A genetic-epidemiologic study of Alzheimer's disease

Alejandro Arias Vásquez

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A genetic-epidemiologic study of Alzheimer's disease

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ziekte van Alzheimer

Proefschrift

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Chapter 2.2

The MAPT and granulin genes are not associated with Alzheimer's disease

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Chapter .2.3

A genome scan in a family with Alzheimer's disease

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Chapter 3.1

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The Cholesteryl Ester Transfer Protein (CETP) gene and the risk of Alzheimer's disease

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Relationship of the ubiquilin 1 gene with Alzheimer's, Parkinson's disease and cognitive function

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Chapter 3.4

A deletion in the DJ-1 gene and dementia – a population-based survey

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Chapter 1

INTRODUCTION

The Genetic epidemiology of Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia, accounting for 50–60% of all cases. The prevalence of dementia is below 1% in persons aged 60–64 years, but shows an almost exponential increase with age, such that in people aged 85 years or older the prevalence is between 24% and 33% in the western world [1]. Besides age, epidemiological studies have suggested several potential risk factors for the disease. Some are related with a decreased reserve capacity of the brain including education [2, 3], others with head injury and repair [4], or vascular pathology including, hypertension, coronary heart disease, atherosclerosis, smoking, hypercholesterolemia, obesity and diabetes [2].

AD is a slowly progressive disorder with insidious onset and progressive impairment of episodic memory. Key signs include aphasia, apraxia, and agnosia, together with general cognitive symptoms, such as impaired judgment, decision-making, and orientation [5]. The brain of an AD patient is characterized by the accumulation of the amyloid ß (Aß) peptide into extracellular plaques and by the formation of tangles inside the neurons as a result of abnormal phosphorylation of the microtubule-associated protein tau (MAPT). Besides Aß deposits and tangles, a marked loss of neurons in the neocortex and hippocampus is part of the neurodegenerative process. This process is estimated to start 20 to 30 years before clinical onset of the disease [6]. Over the years, plaque and tangle load increase and at a certain threshold the first symptoms appear. The pathological changes of AD are not unique but overlaps partly with that of other degenerative disorders, in particular Parkinson's disease. Parkinson's disease is the second common neurodegenerative disorder in the elderly. Parkinson patients often develop dementia while Alzheimer patients may show Lewy body pathology that characterizes Parkinson's disease. Also, both disorders are characterized by disturbances in the ubiquitination machinery [7].

A large proportion of AD patients can be explained by genetic factors [8]. The genes determining risk for AD include (rare) mutations in genes with large effects on the disease risk and one but most likely several common variants in genes with small effects on the risk of AD. The latter variants may show interaction with other genes and environmental risk factors. Variations in four genes have been directly linked to the pathogenesis of AD. Mutations in the amyloid precursor protein (APP), Presenilin 1 (PSEN1) and Presenilin 2 (PSEN2) have a major effect on Aß aggregation, one of the key pathways involved in Alzheimer disease [9] and a common polymorphism (e4) in the gene encoding apolipoprotein E (APOE) increases susceptibility to both early and late onset forms of AD [10, 11].

These four genes together explain less than a quarter of the disease prevalence,

indicating additional genetic risk factors remain to be identified [12, 13]. In addition to APOE, more than three dozens of candidate genes were reported to be associated with AD. None of these, however, has been consistently replicated [14, 15]. Furthermore, genome screens targeting AD loci were conducted. As reviewed by the AlzGene database (www.alzforum.org), the replicated regions from previous genome screens include: 1p36, 1q21-31, 2p23-24, 4q35, 5p13-15, 6p21, 6q15-16, 6q25-27, 9p21-22, 10q21-22, 10q25, 12p11-12, 19q13, 21q21-22, and Xp11-21 [16-31].

In this thesis we investigated the genetic susceptibility to AD employing several strategies to find genes involved in the disease. In chapter two, genomic studies aiming to find genes involved in AD are presented which rely on genome wide analyses and high-throughput genotyping of chromosomal regions. Chapters 2.1 and 2.2 were performed within the Genetic research in Isolated Populations (GRIP) program, which is based in the southwestern part of the Netherlands. The AD GRIP study is a family based study that aims to identify genes involved in complex diseases by selecting affected individuals and their relatives [32]. Chapter 2.1 describes a genome-screen in a large serie of AD patients. Chapter 2.2 describes a detailed study of the chromosome 17 region including the microtubule associated tau protein (MAPT) and granulin (GRN) genes in patients with AD. Chapter 2.3 describes a study in an extended family affected with autosomal dominant AD originating from the east of The Netherlands.

We also performed four candidate gene studies, which are presented in chapter 3. These studies were carried out in the Rotterdam Study and the AD GRIP study. The Rotterdam study is a population-based follow-up study of 7983 persons age 55 years and over who have been followed now for 12 years [33]. The first two chapters (3.1 and 3.2) focus on AD. In chapters 3.3 and 3.4, we are linking our studies of AD to the genetics of Parkinson's disease. Chapter 3.1 describes the association of the cyclin-dependant kinase 5 (CDK5) gene and AD. In chapter 3.2 we describe the association between the cholesteryl ester transfer protein (CETP) and AD. In chapter 3.3 we assessed the relationship of the ubiquilin 1 (UBQLN1) gene and AD, Parkinson's disease and cognitive function. In chapter 3.4 we evaluated the effect of a deletion in the DJ-1 gene in dementia patients. This gene was identified in patients with Parkinson's disease in the GRIP study. Finally, Chapter 4 provides a general discussion of the work presented in this thesis and provides suggestions for future research in AD.

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Chapter 2

Genomic Studies

Chapter 2.1

Genome-wide screen for Alzheimer's disease in a Dutch genetically isolated population

Abstract

Alzheimer's disease (AD) is the most common cause of age-related dementia. AD is characterized by a strong familial aggregation. However, the genetic origin of AD is partly understood. We conducted a genome screen in late onset AD patients from a young, genetically isolated population from the Southwest of the Netherlands. Extensive genealogy was collected, resulting in an extremely large and complex pedigree including 4,645 members and 103 patients. For parametric linkage analysis the pedigree was split into 35 families in which the number of patients is maximized under a family size restriction of maximal 35 bits. This set of sub-pedigrees preserved 87.5% kinship of the patients.

All patients and their 170 closely related relatives were genotyped using 402 microsatellite markers. Simulation analyses suggested that parametric linkage analysis using these sub-pedigrees requires a stringent significance threshold: a LOD of 3.64 corresponds to 5% genome-wide type-I error. The subsequent genome screen multipoint analysis yielded 8 regions with HLOD > 2, which were fine mapped with 45 additional markers. After the fine mapping, four loci were significantly linked and one was suggestively linked to AD.

The strongest evidence of linkage was for chromosome 1q21 (HLOD = 5.20, at marker D1S498), which is a locus identified earlier. We confirmed the AD locus at 10q24 (HLOD = 4.15, at marker D10S185). There is also significant evidence of linkage at chromosomes 3q23 (HLOD = 4.44 at marker D3S1569) and 18q12 (HLOD = 3.68, at marker D18S1152). Several single families contributed strong linkage signals on chromosomes 1, 3 and 18. Multiple families contributed moderate linkage signal to chromosome 10q24 locus. Finally, there is also suggestive evidence of linkage at chromosome 11q24 (HLOD = 3.29, at marker D11S1320).

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that accounts for the vast majority of age-related dementia. The population prevalence of the disease rises steeply with age from below 2% at 65 years to above 35% after the age of 90 [1, 2]. Family history is an important risk factor for AD and in a large number of families the disease segregates as a dominant trait. A recent large twin study confirmed that the heritability for AD was up to 79% [3]. Several dominant mutations for AD genes have been identified including mutations in the presenilin 1 (PSEN1) gene [4], presenilin 2 gene (PSEN2) [5, 6], and Amyloid Precursor Protein (APP) gene [7]. A common polymorphism (e4) in the gene encoding apolipoprotein E (APOE) increases susceptibility to both early and late onset AD [8].

All these four genes together explain less than a quarter of the disease prevalence, indicating additional genetic risk factors remained to be identified [9, 10]. In addition to APOE, more than three dozens of candidate genes were reported to be associated with late onset AD. None of these, however, has been consistently replicated [11, 12]. Furthermore, genome screens targeting AD loci were conducted. As reviewed by the AlzGene database, the replicated regions from previous genome screens include: 1p36, 1q21-31, 2p23-24, 4q35, 5p13-15, 6p21, 6q15-16, 6q25-27, 9p21-22, 10q21-22, 10q25, 12p11-12, 19q13, 21q21-22, and Xp11-21 [13-28].

Loci for the established genes for AD (APP, PSEN1, PSEN2 and APOE) have been initially localized by linkage analysis. Pedigrees suitable to localize genes, however, have become scarce particularly for late onset and sporadic familial diseases. Genetically isolated populations provide excellent material for linkage analysis. Using genealogical records, extended pedigrees can be constructed. This method has been used successfully in Iceland [29, 30]. We have followed this approach in a genetically isolated community from the Southwest of the Netherlands, where church and municipal records were used to connect patients with late onset AD [31]. A total of 103 late onset AD patients were connected into a large pedigree. In this paper, we present the results of a linkage based genome wide screen in these patients.

Material and Methods

Population & Genealogy

This study was performed within the framework of the previously described Genetic Research in Isolated Populations (GRIP) program [32-34]. The Medical Ethics Committee of the Erasmus MC approved GRIP protocols. The GRIP population is a genetically isolated community in the southwest of the Netherlands. Less than 400 individuals founded the population in the middle of the 18th century. Considerable population growth subsequently occurred. Until recently, there was minimal immigration. An estimated 20,000 descendants of this population are now scattered over eight adjacent communities. The genealogical database currently contains information on more than 90,000 people spanning 23 generations. Residents in the GRIP area are generally related via multiple lines of descent and inbred via multiple consanguineous loops.

Patient ascertainment

Patients with AD were traced through general practitioners, neurologists and nursing-home physicians. The diagnosis of AD was verified by two independent neurologists according to the NINCDS ADRDA criteria [35], and then re-evaluated by a research physician and a research neurologist. Data on the presence of AD, parkinsonism, essential tremor and dementia were collected in first, second and third-degree relatives by means of a family-history questionnaire. First-degree relatives also underwent a brief neurological examination. All patients and their relatives who were invited to participate in this study provided informed consent. A total of 112 probable late onset AD patients (age of onset greater than 65 years, mean age of onset = 75 ± 5.3 years) and 170 unaffected relatives were ascertained.

Genotyping

For all patients and their 170 first-degree relatives, DNA was extracted from peripheral leucocytes following a standard protocol [36]. Mutations in the APP, PSEN-1 and PSEN-2 genes were previously excluded [31]. The APOE genotype was determined on all DNA samples using TaqMan allelic discrimination technology on an ABI Prism 7900HT Sequence Detection System with SDS V 2.1 (Applied Biosystems, Foster City, CA). Patients and their first-degree relatives underwent a full genome-screen in two sequential experiments. Both screens were conducted

using the same set of micro-satellite markers, evenly spaced by approximately 10 cM (ABI Prism Linkage Mapping Set MD-10 Versions 2 and 2.5, Applied Biosystems, Foster City, CA, USA). Polymerase chain reactions (PCR) were performed according to the manufacturer's specified conditions. PCR products were separately pooled and analyzed on ABI377 and ABI3100 automated sequencers (Applied Biosystems). Results were pooled using the maximum likelihood method [37]. Two independent technicians read the results from the sequencers; a third reader resolved the discordant results. Only the markers with the discordance proportion less than five percent were selected for further analysis. A total of 402 markers with missing rates less than 5% were included from the initial scan. Regions linked to late onset AD were later fine typed by placing additional 45 markers in between those from the initial set at a distance of one to five cM apart. Genotyping errors leading to Mendelian inconsistencies were detected using PedCheck [38]. Unlikely double recombination events were detected using Merlin [39]. Definitive genotyping errors and unlikely genotypes were rechecked using the data from the laboratory.

Pedigree splitting

To split this large pedigree we used a graph theory approach based on the maximal cliques partitioning, which was successfully applied for pedigree splitting by Falchi et al [43]. This method has been successfully applied in identification of new locus for hypertension [44]. We modified this method by setting the requirement that the resulting sub-pedigrees should have no more than a user-specified bits-size, where the bit-size is computed as (2 * number of founders – number of non-founders). Our software is allows a variable number of relatives to be included in the sub pedigrees. It is freely available at http://mga.bionet.nsc.ru/nlru/.

Evaluation of Type-I error rate

Breaking pedigrees may increase the possibility of spurious linkage findings in patients where multiple distant lines of descent are present and ignored after breaking the pedigree [40]. Therefore we estimated the threshold for statistical significance using simulations.

To evaluate genome-wide type-I error we simulated our genome scan 100 times. We used the complete pedigree including all 4,645 members for marker simulation by dropping unlinked markers down through the pedigree. Number of markers, intermarker distances and maker allele frequencies were simulated according to the typed markers. After each gene-dropping experiment, we performed linkage analysis using the derived sub-pedigrees where disease allele frequency, liability

classes, genetic model and penetrances were the same as we used later in the actual linkage analysis. Genotypes of untyped individuals were set to missing. For each genome-screen the highest HLOD score was stored. Cumulative density function of the obtained 100 maximal HLOD scores approximates the distribution of genome-wide type-I error rates.

Linkage and Haplotype analysis

In linkage analysis, we assumed a dominant model of inheritance with age dependent penetrance. Seven liability classes were defined based on age (years): <65, 65-69, 70-74, 75-79, 80-84, 85-90, and >90. For each age group j, age dependent population prevalence, P_j, was obtained from the Rotterdam Study [1]. The disease gene penetrance, f_j of the jth age group can be estimated:

$$f_j = \frac{PAF \times P_j}{q^2 + 2q(1-q)}$$

where PAF stands for the population-attributable fraction, the proportion of the population prevalence that can be explained by the studied gene (10% assumed), and q is the disease allele frequency (1% assumed). The estimated penetrance for each defined age group is shown in Supplementary Table 1. Marker allele frequencies were estimated based on 144 chromosomes from unaffected elderly GRIP population members. For small pedigrees (bits ≤ 18), we used the exact calculation of multi-locus likelihood, Lander-Green algorithm implemented in GENEHUNTER 2.0 [45]. For larger pedigrees, we used Markov chain Monte Carlo estimation methods implemented in SIMWALK 2.91 [42, 46, 47]. Overall LOD scores and Heterogeneity LOD (HLOD) scores were computed by combining per family results from the two packages using standard formulas below.

$$HLOD = \log_{10}(\max LR)$$

where maxLR is maximized with respect to a, the proportion of the linked families, yielding Maximum Likelihood Estimate $\hat{a}$,

$$\max LR = \prod_{i=1}^{n} (\hat{a} LR_i + 1 - \hat{a})$$

Haplotypes were reconstructed based on the genotypes of patients, spouses of patients and their offspring using MERLIN package [39]. Haplotypes are shown

only for the linked families with the highest LOD scores. These families are further expanded in order to depict the haplotype sharing of other patients who are relatively closely related to the patients in these families.

Association testing

We tested for association six markers on chromosome 3 and four markers on chromosome 18 where we found that 2 haplotypes of these regions were shared by multiple patients from the same family. We used the 112 patients as cases and their 35 age-matched, unaffected spouses as controls, for whom we had both phenotypic and genotypic information. The CC-QLS test proposed by Bourgain et al. was used to test for association adjusting for the inter-relationships among the samples.

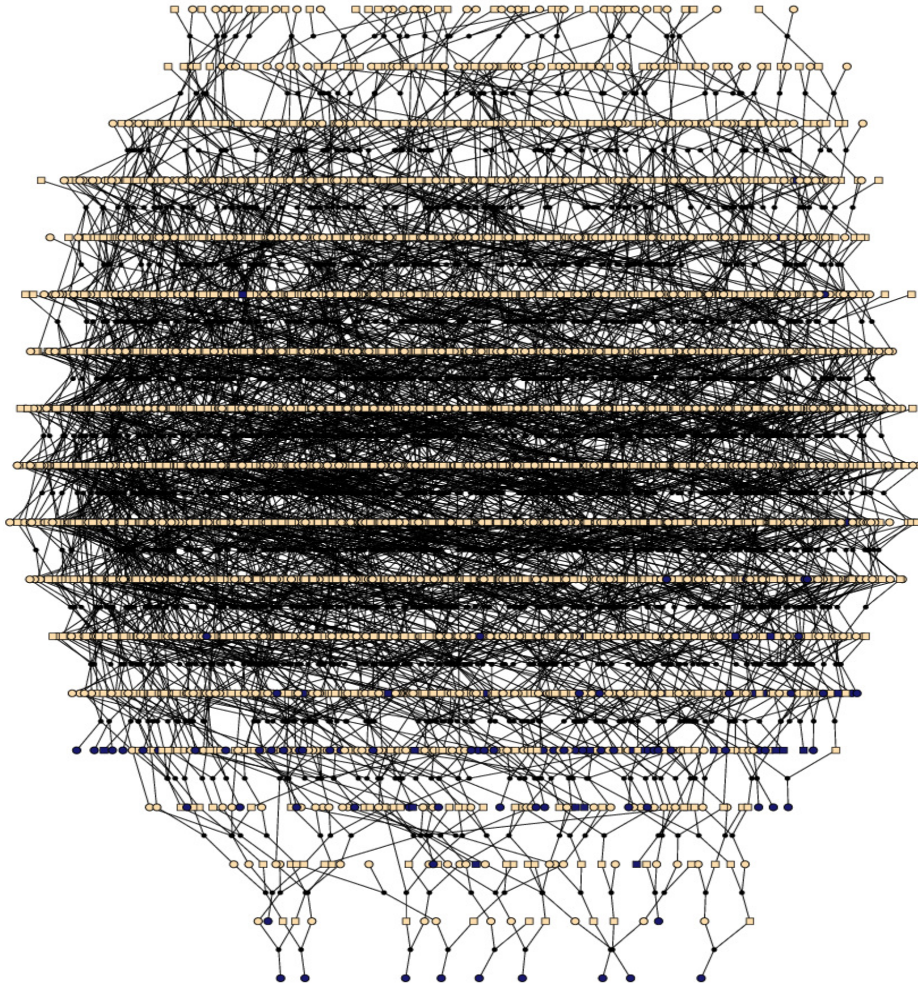
Results

By tracing the genealogy of the 112 probable late onset AD patients, we connected 103 patients to a single pedigree containing 4,645 individuals in 18 generations as depicted in figure 1. The other 9 patients were singletons and, therefore, were not included in the linkage analysis. This large pedigree showed multiple, distant lines of descent and consanguineous loops (table 1). The average kinship coefficient among patients was 0.0018. This value is in between a 3rd cousin once removed and a 4th cousin. Utilizing such a pedigree in linkage analysis is computationally challenging. Using the described algorithm we clustered all patients and 170 first-degree relatives, into 35 sub-pedigrees (supplemental figure 2). These sub-pedigrees contained a total of 1,227 individuals, of whom 630 (51.3%) were female (table 1).

We estimated the threshold for statistical significance using simulation analysis. Our simulations show that a HLOD score of 2.11 corresponds to a genome-wide type-I error rate of 50% (suggestive linkage) and HLOD of 3.64 corresponds to a genome-wide type-I error rate of 5% (significant linkage) (supplementary table 2).

Multipoint LOD and HLOD score plots for the initial scan are shown in supplementary figure 1. A total of eight regions showed suggestive linkage. These were fine typed with 45 additional markers, including: chromosome 1 (14 additional markers), chromosome 3 (10 additional markers), chromosome 5 (2 additional markers), chromosome 6 (5 additional; markers), chromosome 7 (3 additional markers), 0 (6 additional markers), chromosome 11 (2 additional markers), and chromosome 18 (3 additional markers). Table 2 summarizes the results of fine mapping. After fine mapping, we confirmed two regions linked to late onset AD on chromosomes 1

Figure 1. The entire pedigree contains 103 late onset AD patients and 4,645 relatives. Men are represented with squares and women with circles. Black dots represent marriage nodes. Affected individuals are represented with the dark squares and circles. Unknown affection status is represented with grey. For simplicity, unaffected relatives of the patients are not shown.



(figure 2A) and 10 (figure 2C). The maximum HLOD at 1q21 was 5.2 at D1S498. On chromosome 10, the maximum HLOD score was 4.2 at D10S185. There is also suggestive evidence for linkage on chromosome 11 (HLOD = 3.3 at D11S1320, figure 2D). In addition to the confirmed regions, we found significant linkage for a region on chromosome 3 that spanned 18 cM from marker D3S3514 to D3S3626, and reached a maximum HLOD of 4.4 at marker D3S1569 (figure 2B). This is the second highest peak over the genome. We also found significant linkage for a new region on chromosome 18 (HLOD = 3.7 at marker D18S1152, figure 2E).

Table 1. Genealogic characteristics of 103 LOAD patients and their relatives

The full pedigree	Value ±SD	(Range or %)
Family size	4645	
Number of generations	18	
Average number of consanguineous loops per patient	71.7	(0-677)
Average number of meioses in a consanguineous loop	9.9 ± 1.2	(0-29)
Mean inbreeding coefficient × 100	0.39 ± 0.73	(0-3.2)
Average number of lines of descent between a pair of patients	141.7	(0-2673)
Average number of meioses separating a pair of patients	17.1 ± 1.6	(0-34)
Mean kinship coefficient × 100	0.18 ± 1.06	(0-26.4)
The sub pedigrees		
Number of sub-pedigrees	35	
Number of founders	564	(46.0 %)
Number of females	630	(51.3 %)
Mean pedigree size	29.6	(18-75)
Mean number of generations	7.5	(6-10)
Mean number of genotyped individuals per pedigree	7.8	(2-14)
Mean number of patients per pedigree	2.9	(2-6)

Haplotype analysis showed that two haplotypes on chromosome 1 segregate in different families. On chromosome 1q21, a 15 cM region shared by 4 patients in family A and 6 other closely related patients (Figure 3A). The haplotype of the second region on chromosome 1q24 spans 21 cM that segregates in family B and is shared by other four closely related patients (Figure 3B). The haplotype on chromosome 3q23 (18 cM) segregates in some individuals together with the haplotype on

chromosome 18q12 (15 cM) (Figure 3C and 3E). In total 6 patients from families C and E carry both haplotypes. The LOD score from the region on chromosome 10 was based on moderate contributions from multiple families, and, thus, the haplotype segregation is not as obvious as other linked regions. The haplotype of the region on chromosome 11 is shared by 4 patients and 2 other closely related patients (Figure 3 D).

Finally, we tested for association between AD and 6 markers of chromosome 3 and 4 markers of chromosome 18. Association was detected between marker D3S3607 and AD being borderline significant (p-value = 0.05).

Discussion

In this study we confirmed earlier findings suggesting linkage of a wide region on chromosome 1q21-31. This region was initially associated with late onset AD in a genome screen using samples of brain tissue from 50 autopsied AD patients and 50 autopsied controls [24]. A genome-screen in Finland reported linkage to the 1q24.2 region, where the marker D1S1679 was associated with late onset AD [25]. Finally, a genome-scan examined 437 families from the National Institute of Mental Health (NIMH) showed significant evidence for linkage of AD to the 1q23 region [48]. Also our study shows significant evidence of linkage to 1q21 and 1q24. The 1q21 region yielded the most significant evidence of linkage over the genome in our study (HLOD = 5.2). This region contains the gene encoding niscatrin (NCSTN), which binds presenilin and is required for γ-secretase activity and Aβ generation [49]. Mutations in this gene have been found to be related to early onset AD and we have reported association in a sub-group of patients with familial early onset AD, particularly in those who lack the APOE*4 allele [57]. We sequenced all the exons and intron/exon boundaries of this gene, but have not found variants. Another candidate gene in this region is the gene encoding C-reactive protein (CRP), which acts as a scavenger for chromatin released by dead cells during the acute inflammatory process [50]. We also sequenced the exons and intron/exon boundaries of this gene in 7 patients (4 from family 1 and 3 closely related to family 1) and we found 2 variants, SNPs rs1130864 and rs1417938 segregating with 6 of the 7 patients. Interestingly, SNP rs1130864 has been reported as a tagging SNP for a haplotype associated with higher levels of CRP [51, 52].

The second highest linkage signal following chromosome 1q21 region was found at chromosome 3q23. This region was reported to be linked to AD without tau

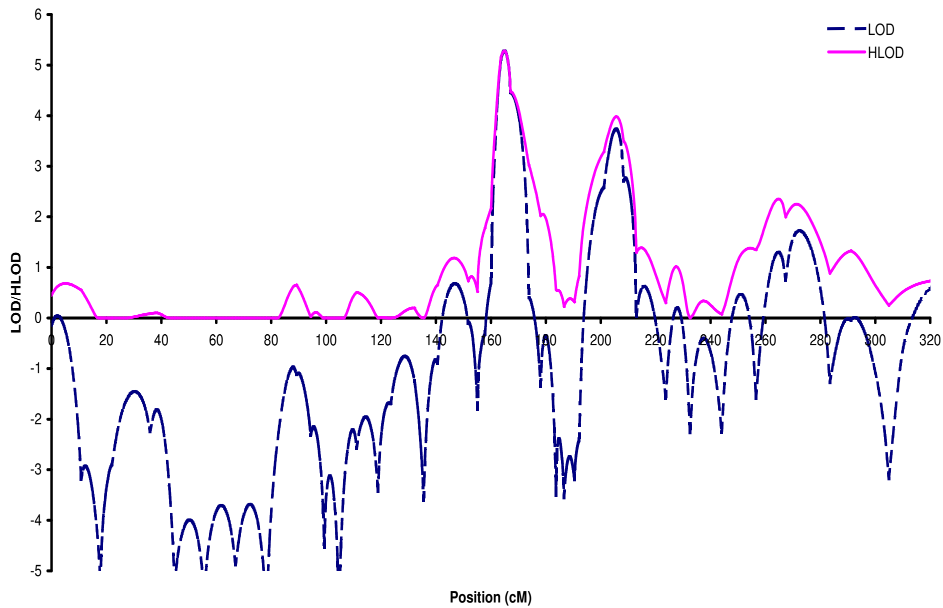
Table 2. Summary of LOD and HLOD scores for regions with significant or suggestive linkage after fine typing

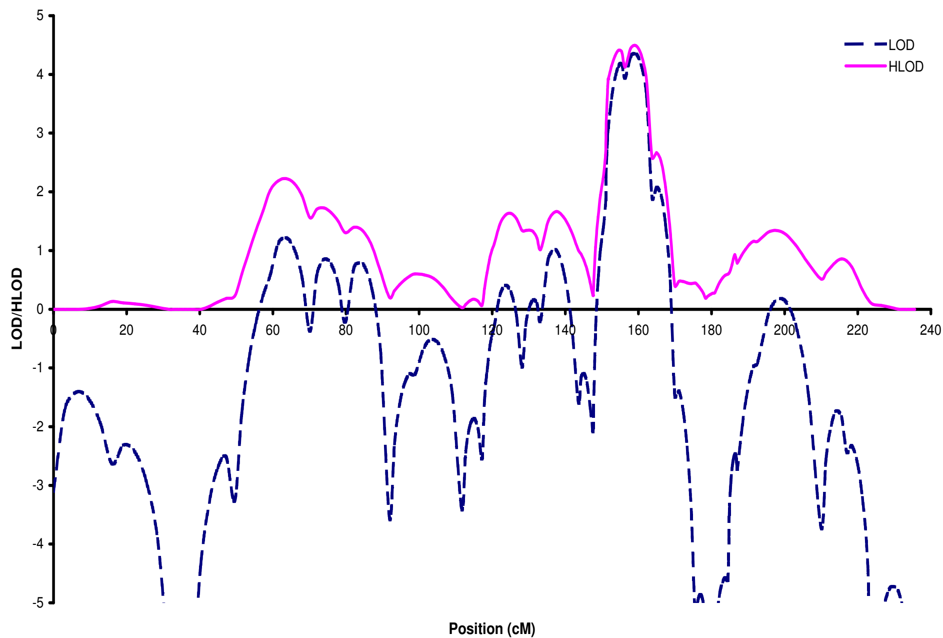
Chromosome	Marker	Position (cM)	LOD	HLOD	α	Regions previously identified[1]
1A	D1S498	164	**5.1**	**5.2**	0.9	A,D,F,G
	D1S305	167	**4.5**	**4.5**	1.0	
	D1S2635	174	0.4	3.0	0.5	
1B	D1S218	201	2.6	**4.0**	0.6	
	D1S466	208	2.7	3.5	0.6	
3	D3S1549	151	2.8	3.6	0.6	B
	D3S1569	158	**4.3**	**4.4**	0.8	
	D3S3626	164	1.9	2.6	0.6	
10	D10S1686	105	**3.7**	**3.7**	1.0	C,E,F,G,H,I
	D10S185	116	**4.2**	**4.2**	1.0	
11	D11S4151	127	0.3	2.8	0.4	G
	D11S4131	138	1.3	3.1	0.5	
	D11S1320	142	1.6	3.3	0.6	
	D11S968	148	0.3	2.0	0.5	
18	D18S1152	89	0.5	**3.7**	0.3	
	D18S64	93	0.2	2.3	0.2	

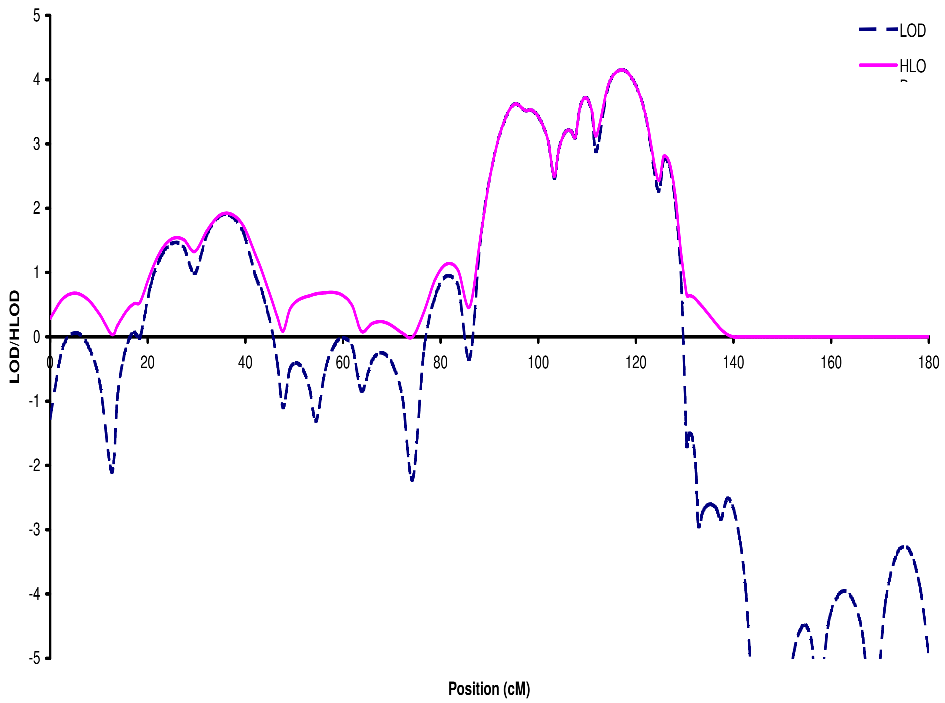
[1] Overlaps with regions reported with suggestive linkage or significant association in previous genome screens, including: A (Zubenko et al. 1998), B (Poduslo et al. 1999), C (Curtis et al. 2001), D (Hiltunen et al. 2001), E (Olson et al. 2002), F (Myers et al. 2002), G (Blacker et al. 2003), H (Farrer et al. 2003), and I (Holmans et al. 2005). Note that B screened for only 2 chromosomes. LOD and HLOD scores with a genome-wide Type I error rate < 0.05 are in bold

