Genetic Determinants of Cognitive Function and Age-Related Brain Changes

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Part I

Introduction

01 General introduction, outline, and scope of the thesis

The brain is by far the most complicated structure of the human being, and its malfunction is characterized by various degrees and types of morbidity. Several brain functions deteriorate with increasing age during life. Cognitive decline and age-related brain pathology are common in the elderly, but these changes may also become manifest early in life and preceding the onset of clinical symptoms of disease. The detection of early changes may be relevant for therapeutic interventions to prevent disease, and are therefore also increasingly targeted in genetic research as endophenotypes. Endophenotypes are defined as heritable phenotypes that are related to the disease of interest, and are typically approached as quantitative outcomes, i.e., instead of hypertension, the endophenotype of interest is systolic or diastolic blood pressure. In contrast to classical risk factors in epidemiology, an endophenotype is by definition not uniquely associated to a single disease. Blood pressure for example, is consistently associated to various clinically relevant outcomes such as stroke, myocardial infarction and heart failure. There is an increasing interest in the genetic research of endophenotypes, and genome-wide association studies of endophenotypes have been very successful [1,2]. In this thesis I focus on cognitive function and age-related brain changes early in life as endophenotypes for late-life brain disease and as targets for early prevention.

Cognitive deterioration can be seen in pre-clinical stages of neurodegenerative and neuropsychiatric disorders like dementia, schizophrenia, bipolar disorder and attention deficit hyperactivity disorder (ADHD) [3-5]. Cognitive function is a broad concept referring to multiple cognitive domains, among which memory, language, executive function and visuospatial ability. Although the domains are highly correlated, it is known that specific domains are related to specific diseases. Cognitive function is in part determined by our genetic make-up. The heritability is estimated to around 40% [6] and there have been various studies that have tried to identify genes explaining the heritability of cognitive functions. These included candidate gene studies [7,8], linkage studies [9-13] and genome-wide association studies [14-17]. The genes and chromosomal regions that have been found so far are partly explained by genes related to neuropsychiatric disease, and partly by genes related to dementia and Alzheimer's disease (AD) with the Apolipoprotein E gene as genetic factor with one of the strongest effects. In the studies presented here, we will focus on a cognitive test battery targeting AD [18]. Dementia is one of the most common causes of morbidity and mortality in the Western society (prevalence of 25 million cases worldwide), in which Alzheimer disease accounts for over 70% of cases [19,20]. Regarding the high prevalence and major impact of these diseases, early diagnosis and treatment strategies have a high priority in neuroscience. Identifying risk factors for cognitive decline would benefit our increasingly elderly population.

At postmortem, AD is characterized by neurofibrillary tangles and amyloid plaques [21]. The major components of the amyloid plaques are amyloid beta (Aβ) proteins, which are formed after

proteolytic processing of the amyloid precursor protein (APP). Aβ pathology is also found in brains of cognitively healthy elderly who may develop the disease later. Aβ levels can be measured in plasma probably due to leakage through the blood brain barrier. It has been suggested that the plasma Aβ42/Aβ40 ratio may be a suitable marker for early AD pathology [22,23]. Various epidemiological studies have show that low Aβ42/Aβ40 ratios in plasma are associated with an increased risk of dementia [22,23]. Since also plasma Aβ levels are heritable with estimates ranging from 54 to 73% [24], they are interesting endophenotypes for genetic research.

Another approach to study early brain changes is magnetic resonance imaging (MRI), which is a sensitive non-invasive imaging technique to visualize brain pathology. Hippocampal atrophy, white matter lesions, microbleeds and lacunar infarcts are the most common age-related brain changes visible on MRI, and have been associated with hypertension, stroke, dementia and cognitive impairment [23-28]. The mechanisms underlying the pathogenesis of these changes are largely unknown. Several studies suggest that structural changes in blood vessels in the brain lead to ischemic damage, causing white matter lesions and lacunar infarcts, and that leakage of red blood cells might lead to microbleeds and subsequent hemosiderin depositions [29,30]. Hypertension and atherosclerosis most likely cause damage to blood vessel walls, but also amyloid angiopathy plays an important role in vascular related events. [31]. The heritability estimates for age-related brain changes are high, ranging from 55 to 71% for white matter lesions [32-35] and ranging from 40 to 69% for medial temporal lobe atrophy [36-40].

