parametric lodscore method 94 and the non-parametric sibpair method 94, 95.
Chromosome 12 was identified in three genomic screens, although one may argue that the regions with significant lodscores do not overlap. In this region the candidate gene alpha-2 macroglobulin (A2M) has been put forward as an AD gene.

This gene has been extensively studied without yielding conclusive findings. Figure 2.2 shows a meta-analysis of the studies conducted to date. The figure and the overall odds ratios show that it is unlikely that A2M is indeed the gene explaining linkage of AD to chromosome 12.

<table>
<thead>
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<th>Ln Odds 95% CI</th>
<th>N</th>
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<td>A2M deletion</td>
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<td>Singleton 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myllkyangas 101</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hu 102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Korovaitseva 103</td>
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<td></td>
</tr>
<tr>
<td>Shibata 104</td>
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<td>Chen 105</td>
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<tr>
<td>Kovacs 106</td>
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<tr>
<td>Crawford 107</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pooled</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>A2M Ile 1000 Val</th>
<th>N</th>
<th>freq GG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liao 108</td>
<td></td>
<td>0.12/0.07</td>
</tr>
<tr>
<td>Wavrant 109</td>
<td></td>
<td>0.11/0.12</td>
</tr>
<tr>
<td>Singleton 100</td>
<td></td>
<td>0.16/0.16</td>
</tr>
<tr>
<td>Myllkyangas 101</td>
<td></td>
<td>0.14/0.17</td>
</tr>
<tr>
<td><strong>Pooled</strong></td>
<td></td>
<td>0.12/0.12</td>
</tr>
</tbody>
</table>

Figure 2.2 Meta-analysis of the A2M deletion and Ile 1000 Val polymorphism of all published case-control studies so far (Ln odds ratio's and 95%).

* Odds ratio 0.99 (0.88-1.12). ** Odds ratio 1.07 (0.76-1.49).

Main problems encountered in genome screens, in particular in late-onset AD, are phenocopies limiting the statistical power of linkage and sibpair studies. The distinction of phenocopies from the genetic form of the disease is clinically impossible at present. When assessing the statistical power, the lack of knowledge on the number of different genes and pathways involved is a major limitation. Although the heritability for AD is high it cannot be excluded that there are a large number of genes with modest effect involved. These genes may be implicated in AD through
Chapter 2.6

complex interactions. This has serious implications for the power of linkage and sib-pair studies, but to what extend cannot yet be overseen.

An opportunity not explored fully in screening the genome are association studies in genetically isolated populations. 93. Chances of success may be better when genetic studies are performed in founder populations which are genetically more homogeneous. 93. This approach has not been taken to its limits in the field of AD. Within these populations, AD genetics may be less complex in terms of number of genes involved due to founder effects and genetic drift. 93, 110. An obvious disadvantage of localising genes in such a population may be the limited relevance in the general population. On the other hand, any gene identified may yield important clues for proteins involved in the AD pathology.

Considerable progress in AD genetics has been made in the last decade. Although the autosomal-dominant mutations do not explain a large proportion of the disease, in particular the discovery of the APP and PSEN1 genes have contributed extensively to our knowledge on the pathogenesis of AD and provided a target for long awaited drug development. Further, identification of these genes has opened opportunities for clinical genetic counselling in families with autosomal-dominant forms of early-onset AD.

From a clinical perspective, findings on APOE have not yielded major implications. Nevertheless, APOE explains a substantial part of the occurrence of disease in the general population. Due to the large proportion of AD explained by this polymorphism, APOE may offer new opportunities for prevention in the future. However, development of effective strategies awaits progress in epidemiological research on environmental factors involved. Particular findings on vascular risk factors, which may be preventable, offer opportunities in this respect.

The challenge for the near future will be to identify new genes involved in the aetiology of AD. There is evidence for an AD locus on chromosome 12, which yields a start for further molecular genetic studies. Although candidate and genome searches have not reached their limits in terms of statistical power and methodology, novel approaches to identify genes are on the way. A possibility to identify new genes is screening all genes identified in the human genome with single nucleotide polymorphisms (SNP's). 111. This approach may be followed in association and sib-pair studies. At present, this approach awaits major progress in technology and development of informative SNP’s in exons. Once these technical problems are
conquered, the next step will be ascertaining AD series large enough for a powerful analysis.

As reviewed AD is a heterogeneous disorder. Development in the clinical diagnosis, in particular of disease phenotypes may enable further progress in unravelling AD genetics. The dissection of late-onset AD into more homogeneous subgroups may lead to progress in genetic epidemiologic research. Although our knowledge of the clinical course of AD is limited, there is growing evidence that the aetiology of AD is strongly linked with that of other neurodegenerative diseases, including vascular disease, tauopathies, and alpha-synucleinopathies. Studies of the genetics of these disorders, in particular brain related atherosclerosis, may open opportunities for the genetic dissection of AD.

The genetic aetiology of AD is not fully understood. As in other complex disorders, major difficulties are encountered in research aiming to further unravel genetics. Methodological and technical developments in the near future may facilitate new breakthroughs.
Chapter 3

Population Based studies

3.1 Introduction
3.2 Variable expression of presenilin 1 is not a major determinant of risk for late-onset Alzheimer’s Disease
3.3 Mutation screening of the tau gene (MAPT) in patients with early-onset Alzheimer’s disease
3.4 The Cystatin C polymorphism is not associated with early-onset Alzheimer’s disease
3.5 The −491 A/T polymorphism in the regulatory region of the Apolipoprotein E gene and early-onset Alzheimer’s Disease
3.6 The APOE −491A/T promoter polymorphism is associated with apolipoprotein E levels but not with Alzheimer’s disease. The Rotterdam Study
Chapter 3.1

Introduction

Genetic research has made a major contribution to our knowledge on the pathophysiology of AD. New proteins involved in AD have been discovered and will be discovered in the future. Opportunities will grow in the near future due to the information becoming available from the human genome project. Together with the increasing knowledge of AD on the protein level, candidate gene studies are a promising tool to discover novel AD susceptibility genes. In this chapter, 5 candidate gene studies are presented. Polymorphisms in these genes are studied in a population-based series of early-onset AD cases and controls and a population-based series of late-onset AD cases and controls. In our studies, we followed a four-step strategy. Firstly, we targeted the PSEN1 gene in which most mutations are found in early-onset AD and studied polymorphisms in this gene in relation to late-onset AD. Secondly, we targeted the tau gene. Tau is a protein which is known to be involved in the pathology of AD and the tau gene is involved in frontotemporal dementia, a disease closely related to AD. Thirdly, we studied the gene coding for cystatin C, a protein which is a component of congophilic amyloid angiopathy. These vascular lesions are often abundantly present in AD brains. Finally, we studied the promoter region of the APOE gene, a well established genetic risk factor for early as well as late-onset AD. As polymorphisms in the promoter region are possibly related to the expression of the protein, they might be associated with AD.
Chapter 3.2

Variable expression of presenilin 1 is not a major determinant of risk for late-onset Alzheimer’s Disease

Recently we described a polymorphism in the promoter region of the presenilin 1 gene (PSEN1) (−48C/T) showing significant association with early-onset Alzheimer’s disease (AD). The CC genotype of −48C/T was associated with a nearly 3 times increased risk for developing early-onset AD. In a systematic screen of 3.5 kb of the upstream regulatory region of PSEN1 we detected additional polymorphisms together identifying a risk haplotype containing the −48C allele. Our results were replicated in a recent study showing a significant overrepresentation of the −48 CC genotype in AD cases with the strongest effect in the early-onset age group and a significantly increased amyloid load in brains of AD patients carrying the −48 CC genotype (J.-C. Lambert and C. Lendon, personal communication). In 1996, Wragg and colleagues described an association between an A to C transversion in intron 8 of PSEN1 and late-onset AD in American Caucasians. Carriers of the AA genotype had a significantly increased risk for developing Late-onset AD. Although the association of PSEN1 intron 8 with late-onset AD was confirmed in different populations, others were unable to replicate these results. We hypothesise that these inconsistent findings may be explained by linkage disequilibrium (LD) of the intron 8 polymorphism to the −48C/T promoter polymorphism. To examine this, we determined the PSEN1 −48C/T and intron 8 polymorphisms in a nested case-control sample of late-onset AD from a large population-based prospective study (The Rotterdam Study). In addition, we reanalysed all published association studies on the PSEN1 intron 8 polymorphism in a meta-analysis to evaluate the contribution of this polymorphism to the risk of late-onset AD.
Chapter 3.2

Methods

Late-onset AD patients were drawn from the Rotterdam Study, a population-based prospective study on residents of a suburb of Rotterdam, The Netherlands. Participants underwent a brief cognitive test for dementia that comprised a combined mini mental state examination (MMSE) and geriatric mental state schedule (GMS-A). Subjects with a MMSE of 25 or less or a GMS-A of 1 or more were subsequently examined by a physician with the CAMDEX (Cambridge examination for mental disorders of the elderly) diagnostic interview. Participants scoring less than 80 on the CAMDEX or who were suspected of dementia on a clinical basis were asked to participate in a third, extensive examination by a neurologist, neuropsychologist, and by MRI imaging. The diagnosis of dementia was assessed by a panel of study physicians, a neurologist, and a neuropsychologist, using the diagnostic and statistical manual of mental disorders (DSM-III-R). A diagnosis of possible or probable AD was based on the National Institute of Neurological and Communicative Diseases and Stroke – Alzheimer’s Disease and Related Disorders Association criteria (NINCDS-ADRDA). By this means, 339 AD patients were ascertained at the start of the study and 116 incident AD patients were diagnosed at the first follow-up period. DNA genotyping was carried out on 258 prevalent cases (mean age at onset 82±7.1 years; 25% men), 98 incident cases (mean age at onset 83±7.3 years; 20% men) and 242 controls (mean age at examination 81±4.9 years; 40% men). Genotyping of the PSEN1 and apolipoprotein E gene (APOE) polymorphisms was performed as described previously.

Hardy-Weinberg equilibrium (HWE) and LD were tested with the HWE and EH programs. For both polymorphisms the genotype distributions in the controls were in HWE. Genotype and allele frequencies were analysed using the \( \chi^2 \) statistic or Fisher’s exact test if appropriate. Results were adjusted for age and gender using logistic regression. Differences in onset ages for the different genotypes were analysed by ANOVA.

For the meta-analysis we searched Medline to collect all published literature on PSEN1 polymorphisms and checked the reference lists of the retrieved articles. We found 27 case-control studies on the PSEN1 intron 8 polymorphism (figure 1) from which three were excluded for further analysis. No studies on the PSEN-48C/T polymorphism other than our own were found. One study and a
subset of another study were excluded since the controls were not in HWE. Two studies were excluded because they were the second publication on the same study population. All AD patients were diagnosed according to international criteria (NINCDS-ADRDA or DSM-III). For the meta-analysis, we used a random effect model. The odds ratio (OR) is estimated by maximising the log likelihood of AA versus AC and CC. The random effect model is also valid when between study variation exists. In case of heterogeneity between studies, the width of the confidence interval of the OR will be too small when the fixed effect model is used.

Results
In the late-onset AD case-control sample no significant association was found with the PSEN1 intron 8 polymorphism, nor with the -48C/T polymorphism (table 3.1). Also, no association was found when incident and prevalent AD patients were analysed separately. No effect on age of onset for the polymorphisms was detected (p > 0.5). Significant LD between the PSEN1 -48C/T and intron 8 polymorphism (p < 0.01) was observed, however, estimated frequencies of the 4 possible haplotypes were not statistically different between cases and controls (p = 0.38).

Table 3.1 Allele and genotype frequencies of PSEN1 intron 8, and PSEN1-48C/T.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>AA</th>
<th>AC</th>
<th>CC</th>
<th>p value</th>
<th>A</th>
<th>C</th>
<th>p value</th>
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<tbody>
<tr>
<td>Intron 8 AD</td>
<td>319</td>
<td>0.36</td>
<td>0.47</td>
<td>0.17</td>
<td>0.28</td>
<td>0.60</td>
<td>0.40</td>
<td>0.23</td>
</tr>
<tr>
<td>Controls</td>
<td>220</td>
<td>0.30</td>
<td>0.52</td>
<td>0.18</td>
<td></td>
<td>0.56</td>
<td>0.44</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>p value</th>
<th>C</th>
<th>T</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-48C/T AD</td>
<td>323</td>
<td>0.84</td>
<td>0.15</td>
<td>0.01</td>
<td>0.32 *</td>
<td>0.91</td>
<td>0.09</td>
<td>0.31</td>
</tr>
<tr>
<td>Controls</td>
<td>221</td>
<td>0.86</td>
<td>0.14</td>
<td>0</td>
<td></td>
<td>0.93</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

All p values are from a χ² statistic with appropriate degrees of freedom except for * which is a Fisher's exact test.

To examine interaction with APOE, we stratified our sample according to APOE genotype. While for PSEN1 intron 8 no interaction with APOE was found, a borderline significant (p = 0.04) interaction was found for PSEN1 -48C/T (table 3.2). This interaction with APOE*4 was the result of genotype frequency shifts in the controls while cases showed no difference. After genotyping 56 additional APOE*4 positive controls, the interaction disappeared (p = 0.21).
Table 3.2 Genotype frequencies of PSEN1 -48C/T stratified for APOE

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOE33</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD</td>
<td>172</td>
<td>0.84</td>
<td>0.15</td>
<td>0.01</td>
<td>0.36</td>
</tr>
<tr>
<td>Controls</td>
<td>131</td>
<td>0.80</td>
<td>0.20</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>APOE*4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD</td>
<td>115</td>
<td>0.81</td>
<td>0.17</td>
<td>0.02</td>
<td>0.04*</td>
</tr>
<tr>
<td>Controls</td>
<td>56</td>
<td>0.95</td>
<td>0.05</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>APOE*2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD</td>
<td>29</td>
<td>0.86</td>
<td>0.14</td>
<td>0</td>
<td>0.70</td>
</tr>
<tr>
<td>Controls</td>
<td>32</td>
<td>0.91</td>
<td>0.09</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

All p values are from Fisher’s exact tests. Seven cases and 2 controls with the APOE 24 genotype were excluded from the stratified analysis.

*Association disappeared when adding 56 APOE*4 positive controls (CC: 0.80; CT: 0.20; TT: 0) (p=0.21)

The 24 published case-control studies on the PSEN1 intron 8 polymorphism, together with the present study, contain a total of 4286 patients and 3759 controls from various ethnic backgrounds (figure 3.1). Two studies show a significant increased risk for the AA genotype. Analysis of the total sample gave an OR of 1.1 (95% CI 1.0-1.1). Allele and genotype frequencies differed substantially between Caucasian and Asian populations. However, stratification according to ethnic background did not result in a statistically significant association with the AA genotype.

Discussion
We recently demonstrated a nearly 3 times increased risk for developing EOAD in individuals homozygous for the C allele at the PSEN1 -48C/T promoter polymorphism. Moreover, we showed that the -48C risk allele is contained in a risk modifying haplotype spanning about 3 kb in the upstream regulatory region of PSEN1 and found a significant allele-specific in vitro effect of -48C/T on PSEN1 promoter activity (143 and unpublished data). This is the first study investigating the role of this functional promoter variation in PSEN1 in relation to the frequent late-onset AD phenotype. In addition, we investigated the PSEN1 intron 8 polymorphism in the same population-based case-control sample as well as in an extensive meta-analysis.
<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>Ln Odds ratio</th>
<th>N</th>
<th>Freq AA genotype AD/ control</th>
<th>AD/ control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled Caucasian</td>
<td>3273/2393</td>
<td></td>
<td></td>
<td>0.33/0.31</td>
<td></td>
</tr>
<tr>
<td><strong>Pooled Asian</strong></td>
<td>777/1042</td>
<td></td>
<td></td>
<td>0.45/0.41</td>
<td></td>
</tr>
<tr>
<td><strong>Mixed ethnic groups</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wragg 87</td>
<td></td>
<td></td>
<td></td>
<td>29/50</td>
<td>0.66/0.66</td>
</tr>
<tr>
<td>Cai 115</td>
<td></td>
<td></td>
<td></td>
<td>122/256</td>
<td>0.30/0.34</td>
</tr>
<tr>
<td>Liao 128</td>
<td></td>
<td></td>
<td></td>
<td>85/18</td>
<td>0.27/0.33</td>
</tr>
<tr>
<td><strong>Total random effect</strong></td>
<td></td>
<td></td>
<td>4286/3759</td>
<td>0.36/0.35</td>
<td></td>
</tr>
</tbody>
</table>

Fig 3.1 This figure shows the frequency of the PSEN1 intron 8 AA genotype in patients and controls as the natural logarithm of the odds ratio with the corresponding 95% confidence intervals. The frequencies of the pooled analyses are crude frequencies. The odds ratio for the pooled analyses are calculated using a random effect model.
For both the PSEN1 -48C/T and intron 8 polymorphisms, we found no evidence for statistically significant differences between our late-onset AD cases and controls. Also, in the meta-analysis no major differences in PSEN1 intron8 AA frequencies between patients (0.36) and controls (0.35) were found (OR 1.1, 95% CI 1.0-1.1).