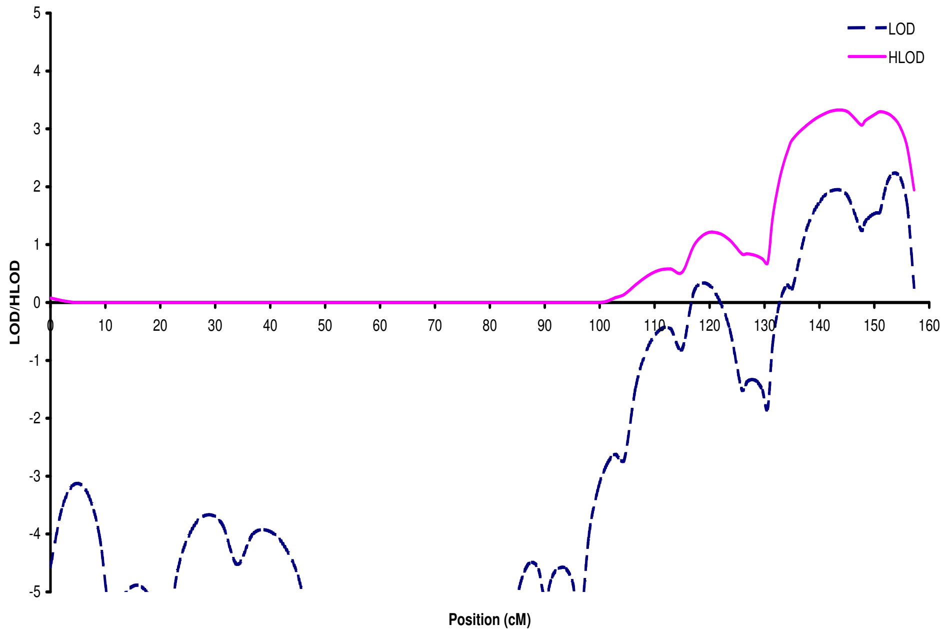
pathology, earlier in a study of a small family with 4 relatives [53]. A significant LOD score of 4.1 between markers D3S1569 and D3S3554 was reported where the marker D3S1569 is the same marker in our study that gives the maximum HLOD over chromosome 3. In the study of Poduslo et al (1999), no genome wide screen was conducted but only chromosomes 3 and 17 were screened since the disease was expected to be related to frontotemporal dementia. As we do not have pathology information of our patients, we cannot exclude that part of our patients also suffer from this atypical form of AD. Furthermore, this region also shows evidence for association to AD in our study. The linked region on chromosome 3 includes obvious candidate genes including the transferrin gene, the gene encoding for butyrilcholinesterase, the neprilysin gene and the somatostatin gene. We screened

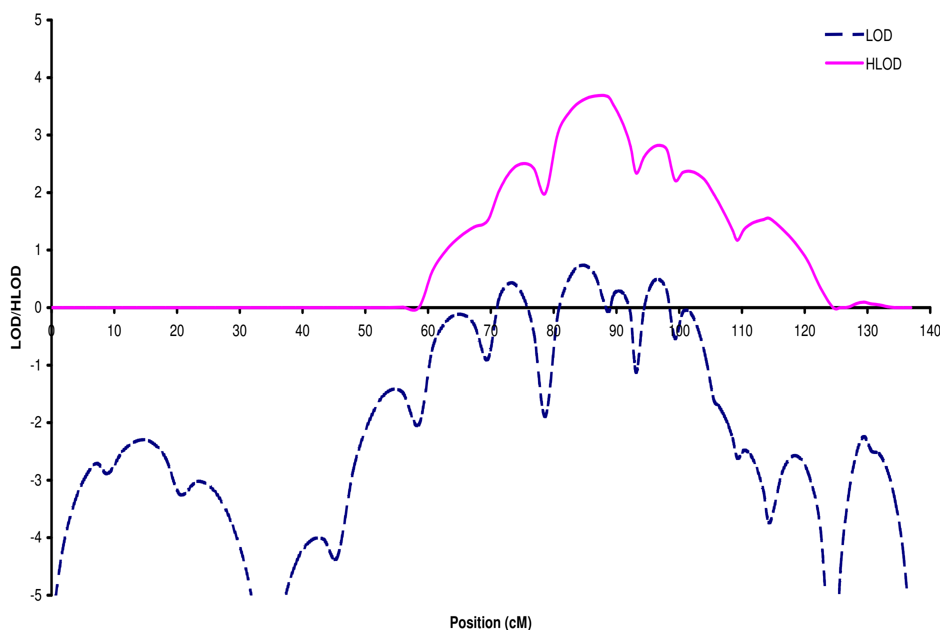
Fig 2. Multipoint LOD (dark) and HLOD (light) scores for chromosomes 1, 3, 10, 11, and 18 in the genome screen of late onset Alzheimer's disease after fine typing. Marker locations are given in Kosambi centi-Morgans.






C. Chromosome 10
LOD
HLOD
LOD/HLOD
Position (cM)
5
4
3
2
1
0
-1
-2
-3
-4
-5
0
20
40
60
80
100
120
140
160
180


D. Chromosome 11
LOD
HLOD
LOD/HLOD
Position (cM)
5
4
3
2
1
0
-1
-2
-3
-4
-5
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160



these genes for mutations but no variants were found. We are currently sequencing more genes in this region.

Our results for chromosome 10q24 are consistent with previous findings. An affected sib-pair analysis using the NIMH sample [13] suggested linkage to chromosome 10p11-q26. A second stage analysis adding additional affected sib-pairs and genetic markers, increased the multipoint LOD score to 3.5 [18]. This same region was linked to plasma amyloid β42 levels with a LOD of 3.9 [54]. Using part of the initial NIMH sample, three recent genome-screens also pointed to the same region (10q21-24) [19, 20, 23]. The region linked to late onset AD in our study (10q24) is the first replication using a fully independent data set from the NIMH sample.

We also found suggestive evidence for linkage on chromosome 11q24. Blacker et al (2003) [20] previously described this region in their study of the NIMH sample, including 437 families with AD.

In our study, chromosome 18 is significantly linked to AD and to our knowledge no candidate gene close to this region has been previously reported in relationship with AD. Linkage of AD to chromosome 18q has been previously reported to a narrow region around 112cM with a multipoint LOD score of 3.37 for D18S541 [55]. This region is 23 cM downstream from our peak. However, for the chromosome 18

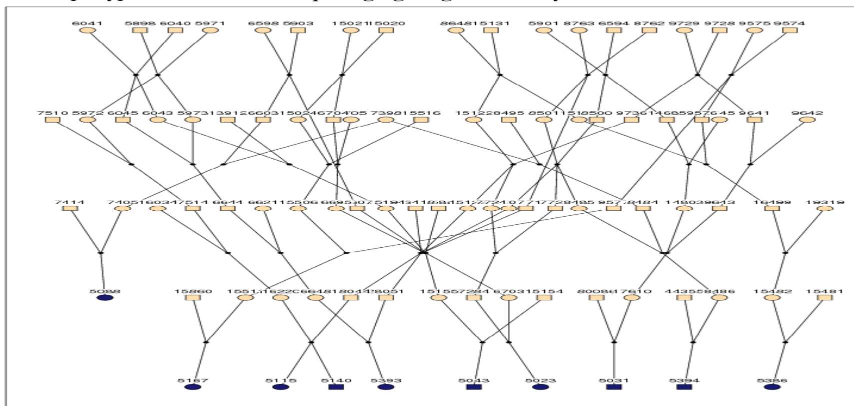
region there is substantial heterogeneity with some families showing negative LOD scores. When testing for association, no evidence of association was found. Since there is a clear overlap in patients (n=6) sharing both the chromosome 3 and 18 haplotypes, it remains to be determined whether one of the two findings is a false positive. This requires sequencing of all genes in both regions. Alternatively, these two regions may be involved in the onset of AD with epistatic effects.

In this study it was necessary to split the extended complex pedigree into small sub-pedigrees for computational reasons. We used an approach for splitting proposed by Falchi et al [43]. In order to optimize the key parameters required by the method of Falchi and to obtain a set of sub-pedigrees which can be efficiently analyzed using currently available software for parametric linkage analysis, we restricted our splitting on maximum bit size and iterated over all the required parameters to find out the best set of sub-pedigrees. Since breaking pedigrees may increase false positives we adjusted our thresholds for linkage based on simulation analysis.

In summary we confirmed two previously well described linkage regions for late onset AD on chromosomes 1q21-25 and 10q24. This is the first genome-wide screen that showed significant linkage to chromosome 3q23 markers. We also found significant linkage to a new locus at chromosome 18q12.

Fig 3. Haplotypes of chromosomes 1, 3, 11 and 18 segregating with AD families from the GRIP population.

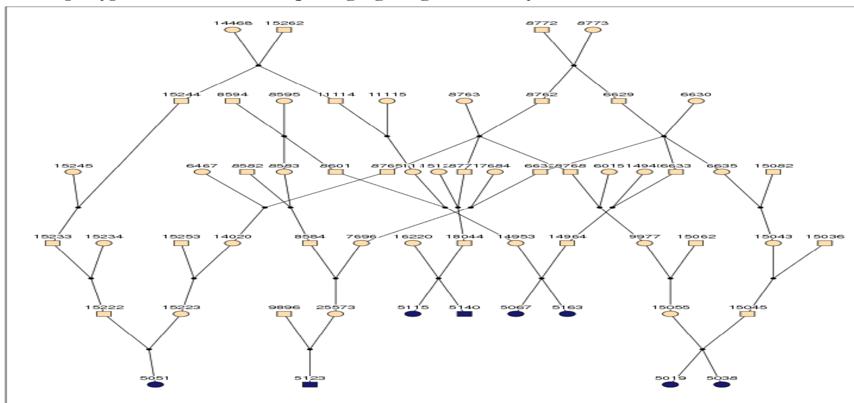
3A. Haplotype of chromosome 1q21 segregating with family A



Markers	5088*		5167		5115		5140		5393*		5043		5023*		5031		5394*		5386	
D1S2746	(1)	(3)	2	1	7	1	7	1	1	3	1	8	7	6	1	1	7	7	7	3
D1S252	2	3	1	4	1	1	1	1	1	2	1	4	1	1	1	1	1	4	1	6
D1S514	(6)	(4)	6	1	6	4	6	7	6	5	6	5	6	4	6	1	6	5	6	3
D1S498	1	9	1	4	1	10	1	6	1	5	1	4	1	2	1	6	1	4	3	9
D1S2635	3	1	3	1	3	3	3	6	3	6	3	4	(3)	(6)	3	3	(3)	(6)	(3)	(1)
D1S484	3	3	3	2	3	3	3	3	3	1	3	1	3	2	3	1	1	3	1	2

Patients with a star are from the sub-family 1, other patients are closely related. Alleles with brackets are inferred. Grey bars depict the shared segment between the patients.

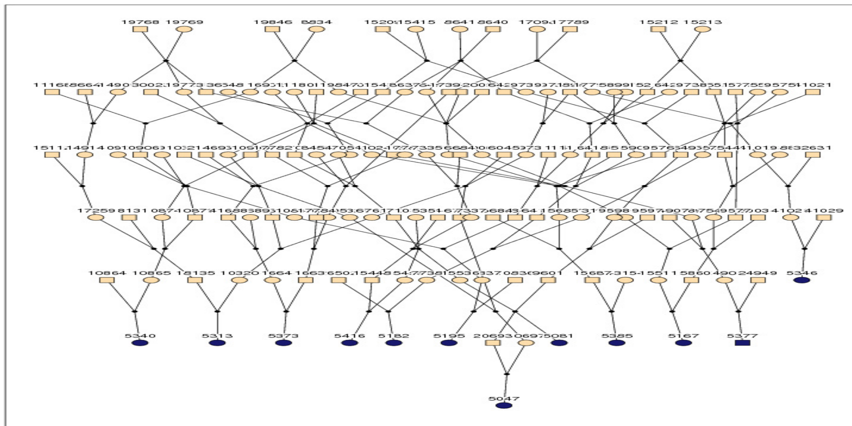
3B. Haplotype of chromosome 1q24 segregating with family B



Markers	5051*		5123		5115*		5140*		5067*		5163*		5019		5038	
D1S2799	3	8	3	4	3	1	4	1	7	1	7	1	1	2	1	2
D1S218	1	1	1	5	5	1	5	1	1	1	1	1	1	2	1	2
D1S466	5	1	1	3	3	1	(1)	(1)	1	1	1	1	1	1	1	1
D1S238	1	2	1	7	2	2	7	2	4	1	4	1	2	1	2	1

Patients with a star are from the sub-family 1, other patients are closely related. Alleles with brackets are inferred. Grey bars depict the shared segment between the patients.

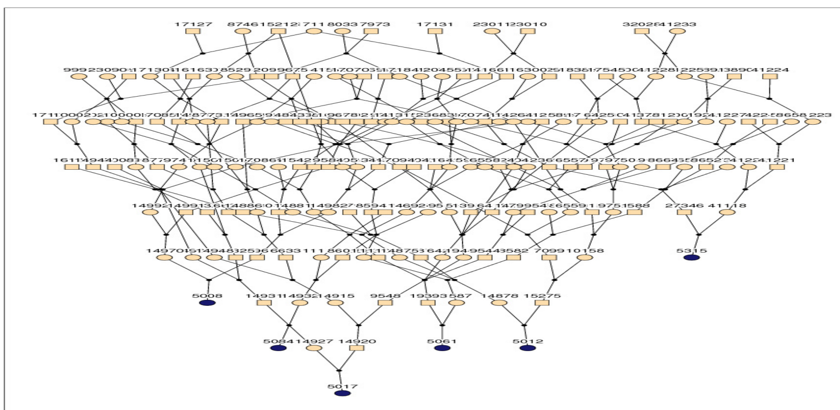
3C. Haplotype of chromosome 3q23 segregating with family C



Markers	5340		5313*		5373		5416*		5182*		5195		5047*		5081*		5385		5167*		5377		5346	
D3S3514	2	3	2	9	4	4	2	2	2	5	2	1	2	2	2	2	2	2	2	1	7	2	2	1
D3S1292	2	9	2	4	5	2	2	2	2	9	2	1	2	2	2	2	2	6	2	1	2	2	2	1
D3S1549	5	2	5	3	5	5	5	2	5	3	5	3	5	2	5	5	5	5	5	8	5	1	5	4
D3S1569	3	1	3	1	3	1	3	2	3	3	3	2	2	6	3	1	3	1	3	1	3	4	3	3
D3S3626	2	8	2	1	3	2	7	3	3	1	2	2	3	3	2	2	2	2	2	8	2	1	2	2

Patients with a star are from the sub-family 1, other patients are closely related. Alleles with brackets are inferred. Grey bars depict the shared segment between the patients.

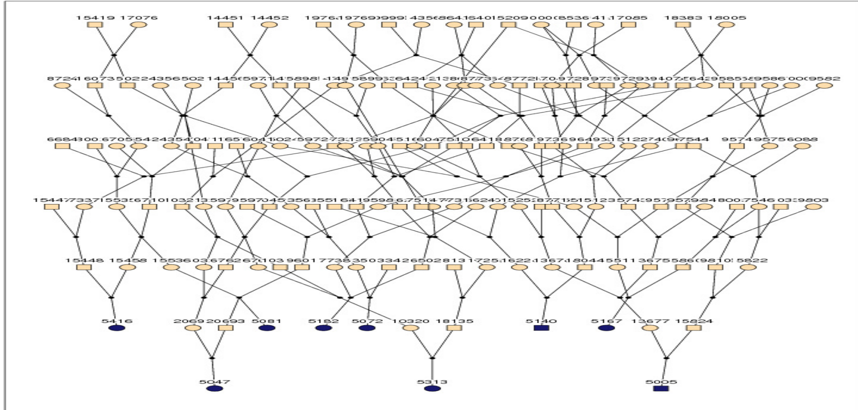
3D. Haplotype of chromosome 11q24 segregating with family D.



Markers	5008*		5084*		5017*		5061		5012*		5315	
D11S925	1	2	8	6	8	3	3	9	8	1	11	2
D11S1328	5	5	3	5	3	5	5	5	3	1	9	3
D11S4151	5	2	2	5	2	2	2	3	2	3	2	4
D11S4131	6	3	6	7	6	3	6	3	6	7	6	3
D11S1320	1	1	1	1	1	1	1	2	1	3	1	3
D11S968	7	1	5	5	5	1	3	1	1	1	5	3

Patients with a star are from the sub-family 1, other patients are closely related. Alleles with brackets are inferred. Grey bars depict the shared segment between the patients.

3E. Haplotype of chromosome 18q12 segregating with family E



Markers	5416*		5047*		5081*		5182*		5072		5313*		5140		5167*		5005	
D18S1102	1	2	1	1	1	1	1	1	3	6	1	5	2	8	8	1	6	3
D18S474	2	5	2	6	2	7	2	9	2	1	2	2	2	5	1	7	2	1
D18S1152	2	4	2	2	2	1	2	2	2	2	2	2	2	3	2	2	2	8
D18S64	3	5	3	4	3	8	3	4	3	4	4	2	3	4	3	4	3	4
D18S1147	12	3	8	5	8	1	8	9	3	9	3	8	12	3	8	2	1	7
D18S68	4	6	4	4	4	4	4	6	3	2	3	1	4	6	4	3	4	6
D18S465	1	7	2	2	2	2	2	1	3	2	1	1	2	5	2	8	9	1
D18S61	2	3	2	7	2	8	2	4	4	7	3	7	2	2	2	10	10	7

Patients with a star are from the sub-family 1, other patients are closely related. Alleles with brackets are inferred. Grey bars depict the shared segment between the patients.

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Supplementary Table 1. Age dependent liability classes and penetrances

Liability Class	Age (years)	Population Prevalence[1]	Penetrance	Number of AD patients	Number of genotyped unaffected relatives
1	< 65		0.00	0	129
2	65-69	0.02	0.09	4	6
3	70-74	0.05	0.23	22	11
4	75-79	0.09	0.46	32	14
5	80-84	0.23	0.99	30	8
6	85-89	0.35	0.99	24	1
7	>=90	>0.35	0.99	0	1

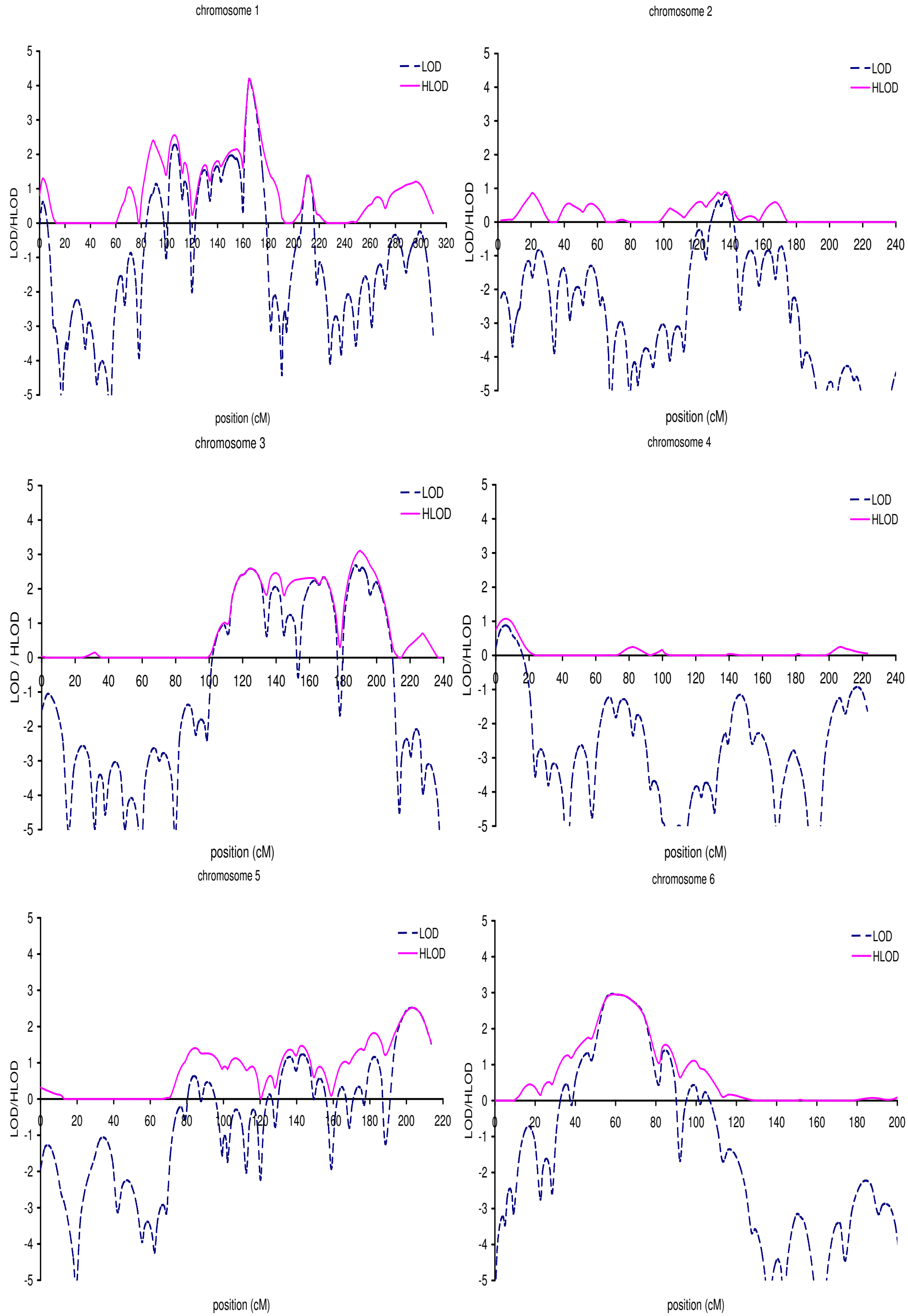
[1] Population prevalence was obtained from the Rotterdam Study (Ott et al. 1995).

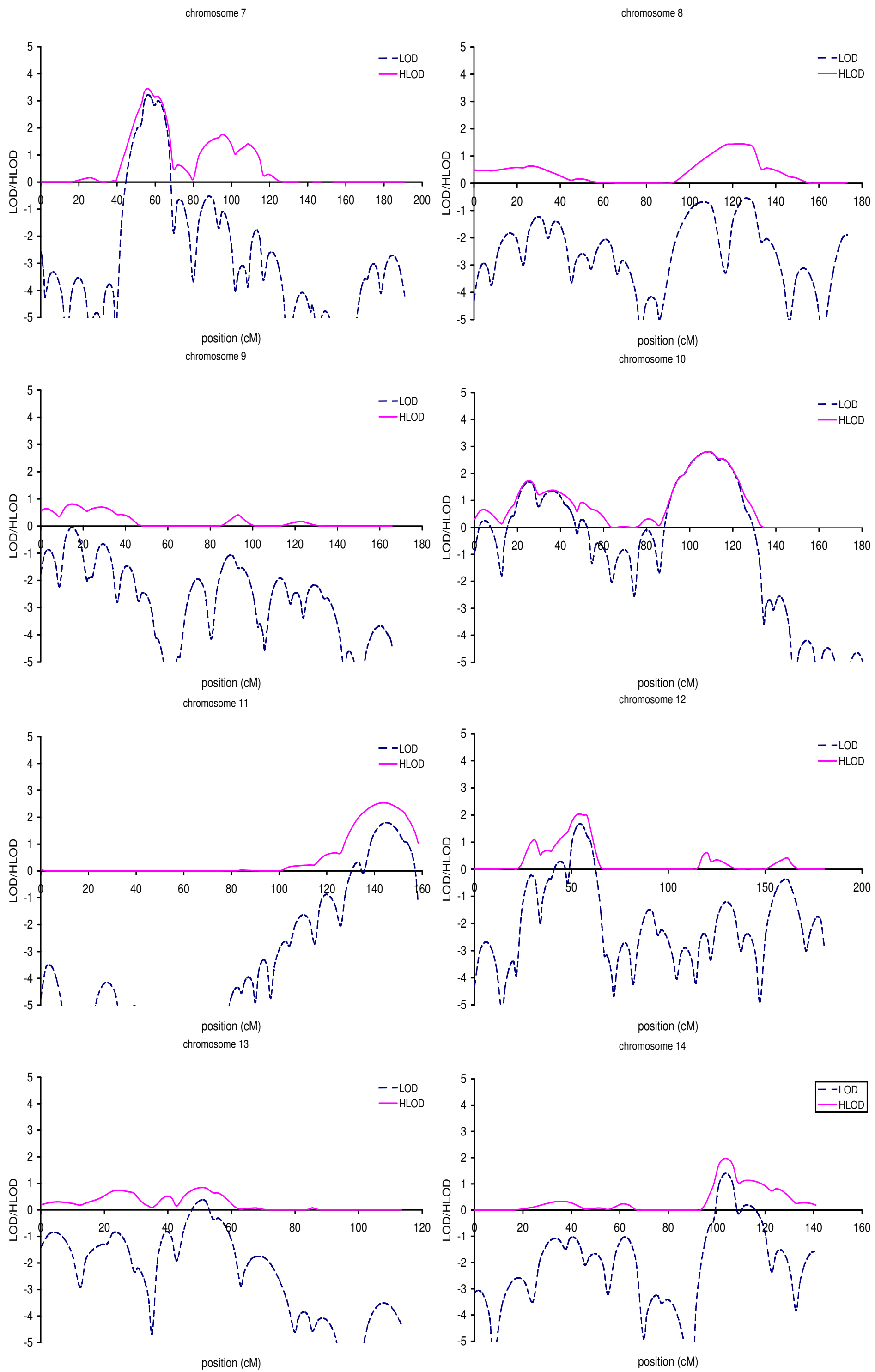
Supplementary Table 2. LOD score and corresponding Type I error, based on 100 genome-wide simulations.

LOD	HLOD	Type I error
4.08	4.08	1%
3.56	3.63	5%
3.24	3.24	10%
2.02	2.11	50%

Supplementary Figures

Supplementary Fig 1. Multipoint LOD and HLOD scores for each autosome in the genome screen of late onset Alzheimer's disease before fine typing. Marker locations are given in Kosambi centimorgans (cM).