The rationale behind the studies presented in this thesis is to use cognitive function, plasma Aβ levels and MRI changes as endophenotypes in the search for new determinants of neurodegenerative and neuropsychiatric diseases. We followed different approaches for our genetic studies. We used a candidate-gene approach by studying genes that have previously been reported in these traits or were involved in a plausible pathway for the disease. In the search for unknown genes, we conducted both genome-wide association studies as well as linkage studies. The underlying hypothesis for the genome-wide association approach is the common-disease common-variant hypothesis. Genome-wide linkage is considered in particular valuable in identifying rare variants with large effects. There is an increasing awareness that a large part of the heritability remains unexplained despite genome-wide association studies. Family-data may contribute to unravelling the genetics of complex traits and add to the data derived from current genome-wide associations studies [43].

The chapters of this thesis are divided in 4 main parts.
Part II focuses on determinants of cognitive function. Chapter 2 describes an epidemiological study on the relation between common cardiovascular risk factors and cognitive function.

Chapter 3 presents a candidate gene study of APOE. Chapters 4 and 5 present genome-wide approaches: a linkage study in chapter 4 and a genome-wide association study in chapter 5.
Part III focuses on genetic determinants of AD. Chapter 6 presents a candidate gene study and meta-analysis of the Cathepsin D gene and chapter 7 describes a collaborative study considering interactive effects of the transferrin and HFE genes. Part IV focuses on determinants of age-related brain changes in hypertensive patients. Here, the results of three candidate gene studies are presented in chapters 8, 9 and 10. Finally, a short overview of all findings, followed by a discussion on the rating of genetic association studies is given in part V.

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Part II

New determinants of cognitive function

02 Insulin-resistance and metabolic syndrome are related to executive function in women in a large familybased study

Abstract

Background While type 2 diabetes is well-known to be associated with poorer cognitive performance, few studies have reported on the association of metabolic syndrome (MetS) and contributing factors, such as insulin-resistance (HOMA-IR), low adiponectin-, and high C-reactive protein (CRP)- levels. We studied whether these factors are related to cognitive function and which of the MetS components are independently associated.

Methods The study was embedded in an ongoing family-based cohort study in a Dutch population. All participants underwent physical examinations, biomedical measurements, and neuropsychological testing. Linear regression models were used to determine the association between MetS, HOMA-IR, adiponectin levels, CRP, and cognitive test scores. Cross-sectional analyses were performed in 1898 subjects (mean age 48 years, 43% men).

Results People with MetS had significantly higher HOMA-IR scores, lower adiponectin levels, and higher CRP levels. MetS and high HOMA-IR were associated with poorer executive function in women ($p=0.03$ and $p=0.009$). The most consistent individual component of MetS, contributing to the association with executive test scores was systolic blood pressure.

Conclusions MetS and HOMA-IR are associated with poorer executive function in women. Of the MetS components, systolic blood pressure is independently associated with executive function.

Introduction

Cognitive impairment is a common problem in the elderly and an important predictor of dementia (DSM-IV). There are multiple risk factors contributing to cognitive decline. For long, type 2 diabetes has been recognized as a major risk factor of dementia [1]. Epidemiological studies indicate that hyperinsulinemia and insulin-resistance, which characterize type 2 diabetes, may cause cognitive dysfunction [2,3]. Insulin-resistance is also a feature of the metabolic syndrome (MetS) and there is increasing interest in the role of MetS in cognition. The syndrome is defined as the presence of at least 3 out of 5 factors, which include central obesity, hyperglycemia, hypertension, hypertriglyceridemia, and low high-density lipoprotein cholesterol (HDL-c) levels [4]. It has a high prevalence of over 22% in Western societies [5], and is an important risk factor of cardiovascular disease [6,7]. Potentially, MetS is an important, modifiable, determinant of cognitive pathology [8-10]. Previous studies have shown associations between cognitive dysfunction and MetS, but results are not consistent and predominantly concern elderly people [11]. Of its individual components, hypertension and hyperglycemia have proven to be the most consistent determinants of cognitive function in a recent review of published studies [12].