There are several possible reasons for the discrepant findings regarding the PSEN1 -48C/T polymorphism in our previous early-onset AD and the present late-onset AD study. First, it can not be excluded that the initial positive association in our early-onset AD series was a false positive finding. However, the association with early-onset AD is supported by functional analysis of variations in the PSEN1 promoter region and by several biological studies demonstrating the importance of PSEN1 levels in APP processing 144, 145. Second, early-onset AD patients might have a different genetic background that enhances the effect of specific promoter variations in PSEN1 leading to an early expression of the disease.

In conclusion, our case-control study, as well as an extensive meta-analysis does not support an association between the PSEN1 intron 8 variation and LOAD. Also, we were not able to extend our observation of an association between the promoter region of PSEN1 and early-onset AD to late-onset AD. More studies on the role of the PSEN1 promoter in early-onset AD are needed.
Chapter 3.3

Mutation screening of the tau gene (MAPT) in patients with early-onset Alzheimer's disease

The two main features characterising Alzheimer's disease (AD) pathology are extracellular plaques composed mainly of the amyloid-β peptide (Aβ) and intracellular neurofibrillary tangles containing hyperphosphorylated tau proteins. In the Aβ precursor protein gene coding for the Aβ peptide, several mutations responsible for early-onset AD were found. No AD-related mutations have been reported in the gene coding for tau (MAPT), a microtubule associated protein. However, recently, mutations in MAPT were found to be involved in the aetiology of frontotemporal dementia (FTD) and related tauopathies. Mutations in MAPT explain about 18% of all FTD cases and 41% of familial FTD cases. Also, a (CA)n-repeat in intron 9 was found associated with progressive supranuclear palsy (PSP). Lilius et al. reported association of a haplotype of MAPT polymorphisms in AD patients carrying an apolipoprotein E (APOE) ε4 allele. In contrast, a recent study of two known polymorphisms in MAPT showed no evidence for association. However, this does not exclude a role of unknown polymorphisms in MAPT. In the light of the neurofibrillary degeneration observed in AD and the involvement of tau in other types of dementia, we performed a molecular genetic analysis of MAPT in early-onset AD patients.
Chapter 3.3

Methods

Patients were derived from a population-based epidemiological study of early-onset AD in four northern provinces of The Netherlands and the area of metropolitan Rotterdam. The study aimed at the complete ascertainment of all early-onset AD patients with a disease onset before the age of 65 years. The diagnosis of probable AD was independently confirmed by two neurologists using a standardised protocol according to the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) criteria for AD. Of the 198 patients ascertained, 101 blood samples were available for DNA analysis. The mean age at onset of the 101 patients was 57 years (SD=5.0), while the mean age at the time of study was 63 years (SD=4.9). Patients were compared to a control series in the same age range (n=117; mean age 61 years, SD=3.3) that was drawn randomly from the Rotterdam Study. All controls were screened on cognitive function and none of them showed symptoms of dementia.

Single stranded conformational polymorphism (SSCP) analysis of MAPT exons 9 to 13 encoding the microtubule-binding domains of tau containing all known FTD mutations, was performed in all patients. Each exon was PCR amplified under standard conditions using intronic primers flanking each exon. The PCR products were heat denatured, renatured on ice and separated at room temperature on precast ExcelGel gels (Pharmacia, Uppsala, Sweden) or 1 X Hydrolink MDE gels (J.T. Baker, Phillipsburg, USA) with 5% glycerol by electrophoresis using the MultiphorII system (Pharmacia). Bands were visualised by silver staining. Sequence analyses of aberrant SSCP patterns were performed using the ‘Taq-dye terminator kit’ (Applied Biosystems, Foster City, USA) and analysed on a 373A automated DNA sequencer (Applied Biosystems).

Results

SSCP analysis of exons 11, 12 and 13 did not show variations. SSCP analysis of exon 10 identified 1 patient with an aberrant pattern in the heterozygous state. Subsequent SSCP analysis of the control population revealed 1 control individual with the same SSCP pattern. Also, another control sample was identified with a different heterozygous SSCP pattern. Sequence analyses of MAPT exon 10 showed that both aberrant patterns were caused by intronic sequence variations. Observed were a C to T transition at nucleotide 25 of intron 10 (IVS10+25C>T) in a patient and control.
individual and a G to A transition at nucleotide 29 of the same intron (IVS10+29G>A) in one control (Table 3.3). The IVS10+29G>A polymorphism is located outside the stem-loop structure affecting the alternative splicing of exon 10 in FTD patients and was demonstrated not to alter the splicing of exon 10. Although this does not strictly exclude an alternative aberrant splicing mechanism in AD, the intronic location as well as the occurrence of the substitution in a case and a control makes a causative relation unlikely.

SSCP analysis of exon 9 revealed complex altered mobility patterns suggesting the presence of more than 1 sequence variation in both patients and controls. By sequence analysis, the SSCP patterns could be explained by 2 silent mutations: an A to G transition at codon Ala169 abolishing a TspRI restriction site and a T to C transition at codon Asn197, creating a MaeII restriction site (Table 3.3). The codons are numbered following the longest transcript of the tau protein. Using PCR-RFLP analysis, the allele frequencies of the Ala169 polymorphism were determined at 0.77 (A) and 0.23 (G) in the control population. The Asn197 polymorphism was in complete linkage disequilibrium with Ala169 in 10 AD cases analysed by PCR-RFLP analysis.

Table 3.3 MAPT polymorphisms.

<table>
<thead>
<tr>
<th>Location</th>
<th>Variation</th>
<th>Detection method</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 9</td>
<td>c.507A&gt;G (Ala169)</td>
<td>TspRI PCR-RFLP</td>
<td>G:271 + 108 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A: 165 + 108 + 106 bp</td>
</tr>
<tr>
<td>Exon 9</td>
<td>c.591T&gt;C (Asn197)</td>
<td>MaeII PCR-RFLP</td>
<td>T: 379 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C: 243 + 136 bp</td>
</tr>
<tr>
<td>Intron 10</td>
<td>IVS10+25T&gt;C</td>
<td>HhaI PCR-RFLP</td>
<td>T: 200 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C: 157 + 43 bp</td>
</tr>
<tr>
<td>Intron 10</td>
<td>IVS10+29G&gt;A</td>
<td>SSCP</td>
<td></td>
</tr>
</tbody>
</table>

The Ala169 polymorphism and the 7-allelic (CA)9 repeat polymorphism in intron 9 that was previously associated with PSP were further analysed in the patient and control series (Table 3.4). Allele and genotype frequencies of both polymorphisms were in Hardy-Weinberg equilibrium. Linkage disequilibrium was
Chapter 3.3

tested using the EH programs described by Terwilliger and Ott \(^{122}\), showing tight linkage disequilibrium with the 142 bp and 148 bp alleles of the (CA)\(_n\)- repeat polymorphism linked to the A-allele, and the 150 bp and 154 bp alleles linked to the G-allele of the Ala169 polymorphism, showing only 13 recombinants in patients and controls (n=228). This is in accordance with the observation that only two haplotypes in the MAPT region exist, suggesting that these haplotypes were established early in the history of the Caucasian population \(^{157}\). No significant differences in allele or genotype distributions were observed when adjusting for age and gender. Also, there was no significant evidence for interaction with the APOE4 genotype as reported by Lilius \(^{82}\) in a stratified analysis.

Conclusion

Although MAPT is an obvious candidate gene for AD, our mutation analysis of the region encoding the microtubule-binding domains involved in FTD and other tauopathies showed no evidence for mutations or polymorphisms causally related to early-onset AD.
Table 3.4 Genotype and allele distribution of the MAPT Ala169 and the intron 9 (CA)$_n$ repeat polymorphisms in Alzheimer cases and controls

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th></th>
<th>Controls</th>
<th></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Exon 9 Ala169</td>
<td></td>
<td></td>
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<td>0</td>
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</tbody>
</table>

* p-value using a Chi-square statistic. ** p-value using Likelihood ratio test. # p-value after correcting for age and gender using a logistic regression model.
Chapter 3.4

The Cystatin C polymorphism is not associated with early-onset Alzheimer's disease

There is growing evidence that vascular factors are involved in Alzheimer's disease (AD) \(^{13}\). In light of these findings, a polymorphism in the cystatin C gene (CST3) that was recently found to be associated with AD, is a promising candidate gene \(^{158}\). Cystatin C is an amyloidogenic protein found together with β-amyloid in the arteriolar walls of AD patients and patients with congophilic amyloid angiopathy (CAA) \(^{159}\). In contrast to other AD genes like apolipoprotein E (APOE) and presenilin-1, which have the largest impact on early-onset AD \(^{7,160}\), the cystatin C gene was found to be associated with AD with a very late-onset age only \(^{158}\). As CAA is also a prominent feature in early-onset AD and Down syndrome patients \(^{161}\) and a mutation in cystatin C leads to CAA with pathology at a very early age \(^{162}\) we investigated the role of the CST3 polymorphism in early-onset AD.
Chapter 3.4

Methods

Patients were derived from a population-based study of early-onset AD (defined as onset before 65 years) in four northern provinces of the Netherlands and the area of Rotterdam, as described in detail before 121. AD was diagnosed using a standardised protocol in accordance with the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) criteria 12. Of the 198 patients ascertained, 101 blood samples were available for DNA analysis.

Controls were derived from the Rotterdam Study, a population-based study in subjects aged 55 years or over 13. They were randomly drawn from subjects in the age category of 55 to 65 years. All controls were screened on cognitive function with the Mini Mental State Examination and the Geriatric Mental Schedule. Persons who were suspected of dementia were excluded 13. For this study 117 control subjects were genotyped.

The CST3 and APOE polymorphisms were determined as described previously 121, 163. Genotype and allele frequencies were compared between cases and controls with the chi-square statistic. The odds ratio for AD associated with the CST3 GG genotype was computed using logistic regression analysis, using the GA and AA groups as a reference. To adjust for confounding, age, gender, and education level were entered in the model. In order to facilitate comparisons with the study by Crawford et. al. 158, possible interaction with age or APOE were explored similarly, i.e. by stratification (median age of 58 used as cut-off) and by testing for multiplicative interaction, using a product term in the logistic regression model.

Results

The mean age at AD onset was 57 years (SD=5.0) which was lower than the age in the controls (mean 61 years, SD=3.3, t-test, P < 0.01). The patient group included a larger proportion women compared to controls (77% versus 55%, $X^2_{1df} = 12.1$, P < 0.01). The CST3 genotype frequencies were, in both cases and controls, in Hardy Weinberg equilibrium (controls: $X^2_{1df} = 0.07$, P = 0.8; cases $X^2_{1df} = 0.9$, P = 0.3).
Table 3.5 Cystatin C in Alzheimer cases and non-demented controls

<table>
<thead>
<tr>
<th>genotypes</th>
<th>alleles</th>
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<th></th>
<th></th>
<th></th>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>0.62</td>
<td>0.33</td>
<td>0.05</td>
<td>0.26 (0.9)</td>
<td>0.78</td>
<td>0.22</td>
</tr>
<tr>
<td>(n=118)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td>0.65</td>
<td>0.31</td>
<td>0.06</td>
<td>0.78</td>
<td>0.22</td>
<td></td>
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<tr>
<td>(n=95)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Table 3.5 shows the genotype and allele frequencies of the CST3 polymorphism. We found no association between the CST3 genotype and early-onset AD overall ($\chi^2_{2 df} = 0.26$, $P = 0.9$). The age, gender and education adjusted odds ratio for early-onset AD associated with the GG genotype was 1.1 (95% Confidence Interval 0.5-2.4). There was no evidence for a statistical interaction between the CST3 polymorphism and age (product term: $P = 0.82$). After stratification according to age, the GG frequency was slightly higher in older cases (table 3.6), but far from statistically significant ($P = 0.52$). There was also no evidence for a statistical interaction between the CST3 and APOE polymorphisms (product term for any APOE*4 allele and CST3 GG genotype: $P = 0.69$).

Table 3.6 Cystatin C genotype in AD cases and non-demented controls by age

<table>
<thead>
<tr>
<th>Age &lt; 58 (years)</th>
<th>GG</th>
<th>GA</th>
<th>AA</th>
<th>$\chi^2_{2 df}$ (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n=30)</td>
<td>0.63</td>
<td>0.33</td>
<td>0.03</td>
<td>0.39 (0.8)</td>
</tr>
<tr>
<td>Cases (n=52)</td>
<td>0.58</td>
<td>0.37</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Age ≥ 58 (years)</td>
<td>Controls (n=88)</td>
<td>0.61</td>
<td>0.33</td>
<td>0.06</td>
</tr>
<tr>
<td>Cases (n=43)</td>
<td>0.70</td>
<td>0.23</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

In this population-based study on early-onset AD, we did not find an association with the CST3 GG genotype. In a previous study the CST3 GG genotype was found to be associated with very late-onset AD $^{158}$. AD aggregates strongly in families even at very old age. This is only partly explained by APOE*4 $^7$ suggesting that there are unknown late-onset AD genes. However, it is doubtful whether CST3 is one of these genes. In the study of Crawford et. al. there was no overall effect, no trend across age strata, and only a borderline significant effect in the oldest age group $^{158}$. In this
Chapter 3.4

study, the GG genotype frequency increased only slightly in the oldest age group and the association resulted mainly from a decrease in GG frequency in the controls. This suggests a role of the gene in another competing disorder rather than a role in AD. However, the control group in the oldest age group was small (n=49), and the risk estimate therefore unreliable. Since Cystatin C is a protein involved in CAA, which is also present in early-onset AD, there is no a priori hypothesis that the Cystatin C gene is more important in late-onset AD. Furthermore, although the CST3 polymorphism is located in the coding region of the gene, up until now no functionality of this polymorphism has been described.

We found no association of CST3 with early-onset AD. However, we can not exclude that the CST3 genotype is specifically related to AD with very late-onset. This should be investigated in other late-onset AD series.
Chapter 3.5

The -491 A/T polymorphism in the regulatory region of the Apolipoprotein E gene and early-onset Alzheimer’s Disease

Recently several new polymorphisms within the transcriptional regulatory region of the apolipoprotein E gene (APOE) were reported. One of these new polymorphisms (-491A/T) was found to be associated with the late-onset form of Alzheimer’s disease (AD) in two populations, one of Spanish and one of North American descent. An increased frequency of homozygosity of the A allele independent of APOE*4 status was observed. We have studied this polymorphism in a series of 99 Dutch and 78 Spanish early-onset AD patients.
Chapter 3.5

Methods

The Dutch patients were derived from a population based epidemiological study of early-onset AD in four northern provinces of the Netherlands and the area of metropolitan Rotterdam \(^{121}\). The study aimed at a complete ascertainment of all early-onset AD patients in whom the onset of AD was before the age of 65 years. The diagnoses of probable AD was independently confirmed by two neurologists using a standardised protocol according to the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) criteria for AD \(^{12}\). In this study 198 patients were ascertained and for 99 patients blood was available for genotyping the -491A/T polymorphism. The mean age at onset of the 99 patients was 57 years (SD = 5.0), while the mean age at the time of study was 63 (SD = 4.9). Patients were compared to an age-matched control series (n = 109; mean age 61 years SD = 3.3) that was drawn randomly from the Rotterdam Study \(^{118}\). All controls were screened on cognitive function and none of them showed symptoms of dementia.

The Spanish sample is a clinic-based series of patients who were recruited from the Neurology Service of the Hospital La Paz of Madrid. The NINCDS-ADRDA criteria were also used for the diagnosis of AD in the Spanish patients. Blood for genotyping was available for 78 patients. The mean age at onset in the Spanish patients was comparable to that found in the Dutch patients (57 years SD = 5.8) with a mean age at time of study of 63 years (SD = 6.0). Controls were derived from the Madrid region of Spain. They were matched for age and screened for cognitive function. Blood for genotyping was available for 132 controls with a mean age of 64 years (SD = 8.2).

APOE genotyping has been described earlier for the Dutch \(^{121}\) and Spanish patients \(^{164}\). The -491A/T polymorphism in the regulatory region of APOE was performed as described by Bullido et al. \(^{90}\).

Hardy-Weinberg equilibrium and linkage disequilibrium were tested using the programs of Terwilliger and Ott \(^{122}\). Genotype and allele distributions were analysed using a \(\chi^2\) test, and if appropriate a Fisher exact test. When the \(\chi^2\) or Fisher exact test was significant, the strength of association between the genotypes/alleles and AD was estimated with the odds ratio (OR). OR’s are presented with 95% confidence intervals (95% CI). The results were corrected for age and gender using logistic regression.
Results

For both populations, there was no evidence for significant deviations in \(-491\) A/T genotype distribution from Hardy-Weinberg equilibrium in patients or controls.