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LOD
HLOD
LOD/HLOD
position (cM)
chromosome 8
LOD
HLOD
LOD/HLOD
position (cM)
chromosome 9
LOD
HLOD
LOD/HLOD
position (cM)
chromosome 10
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HLOD
LOD/HLOD
position (cM)
chromosome 11
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HLOD
LOD/HLOD
position (cM)
chromosome 12
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LOD/HLOD
position (cM)
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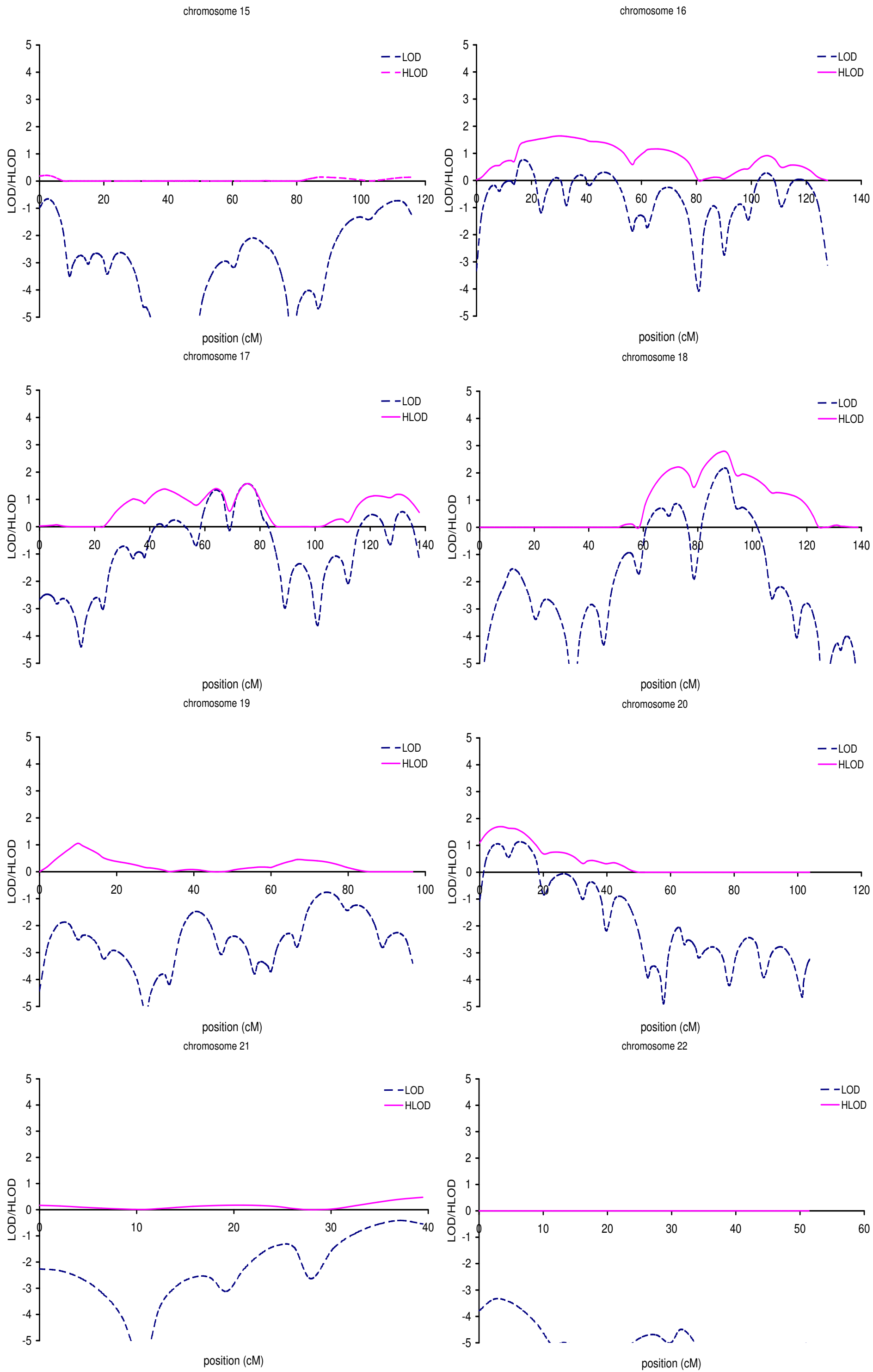
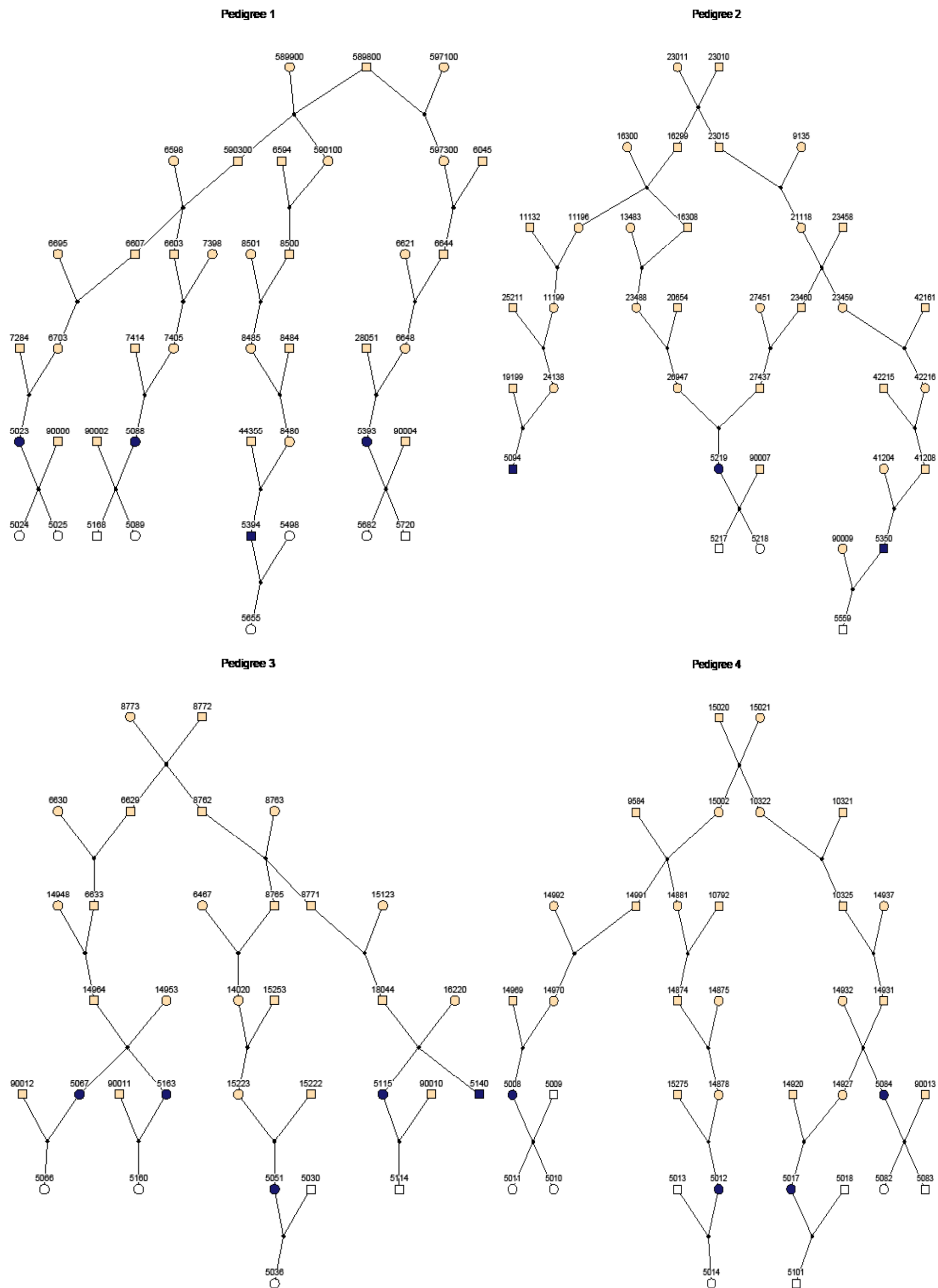
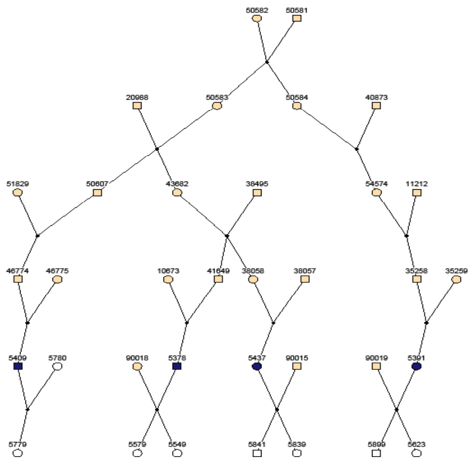
HLOD
LOD/HLOD
position (cM)


chromosome 15
chromosome 16
chromosome 17
chromosome 18
chromosome 19
chromosome 20
chromosome 21
chromosome 22
LOD
HLOD
LOD/HLOD
position (cM)

Supplementary Fig 2. The 35 sub-pedigrees obtained by applying the clustering algorithm to the entire pedigree. Affected individuals are shown with b;lack squares and circles, unaffected first-degree relatives of the patients, ancestors from the patients and relatives are shown with grey squares and circles.

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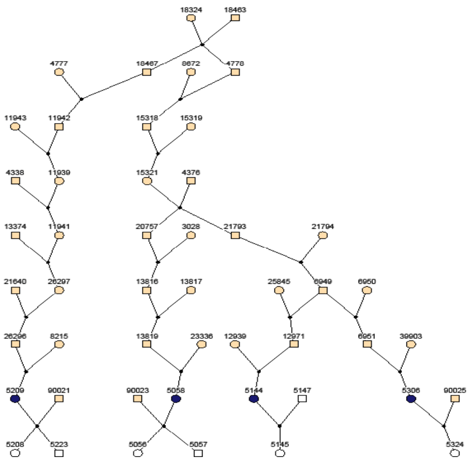
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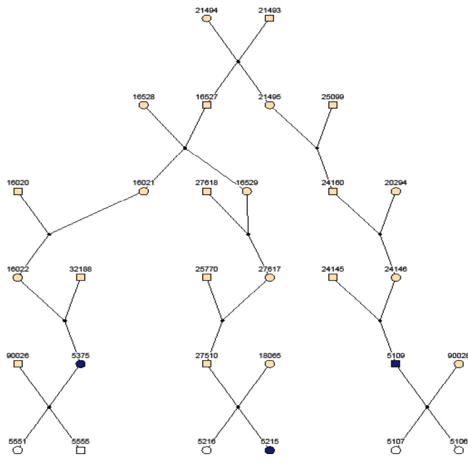
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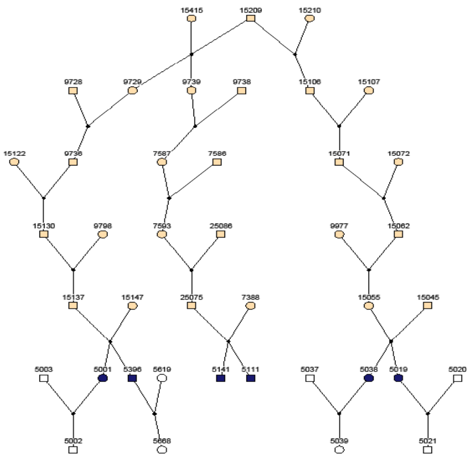
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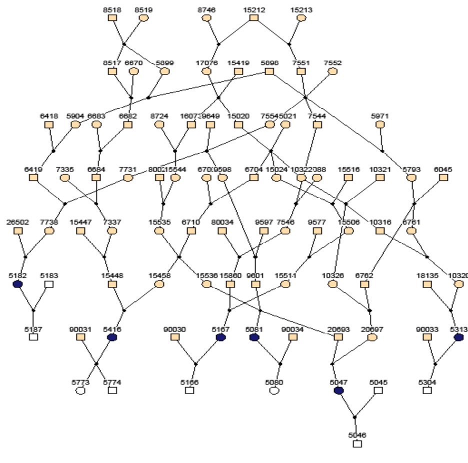
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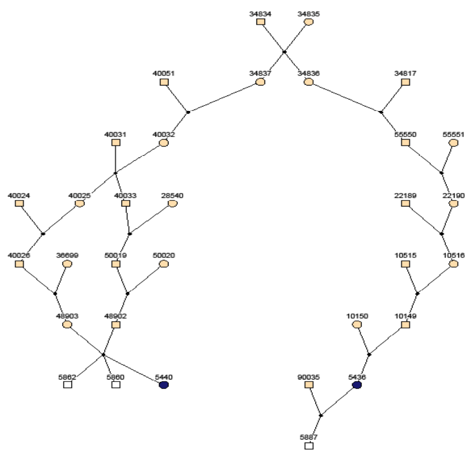
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Pedigree 11

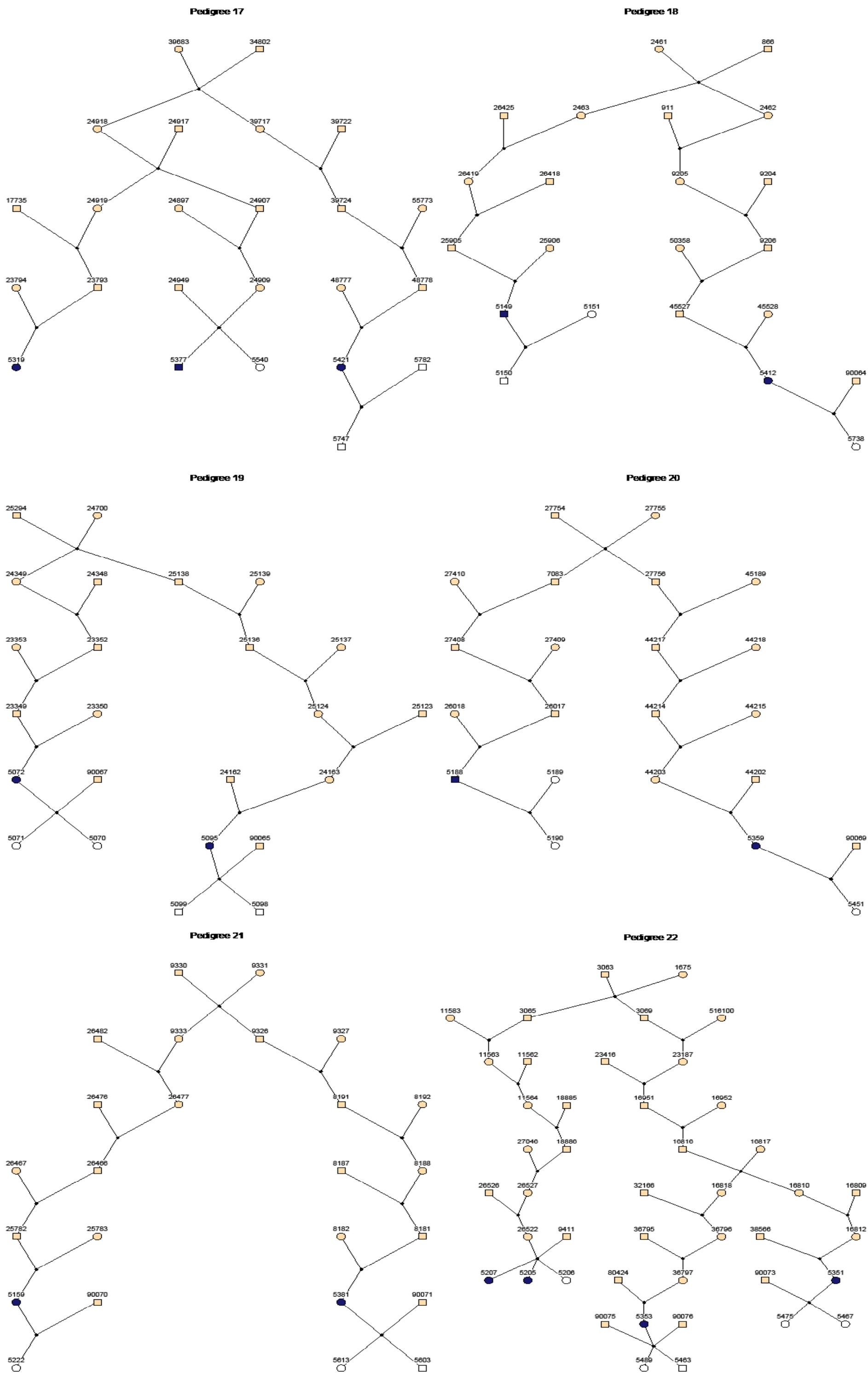
Pedigree 12

Pedigree 13

Pedigree 14

Pedigree 15

Pedigree 16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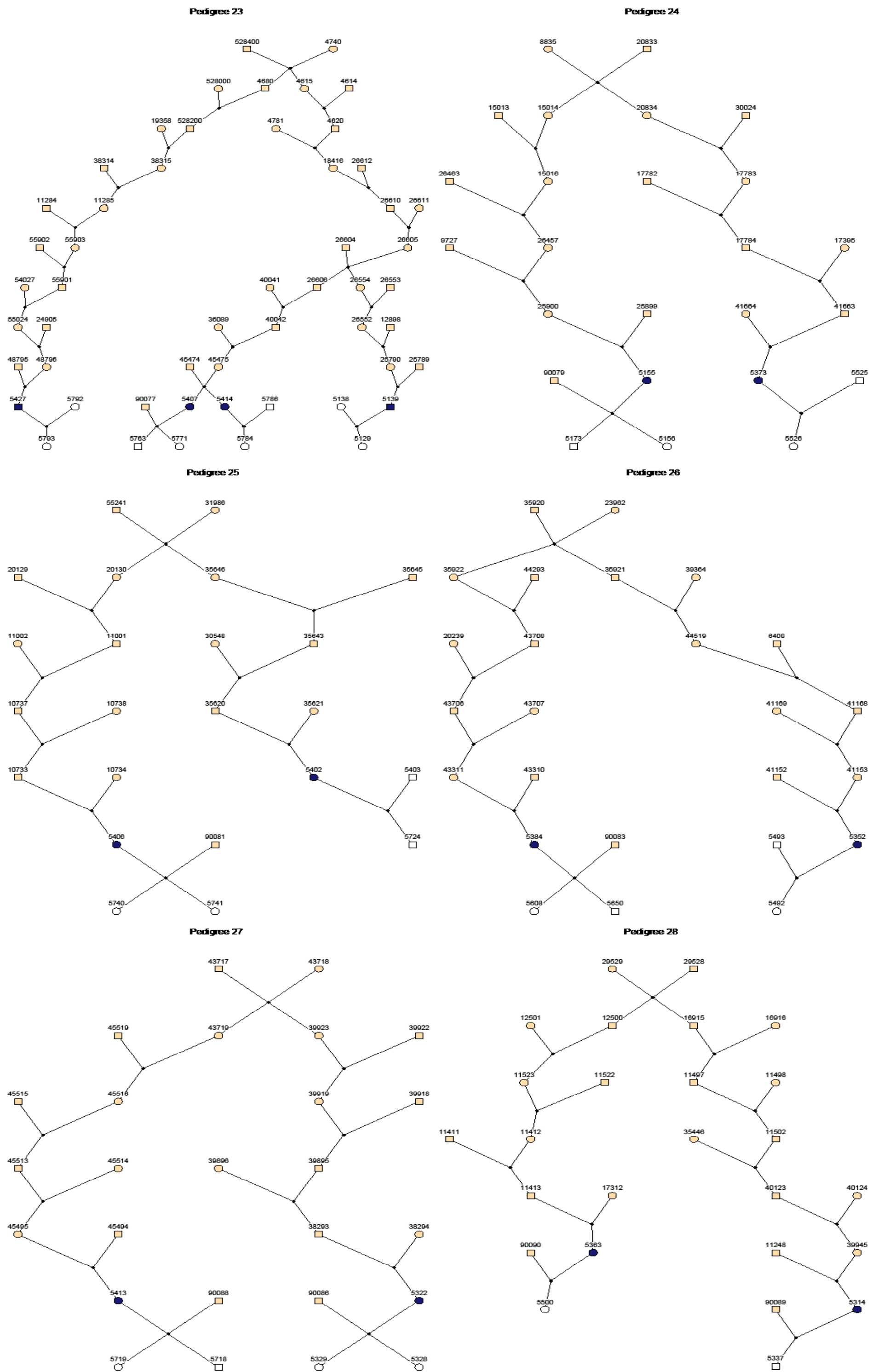
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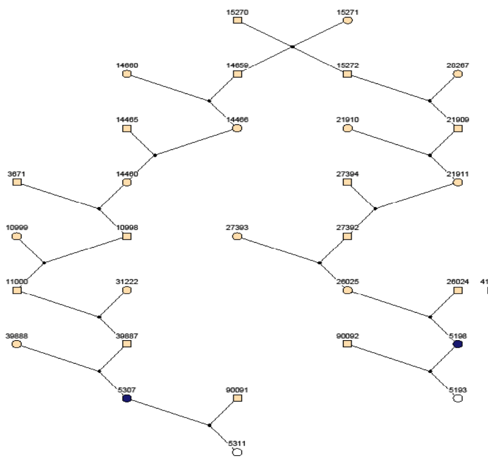
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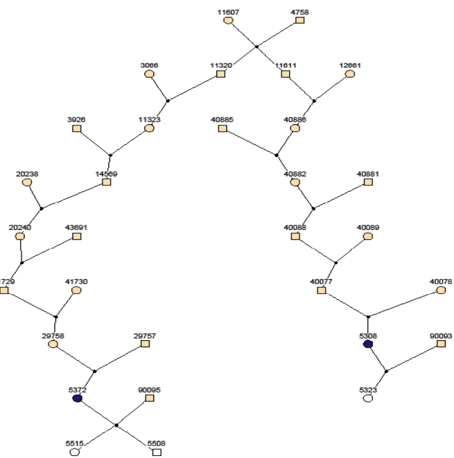
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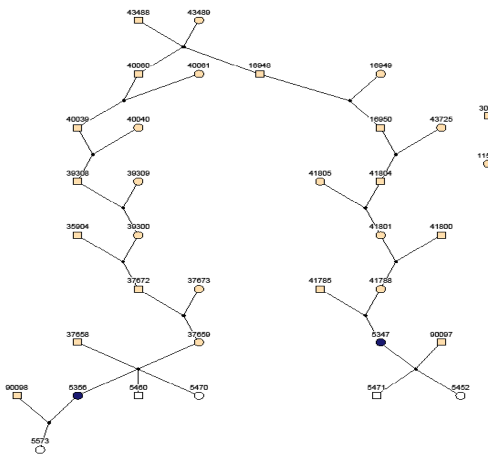
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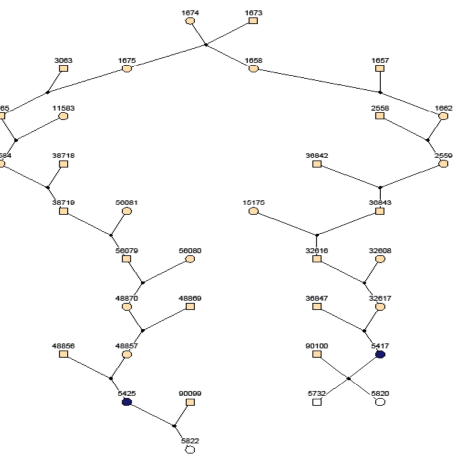
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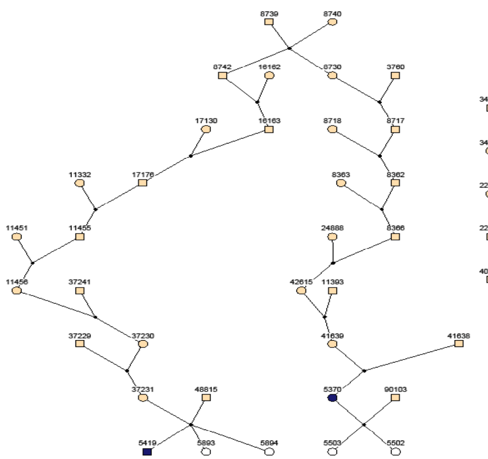
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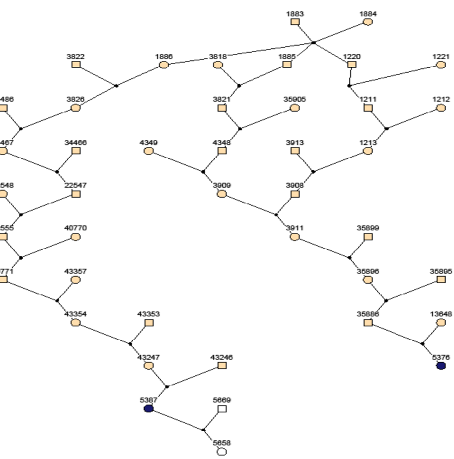
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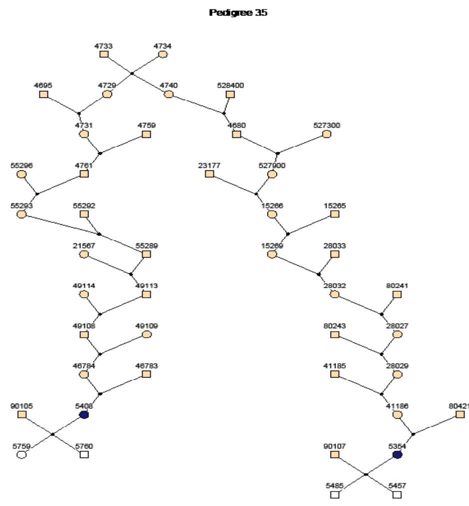
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Chapter 2.2

The MAPT and Granulin genes are not associated with Alzheimer's disease

Abstract

The microtubule associated protein tau plays a pivotal role in Alzheimer's disease (AD) pathogenesis but findings on the MAPT gene, encoding for the tau protein, have been controversial. The discussion about the role of this gene was recently fuelled by the identification of new MAPT haplotypes associated with AD and a new gene, granulin (GRN) 1.4 Mb upstream of MAPT associated with fronto-temporal dementia. We have investigated the association between AD and the region including MAPT and GRN in patients from the Genetic Research in Isolated Populations (GRIP) program. We studied 122 AD patients and 85 control subjects using an extensive Single Nucleotide Polymorphism (SNP) panel. No association was found for any individual SNP of the MAPT gene, H1/H2 haplotype, or H1c haplotype sub-clade. The interaction with APOE*4 was considered by stratified analysis but no evidence for association was found. Also, the MAPT gene was excluded by linkage analysis. We also performed a meta analysis of all studies conducted to date on the associations between the MAPT gene and AD stratified by APOE*4. The meta analysis showed a very modest non-significant association (OR = 1.20, 95% CI 0.93-1.55) for carriers of the H2 haplotype compared to H1/H1 carriers, only in those with the APOE*4 allele. Nominally significant association was found for two SNPs located 722 kb upstream the MAPT locus in the NMT1 gene, but findings did not sustain adjustment for multiple testing. The GRN gene was sequenced in 17 patients of families showing evidence of linkage to this region but no mutations were found. These results suggest that variations in the MAPT gene and other genes in the region including GRN have little or no effect with Alzheimer's disease in our population.

Introduction

Alzheimer's disease (AD) is a complex genetic disorder, which is characterized by amyloid deposition and hyperphosphorylation of the microtubule associated protein tau in the brain [1, 2]. Several genes have been implicated in the dominant form of early onset AD including the amyloid precursor protein gene (APP) [3], presenilin-1 (PSEN-1) [4-8], and presenilin-2 (PSEN-2) [9, 10]. The e4 allele of APOE gene has been associated with early and late onset AD [11, 12]. Follow up studies have shown that individuals carrying one copy of this allele have a 1.5-2 fold increase of risk of the disease and those carrying two copies have a 6-15 fold increase of risk [1, 13]. Each of these 4 genes is implicated in the amyloid pathology involved with AD rather than to the tau pathology characteristic of AD.

Despite the fact that the microtubule associated protein tau plays a key role in AD, findings on the gene encoding for the tau protein (MAPT gene ID:4137) are inconsistent. While specific mutations and polymorphisms in this gene are known to lead to several tauopathies including fronto-temporal dementia, progressive supranuclear palsy, corticobasal degeneration, and argyrophilic grain disease [14, 15], the relationship between MAPT polymorphisms and AD remains controversial [15].

In brains of AD patients, the tau protein is hyperphosphorylated, truncated, and aggregated into neuro fibrillary tangles (NFTs). In vitro studies suggest that the tangle formation is partially controlled by the tau protein itself [16]. The MAPT gene is located on chromosome 17q21.1 and in the human brain it codes for 6 different isoforms, which contain 12, 11, 10, or 9 exons due to complex alternative splicing. Some studies found association of polymorphisms in MAPT with AD [17, 18]. The new Single Nucleotide Polymorphism (SNPs) of the MAPT gene identified in the HapMap project [24] and other sequencing efforts have generated new opportunities to study this gene in depth. However, these findings have not been consistent with five studies failing to support association of MAPT to AD [19-23]. There is some evidence for modification of the effect of MAPT by APOE but these findings have also been inconsistent. Three studies found an association between MAPT and AD in APOE*4 carriers [17, 25, 26] indicating a possible interaction between the APOE gene and the tau protein. In contrast, the H1c variant of the H1 haplotype was over represented in AD cases without the APOE*4 allele [27]. Finally, mutations in the granulin (GRN) gene, located 1.4Mb upstream of MAPT [28, 29] and leading to frontal lobe dementias, may explain the association between the MAPT region and AD.

We investigated 43 SNPs spanning a 1.85 Mb region including the MAPT and GRN genes in an extended family-based study including 122 AD patients, allowing both linkage and association analyses [30, 31]. We further evaluated the interaction between MAPT SNPs and the APOE*4 allele. Since studies of interaction require large numbers of patients and controls, we evaluated the evidence for the interaction between the APOE gene and the MAPT haplotypes in a meta-analysis of all studies published to date.

Materials and Methods

Study population

AD patients (n=122) and their relatives were derived from a study of dementia, performed within the Genetic Research in Isolated Populations (GRIP) study [32]. The GRIP population is a genetically isolated community in the Southwest of The Netherlands. This community expanded from a founding population of about 150 ancestors to a current number of approximately 20,000 inhabitants. Since there has been little inward migration, a genetically homogeneous population arose from the founder population. In this isolate, all patients with dementia were ascertained through local general practitioners, neurologists and nursing-home physicians. Genealogy data up to 23 generations was collected for all patients. Clinical diagnoses of AD were confirmed by an independent neurologist according to the NINCDS-ADRDA criteria [33]. Control individuals were ascertained from the

Table 1. Description of cases and controls

	AD patients	Controls
Number	122	85
Mean age at onset (years)	75.0	-
Female (%)*	94(76.4)	44(51.8)
Average Kinship*	0.001953 - 0.000977	0.000488 - 0.000244
APOE*4 carriers (%)*	72/116(62.1)	30/85 (35.3)

GRIP population. Informed consent was obtained from first-degree relatives or spouses and the patient if possible. The Medical Ethical Committee of the Erasmus Medical Center approved the study protocol. More detailed descriptions of the clinical diagnosis re-evaluation and genealogy data retrieval can be found in our previous report [32].