Adipose tissue also plays an important role in the etiology of MetS. Adipose tissue functions as endocrine tissue, among which secretion of adipocytokines and cytokines, which indirectly affect C-reactive protein (CRP) levels [13-15]. The visceral fat depot is thought to play an important role in adipocytokine secretion and MetS [16]. Several groups have suggested that in particular adiponectin

might be a promising biomarker for MetS [17-19]. Whether it is related to cognitive function in humans is not known, but animal studies indicate that adiponectin may influence brain metabolism [20]. Regarding CRP levels, it has been reported that the association of MetS with cognitive function is stronger in those with high levels of CRP [21,22].

In this study, we examined whether MetS, insulin-resistance, plasma adiponectin and CRP- levels are related to cognitive function. In addition, we investigated which MetS components attributed independently to the association.

Methods

Study population

The study was performed in the Erasmus Rucphen Family Study (ERF), which is an ongoing family-based cohort study in a genetically isolated population [23]. The ERF population includes around 3000 individuals, all living descendents of 22 couples who, at the end of the 19th century had at least six children baptized in the community church. Extensive data on cardiovascular risk factors, body composition, cognitive functioning, and blood chemistry are available. These data were collected between 2002 and 2005. All participants gave informed consent and the study was approved by the Medical Ethics Committee at ErasmusMC.

Clinical and laboratory assessments

To define MetS, we used the definition proposed by the National Cholesterol Education Program Adult Treatment Panel (ATPIII) [4]. Waist circumference was measured exactly halfway the distance between the lower costal margin and the iliac crest [24]. Blood pressure was measured twice with an automatic device in a sitting position [25].

Blood was taken after fasting of at least 8 hours and serum triglycerides (TG), serum HDL-c, and plasma glucose (FPG) were assessed. Fasting plasma insulin was analyzed with the INS-Irma kit of Biosource (cat.#: KIP1254). Insulin-resistance was determined as homeostasis model assessment insulin-resistance (HOMA-IR) [26], which was computed by multiplying fasting plasma insulin (micro-international units per milliliter) and fasting plasma glucose (millimoles per liter)/22.5. Total plasma adiponectin was analyzed with the Human adiponectin RIA kit (cat.#: HADP-61HK) of Linco Research. To measure total plasma CRP, we used the US C-reactive protein ELISA (cat.# DSL-10-42100) of Diagnostic Systems Laboratories, Inc (expected range within 90th percentile: 254-16104 ng/ml).

We considered age, gender, smoking, alcohol use, education, depression, and Apolipoprotein E genotype (APOE) as possible confounders. Use of alcohol was defined as drinking at least 1 glass each day. All subjects were categorized into four groups with respect to level of education: 1. primary education; 2. lower vocational education; 3. intermediate vocational education and secondary education; and 4. higher vocational education and university training. The years of schooling in the first category corresponded to 6 years; in the second to 7-10 years; in the third to 11-14 years; and

in the fourth to 15-18 years.

Depressive symptoms were measured with the Hospital Anxiety and Depression Scale (HADS-D). Genomic DNA was extracted from whole blood samples, utilizing the salting out method. Samples were genotyped for APOE with a Taqman allelic discrimination Assay-By-Design (Applied Biosystems, Foster City, CA). The measurements were performed conform the manufacturers protocols. Clinical and laboratory data was not available for 721 participants.