**Table 3.7 APOE \(-491\) A/T genotype and allele frequencies**

<table>
<thead>
<tr>
<th>Population</th>
<th>Cases</th>
<th>Controls</th>
<th>(\chi^2)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Dutch</td>
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</tr>
<tr>
<td>Allele</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>178</td>
<td>90</td>
<td>198</td>
<td>91</td>
</tr>
<tr>
<td>T</td>
<td>20</td>
<td>10</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>AA</td>
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<td>81</td>
<td>91</td>
<td>83</td>
</tr>
<tr>
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<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Spanish</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Allele</td>
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<td></td>
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</tr>
<tr>
<td>A</td>
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<tr>
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<td>2</td>
<td>3</td>
<td>5</td>
<td>4</td>
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</table>

† P-values corrected for age and gender

Table 3.7 shows allele and genotype distribution of the \(-491\)A/T polymorphism in AD patients and controls. Both in the Dutch and Spanish population there was no evidence for association in the overall analysis based on genotype distribution. In the Spanish population the frequency of the A allele was borderline significantly increased (p = 0.05) when comparing allele frequencies between patients and controls. There was a significant difference in allele and genotype frequencies between the two populations, which makes pooling inappropriate.

Given the small genetic distance between the regulatory region of APOE and the APOE gene, recombination is unlikely to occur during meiosis. As a consequence, combinations of the \(-491\)A/T and APOE alleles may be found more often than expected by chance. In genetic terms this is referred to as linkage disequilibrium. There was significant evidence for linkage disequilibrium in the Dutch population (patients p = 0.0002, controls p = 0.002) as well as in the Spanish patients (patients p = 0.012, controls p=0.12). In both populations allele T of the \(-491\)A/T polymorphism was coupled with APOE*2 in patients as well as controls, i.e., carriers of the APOE*2
allele more often carried the T allele than expected by chance (see also table 3.8 and 3.9). This effect was most pronounced in the Dutch population. When stratifying for APOE, a significant difference in the -491A/T polymorphism genotype distribution between AD patients and controls was found in those with the APOE 33 genotype (p = 0.004 see table 3.8). A six fold increase in the frequency of AT genotype compared to AA genotype was observed in patients (OR = 6.5; 95% CI 1.4-30.0). In the Dutch APOE*2 carriers also the frequency of the AT genotype and the T allele was increased. However, the number of subjects was too small to allow for conclusions. In the APOE stratified analysis for the Spanish population, no statistically significant association between the polymorphism and AD was found (table 3.9). In Spanish patients with APOE*4, there was a tendency of an increased frequency of the -491 AA genotype in patients.

Table 3.8 APOE -491 A/T genotype and allele distributions in APOE strata for the Dutch study group *

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<th>Controls</th>
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<td>%</td>
<td>N</td>
<td>%</td>
<td>$\chi^2$</td>
</tr>
<tr>
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<tr>
<td>Allele</td>
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<td></td>
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<td>94</td>
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<td>4</td>
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<td></td>
</tr>
<tr>
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</tr>
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</table>

* Excluded are 1 case and 2 controls with the APOE 24

†† Excluding the TT genotype from the analysis

† p-values corrected for age and gender
Table 3.9 APOE -491 A/T polymorphism in early-onset AD for the Spanish study group

<table>
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<th>P-value</th>
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<td>%</td>
<td>N</td>
<td>%</td>
<td>χ²</td>
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<td>89</td>
<td>27</td>
<td>79</td>
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<td>0.17</td>
</tr>
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</tr>
<tr>
<td>APOE *2 Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>4</td>
<td>67</td>
<td>16</td>
<td>57</td>
<td>0.18</td>
<td>0.67</td>
</tr>
<tr>
<td>T</td>
<td>2</td>
<td>33</td>
<td>12</td>
<td>43</td>
<td></td>
<td>0.56†</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>1</td>
<td>33</td>
<td>5</td>
<td>36</td>
<td>0.94</td>
<td>0.62</td>
</tr>
<tr>
<td>AT</td>
<td>2</td>
<td>67</td>
<td>6</td>
<td>43</td>
<td></td>
<td>0.75†</td>
</tr>
<tr>
<td>TT</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Excluded are 2 cases with the APOE 24 genotype
** Excluding the TT genotype from the analysis
† p-values corrected for age and gender

Discussion

We did not find a consistent relationship between the -491A/T polymorphism in the regulatory region of APOE and early-onset AD in the Dutch or Spanish case-series studied. In the Spanish population there was evidence of an increased frequency of the A allele in patients. In the analysis stratified for the APOE genotype, a statistically significant increased frequency of the T allele was found in carriers of the APOE33 genotype in the Dutch population, but not in the Spanish one. The increased frequency of the T allele was fully determined by an increased frequency of the AT genotype in the Dutch patients compared to the AA genotype. The finding in Dutch patients suggests that not the A allele but rather the T allele is associated with an increased risk of the early-onset form of AD. There may be several explanations for this discrepancy. Bias may have occurred in association studies because of population admixture, in which the population consists of several sub-populations with different allele frequencies and different risks of disease. Indeed, there are significant differences in allele frequencies among the control series derived from the Dutch,
Chapter 3.5

Spanish and US population (as published by Bullido et al., 1997) 90. Although population admixture is difficult to disqualify, this explanation seems unlikely in the Dutch population because patients and controls are both drawn from a Caucasian Dutch population. Our finding in the APOE stratified analysis of the Dutch population may be due to chance as an association was only found in a small subgroup and not in the overall analysis. In particular the increased frequency of the AT genotype in cases without an increased frequency of early-onset AD patients with the TT genotype is unexpected. However, part of the explanation for this observation may be the low frequency of this genotype (0.02 in controls). An alternative explanation for the differences in the −491A/T allele associated to AD in different populations may be that the −491A/T polymorphism is not functionally related to the risk of AD but is in linkage disequilibrium with another polymorphism in APOE or its regulatory region. A strong linkage disequilibrium between the −491A/T and APOE polymorphism was found in late-onset patients, and explains most of the association with the −491A allele in this population 165. This finding is compatible with the increased frequency of the T allele (AT compared to the AA genotype) observed in the Dutch early-onset AD patients and the increased frequency of the A allele in the Spanish patients. However, this explanation implies that there are major differences in haplotypes associated to the early-onset form of AD in different populations. Up until now there has been little evidence for this in studies of the APOE genotype, with the exception of the inconsistent findings in patients of African descent 166. However, the findings of the present study of a stronger association between the APOE*2 and −491T allele in the Dutch population (p=0.002 in controls) than in the Spanish one (p=0.07 in controls) supports the presence of population specific disease haplotypes in the APOE regulatory region. If there is another AD polymorphism in the APOE regulatory region, the strong linkage disequilibrium between the −491T and APOE*2 allele may explain the increased risk of AD associated with the APOE*2 allele found in the Dutch early-onset population that was not found in other populations 52, 53, 55. In summary, in the present study of early-onset AD we did not find evidence supporting the earlier observation of an increased frequency of the −491 AA genotype in patients with late-onset AD. Findings of our study are compatible with population-specific disease haplotypes in the APOE regulatory region that are associated to the AD risk.
Chapter 3.6

The APOE −491A/T promoter polymorphism is associated with apolipoprotein E levels but not with Alzheimer’s disease. The Rotterdam Study

The apolipoprotein E gene (APOE) is a gene with 3 common alleles, involved in lipid transport. These alleles, APOE*2, APOE*3, and APOE*4 influence lipid levels. The APOE*2 results in lower levels of total and low density lipoprotein cholesterol and higher levels of high density lipoprotein cholesterol and plasma apo e. APOE*4 shows opposite effects and APOE*3 intermediate effects 167, 168. Besides its role in lipid metabolism, APOE is a well established genetic risk factor for Alzheimer’s disease (AD) with APOE*4 showing an increased risk and APOE*2 a protective effect 166.

Besides the APOE polymorphism several polymorphisms within the promoter region of APOE were reported to be associated with a higher transcriptional activity and an increased risk for AD 90, 169, 170. In humans, plasma apo e levels were found to be influenced by promoter polymorphisms in case-control studies of myocardial infarction 171 and AD 172. Case-control studies trying to confirm the initial association of the promoter polymorphisms with AD reported inconsistent findings 91, 105, 165, 170, 173. A possible explanation for these inconsistent findings might be the presence of linkage disequilibrium between these promoter polymorphisms and the APOE polymorphism.

In this study we describe the association between the −491A/T promoter polymorphism and plasma apo e levels and AD in a population based sample of AD cases and controls. Furthermore, we analyses whether there was linkage disequilibrium between the APOE and the −491A/T promoter polymorphism and whether interaction between these loci influenced apo e plasma levels or AD risk.
Chapter 3.6

Methods

This study is performed as part of the Rotterdam Study, a population-based cohort study of 7983 subjects aged 55 years and over living in a suburb of Rotterdam, the Netherlands. AD was diagnosed as described previously, using the National Institute of Neurological and Communicative Diseases and Stroke – Alzheimer's Disease and Related Disorders Association criteria (NINCDS-ADRDA). By this means, 339 prevalent AD patients were ascertained at the baseline of the study and 116 incident AD patients were diagnosed at the end of the first follow-up period.

DNA was available for 360 AD cases (263 prevalent and 97 incident cases). For the genotype comparison a random sample of subjects without dementia was drawn from the baseline cohort (n=247). Non-fasting blood samples were used for lipid level measurements and DNA extraction. Plasma apoE levels were measured at baseline using standard methods. APOE and −491A/T genotyping was performed as described previously.

Hardy-Weinberg equilibrium and linkage disequilibrium were tested using the HWE and EH programs. For the statistical analyses of plasma apoE levels we used log-transformed values. The adjusted mean plasma apoE levels for APOE and −491A/T genotypes were determined using a general linear regression model. The means were adjusted for the effect of the other locus, age at venapuncture, gender, disease status (non-demented, incident AD, or prevalent AD), and body mass index (BMI) (Kg/m²). Mean levels are presented for APOE*3 homozygotes, APOE*4 carriers (APOE 34 and 44), and APOE*2 carriers (APOE 23 and 22). Subjects with the APOE 24 genotype were excluded from the analysis because of the combination of two alleles with opposite effects. For the −491AT polymorphism mean apoE levels were given for the 3 possible genotypes. Interaction between the APOE and −491AT genotypes was analysed using an additive model. Briefly, interaction between APOE and −491AT was considered to be a departure from additivity, and judged to be present if the interaction term was not equal to zero using formula 1:

\[
\text{formula 1: } (\beta_{A+B} - (\beta_{A+}) - (\beta_{B-}) \neq 0)
\]

In this formula, \(\beta_{A+B}\) is the regression coefficient of the group carrying an APOE*4 allele and the −491AA genotype, \(\beta_{A+}\) of the group with an APOE*4 allele and without the −491AA genotype, and \(\beta_{B-}\) of the group without an APOE*4 allele and with the −491 AA genotype. To test for statistical significance a 95% confidence
interval (CI) around the interaction term was calculated using the variances and covariances of the regression coefficients. Interaction was considered statistically significant if the 95% CI did not contain zero.

Genotype and allele frequencies of AD cases and controls were compared using the χ² statistic. Results were adjusted for age, gender, and the APOE polymorphism using logistic regression. In line with previous publications the odds ratio for the AA genotype was calculated with the AT and TT as a reference. Interaction between the −491AT and APOE polymorphisms was again tested using an additive model, and judged to be present if the interaction term was not equal to zero using formula 2.

\[ \text{formula 2: } (\text{OR}_{\text{A+T}}) - (\text{OR}_{\text{A+B}}) - (\text{OR}_{\text{A,B}}) + 1 \neq 0 \]

The 95% CI of the interaction term was calculated from the variances and covariances of the logarithm of the odds ratios using the standard delta method ¹⁷⁷.

**Results**

The characteristics of the study population are given in table 3.10. AD patients were significantly older and were more often male than controls. Furthermore, cases showed a significantly lower BMI and plasma apoe levels. Both the APOE and −491A/T genotype frequencies in controls were in Hardy-Weinberg equilibrium. There was a strong linkage disequilibrium between the APOE and −491A/T polymorphism in both cases and controls (p < 0.001). The −491 A allele was linked to APOE*4 and the −491 T allele to APOE*2.

For the three APOE genotype groups the expected trend of plasma apoe levels were found with the highest levels for APOE*2 carriers, the lowest for APOE*4 carriers, and intermediate levels for APOE*3 homozygotes (table 3.11). The APOE genotypes showed a highly significant contribution to the plasma apoe levels and explained 22% of the variance of the adjusted plasma APOE levels. In the analysis of the −491A/T polymorphism there was a borderline significant contribution to the plasma apoe levels (p=0.05). This contribution was however minimal and explained no more than 1% of the variance of the adjusted plasma APOE levels (table 3.11).

The plasma apoe levels indicated there was an interaction between the APOE and −491A/T polymorphism. Subjects with neither an APOE*4 allele nor the −491 AA genotype had a mean plasma level of 2.59, subjects with only the APOE*4 allele 2.36, subjects with only the −491 AA genotype 2.47, and subjects with both an APOE*4 allele and the −491 AA genotype 1.85. Using the formula of additive interaction with
Chapter 3.6

the regression coefficients we found a non-significant interaction term of −0.2 with a 95% CI from −0.49 to 0.09.

Table 3.10 Characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>AD cases</th>
<th>Controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=360</td>
<td>N=247</td>
<td></td>
</tr>
<tr>
<td>Age in years (SD)</td>
<td>82.4 (7.1)</td>
<td>74.7 (3.6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Female (%)</td>
<td>76</td>
<td>85</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Body mass index (kg/m²) (SD)</td>
<td>25.7 (3.8)</td>
<td>27.4 (4.5)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Plasma apoE (SD)</td>
<td>2.3 (1.6)</td>
<td>2.7 (1.6)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Table 3.11 Adjusted mean plasma apoE levels for APOE and −491A/T genotypes

<table>
<thead>
<tr>
<th></th>
<th>APOE*2</th>
<th>APOE33</th>
<th>APOE*4</th>
<th>p-value</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=64)</td>
<td>(n=195)</td>
<td>(n=119)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean apoE levels for</td>
<td>4.02 (1.1)</td>
<td>2.83 (1.1)</td>
<td>2.18 (1.1)</td>
<td>&lt;0.001</td>
<td>0.22</td>
</tr>
<tr>
<td>APOE genotypes (SE)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>-491 AA</th>
<th>-491 AT</th>
<th>-491 TT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=281)</td>
<td>(n=87)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Mean apoE levels for</td>
<td>2.54 (1.1)</td>
<td>2.70 (1.1)</td>
<td>3.64 (1.2)</td>
</tr>
<tr>
<td>491A/T genotypes (SE)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Genotype and allele frequency distributions of the −491A/T polymorphism in AD cases and controls are given in table 3.12. In accordance with previous reports the AA genotype and A allele are more frequent in cases. This minor difference was not statistically significant. In an age and gender adjusted logistic regression analysis the OR for AD of the AA genotype versus the AT/TT genotypes was borderline significantly increased (OR = 1.6, 95% CI 1.0-2.5). However after adjusting for APOE genotypes this association disappeared (OR = 1.2, 95% CI 0.7-1.9). There was no evidence for additive interaction between the APOE and −491A/T polymorphisms on AD risk. Subjects with only APOE*4 and without the −491 AA genotype had an OR of 1.7, subjects with only the −491 AA genotype had an OR of 1.08, and subjects with both APOE*4 and the −491 AA genotype an OR of 1.86. Using the formula of
additive interaction with the odds ratios we found a non-significant interaction term of 0.07 with a 95% CI from −1.95 to 2.09.

### Table 3.12 Genotype and allele frequencies of the −491A/T polymorphism

<table>
<thead>
<tr>
<th></th>
<th>AA</th>
<th>AT</th>
<th>TT</th>
<th>( \chi^2 ) (p-value)</th>
<th>A</th>
<th>T</th>
<th>( \chi^2 ) (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n=214)</td>
<td>0.71</td>
<td>0.27</td>
<td>0.02</td>
<td>2.3 (0.3)</td>
<td>0.84</td>
<td>0.16</td>
<td>1.42 (0.23)</td>
</tr>
<tr>
<td>AD cases (n=349)</td>
<td>0.76</td>
<td>0.22</td>
<td>0.03</td>
<td></td>
<td>0.87</td>
<td>0.13</td>
<td></td>
</tr>
</tbody>
</table>

### Discussion

In this study we demonstrated that the A allele of the −491A/T polymorphism in the regulatory region of APOE is associated with lower levels of plasma apoe independent of the APOE genotype. There was a statistically non-significant additive interaction between the APOE and −491A/T polymorphisms in determining the apoe plasma levels. There was a borderline significant association of the AA genotype with AD but after adjusting for the APOE genotype the association disappeared. No interaction between the APOE and −491A/T locus on AD risk could be detected.

In our study both the AA and the APOE*4 genotypes are associated with lower apoe levels. The −491A/T polymorphism explained only 1% of the variance of apoe levels while the APOE locus was more important, explaining 22% variance. There are only two previous reports on the association between APOE promoter polymorphisms and apoe plasma levels. One study showed an association with the −219G/T polymorphism and plasma apoe levels. The T allele of this polymorphism was associated with lower apoe levels and an increased risk for myocardial infarction. They could not find a similar effect of the −491A/T polymorphism. It is possible that neither the −491A/T nor the −219G/T polymorphism directly influence the apoe levels but the physically close APOE polymorphism in linkage disequilibrium with these polymorphisms. This could explain that different markers are associated in different populations. The second study reported an association between the −491A/T polymorphism and apoe plasma levels. However, they reported the highest apoe plasma levels for the AA genotype and this genotype was also associated with an increased risk for AD.