Genotyping

Blood was drawn from patients and their first-degree relatives. DNA was extracted from peripheral leucocytes according to a standard protocol [34]. A total 43 of SNPs spanning a 1.9 Mb region around the tau gene on chromosome 17 were selected, with an average distance of 40.5 kb between adjacent SNPs. The selection of the SNPs for this study was based on their physical location in the MAPT gene and its flanking region. 10 SNPs were located in the gene's transcriptional region. SNP genotyping was performed using a SNPlex assay (Applied Biosystems). The SNPlex assay was conducted following the manufacturer's recommendations (Applied Biosystems, Foster City, CA). Stock genomic DNA (gDNA) solutions (50 ng/µl) were diluted after fragmentation to a final concentration of 18.5 ng/µl. Diluted gDNA aliquots (2 µl) were spotted and dried down in 384 well plates (Bioplastics). Prior to the oligo ligation assay (OLA) reaction, reagents were phosphorilated and diluted (1:1). The OLA reaction was performed in 5 µl volumes, which contained 37 ng of gDNA, (0.5 µl) ligation buffer, (0.025 48-SNPlex ligase and (l µl) activated ligation probe pool). The PCR conditions were: 3 minutes at 90°C, 30 cycles of 15 seconds at 90°C, 30 seconds at 60°C and 30 seconds at 51°C (2% ramp), followed by denaturation step at 99°C for 10 minutes. After this step, a purification (digestion) step was conducted after which OLA products were ready to be amplified in a final volume of l0 µl. Exonuclease I (4l µl) and Lambda Exonuclease (82 µl) (Applied Biosystems) were added, and incubated first at 37°C for 90 minutes and then at 80°C for 10 minutes. Purified OLA products were diluted (2:3) for further amplification. Amplification of OLA products was performed in l0 µl volumes, which contained 2 µl of diluted OLA reaction, (Ix) SNPlex amplification master mix and (20x) SNPlex amplification primers. The OLA amplification conditions were: 95°C for 10 minutes, followed by 95°C for 15 seconds, 63°C for 60 seconds for 30 cycles. After hybridization step, analysis of the fluorescence intensity was performed in an aliquot (7.5 µl) using ABI Sequencer 3730 (Applied Biosystems, Foster City, CA). All pre-PCR steps were performed on a cooled block. Reactions were carried out in Gene Amp 9700 Thermocycler (Applied Biosystems, Foster City, CA). Data was analyzed using Genemapper v3.7 (Applied Biosystems, Foster City, CA). The APOE genotypes were determined on 5 ng/ul dry DNA samples using TaqMan allelic discrimination technology on an ABI Prism 7900HT Sequence Detection System with SDS v 2.1 (Applied Biosystems, Foster City, CA). All samples were

Table 2. Description of the 37 SNPs studied

	Marker	Position	rsnumber	Gene	Location:
1	hCV2536908	40526680	rs7219596	NMT1	exon 4*
2	hCV341577	40538554	rs1053733	NMT1	3' UTR
3	hCV9254243	40571807	rs4986172	ACBD4	intron 7
4	hCV2032862	40598477	rs11657325	FLJ32384	intron 1
5	hCV2032865	40603713	rs7208548	Intragenic	-
6	hCV2554844	40717672	rs2074291	MAP3K14	intron 5
7	hCV2541205	40828104	rs1059504	LOC201176	3' UTR
8	hCV2265271	41070456	rs241039	LOC401884	3' UTR
9	hCV2544843	41235818	rs110402	CRHR1	intron 1
10	hCV2257689	41241147	rs242924	CRHR1	intron 2
11	hCV2544830	41256855	rs173365	CRHR1	intron 4
12	hCV2257669	41301901	rs1078830	Intragenic	-
13	*hCV7450857*	41340226	rs1467966	MAPT	5' UTR
14	*hCV3202946*	41350591	-	MAPT	5' UTR
15	*hCV3202949*	41352389	rs1560313	MAPT	5' UTR
16	*hCV1016016*	41375573	rs242557	MAPT	5' UTR
17	*hCV3202956*	41381748	rs242559	MAPT	5' UTR
18	*hCV7563692*	41407682	rs1800547	MAPT	intron 3
19	*hCV3202960*	41424176	rs2435214	MAPT	intron 5
20	*hCV2042903*	41424329	rs11656151	MAPT	intron 5
21	*hCV11936104*	41439239	rs1991556	MAPT	intron 7
22	hCV2560317	41461242	rs7521	Intragenic	-
23	hCV2264293	41465690	rs1078997	LOC284058	intron 12
24	hCV2560314	41472690	rs16940845	LOC284058	intron 7
25	hCVl1936132	41497167	rs2838	LOC284058	intron 5
26	hCVl5858203	41511550	rs2066899	LOC284058	intron 3
27	hCV7563831	41551932	rs1468241	KIAA1267	intron 1
28	hCV2560260	41560151	rs2016730	KIAA1267	intron 2
29	hCV338624	41604276	rs2240758	KIAA1267	exon 1*
30	hCV2598655	41615467	rs2240756	Intragenic	-
31	hCV2554114	42150418	rs142167	NSF	intron 15
32	hCV2261778	42164185	rs199448	NSF	intron 6
33	hCV2261785	42184098	rs199533	NSF	exon 8*
34	hCV2261819	42220763	rs199498	WNT3	intron 1
35	hCVl1623713	42227151	rs111769	WNT3	intron 1
36	hCVl139279	42248220	-	*Intragenic*	-
37	hCV2275279	42372240	rs758391	GOSR2	5'UTR

Position: position in bp of each SNP, *SNP names in italics* show the SNPs located on the MAPT gene and on its 3 kb-flanking region, *: Indicates a SNP that generates a synonymous change

Table 3. Comparison of the SNP allele frequencies between cases and controls

		MAF		Single SNP	2SNPs haplotype	3SNPs haplotype
	Marker	**Cases**	**controls**	**P**	**P**	**P**
1	hCV2536908	0.2371	0.2099	0.509	0.334	**0.046**
2	hCV341577	0.4454	0.4146	0.508	**0.017**	**0.044**
3	hCV9254243	0.3683	0.3736	0.954	0.206	0.084
4	hCV2032862	0.233	0.2813	0.262	**0.013**	**0.028**
5	hCV2032865	0.4187	0.4999	0.094	0.120	0.532
6	hCV2554844	0.4968	0.4938	0.890	0.949	0.978
7	hCV2541205	0.4708	0.4389	0.571	0.778	0.664
8	hCV2265271	0.1755	0.2126	0.384	0.264	0.272
9	hCV2544843	0.4495	0.3671	0.109	0.153	0.360
10	hCV2257689	0.459	0.3671	0.074	0.288	0.470
11	hCV2544830	0.446	0.4631	0.766	0.736	0.610
12	hCV2257669	0.1837	0.2049	0.643	0.859	0.702
13	*hCV7 450857*	0.1887	0.235	0.288	0.287	0.431
14	*hCV3202946*	0.1347	0.1357	0.930	0.763	0.348
15	*hCV3202949*	0.4547	0.4368	0.690	0.130	0.347
16	*hCV1016016*	0.383	0.3536	0.597	0.550	0.650
17	*hCV3202956*	0.1863	0.2346	0.261	0.368	0.336
18	*hCV7563692*	0.1808	0.2135	0.443	0.450	0.411
19	*hCV3202960*	0.1695	0.1446	0.463	0.461	0.245
20	*hCV2042903*	0.2682	0.3078	0.396	0.189	0.414
21	*hCV11936104*	0.1734	0.2376	0.131	0.158	0.152
22	hCV2560317	0.4803	0.4819	0.946	0.784	0.982
23	hCV2264293	0.194	0.2194	0.497	0.669	0.315
24	hCV2560314	0.4335	0.4405	0.867	0.507	0.482
25	hCVl1936132	0.1745	0.2074	0.442	0.629	0.180
26	hCVl5858203	0.1912	0.2188	0.544	0.650	0.366
27	hCV7563831	0.1776	0.2084	0.467	0.281	0.252
28	hCV2560260	0.1659	0.125	0.243	0.338	0.368
29	hCV338624	0.1565	0.1125	0.193	0.208	0.152
30	hCV2598655	0.1543	0.1325	0.510	0.450	0.162
31	hCV2554114	0.2083	0.2013	0.801	0.149	0.596
32	hCV2261778	0.1703	0.2013	0.479	0.478	0.298
33	hCV2261785	0.1733	0.2049	0.469	0.566	0.520
34	hCV2261819	0.174	0.2063	0.411	0.414	0.398
35	hCVl1623713	0.3827	0.45	0.191	0.594	0.372
36	hCVl139279	0.2389	0.1976	0.329	0.790	-
37	hCV2275279	0.4759	0.475	0.998	-	-

MAF: Minor allele frequency, p: nominal p values from CC-QLS test, (X) **SNPs haplotype:** haplotype-wise case-control comparison by CC-QLS. Bold P values indicate nominally significant CC-QLS p values.

genotyped using 15 flourescently labelled microsatellite markers from chromosome 17 which were, on average, 10 centimorgans apart (ABI Prism Linkage Mapping Set MD-10 Versions 2.5, Applied Biosystems, Foster City, CA, USA). The genotyping experiments were done following manufacturer instructions (Applied Biosystems, Foster City, CA).

Mutation Screening

Mutations in the APP, PSEN-1 and PSEN-2 genes were previously excluded [32]. For mutation screening, of the GRN gene, sequences were obtained from the UCSC database (http://genome.ucsc.edu/) assembly March 2006. A touchdown PCR was designed to amplify all the amplicons. Direct sequencing of both strands was performed using Big Dye Terminator V 3.0 chemistry (Applied Biosystems). Fragments were loaded on an ABI 3100 automated sequencer and analysed with DNA Sequencing Analysis V 3.7 and SeqScape V 2.5 packages (Applied Biosystems). Information on amplicons, primers, and size of fragments is available on request.

Statistical Analyses

Genotyping errors leading to Mendelian inconsistencies were screened using Mega2 [35] and PedCheck [36]. To identify unlikely double recombination events on the same chromosome we used the Merlin package [37]. We used the exact test implemented in HAPLOVIEW [38] to examine whether genotype and allele proportions were in Hardy-Weinberg equilibrium. Only those SNPs that had genotype data available for more than 90% of all samples, and that the genotype proportions were in Hardy-Weinberg equilibrium in controls ($p>0.05$) were selected for the analysis. The haplotype block structure of the MAPT region was determined using HAPLOVIEW. Linkage disequilibrium (LD) blocks were defined using the Gabriel method [39]. The SNP hCV7563692 which is in LD ($r2>0.96$) with the H1 haplotype tagging SNP [40], was used to infer the H2/H1 haplotype. The Hlc haplotype, which has been recently associated with AD [27], was inferred by the combination of the hCV1016016 and hCV2560317 SNPs. In order to test differences in the extent of LD between cases and controls, we applied the LD contrast test [41]. Briefly, this test provides a comparison of pair wise matrices of LD between cases and controls. Haplotypes were estimated for all SNPs and sliding windows of two and three adjacent SNPs using the program PHASE 2.0 [42, 43].

The Quasi-Likelihood Score (QLS) test of the CC-QLS package [44] was used for the association analysis to correct for relationships between patients. For the CC-QLS test, kinship and inbreeding coefficients were calculated using our genealogy

database. For those individuals whose genealogy was not available, the average kinship and inbreeding coefficients were used. False-positive linkage peaks may result from linkage disequilibrium among tightly linked SNPs [45] therefore, we selected tagging SNPs for the linkage analysis which showed a pair wise D' value of less than 0.4. Linkage analysis was conducted using SimWalk2 V.2.91 [46-48]. Parametric linkage analysis was performed under a dominant model. Age dependent penetrance was estimated based on population prevalence [49]. The disease gene frequency was set to one percent. Allelic frequencies for microsatellite markers were estimated from unrelated controls.

Meta Analysis

We performed an internet search through the Alzheimer Research Forum (ARF) web page (http://www.alzforum.org/) [50] for all studies assessing the association of the MAPT haplotypes and the risk of AD. From this list we selected all papers studying the association between the H1/H2 haplotype of the MAPT gene and AD in Caucasian populations. In our meta-analysis we used the original papers to derive the risk for AD stratifying by APOE*4 carrier status. Four papers that did not provide information on APOE genotype were excluded [20, 51-53]. We excluded two more papers that did not study Caucasian populations [18, 54]. Finally, we used 8 studies in our analysis [17, 19, 21-23, 25, 27, 55]. The meta-analysis was carried out using the Review Manager V 4.2 package [56].

Results

Basic characteristics of cases and controls are shown in Table 1. There were significantly more females among the AD patients compared to controls (p=0.0002) and the average kinship coefficient was significantly higher in patient pairs than in control pairs (P<0.0001). Also, the APOE*4 frequency was significantly increased in cases p=0.0001.

Out of the total number of SNPs genotyped (n=43), six had a failure rate over 10%. The genotypes of the remaining SNPs were in Hardy-Weinberg equilibrium (p > 0.05). The resulting 37 SNPs were located between the chromosomal positions 40526680 bp (hCV2536908) and 42372240 bp (hCV2275279), based on contig NT 010783.14, build 36.1 of NCBI SNP database http://www.ncbi.nlm.nih.gov/projects/SNP/. Out of the 37 SNPs included in the analyses, 22 were distributed in eight confirmed genes, 10 in putative genes and five were on intragenic segments (Table 2).

Figure 1 a. LD of 37 SNPs in the MAPT region for AD cases (The MAPT gene is flanked by SNPs hCV3202946 and hCV2560317 solid box). Dark squares represent high LD values between SNPs while the white ones low. The numbers inside the squares indicate the SNP pair wise D' values (in empty squares LD=1). Light gray squares show SNPs with high D' values but a low measure of confidence in this value

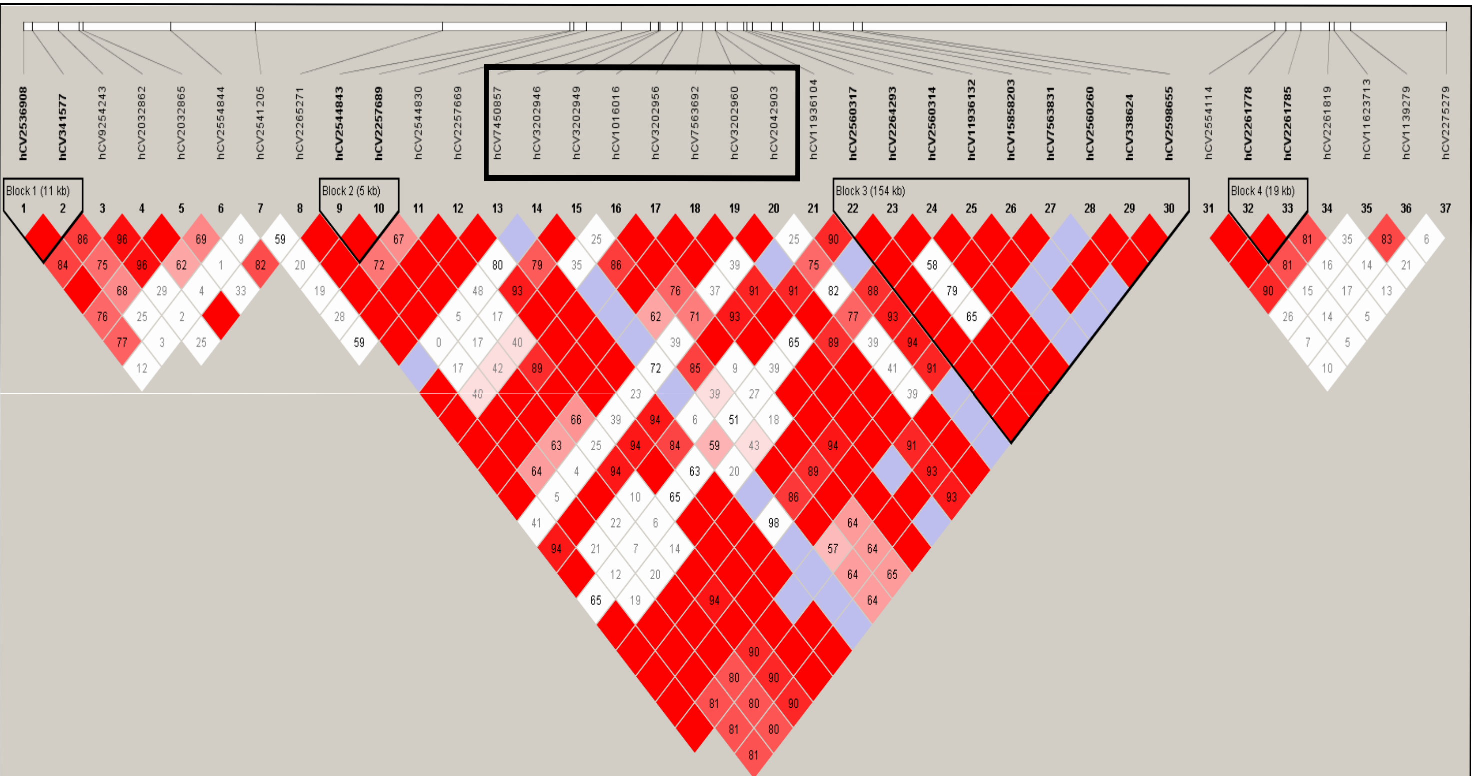
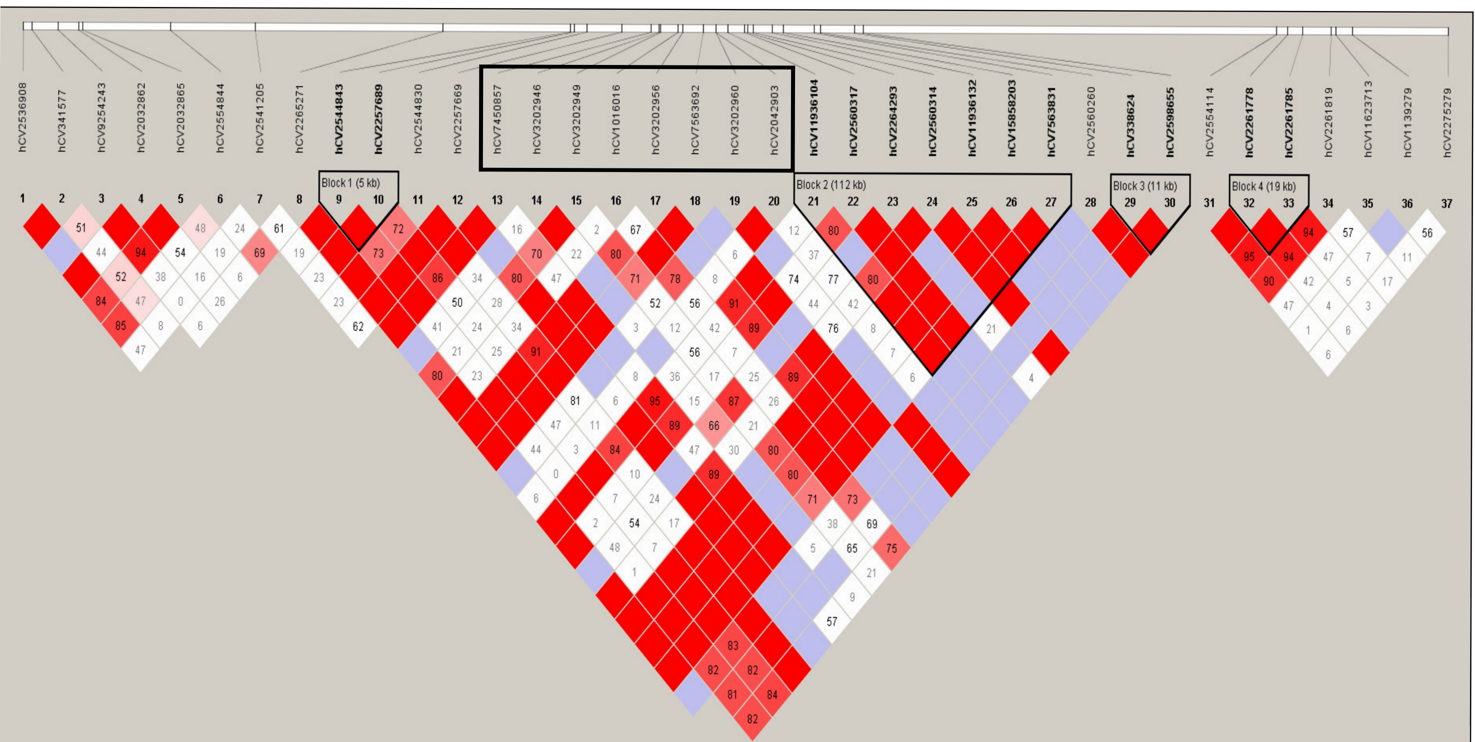


Figure 1 b. LD of 37 SNPs in the MAPT region for controls (The MAPT gene is flanked by SNPs hCV3202946 and hCV2560317 solid box). Dark squares represent high LD values between SNPs while the white ones low. The numbers inside the squares indicate the SNP pair wise D' values (in empty squares LD=1). Light gray squares show SNPs with high D' values but a low measure of confidence in this value



The LD structure of all the SNPs in cases and controls is shown in Figure 1. The cases (Figure 1a) showed a small LD block 722kb upstream the MAPT gene. This block was not detected in the control sample (Figure 1b). These two SNPs are located in the N-myristoyltransferase 1 (NMT1) gene. SNP hCV2536908 (rs7219596) is a synonymous C to T substitution at codon 163 and SNP hCV341577 (rs1053733) is a G to A substitution at the 3' untranslated region of the NMT1 gene. The NMT1 protein is involved in protein myristoylation, proliferation, and apoptosis [57]. We compared the LD between cases and controls using the LD contrast test and found a statistically significant difference between the two groups (p=0.03) for this region. The patient group showed a larger LD block than controls spanning 154kb between SNPs hCV2560317 to hCV2598655. However, the difference in the length of the block was not statistically significant (p=0.20). We also tested the extent of LD in the region of the MAPT gene (between SNPs hCV3202946 and hCV11936104) and found no significant difference between cases and controls (p=0.70).

Linkage analysis of the microsatellite markers only, showed a positive LOD score of 1.35 for the MAPT region. After inclusion of the selected tagging SNPs (7), the LOD score between SNPs hCV341577 (722kb upstream the MAPT gene) and hCV2275279 dropped below –2, excluding linkage of AD to this region. LOD scores corresponding to the flanking microsatellite markers of the MAPT region were –1.78 for marker D17S798 and –2.27 for marker D17S1868 (Figure 2).

We did not find significant evidence for association with AD for any of the studied SNPs (all nominal p values > 0.05) in the single SNP analysis (Table 3). Using haplotype analysis with two SNPs sliding windows, we detected nominal p values < 0.05 for the hCV341577 - hCV9254243 haplotype (p= 0.02) and the hCV2032862 - hCV2032865 haplotype (p= 0.01). The 3 SNPs sliding window analysis showed nominal p values < 0.05 for the hCV2536908 - hCV2554844 haplotype (first 6 SNPs). These significant p-values did not sustain when adjusting for multiple testing. No significant evidence for association was found for the MAPT gene itself or its transcript region (SNPs indicated in italics, Table 3). Also, when stratifying for APOE*4, the MAPT region was not associated to AD (data not shown). When testing the H1/H2 haplotype in our population, we found that the H1 haplotype frequencies were 83% for cases and 79% for controls (p=0.61). The frequencies for the Hlc haplotype were 31% in cases and 30% in controls (p=0.84).

Since we found nominal evidence for association 722 kb upstream of the MATP gene, which is 716 kb downstream from GRN, we sequenced all the exons and

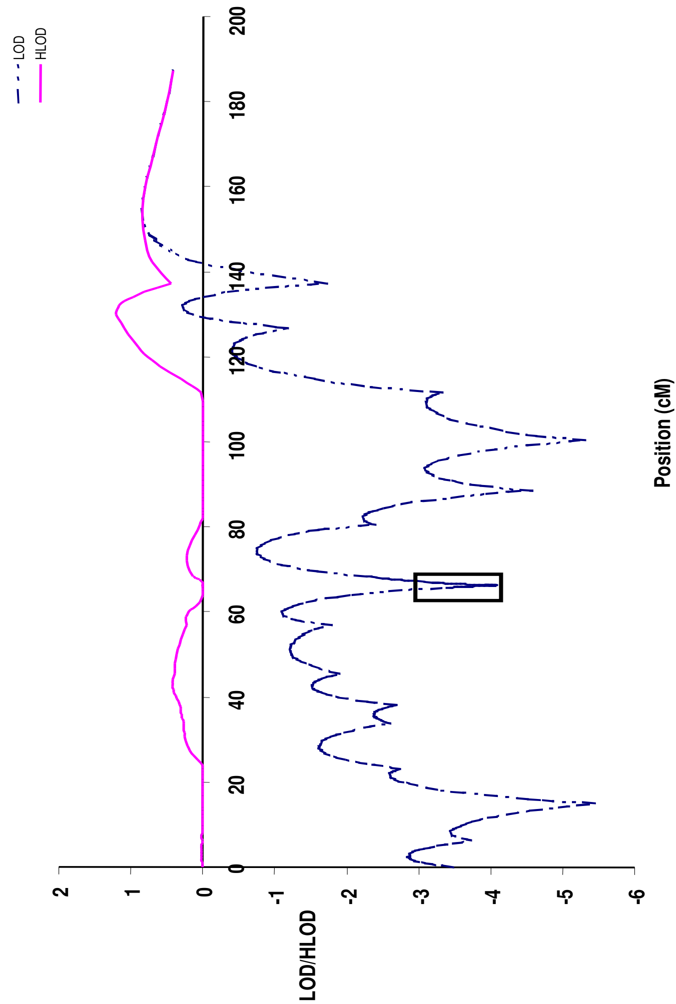


Figure 2. Linkage analysis of AD patients on chromosome 17. Linkage analysis was performed using 15 microsatellite markers through out the complete chromosome and 7 tagging SNPs and covering the MAPT region (boxed LOD curve). These SNPs were selected using a D' value for each SNP-SNP pair of less than 0.4.

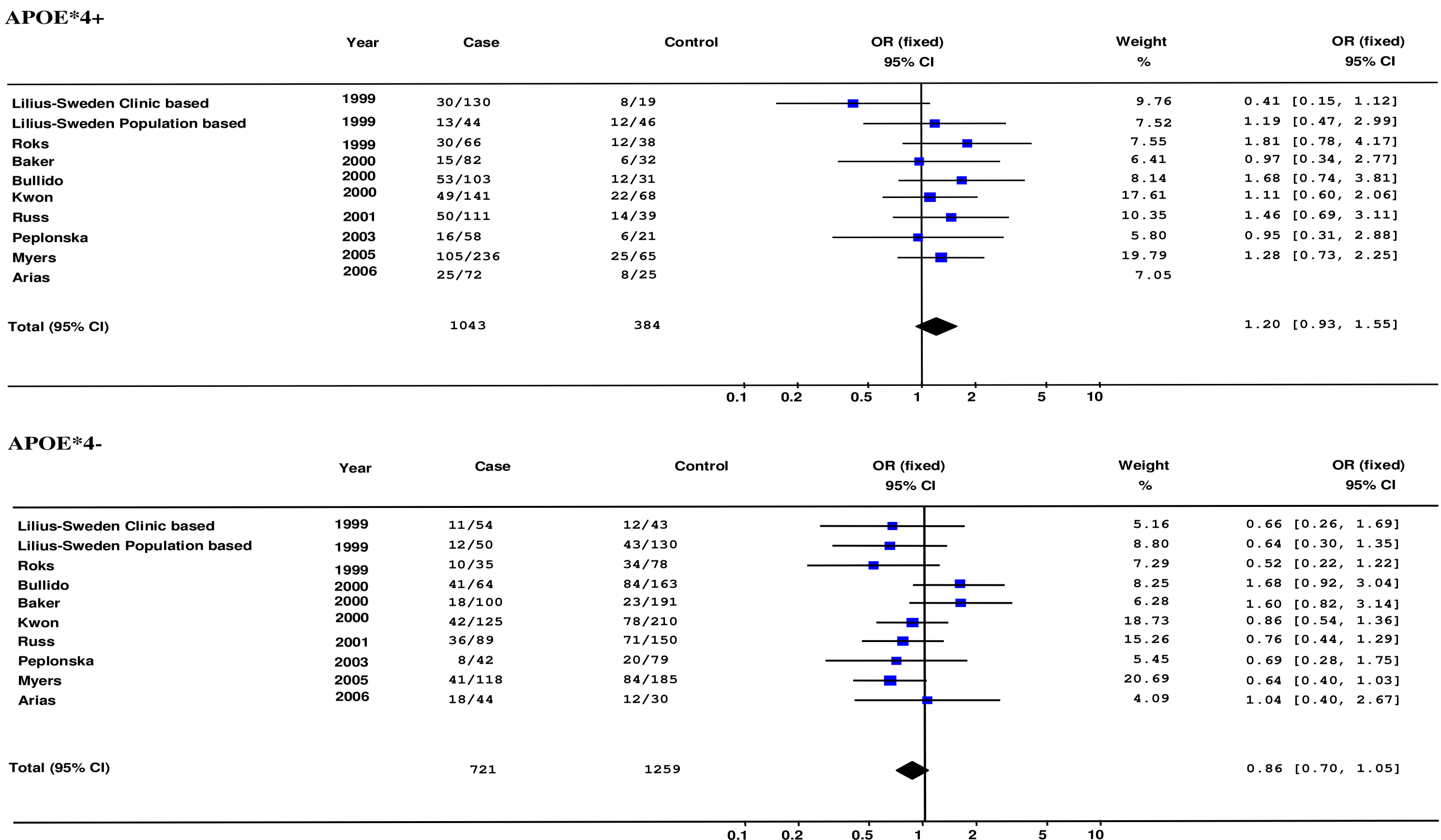
splice sites of this gene in 17 patients ascertained from the GRIP program who were from families contributing to the LOD score. No mutations on the GRN gene were found in these patients.

Finally, we performed a meta analysis of the H1/H2 haplotypes by APOE*4. The studies are shown in Table 4. Overall, 12 studies have addressed the role of MAPT in AD. When analysing all studies, no significant association of the MAPT H1/H2 haplotype and AD was found (OR=0.95, 95%CI 0.86-1.04, source: www.alzforum.org) [50]. Our meta analysis showed that the frequency of the H2 haplotype in those

Table 4. Distribution of the MAPT HI and H2 haplotypes in the studies included in the meta analysis by APOE*4

			TAU haplotypes-Number of Cases/Controls (%)			
Study	**Population**	**Number Cases/Controls**	**H1H1**	**H1H2**	**H2H2**	**H2 frequency**
APOE*4+						
Lilius, 1999	Sweden Clinical	130/19	100(15.2) /1 1(4.2)	28(8) / 8(7.8)	2(5.7) / 0(0)	12.3/21.1
Lilius, 1999	Sweden Population based	44/46	31(4.7) / 34(13.1)	13(3.7) /11(10.7)	0(0) /I (4.5)	14.8/14.1
Roks, 1999	Netherlands	66/38	36(5.5) / 26(10)	27(7.7) / 10(9.7)	3(8.6) / 2(9.1)	25.0/18.4
Baker, 2000	Finland	82/32	67(10.2) / 26(10)	14(4) / 6(5.8)	1 (2.9) / 0(0)	9.8/9.4
Bullido, 2000	Spain	103/31	50(7.6) /1 9(7.3)	47(13.4) / 6(5.8)	6(17. 1)/ 6(27.3)	28.6/29.0
Kwon, 2000	USA Washington	141/68	92(14) / 46(17.8)	43(12.3) / 17(16.5)	6(17.1) / 5(22.7)	19.5/19.9
Russ, 2001	United Kingdom	111/39	61(9.3) / 25(9.7)	47(1 3. 4) /1 0(9.7)	3(8.6) / 4(1 8.2)	23.9/23.1
Peplonska, 2003	Poland	58/21	42(6.4) /1 5(5.8)	15(4.3) / 6(5. 8)	1 (2.9) / 0(0)	14.7/14.3
Myers, 2005	USA+UK	236/65	131(19.9) / 40(15.4)	93(26.5) / 22(21.4)	12(34.3) / 3(13.6)	24.8/21.5
Arias, 2006	Netherlands	72/25	47(7.2) /1 7(6.6)	24(6.8) / 7(6.8)	1(2.9) /I (4.5)	18.1/18.0
Total		**1043/384**	**657/259**	**351/103**	**35/22**	**20.2/19.1**
APOE*4-						
Lilius, 1999	Sweden Clinical	42/43	34(7.2) / 3 1(3. 8)	9(4.2) / 10(2.4)	2(8) / 2(3. 8)	14.4/16.3
Lilius, 1999	Sweden Population based	50/130	38(8) / 87(10.5)	1 1(5.2) / 38(9 .2)	1 (4) / 5(9.6)	13.0/18.5
Roks, 1999	Netherlands	35/78	25(5.3) / 44(5.3)	9(4.2) / 29(7)	1 (4) / 5(9.6)	15.7/25.0
Baker, 2000	Finland	100/191	82(17.3) / 194(23.5)	1 8(8.5) / 28(6.8)	0(0) /I (1.9)	9.0/6.7
Bullido, 2000	Spain	64/163	23(4.8) / 79(9.6)	36(17) / 75(18.2)	5(20) / 9(17.3)	35.9/28.5
Kwon, 2000	USA Washington	125/210	83(17.5) / 132(16)	39(18.4) / 68(16.5)	3(12) / 10(19.2)	18.0/21.0
Russ, 2001	United Kingdom	89/150	53(11. 2) / 79(9.6)	33(15.6) / 63(15.3)	3(12) / 8(15.4)	21.9/26.3
Peplonska, 2003	Poland	42/79	34(7.2) / 59(7.1)	8(3.8) / 17(4.1)	0(0) / 3(5.8)	9.5/14.6
Myers, 2005	USA+UK	118/187	77(16.2) / 101(12.2)	31(14.6) / 77(18.6)	10(40) / 7(13.5)	21.6/24.6
Arias, 2006	Netherlands	44/30	26(5.5) / 20(2.4)	1 8(8.5) / 8(1. 9)	0(0) / 2(3. 8)	20.5/20.0
Total		**712/1261**	**475/826**	**212/413**	**25/52**	**18.4/20.5**

Figure 3: Weighted Odds Ratios and 95% confidence intervals for the risk of AD for carriers of the H2 haplotype of the MAPT gene, in APOE*4 carriers (above) and non-carriers (below).



with APOE*4 was slightly increased in the patient group (p=0.11) (Table 4 above), resulting in an odds ratio of 1.20 (95% CI = 0.93-1.55) as shown in Figure 3. In the APOE*4 non-carriers (Table 4 below) the frequency of the H2 haplotype was higher in controls than in cases (p=0.23), resulting in an odds ratio of 0.86 (95% CI = 0.70-1.05). There was no significant evidence for interstudy heterogeneity in either of the groups (p=0.67 APOE*4 carriers, p=0.19 APOE*4 non-carriers).