Cognitive function

The battery of neuropsychological tests measured different cognitive domains and a Dutch validated version was available [27,28]. General cognitive ability was measured with the Dutch Adult Reading Test (DART). Memory function was measured with a word learning test with 5 exposure trials from which four scores were derived: immediate memory, defined as the number of correctly recalled words in trial 1; learning, defined as the total number of recalled words in trials 2 to 5; delayed recall, defined as the number of correctly recalled words after 20 minutes; and recognition, defined as the number of correctly recognized and correctly rejected words. Executive function was assessed with the Trail Making Test parts A and B (TMT), the Stroop Color and Word Test cards I, II and III, and verbal fluency tests. TMT and Stroop are time-demanding tasks in which participants had to complete the test as quickly as possible either by connecting letters and numbers (TMT) or by naming words and colors (Stroop) [29,30]. For analyses, we used time in seconds depicted as the ratios of TMT-B/TMT-A and Stroop-III/Stroop-II. These ratios were used to adjust for mental slowness, which is mainly reflected in TMT-A and Stroop-II. When participants timed out, scores were imputed for TMT-B and Stroop-III (3-8%). The imputation was performed using missing value analysis in SPSS through expectation maximization algorithms using age, sex, education and either score on TMT-A or Stroop-I and Stroop-II as predictor variables. For the verbal fluency tests, participants had to name as many animals and as many words starting with the same letter (D, A and T), each in one minute. The score was defined as the total number of correct words on both tasks. Lastly, visuo-spatial ability was assessed with the WAISIII block-design subtest.

In addition to analyzing individual test scores, three composite scores were used: 1. memory (z-memory); 2. executive function (z-executive); 3. global cognition (z-global). These scores were computed by taking the average of z-scores of the individual tests, which were computed by subtracting the mean from the test score divided by the standard deviation [31]. For time-demanding tasks, the test score was subtracted from the mean. The composite scores were only computed when at least 3 memory scores (z-memory), 2 executive scores (z-executive) and 6 total test scores (z-global) were available. In the current study, we excluded participants who had a history of stroke or dementia (N=21) or were physically not able to perform a neuropsychological test (e.g. sensory handicaps (N=6), illiteracy (N=28), other (N=10)). There were 10 participants with missing test scores due to technical problems.

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Statistical analysis

One large pedigree was extracted from the genealogical database for eligible participants. The pedigree was cut into smaller pedigrees before analyses with the software PedCut [32]. In total, 1898 people could be linked to pedigrees with a maximum bit size of 18 and these were included in the analyses.

Baseline descriptive analyses were performed with SPSS version 15.0, using the independent-samples T-test (continuous variables) and Chi-square statistics (categorical data). Deviations from normality were checked using P-P plots and histograms. C-reactive protein levels were transformed by taking the common logarithm. Because high levels of CRP could have been caused by acute inflammation, all values of the log-transformed variable greater than three times the standard deviation above the mean were excluded.

Possible confounders were first analyzed as single covariates in two regression models with either MetS or cognitive test scores as dependent variables. Also, interaction terms of MetS, HOMA-IR, adiponectin and CRP with age and sex were analyzed in these models. Covariates that were significantly associated were then entered simultaneously into a model using backward regression. We removed all covariates with a p-value > 0.10. The final model included age, level of education, HADS-D score, alcohol use and inbreeding coefficient. The analyses were stratified by sex, because the interaction terms of MetS with sex were significant.

To identify associations between cognitive test scores and MetS, HOMA-IR, adiponectin, and CRP and to adjust for family relationships, we performed regression analyses using the SOLAR software version 4.1.0 [33]. The MetS was included in the model as a binomial variable, and additionally as a continuous variable ranging from 0 to 5 to test the effect of increasing number of MetS components. To determine to what extend the association of MetS with cognitive test scores was accounted for by its individual components, all individual MetS components were analyzed simultaneously in one regression model. The components were entered in the model as continuous variables. We used quartiles of HOMA-IR, adiponectin and CRP with the lowest quartile as a reference category to estimate the association with adipose tissue endocrine function.

Results

The mean age of the study population was 47.7 (±14.3) years in women and 49.2 (±14.1) years in men, with a range of 18 to 86 years and less than 10% older than 67. Metabolic syndrome was present in 227 (21%) women and in 207 (25%) men. Of these, 155 (68%) women and 150 (73%) men fulfilled three of the MetS diagnostic criteria, 63 (28%) and 54 (26%) fulfilled four and 9 (4%) and 3 (1%) fulfilled all five. Null criteria were fulfilled by 282 women and 153 men. One and two criteria were fulfilled by respectively 385 and 305 women and 182 and 157 men. With regard to the individual MetS components in the total population, central obesity was present in 28% of women and 24% of men, hypertension in 49% and 70%, dyslipidemia in 47% and 43%, and finally increased glucose levels in 5% and 7%. A comparison of the individual MetS components is given in Table 1.