Although the −491A/T polymorphism was associated with apoe plasma levels we could not detect an APOE independent association with AD. This is in accordance
with several studies 91, 105, 165 but in contradiction with others 90, 178. A problem in some studies is that the analyses are stratified for APOE*4 carriers and non-carriers. This does not take into account the possible role of the APOE*2 allele, which is in linkage disequilibrium with the −491 T allele, and the dose-dependent effect of APOE*4. This is a possible cause of the contradicting findings. In our analysis of interaction between APOE and −491A/T we also took into account the effect of APOE*2. The best way to disentangle the role of each of the 2 loci is to analyse haplotypes in families.

Another explanation for the contradicting findings might be population specific effects due to linkage disequilibrium. It could be that not the −491 A allele but a locus in linkage disequilibrium with it is associated with apoE plasma levels and AD. Linkage disequilibrium is easier to detect in populations where the A allele is less frequent. This is in concordance with the fact that studies of populations with a high frequency of the −491 A allele, such as this study, show no association of this polymorphism with AD 105, 179, 180.

In conclusion, data from the Rotterdam study suggest a minor but statistically significant impact of the APOE −491A/T locus on plasma apoE levels. There is however no evidence for an APOE independent association of the −491A/T locus with AD.
Chapter 4

Family-based studies

4.1 Introduction

4.2 Presentation of amyloidosis in carriers of the codon 692 mutation in the amyloid precursor protein gene (APP692)

4.3 Alzheimer’s disease in a Dutch recent genetically isolated population. The GRIP Study

4.4 Linkage-disequilibrium mapping in an isolated population; a study of chromosome 10 and 12
Chapter 4.1

Introduction

Family-based studies have proven to be a powerful approach to identify genes involved in a disease. The most powerful design used in family-based studies is the linkage analysis. In this approach anonymous genetic markers equally distributed across the genome are genotyped. In linkage analysis cosegregation of a marker with the disease is examined in a family. In 4.2 a family-based genotype-phenotype study is presented of a family which was found to be linked to a mutation in the amyloid precursor protein gene after a classical linkage study. This complex phenotype of a family with an APP gene mutation is discussed to illustrate the importance of even very rare mutations for our knowledge on the pathophysiology of complex disorders. The limitation of linkage analysis is that for late-onset disorders like Alzheimer’s disease, families with a clear inheritance pattern are limited. Furthermore, it is difficult to sample multiple affected subjects in 2 or more generations. These problems can be dealt with in genetically isolated populations. Since subjects sampled from these populations have a high probability of being related to each other extensive pedigrees can be created after genealogical research. In 4.3 a recently isolated population in the Southwest of the Netherlands is presented. Since susceptibility genes are inherited from the same founders and the number of recombinations is limited if the population is of recent origin, patients carrying the susceptibility allele will share a considerable piece of DNA around the disease allele. In 4.4 this linkage disequilibrium around disease alleles is used to study the chromosome 10 and 12 regions in the Dutch isolated population.
Chapter 4.2

Presentation of amyloidosis in carriers of the codon 692 mutation in the amyloid precursor protein gene (APP692)

A common pathological hallmark of Alzheimer's disease and cerebral amyloid angiopathy (CAA) is the deposition of amyloid β (Aβ) in parenchymal senile plaques and cerebral blood vessel walls. Several mutations in exons 16 and 17 of the amyloid precursor protein (APP) gene cause autosomal dominant forms of early onset Alzheimer's disease \(^3, 181\). However, APP mutations may alternatively cause CAA with cerebral haemorrhages without Alzheimer's disease pathology. A glutamic acid to glutamine mutation at codon 693 of APP (APP693) is associated with hereditary cerebral haemorrhage with amyloidosis of the Dutch type (HCHWA-D) \(^38, 182\). The pathology in carriers of APP693 shows extensive CAA but no lesions characteristic of Alzheimer's disease \(^183\). Our group described a mutation at codon 692 of the APP gene (APP692) presenting with either a presenile dementia or cerebral haemorrhage \(^39\). Little is known about the natural history of disease in carriers of APP692. We have conducted a follow-up study of the patients with Alzheimer's disease and CAA as well as their offspring. The disease progression and pathology was studied in 8 patients carrying the APP692 mutation. Further, 21 first-degree relatives at 50% risk were tested for the APP692 mutation and studied for presymptomatic signs by neurological examination, neuropsychological testing, and brain MRI.
Chapter 4.2

Methods

Family ascertainment

This family was brought to our attention by a 51 years old patient who presented with presenile dementia. A sibling was known with cerebral haemorrhage caused by CAA. Family history showed a genetic disorder segregating in this family either manifesting as presenile dementia or cerebral haemorrhage (figure 4.1). In four cases, cerebral haemorrhage occurred in the offspring of a patient with dementia whereas one dementia case was reported in the offspring of a haemorrhage patient. Both traits could be linked to the APP 692 mutation 39.

The disorder could be traced back to an ancestor born 3 generations back in 1866, who was affected with a presenile dementia syndrome according to heteroanamnestic data. All offspring of the siblings of this ancestor were traced using municipal genealogy records and living descendants were contacted. None of the 81 descendants of these siblings were known with early onset dementia or cerebral haemorrhage. Figure 4.1 shows the only branch of the family segregating the APP692 mutation.

Subjects

Clinical data of 9 patients with a cerebral haemorrhage, dementia, or both could be retrieved. Seven patients were clinically examined. Two patients were described using records from other hospitals. Twenty-one healthy individuals at 50% risk participated in our study of asymptomatic carriers, 10 from generation IV, and 11 from generation V. All clinical, neuropsychological and MRI studies were conducted blinded for the APP692 status. The physical examination and MRI studies were performed in all respondents. The neuropsychological test battery was completed by 18 subjects.

Of the 21 subjects tested for the APP692 mutation, 5 carried the mutation. Characteristics of carriers and non-carriers participating in the study are given in table 4.1. Carriers and non-carriers differed significantly in age (P = 0.02). Age is an important determinant of neurological performance and white matter lesions (WML). For the MRI studies, an age-matched control group was drawn (Table 4.1). Subjects without known or suspected pathology involving white matter were derived from the neurological outpatient clinic. To adjust for the difference in age distribution between carriers and non-carriers, age and sex standardised scores for neuropsychological tests were used.
Figure 4.1 Updated pedigree of family segregating the APP692 mutation. The pedigree is disguised for reasons of confidentiality. The filled symbols represent the patients presenting with presenile Alzheimer's disease, the half-filled symbols the patients presenting with cerebral haemorrhage. Of the subjects alive in generations IV and V, only the people who participated in this study after informed consent are indicated.
Table 4.1 Participants-characteristics of the study of asymptomatic carriers

<table>
<thead>
<tr>
<th></th>
<th>APP692 carriers</th>
<th>Non-carriers</th>
<th>Age matched unrelated controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MRI studies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of subjects</td>
<td>5</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Male / Female</td>
<td>4 / 1</td>
<td>8 / 8</td>
<td>5 / 7</td>
</tr>
<tr>
<td>Age (years) (Range)</td>
<td>26.4 (21-30)</td>
<td>37.8 (24-60)</td>
<td>25.5 (19-31)</td>
</tr>
<tr>
<td><strong>Neuropsychology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of subjects</td>
<td>4</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>Male / Female</td>
<td>3 / 1</td>
<td>7 / 7</td>
<td></td>
</tr>
<tr>
<td>Age (years) (Range)</td>
<td>26.8 (21-30)</td>
<td>38.2 (24-60)</td>
<td></td>
</tr>
</tbody>
</table>

From all participants informed consent was obtained and the protocol of this study was approved by the Medical Ethics Committee of the Erasmus Medical Centre Rotterdam. According to the study protocol the mutation tests were not disclosed to the participants. All subjects irrespective of APP692 status were offered individual genetic counselling and presymptomatic testing. The results of the MRI studies and neuropsychological tests were reported to participants on a group level; no individual scores were given.

**Clinical and neuropsychological examination**

All subjects at risk and 7 out of 9 patients diagnosed with cerebral haemorrhage or dementia underwent neurological examination. The examination included medical history, assessment of the mental status, cranial nerves, muscle strength and tone, sensation, co-ordination, fine motor skills, tendon reflexes, primitive reflexes, posture, and movement.

The neuropsychological evaluation of subjects at risk was performed by a neuropsychologist. The test battery included 9 domains. The condensed version of the Groningen Intelligence Test (GIT) \(^ {184} \), a Dutch intelligence test. The expected premorbid intelligence quotient (IQ) was calculated from level and years completed of education for all persons, based on standardised scores for the Dutch population \(^ {185} \). The ratio of the current IQ to expected premorbid IQ was used as estimation for deterioration in IQ. Tests for measuring attention and concentration included part A of the Trail Making Test \(^ {186} \), and the ‘substitution’ test of the Wechsler Adult
Intelligence Scale (WAIS) \(^{187}\). We used digit span forward and backward version of the WAIS \(^{187}\) to assess the span of immediate verbal recall and also as a measure of attention capacities. Auditory verbal memory was examined by the Dutch validated and standardised version of the California Verbal Learning Test (CVLT) \(^{188, 189}\). The total score is the number of words correctly recalled in five trials. Recognition score is related to the score after five presentations, represented with "hits" and "false positives". Recall of the complex figure of Rey was used for measuring nonverbal memory \(^{190}\). The subtest ‘Similarities’ of the WAIS provided a measure for abstraction and verbal concept formation \(^{187}\). Furthermore, executive (control) functions were examined with part B of the Trail making test \(^{186}\), and verbal fluency \(^{184}\). Visuoconstructive performance was assessed by the Rey-Osterrith complex figure test \(^{190}\) and visuospatial abilities were examined with the Line Orientation test \(^{191}\).

**Table 4.2 Semiquantitative MRI scores for white matter lesions**

<table>
<thead>
<tr>
<th>Periventricular</th>
<th>Range</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caps: occipital</td>
<td>0-2</td>
<td>0 = absent</td>
</tr>
<tr>
<td>Caps: frontal</td>
<td>0-2</td>
<td>1 = &lt; 4 mm</td>
</tr>
<tr>
<td>Lines: lateral ventricle</td>
<td>0-2</td>
<td>2 = &gt; 4 mm</td>
</tr>
<tr>
<td>Total periventricular</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Subcortical**

| Frontal lobe          | 0-6     | 1 = < 4 mm, n < 6 |
| Parieto-occipital lobe | 0-6     | 2 = > 4 mm, n > 6 |
| Total white matter    | 0-12    | 4 = 4-10 mm, n < 6|
|                       |         | 5 = > 10 mm    |
|                       |         | 6 = confluent  |

**Total score**

0-18

**Neuro-imaging**

In the subjects at 50% risk MRI scanning was performed on a Gyroscan T5-II, using T1 and T2 weighted series in the axial plane. A neurologist and a research physician scored the MRI scans with a semiquantitative scale separately for periventricular and
Chapter 4.2

subcortical WML, blinded to the clinical and genetic data. This scale produces a score related to the size and number of the WML (Table 4.2). Lesions were defined as areas of higher signal intensity compared to surrounding brain tissue on T2 weighted scans.

**DNA testing**
Genomic DNA was extracted from peripheral blood leucocytes. APP mutation screening was performed either by PCR-RFLP or by SSCP analysis as follows. Exon 17 was amplified and the PCR product was loaded on a 1x HydroLink MDE gel (J.T. Baker Inc, Philipsburg, New York) with 10% glycerol and the gel was silverstained. The analysis of the apolipoprotein E genotypes (APOE) was performed as described elsewhere.

**Statistical analysis**
The differences between mutation carriers, non-carriers, and the unrelated controls were analysed with the non-parametric Mann Whitney test. Exact one-sided P-values were used because our hypothesis is that mutation carriers are more likely to score worse on psychometric tests and MRI.

**Results**

**Clinical course and pathology affected subjects**
The clinical characteristics of the 9 patients are summarised in table 4.3; a detailed report on the clinical course for each patient is given in the appendix. Three patients presented with cerebral haemorrhage (patient 2, 3 and 5). One of these patients was diagnosed with probable Alzheimer’s disease 6 years after the haemorrhage (see appendix patient 3). Cognitive function could not be assessed in the other 2 patients presenting with cerebral haemorrhage because one patient reached a vegetative state within one year after the first stroke, while the other refused further participation. All patients presenting with cerebral haemorrhage carried the APP692 mutation and the APOE genotype 34. In patient 3 a brain biopsy was taken during surgery at the initial stroke. The biopsy showed extensive amyloid deposition in the blood vessel walls and in parenchymal senile plaques. The senile plaques were predominantly of the diffuse type, with a few neuritic plaques but without neurofibrillary tangles.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Onset age (years)</th>
<th>Age of first Haemorrhage (years)</th>
<th>Age at death (years)</th>
<th>First symptoms</th>
<th>Disease course</th>
<th>Behavioural problems</th>
<th>Neuro-imaging</th>
<th>APP692 mutation</th>
<th>APOE</th>
<th>Pathology</th>
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<tr>
<td>1</td>
<td>48</td>
<td>-</td>
<td>57</td>
<td>Memory loss</td>
<td>Gradual decline cognitive function L hemiplegia</td>
<td>+</td>
<td>Cortical + central atrophy, WML</td>
<td>+</td>
<td>34</td>
<td>SP, NFT, CAA #</td>
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<td>-</td>
<td>43</td>
<td>-</td>
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<td>-</td>
<td></td>
<td>Haematoma, WML</td>
<td>+</td>
<td>34</td>
<td>-</td>
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<tr>
<td>3</td>
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<td>42</td>
<td>-</td>
<td>L hemiplegia</td>
<td>Gradual decline cognitive function Vegetative state</td>
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<td>+</td>
<td>34</td>
<td>CAA ##</td>
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<td>4</td>
<td>49</td>
<td>-</td>
<td>-</td>
<td>Memory loss</td>
<td>Gradual decline cognitive function</td>
<td>+</td>
<td>Cortical + central atrophy, WML</td>
<td>+</td>
<td>34</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>?</td>
<td>44</td>
<td>-</td>
<td>R hemiplegia</td>
<td>?</td>
<td></td>
<td>Haematoma</td>
<td>+</td>
<td>34</td>
<td>-</td>
</tr>
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<td>Normal</td>
<td>+</td>
<td>33</td>
<td>SP, NFT, CAA #</td>
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<td>Gradual decline cognitive function</td>
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<td>+</td>
<td>?</td>
<td>-</td>
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<td>Cortical + central atrophy</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>9</td>
<td>41</td>
<td>46</td>
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<td>Memory loss</td>
<td>Gradual decline cognitive function L hemiplegia</td>
<td>-</td>
<td>Cortical + central atrophy, WML</td>
<td>+</td>
<td>34</td>
<td>-</td>
</tr>
</tbody>
</table>

APOE = apolipoprotein E genotype, +/-? = present/not present/unknown, R/L = right/left, SP = senile plaques, NFT = neurofibrillary tangles, # autopsy ## Biopsy
In total 6 patients presented with dementia. In these patients, the clinical course of disease was compatible with probable Alzheimer’s disease. One patient developed a haemorrhage after the diagnosis of Alzheimer’s disease (patient 9). Each patient was screened for the APP692 mutation, and except for patient 8, all were carriers. As shown in figure 4.2, the clinical course of patient 8 differs in that the age at onset and death is outside the range of patients carrying the mutation. The diagnosis of Alzheimer's disease was pathologically confirmed for patient one and six. Both patients showed cortical and subcortical neuronal loss, which was accompanied by the presence of numerous senile plaques and neurofibrillary tangles and congophilic angiopathy. The senile plaques were larger than reported for classical Alzheimer’s disease. Of the 5 patients diagnosed with dementia with a known APOE genotype, 4 carried the APOE*34 genotype.

When comparing the APP692 carriers presenting with haemorrhage and dementia, the vascular pathology occurred at an earlier age (mean age 43 years) than the dementia syndrome (mean age 52 years) (see also figure 4.2). However, both patient 3 and 9 show that the vascular and Alzheimer’s disease pathology may occur in a single patient, presenting with either haemorrhage or with dementia. WML were found at neuroimaging in 2 out of the 3 patients presenting with cerebral haemorrhage while 3 out of 5 patients presenting with dementia showed WML (for one patient, no scan was available). There was no evidence that APOE influences the age at onset of dementia or cerebral haemorrhage.

**Pre-clinical signs in subjects at risk**

None of the 21 subjects at risk studied showed abnormalities at the neurological examination. Scores of the neuropsychological tests were within the normal range. The 4 APP692 carriers tended to score lower on tests for intelligence, short-term memory, abstraction, and visuospatial abilities than the 14 non-carriers (table 4.4).
Figure 4.2 duration of follow-up of patients. Mean age of first symptoms and death are calculated for APP692 carriers. Open bars are patients initially presenting with dementia and filled bars are patients presenting with haemorrhage.