Discussion

In our study including 37 SNPs we did not find evidence for association between AD and MAPT using single SNP and haplotype analysis. Moreover, linkage analysis including 7 tagging SNPs in MAPT excluded linkage. In addition, our meta analysis, stratified by the APOE*4, did not yield significant evidence for association between the H2 haplotype of MAPT and AD, making it unlikely that MAPT itself or in interaction with APOE is involved in the pathogenesis of AD. In the upstream region of the MAPT gene, several SNPs showed nominally significant evidence for association in the haplotype analyses (nominal p=0.01). The same was seen for the haplotype hCV2032862-hCV2032865 (nominal p = 0.01). SNPs hCV2536908 and hCV341577 are located 722 kb upstream from the MAPT gene within the NMT1 gene. This region showed more LD in cases than in controls. NMT1 is located 716 kb downstream from the GRN gene, which was recently associated with frontotemporal dementia [28, 29]. We sequenced the GRN gene in 17 AD patients from families suggesting linkage to this region but did not find any mutation. When adjusting our association analyses for multiple testing, none of the association of the haplotypes upstream of MAPT remained significant.

Previous studies, including one study on the MAPT H1 haplotype and two studies of newly identified SNPs, found evidence for interaction between MAPT polymorphisms and APOE*4 allele [17, 18, 25]. Our study showed no evidence of association with any of our SNPs when considering this interaction. An increase of risk of AD was found for carriers of the Hlc haplotype in those without the APOE*4 allele [27]. We were not able to replicate this finding in our study in the isolated population. In our meta analysis of 1755 patients and 1645 controls, a weak association in carriers of the H2 haplotype to AD was found in APOE*4 carriers but the odds ratio was non-significantly increased (OR = 1.20, 0.93-1.55, p=0.22) compared to H1/H1 carriers. Although one may argue that even this meta-analysis lacks statistical power to detect an odds ratio of 1.2, it is also important to realise that studies of interaction are susceptible to false positive and false negative findings. The absence of an overall effect and the opposite trend observed in those with and without APOE*4 suggests the association in subgroups is most likely explained

by random fluctuation.

There is no doubt that the tau protein plays a pivotal role in the development of Alzheimer's disease [16, 58]. However, our series of patients from the isolated population did not show evidence for association, nor linkage to AD. Our meta-analysis of all studies conducted to date also failed to show an effect of MAPT on AD. Our study describes some increase in linkage disequilibrium upstream of MAPT, which was not explained by GRN or MAPT mutations. The association did not sustain when adjusting for multiple testing. None of the SNPs studied in the region showed strong association to AD nor was there evidence for linkage to this region making the presence of an AD gene very unlikely.

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Chapter 2.3

A genome scan in a family with Alzheimer’s disease

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by global cognitive decline. We conducted a genome screen in a family including 9 affected individuals with AD using 420 markers. The data were analysed with parametric linkage analysis. The highest multipoint LOD scores were 1.15 for markers D1S450, D7S2465 and D19S571. The latter LOD score was most likely explained by the presence of APOE*4 in the patients. A two locus linkage analyses for the chromosomes 1 and 7 regions yielded a parametric LOD score of 2.92. The most promising candidate genes in the two regions are CASP9, TNFRSF1B and MTHFR on chromosome 1 and CDK5 and NOS3 on chromosome 7 which were selected for sequencing. No sequence variations were observed in the coding regions of the CASP9 and CDK5 genes but we found that allele A of the SNP rs9278 in the 3'UTR of the CDK5 gene was present in all affected individuals. The CDK5 gene may explain the linkage of AD to chromosome 7 in this family. However, we cannot exclude epistatic effects of this or other genes on chromosome 7 with a gene on chromosome 1 or with APOE.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by global cognitive decline involving memory, orientation, judgment, and reasoning [1]. Dominant mutations leading to the early onset form of AD are present in the Amyloid Precursor Protein (APP) gene [2-7], Presenilin 1 (PSEN-1) [3-7] and Presenilin 2 (PSEN-2) [8-10] genes. Although these genes play an important role in families affected with early onset AD, mutations are rare in the general population and explain a small number of cases [11, 12]. The e4 allele of APOE gene (APOE*4) has been associated with early and late onset AD [13, 14].

The major pathway involved in disease development is the amyloid beta cascade [15]. Aggregation of the Amyloid ß (Aß) protein is one of the key features of AD [16]. The second key pathway involved in AD pathology is the microtubule associated protein tau (MAPT) pathway [17]. In AD brains, the tau protein is hyperphosphorylated, truncated, and aggregated into neurofibrillary tangles (NFTs).

The genes identified to date explain less than 20% of all AD patients [12]. It has been proven difficult to identify new AD genes [18]. In the past decade, various genes have been implicated in AD by either association or linkage. However, few of these findings have been confirmed. Up to September 2006, the AlzGene database (http://www.alzforum.org) has recorded 360 genes studied in Alzheimer's disease [19]. Convincing evidence for linkage of AD has been reported on chromosomes 9, 10, and 12 [20-27] but until now there is disagreement on the genes underlying the linkage results. The reason may be related to the fact that AD has a complex aetiology. Moreover, the disease may be the consequence of interactions between genes, in particular in small families with multiple affected relatives in which the disease only clusters in 2 or 3 generations. This has been suggested for other complex disorders such as breast cancer [49].

There are still families segregating AD for which the gene responsible has not been localized. Given the strong clustering of this disease, a single or a limited number major genes are expected to explain the disease in these families. In this study we used a multilocus model to evaluate the evidence for epistatic effects in a single family segregating AD.

Materials and Methods

Family Description

Family 3355 includes 31 relatives in three generations. We identified nine individuals with probable AD and were able to collect detailed clinical data on six of them. The clinical diagnosis of AD was based on clinical history, reviewing all medical records, neurological examination, neuropsychological tests, and neuroimaging data. Based on this information, a second physician verified the clinical diagnosis of the treating physician. Clinical diagnoses of AD were performed by a neurologist according to the NINCDS–ADRDA criteria [28]. The age at onset for the affected individuals ranged from 56 to 70 years with a mean at onset of 65 years. Blood samples were obtained for 24 individuals. All members of the family gave informed consent prior to participation and the medical ethics committee of the Erasmus MC approved this study.

Genetic analyses

DNA was extracted using the phenol-chloroform isolation method. We performed a genome screen of AD in this family using 420 fluorescent microsatellite markers (Applied Biosystems linkage mapping set version 2, Foster City, CA). The APP, PSEN1, and PSEN2 genes were screened for mutations by direct sequencing. APOE genotyping was done using TaqMan allelic discrimination technology. The TaqMan assay utilized 5 nanograms of genomic DNA and 2 microliter reaction volumes. The amplification and extension protocol was as follows: an initial activation step of 10 min at 95 deg. preceded 40 cycles of denaturation at 95 deg. for 15 s. and annealing and extension at 50 deg. for 60 s. Allele-specific fluorescence was then analyzed on an ABI Prism 7900HT Sequence Detection System with SDS v 2.1 (Applied Biosystems, Foster City, CA).

Linkage Analyses

Parametric linkage analysis and haplotype reconstruction were done using SimWalk2 v 2.91 [29-31]. Disease model was set to dominant; age dependent penetrances were computed based on population prevalence [32]. Disease allele frequency was set to 0.01 and marker allele frequencies were estimated using all samples. APOE was included as a marker in the linkage analyses. Parametric two-locus linkage analysis was performed with GeneHunter–two locus parallel program (GHT) [33, 34].

Table 1. Clinical features of patients

ID	AAO (years)	APOE*4 carrier	Disease Duration (yrs)
1	N/A	N/A	N/A
4	65	+	14
5	70	+	4
6	65	+	12
10	59	+	19
12	67	+	9
13	70	+	5
14	56	+	18
16	65	+	8

AAO = Age At onset of disease, N/A = Not Available

Disease model was set to dominant, with penetrance 0.99 and phenocopy rate 0.001. Because of the computational limitations of GHT, only the founders and affected persons of the second generation were used. Haplotypes of the two chromosomal regions for these individuals were transformed into genotypes as single markers, and used in the LOD score calculation by GHT.

Candidate gene selection by text mining

We developed a computational biology tool to prioritise AD candidate gene selection for sequence analysis. Our tool is based on the analysis of the Entrez-Genes database from the NCBI website (http://www.ncbi.nlm.nih.gov/). For each gene included in the Entrez-Genes database, the list of papers that is used as Gene References into Function (GeneRIF) is retrieved. The title, abstract, and MeSH headings of these papers are concatenated into one long text. This text is then indexed using the MeSH as well as the GO thesauri. Therefore, for each gene that contains reference literature in the database, a fingerprint pattern is constructed and stored in a database for future use [35]. A list of query concepts, in this case genes, needs to be input into the program. The query list selected was the list of genes described in the AD pathway included in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.ad.jp/kegg/). The query genes were: A2M, LRP, LPL, APOE, APP, APBB1, APPBP1, BAC, GAPD, CASP3, PEN2, PSEN1, PSEN2, NCSTN, APH-1, IDE, MME, GSK3B, MAPT, CASP7 and SNCA [36, 37]. We matched our input list with all genes known in the NCBI database (http://www.ncbi.nlm.nih.gov/) using a matching algorithm based on a conceptual similarity score. Genes are then ranked on the conceptual similarity score (between zero and one), which is assigned to each gene. This similarity score is basically a measurement of how concordant the fingerprint patterns of the genes in the NCBI database are compared to input list genes. The output is a list of genes ranked from the most to the less similar. From this output list, we then select candidate genes by the similarity score and their chromosomal location.

Mutation Screening

For mutation screening, the gene sequences were obtained from the UCSC database (http://genome.ucsc.edu/) assembly March 2006 [38, 39]. To facilitate amplification, the exons were divided in amplicons when the final sequenced product exceeded 600 bp. A touchdown PCR was designed to amplify all the segments. PCR cycle conditions were: 7:30 min at 94°C; 10 cycles with 30 s denaturation at 94°C, 30 s annealing temperatures ranging from 68°C to 59°C (-1°C per cycle) and 60 s extension at 72°C. Then 25 cycles with 30 s denaturation at 94°C, 30 s at 58°C

annealing temperature, and 60 s extension at 72°C. Final extension was set at 72°C for 5 min. Primers were designed using Primer3 software [40]. Direct sequencing of both strands was performed using Big Dye Terminator V 3.0 chemistry (Applied Biosystems). Fragments were loaded on an ABI 3100 automated sequencer and analyzed with DNA Sequencing Analysis V 3.7 and SeqScape V 2.5 packages (Applied Biosystems). Information on amplicons, primers, and size of fragments is available on request.

Results

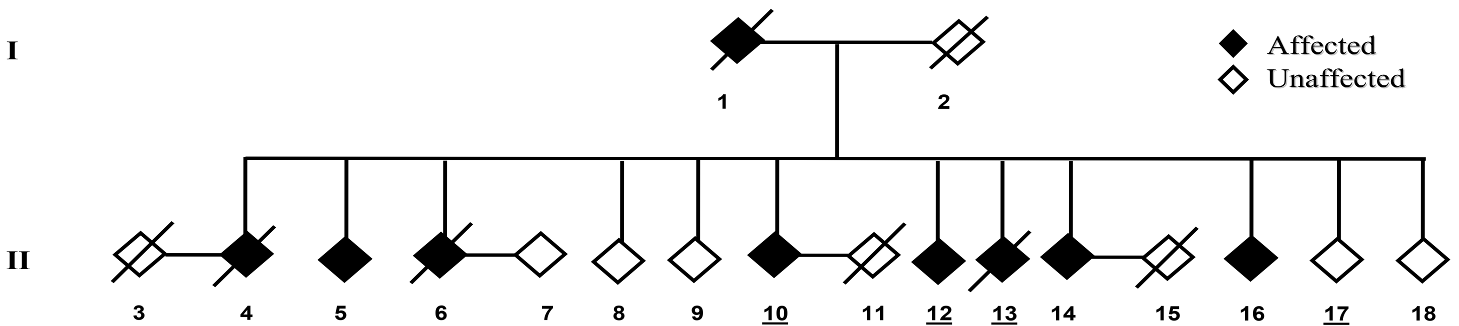
Study Family

Figure 1 shows the pedigree of the study family and Table 1 shows the clinical characteristics of the patients. The numbers used in figure 1 refer to the same individuals in Table 1. Mean age at onset of disease was 65 years, ranging from 56 to 70. All of the genotyped patients were APOE*4 carriers and seven out of nine patients were women. Mutations on APP, PSEN1, and PSEN2 were excluded by direct sequencing. In Figure 1 only the first two generations are shown, the third one is omitted for confidentiality of the participants.

Linkage Analyses

The parametric linkage analysis yielded 3 regions through out the whole genome with a LOD score greater than 1.0. Our analysis yielded a LOD score of 1.15 for marker D1S450, 1.15 for marker D7S2465 and 1.15 for marker D19S902. Haplotype analyses showed a ~17 cM shared region in chromosome 1 (including markers D1S450, D1S2667 and D1S2697), a ~25 cM shared region in chromosome 7 (including markers D7S661, D7S798 and D7S2465), and a ~20 cM shared region in chromosome 19 (APOE, D19S902, D19S571 and D19S418). Since the APOE gene is included in this region, the linkage signal on chromosome 19 is most likely attributed to the effect of this gene. We next studied whether there is an epistatic effect of the loci on chromosomes 1 and 7. The two locus linkage analysis yielded a parametric LOD score of 2.92 for the combination of chromosomes 1 and 7. The co-segregation pattern of the haplotypes in the affected individuals of the first generation can be seen in Figure 2. Affected family members share all the chromosomal regions, while the unaffected siblings miss some (9) or all co-segregating haplotypes (17 and 18). Individual 8 is not affected but shares the same haplotypes as the affected sibs.

Figure 1. Pedigree of the study family. Underlined numbers show individuals initially sequenced for the two candidate genes. The third generation is omitted



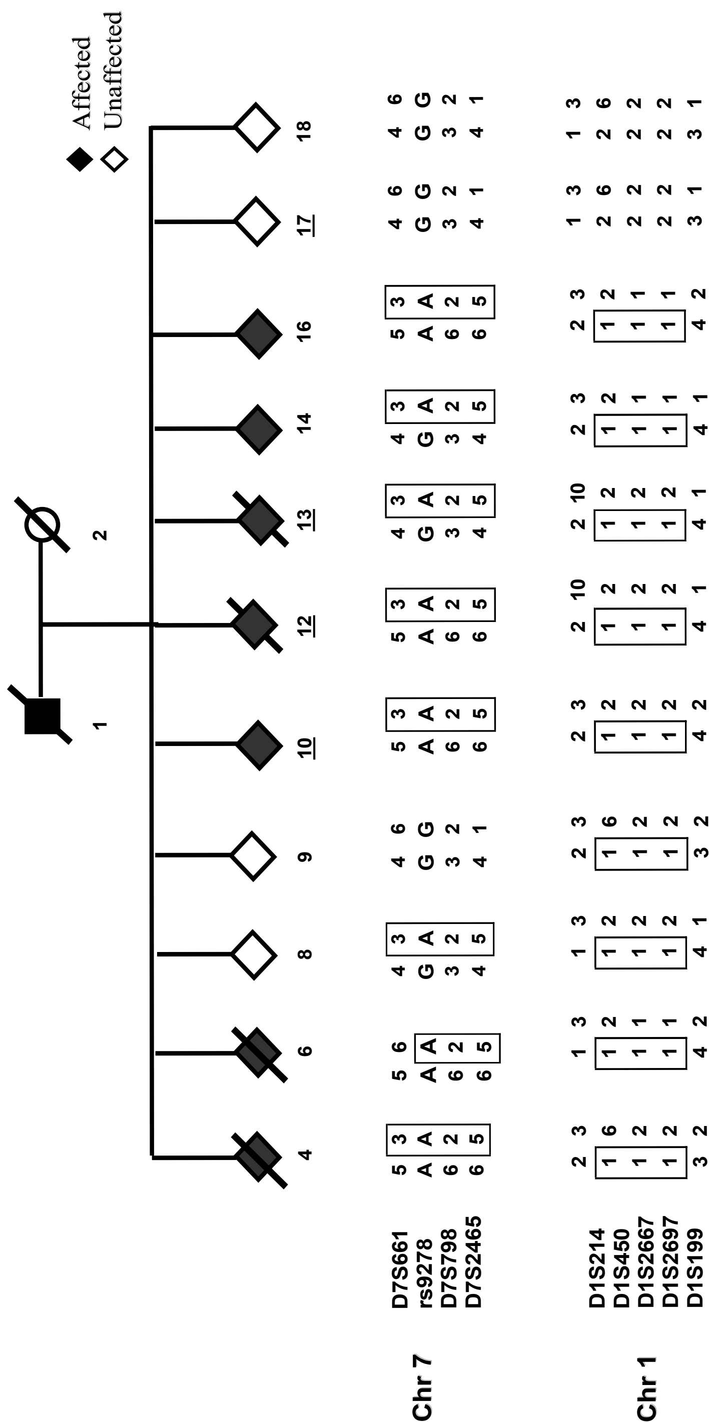


Figure 2. Haplotype co-segregation between members of the second generation of the study family for the two linked regions. Boxed haplotypes show the chromosomal regions segregating with AD. Underlined numbers show individuals initially sequenced for the two candidate genes

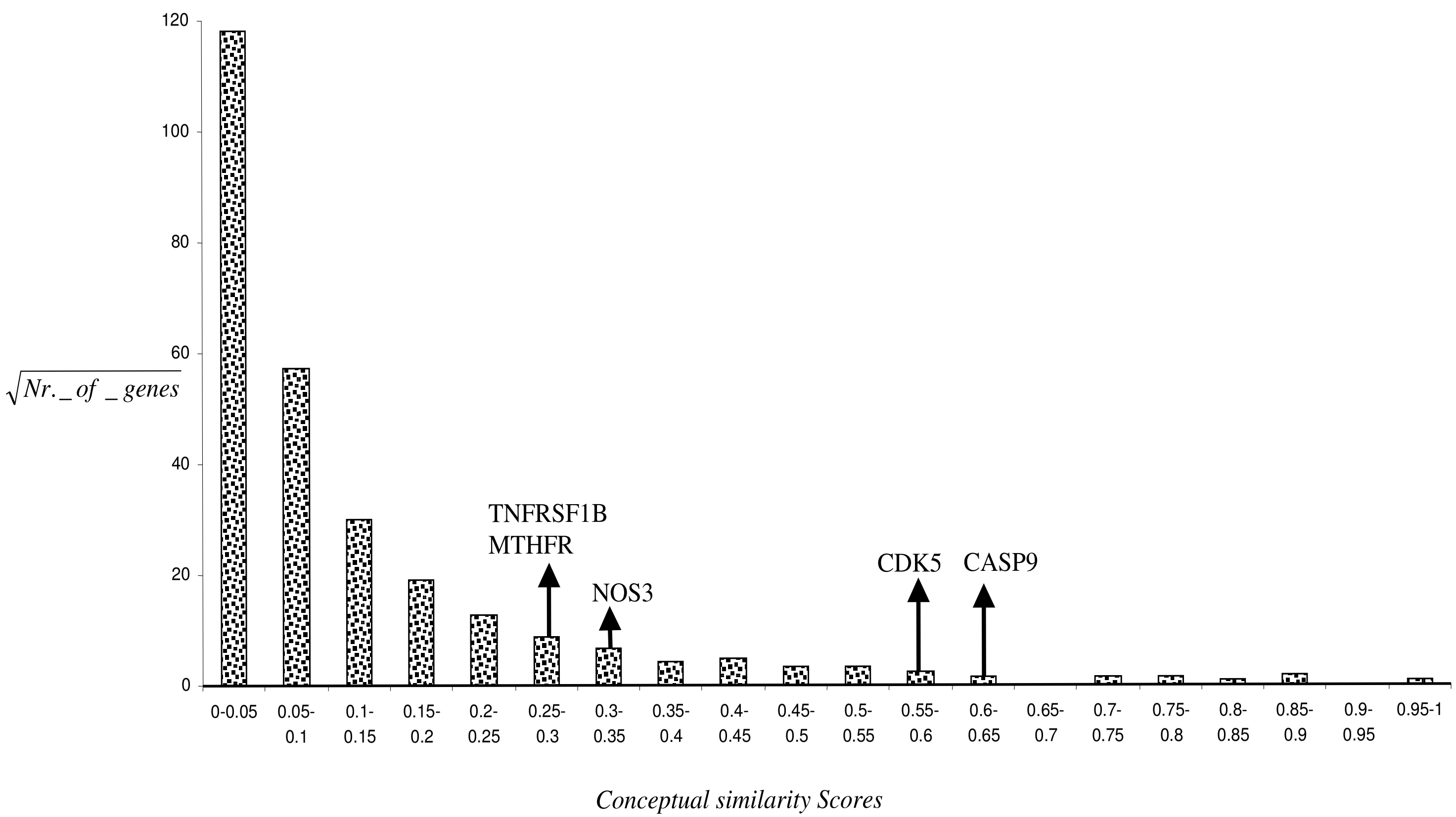
Candidate gene selection by concept profiling

There were 18913 genes with conceptual similarity scores ranging from 0 to 1. The distribution of the similarity indexes is plotted in figure 3. From this figure, we can see that 99.3% of the genes have a similarity index less than 0.25. Along with the increase of the similarity index, the number of genes decreased sharply and a plateau was encountered around 0.25. Therefore, we used this similarity score as a first stringent threshold for candidate gene selection. In the locus on chromosome 1, we identified 112 genes in a 17cM region between markers D1S450 and D1S2697. 123 genes were identified between markers D7S661 and D7S2465 in chromosome 7. We ranked all these genes according to their similarity score and found that the genes in the locus of chromosome 1 with similarity scores above 0.25 were CASP9, TNFRSF1B and MTHFR. The genes on chromosome 7 with similarity scores above the threshold were CDK5 and NOS3. These genes have been previously associated with AD, [41-44] or apoptosis [45, 46].

Mutation Screening

Based on the results provided by the linkage analyses and the conceptual similarity scores, we selected the two top genes in the loci to perform mutation screening. We screened all the exons and plice sites of the CASP9 and CDK5 genes initially in four individuals of the study family. Individuals II-10, II-12, II-13, and, II-17 were initially selected due to the fact that they include the four haplotypes present in the family. No sequence variations were observed in the exons of the CASP9 and CDK5 genes but we found the Single Nucleotide Polymorphism (SNP) rs9278 in the 3'UTR of the CDK5 gene. This is a G>A substitution at 116 bp downstream from the stop codon in exon 12. Two of the affected individuals (II-10, II-12) selected for initial sequencing were homozygotes for this SNP and the other one was heterozygote (II-13). The unaffected sib (II-17) was homozygote for the common variant G (figure 2). We later sequenced both genes in the other members of the family and confirmed the presence of allele A of the rs9278 SNP in all the affected individuals and also in individual II-8 who is unaffected. We did not detect any variations in the CASP9 gene in any of the family memebers screened.

Figure 3. Distribution of conceptual similarity scores. The top three candidate genes in each locus are shown according to their conceptual similarity score



Discussion

In this study, we performed a whole genome scan in a family with 9 patients affected with AD. Three loci with LOD scores above 1.0 were found on chromosomes 1, 7 and 19. All patients carry the APOE*4 allele which most likely explains the linkage to chromosome 19. A further two locus linkage analysis using only affected individuals yielded a LOD score of 2.92 for the combination of the loci of chromosomes 1 and 7. The two locus model could only be studied using the first 2 generations due to computational limitations. It is expected that if all the unaffected persons in second generation are included in the analyses, the 2 locus LOD scores should be higher. We prioritised candidate gene selection using a computerized data mining strategy leading to CASP9 and CDK5 as the most promising candidate genes. The protein encoded by CASP9 is one of the proteins that activate Caspase 3, which is a key factor in the cleavage of Tau protein [46] and the Cdk5 protein is a key protein involved in Tau phosphorylation and AD [47, 48]. We did not find any mutation in the CASP9 or CDK5 coding exons but all patients shared the same allele of SNP rs9278 on the CDK5 gene. One individual who is unaffected carried the same haplotype. This may be the result of reduced penetrance of the gene responsible for AD in this family.

Five SNPs in the CDK5 gene were associated with early onset in two AD series and we found that SNP rs9278 is a tagging SNP for a haplotype that is over presented in early-onset AD patients. In that, our findings corroborate with our earlier findings reported [44]. However, it is also possible that one of the 2 regions is a false positive finding. Of note is the fact that all patients carry the APOE*4 allele suggesting that this gene may partly determine the presence of the disease in this family. The co-segregation of the chromosome 1 and 7 regions with AD may be also be explained by a synergistic interaction between two genes involved in the aetiology of AD in this family. SNP rs9278 is located in 3' UTR of the CDK5 gene. The functional role of this SNP is unknown and we could speculate that it could be in linkage disequilibrium with a functional polymorphism present elsewhere in the gene affecting, for example, exon splicing or mRNA stability. Alternatively, other genes may explain the linkage on chromosome 7. NOS3 is another obvious candidate gene for AD [42]. At chromosome 1, the TNFRSF1B and MTHFR genes are also candidates as they have been previously associated with AD [41, 43].

We developed a tool to select candidate genes for AD. CDK5 and CASP9 were identified as having the highest conceptual similarity scores followed by NOS3, TNFRSF1B and MTHFR. However, a limitation of this approach is that it makes

it impossible to identify those genes that are involved in unknown pathways of AD unless there are publications connecting the genes or proteins to those specified in the pathways.

In conclusion, our results suggest that the CDK5 gene may be implicated in AD in this family but we cannot exclude another locus. Our data are compatible with epistatic effects of genes on chromosome 1 and 7 which perhaps together with APOE*4 increase the risk of AD.

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Chapter 3

Candidate Genes Studies

Chapter 3.1

The cyclin-dependant kinase 5 gene is associated with Alzheimer's disease

Abstract

Although the role of the Cdk5 protein in Alzheimer's disease (AD) is well recognized, there have been relatively few studies investigating genetic variants in the CDK5 gene in AD. In this study we assessed the association between five previously described Single Nucleotide Polymorphisms (SNPs) in the CDK5 gene and AD by means of logistic regression and haplotype association analysis. Including all prevalent and incident AD cases, we found a significantly increased risk of AD for carriers of the GG genotype of SNP rs2069442 (OR=1.8, 95% CI 1.16-2.79, p=0.009) in those without APOE*4. When limiting the analysis to incident cases without APOE*4, carriers of the GG genotype showed a 2-fold increase of risk for AD (95%CI 1.28-3.12, p=0.02). Variations in the CDK5 gene can be described in 5 haplotype blocks. In our analysis, the haplotype tagged by the G allele of SNP rs2069442, was significantly associated to AD (p=0.05). In conclusion, our study suggests that CDK5 may be associated to AD.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by loss of neurons, cognitive impairment and dementia. The characteristic pathology of the brains of patients includes extracellular Amyloid ß (Aß) plaques aggregation and intracellular neurofibrillary tangles (NFTs) comprised of the hyperphosphorylated Tau protein.

There is growing evidence that cyclin-dependent kinase 5 (Cdk5) is a key protein involved in the pathogenesis of AD [1-3]. Cdk5 is composed of 293 amino acid residues and exhibits serine/threonine kinase activity [4, 5]. The interaction between Cdk5 and its regulatory subunits p35/p39 plays a key role in neuronal growth and migration processes [3]. Cdk5 is critical to synapse formation and synaptic plasticity, pivotal processes for learning and memory [6-8]. Deregulation of Cdk5 activity and calpain-mediated cleavage of p35 in to p25 has been observed in AD brains [9]. It was recently demonstrated that the prolonged expression of p25 results in impaired spatial learning, brain atrophy and hippocampus neuronal loss [10].

Cdk5 may also be involved in the abnormal phosphorylation of tau, contributing to the formation of NFTs [11] and may be a key protein linking NFTs and senile plaques [1, 9]. Cdk5 co-localizes with filamentous tau deposits and has increased activity in several tauopathies and AD [3, 12-14]. Deficiency in APP results in higher levels of tau phosphorylation and Cdk5 kinase activity [15]. Cdk5 is not only involved in hyperphosphorylation of tau, but also contributes to increased susceptibility to neuronal death.

Although the role of the Cdk5 protein in AD is well recognized, there have been relatively few studies investigating the role of genetic variants in the CDK5 gene in AD [16]. This gene is located in chromosome 7q36, is a 4089 bp long gene and includes 13 exons with only one transcript described (www.ensembl.org). Our group sequenced the CDK5 gene and its regulatory elements and studied five tagging Single Nucleotide Polymorphisms (SNPs) in a series of 85 early onset AD patients from Sweden and 104 early onset patients from The Netherlands [17]. These relatively small series suggested an association between CDK5 and AD. The aim of this study is to assess the five previously described SNPs in the CDK5 gene in the Rotterdam Study, a population based follow up study in which 7983 individuals have been followed for 12 years [18].

Materials and Methods

Patient ascertainment

The study is part of the Rotterdam Study, a Dutch population-based cohort study that investigates the occurrence and determinants of diseases in the elderly [18]. Baseline examinations, including a detailed questionnaire, physical examination, and blood collection, were conducted between 1990 and 1993. The medical ethics committee of Erasmus Medical Centre approved the study protocol.

The diagnosis of dementia was made following a stringent three-step protocol [19, 20]. Briefly, all subjects were screened at follow-up visits (1997-1999, 1999-2000 and 2000-2003) using two tests of cognition: the Mini-Mental State Examination (MMSE) [21] and Geriatric Mental State Schedule (GMS) [22]. Participants that were screen-positives (MMSE score < 26 or GMS organic level > 0) underwent the Cambridge examination for mental disorders of the elderly (CADMEX). A neuropsychologist examined subjects who were suspected of having dementia if additional neuropsychological testing was required for diagnosis. In addition, the total cohort was continuously monitored for incident dementia through a computerized link between the study databases and digitalized medical records from general practitioners and the Regional Institute for Outpatient Mental Health Care until January 1, 2005. The diagnosis of dementia and subtypes of dementia was made in accordance with internationally accepted criteria for dementia (DSM-III-R) [23] and Alzheimer disease (NINDS-ADRDA) [24] by a panel of a neurologist, neuropsychologist and research physician.