Table 2 shows the baseline characteristics of men and women with and without MetS regarding determinants of cognitive function and vascular and endocrine factors. Individuals with MetS were older, had lower education and had higher scores on the depression scale. Men with MetS drank alcohol more frequently, whereas women with MetS drank alcohol less frequently. The endocrine

Metabolic Syndrome in the Erasmus Rucphen Family study (n=1898)

	Men		Women	
	MetS absent (n=615)	**MetS present** (n=207)	**MetS absent** (n=849)	**MetS present** (n=227)
MetS components				
Waist circumference (cm)	90.1 (9.5)	105.1 (9.3)	78.2 (9.5)	94.5 (10.2)
SBP (mm Hg)	140.7 (17.2)	151.2 (17.3)	131.2 (18.4)	152.9 (21.0)
DBP (mm Hg)	80.3 (9.5)	85.5 (9.4)	76.8 (9.3)	84.0 (10.0)
Use of antihypertensive medication (%)	11.7	34.3	9.0	38.3
Glucose (mmol/L, FPG)	4.6 (0.8)	5.2 (1.4)	4.3 (0.7)	5.1 (1.1)
Use of antidiabetic medication (%)	0.7	9.7	0.2	8.8
HDL-cholesterol (mmol/L)	1.2 (0.3)	0.9 (0.2)	1.5 (0.4)	1.2 (0.3)
Triglycerides (mmol/L)	1.2 (0.6)	2.3 (1.0)	1.1 (0.5)	1.8 (0.8)
Use of statins (%)	11.4	25.6	5.9	25.6

Values presented as mean (standard deviation). MetS= metabolic syndrome

Table 1

factors were also significantly different between individuals with and without MetS. MetS was associated with higher levels of insulin, higher HOMA-IR scores, lower adiponectin levels and higher CRP levels.

Next, we performed regression analysis to study the associations between MetS and cognitive test scores. Although all mean test scores which are shown in Table 3 were lower in individuals with MetS, the adjusted analysis was significant only for Stroop-ratio and z-executive in women (p=0.03 and p=0.02). Increasing number of MetS components was associated to lower verbal fluency and z-executive scores (p = 0.04 and 0.02) in women. The same trend, however non-significant (p=0.10), was seen for Stroop-ratio.

Metabolic Syndrome in relation to vascular and endocrine factors and determinants of cognitive function

	Men		Women	
	MetS absent (n=615)	**MetS present** (n=207)	**MetS absent** (n=849)	**MetS present** (n=227)
Vascular and endocrine factors				
Smoking (%)	32.7	33.5	46.5	45.1
Insulin (µU/ml, FPI)	11.8 (5.6)	19.1 (11.1) **	11.5 (4.7)	18.0 (9.2) **
HOMA-IR	2.4 (1.4)	4.5 (2.9) **	2.2 (1.0)	4.1 (2.4) **
Adiponectin (mg/L)	8.5 (4.2)	6.7 (3.7) **	13.1 (5.8)	9.5 (4.6) **
CRP (mg/L)	3.1 (6.2)	3.5 (5.8) **	3.4 (5.4)	5.1 (7.4) **
Determinants of cognitive function				
Age (year)	47.4 (14.3)	54.4 (11.9) **	45.3 (13.7)	56.7 (12.6) **
Education level (%)		**		**
Low	30.0	37.7	26.2	46.9
Low-intermediate	37.9	44.4	42.0	40.7
High-intermediate	24.1	14.5	27.3	11.5
High	8.0	3.4	4.5	3.7
Alcohol (% frequent users) §	45.0	36.6 *	19.9	12.9 *
HADS-D score	5.7 (4.0)	6.4 (3.8) *	5.4 (4.2)	7.6 (4.6) **
Apoe 4 allele carriers (%)	36.3	42.6	35.5	40.3

Values presented as mean (standard deviation).
MetS= metabolic syndrome, FPG = fasting plasma glucose, FPI= fasting plasma insulin, HOMA-IR = homeostatic assessment –insulin resistant model.
HADS-D: Hospital Anxiety and Depression Scale. § defined as drinking at least one unit of alcohol per day.
* unadjusted p-value <0.05; ** unadjusted p-value <0.01.