† age at death, * age at end of follow up
Table 4.4 Scores of the neuropsychological testing

<table>
<thead>
<tr>
<th></th>
<th>Mutation (n=4)</th>
<th>No Mutation (n=14)</th>
<th>P-value</th>
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<tr>
<td></td>
<td>Median</td>
<td>Range</td>
<td>Median</td>
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<tr>
<td><strong>Intelligence</strong></td>
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<tr>
<td>GIT-IQ</td>
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<td>79-107</td>
<td>103</td>
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<tr>
<td>Expected IQ</td>
<td>108</td>
<td>86-108</td>
<td>108</td>
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<tr>
<td>Ratio GIT/expected IQ (%)</td>
<td>80</td>
<td>73-99</td>
<td>99</td>
</tr>
<tr>
<td><strong>Attention and concentration</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Trail making A *</td>
<td>50-75</td>
<td>50-100</td>
<td>50-75</td>
</tr>
<tr>
<td>Substitution WAIS *</td>
<td>54</td>
<td>36-93</td>
<td>83</td>
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<tr>
<td><strong>Auditory verbal memory</strong></td>
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<tr>
<td>Total score CVLT ** †</td>
<td>-2</td>
<td>-5 to 0</td>
<td>-1</td>
</tr>
<tr>
<td>Recognition CVLT ** ††</td>
<td>+2</td>
<td>+1 to +3</td>
<td>+1</td>
</tr>
<tr>
<td><strong>Short term memory</strong></td>
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<tr>
<td>Digit span WAIS *</td>
<td>38</td>
<td>14-66</td>
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<td><strong>Non verbal memory</strong></td>
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<td>10-100</td>
<td>80-90</td>
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<tr>
<td><strong>Abstraction</strong></td>
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<tr>
<td>Similarities WAIS *</td>
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<td>14-95</td>
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<tr>
<td><strong>Executive control functions</strong></td>
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<tr>
<td>Trail making B *</td>
<td>50-75</td>
<td>25-75</td>
<td>50-75</td>
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<tr>
<td>Word fluency test **</td>
<td>28</td>
<td>26-30</td>
<td>31</td>
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<tr>
<td><strong>Visuoconstructional</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Rey complex figure *</td>
<td>80-90</td>
<td>20-100</td>
<td>90-100</td>
</tr>
<tr>
<td><strong>Visuospatial</strong></td>
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<td></td>
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</tr>
<tr>
<td>Line orientation test *</td>
<td>48</td>
<td>22-100</td>
<td>74</td>
</tr>
</tbody>
</table>

* Percentile score ** Standardised score
† Number of words learned in 5 trials of 16 words
†† Difference between recognition and long term free recall

MRI did not show evidence for cerebral haemorrhage in any of the asymptomatic family members. The results of the semiquantitative score of the MRI scans were computed for the periventricular and subcortical region (table 4.2). The five mutation carriers had a statistically higher score of total periventricular WML (median = 1) than the 16 non-carriers (median = 0; P = 0.001) and the clinic-based control group (median = 0; P = 0.001). The median scores for the subcortical region did not differ between carriers (median = 1) and non-carriers (median = 1), but differed borderline significantly from the age matched controls (median = 0; P = 0.04). The total WML score for the periventricular and subcortical region in mutation carriers (median = 2) was not different from scores in related non-carriers (median = 1), but differed
significantly from those in the age-matched hospital controls (median = 0, \( P = 0.001 \)). APOE 4 was not related to the load of WMH. However, as in the affected relatives a high proportion of subjects at risk carried the APOE E4 allele (15 out of 21).

**Discussion**

Our study of eight patients with the APP692 mutation showed variable onset and progress of disease. Patients with APP692 presented with haemorrhage, dementia, or both diseases. The dementia in patients with the APP692 mutation was compatible with Alzheimer’s disease both clinically and neuropathologically. The presymptomatic carriers showed a subtle, non-significant, impairment of cognitive function compared to relatives without APP692. A significant increase in the number of periventricular and subcortical white matter lesions at early age was seen in presymptomatic carriers (mean age of 26.4 years).

Although both the APP692 and APP693 mutations involve missense mutations at neighbouring codons, the clinical expression is very different. Patients with the APP693 mutation diagnosed with HCHWA-D suffer from recurrent strokes with a high mortality 199. Mortality after haemorrhage in carriers of the APP692 mutation is low; all patients described here are still alive even after multiple haemorrhages. Few patients with the APP693 mutation diagnosed with HCHWA-D develop dementia 199. If dementia occurs, the clinical course is compatible with vascular dementia rather than with Alzheimer’s disease 199. No neurofibrillary tangles have been observed at pathological examination in patients with the APP693 mutation 183. Although it is difficult to disentangle the cause of dementia in the presence of CAA, the clinical course of dementia was compatible with Alzheimer’s disease in the patients with APP692. For 2 patients with the APP692 mutation presenting with dementia, the clinical diagnosis of Alzheimer’s disease was confirmed pathologically 197. At the level of pathology, senile plaques and neurofibrillary tangles were present. Also in the biopsy sample from patient 3 which was taken during vascular surgery, senile plaques were found but no neurofibrillary tangles 39. These pathological lesions did not fulfil the Alzheimer’s disease criteria due to the absence of neurofibrillary tangles 9. This patient was diagnosed with Alzheimer’s disease 6 years after the haemorrhage. Since there was no evidence for subsequent vascular events, and the cognitive deficits gradually progressed, we hypothesise that the patient’s cerebral pathology has
developed further towards Alzheimer’s disease. An implication of our hypothesis is that the vascular pathology and Alzheimer’s disease are processes occurring simultaneously in carriers of APP692.

The APP 692 mutation leads to mutated forms of secreted Aβ peptides Aβ 40 and Aβ 42. The expression of APP692 as Alzheimer’s disease or cerebral haemorrhage may be explained by the effect of the mutation on the levels of Aβ 40 and Aβ 42 \(^{146}\). The Aβ 42 peptide is predominantly deposited in senile plaques and is selectively increased in Alzheimer’s disease patients carrying APP mutations \(^{146}\), while increased Aβ 40 levels may lead to vascular amyloid depositions \(^{200}\). In contrast to other APP mutations, in vitro cDNA transfection studies show that the APP692 mutation leads to both increased Aβ 42 and Aβ 40 secretion \(^{201}\), which is compatible with an increased risk of Alzheimer’s disease pathology and vascular amyloid depositions.

Although findings of neuropsychological tests in presymptomatic carriers were not conclusive statistically, overall carriers appear to perform worse on the intelligence, short-term memory, visuospatial, and abstraction tests at early age (mean age 26.4 years). However, the differences between carriers and non-carriers are small and as the findings are based on only four mutation carriers who completed the neuropsychological testing, no conclusions can be drawn based on this sample.

White matter pathology was found more frequently in asymptomatic mutation carriers in the periventricular regions, as compared to the related and the age-matched controls. These findings suggest that WML are early manifestations of APP692 related pathology. Also in the general population, the strongest relation between WML and cognitive function is observed in the periventricular region \(^{202}\). However, compared to age-matched hospital controls, also in the subcortical region WML were more frequently present in pre-symptomatic carriers of the APP692 mutation. WML were also found in 60% of the patients described in this paper. The pathophysiology of WML lesions as well as their role in the development of dementia is unclear. None of the subjects studied suffered from hypertension, the most important risk factor for WML in the general population \(^{203}\). Vascular amyloid deposits leading to stenosis of arterioles may cause chronic hypoperfusion leading to WML as well as Alzheimer’s disease pathology \(^{204}\). Patient 9 showed WML adjacent to previous haemorrhages (see figure 4.4) which is in line with the observation that WML are predominantly the result of secondary damage after cortical haemorrhages and/or infarcts \(^{205, 206}\).
Recently the E4 and E2 allele of the APOE gene have been found to modify the clinical presentation of CAA. We did not find any association of the E4 allele with age at onset and the presence of dementia in symptomatic carriers of APP692 nor with the presence of WML or cognitive (dys)function in the asymptomatic APP692 carriers. Also for the APP693 mutation no association with the APOE gene was found. However, 75% of the subjects in the present study carried the APOE E4 allele and none carried the E2 allele. The statistical power to analyse the effect of APOE on the clinical expression of the APP692 mutation was therefore low in our study.

The findings in our family segregating the APP692 mutation suggest that a single mechanism may underlie the pathology of Alzheimer’s disease and CAA. Further the findings in asymptomatic carriers suggest that these diseases are subclinically manifested by white matter pathology at early age. These observations suggest that preventive strategies in these high risk subjects are to start at early age. It remains to be determined whether our findings in carriers of the APP692 mutations can be extrapolated to the vast majority of patients with sporadic Alzheimer’s disease. Given the presence of CAA in sporadic patients with Alzheimer’s disease, further studies of the pathophysiology of the APP692 mutation may be relevant for our understanding of the common origin of both the CAA and Alzheimer’s disease lesions.

Appendix

Patient description

Patient 1 was seen at age 51 years because of headaches and behavioural problems. The spouse reported memory loss, aggressive behaviour, loss of initiative, and gradual cognitive decline since 2 to 3 years. No epileptic seizures or myoclonic jerks were reported. Neurological exam was normal. Neuropsychological testing showed cognitive dysfunction (Cognitive Screening Test 6/20), with disorientation in time, memory problems (immediate and delayed recall 0/15 words), impaired visuospatial skills (unable to draw or copy a clock), and language disturbances (resembling a transcortical sensory aphasia on the Achener Aphasia Test). Blood and CSF tests were normal. EEG showed a low dominant rhythm with episodes of delta activity bilaterally in the frontal regions. On CT scan marked cortical and central atrophy, with multiple WML was seen. Single photon emission computed tomography (SPECT) scanning showed symmetric temporo-parietal hypoperfusion. The clinical
Chapter 4.2

diagnosis was probable Alzheimer's disease according to the NINCDS-ADRDA criteria (National Institute of Neurological and Communicative Diseases and Stroke – Alzheimer's Disease and Related Disorders Association) 12. Cognitive function and capabilities declined gradually during the disease course leading to nursing home admission and death at age 57 years following pneumonia.

At autopsy, a moderate cortical and central atrophy was found 197, 198. The meninges were thickened and brown discoloured at the frontal and temporal lobes. Both in the cortical and subcortical white matter small cavities were found often surrounded by a brownish hue. Numerous leptomeningial vessels with amyloid deposits were present. Throughout the whole cortex, senile plaques of different types were found (see figure 4.3). Several of these plaques had a larger core than in classical Alzheimer's disease. Lumen reducing amyloid deposits were found in vessels. Neurofibrillary changes were numerous especially in the superior temporal gyrus. In areas CA1 and CA2, there was extensive neuronal loss while area CA3 and CA4 were relatively spared. Immunohistochemistry showed numerous amyloid deposits, dystrophic neurites and neurofibrillary tangles (see figure 4.3). In the subcortical areas few abnormalities were found.

*Patient 2* was successfully treated with sodium valproate for generalised epileptic seizures at age 30 years. Neurological examination nor EEG showed evidence for focal pathology. Seizures recurred at age 41 years, with left-sided symptoms. Neurological examination was normal. Paroxysms of theta and delta activity in the central temporal region were seen on EEG. CT scan revealed a hypodensity in the right basal ganglia and in the left parietal region. Calcifications were present in the white matter of the centrum semiovale on the right side. At age 43 years, the patient was admitted with a right-sided hemiparesis, aphasia, and a right-sided homonymous hemianopia. Tendon reflexes in the right extremities were increased with an ankle clonus and a right-sided extensor plantar response. CT scan revealed a large parieto-temporal occipital haematoma, with extension into the lateral ventricle. A second stroke one year later resulted in a left-sided paresis and complete loss of consciousness. The patient has remained in a vegetative state up till last follow-up (December, 1999).
Fig. 4.3a Numerous dense-core (arrows) and diffuse amyloid plaques (white arrows) in the neocortex stained by monoclonal antibody 4G8 directed to Aβ (Senetek, Maryland Heights, MO). Note also the presence of congophilic angiopathy (arrowhead).

Fig. 4.3b Numerous neurofibrillary tangles and dystrophic neurites in neocortex, immunostained by monoclonal antibody AT8 (for hyperphosphorylated tau; Innogenetics, Belgium). Arrow indicates the core of a cored plaque.
Chapter 4.2

Patient 3 was admitted to the neurosurgery department because of headache, nausea, vomiting, and loss of consciousness at age 42 years. Glasgow Coma Scale was E3M5V1. The patient was hemiplegic with a dilated pupil (left). CT scan showed a large left-sided parieto-occipital haematoma with midline shift. The haematoma was successfully surgically removed. A brain biopsy showed extensive amyloid deposition in the blood vessel walls and in parenchymal senile plaques 39. The senile plaques were predominantly of the diffuse type, with a few neuritic plaques but without neurofibrillary tangles. The patient was discharged with dysphasia, hemianopia, and a Gerstmann syndrome, without cognitive dysfunction.

The cognitive function stayed stable over the next three years, but declined afterwards resulting in dementia six years after the haemorrhage. Neurological evaluation showed an insidious onset of cognitive decline more than 3 years after the haemorrhage without evidence for a stepwise progression. At MRI, no evidence was found for a second haemorrhage. Neurological evaluation and neuropsychological screening tests resulted in the diagnosis probable Alzheimer's disease based on NINCDS-ADRDA criteria.

Patient 4 had symptoms since age 49 years. Neurological examination showed bradyphrenia, dysphasia, and severe memory disturbances. Neuropsychological testing revealed a decline in intellectual performance (GIT IQ 57), memory loss (Rivermead Behavioural Memory Test 2/12), reduced verbal (immediate and delayed recall 0/15 words) and visual recall (recall Rey figure 10th percentile), time orientation problems, moderate aphasia, severe disturbances of executive functions (semantic word fluency standardised score 17, mean population standardised score 25), and severe visuospatial problems (copy of Rey figure < 10th percentile). Routine blood and CSF tests were normal. CT scan showed WML, and both cortical and central atrophy. Reduced perfusion in all cortical regions, white matter and the left thalamus was revealed with SPECT, while EEG showed diffuse slowing of activity. According to the NINCDS-ADRDA criteria the diagnosis probable Alzheimer's disease was made. Cognitive function gradually declined and the patient was admitted to a psychogeriatric nursing home and is still alive at last follow-up (December, 1999).
Patient 5 was referred to emergency unit at age 44 years with a severe headache, nausea, vomiting, and right-sided hemiplegia. Memory and orientation were normal. The patient reported visual complaints 2 years earlier. Neurological examination showed paralysis of the right extremities with right-sided homonymous hemianopia, increased tendon reflexes and extensor responses bilaterally. CT scan showed a subcortical haemorrhage in the left fronto-parietal region with rupture into the ventricles. The patient was lost to follow up after admission to a rehabilitation centre.

Patient 6 had increasing memory problems since age 46 years and was examined at age 49 years. Neurological examination was normal. Time orientation and memory were slightly disturbed, masked by confabulations. Blood tests and CT scan were normal; EEG showed diffuse cortical disturbances. The diagnosis probable Alzheimer’s disease was made based on NINCDS-ADRDA criteria. Within one year, the patient was admitted to a nursing home, where mood disturbances were treated with anti-depressives. During the admission cognitive function gradually deteriorated. The patient developed hypokinesia and severe rigidity. Parkinsonism was ascribed to use of neuroleptics. Behavioural problems were marked, with screaming, aggression, agitation, and obsession. At 55 years the patient died from pneumonia and bowel obstruction. Macroscopic examination of the brain showed temporal, frontal cortical and subcortical atrophy, and a pale substantia nigra. Microscopy findings fulfilled the criteria for definite Alzheimer’s disease, with amyloid plaques, numerous neurofibrillary tangles, and congophilic amyloid angiopathy. A few Lewy bodies were present in the substantia nigra.

Patient 7 developed cognitive dysfunction insidiously at age 46 years. In the beginning of the 20th century, the patient was diagnosed with presenile dementia at age 51 years. EEG showed no θ rhythm with bilateral synchronous, often intermittent theta activity, and diffuse delta activity. A pneumo-encephalography revealed dilated lateral ventricles, predominantly in the right frontal and occipital lobes. At age of 59 years the patient died of bronchopneumonia. Autopsy was not performed.

Patient 8 first presented with memory problems, initiative loss, roaming, and depressive mood at age 59 years. At age 63 years, a presenile dementia syndrome was
diagnosed. The patient was known with glaucoma and diabetes mellitus type II. Besides loss of ankle reflexes, the neurological examination was normal, as were laboratory studies. EEG showed a diffuse slow rhythm with more abnormalities in the left fronto-temporal region. CT scan showed cortical and central atrophy. The diagnosis Alzheimer’s disease was made (according to NINCDS-ADRDA criteria). The patient was admitted to a psycho-geriatric institution at age 64. Cognitive functions declined rapidly. The patient developed severe rigidity and died at the age of 68 years. No autopsy was performed.