Genotyping

Genomic DNA was extracted from whole blood samples using standard methods [25]. The apolipoprotein E (APOE) gene was genotyped using the primer and amplification conditions described by Wenham et al. [26]. The SNPs in the CDK5 gene selected to be analyzed in this study were previously described as tagging SNPs in this gene. [17]. The SNPs were selected after sequencing 70 familial Dutch early onset AD patients. We genotyped 318 samples twice for every SNP and used them as controls by comparing both genotypes, discordant genotypes were excluded. The percentage of concordant sample pairs tested was 100% for SNPs rs2069454, rs891507 and rs9278 and 99.7% (1 discordant sample) for SNPs rs2069442 and rs2069459. The general description of the SNPs selected for this study is shown in Table 1.

Table 1. Description of the 5 CDK5 SNPs

rsnumber	Substitution	Chromosomal Position*	Position relative to CDK5 gene
rs2069442	C/G	150386138	269bp upstream transcription start site
rs2069454	G/C	150383915	Intron 5
rs891507	G/A	150382999	Intron 9
rs2069459	G/T	150382523	Intron 9
rs9278	G/A	150381913	3'UTR

*According to accession number NT007914.14 from NCBI (www.ncbi.nlm.nih.gov/SNP/)

Samples were genotyped with TaqMan allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA). Forward and reverse primer sequences were GGGCACACTGGGAACCA and GCGGAGGCAAACCTTGGA for SNP rs2069442, GAGCCGACCCGTTGCT and GAGAGGGAGAAGGGAGTCAGA for SNP rs2069454, CCTTGCTTTCCTCTGCCTTGA and ACTGCCCTCACCCATTGTG for SNP rs819507, CACCCTGCCCCTGAAGATG and CCACGGAGTCAAGGATCACTT for SNP rs2069459 and TGTGCTCCAGCAGTGCT and TCCACAAAGGGAGTGAGAAATTCG for SNP rs9278. The minor groove binding probes used were: TTCCTGGGACTTGAAG-VIC and CCTGGGAGTTGAAG-FAM for SNP rs2069442, CATGCAACTGTGCCC-VIC and ATGCAAGTGTGCCC-FAM for SNP rs2069454 (designed using the reverse strand), CAAAACTCCACGGACTC-VIC and CAAAACTCCATGGACTC-FAM for SNP rs819507 (designed using the reverse strand), CAGCTGTGGCCCTT-VIC and CAGCTTTGGCCCTT-FAM for SNP rs2069459 and, CCACCCCGGCTGG-VIC and CCCACCCTGGCTGG-FAM for SNP rs9278 (designed using the reverse strand). The assays utilized 5 nanograms of genomic DNA and 2 microliter reaction volumes. The amplification and extension protocol was as follows: an initial activation step of 10 min at 95 deg. preceded 40 cycles of denaturation at 95 deg. for 15 s. and annealing and extension at 50 deg. for 60 s. Allele-specific fluorescence was then analyzed on an ABI Prism 7900HT Sequence Detection System with SDS v 2.1 (Applied Biosystems, Foster City, CA).

Statistical Analysis

Hardy-Weinberg equilibrium (HWE) was assessed for all genotypes using the $\chi 2$ statistic. Linkage disequilibrium (LD) pattern of the CDK5 SNPs was determined using HAPLOVIEW [27]. Also, genotype frequencies of the CDK5 SNPs were compared between cases and controls using the $\chi 2$ test. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using multivariate logistic regression adjusting for age, gender and the APOE*4 allele using all available AD cases (prevalent and incident). The APOE*4 stratified analyses were adjusted for age and gender. Haplotypes were estimated using the haplo.em function implemented in the haplo.stats package [28], which computes maximum likelihood estimates of haplotype probabilities. Posterior probabilities of haplotype pairs for each subject are also computed to account for the fact that there may be more than one pair of haplotypes that are consistent with the observed marker phenotypes. Haplotype association analyses were done using the haplo.score [29] function implemented in haplo.stats. Briefly, this package computes score statistics to test associations between haplotypes and a wide variety of traits, including binary, allowing for adjustment for other determinants.

Table 2. General characteristics of the study population

	Cases[a]	**Controls**
Number of participants	549	5728
Mean Age of Entry (SD)*	78.81 (8.27)	68.50 (8.70)
Female (%)*	415 (75.6)	3317 (57.9)
APOE*4 carriers (%)*	217 (40.9)	1497 (27.3)
Total typed (% of total)		
rs2069442	543 (98.9)	5648 (98.6)
rs2069454	544 (99.1)	5673 (99.0)
rs891507	542 (98.7)	5640 (98.5)
rs2069459	543 (98.9)	5648 (98.6)
rs9278	536 (97.6)	5566 (97.1)

[a] 389 incident and 160 prevalent
* $p < 0.01$ = for cases vs. controls

Results

General characteristics of the AD patients and non-affected individuals are shown in Table 2. Cases were significantly older than control subjects ($p < 0.01$). Also, there were significantly more women ($p< 0.01$) and APOE*4 carriers in the cases ($p < 0.01$). No deviations from Hardy-Weinberg Equilibrium were found for any of the CDK5 SNPs (p values ranging from 0.21 to 0.96). No differences were found in the proportion of successfully genotyped individuals between cases and controls for any of the CDK5 SNPs.

The distribution of the CDK5 SNPs in AD cases (prevalent and incident) and controls is shown in Table 3. Overall, there was no difference in the genotypic distribution of any of the SNPs between AD cases and controls. After stratifying by APOE*4, we found a statistically significant difference in the distribution of SNP rs2069442 between cases and controls in those without APOE*4 ($\chi 2 = 9.68$, $p = 0.008$). This association was explained by an increased frequency of the GG genotype in cases. After adjustment for age, gender and APOE*4, a significant trend was seen for SNP rs9278 in the overall group (pfor trend = 0.04) in which the frequency of the rare allele (A) was decreased in patients. No further differences in the genotypic distribution of any of the other CDK5 SNPs were seen overall or in the analysis stratified by APOE*4.

Table 4 shows the odds ratios overall and stratified by APOE*4 including all prevalent and incident cases. For SNP rs2069442 a non-significant increase in the odds ratio was seen in the overall analysis for the GG carriers compared to the CC carriers (OR = 1.33, 95% CI 0.92-1.93, p=0.11). In those without APOE*4, the OR increased to 1.8 (95% CI 1.16-2.71, p=0.009). When limiting the analysis to incident cases only, carriers of the GG genotype without the APOE*4 allele showed a 2-fold increase of risk for AD (95%CI 1.28-3.12, p=0.02). The difference in the relationship of the CDK5 gene and AD between APOE*4 carriers and non-carriers was statistically significant (p for interaction = 0.05).

For SNP rs9278, which showed a significant trend, the odds ratios were 0.65 (95% CI 0.37-1.15) for the AA carriers and 0.84 (95% CI 0.68-1.04) for the GA carriers compared to GG carriers in the overall group (Table 4).

Overall, the mean age at onset in patients carrying the GG genotype of SNP rs2069442 (89.65 years, SD = 8.44) did not differ significantly from that of the other patients (88.51 years, SD = 7.80 for CG carriers and 87.86 years, SD = 8.02 for CC carriers, p=0.41). Also, when comparing the age at onset of those patients carrying the GG genotype of SNP rs2069442 to all other patients in those without

Table 3. Distribution of AD cases and controls by CDK5 SNPs overall and stratified by APOE*4

SNP	Overall		APOE*4+		APOE*4-	
	Cases (%)	Controls (%)	Cases (%)	Controls (%)	Cases (%)	Controls (%)
rs2069442						
CC	295 (54.6)	3119 (55.2)	126 (58.9)	827 (56.4)	159 (51.6)	2172 (55)
CG	201 (37.2)	2169 (38.4)	79 (36.9)	541 (36.9)	115 (37.3)	1524 (38.6)
GG	44 (8.1)	367 (6.5)	9 (4.2)	99 (6.7)	* 34 (11.0)	254 (6.4)
Total	540	5655	214	1467	308	3950
rs2069454						
GG	489 (89.9)	5127 (90.4)	192 (89.7)	1334 (90.1)	282 (90.4)	3577 (90.4)
GC	55 (10.1)	525 (9.3)	22 (10.3)	143 (9.7)	30 (9.6)	363 (9.2)
CC	0 (0)	21 (0.4)	0 (0)	4 (0.3)	0 (0)	16 (0.4)
Total	544	5673	214	1481	312	3956
rs891507						
GG	397 (73.2)	3980 (70.6)	152 (71)	1060 (71.9)	231 (74.5)	2750 (70)
GA	132 (24.4)	1512 (26.8)	55 (25.7)	366 (24.8)	73 (23.5)	1081 (27.5)
AA	13 (2.4)	148 (2.6)	7 (3.3)	49 (3.3)	6 (1.9)	96 (2.4)
Total	542	5640	214	1475	310	3927
rs2069459						
GG	193 (35.5)	2222 (39.3)	71 (32.9)	573 (39)	120 (38.8)	1568 (39.8)
GT	269 (49.5)	2635 (46.7)	112 (51.9)	670 (45.6)	145 (46.9)	1839 (46.7)
TT	81 (14.9)	791 (14)	33 (15.3)	225 (15.3)	44 (14.2)	535 (13.6)
Total	543	5648	216	1468	309	3942
rs9278[a]						
GG	361 (67.4)	3526 (63.3)	140 (66.4)	919 (63.6)	206 (67.1)	2447 (62.9)
GA	160 (29.9)	1804 (32.4)	67 (31.8)	465 (32.2)	90 (29.3)	1275 (32.8)
AA	15 (2.8)	236 (4.2)	4 (1.9)	61 (4.2)	11 (3.6)	166 (4.3)
Total	536	5566	211	1445	307	3888

* $p = 0.008$, [a] ptrend = 0.04

Table 4. The relationship between CDK5 SNPs and AD in the Rotterdam Study

SNP	Overall[a] OR (95% CI)	APOE*4+† OR (95% CI)	APOE*4-† OR (95% CI)
rs2069442			
CC	reference	reference	reference
CG	0.98 (0.80-1.21)	0.94 (0.68-1.29)	1.02 (0.78-1.34)
GG	1.33 (0.92-1.93)	0.66 (0.32-1.37)	1.79 (1.16-2.79)
rs2069454*			
GG	reference	reference	reference
GC	0.93 (0.67-1.29)	0.84 (0.63-1.75)	1.05 (0.55-1.30)
CC	NA	NA	NA
rs891507			
GG	reference	reference	reference
GA	0.91 (0.73-1.14)	1.03 (0.72-1.47)	0.85 (0.63-1.13)
AA	0.91 (0.49-1.70)	1.35 (0.57-3.20)	0.62 (0.25-1.53)
rs2069459			
GG	reference	reference	reference
GT	1.10 (0.89-1.36)	1.29 (0.93-1.82)	0.98 (0.75-1.30)
TT	1.14 (0.84-1.54)	1.20 (0.75-1.92)	1.09 (0.74-1.62)
rs9278			
GG	reference	reference	reference
GA	0.84 (0.68-1.04)	0.88 (0.63-1.22)	0.82 (0.63-1.22)
AA	0.65 (0.37-1.15)	0.45 (0.16-1.30)	0.78 (0.40-1.52)

* OR is calculated for GC compared to GG as ref. (NA = not applicable)
a Models adjusted for age, sex and APOE*4
† Models adjusted for age and sex

APOE*4, we found no significant differences (90.29 years, SD = 8.43 versus 88.13 years, SD = 7.95).

There is strong LD between the SNPs (D' values ranging from 0.96 to 1.00). Haplotype frequencies are shown in Table 5. Five haplotypes (Haplotype A – Haplotype E) comprised >98% of all observed variability and have been previously described [17]. The overall haplotype distributions were not significantly

different between cases and controls. After stratification by APOE*4, we found an increase in frequency of haplotype C in cases in those without the APOE*4 allele (p = 0.05). Haplotype C is tagged by the G allele of SNP rs2069442, which is also over represented in the single SNP association analysis. We next examined the association between the CDK5 haplotypes with AD using the haplo.score function from haplo.stats [28] controlling for age sex and APOE*4. We did not see an association between any of the CDK5 haplotypes and AD in the overall analysis. After

Table 5. Haplotype association analyses between CDK5 gene and AD in the Rotterdam Study

Name	Haplotype[a]	Overall[b]			APOE*4+[†]			APOE*4-[†]		
		% Cases (N=549)	% Controls (N=5278)	p¶	% Cases (n=217)	% Controls (n=1497)	p¶	% Cases (n=313)	% Controls (n=3989)	p¶
HAP A	(CGGTG)	33.8	31.8	0.24	35.4	32.7	0.30	31.8	31.3	0.49
HAP B	(CGGGA)	18.1	20.4	0.06	18.5	20.4	0.27	18.3	20.5	0.14
HAP C[‡]	(GGGGG)	25.9	24.7	0.27	22.3	24.6	0.43	**28.5**	**24.7**	**0.05**
HAP D	(CGAGG)	14.7	15.9	0.58	16.4	15.5	0.49	13.8	16.1	0.20
HAP E	(CCGTG)	5.0	4.9	0.49	4.8	4.9	1.00	5.0	4.9	0.34

a Haplotypes estimated using the haplo.em function from haplo.stats
bModel adjusted for age, sex and APOE*4 carriership
† Model adjusted for age and sex
‡ Haplotype tagged by SNP rs2069442
¶ p-value estimated using the haplo.score function from haplo.stats

stratification by APOE*4, we found a statistically significant association between haplotype C and the disease (p =0.05, Table 5). For haplotype B, tagging the A allele of SNP 9278, borderline association was found in the overall analysis (p = 0.06).

Discussion

In this population-based study, we observed a 2-fold risk of AD for carriers of the GG genotype of the SNP rs2069442 of the CDK5 gene in non-carriers of the APOE*4 allele. Our haplotype analysis showed a statistically significant increase in frequency of haplotype C in AD cases, which is tagged by SNP rs2069442. SNP rs9278 showed a trend, which was explained by a reduced frequency of the rare allele (A) in AD cases and the association was borderline significant in the haplotype analysis.

There have been few studies conducted on the relationship of the CDK5 gene with AD [16]. In our previous association study [17], focusing on early onset AD, we found SNP rs2069454 (which tags haplotype E) to be associated with the disease. In this study, we did not detect an effect of this specific haplotype with AD in our overall and stratified analyses. Although we cannot exclude spurious findings in either studies, the difference in the findings between them may also be explained by the age of onset of the patients.

We found a significant association between SNP rs2069442 and AD in the APOE*4 non-carriers. This SNP is located in the regulatory region of the CDK5 gene making the finding interesting for future functional studies. SNPs in the regulatory region are in particular of interest for late onset AD as mild effects may not lead to major pathology early in life but may result in later accumulating problems over age and ultimately leading to disease.

Cdk5 is one of the key proteins involved in AD [30] and has been identified as a major candidate mediating tau hyperphosphorylation at specific protein sites characteristic for neurodegenerative tauopathies [31-34]. It has been suggested that Cdk5 can directly induce hyperphosphorylation of tau at disease-associated protein sites and that this may subsequently trigger neurofibrillary tangle formation in tauopathies [2, 14].

In order to become active, inactive monomeric Cdk5 needs to bind with its regulatory subunits p35 and p39, which are only expressed in the brain [3]. This neuron-specific expression of the Cdk5 protein complex is essential for neuronal

growth, development and migration. It has been previously demonstrated that Aß could provoke the conversion of the p35 and p39 subunits to p25 and p29 which are truncated proteins that nevertheless maintain the ability to bind with Cdk5 activating it [11]. The aberrant activation of the Cdk5 protein by p25 is linked to neurodegeneration and the formation of neurofibrillary tangles [2].

In conclusion, our population-based study suggests that the CDK5 gene may be an independent risk factor for AD. We found an effect of SNP rs2069454 in the regulatory region of the CDK5 gene, suggesting that alterations in the expression of the protein may be relevant in neurodegenerative processes. The relation was most pronounced and significant in those without the APOE*4 allele, suggesting the effect is independent of the APOE gene.

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Chapter 3.2

The Cholesteryl Ester Transfer Protein (CETP) gene and the risk of Alzheimer's disease

Abstract

Similar to the apolipoprotein E (APOE) gene, the most common genetic determinant for Alzheimer's disease (AD), the cholesteryl ester transfer protein (CETP) is involved in lipid metabolism. We studied the I405V polymorphism of the CETP gene in relation to AD. We genotyped 525 AD cases and 5404 controls from the Rotterdam Study using a TaqMan allelic discrimination assay. Odds ratios (OR) for AD were estimated using logistic regression analysis. CETP VV carriers showed significantly increased HDL levels compared to the IV and II carriers. In the overall analysis of AD, the frequency of the VV carriers of the CETP polymorphism was non-significantly increased in patients OR = 1.33, 95% confidence interval 0.96-1.90 p = 0.08. In those without the APOE*4 allele, the risk of AD for VV carriers was increased 1.67 fold (95% CI 1.11-2.52, p = 0.01). The difference in the relationship between CETP and AD between APOE*4 carriers and APOE*4 non-carriers was statistically significant (p for interaction = 0.04). Our results suggest that the CETP gene increases the risk of AD in the absence of the APOE*4 allele, probably through cholesterol metabolism in the brain.

Introduction

Alzheimer's disease (AD) is a complex neurodegenerative disorder characterized by progressive memory loss and deterioration of cognitive function. Of the many genes studied thus far, the only consistently replicated genetic risk factor for AD has been the apolipoprotein E (APOE) gene. APOE, which transports cholesterol in the brain [1], may relate to AD by modifying the production of Amyloid β (Aβ). High cellular cholesterol may promote Aβ production and senile plaque deposition thus, increasing the risk of AD [2-7]. Cholesterol dysregulation is linked to the pathogenesis of AD [8, 9]. Lack of cholesterol supply to neurons impairs neurotransmission and synaptic plasticity [10] and induces neurodegeneration and tau pathology [11]. Also, there is some evidence that patients treated with cholesterol lowering drugs, such as statins, have a reduced prevalence of AD [12].

High Density Lipoprotein (HDL) is essential for the removal of excess cholesterol from cells exerting several potentially anti-atherogenic effects including reverse cholesterol transport from peripheral cells to the liver [13]. Cholesteryl ester transfer protein (CETP) is a key player in lipid metabolism by catalysing the transfer of cholesteryl ester from HDL particles to triglyceride-rich lipoproteins in exchange for triglycerides [14]. CETP is a protein composed of 439 amino acid residues. It is coded by the CETP gene, which is located on chromosome 16q21 and has 14 exons. The 405V allele of the CETP I405V polymorphism in exon 14 has been associated with lower levels of CETP protein, higher levels of circulating HDL [15], a lower incidence of cardiovascular disease [15] and a longer survival [16].

Studies evaluating the role of the CETP gene in AD have shown contradictory results. While one previous study [17] showed evidence that the C-629A polymorphism of the CETP gene modifies the risk of AD in association with the APOE gene by reducing the risk of AD, another study found no effect of this gene on the disease [18]. We studied the I405V polymorphism of the CETP gene in relation to AD in a large population based study and investigated whether this polymorphism is independently associated with AD, or acts in concert with the APOE gene conferring risk for the disease.

Materials and Methods

Study Population

Our study is part of the Rotterdam Study, a population-based follow-up study of determinants of diseases in the elderly. All inhabitants of Ommoord, a suburb of Rotterdam, aged 55 years or over, were invited to participate. The design of the study has been previously described [19]. From all subjects, informed consent was obtained and the Medical Ethics committee of the Erasmus Medical Center approved the study. 7.983 participants (response rate of 78%) were examined at baseline (1990 to 1993). Information on age, smoking behavior and medical history was obtained using a computerized questionnaire.

Patient ascertainment

Dementia was diagnosed following a three-step protocol [20, 21]. All participants were screened at follow-up visits using two tests of cognition: the Mini-Mental State Examination (MMSE) [22] and Geriatric Mental State Schedule (GMS) [23]. Participants that were screen-positives (MMSE score < 26 or GMS organic level > 0) underwent the Cambridge examination for mental disorders of the elderly (CADMEX). In addition, the cohort was continuously monitored for incident dementia through computerized linkage between the study databases and medical records from general practitioners and the Regional Institute for Outpatient Mental Health Care until January 1, 2005. The diagnosis of dementia and subtype of dementia was made in accordance with accepted criteria for dementia (DSM-III-R) [24] and Alzheimer disease (NINDS-ADRDA) [25] by a panel of a neurologist, neuropsychologist and a research physician.

Genotyping

DNA was extracted from whole blood samples using standard methods [26]. The APOE gene was genotyped using the conditions described by Wenham et al. [27]. Samples were genotyped for the CETP I405V polymorphism using TaqMan allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA). We genotyped 317 samples twice and used them as controls by comparing both genotypes, and if present, samples with discordant genotypes were excluded. Forward and reverse primer sequences are available on request. The assays utilized 5 nanograms of genomic DNA and 2 microliter reaction volumes. The amplification and exten-

sion protocol was as follows: an initial activation step of 10 min at 95° C preceded 40 cycles of denaturation at 95° C for 15 s. and annealing and extension at 50° C for 60 s. Allele-specific fluorescence was then analyzed on an ABI Prism 7900HT Sequence Detection System with SDS v 2.1 (Applied Biosystems, Foster City, CA).

Statistical Analysis

Genotype frequency of the CETP I405V was compared between cases and controls and Hardy-Weinberg equilibrium (HWE) were assessed for all genotypes using the $\chi 2$ test. Odds ratios (OR) and 95% confidence intervals (CI) were estimated using multivariate logistic regression adjusting for age, gender and APOE*4 status using all available AD cases (prevalent and incident). The APOE*4 stratified analyses were adjusted for age and gender. All analyses were performed using SPSS 11.0.

Table 1. General characteristics of the study population stratified by CETP genotype

Genotype	II	IV	VV
Total typed (%)	2949 (36.9)	2826 (35.4)	646 (10.1)
Mean age of entry in years (SD)	69.4 (9.2)	69.5 (9.2)	69.1 (9.0)
Female (%)	1702 (59.9)	1608 (59.1)	369 (59.0)
Mean HDL levels mMol/L (SD)*	1.33 (0.37)	1.34 (0.36)	1.39 (0.39)
Mean total cholesterol levels mMol/L (SD)	6.61 (1.21)	6.60 (1.24)	6.61 (1.23)
APOE*4 carriers (%)	790 (29.3)	733 (28.2)	156 (26.8)

* $p = 0.001$

Results

Table 1 shows the general characteristics of the study population. The distribution of the CETP I405V genotypes was in Hardy Weinberg Equilibrium (p= 0.38). The mean serum HDL level of the VV carriers was significantly increased compared to the IV and II carriers (p = 0.001). There were no statistically significant differences in the distribution of the other variables between the genotype groups.

The genotype frequencies of the CETP I405V polymorphism are shown in Table 2. Overall, we did not find any significant difference in the genotypic distribution of the CETP I405V polymorphism between AD cases (prevalent and incident) and controls. After stratifying for APOE*4, we found a statistically significant difference in the distribution between cases and controls in those without APOE*4 ($\chi2 = 6.62$, p = 0.04). This difference was explained by an increased frequency of the VV genotype in cases.

Table 3 shows the odds ratios overall and stratified by APOE*4. Including all prevalent and incident cases, a non-significant increase in the odds ratios was seen in the overall analysis when comparing the VV carriers to the II carriers of the

Table 2. Distribution in cases and controls by CETP I405V polymorphism overall and stratified by APOE*4

	Overall		APOE*4+		APOE*4-	
Genotype	**Cases (%)**	**Controls (%)**	**Cases (%)**	**Controls (%)**	**Cases (%)**	**Controls (%)**
II	236 (43.4)	2604 (46.1)	106 (49.8)	684 (46.7)	121 (38.8)	1806 (45.9)
IV	249 (45.8)	2474 (43.8)	89 (41.8)	644 (43.9)	151 (48.4)	1738 (44.1)
VV	59 (10.8)	566 (10.0)	18 (8.5)	138 (9.4)	*40 (12.8)	394 (10.0)
Total	544	5644	213	1466	312	3938

* p = 0.04

I405V polymorphism, (OR = 1.33, 95% CI 0.96-1.90, p = 0.08). In those without APOE*4, the OR increased to 1.67 (95% CI 1.11-2.52, p = 0.01). The IV carriers showed a non-significant increase in risk of AD compared to II carriers in those

without APOE*4, OR = 1.26 (95% CI 0.96-1.64, p = 0.09). These estimates did not change after adjusting for serum HDL levels. Serum HDL level itself was not associated to AD. We did not detect an effect of the CETP gene on the risk of AD in those with APOE*4 (p VV carriers = 0.65) (Table 3). The difference in the relationship of the CETP gene and AD between APOE*4 carriers and non-carriers was statistically significant (p for interaction = 0.04). The mean age at onset in patients carrying the VV genotype of the CETP I405V polymorphism (88.64 years, SD = 7.19) did not differ significantly compared to that of the II and IV carriers (88.75 years, SD = 8.02 for IV carriers and 87.75 years, SD = 817 for II carriers, p=0.41). In those without the APOE*4 allele, the difference in age at onset between VV carriers and non-carriers was also not significant (p = 0.48).

Table 3. Association analyses between CETP I405V polymorphism and AD

	Overall¶	**A POE*4+†**	**APOE*4-†**
OR IV (95% CI)	1.11 (0.91-1.36)	0.91 (0.66-1.26)	0.91 (0.51-1.60)
OR VV (95% CI)	1.33 (0.96-1.90)	1.26 (0.96-1.64)	1.67 (1.11-2.52)

¶ Models adjusted for age, sex and APOE*4, † Model adjusted for age and sex

Discussion

Our results suggest an increased risk of AD for the carriers of the VV genotype of the I405V polymorphism of the CETP gene. The increase in risk was 1.67-fold in non-carriers of the APOE*4 allele. No association was observed between this polymorphism of the CETP gene and AD in those with the APOE*4 allele. We detected a statistically significant difference in the relationship between CETP and AD in APOE*4 carriers and non-carriers (p for interaction = 0.04). The association was independent of serum HDL levels, which by itself, was not associated to AD.

The relationship of lipid transfer proteins as CETP and AD has been previously studied. In an experimental study, Yamada et al. showed that in brains of patients with AD, reactive astrocytes in grey and white matter exhibit CETP-like immunoreactivity. Thus, CETP-positive astrocytes may play a role in the pathology of

Alzheimer's disease, in particular tissue repair [28]. Our findings partly overlap with those of other genetic studies. The CETP C-629A and the TaqI B polymorphisms have been studied in relation with AD. A study in a Spanish population of the relationship between these two polymorphisms and the APOE*4 allele indicated that the CETP gene modifies Alzheimer's disease risk possibly through modulation of brain cholesterol metabolism [17]. On the other hand, the studies of Zhu et al., which evaluated the effect of two promoter and the TaqI B polymorphisms [18] and the study of Fidani et al, which also evaluated the effect of the TaqI B polymorphism found no effect of this gene on the disease [29].

Since the VV genotype is associated with lower levels of CETP and higher HDL, our study is compatible with the view that low CETP levels may increase the risk of AD through a reduction in neuronal repair capacity. An alternative mechanism is related to cholesterol removal by HDL, this is one of the mechanisms that the cell uses to keep the levels of cholesterol in their membranes fairly constant [9, 13, 30], this would imply that VV carriers could be at lower risk of AD. The fact that we found this group (VV carriers) at a higher risk of AD could be explained by previous findings that suggest that the actions of the HDL in the transfer of lipids in brain may differ from these in the peripheral system [31]. In fact, the processing of cholesterol in brain is restricted in the central nervous system by the blood-brain barrier, which is impermeable to plasma lipoproteins [32]. Unlike fatty acids that must be transported to the central nervous system from the periphery, brain cholesterol is mainly independent of dietary uptake or synthesis in the liver and is synthesized almost all in the brain [33].

Taken all together, our results suggest that the CETP gene increases the risk of AD independently of the APOE gene, probably through the regulation of cholesterol metabolism in the brain. However, our data are not supported by the literature. More studies are needed to clarify the role of the CETP gene as well as the CETP protein and HDL cholesterol in AD.

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Chapter 3.3

Relationship of the ubiquilin 1 gene with Alzheimer's and Parkinson's disease and cognitive function

Abstract

Ubiquilin 1 (UBQLN1) is involved in the ubiquitination machinery, which has been implicated in Alzheimer's disease (AD) as well as Parkinson's disease (PD). The gene encoding for UBQLN1 has been previously associated with AD. We studied the role of the SNP rs12344615 on the UBQLN 1 gene in AD, PD and cognitive function in a population based study, the Rotterdam Study, and a family based study embedded in the Genetic Research in Isolated Population (GRIP) program. The Rotterdam study includes 549 patients with AD and 157 patients with PD. The GRIP program includes a series of 123 patients with AD and a study of 1049 persons who are characterized for cognitive function. Data were analysed using logistic and multiple regression analysis. We found no significant difference in risk of AD or PD by the UBQLN1 SNP 12344615 in our overall and stratified analyses in the Rotterdam Study. In our family based study, we did not find evidence for linkage of AD to the region including the UBQLN1 gene. In the family-based we also failed to detect an effect of this polymorphism on cognitive function. Our results suggest that it is unlikely that the SNP 12344615 of the UBQLN1 gene is related to the onset of AD, PD or cognitive function.

Introduction

Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common neurodegenerative disorders in western societies. Familial aggregation of these two disorders has been observed suggesting a common genetic cause [1-3]. The ubiquilin1 gene (UBQLN1, GeneID 29979) may be involved in both diseases. A single intronic Single Nucleotide Polymorphism (SNP) located downstream of exon 8 (rs12344615) of the UBQLN1 gene has been associated with AD [4]. UBQLN1 is a 48075 bp gene that includes 11 exons and for which two transcripts have been described (www.ncbi.nlm.nih.gov).

UBQLN1 specifically interacts with presenilin 1 and 2 apparently increasing the level of these proteins [5], making the gene encoding for UBQLN1, which is located in a region showing linkage to AD, an interesting candidate for AD. UBQLN1 is also involved in the ubiquitination machinery, related with general protein degradation [6]. There is substantial evidence for disturbances in the ubiquitinization machinery in PD. This makes the UBQLN1 gene also of interest for PD [7]. Previous studies on the relationship of this gene and AD have shown contradicting results. While some studies found an association with AD or related quantitative traits for SNP rs12344615 [8, 9], other studies did not find any evidence for association [10-13]. No studies have been performed so far evaluating the relationship between the UBQLN1 gene and PD. We studied the role of the rs12344615 polymorphism in the UBQLN 1 gene in AD, PD and cognitive function in population and family based studies.