Table 2

We followed up on these associations to see which individual MetS components attributed independently to our findings (Table 4). Higher systolic blood pressure was related to lower scores on Stroop-ratio, verbal fluency and z-executive. Lower HDL-c levels were also associated with lower verbal fluency scores. Diastolic blood pressure showed an opposite effect with Stroop-ratio

and z-executive, but the association with z-executive disappeared when excluding persons using antihypertensive medication. The other associations remained unaltered when excluding people using medication.

Association metabolic syndrome and cognitive function in men and women

Cognitive test	Men			Women		
	n	MetS absent	MetS present	n	MetS absent	MetS present
General cognitive ability						
Dutch Adult Reading Test	750	62.1 (19.0)	58.3 (18.5)	993	60.4 (18.2)	52.6 (18.1)
Memory						
AVLT – Working memory	757	4.3 (1.7)	3.9 (1.5)	999	4.6 (1.7)	4.0 (1.6)
AVLT – Learning	757	32.1 (8.9)	29.2 (8.3)	999	35.5 (8.9)	31.8 (8.9)
AVLT – Recognition	759	27.7 (2.2)	27.1 (2.5)	1000	28.3 (2.2)	27.7 (2.4)
AVLT – Delayed recall	759	7.1 (2.9)	6.4 (2.8)	1000	8.3 (2.9)	7.2 (2.9)
Executive						
Ratio TMT-B / TMT-A †	752	2.7 (1.0)	2.8 (1.0)	996	2.6 (1.0)	2.9 (1.1)
Ratio Stroop III / Stroop II †	734	1.7 (0.4)	1.8 (0.4)	996	1.7 (0.3)	1.9 (0.6) *
Verbal fluency	758	63.7 (18.0)	60.0 (17.1)	998	63.0 (17.9)	53.5 (18.7)
Visuospatial						
Block design	756	30.8 (15.6)	25.7 (13.9)	998	28.7 (15.1)	20.8 (11.2)
Composite scores						
Memory function	757	0.1 (0.8)	-0.2 (0.8)	999	0.1 (0.8)	-0.3 (0.9)
Executive function	759	0.1 (0.7)	-0.1 (0.7)	998	0.1 (0.6)	-0.4 (0.8) *
Global cognitive function	758	0.1 (0.7)	-0.2 (0.6)	998	0.1 (0.7)	-0.3 (0.7)

Values presented as mean (standard deviation). AVLT=Adult Verbal Learning Test, TMT=Trail Making Test, Stroop=Stroop Color and Word Test; † time demanding task: high values represent low test scores.
p=p-value derived from regression analysis adjusted for age, education, depressive score, alcohol use and family-relationship.
* $p < 0.05$

Table 3

Contribution of individual metabolic syndrome components to executive function in women