*Patient 9* presented at the age of 41 years with memory complaints. Cognitive screening only showed slight disturbances in the immediate and delayed recall (Rivermead Behavioural Memory Test 9/12). Neurological exam and laboratory studies were all normal. CT showed WML and a small hypo-dense lesion in the frontal cortex. EEG showed diffuse slow activity. Six months later, a marked decline in non-verbal memory (recall Rey figure < 10th percentile) was found. At that time the patient was diagnosed as probable Alzheimer’s disease (NINCDS-ADRDA criteria). By age 42 the patient could only perform simple tasks at work and showed a marked decline in cognitive function when tested. At follow-up, MRI showed extensive white matter lesions, two haemorrhages (both old, one not noticed earlier on CT), and cortical and central atrophy (see *figure 4.4a*). The hippocampus was symmetrically reduced in volume. At age 46 years the patient developed a left-sided hemiplegia. CT scanning revealed an intracerebral haemorrhage right parietal (see *figure 4.4b*). During recovery partial epileptic insults occurred that were successfully treated with anti-epileptic drugs. The patient was still alive at last follow-up (December, 1999).
Figure 4.4a MRI scan typical for patients with dementia carrying the APP 692 mutation. There is cortical and central atrophy, numerous WMLs most prominent adjacent to old haemorrhages in the frontal and occipital cortex.

Figure 4.4b Circumscribed haemorrhage in the right parietal cortex and global cortical atrophy.
Chapter 4.3

Alzheimer’s disease in a Dutch recent genetically isolated population. The GRIP Study.

In recent years, considerable progress has been made in unravelling the genetic aetiology of Alzheimer’s disease (AD)\textsuperscript{209}. Dominant mutations have been identified, in the amyloid precursor protein gene (APP), and in two homologous genes presenilin 1 (PSEN1) and presenilin 2 (PSEN2) \textsuperscript{210}. However, these causal mutations are rare and only present in a minor proportion of families with early-onset AD. A genetic risk factor more important on the population level is the apolipoprotein E gene (APOE) that may explain up to 17\% of the prevalence of AD in the general population \textsuperscript{8}, leaving the majority of patients with AD unexplained.

Several strategies to identify novel genes have been followed including candidate gene approach and genomic screening. None of these approaches yielded equivocal results so far. Up until now, all AD genes including APOE were identified using a linkage approach in large family based studies. However, the number of such pedigrees available is limited for a late-onset disease such as AD. There is increasing interest in using genetically isolated populations as an alternative to identify new genes for complex disorders. In genetically isolated populations the chances of localising genes may be more favourable. Since patients sampled from a genetically isolated population have a high probability of being related to each other, extensive pedigrees of these patients can be constructed by means of genealogical research. Up until now, the focus has been on populations of prolonged isolation such as the Finnish and Icelandic \textsuperscript{211, 212}. We present a study in a more recently isolated population for genetic studies of AD. We have examined to what extent apparently unrelated patients sampled from an isolated population can be connected to each other by genealogical research. Furthermore, we examined to what extent the occurrence of AD in this population may be explained by the known AD genes (APP, PSEN1, PSEN2, and APOE).
Chapter 4.3

Materials and methods

Study population

The study population comprises a genetically isolated population of about 20,000 inhabitants in the Southwest of the Netherlands. The founding of this population dates back to the middle of the 18th century. In this period a community of about 150 people settled in a small area in the Southwest of the Netherlands. From the initial settlement until the beginning of this century descendants of these families have lived in social isolation, without substantial integration with inhabitants of the surrounding area. The demography of this population has been characterised by minimal immigration. Immigration has been estimated to be 5.8% until 1940. The baptismal register reports 175 inhabitants in the year 1793 and 350 in the year 1820. The first official demographic documentation dates back to 1848. From this year on the population has expanded from 700 up to 20,000 inhabitants. This extensive growth was mainly a consequence of an increasing birth rate and improved life-expectancy. Population growth follows the curve of the general Dutch population.

Ascertainment of AD patients

The study of AD is part of a larger research program entitled; "Genetic Research in Isolated Populations (GRIP)". The medical ethical committee has approved this research program. Patients were recruited through general practitioners, nursing home physicians, and neurologists working in the catchment area comprising the isolated village. These physicians were asked for all patients suspected of dementia. Patients or caregivers first gave consent to the treating physician and after that to the research physician. All patients suspected or diagnosed with a dementia syndrome were visited by a research physician. Clinical information was collected using a standardised disease history, clinical examination, family history, and a review of the patient’s medical records. The diagnosis AD was made by the research physician and a neurologist according to the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) criteria for AD. In case of an uncertain diagnosis the patient was re-evaluated a year later. The degree of dementia was rated using the clinical dementia rating scale (CDR).
Collection of genealogical data

Genealogical data comprising the name, date, and place of birth of parents and grandparents were collected at a home interview. This genealogical information was extended up to 22 generations using municipal registers and data from a large genealogy database that holds genealogical information on 60,000 individuals from this region of The Netherlands. Genealogical relationship between 2 patients was expressed as the kinship coefficient. This is the probability that a randomly drawn allele from one patient is identical by descent to a randomly drawn allele at the same locus of the other patient. For example in a sib-pair the kinship coefficient is 0.25 and between cousins the kinship coefficient is 0.125. This means that the probability of a random allele genotyped in a sib-pair or cousin pair to be identical by descent is 0.25 and 0.125 respectively.

DNA analysis

Genomic DNA was extracted from peripheral blood leucocytes from all patients and if possible two first-degree relatives to reconstruct haplotypes. APOE genotyping was performed on all AD patients using previously described methods. Results were compared with Caucasian genotype frequencies from a large meta-analysis. All patients were screened for mutations in APP, PSEN1, and PSEN2. Exons 16 and 17 of APP, exon 8 of PSEN1, and exons 3 and 4 of PSEN2 were sequenced using the ABI PRISM Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, USA) and the sequence was analysed on automated ABI377 fragment analyser (Applied Biosystems). Single stranded conformational polymorphism (SSCP) analysis of all coding exons of the presenilin genes was performed. Each exon was PCR amplified under standard conditions using intronic primers flanking each exon. The PCR products were heat denatured, renatured on ice and separated at room temperature on precast ExcelGel gels (Pharmacia, Uppsala, Sweden) or 1 X Hydrolink MDE gels (J.T. Baker, Phillipsburg, USA) with 5% glycerol by electrophoresis using the MultiphorII system (Pharmacia). Bands were visualised by silver staining. When aberrant patterns were observed, the DNA sequence of the PCR product was determined as described above.

Statistical analysis

Pairwise kinship coefficients were calculated for all pairs of patients with the
Chapter 4.3

Kinship programme, written by L.A. Sandkuijl. A chi square statistic was used to compare the APOE genotype frequencies between cases from the genetically isolated population and cases from the general Caucasian population.

Results

Patients

In total 81 patients participated in the study after being invited by their treating physician. In 4 patients the diagnosis dementia could not be made since there were only memory complaints and memory deficits on neuropsychological testing. Clinical characteristics of the dementia patients are given in table 4.5. Of all patients with dementia 62 were diagnosed as probable AD, and 11 as possible AD. Of all AD patients 42 (57%) had at least 1 first-degree relative with AD (28 with 1, 6 with 2, 4 with 3, 2 with 4, and 2 AD patients with 5 first-degree relatives). The mean age at onset of the AD patients was 73.1 years and 69% of the patients was female. Most AD patients were included at a late stage of the disease (median CDR 3). In four patients other dementia syndromes were diagnosed; one frontotemporal dementia patient, one vascular dementia patient, one dementia due to neurosyphilis, and for one patient no further subdiagnosis was possible.

Table 4.5 Clinical characteristics of the 81 dementia patients

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>% female</th>
<th>% positive family history</th>
<th>Mean age at onset</th>
<th>Median CDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probable AD</td>
<td>62</td>
<td>67.7</td>
<td>60.0</td>
<td>73.2 ± 8</td>
<td>3 (1-5)</td>
</tr>
<tr>
<td>Possible AD</td>
<td>11</td>
<td>72.7</td>
<td>46.0</td>
<td>72.8 ± 5.4</td>
<td>3 (1-4)</td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
<td>25.0</td>
<td>50.0</td>
<td>70.8 ± 6.2</td>
<td>2.25 (2-4)</td>
</tr>
</tbody>
</table>

Genealogical studies

Genealogical studies revealed that 70 out of 73 AD patients (95% of the AD patient group) were related within 13 generations. These relationships included 6 affected sibling-pairs. Most patients could be linked to another AD patient within 3 generations. For the patients a pedigree was constructed aiming to maximise the number of patients that could be linked to the nearest common ancestor. The relationships of 63 AD patients based on this condition is given as a simplified pedigree in figure 4.5. In this pedigree multiple links between patients exist but only the shortest connection between patients is shown. Kinship coefficients were
Alzheimer's disease in a Dutch recent genetically isolated population

calculated for 1540 pairs of patients (excluding sibling-pairs). The mean kinship coefficient of these 1540 patient pairs was 0.003. So the a priori probability of a random allele genotyped in a patient couple to be identical by descent is 0.003.

DNA analyses

Mutation screening of APP, PSEN1, and PSEN2 did not yield any evidence for a functionally relevant mutation that could explain the disease in the population. The APOE genotypes of the cases from the isolated village and that of the Caucasian cases reported in the literature are given in table 4.6. The frequency of the APOE*4 allele in patients from the isolated population was somewhat higher than the frequency of the Caucasian patients but this difference was not statistically significant.

<table>
<thead>
<tr>
<th>Apoe</th>
<th>Patients from isolate</th>
<th>Caucasian patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>19 (0.27)</td>
<td>1860 (0.37)</td>
</tr>
<tr>
<td>*2</td>
<td>3 (0.04)</td>
<td>257 (0.05)</td>
</tr>
<tr>
<td>*4</td>
<td>49 (0.69)</td>
<td>2857 (0.57)</td>
</tr>
</tbody>
</table>
Fig 4.5: Simplified pedigree of 65 out of 70 AD patients with a common ancestor within 13 generations. Filled symbols represent the AD patients, the phenotype of ancestors has not been included in this pedigree.
Alzheimer’s disease in a Dutch recent genetically isolated population

Discussion
In a recently genetically isolated population we identified a total of 73 patients with AD. Almost all of these patients could be linked to a common ancestor within 13 generations. There was no evidence for a role of APP, PSEN1 and PSEN2 underlying the clustering of patients. APOE*4 was found to be associated with AD as in other populations.

Most of the patients were ascertained at a relatively late stage of the disease (median CDR=3). Of all AD patients ascertained in the isolate 42 (57%) had at least 1 first-degree relative with AD. This is significantly higher compared to the 37% found in a meta-analysis of 7 population-based studies. In the isolate the mean age at onset of the AD patients (73.2 years, SD = 7.7) was lower than the AD patients in the Rotterdam Study (82 years, SD = 7.1), a population-based study of 8000 subjects aged 55 years and over. The percentage of women with AD in the isolate (69%) was lower than that in the Rotterdam Study (78%). These differences may in part be explained by selection during the sampling of the patients rather than a true difference between the isolate and the general Dutch population.

Our genealogical studies revealed that at least 70 patients with AD living in this village (95% of all AD patients) were related within 13 generations. This resulted in an extended pedigree with multiple connections between patients. We found the mean kinship coefficient of all possible patient-pairs to be 0.003. However for patients being close together in the pedigree the probability of sharing alleles around a disease gene will be much higher. The four known AD genes could not explain the prevalence of AD in this population fully. The modest increase in the APOE*4 allele frequency may be explained by genetic drift. However, the distribution of APOE was not significantly different from Caucasian patients. Based on the APOE*4 allele frequency and that in the general Caucasian population APOE*4 is expected to explain 52% of the prevalence of AD, while mutation in PSEN1, PSEN2 and APP were not found. Part of the APOE*4 effect may be explained by interactions with other (un)known AD genes.

Several approaches can be followed to identify new AD genes in this population. One approach may be to break down the pedigree in small pedigrees which may be analysed by linkage analyses allowing for heterogeneity between branches. This approach is followed in the studies in Iceland. An alternative approach to follow is an association-based approach. The basic rationale for this approach is that a common
mutation is transmitted from a common ancestor together with the surrounding haplotype. Markers located on this haplotype will flag the mutation in a genome screen. In this genetic isolate the linkage approach will not likely be successful since families are too small to give enough linkage information. Given the short coalescence time and the large mean kinship coefficient between patient pairs the association-based approach might be more fruitful in this recent isolate.

Although the usefulness of old genetically isolated populations such as the Finish and Icelandic is an issue of debate 217, 218, our study in a Dutch recently genetically population shows this approach may be valuable to study a late-onset disease such as AD. Due to the specific structure of this population genetic complex disorders become 'less complex'. As is the case for many isolated populations, the use of this population also has its limitations. Causal mutations or susceptibility genes identified in this specific population may be less important predictors of common disorders in larger, more outbred populations. However, rare mutations may have a major spin-off as they result in a better insight in the aetiology and pathophysiology of common diseases, as has been demonstrated by the discovery of the AD causing mutations in APP, and the presenilin genes.
Chapter 4.4

Linkage-disequilibrium mapping in an isolated population;
a study of chromosome 10 and 12

Several studies suggested linkage of Alzheimer’s disease (AD) to chromosome 12 and
to chromosome 10. Up until now three groups have found evidence for an AD locus
on chromosome 12 but no conclusive evidence for a more precise location of the AD
gene on chromosome 12 is available. The first report of linkage to chromosome 12
was based on a genome screen using a classical linkage and sibpair approach in
multiple affected families 94. The authors described strong evidence for linkage
(maximal multipoint lodscore of 3.5) with a 30 centimorgans (cM) region including
the centromere of chromosome 12. This locus was confirmed in a similar study which
also found evidence for a locus on 12p 96 (see figure 4.6). The centromeric locus was
not confirmed in a third study, which did show weak evidence for a locus on 12p 97.
Both the lipoprotein related protein (LRP), which is a neuronal receptor for the APOE
protein 219, and the α2-macroglobulin gene (A2M), a ligand of LRP 220, have been
put forward as candidate genes located on chromosome 12. However, association
studies testing polymorphisms in these candidate genes have been controversial 71-74.
Meta-analyses failed to confirm the association with LRP 221 or A2M 216.

Recently, three studies reported linkage with chromosome 10. The first study
reporting on linkage of AD to chromosome 10 was a genome-wide search 95. In this
sib-pair study 4 chromosomal regions suggestive for linkage were identified of which
the chromosome 10 region was most promising. In stage 2 of the study the
chromosome 10 region was analysed using additional sibpairs and a denser marker set
with an average marker-spacing of 5 cM 222. The most likely position of the AD locus
was found to be located between marker D10S1227 and D10S1225 (maximal
multipoint lodscore 3.83) (see figure 4.7). The second study on chromosome 10 used
plasma amyloid beta-42 (Ab42) as a quantitative biomarker for AD. Using a
quantitative trait approach in first-degree relatives of AD patients with high Ab42, high
Ab42 levels were linked to a region on chromosome 10 with a maximum lodscore
between D10S1227 and D10S1211 (maximal multipoint lodscore 3.93) 223. The third
study targeted the insulin-degrading enzyme gene (IDE), an enzyme involved in degradation and clearance of amyloid. There was evidence for an AD locus close to the IDE gene but no evidence was found for association of a polymorphism in IDE with AD.

In preparation of a genome-wide screen, we investigated the regions of chromosome 10 and 12, which were linked to AD and Aβ42 levels, in 73 patients derived from a genetically isolated population.

**Methods**

**Subjects**

Patients with AD were ascertained from a genetically isolated village in the Southwest of the Netherlands. AD patients were recruited through general practitioners, nursing home physicians, and neurologists working in the catchment area of the isolated village. All patients suspected or diagnosed with a dementia syndrome were visited by a research physician. Clinical information was collected using a standardised disease history, clinical examination, family history, and a review of the patient’s medical records. The diagnoses AD was made by the research physician and a neurologist according to the NINCDS-ADRDA criteria for AD. In order to determine which subjects descended primarily from the original founders of the isolated village, a genealogical search was completed for each patient (see chapter 4.2). Partners of patients diagnosed with AD, or Parkinson’s disease or diabetes mellitus (n = 100) were used to obtain control allele frequencies.

**Genotyping**

DNA samples were obtained from peripheral white blood cells. Short-tandem-repeat markers from Applied Biosystems version 2 located in or close to the regions identified on chromosome 10 and 12 were genotyped. For chromosome 12, D12S99, D12S336, D12S364, D12S310, D12S1617, D12S345, D12S85, D12S368, and D12S83 were genotyped (see figure 4.6), and for chromosome 10, D10S196, D10S1652, D10S537, D10S1686, D10S185, D10S192, and D10S597 were genotyped (see figure 4.7). PCR reactions were carried out as described by the manufacturer and PCR products were pooled and loaded on an ABI377 automated sequencer (filterset
Linkage-disequilibrium in an isolated population: chromosome 10 and 12

D; 6.25% denaturing FMC LongRanger acrylamide gel) and data were analysed using ABI Genescan3.1 and ABI Genotyper2.1 software.