Materials and Methods

Study populations

Rotterdam Study

This is a population-based follow-up study of determinants of diseases in the elderly. The design of the study has been previously described [14]. From all subjects, informed consent was obtained and the Medical Ethics committee of the Erasmus Medical Center approved the study. The diagnosis of dementia was made following a three-step protocol [15, 16]. Briefly, all subjects were screened during follow-up

visits (1997-1999, 1999-2000 and 2000-2003) with two tests of cognition (Mini-Mental State Examination (MMSE) [17] and Geriatric Mental State Schedule (GMS) [18]). Screen-positives (MMSE score < 26 or GMS organic level > 0) underwent the Cambridge examination for mental disorders of the elderly (Camdex). A neuropsychologist examined persons who were suspected of having dementia if additional neuropsychological testing was required for diagnosis. The diagnosis of dementia and subtypes of dementia was made in accordance with internationally accepted criteria for dementia (DSM-III-R) [19] and Alzheimer disease (NINCDS-ADRDA) [20] by a panel of a neurologist, neuropsychologist, and a research physician.

We also used a two-step design to identify subjects with PD [21]. Participants were screened for parkinsonian signs (resting tremor, rigidity, bradykinesia, or impaired postural reflexes) in a standardized way. Individuals who screened positive received a diagnostic workup including the Unified Parkinson's Disease Rating Scale (UPDRS) [22] and neurological examination. In addition, the cohort was continuously monitored through computerized linkage to general practitioners' medical files. PD was diagnosed if two or more parkinsonian signs were present in a person not taking antiparkinsonian drugs, or if at least one sign had improved after medication was started, and when all causes of secondary parkinsonism

Table 1. General characteristics of the Rotterdam study population stratified by SNP rs12344615 genotype

Genotype	TT	CT	CC
Total Typed (%)	4142 (66.0)	1923 (30.6)	212 (3.4)
Mean age at entry in years (SD)	69.3 (9.1)	69.7 (9.3)	68.8 (8.7)
Female (%)	2448 (59.1)	1164 (60.5)	120 (56.6)
APOE*4 carriers (%)	1156 (29.0)	499 (27.3)	59 (28.8)
Number of AD cases[a]	353 (8.5)	178 (9.3)	18 (8.5)
Number of PD cases[b]	105 (2.5)	45 (2.3)	7 (4.5)
Number of Parkinsonism cases	171 (4.1)	77 (3.0)	9 (3.5)

Parkinsonism cases include all PD cases
a 389 incident and 160 prevalent
b 89 incident and 68 prevalent

(dementia, use of neuroleptics, cerebrovascular disease, multiple system atrophy, or progressive supranuclear palsy) could be excluded [23].

Genetic Research on Isolated Populations (GRIP) study

The GRIP study is a family based study that aims to identify genes involved in complex diseases by selecting affected individuals and their relatives. The population in which the study was conducted is a genetically isolated community in the Southwest of The Netherlands [24, 25]. The Medical Ethics Committee of the Erasmus Medical Center approved this study. The patient ascertainment was described previously [2]. Briefly, all AD patients (n = 123) were examined by one of two research physicians to re-evaluate the previous clinical diagnosis. Examination consisted of neurological examination and brief neuropsychological testing. A standard interview was performed with close relatives concerning symptoms, disease course, medical, social and family history [2]. Dementia was classified using the Clinical Dementia Rating Scale (CDR). We also reviewed all available medical records, neuropsychological test results and neuroimages to establish a diagnosis according to the NINCDS–ADRDA criteria [20].

Erasmus Rucphen Family (ERF) study

This is a family-based study of a recent genetically isolated population embeded in the GRIP study. The ERF study aims to identify susceptibility genes for complex disorders by studying quantitative traits. Its participants are all the descendants of 22 families that had at least 5 children baptized in the community church between 1850 and 1900. All living descendants of these families and their spouses, aged 18 and older were invited to participate [24]. The Medical Ethics Committee of Erasmus Medical Center approved the study.

A battery of neuropsychological tests was administered to the ERF participants [24]. Five neuropsychological tests adapted to the Dutch language were used to evaluate different cognitive domains related to neurodegenerative disorders [26-28], namely: the Dutch version of the auditory verbal learning test (AVLT) [29, 30], the Trail Making Test (TMT) [31], the Stroop Color-word Test [32, 33], the verbal fluency test [34, 35], and the Block Design subtest of the Wechsler Adult Intelligence Test (WAIS) [36]. Individuals with a previous diagnosis of neurological or psychiatric disorders were excluded (n= 47). A more detailed description of these cognitive tests is included in our previous report [24].

Genotyping

Genomic DNA was extracted from whole blood samples using standard methods [37]. All samples were genotyped with TaqMan allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA). Primer sequences are available on request. The amplification and extension protocol was as follows: an initial activation step of 10 min at 95°C preceded 40 cycles of denaturation at 95°C for 15s and annealing and extension at 50°C for 60s. Allele-specific fluorescence was then analysed on an ABI Prism 7900HT Sequence Detection System with SDS v 2.1 (Applied Biosystems, Foster City, CA). We genotyped 318 samples twice for SNP rs12344615 and used them as controls by comparing both genotypes, discordant genotypes were excluded. The percentage of concordant sample pairs tested was 100%.

All samples from the GRIP study were genotyped using 18 flourescently labelled microsatellite markers from chromosome 9 which were, on average, 10 centimorgans apart. The genotyping experiments were done following manufacturer instructions (Applied Biosystems, Foster City, CA).

For the Rotterdam study, the APOE gene was amplified using the primer and amplification conditions described by Wenham et al. [38]. For the GRIP and ERF studies, samples were genotyped for the APOE polymorphisms with TaqMan allelic discrimination Assay-By-Design (Applied Biosystems, Foster City, CA). Primer sequences are available on request. The assays utilized 5 nanograms of genomic DNA and 2 microliter reaction volumes. Given the characteristics of the chemistry used for designing the TaqMan primers and probes, PCR conditions and analysis methods were the same used for the rs12344615 SNP.

Statistical analyses

Hardy-Weinberg equilibrium (HWE) for all genotypes and genotype frequencies of the rs12344615 polymorphism were compared between cases and controls using the $\chi 2$ test. For the patients of the Rotterdam Study, odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using multivariate logistic regression adjusting for age, gender and APOE*4 using the SNP rs12344615 TT genotype as reference. We assessed the effect of the rs12344615 SNP on the age at onset of AD and PD using incident cases only using multiple regression analysis. All analyses were performed with SPSS 11.0 for windows package.

For the GRIP study the Quasi-Likelihood Score (QLS) test of the CC-QLS package [39], was used for the association analysis to correct for familial relationships. For the CC-QLS test, kinship and inbreeding coefficients were calculated using our genealogy database. For those individuals whose genealogy was not available, the average kinship and inbreeding coefficients were used. Parametric linkage analysis for AD was performed using a dominant model. Age dependent penetrance was estimated on population prevalence [40]. The disease gene frequency was set to one percent. Allelic frequencies for microsatellite markers were estimated from unrelated controls. Linkage analysis was conducted using SimWalk2 V.2.91 [41-43].

We evaluated the association between SNP rs12344615 and cognitive function [24]. Given the skewed distributions of the AVLT-Trial VII, TMT-A, TMT-B, cards I, II and III of the Stroop colour-word and Block design tests, the data were log-transformed to achieve normality. Data were analysed using the linear mixed model implemented in the SOLAR package [44]. The models were adjusted for age, age2, sex, inbreeding and general cognitive ability.

Table 2. Distribution of Alzheimer's and Parkinson disease cases and controls by SNP rs12344615 in the Rotterdam Study stratified by APOE*4

	Overall		**APOE*4+**		**APOE*4 -**	
	Cases (%)	**Controls (%)**	**Cases (%)**	**Controls (%)**	**Cases (%)**	**Controls (%)**
AD						
rs12344615						
TT	353 (64.3)	3789 (66.1)	137 (63.1)	1019 (68.1)	209 (66.8)	2615 (65.6)
TC	178 (32.4)	1745 (30.5)	75 (34.6)	424 (28.3)	91 (29.1)	1241 (31.1)
CC	18 (3.3)	194 (3.4)	5 (2.3)	54 (3.6)	13 (4.2)	133 (3.3)
Total	549	5728	217	1497	313	3989
PD						
rs12344615						
TT	105 (66.9)	4035 (66.0)	33 (78.6)	1123 (67.2)	68 (63.6)	2754 (65.7)
TC	45 (28.7)	1877 (30.7)	7 (16.7)	492 (29.4)	34 (31.8)	1297 (30.9)
CC	7 (4.5)	205 (3.4)	2 (4.8)	57 (3.4)	5 (4.7)	141 (3.4)
Total	157	6117	42	1672	107	4192

Results

Rotterdam Study

The general description of the participants of the Rotterdam Study stratified by rs12344615 genotype is shown in table 1. The distributions of the genotype frequencies were in Hardy Weinberg equilibrium ($p = 0.70$). No significant differences between the genotype groups were found in mean age at entry, gender, or APOE*4 distribution. The total number of affected individuals with AD, PD or Parkinsonism was not significantly increased in carriers of the C allele, although the number of PD patients tended to be increased in those homozygous for this allele.

The distribution of rs12344615 in AD and PD cases and controls, overall and stratified by APOE*4, is shown in Table 2. We did not find a statistically significant difference in the distribution of this SNP between patients and controls. There was a modest non-significant increase in the frequency of CC genotype in AD patients in those without APOE*4 allele. We also found a non-significant increase in CC carrier frequency in the PD cases both with and without APOE*4 (Table 2).

We assessed the association between the rs12344615 polymorphism and the risk of AD and PD by means of logistic regression adjusting for age, sex and APOE*4 (Table 3). There was a non-significant increase in risk of AD and PD for CC carriers of SNP rs12344615 in those without APOE*4 in the additive and recessive models. The difference in the relationship of the UBQLN1 gene and AD and PD between APOE*4 carriers and non-carriers was not statistically significant (p for interaction = 0.97 for AD and p for interaction = 0.83 for PD).

We tested the effect of SNP rs12344615 on age at onset of AD and PD patients. Overall, the mean onset of AD in patients carrying the rs12344615 CC genotype (86.59 years, SD = 8.88) was 2.8 lower than that of CT carriers (89.40 years, SD = 8.76) and 1.2 years lower for TT carriers (87.80 years, SD = 7.50 for TT carriers) but this difference was not statistically significant ($p = 0.14$). When comparing the onset of PD patients carrying the CC genotype of SNP rs12344615 to all other PD patients we found no significant differences between the groups (CC genotype carriers= 86.59 years, SD = 8.88 versus other genotype carriers = 88.32 years, SD = 7.96).

GRIP study

In this population there are more females in the AD cases compared to controls (p=0.0002) and the average kinship coefficient is higher in case pairs than in control pairs (P<0.0001). The APOE*4 frequency was significantly increased in cases compared to controls (p=0.0001) and the mean onset of AD was 74.8 ± 5.3 years. There was no significant difference in the onset of AD between the genotype groups (74.01 years, SD = 4.97 for CC carriers, 74.84, SD = 4.97 for TC carriers and 74.83, SD = 5.47 for TT carriers, p = 0.33).

There was some evidence for association between AD and marker D9S164 (nominal p = 0.04 without adjustment for multiple testing). Also, marker D9S285 showed a borderline nominally significant p value (nominal p = 0.07).

Table 3. Association analyses of SNP rs12344615 with Alzheimer's and Parkinson's disease in the Rotterdam Study

	Overall¶	**APOE*4+†**	**APOE*4-†**
	OR (95% CI)	**OR (95% CI)**	**OR (95% CI)**
AD			
TC[a]	0.96 (0.78-1.19)	1.14 (0.82-1.58)	0.86 (0.65-1.14)
CC[a]	1.17 (0.69-2.00)	0.68 (0.25-1.83)	1.60 (0.86-2.99)
CC[b]	1.19 (0.70-2.02)	0.65 (0.24-1.74)	1.68 (0.90-3.11)
PD			
TC[a]	0.85 (0.59-1.23)	0.42 (0.18-1.02)	1.05 (0.69-1.60)
CC[a]	1.44 (0.66-3.15)	1.26 (0.29-5.49)	1.52 (0.60-3.84)
CC[b]	1.51 (0.69-3.27)	1.55 (0.36-6.71)	1.49 (0.60-3.73)

a OR estimated using the TT genotype carriers as reference group
b OR estimated using the TT/TC genotype carriers as reference group
¶ Models adjusted for age, sex, and APOE*4, † Models adjusted for age and sex

SNP rs12344615 itself showed no association with AD in this population (nominal p = 0.74) (Table 4). Stratified analysis by APOE*4 showed no association between the tested markers and AD (data not shown). Linkage analysis for AD using the microsatellite markers and SNP rs12344615 yielded negative LOD scores. For SNP rs12344615, the LOD score was below –2.0, excluding linkage and LOD scores

Table 4. Comparison of microsatellite markers and SNP rs12344615 allele frequencies between AD cases and controls using CC-QLS on the GRIP population

Marker	Position	Cases (n=123)	Controls (n=58)	QLS	P
D9S288	9.83	121	57	9.90	0.54
D9S286	18.06	121	56	5.64	0.93
D9S285	29.52	119	57	17.39	0.07
D9S157	32.24	119	51	8.47	0.29
D9S171	42.73	118	56	10.76	0.22
D9S161	51.81	120	54	5.63	0.69
D9S273	65.79	114	54	2.92	0.97
D9S175	70.33	117	56	18.75	0.13
D9S167	83.41	113	57	18.23	0.20
rs12344615	85.21[a]	120	58	0.11	0.74
D9S283	94.85	121	55	13.89	0.31
D9S287	103.42	115	57	7.33	0.50
D9S1690	106.63	120	54	6.45	0.49
D9S1677	117.37	116	56	11.49	0.40
D9S1776	123.33	121	56	4.46	0.88
D9S1682	132.09	120	54	5.31	0.38
D9S290	140.86	121	57	7.96	0.34
D9S164	147.91	121	54	18.87	**0.04**
D9S1826	159.61	120	57	4.04	0.78

Position: position in centimorgans of each marker (according to NCBI build 36.1), p: nominal p values from CC-QLS test, Italic marker name: UBQLN1 rs12344615 SNP, [a] genetic position estimated using physical position, Bold P indicate nominally significant CC-QLS p values

Table 5. Effects of SNP rs12344615 of the ubiquilin1 gene on cognitive tests on the ERF population

Cognitive Tests	Overall	APOE*4+	APOE*4-
AVLT- Trail I (short-term memory)	0.016(0.079)	0.056(0.130)	0.005(0.103)
AVLT-Trail II- V (learning)	0.456 (0.422)	0.066(0.724)	0.761(0.537)
AVLT- Trail VI (delayed recall)	0.111 (0.145)	-0.166(0.237)	0.275(0.186)
AVLT- Trail VII † (recognition)	0.006 (0.119)	0.077(0.214)	-0.027(0.143)
Semantic fluency (verbal fluency)	0.124 (0.490)	0.724(0.844)	-0.119(0.603)
Phonological fluency (verbal fluency)	0.633 (0.525)	1.412(0.915)	0.238(0.659)
TMT-A† (psychomotor speed)[a]	0.466(2.014)	2.231(3.503)	-1.221(2.604)
TMT-B† (cognitive flexibility)[a]	2.623(2.657)	0.206(4.768)	2.848(3.104)
Stroop Card I† (reading abilities)[a]	0.006 (0.038)	0.384(1.908)	-0.059(1.438)
Stroop Card II† (reading abilities)[a]	0.042 (0.035)	1.268(1.931)	1.425(1.260)
Stroop Card III† (interference)[a]	0.081 (0.043)	1.574(2.200)	2.519(1.624)
Block design†[b] (visuospatial abilities)	0.710(2.510)	-0.933(4.324)	2.080(3.055)

Values are regression coefficients (se) of the SNP rs12344615 on cognitive tests Models adjusted for age age2, sex, inbreeding education, and general cognitive ability, † Log-transformed variables, [a] Positive effects correlate with worse cognitive performance, [b] WAIS III

corresponding to the microsatellite markers flanking the SNP rs12344615 ranged from –3.6 for marker D9S167 to –2.5 for marker D9S283.

ERF study

In the ERF study, for 1049 individuals, phenotypic and genotypic information was available. The frequencies of SNP rs12344615 genotypes were 54% for the TT genotype, 38% for the TC genotype and 8% for the CC genotype. These genotypes were in Hardy-Weinberg equilibrium ($p = 0.18$). There was no significant difference in the distribution of age, gender and inbreeding between the genotype groups ($p > 0.05$). We found no significant differences in the median scores of the cognitive tests by rs12344615 genotype (data not shown). We tested the association between SNP rs12344615 and the cognitive tests applied and found no statistically significant relationship (Table 5).

Discussion

We found no significant difference in risk of AD or PD for carriers of the CC genotype of SNP 12344615 in our overall and stratified analyses in the Rotterdam Study. We did not detect a significant effect on the onset of AD or PD in this population-based sample. In our family based analysis in the GRIP study, we did not find a convincing significant association between SNP rs12344615 and AD. Marker D9S164 was associated with AD ($p = 0.04$) and marker D9S185 showed borderline association with AD ($p = 0.07$), however, these associations are nominally significant and did not sustain when adjusting for multiple testing. Moreover, the parametric LOD curve based on 18 microsatellite markers and SNP rs12344615, excluded linkage. We detected no effect of this polymorphism on cognitive function.

The ubiquilin protein has been shown to interact with presenilin 1 and 2 [5] and overexpression of ubiquilin results in a decrease in pen-2 and nicastrin levels. These are essential components of the γ-secretase complex, suggesting that ubiquilin expression may regulate γ-secretase activity [45]. The UBQLN1 gene is located in a region that has been linked to AD (chromosome 9) [4]. In that, the association of this gene to AD is plausible. However, studies assessing the relationship between the UBQLN1 gene and AD [8-12] have been contradictory. While the studies of Silfer [9] and Kamboh [8] found an association between SNP 12344615 and AD, age at onset and cognitive function, the studies of Smemo [11] (which analysed

seven SNPs including rs12344615), Silfer [10], Browers [12] (which included AD cases with onset before 70 years) and more recently the study from Bensemain, found no association with AD [13].

Our results do not support a role for UBQLN1 in AD. We studied the relationship of SNP rs12344615 both in a large outbred and in an isolated, inbred, populations. The Rotterdam Study has a statistical power of 100% to detect a 1.5-2 fold increased risk of AD ($p = 0.05$). The study has a 70% power to detect a similar effect on PD assuming an α level of 0.05. Given the lower statistical power for PD studies it is of interest that we found a non-significant association to PD. We detected a 1.5-fold increase in risk of PD for carriers of the CC genotype of SNP 12344615. This may be of interest for further research.

In summary, our results suggest that it is unlikely that the SNP 12344615 of the UBQLN1 gene is related to the onset of AD, PD or cognitive function. However, our results do not exclude a small effect of the protein on the risk of PD.

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Chapter 3.4

A deletion in the DJ-1 gene and dementia – a population-based survey

Abstract

The DJ-1 gene is associated with autosomal recessive early-onset parkinsonism, most likely through its role in defense against oxidative stress. Oxidative stress is not only involved in Parkinson's disease, but also in other neurodegenerative disorders, such as dementia. We assessed the presence of a 14 kb DJ-1 deletion in 191 patients with dementia, ascertained from the genetically isolated population where the first kindred with DJ-1 related parkinsonism was originally identified. The control group consisted of 129 non-demented subjects. We found the deletion in two patients and one control. There was no evidence for an increased risk of dementia in carriers. All subjects were heterozygous for the deletion and related to a common ancestor within 8 generations. Our results suggest it is unlikely that haploinsufficiency in the DJ-1 gene imparts an increased risk for dementia.

Introduction

We recently reported mutations in the DJ-1 gene in autosomal recessive early-onset parkinsonism [2]. The functional properties of the DJ-1 protein are not fully elucidated. Unlike the three other genes for familial Parkinson's disease (PD) [9, 11, 18], the involvement of DJ-1 in the ubiquitin protein degradation pathway, presently considered central in the pathophysiological model for PD [3, 4], is still a matter of debate. Previous studies have suggested that DJ-1 plays a role in the cellular defense against oxidative stress [16] and Yokota et al. suggested a protective effect of DJ-1 against proteasome stress, although these results have not been replicated [23]. Likewise, in other neurodegenerative conditions, such as Alzheimer's disease (AD) and related disorders, oxidative damage is implicated [7]. Alzheimer's disease shows a considerable overlap with PD pathology. In PD as well as in AD, Lewy bodies can be found at postmortem examination [1], while a high number of PD patients develop dementia [6, 8]. Inhibition of proteasome function in the pathological state, as has been reported in both Alzheimer's and Parkinson's disease, could therefore contribute both to accumulation of non-ubiquitylated forms of aggregation-prone neuronal proteins, as well as impaired clearance of ubiquitylated aggregates [10]. There is some evidence of familial aggregation of AD and PD suggesting a common genetic origin [5]. Li et al showed evidence of a possible overlap of these two entities through a role of inflammation in these two neurodegenerative disorders [12]. Also Rizzu et al. demonstrated the colocalization of DJ-1 protein within a subset of pathological tau inclusions in a diverse group of neurodegenerative disorders known as tauopathies [19]. We hypothesized that mutations in DJ-1 may not only concern the strictly defined disease entity AD, but rather a broader range of dementias. We assessed the role of DJ-1 mutations in dementia by performing a case-control study of dementia patients and controls ascertained from the genetically isolated population in which the deletion in DJ-1 gene was originally detected in a kindred with early-onset parkinsonism [2, 22].

Materials and Methods

Study population

Patients were derived from a study of genetic susceptibility for dementia, performed within the framework of the GRIP (Genetic Research in Isolated Populations) study [21]. The population in which the study was conducted is a genetically isolated community in the Southwest of The Netherlands. From a founding population of about 150 ancestors, this community expanded to a current number of approximately 20,000 inhabitants. Since there has been little inward migration, a genetically homogeneous population arose from the founder population. In this isolate all patients with dementia were ascertained through local general practitioners, neurologists and nursing-home physicians.

Patient ascertainment

The diagnoses of dementia type (AD, FTD, LBD, and VaD) were made by a research physician and two neurologists according to the international NINCDS-ADRDA criteria for AD [14], consensus criteria for frontotemporal dementia [17], Lewy body dementia [13], and vascular dementia [20]. Genealogical information including name, date and place of birth of parents and grandparents was obtained from a close relative. These lineages were extended by means of community and church registers and a computerized genealogy database. A family-history questionnaire was administered in order to ascertain any family history for neurological, vascular or psychiatric disease.

The control group was composed of 129 individuals selected from studies on dementia, cardiovascular disease and diabetes mellitus type 2 conducted within GRIP. Control individuals with a history of PD, parkinsonism or other neurodegenerative disease were excluded [1]. All patients and controls provided informed consent prior to participation. The Medical Ethics Committee of the Erasmus MC approved this study.

Statistical and genetic analyses

Presence or absence of the deletion and the clinical diagnosis of dementia were coded as dichotomous variables. To examine the association between the DJ-1 deletion and the presence of dementia, Chi-square and Fisher exact tests were performed with two-sided significance thresholds of 0.05. Analyses were carried out using

SPSS for Windows, V. 11.0. Kinship coefficients between individual carriers of the DJ-1 deletion were calculated using the PEDIG software package (http://dga.jouy.inra.fr/sgqa/diffusions.htm). DNA was isolated from the blood samples using standard procedures (salting out method) [15]. All subjects were screened for the 14-kb deletion extending into the DJ-1 gene, which has been reported in this isolate before [2]. In order to establish if the carriers of the deletion were homozygous or not, these subjects were genotyped for one of the deleted DJ-1 exons (exon 5 – 354 bp band). Primers and general PCR conditions are reported elsewhere [2].

Results

In the genetically isolated population, 191 patients with dementia were ascertained from 188 independent nuclear families. The diagnosis of probable AD was established in 139 patients (122 with probable late onset and 17 with early onset). Possible late-onset AD was diagnosed in 18 patients, and clinically possible early-onset AD in four patients. Vascular dementia was clinically diagnosed in 10 patients, Lewy body dementia in nine patients, frontotemporal dementia in six patients, and the type of dementia was undetermined in five patients.

Through mutation screening we detected the deletion in DJ-1 in two cases, and also in one control (Table 1). All three were heterozygous for the deletion. Our data shows that there is no evidence for association between the DJ-1 deletion and the presence of dementia (OR= 1.35 (95% CI 0.12-15.10, p = 0.80).

Table 1. Mean age and DJ-1 deletion in patients with dementia and controls

	Mean Age (±sd)	DJ-1 Deletion carriers (%)	DJ-1 Deletion non-carriers (%)	Total
Dementia Cases	73.0 (7.7)*	2 (1.04)	189 (98.96)	191
Controls	77.3 (6.3)	1 (0.78)	128 (99.22)	129
Total	-	3 (0.94)	317 (99.06)	320

* Mean age at onset

Of the two cases who carried of the deletion, one was diagnosed with probable AD, and the other with amnestic syndrome. The second one, a man aged 90 years started to show signs of forgetfulness at 82 years, with slow deterioration of memory in subsequent years. The severity of memory loss showed periodic fluctuations. Twice he experienced a transient episode of loss of consciousness. He has no hallucinations and no signs of parkinsonism. According to his family history, his sister had dementia.

The other patient, an 86 years old woman, showed a mild and slowly progressive AD with onset at 74 years. The disease progressed slowly, with daily fluctuations of severity, gradually affecting more areas of cognition. She had no signs of parkinsonism or hallucinations. Her family history was negative for dementia.

We found one control to be a DJ-1 deletion carrier. At 67 years, he showed no signs of cognitive impairment, dementia or parkinsonism. Apart from diabetes mellitus type II, his medical history is negative, and he has no family history of dementia.

By means of genealogical research, 134 patients with dementia of any kind could be connected to a common ancestor within 11 generations. The three DJ-1 deletion carriers were related within 8 generations, their average kinship coefficient was 0.0036.Individuals one and three are closely related: the kinship coefficient among them is between (1/2) 6 (corresponding to 6 meiosis). Individual number two is more distantly related, showing a kinship coefficient with the other carriers that is between (1/2) 10 and (1/2) 13 (ten and thirteen meiosis).

Discussion

This case control analysis was designed to assess the association between a deletion in the DJ-1 gene and the presence of dementia. Here, we analysed 191 demented patients and 129 non-demented controls. We found no association between this deletion and the presence of dementia. In this study we found 3 heterozygous carriers of the DJ-1 gene deletion (two affected individuals and one control) that were related within 8 generations suggestion a common origin of the deletion.

The deletion in the DJ-1 gene was recently associated with autosomal recessive early onset parkinsonism in an isolated population in the Netherlands [2] and, even though the function of the DJ-1 protein is still unknown, this gene was previously described as a possible endogenous indicator of oxidative stress status in vivo [16]. The patient series of this study did not include the previously ascertained early onset PD patients present in this isolate [2]. At clinical examination, these four

patients were cognitively normal but neuropsychological tests were not performed. Therefore we cannot exclude the presence of minor neuropsychological abnormalities. Moreover, these individuals are still young to present with dementia [22].

Even though the number of heterozygous carriers in our sample is low and this could affect the way we can be able to adequately assess whether there is an association with dementia, the present data did not show evidence for an association between the DJ-1 deletion and dementia. Most likely, the functional copy of the gene present in these carriers supersedes the deleted one. One of the three carrier individuals was a cognitively healthy control. Given the age at onset of disease in the other two carriers, we cannot exclude that this person is still at risk to develop dementia.

In conclusion, we cannot exclude that carriers of this deletion in the DJ-1 gene have a higher risk of developing late onset Alzheimer's disease. Variations in the DJ-1 gene different from the deletion we found in this isolate may be associated with the risk of dementia. However, our results show that haploinsufficiency in the DJ-1 gene is not a major risk factor for dementia in this isolated population.

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Chapter 4

General Discussion

Alzheimer's disease is complex

Alzheimer's disease (AD) is a heterogeneous disorder and the process of finding genes involved in its pathophysiology is a challenge. Four genes are known to be implicated in the onset of AD, the amyloid precursor protein gene (APP) [1], presenilin-1 (PSEN-1) [2-6], and presenilin-2 (PSEN-2) [7, 8]. The other gene involved in AD pathology is the apolipoprotein (APOE) gene [9-12]. After decades of genetic research and hundreds of genes studied in relationship with AD [13], no new genes have been consistently associated with the disease.

AD is characterized by Amyloid β (Aβ) aggregation, the main component of plaques and, tau hyperphosphorylation and subsequent tangle formation. Further, the pathology of AD includes neurovascular dysfunction, cell-cycle abnormalities, inflammatory processes, oxidative stress, mitochondrial dysfunction and cholesterol metabolism in the brain, among others. The amyloid cascade hypothesis assumes that an imbalance between the production and clearance of Aβ in the brain is the initiating event ultimately leading to neuronal degeneration and dementia [16]. This hypothesis has dominated AD research and subsequent strategies for therapeutic drug development for over two decades. Key genetic findings that support this hypothesis include: a) mutations in APP, encoding the amyloid precursor protein leading to early onset AD b) people with Down's syndrome, who carry an extra copy of the APP gene, develop Aβ plaques and dementia early in life and c) the presence of a duplication of the APP locus in families with early onset AD [17] results in accumulation of Aβ peptides.

Another key player in AD pathology is the microtubule associated protein tau (MAPT). In the brains of AD patients, this protein is hyperphosphorylated, truncated and aggregated into neurofibrillary tangles (NFTs). The accumulation of abnormally hyperphosphorylated tau is associated with neurofibrillary degeneration and dementia. In vitro studies suggest that the tangle formation is partially controlled by the tau protein itself [18]. While various tauopathies have been explained by specific mutations in the MAPT gene encoding for tau [19, 20] the relationship between MAPT polymorphisms and AD is still not clear.

Chapter 2.2 describes a linkage and association analysis of 37 Single Nucleotide Polymorphism (SNPs) in the region including the MAPT gene, seven other confirmed genes and five putative genes in a family based study including 122 AD patients from the Genetic Research in Isolated Populations (GRIP) program. GRIP is conducted in an isolated population in the southwestern part of the Netherlands. In this population, we did not find any evidence of a role of MAPT in AD (chapter 2.2). We also performed a meta analysis of all studies conducted to date on the

MAPT gene and AD stratified by APOE*4 but also this analysis did not yield evidence for a role of MAPT. We did find higher linkage disequilibrium in cases than in controls for two SNPs 722 kb upstream of MAPT in the N-myristoyltransferase 1 (NMT1) gene and also nominally significant association for the haplotypes in this region and AD (p=0.02 before multiple testing correction). This result must be evaluated further but may be a false positive finding. Finally, our results suggest that is unlikely that MAPT is involved in AD.