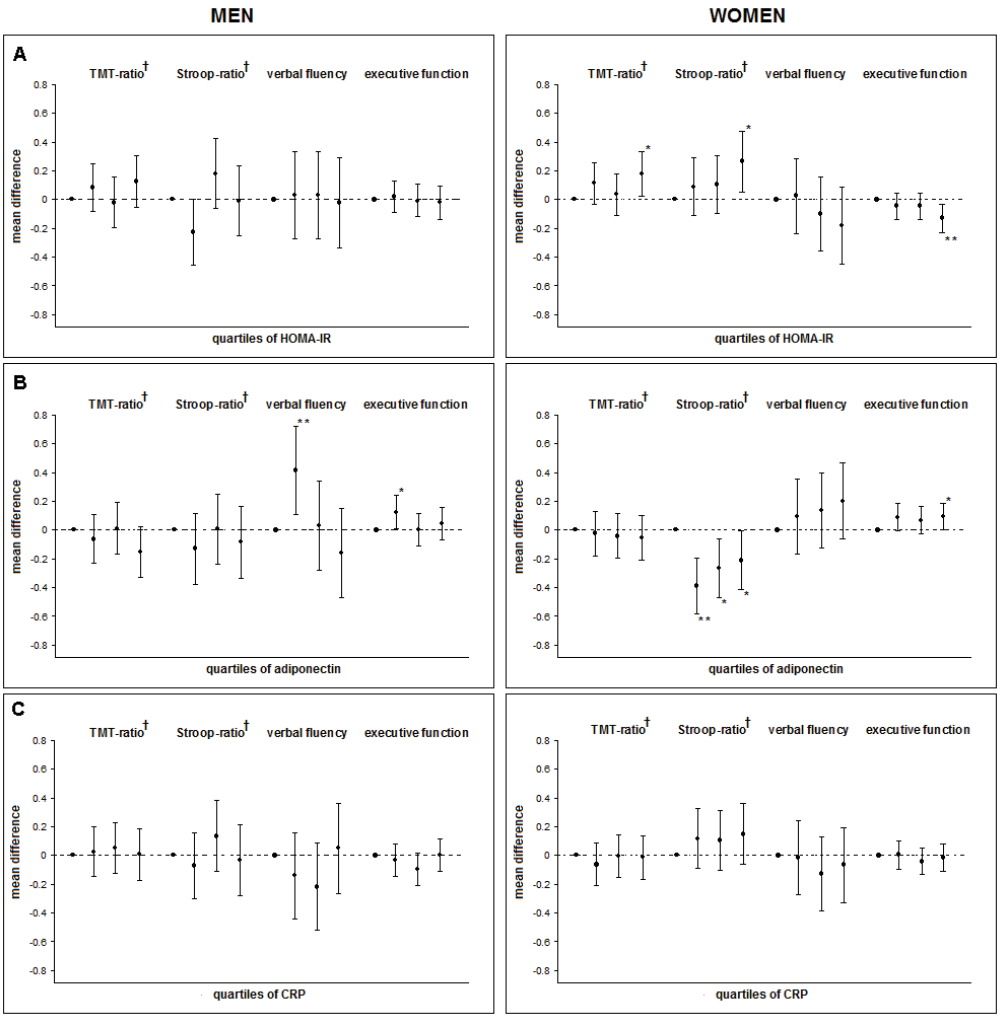
	Ratio TMT-B / TMT-A †	Ratio Stroop III / Stroop II †	Verbal fluency	z-executive
Waist circumference	0.006 (0.003)	0.005 (0.004)	0.009 (0.049)	-0.002 (0.002)
SBP	0.002 (0.002)	0.009 (0.003) **	-0.120 (0.035) **	-0.005 (0.001) **
DBP	-0.001 (0.004)	-0.016 (0.005) **	0.081 (0.063)	0.005 (0.002) *
TG	-0.001 (0.048)	0.050 (0.066)	1.342 (0.807)	0.012 (0.029)
HDL-c	0.048 (0.080)	0.005 (0.110)	3.277 (1.423) *	0.064 (0.051)
FPG	0.005 (0.036)	0.024 (0.054)	0.765 (0.630)	0.009 (0.023)

All individual MetS components were entered as continuous variables to the regression model. Results are presented as betas (standard errors).
TMT=Trail Making Test; Stroop II=Stroop Color Card; Stroop III=Stroop Color and Word card; z-executive= composite score of executive tests;
SBP=systolic blood pressure; DBP=diastolic blood pressure; TG=Triglycerides; HDL-c=High Density Lipoprotein Cholesterol; FPG=Fasting Plasma Glucose.
† time-demanding task: positive beta represents negative association.
* p-value < 0.05; ** p-value <0.01

Table 4

The findings related to adipose tissue endocrine function are given in Figure 1 with analyses limited to executive function. Women with high HOMA-IR scores had lower scores on TMT-ratio, Stroop-ratio and z-executive than women with low HOMA-IR scores. Higher adiponectin levels were associated with higher scores on z-executive in both men and women and with Stroop-ratio in women only. The associations of HOMA-IR with Stroop-ratio and z-executive were more significant (p=0.004 and 0.007) when analyzing HOMA-IR as continuous variables instead of quartiles. The other associations, however, became non-significant. No association between CRP and executive function was found in this population.