Statistical analysis

The chromosome 10 and 12 marker data were analysed using a test for combined linkage and association of single markers with AD\textsuperscript{226}. This test is based on the method as described by Terwilliger\textsuperscript{227} and assumes that some marker alleles will be over-represented on chromosomes that carry the disease mutation, when many of these chromosomes descend from a single ancestor. The proportion of disease chromosomes with this ancestral allele is represented by the parameter lambda. The test considers each of the marker alleles separately as potential founder alleles. Consequently, a total likelihood is obtained for a given value of lambda by computing the likelihood on the data for each potential founder allele, and summing those likelihoods, weighted for the population frequency of the respective founder allele. While the procedure was originally applied to genotype data in samples of affected and unaffected individuals, we have used a generalised version for pedigree data\textsuperscript{226}. In the estimation procedure the recombination fraction was fixed at 0.01 and the disease gene frequency was kept constant at 5% with a penetrance of 70%. Alleles with a frequency of less than 5% were pooled. Only markers with a one-sided p-value of less than 0.05 and corresponding values of lambda are further analysed by calculating odds ratios for the ancestral allele with a 95% confidence interval.
Fig 4.6 The markers of chromosome 12 we used in the present study, and the ones reported to be linked with AD in previous studies (in bold). The markers are given with their map position according to the Marshfield database. The regions identified in previous studies are marked with the black bars. Also the position of A2M and LRP is given.

Fig 4.7 The markers of chromosome 10 we used in the present study, and the ones reported to be linked to AD in previous studies (in bold). The markers are given with their map position according to the Marshfield database. The regions identified in previous studies are marked with the black bars. Also the position of the insulin-degrading enzyme gene (IDE) is given.
Chapter 4.4

Results and discussion

We ascertained 81 patients with dementia in the catchment area of the isolated village. After clinical evaluation 73 were diagnosed as possible or probable AD patients. Genealogical information was collected up to 15 generations for all patients and revealed that 70 AD patients (95% of the AD patients) could be traced back to a common ancestor within 13 generations (see chapter 4.3). The analyses are restricted to these 73 patients.

Table 4.7 associated marker on chromosome 12

<table>
<thead>
<tr>
<th>Marker</th>
<th>Frequency in controls (p-value)</th>
<th>Frequency in cases (p-value)</th>
<th>Lambda</th>
<th>p</th>
<th>risk for heterozygotes (CI)</th>
<th>risk for homozygotes (CI)</th>
<th>trend (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D12S336</td>
<td>0.51</td>
<td>0.73</td>
<td>0.44</td>
<td>0.03</td>
<td>1.3 [0.5-3.3]</td>
<td>2.1 [0.8-5.7]</td>
<td>1.5 [0.9-2.4]</td>
</tr>
<tr>
<td>2</td>
<td>0.06</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.10</td>
<td>0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.13</td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.14</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pooled</td>
<td>0.06</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

On chromosome 12, one marker, D12S336, showed evidence for a common ancestral disease allele (lambda = 0.44, p-value = 0.03) (see table 4.7). The flanking marker (D12S364) showed, although far from significant, some evidence for a common ancestral disease allele (lambda = 0.12). The region between D12S336 and D12S364 includes the A2M region and although the odds ratios for these markers do not reach statistical significance, we cannot exclude that the A2M region plays a role in this population. Given the number of patients available, the frequency of the A2M deletion allele (15% in controls), and the effect of the locus (OR = 1.5), it is unlikely that we can show an effect using this series. To further study the region and to test whether there is indeed an AD locus the number of patients has to be increased.

For chromosome 10, only marker D10S537 showed some evidence for a common ancestral disease allele (lambda of 0.21). However, the association with this allele was not significant (p-value of 0.20). Markers D10S196, D10S1652, D10S1686, D10S185, D10S192, and D10S597 showed no evidence for a common ancestral disease allele (lambda = 0, p-value = 0.5). Although in the literature evidence for an AD locus on chromosome 10 is quite convincing 222, 223, 225, we cannot confirm this
locus in our isolated population. Because of the specific genetic make up of an isolated population it is possible that genetic factors that play a substantial role in the general population are of minor or no importance in an isolated population. This is also illustrated in a study on AD in the Amish, an isolated population in the United States of America, where APOE*4 had no effect on the occurrence of AD.

Given the absence of evidence for a locus on chromosome 10 and 12 and the absence of a role for presenilin 1 and 2 and the amyloid precursor protein gene (chapter 4.3) we will extend our study to a full genome screen. An alternative approach is to study Aβ42 in relatives to improve the statistical power of our study on chromosome 10 and 12.
Chapter 5

Discussion

5.1 Introduction
5.2 Guidelines for genetic case-control studies
5.3 Main findings and clinical relevance
Chapter 5.1

Introduction

In this thesis several approaches to unravel the genetics of AD are presented. In the chapter 2 the genetic epidemiology of AD is reviewed. Chapter 3 describes five candidate gene studies and chapter 4 describes family-based approaches to study the genetics of AD. In chapter 5.2 we discuss the possible explanations for, and propose a strategy to prevent, inconsistent findings in genetic case-control studies. In chapter 5.3 we discuss the main findings and clinical relevance of this thesis.
Chapter 5.2

Guidelines for Genetic case-control studies

In ancient Greece, illness was considered to result from an imbalance of blood, phlegm, yellow and black bile. According to today’s dogma, diseases are caused by an interaction of genetic and/or environmental factors. A frequently used design to prove that genes play a role in disease aetiology is the genetic case-control study using the candidate gene approach. The rationale of this approach is that a gene is expected to determine the risk for a disease because the corresponding protein (the gene product) is involved in the pathophysiology. However, genetic case-control studies are notorious for their inconsistent findings. This paper aims to offer a framework for a valid design and data-analysis in order to limit the chance of false-positive and negative results and biased risk estimates.

Lack of reproducibility is often due to a false-positive first observation caused by multiple testing. Although multiple testing is usually not regarded to be a crucial issue in clinical and epidemiological research, in genetic studies it may be a major problem. The reason for this is that a priori it is usually not clear which gene is involved in a disease. It is often suggested that candidate genes lie in the eye of the beholder. However, if the findings on one candidate gene are not convincing, this gene is easily replaced by another promising candidate gene. The total human genome consists of 30,000-40,000 genes resulting in 30,000-40,000 tests if only one marker per gene is tested, but usually more markers can be used. Given an alpha level of 5%, the null hypothesis is incorrectly rejected in, on average, one out of 20 unbiased studies. The number of false-positive findings becomes tremendous if more than 30,000 tests are conducted. Even if there is strong evidence for an association between a gene and a disease, one often tests without an a priori hypothesis about which variant (allele) of the gene is associated. For instance, one may test a DNA variant which is present in the forms A, B, C, and D. In this case 10 genotypes are possible. In the stage of gene identification we have no a priori hypothesis which of the four alleles (or which of the 10 genotypes) is (are) associated with the disease. This opens the opportunity for further false-positive findings for each gene.
Other causes of false-positive results may be sought in the design and analysis phase of the study. The choice of controls is crucial. If disease-free subjects are not chosen from the same population source as the cases, selection bias may be introduced. Often in genetic case-control studies the cases are selected from a specialised centre without defining the source population. The controls are chosen from other clinical departments, the general population, or sometimes the selection of controls is not described at all. Control selection is particularly tricky in studies of a certain gene involved in different common disorders. For example polymorphisms in the apolipoprotein E gene and angiotensin converting enzyme gene are not only implicated in cardiovascular disease but may also be involved in neurodegenerative disorders.\(^7, ^{230-232}\) This limits the possibility of using hospital-based controls as they may suffer from another disorder that is also related to the gene under study. A tool for detecting selection bias in the ascertainment of controls is testing for Hardy Weinberg equilibrium (HWE) of the allele and genotype frequencies.\(^92\) It is remarkable that this straightforward tool is not used in some genetic case-control studies to evaluate the control ascertainment and genotyping quality. Even in highly rated journals papers are published in which the genotype frequencies of controls deviate considerably from HWE.\(^90\) A problem of the HWE test is that the power to detect deviations from HWE is dependent on the number of subjects and the number of alleles at the marker under study. Therefore the test may fail in small studies and for markers with many alleles.

Population stratification (or admixture) is widely regarded as the most important confounding factor in genetic case-control studies. Population stratification occurs when the population under study is actually a combination of two or more sub-populations with a different genetic make-up and a different disease risk. When cases and controls are not matched for genetic background a spurious association may be found. In order to detect the presence of population stratification, a specific test has been developed using several markers which are not linked to the disorder under study.\(^233\)

Further, false-positive findings frequently occur in the analysis phase of putative gene-gene interaction. As mentioned before, given the large number of genes there are infinite numbers of interactions to be tested which introduces the possibility for “data driven stratification”. Candidate gene studies often report interaction with known
susceptibility genes. However, these interactions have rarely been replicated consistently in subsequent studies. For most genes there is a priori no biological reason to assume interaction. Clear-cut false-positive findings occur when stratifying the control sample in strata containing too few cases or controls. In a study on the association between the presenilin-1 (PSEN1) promoter polymorphism and Alzheimer's diseases we found significant evidence for interaction with the APOE*4 allele, a well known risk-increasing allele (see chapter 3.2). However, this resulted from a shift in PSEN1 promoter genotype frequency in controls carrying the APOE*4 allele. Biologically it is unlikely that interaction in controls will explain the risk of disease. Since most controls are APOE*4 negative, the APOE*4 positive control group is small and subject to random errors. We therefore genotyped additional APOE*4 positive controls for PSEN1. In the analysis with the extended control group the association disappeared showing that our earlier finding was a false-positive one. This example indicates that interaction should be interpreted with caution if it is caused by genotype frequency shifts in small subgroups.

Last but not least, inconsistent findings may occur because there is only a very minor effect of the genetic determinant. Studies are often too small to detect a statistically significant effect, leading to false-negative reports. It is crucial to evaluate a role of a gene in the disease aetiology in light of all studies available in the literature. A meta-analysis of all conducted studies will increase the power, as is the case in every epidemiological study. Of course, studies included in a meta-analysis have to fulfil the criteria proposed in the previous sections.

Genetic case-control studies have been widely criticised because of the repeated failure to replicate results. We propose straightforward guidelines for good genetic-epidemiological practice. Proper case and control selection and description, test samples for HWE and population stratification, interpret interaction with caution, and perform a meta-analysis of all available studies if small effects are expected. When conducted with valid methods the candidate gene approach has a promising future as our knowledge on the pathogenesis of diseases and on localisation of genes will increase dramatically in the near future.
Chapter 5.3

Main findings and clinical relevance

Chapter 3 describes 5 candidate gene studies using the case-control design. For the various case-control studies two population-based samples were used. The first sample was a population-based sample of early-onset AD cases ascertained in the 4 Northern provinces of the Netherlands and the area of the metropolitan Rotterdam. Control subjects for this study were randomly drawn from the Rotterdam study, a population-based prospective cohort study among 7,983 persons aged 55 years and older. The second case-control series was derived from the Rotterdam study and targeted late-onset AD. All subjects were screened for dementia by an extensive three-step examination. For the genetic case-control studies prevalent AD patients from the first screening phase and incident AD patients from the first follow-up period were used. The AD patients were group-matched to a series of dementia-free subjects. For both the early and late-onset case-control study, the diagnosis AD was made by a panel according to the NINCDS-ADRDA criteria.

There can be several arguments to consider a gene as a candidate susceptibility factor for AD. We choose our candidate genes for 4 reasons (chapter 3.2). Firstly, we targeted the PSEN1 gene in which most mutations are found in early-onset AD and we studied polymorphisms in this gene in relation to late-onset AD (chapter 3.2). Secondly, we targeted the tau gene (chapter 3.3). Tau is a protein which is known to be involved in the pathology of AD and the tau gene is involved in frontotemporal dementia, a disease closely related to AD. Thirdly, we studied the gene coding for cystatin C, a protein which is a component of congophilic amyloid angiopathy (chapter 3.4). These vascular lesions are often abundantly present in AD brains. Finally, we studied the promoter region of the APOE gene (chapter 3.5 and 3.6), a well established genetic risk factor for early as well as late-onset AD. As polymorphisms in the promoter region are possibly related to the expression of the protein, they might be associated with AD. Although several previous studies reported associations with these genes, we found no effect of any of the polymorphisms studied on the risk for AD. There are several explanations for the inconsistent findings.
in genetic case-control studies. Not the locus under study may be associated with the disease but a nearby located locus which is in linkage disequilibrium with the locus under study. In this case, associations can lead to inconsistent results between different populations and also to association with different alleles in different populations. Furthermore, multiple testing, selection bias, population stratification, and lack of power can cause false-positive or false-negative results. These methodological issues regarding genetic case-control studies were already discussed in depth in chapter 5.2.

In AD research, the most successful candidate gene studies in AD were those on APOE. The success on APOE started with a report of linkage to a region on chromosome 19 in families with late-onset AD. APOE was tested as a candidate because of the presence of apolipoprotein E in senile plaques and its affinity to amyloid. The APOE*4 allele was found to be associated with AD. Ever since, the number of studies that confirmed the association between APOE*4 and AD is overwhelming. Given the example of APOE, a powerful strategy is to first physically locate regions of interest and subsequently analyse candidate genes in this region. In this thesis we present a Dutch isolated population as a possible population to search for novel AD genes in a genomic screen. As a result of reduced genetic variability in genetically isolated populations, there is a higher probability that patients carry a common gene variant inherited from a common ancestor. Because these patients can be selected on the fact that they are closely related (see chapter 4.3) they are likely to share a substantial part of DNA surrounding a disease related gene variant. Recent studies have challenged the idea that linkage disequilibrium is larger in genetically isolated populations. However, studies in Costa Rica found linkage disequilibrium over a region of 7 cM and preliminary findings in the GRIP population suggest that linkage disequilibrium extents over a region of at least 15 cM (personal communication J.J. Houwing-Duistermaat). This suggests that genetic homogeneous populations might open opportunities to map new AD loci. In our isolated population, we have studied the effect of the known genes (APP, PSEN1, PSEN2, and APOE) as well as the regions on chromosome 10 and chromosome 12 which were recently found to be linked with AD. APOE showed an association comparable with the effect of APOE in the general population. However, the other AD genes and the chromosome 10 and 12 loci had no effect on the occurrence and
familial clustering in this isolated population. Therefore, other genes may play a role in the occurrence of AD in the GRIP population. To discover novel genes a genome-wide search will be performed in the near future.

Besides studying genetically homogeneous groups of AD patients, the collection of a phenotypically homogeneous patients group might help in locating susceptibility genes. On the other hand, in this thesis a family with the APP692 mutation (chapter 4.2) is described in which one single mutation can cause very different clinical pictures compatible with multiple diagnoses. Some patients presented with a cerebral haemorrhage while others presented with a dementia which was clinically and neuropathologically compatible with AD. Both the cerebral haemorrhage patients and the dementia patients show extensive congophilic amyloid angiopathy in pathological studies suggesting a common pathogenesis.

Although since the discovery of the four AD genes (APP, PSEN1, PSEN2, and APOE), no other gene has been definitely linked to AD there have been important breakthroughs in our knowledge of the molecular biology of AD. Especially the discovery of AD-causing mutations in APP has had a major impact. It has become clear that the amyloid beta protein (Aβ), a protein that is generated after two proteolytic cleavages of APP, first by the α or β secretase and subsequently by the γ-secretase, has a central role in the pathogenesis of AD 146. Aβ is found in multiple amino-acid lengths. The most frequent is Aβ1-40 (60 - 70%) although some Aβ1-42 (15%) is present. It has been found that all mutations in the APP gene are located near the secretase sites 146 and that they all influence Aβ production either by increasing the total Aβ production or specifically increasing Aβ1-42 45, 201, 236, 237. Aβ1-42 forms insoluble aggregates much faster than Aβ1-40 238, 239. Not only APP mutations but also the PSEN1 and PSEN2 mutations result in a higher Aβ1-42 production 45. Recently, it has been shown that most likely the presenilins are the γ-secretase or are at least a component of the γ-secretase complex 240, 241.

A promising new opportunity in genome screens is using plasma Aβ42 levels as a surrogate quantitative biomarker for AD. In a recent study this has shown to be a fruitful approach 223. Using this quantitative approach the youngest generation may contribute information while previously these subjects could only be used to determine the haplotype phase in patients. Studying quantitative measures instead of a binary trait increases the statistical power. This approach can be used in population-
based approaches, but it also provides the opportunity to localise genes in families which at present do not have enough patients to find linkage.

The most appealing and important consequence of increasing knowledge on the molecular biology of AD will be the development of therapeutic strategies. These strategies can develop interventions at different points in the pathways to the production of harmful components. First, the production of Aβ1-42 can be influenced, for example by influencing the (level of) activity of one of the 3 secretases. Decreasing β- or γ-secretase activity or increasing α-secretase activity potentially decreases Aβ production. Another option is preventing Aβ1-42 to form deposits. This can be achieved by clearing Aβ1-42. Immunisation with Aβ reduces the aggregation of this protein in a mouse model of AD 242. In another mouse model for AD immunisation with Aβ results in a significant reduction in the cognitive defects 243, 244.