Another gene, linked recently to tauopathies and of interest to AD is the granulin (GRN) gene. This gene was recently associated with tau-negative frontotemporal dementia linked to chromosome 17 (FTDP-17) located 1.4 Mb upstream from MAPT [21, 22]. Even though its function in the brain is not well understood, pathological studies that followed the identification of this gene suggest that the protein encoded by the GRN gene is essential for neuronal survival and even partial loss of GRN protein eventually leads to neurodegeneration [55, 56]. It has also been demonstrated that GRN activates mitogen-activated protein kinase (MAPK) signaling cascades modulating inflammation processes [26]. In chapter 2.2 we sequenced this gene in set of 17 AD patients from the GRIP study that show modest evidence for linkage to the region in chromosome 17 where GRN and MAPT are located; we did not find any sequence variations in the GRN gene in these patients.

The cyclin-dependant kinase 5 (Cdk5) protein is involved in both the Aβ and tau pathways. Evidence is accumulating that Cdk5 is involved in the abnormal phosphorylation of tau, contributing to the formation of NFTs [23]. Also, the Cdk5/p35 complex is a regulator of PSEN-1 metabolism and may be a key protein linking NFTs and senile plaques [24]. The evidence for a role of the CDK5 gene in AD is scarce and has been studied only in early onset AD. In chapter 2.3 we sequenced the CDK5 gene in a family with nine affected individuals and found that the rare allele of the SNP rs9278 is present in all affected individuals. This SNP, located 116 bp downstream the stop codon, was also over represented in early onset AD patients of Dutch and Swedish origin [25]. In chapter 3.1 we studied the association between the CDK5 tagging SNPs in the Rotterdam study. This is a population-based study of 7983 persons aged 55 or over. The study includes 389 prevalent and 160 incident patients with AD. In this population, an association was found between AD and another SNP rs2069442, (in single SNP and haplotype analyses) located 269 bp upstream the transcription start site. Although the AD GRIP and Rotterdam study differ in that the GRIP study is based in an isolated population while the Rotterdam Study is based in an outbred population, the finding of association to different SNPs in CDK5 is unexpected. Our earlier simulation studies suggest that genetic variants with a frequency of 1% or higher are unlikely to disappear from the GRIP population [57]. Although the functional effects of these variants is far from clear and the association described in chapters 3.1 and 2.3 remains to be confirmed, our

studies of this gene suggests that it may be involved in AD.

In this thesis, we studied various other candidate genes, including the gene encoding for the cholesteryl ester transfer protein (CETP). Similar to APOE, CETP is involved in the regulation of lipid metabolism. There is some evidence that CETP may interact with APOE [58]. In chapter 3.2 we studied the I405V polymorphism of CETP gene in relation to AD and investigated whether this polymorphism is independently associated with AD, or acts in concert with the APOE gene. We found a 1.8-fold increase in AD risk for VV carriers of the I405V polymorphism in those without the APOE*4 allele. No association was observed between this polymorphism and AD in those with the APOE*4 allele. The difference in the relationship between CETP and AD between APOE*4 carriers and non-carriers was statistically significant (p for interaction = 0.04). Our results suggest that the CETP gene increases the risk of AD independently of the APOE gene, probably through the regulation of cholesterol metabolism in the brain.

Another candidate gene study performed targeted the ubiquilin1 (UBQLN1) gene (chapter 3.3). A recent study suggested that the UBQLN1 gene is the gene explaining the linkage of AD to chromosome 9. SNP rs12344615, located downstream of exon 8 on the gene, was associated with AD in a family based sample that showed evidence for linkage to chromosome 9 [30]. As this gene is involved in the ubiquitination machinery, it may also play a role in Parkinson's disease (PD). In chapter 3.3 we analyzed the association of this SNP with AD, PD and cognitive function. We studied this SNP in a large population based study (the Rotterdam Study) and the GRIP study [31]. We did not find any significant increase in the risk of AD or PD for carriers of the CC genotype of SNP 12344615 in our overall and stratified analyses by APOE*4. Further, we did not detect an effect on age at onset of AD or PD in. Moreover, we detected no effect of this polymorphism on various cognitive tests. In our population there is no evidence for a role of UBQLN1 in AD. Of interest for future research is the non-significant association observed between the SNP rs1234615 and PD, which may be followed-up further.

The last candidate gene study concerns the DJ-1 gene. Mutations in DJ-1 lead to autosomal recessive early-onset parkinsonism, most likely through oxidative stress. Oxidative stress is not only involved in PD, but also in dementia. We evaluated the relationship between the DJ-1 gene and dementia in chapter 3.4. This case control analysis was based in the GRIP population where the first kindred with DJ-1 related to PD were originally identified [32]. We found no association between the DJ-1 deletion involved in PD and the presence of dementia (OR= 1.35, 95% CI 0.12-15.10, p = 0.80). We found 3 heterozygous carriers of the deletion (two affected individuals and one control) that were related within 8 generations suggestion a common origin of the deletion. Even though we cannot exclude that carriers

of this deletion in the DJ-1 gene have a higher risk of developing late onset AD, our results suggest that the PD related deletion in the DJ-1 gene is not a major risk factor for dementia in this isolated population.

In this thesis, 2 genome scans are described. The first genome scan was embedded in the isolated population (GRIP). These populations have been shown to be a powerful setting for finding genes for both Mendelian and complex disorders. One of the most important advantages of the GRIP study population is the availability of genealogic database of the community. Currently, the genealogy database includes data on over 90,000 people. In chapter 2.1 we performed a genome wide screen in the late-onset AD from the GRIP program including 112 AD patients. We detected evidence for linkage for previously established loci on chromosomes 1q23 [33-36] and 10 q23 [34, 35, 38-41]. The genes responsible for linkage are yet not known at either locus. In the region of chromosome 1 there are two obvious candidate genes for AD, the C-reactive protein (CRP) gene [42] and the nicastrin (NCSTN) [43] gene. We sequenced these genes but we did not find any mutation. We have now begun with the selection of other candidate genes in this region. Further, there was significant evidence for linkage of AD to chromosomes 3q23 and 18q21. At this stage, we have not resolved whether there are 2 loci or whether one of the two regions is a false positive. The linkage of AD to chromosome 3q23 was accompanied by association to this region, while this was not the case for chromosome 18. We therefore focused on sequencing the chromosome 3 region first. In this region there are evident candidate genes including the transferrin (TF) gene [44], the gene encoding for butyrilcholinesterase (BCHE) [45], the neprilysin (MME) gene [46] and the somatostatin (SST) gene. These genes were sequenced first but showed no mutations in the coding region or the intron exon boundaries. We started sequencing the region between markers D3S1587 (the right border of the linked region) and D3S1569 (marker with the highest LOD score). Based on the information provided on NCBI's build 36.1 (http://www.ncbi.nlm.nih.gov/) we identified 106 genes (80 confirmed and 26 putative) between these markers. A complete overview of the other genes selected for sequencing in this locus is shown in Table 1. Up to now we have not been able to find a gene that harbors a mutation. In the region on chromosome 18 we did not find any obvious candidate gene for mutation screening [13]. According to NCBI's build 36.1, between markers D18S1152 and D18S64 (region with the highest LOD score) there are 16 confirmed and five putative genes described which will be used as a starting point for the sequencing analysis of this region.

In chapter 2.3 we present the second genome scan. In a family including 9 AD patients, who all carried the APOE*4 allele, there was evidence of 2 regions segregating with the disease on chromosomes 1 and 7. We identified 112 genes in the region linked to AD in chromosome 1. We further identified 123 genes between

markers D7S661 and D7S2465 in chromosome 7. For chromosome 1, the CASP9, TNFRSF1B, and MTHFR genes were identified as the most interesting candidates after data mining the literature. The best candidate genes on chromosome 7 were CDK5 and NOS3. These genes have been previously associated with AD, [25, 47-49] or apoptosis [50, 51]. The CDK5 and CASP9 genes were selected first for mutation screening. No sequence variations were observed in the coding regions of the CASP9 and CDK5 genes but we found that allele A of the SNP rs9278 in the 3'UTR of the CDK5 gene present in all affected individuals. The CDK5 gene may explain the linkage of AD to chromosome 7 in this family; however, we cannot exclude epistatic effects of this or other genes on chromosome 7 with a gene on chromosome 1 or with APOE.

In this thesis we have performed 5 candidate gene studies and two genome screens. Although there is strong evidence for a locus on chromosome 3, the findings of our studies are either negative (MAPT, GRN, DJ-1, UBQLN1) or ambiguous (chromosome 18, CDK5). These findings reflect those of others working in the field of AD. The last decade of AD research has provided a great number of candidate genes that have been studied. Despite this enormous effort, many of these results could not be replicated. It is important to realize that there have been major limitations of the studies conducted to date. Often, the analyses have been based on only one SNP, which does not exclude a role of other polymorphisms in the gene. Also the studies have been frequently underpowered due to the small number of patients, making it difficult to detect genetic variants with only small effects [30]. Finally, a major problem encountered in genetic research is that of multiple testing. Since a large number of tests are performed in genetic research, the probability that a significant association is a false positive is high, in particular if one uses a type 1 error of 0.05 for statistical significance [59]. Further, as the a priori probability that a gene is involved in a disease is low, even for candidate genes, the posterior probability that a significant association is true positive is very low. These limitations also concern our studies, both in GRIP and the Rotterdam study. With the increasing number of SNPs available from the high-throughput sequencing efforts that have been conducted as part of the HapMap consortium [52, 53] and the increasing technological developments, a more detailed characterization of candidate genes by multiple SNPs is feasible in large series of patients. An important question is how large future studies have to be. Association studies should include at least 550 cases and 550 controls to detect effects of 1.5-2 explained by variants with allele frequencies of 10% or higher. To detect genes with smaller effects may require series of thousands of patients if not 10,000.

An important new development that spun-off from the HapMap project is the possibility to conduct genome wide association studies using SNP arrays including 500,000 to 1,000000 SNPs covering the full genome. The prospects of genome

wide association studies aiming to detect genetic variants with small effects, also ask for even larger cohorts ranging from 3000 to 10,000 cases. Genome wide association analysis will allow the identification of new genes and detailed evaluation of candidate genes. The major limitation at present is the costs related with the genotyping assays, but prices are expected to go down. Also there are a number of methodological problems to be tackled, in particular when targeting common variants associated with a small increase in risk. For these variants, it will be very difficult to distinguish those from the large number of false positive associations that are inevitability to occur in genome wide analysis of large series [60]. Replication studies may be a way to distinguish the true and false positive findings. This asks for multicenter collaborative studies. A key issue will be to standardize studies a priori to allow for replications.

Another important development in technology concerns the production of a technology capable of sequencing the human genome in a fast, cheap and reliable way. The new sequencing technologies are based on different approaches including parallel bead arrays, capillary electrophoresis and biochips among others. These will allow sequencing of up to 90 Mb with high quality results (accuracy higher than 99.99%) but at a high price. Next generation sequencers offer promising prospects of being able to screen up to 1 Gb of DNA but still these methodologies are not precise enough which limits the use of this technology in available populations. These developments are particular useful in the identification of genes, in regions identified in linkage studies. So far, several AD loci have been localized (chromosomes 1, 9, 10 and, 12) but it has been difficult to identify the causal genes in this region. High-throughput sequencing technologies are a very important tool to resolve this problem in the future.

Finally, the implementation of systems biology in research will be important in future research. Systems biology seeks to integrate different levels of information to understand how biological systems function by the study of the relationships and interactions between its various parts. It offers the potential to understand the complexity of human brain aging and neurodegenerative diseases. Systems biology based bioinformatic tools will help not only in the selection of suitable candidate genes or rather candidate gene pathways that can be studied simultaneously but also allows to evaluate and rank candidate genes in regions linked or associated to AD. Systems biology may form a biological basis for studies of interactions of genes. A major problem in complex traits such as AD is that many interactions between genes and environmental risk factors may occur. Although statistical interactions are found, these have been extremely difficult to replicate. Systems biology may provide a valuable tool in the future for the evaluation of the biological plausibility of these interactions.

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Table 1. Description of the genes selected for mutation screening in the locus on chromosome 3

Gene Symbol	Start*	End*	Size (bp)	Cytogenetic band	Amplicons Sequenced	Distance from marker D3S1587 (bp)
PLXND1	130808351	130756708	51643	3q21.3	43	-1473177
CPNE4	132736262	133236534	500272	3q22.1	26	454734
UBE1DC1	133861836	133879312	17476	3q22.1	16	1580308
TOPBP1	134802177	134863380	61203	3q22.1	27	2520649
TF	134947925	134980325	32400	3q22.1	17	2666397
SRPRB	135007367	135022213	14846	3q22.1	11	2725839
SLCO2A1	135134230	135231418	97188	3q21	19	2852702
RYK	135358667	135452276	93609	3q22	18	3077139
AMOTL2	135556880	135576096	19216	3q21-q22	17	3275352
EPHB1	135996950	136461999	465049	3q21-q23	22	3715422
PPP2R3A	137167257	137349423	182166	3q22.1	34	4885729
MSL2L1	137350450	137397378	46928	3q22.2	36	5068922
PCCB	137451838	137531695	79857	3q21-q22	15	5170310
TMEM22	138020668	138057420	36752	3q22.3	12	5739140
NCK1	138063763	138150658	86895	3q21	6	5782235
RASA2	142688616	142813887	125271	3q22-q23	25	10407088
RNF7	142939741	142947933	8192	3q22-q24	8	10658213
SLC9A9	144466754	145049979	583225	3q24	22	12185226
MME	156280153	156384186	104034	3q25.1-q25.2	36	23998625
BCHK	167037944	166973387	64557	3q26.1-q26.2	9	34756416
SST	188869388	188870895	1508	3q28	3	56589367

*Gene positions were obtained from build 36.1 from NCBI

Minus sign indicate upstream from the marker star point at 132281528 bp (build 36.1 from NCBI)

Summary

Summary

Alzheimer's disease (AD) is the most frequent cause of dementia and thus is a major public-health problem. Age and genetic predisposition to the disease are the most important risk factors. In 2001 more than 24 million people in the western world had dementia. This number is expected to double every 20 years up to 81 million in 2040 because of the anticipated increase in life expectancy. Genetically, AD is a heterogeneous disorder with both familial and sporadic forms.

Chapter 1 is a general introduction on epidemiological and genetic factors of AD and also describes the different studies performed in this thesis.

In chapter 2 the linkage studies are presented. Chapter 2.1 describes a whole genome screen on 112 AD patients and their first-degree relatives from the Genetics Research in Isolated Populations (GRIP) study. Of the 112 patients, 103 could be connected into an extremely large and complex pedigree. This pedigree cannot be analyzed with available linkage software. In this study, we developed an algorithm for splitting complex pedigrees to allow us to conduct linkage analysis. Then we determined genome wide significance thresholds for linkage analysis using the sub pedigrees obtained by our pedigree cutting algorithm and finally, we performed linkage analysis sing these sub pedigrees. We localized 3 loci previously described to be involved in AD on chromosomes 1, and 10 yielding a strong proof of principle of our splitting method. We also identified genome wide significant novel loci for AD on chromosomes 3q23 and 18q21, since the same family contributes to both loci we must stablish if our findings represent false positives. We sequenced the obvious candidate genes on chromosome 1, nicastrin (NCST) and C-reactive protein (CRP) but no mutations on these genes were found. Since we found evidence for association with AD to the locus on chromosome 3, we also sequenced 21 genes on this locus including the transferrin gene, the gene encoding for butyrilcholinesterase, the neprilysin gene and the somatostatin gene. We screened these genes for mutations but nohave been found.

In chapter 2.2 we present a combined linkage and association study between the microtubule associated tau protein (MAPT) and granulin (GRN) genes and AD. In this study we investigated the association between the region including MAPT plus other eight confirmed genes (including the GRN gene) and five putative genes and AD in patients from the GRIP study. We studied 122 AD patients and 85 control subjects using an extensive Single Nucleotide Polymorphism (SNP) panel. No association was found for any individual SNP of the MAPT gene and linkage analysis including 7 tagging SNPs in MAPT excluded linkage. Nominally significant association was found for two SNPs located 722 kb upstream the MAPT gene

in the N-myristoyltransferase 1 (NMT1) gene. The GRN gene was sequenced in 17 patients but no mutations were found. We also performed a meta analysis of all studies conducted to date on the associations between the MAPT gene and AD stratified by APOE*4. We found a very modest non-significant association for carriers of the H2 haplotype compared to H1/H1 carriers, only in those with the APOE*4 allele. These findings suggest that the MAPT and GRN genes do not play a role in AD in our population.

In AD, in chapter 2.3 we performed a genome wide linkage analysis on an extended Dutch family with nine affected individuals. Two linkage peaks above 1.0 were found on chromosomes 1 and 7. A further two locus linkage analyses using only affected individuals yielded a LOD score of 2.92 for the combination of these 2 loci suggesting an epistatic effect between these two loci. We used a literature mining strategy to prioritize candidate gene selection from the genes present in the defined loci. Our literature mining approach suggested as potential candidate genes in these two regions the CASP9, TNFRSF1B and MTHFR genes on chromosome 1 and CDK5 and NOS3 on chromosome 7. These genes have been previously associated with AD and apoptosis. CASP9 on chromosome 1 and CDK5 on chromosome 7 were selected for sequencing. We found no variants in the coding exons or the splice sites of these genes but we found the rare allele of SNP rs9872 on the CDK5 gene present in all affected individuals suggesting that this gene maybe involved in AD in this family.

Chapter 3 describes the genetic association studies performed in this thesis. Three studies were carried out using the Rotterdam Study (chapters 3.1-3.3) a large prospective study of determinants of diseases in the elderly and one in the GRIP study (chapter 3.4). In chapter 3.1 we tested the association between 5 SNPs on the CDK5 gene and AD. We found a significantly increased risk of AD for carriers of the GG genotype of SNP rs2069442 (OR=1.8, 95% CI 1.16-2.79, p=0.009) in those without APOE*4.. Our haplotype analysis showed that haplotype C, tagging the G allele of SNP rs2069442, was significantly associated (p=0.05) to AD. These result suggest that alterations in the expression of the Cdk5 protein may be relevant in neurodegenerative processes Taken together, the results of chapters 2.3 and 3.1 underscore the relevance of this gene on AD.

We also evaluated the relationship between the cholesteryl ester transfer protein (CETP) and AD in chapter 3.2. Here we show that there is a positive association between the VV carriers of the I405V polymorphism of the CETP and AD independently of the APOE gene and systemic HDL levels. Since the VV genotype is associated with lower levels of CETP and higher HDL, our study is compatible with the view that low CETP levels may increase the risk of AD through a reduction in neuronal repair capacity.

In chapter 3.3 we investigated the relationship between the ubiquilin 1 (UBQLN1) gene and AD, Parkinson's disease (PD) and cognitive function. This gene is involved in the ubiquitination machinery, related with general protein degradation. A SNP located downstream of exon 8 (rs12344615) of the UBQLN1 gene has been associated with AD. We found no significant difference in risk of AD or PD for carriers of the CC genotype of SNP 12344615 in our overall and stratified analyses in the Rotterdam Study. We did not find an association of this SNP and AD in our family based study, we did not find linkage of AD to the region including the UBQLN1 gene, and we detected no effect of this polymorphism on cognitive function. Our data suggest that it is unlikely that the SNP 12344615 of the UBQLN1 gene is related to AD, PD or cognitive function.

Chapter 3.4 shows the relationship between a 14-kb deletion extending into the DJ-1 gene and dementia using a case-control study of dementia patients and controls ascertained from GRIP study. We analysed 191 demented patients and 129 non-demented controls. We found no association between this deletion and the presence of dementia. There were 3 heterozygous carriers of the DJ-1 gene deletion (two affected individuals and one control) that were related within 8 generations suggestion a common origin of the deletion. Form our results we cannot exclude that carriers of this deletion in the DJ-1 gene have a higher risk of developing AD. Variations in the DJ-1 gene different from the deletion may be associated with the risk of dementia. However, our results show that haploinsufficiency in the DJ-1 gene is not a major risk factor for dementia in this isolated population.

Finally, chapter 4 provides a general discussion on the results obtained on the studies on AD presented in this thesis and all findings are put into perspective for future studies.

Samenvatting

Samenvatting

De ziekte van Alzheimer (AD) is de meest voorkomende oorzaak van dementie en daarmee een belangrijk public health probleem. De belangrijkste risicofactoren voor AD zijn leeftijd en genetische aanleg. In 2001 leden in Westerse landen meer dan 24 miljoen mensen aan dementie. Door de voorspelde toenames in de levensverwachting (vergrijzing) zal naar verwachting zal dit aantal elke 20 jaar verdubbelen en toenemen tot 81 miljoen patiënten in het jaar 2040. Vanuit genetisch oogpunt bezien is AD een heterogene ziekte, met zowel familiaire als sporadische varianten.

Hoofdstuk 1 is een algemene inleiding waarin achtergrondinformatie over epidemiologische en genetische invloeden op AD wordt gegeven. Ook worden in dit hoofdstuk de verschillende studies die aan dit proefschrift ten grondslag liggen beschreven. In hoofdstuk 2 worden de resultaten van koppelings analyses beschreven. We voerden een koppelings analyse uit op de volledige genomische data van een groep van 112 AD patiënten en hun eerstegraads familieleden die deelnamen aan de Genetics Research in Isolated Populations (GRIP) studie; de resultaten hiervan staan beschreven in hoofdstuk 2.1. Van de 112 patiënten konden er 103 teruggeleid worden naar een zeer grote en complexe stamboom, die niet met de beschikbare linkage programmatuur geanalyseerd kon worden. Daarom ontwikkelden we een algoritme waarmee complexe stambomen opgesplitst kunnen worden. Gebruik makend van deze sub-stambomen werden voor de volledige genomische data significantie-drempelwaarden bepaald voor koppelings analyse, waarna we de linkage-analyses konden uitvoeren.

We lokaliseerden 3 loci op de chromosomen 1, 3 en 10, waarvan al eerder een verband met AD beschreven was. Dit gaf sterke aanwijzingen dat ons algoritme in principe werkte. We ontdekten ook een nieuw locus voor AD op chromosoom 18q21. Bij sequencing van de voor de hand liggende kandidaat-genen op chromosoom 1, nicastrin (NCST) en C-reactive protein (CRP) vonden we geen mutaties binnen deze genen. Op het locus op chromosoom 3 doorzochten we de hele (exonische en splice donor sites) DNA volgorde van 21 genen, waaronder het transferrine gen, het gen dat codeert voor butyrilcholinesterase, het neprilysine gen en het somatostatin gen. Bij het screenen van deze genen op mutaties werden geen varianten gevonden.

In hoofdstuk 2.2 wordt een gecombineerde koppelings- en associatie studie tussen de microtubule-geassocieerde tau proteïne (MAPT) en granuline (GRN) genen en AD beschreven. Voor deze studie onderzochten we de associatie tussen de regio waarop MAPT en acht andere bevestigde genen (waaronder het GRN gen) liggen

en vijf mogelijke genen en AD bij patiënten uit de GRIP studie. We onderzochten gegevens van 122 AD patiënten en 85 controle-personen, gebruik makend van een uitgebreid Single Nucleotide Polymorphism (SNP) panel. Voor geen enkele SNP van het MAPT gen werd een associatie gevonden. Koppelings analyse van 7 tagging SNPs in MAPT maakte duidelijk dat er geen sprake van koppeling was. Een nominaal significante associatie werd gevonden voor twee SNPs die 722 kb upstream liggen van het MAPT gen binnen het N-myristoyltransferase 1 (NMT1) gen. Het GRN gen werd bij 17 patiënten gesequenced maar er werden geen mutaties gevonden. We voerden een meta-analyse uit van alle studies die tot dan toe waren uitgevoerd met betrekking tot de associatie tussen het MAPT gen en AD, gestratificeerd voor APOE*4. Er werd een heel klein, niet-significant verband gevonden voor dragers van het H2 haplotype in vergelijking met de H1/H1 dragers, uitsluitend bij diegenen met het APOE*4 allel. Deze resultaten suggereren dat de MAPT en GRN genen geen rol spelen bij AD binnen onze populatie.

Het doel van het onderzoek dat ik hoofdstuk 2.3 beschreven is was om genen te ontdekken die betrokken zijn bij AD. We voerden een koppelings analyse uit op de volledige genomische data binnen een uitgebreide Nederlandse familie, waarvan 9 personen AD hadden. Er werden twee koppelingspieken groter dan 1.0 gevonden op chromosomen 1 en 7. Vervolgens werden in twee koppelings analyses waarbij alleen de gegevens van aangedane personen werden geanalyseerd resulteerde in een LOD score van 2.92 voor de combinatie van deze 2 loci, wat een epistatisch effect tussen deze loci suggereert. Een literatuur mining strategie om prioriteiten te kunnen stellen tussen de potentiele kandidaat genen die op de gedefinieerde loci liggen. Deze benadering suggereerde dat de potentiele kandidaatgenen in de regio op chromosoom 1 de genen CASP9, TNFRSF1B and MTHFR waren. Voor chromosoom 7 waren dit de genen CDK5 en NOS3. Van deze genen zijn al eerder associaties met AD en apoptose beschreven. We selecteerden CASP9 op chromosoom 1 en CDK5 op chromosoom 7 om te sequencen. Er werden geen varianten in de coderende exonen of de splice sites van deze genen, maar we ontdekten dat het zeldzame allel van SNP rs9872 van het CDK5 gen aanwezig was bij alle aangedane personen, wat erop wijst dat dit gen betrokken zou kunnen zijn bij AD in deze familie.

Hoofdstuk 3 beschrijft de genetische associatie studies die binnen het kader van dit proefschrift werden uitgevoerd. Er werden drie studies uitgevoerd (hoofdstuk 3.1-3.3) binnen het cohort van de Rotterdam Studie; een groot prospectief onderzoek dat is opgezet om ziekten bij ouderen te bestuderen. Ook werd een onderzoek uitgevoerd binnen de GRIP (Genetic Research in Isolated Populations) studie (hoofdstuk 3.4). In hoofdstuk 3.1 testten we of er een associatie was tussen 5 SNPs op het CDK5 gen en AD. Binnen de groep mensen zonder APOE*4 bleken dragers van het GG genotype van SNP rs2069442 een significant verhoogd risico voor AD te hebben (OR=1.8, 95% BHI 1.16-2.79, p=0.009). De haplotype analyse demon-

streerde dat haplotype C, die het G allel van SNP rs2069442 markeert, significant geassocieerd was met AD (p=0.05). Deze resultaten suggereren dat veranderingen in de expressie van het CDK5 eiwit relevant zou kunnen zijn voor neurodegeneratieve processen. De resultaten van hoofdstuk 2.3 en 3.1 illustreren hoe belangrijk dit gen is voor AD.

In hoofdstuk 3.2 evalueerden we het verband tussen het cholesteryl ester transfer proteïne (CETP) en AD. Er werd een positief verband gevonden tussen de VV dragers van het I405V polymorfisme van het CETP en AD dat onafhankelijk was van het APOE gen en systemische HDL niveaus. Aangezien het VV genotype geassocieerd is met lagere CETP en hogere HDL spiegels, passen deze bevindingen binnen de theorie dat lage CETP spiegels de kans op het krijgen van AD kunnen verhogen door middel van een reductie in de neurale repair capaciteit.

In hoofdstuk 3.3 onderzochten we het verband tussen het ubiquiline 1 (UBQLN1) gen en AD, de ziekte van Parkinson (PD) en cognitieve functie. Dit gen speelt een rol in de ubiquitinatie macherinerie en is gerelateerd aan algemene eiwit degradatie. Een SNP die downstream van exon 8 (rs12344615) op het UBQLN1 gen ligt, was al eerder geassocieerd met AD. We vonden geen significant verschil in de kans op het krijgen van AD of PD voor dragers van het CC genotype van SNP 12344615 in onze algemene en gestratificeerde analyses binnen de Rotterdam Studie. Er werd geen verband gevonden tussen deze SNP en AD in onze family based studie, vonden geen linkage van AD met de regio waarin het UBQLN1 gen ligt, en vonden geen effect van dit polymorfisme op cognitieve functie. Uit onze gegevens blijkt dat SNP 12344615 van het UBQLN1 gen waarschijnlijk niet gerelateerd is aan AD, PD of cognitieve functie.

Hoofdstuk 3.4 beschrijft de relatie tussen een 14-kb deletie die reikt tot binnen het DJ-1 gen en dementie, zoals die gevonden werd in een patiënt-controle studie van dementie patiënten en controlepersonen uit de GRIP studie. We analyseerden de gegevens van 191 patiënten met dementie en 129 controlepersonen die niet leden aan dementie. Er werd geen verband gevonden tussen deze deletie en dementie. Er waren 3 heterozygote dragers van de DJ-1 gen deletie (twee aangedane patiënten en één controlepersoon) die binnen 8 generaties gerelateerd waren, wat een gemeenschappelijke oorsprong van de deletie suggereert. Gebaseerd op deze resultaten kunnen we niet uitsluiten dat dragers van deze deletie in het DJ-1 gen een verhoogde kans hebben om AD te krijgen. Variaties binnen het DJ-1 gen, anders dan de deletie, kunnen geassocieerd zijn met het risico op dementie. Onze resultaten tonen wel dat haploinsufficientie in het DJ-1 gen geen belangrijke risicofactor voor dementie is binnen deze geïsoleerde populatie.

In het laatste hoofdstuk, hoofdstuk 4, worden de resultaten van deze studies

naar AD bediscussieerd. Ook worden alle bevinden in perspectief geplaatst voor toekomstige studies.

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About the author

Alejandro Arias was born June 22nd, 1975 in Bogotá, Colombia. One year later his parents moved to the coastal city of Barranquilla where he attended school at the Marymount School Barranquilla and graduated in June 1992. Following his graduation, he entered the obligatory military service from August of that same year until August 1993. After finalizing his military duties, he entered the Pontificia Universidad Javeriana to receive training as a biologist. In the year 1998 he started his work as a trainee in the Human Genetics Institute from the same university. Here he carried out the study included in his bachelor degree thesis and he graduated in the year 2000. In 1998 he also started working as a high school teacher in the Fundación Colombia School, where he worked until July 2001. That year he received a scholarship from the Netherlands University Foundation for International Co-operation (NUFFIC) to start a Master of Science program from the Netherlands Institute for Health Sciences (NIHES) at the genetic epidemiology unit of the department of Epidemiology & Biostatistics of ErasmusMC. He obtained his masters degree in June 2002 and immediately started a Doctor in Science program, which he finalized in June 2003. Later that year he started the work described in this thesis under the supervision of professor Cornelia van Duijn and professor Ben Oostra. He is currently the laboratory manager of the Department of Epidemiology & Biostatistics of ErasmusMC.

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