As is shown by the example of APP and the presenilins, discovery of new genes will have a major impact on molecular biological research. The identification of AD disease genes has already resulted in the development of novel diagnostic markers, an innovative quantitative trait approach to AD, and novel therapeutic strategies. Although it is a long way from curing AD mice to curing AD patients there is a reasonable hope for therapeutics in the future.
Summary

Dementia is a frequent neurodegenerative disorder mainly affecting the elderly (see chapter 2). The most frequent subtype of dementia in the elderly is Alzheimer's disease (AD) which is clinically characterised by an insidious onset of decline in memory and problems in at least one other area of cognition. The disease is gradually progressive which will ultimately lead to a state of complete dependency. Because it is a disease which presents mainly at old age, the prevalence of dementia will increase rapidly in the near future. From epidemiological studies it has become clear that genetic factors play an important role in the pathogenesis of AD. The objective of this thesis was to identify novel genetic susceptibility factors for AD. For this purpose both population-based and family-based studies are used.

Chapter 2 provides a review on the genetic epidemiology of AD describing the state of the art of genetic research of AD. In Chapter 3 several population-based candidate gene studies are presented. In chapter 3.2 our findings on 2 PSEN1 polymorphisms are described. Previously a significant association between early-onset AD and an allele in the promoter of PSEN1 was described. For late-onset AD, numerous studies have reported inconsistent associations with a PSEN1 intron 8 polymorphism. We therefore hypothesised that linkage disequilibrium between the intronic PSEN1 polymorphism and the functional promoter polymorphism might explain the conflicting reports in late-onset AD. We analysed both variations in 356 late-onset AD patients and 230 controls in a population-based case-control study. In addition, we re-analysed all published literature on the PSEN1 intronic polymorphism in a meta-analysis. In our case-control sample no significant association was found with the PSEN1 intronic and promoter polymorphism, and in the meta-analysis no association with the PSEN1 intronic variation was found, suggesting that there is no major effect of variable PSEN1-expression in late-onset AD. Chapter 3.3 describes the findings on the tau gene. Hyperphosphorylated microtubule associated protein tau, present in neurofibrillary tangles, is a prominent pathological feature of AD. The gene encoding tau (MAPT) was recently found mutated in frontotemporal dementia (FTD) and other tauopathies. In this chapter MAPT was studied as a candidate gene in the aetiology of AD. The study population consisted of 101 early-onset AD patients and 117 controls. Mutation analysis did not detect causal mutations in exons 9 to 13.
encoding the microtubule-binding domains involved in FTD. However, 2 novel polymorphisms were detected in exon 9. Using the Ala169 polymorphism in exon 9 and a previously reported (CA)$_n$ repeat polymorphism in intron 9, an association study was performed. No association with early-onset AD was detected. Together, these data indicate that MAPT does not play a role in early-onset AD. In Chapter 3.4 we studied the role of the cystatin C gene in AD. A recent publication suggested a new genetic risk factor for very late-onset AD, the GG genotype of the cystatin C gene (CST3). We examined the role of this polymorphism in a population based study on AD with an early-onset age, and did not find an association of CST3, nor could we confirm the proposed interaction with age and the apolipoprotein E gene. In Chapter 3.5 and 3.6 the role of the apolipoprotein E gene (APOE) promotor in early-onset and late-onset AD is described. The -491A/T polymorphism in the promotor region of APOE has been suggested to be associated with increased risk for AD independent of APOE status. We studied the association between the -491A/T polymorphism and risk for early-onset AD in Dutch and Spanish early-onset patients and in late-onset patients derived from the Rotterdam study. For both early and late-onset AD we found no consistent relationship with a single allele of the -491A/T polymorphism. We also studied the of the APOE promotor on plasma apoE levels. We found a modest but significant effect of the -491A/T polymorphism on plasma apoE levels independent of the APOE gene polymorphism. In conclusion, our data suggest that the -491A/T polymorphism has an APOE genotype independent effect on plasma apoE levels but no APOE independent effect on AD risk.

In chapter 4 family-based studies are described. Chapter 4.2 comprises an extensive description of the phenotype presented by a family with a mutation in the APP gene. Several mutations in APP may lead to either AD or cerebral haemorrhage due to congophilic amyloid angiopathy (CAA). A single family is known to express both types of pathology due to a missense mutation at codon 692 of the APP gene (APP692). The clinical and pathological expression of APP692 in 8 patients with the mutation is described. Further, 21 first-degree relatives with an a priori risk of 50% to be carrier were tested for the APP692 mutation and studied for presymptomatic signs by neurological examination, neuropsychological testing, and brain MRI. Patients with APP692 presented with either haemorrhage, dementia, or both diseases. The dementia in patients with the APP692 mutation was compatible with AD both clinically and neuropathologically. Of the 21 healthy relatives, 5 carried the APP692
Summary

mutation. The presymptomatic carriers showed a subtle, non-significant, impairment of cognitive function compared to relatives without APP692. A significant increase in the number of periventricular and subcortical white matter lesions at young age was seen in presymptomatic carriers (mean age of 26.4 years). The findings of this study suggest that a single (genetic) mechanism may underlie the pathology of AD and CAA. In chapter 4.3 we present a series of 73 patients derived from an isolated population of whom 95% could be linked to a common ancestor within 13 generations. There was no evidence for a role of APP, PSEN1, or PSEN2 underlying the clustering of patients. APOE*4 was found to be associated with AD as in other populations. Due to the specific structure of this population genetic complex disorders become 'less complex'. Given the short coalescence time and the large mean kinship coefficient between patient pairs the association-based approach might be a fruitful method to identify novel susceptibility genes for AD in this isolate. Finally, in chapter 4.4 data on chromosome 10 and 12 from the isolated population are presented. Recently several independent genomic screens found linkage of AD to regions on chromosome 10 and 12. The patients described in our study could not be explained by these 2 regions. Further screening of the genome will be necessary to identify the AD genes involved in these families.

Chapter 5 discusses our findings. Chapter 5.2 emphasises on the methodological issues of genetic case-control studies and chapter 5.3 discusses our main findings and relevance in clinical practice.
Samenvatting

Dementie is een neurodegeneratieve aandoening die vooral in de oudere bevolking frequent voor komt (zie hoofdstuk 2). De meest frequentte vorm van dementie bij ouderen is de ziekte van Alzheimer (AD). Deze ziekte vertoont een sluipend begin met geheugenproblemen en een soortgelijk een andere cognitieve functie. De ziekte is langzaam progressief en leidt uiteindelijk tot een toestand waarin patiënten volledig afhankelijk zijn. Omdat het een ziekte is die zich vooral bij ouderen manifesteert zal de prevalentie van AD drastisch toenemen in de nabije toekomst. Uit epidemiologisch onderzoek is gebleken dat genetische factoren een belangrijke rol spelen bij het ontstaan van AD. Het doel van dit promotieonderzoek was het identifieren van nieuwe gevoeligheidsgenen voor AD. Hiervoor werden zowel populatiestudies als familiesudies gebruikt.

Hoofdstuk 2 geeft een overzicht van de genetische epidemiologie van de ziekte van Alzheimer. In hoofdstuk 3 worden verschillende kandidaat gen studies gepresenteerd. Hoofdstuk 3.2 beschrijft de resultaten van een studie naar een tweetal polymorfismen in het preseniline-1 gen (PSEN1). In een voorgaande studie is een significante associatie gevonden tussen een polymorfisme in de promotor van PSEN1 en de vroege vorm van AD (begin van de ziekte voor het 65e levensjaar). Voor de late vorm van AD (begin van de ziekte na het 65e levensjaar) hebben verschillende studies een associatie met een polymorfisme in intron 8 van PSEN1 beschreven. Wij hebben beide polymorfismen bestudeerd in 356 patiënten met de late vorm van AD en 230 controle personen. Verder zijn alle beschikbare studies over het PSEN1 intron 8 polymorfisme geanalyseerd in een meta-analyse. In ons patiënt-controle onderzoek vonden we voor zowel het promotor als het intron 8 polymorfisme geen associatie met de late vorm van AD. Ook uit de meta-analyse bleek dat het PSEN1 intron 8 polymorfisme niet is geassocieerd met de late vorm van AD. Hoofdstuk 3.3 beschrijft onze bevindingen betreffende het tau gen (MAPT). Hypergefosforyleerd microtubuli geassocieerd eiwit is aanwezig in neurofibrillaire tangles (een belangrijk neuropathologisch kenmerk van AD). Voor frontotemporale dementie zijn recent mutaties gevonden in het MAPT gen. Wij hebben het MAPT gen bestudeerd als kandidaat gen voor AD. Voor deze studie hebben we 101 patiënten met de vroege vorm van AD en 117 controle personen gebruikt. Met mutatie analyse werden geen
causale mutaties gevonden in het MAPT gen. Wel hebben we twee nieuwe polymorfenismen in exon 9 van MAPT gevonden. In een patiënt-controle onderzoek is geen associatie met deze polymorfenismen en een eerder beschreven polymorfisme in exon 9 met de vroege vorm van AD gevonden. Dus hoewel het tau eiwit een belangrijk neuropathologisch kenmerk van AD is, lijkt het gen dat voor dit eiwit codeert geen rol te spelen in het ontstaan van AD. In Hoofdstuk 3.4 is de rol van het cystatine C gen (CST3) in AD bestudeerd. Een recente publicatie heeft het GG genotype van CST3 beschreven als een nieuwe genetische risicofactor voor de late vorm van AD. Wij hebben de rol van dit CST3 polymorfisme bestudeerd voor de vroege vorm van AD. Er werd geen associatie gevonden met de vroege vorm van AD en ook geen interactie met het apolipoproteine E gen. Hoofdstuk 3.5 en 3.6 beschrijven de rol van de promotor van het apolipoproteine E gen (APOE) in zowel de vroege als de late vorm van AD. Het –491A/T polymorfisme in de APOE promotor is in verschillende studies beschreven als een risicofactor voor AD. Wij hebben dit polymorfisme bestudeerd in Spaanse en Nederlandse patiënten met de vroege vorm van AD en in Nederlandse patiënten met de late vorm van AD. Voor zowel de Spaanse als de Nederlandse patiënten konden we geen consistente associatie vinden tussen het –491A/T polymorfisme en AD. Ook is het effect van het –491A/T polymorfisme op plasma apoE spiegels bestudeerd. Er was een klein maar significant effect van het –491A/T polymorfisme op plasma apoE spiegels. Onze data suggereren dat het –491A/T polymorfisme geen effect heeft op het ontstaan van AD maar wel een beïnvloedend effect op de apoE plasma spiegels.

In hoofdstuk 4 zijn de familie studies beschreven. Hoofdstuk 4.2 geeft een uitgebreide beschrijving van het fenotype van een autosomaal-dominante mutatie in het amyloid precursor eiwit gen (APP). Mutaties in APP kunnen leiden tot of AD of hersenbloedingen als gevolg van congofilie amyloid angiopathie. De familie die wij beschrijven is bekend met een mutaties in codon 692 van het APP gen (APP692) en presenteren zich met zowel AD als hersenbloedingen. Het klinisch en neuropathologisch beeld van 8 patiënten met de APP692 mutatie wordt beschreven. Verder werden 21 eerstegraads familieleden met een a priori risico van 50% op het dragen van de mutatie getest voor de APP692 mutatie en met neurologisch, neuropsychologische en MRI onderzoek onderzocht op presymptomatische verschijnselen. Patiënten met de APP692 mutatie presenteerden zich met hersenbloedingen, dementie of beide ziektebeelden. Het dementie beeld in deze
Samenvatting

patiënten voldeed aan de klinische en neuropathologische criteria voor AD. Bij 5 van de 21 familieleden met het risico op het dragen van de mutatie hebben we de mutatie gevonden. Deze presymptomatische mutatie dragers scoorden minimaal, niet significant, slechter op de neuropsychologische testen in vergelijking tot familieleden zonder de mutatie. Bij de MRI studie van de mutatie dragers werd op zeer jonge leeftijd (gemiddeld 26.4) een significant verhoogde hoeveelheid witte stof afwijkingen gevonden. Deze studie laat zien dat één enkel genetisch mechanisme aan de grondslag kan liggen van het ontstaan van zowel AD als congofiele amyloid angiopathie. In hoofdstuk 4.3 presenteren we een groep van 73 Alzheimer patiënten uit een genetisch geïsoleerde populatie waarvan 95% gekoppeld kon worden aan een gezamenlijke voorouder. Er was geen aanwijzing dat de bekende Alzheimer genen (PSEN1 en 2, en APP) deze clustering van Alzheimer patiënten kon verklaren. Het APOE E4 alleel was geassocieerd met AD in een mate vergelijkbaar met andere populaties. Door de specifieke structuur van deze populatie zijn genetisch complexe aandoeningen minder complex. Gegeven het feit dat patiënten nauw aan elkaar verwant zijn zou de associatie strategie een goede mogelijkheid zijn om genen te lokaliseren in deze populatie. Tot slot beschrijven we in hoofdstuk 4.3 de resultaten van een associatie studie met markers op chromosoom 10 en 12 in deze geïsoleerde populatie. Chromosoom 10 en 12 zijn onderzocht omdat recent in andere studies aanwijzingen zijn gevonden dat in deze regio’s Alzheimer genen zijn gelokaliseerd. De patiënten uit de geïsoleerde populatie waren echter niet met chromosoom 10 of 12 geassocieerd. Dit betekent dat andere, nog onbekende, genen de clustering van AD in deze geïsoleerde populatie veroorzaken. Een analyse van het totale genoom zal verrichten worden om deze genen te lokaliseren.

In hoofdstuk 5 worden onze bevindingen bediscussieerd met in hoofdstuk 5.2 aandacht voor de methodologie in genetisch patiënt–controle onderzoek en in hoofdstuk 5.3 de belangrijkste resultaten en klinische relevantie.
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Dankwoord

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Curriculum Vitae

Gerwin Roks was born on August 8, 1971 in Rucphen. He graduated in 1989 from secondary school (VWO katholieke scholengemeenschap Etten Leur), and started his medical studies at the university of Nijmegen. During this period he participated in experimental research on the side-effects of anti-epileptic drugs (Prof. dr. H. Meinardi). In 1997 he obtained his medical degree and started working at the genetic epidemiological unit, departments of epidemiology & biostatistics (Prof.dr.ir. C.M. van Duijn) and clinical genetics (Prof.dr. B.A. Oostra), of the Erasmus Medical Centre Rotterdam. In 2000 he obtained a master of science degree in genetic epidemiology at the Netherlands Institute for Health Sciences in Rotterdam. From January 2001 he is working as a resident neurology at the St Elisabeth Hospital in Tilburg (Dr. C.C. Tijssen).

Other publications


I
Er zijn geen aanwijzingen dat variaties in het preseniline-1 gen betrokken zijn bij het ontstaan van de ziekte van Alzheimer op late leeftijd.
(Dit proefschrift)

II
Hoewel dragers van de APP692 mutatie vaak meerdere hersenbloedingen doormaken past het dementie syndroom bij deze dragers zowel klinisch als pathologisch bij de ziekte van Alzheimer.
(Dit proefschrift)

III
Inconsistente resultaten van kandidaat-gen studies zijn vaak toe te schrijven aan tekortkomingen in de opzet en analyse van deze studies.
(Dit proefschrift)

IV
De centrale rol van het beta-amyloid eiwit in het ontstaan van de ziekte van Alzheimer wordt ondersteund doordat immunisatie met beta-amyloid-42 de ontwikkeling van plakken in de PDAPP transgene muis voorkomt.
(D. Schenl, Nature 1999, 400-6740; 175-177)

V
Ondanks het feit dat bij weinig patiënten met een herseninfarct acute trombolyse mogelijk is, heeft opname op een stroke-unit grote voordelen voor de patiënt.
(L. Kalra, The Lancet 2000, 356;894-899)

VI
Het post-hoc bepalen van de power van een studie ten behoeve van de interpretatie van de resultaten is misbruik van de power-analyse.

VII
De kwaliteit van het peer-review proces van wetenschappelijke artikelen zal sterk verbeteren als ook de ruwe gegevens tot beschikking van de reviewers staan.

VIII
Dankzij de ontwikkelingen in de genetica is binnen de neurologie meer aandacht voor de verschijnselen in neurodegeneratieve ziekten ontstaan.
IX
De mogelijkheid om voor een individu een uitspraak te doen over het risico op het ontwikkelen van een multi-factoriële aandoening op basis van het genetisch profiel wordt sterk overschat.

X
De aandacht voor de nieuwe variant van de ziekte van Creutzfeldt Jakob overschaduwt ten onrechte de aandacht voor de iatrogene vorm van de ziekte.

XI
Genetische isolaten in Nederland worden gekenmerkt door het relatief hoge niveau van de plaatselijke voetbalvereniging.

XII
Met de introductie van de euro wordt een nieuwe cognitieve test geïntroduceerd.

XIII
Gezien de talrijke mogelijkheden is het onnodig twee maal dezelfde fout te maken. (naar Robert Lemke)

Rotterdam, 6 juni 2001