Associations of HOMA-IR (A), adiponectin (B), and CRP (C) with cognitive function in men and women



Results are presented as mean differences compared to quartile 1.
The differences are adjusted for age, education, depressive score, alcohol use and family-relationship.
HOMA-IR=homeostatis model assessment insulin-resistance, CRP=C-reactive protein
* p-value < 0.05; ** p-value <0.01

Figure 1

Discussion

The main finding of the current study is that MetS and HOMA-IR is associated with poorer executive performance in women. Of the MetS components, systolic blood pressure is independently associated with executive function.

Our findings of association of MetS with cognitive performance confirms previous cross-sectional and longitudinal studies [22,34,35]. Although we studied different cognitive domains, mainly executive function was associated with MetS. This is in line with two recent studies that found significant differences in processing speed, semantic fluency and executive function between persons with and without MetS [36,37]. Associations of the MetS with fronto-subcortical syndrome and with frontal white matter changes were found by others [10,38]. Damage to cerebral small vessels could be one of the explanations for the observed associations in the current study [39-41], which is supported by the independent association of systolic blood pressure with cognitive function [12,34,42]. Whereas diabetes has been reported to be associated with cognitive function [12], the low prevalence of hypergelycemia in our population could explain the lack of association with cognitive tests. Insulin-resistance as an early sign of diabetes pathology was more common in our mainly middle aged population and was associated with cognitive tests. Although findings on the individual MetS components vary, a number of studies find that an increasing number of MetS components is associated with lower cognitive scores, which is also confirmed by our observations [10,35,42-44].

An effect of MetS components on the early development of Alzheimer's disease (AD) pathology may be another possible mechanism underlying our observations, since the insulin-degrading enzyme (IDE) functions less well in the presence of insulin-resistance, resulting in higher levels of amyloid β-peptide (Aβ) [45,46]. Long-term exposure to insulin due to insulin-resistance, may by itself also have a direct damaging effect on neurons [47]. Our observed association of HOMA-IR and executive function is in line with this. Previous studies on insulin-resistance measured as HOMA-IR have been inconsistent [3,48,49].

Despite possible pathways through which adiponectin could be linked to cognition, such as improvement of insulin-sensitivity, anti-inflammatory effects and effects on brain metabolism and the vascular system, our results do not show a consistent association of adiponectin with cognition [50-55] . Adiponectin levels are however, lower in individuals with MetS in our study and a trend was seen with executive function in women, which supports further study on the role of adiponectin in cognition.

Levels of CRP were not related to cognition in the present study, however, data on other markers, such as interleukin-6 (Il-6) and α1-antichymotrypsin (ACT) were not available. These markers have been related to cognitive decline and increased systemic inflammation has been proposed as a possible modifier between MetS and cognition [22,54]. We could not confirm this, which may also be due to a more pronounced effect of inflammation on cognition in the elderly than in middle aged individuals.

Our study shows that there are gender differences in the association of MetS with cognitive function. Gender-specific effects of metabolic components on cognitive function have not extensively been addressed in previous studies [56], while there is evidence that MetS has greater effect on cardiovascular and cerebrovascular disease in women than in men, indirectly supporting our observed differences [57-60]. Furthermore, there is evidence that men are treated more aggressively for cardiovascular risk factors [61]. As a consequence, men may be less likely to experience the adverse effects of MetS. Another factor that could explain gender differences is depression, which is highly correlated to cognition and more common in women. Since our analyses were adjusted for depressive symptoms, however, we do not think that depression explains the observed differences in our study.

The strength of this study is the population-based design in which participants were not selected on the disease of interest. Also, the inclusion of a large sample size and a range of cognitive tests allowed detailed study of cognition. The cross-sectional design is a limitation of our study, and it does not allow claiming causality. Previous longitudinal studies, however, suggest that MetS might be a causative factor in the relationship with cognition [62]. Another limitation is that some of the significant p-values could be false-positive findings due to the large number of tests that we performed. However, the consistent associations argue against this.

In summation, the most important findings of our study are that MetS and HOMA-IR are associated with executive function in women. Of the MetS components, systolic blood pressure is independently associated with executive function.

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03 The apolipoprotein E gene and its age-specific effects on cogntive function

Abstract

The ε4 allele of the apolipoprotein E gene (APOE) is a well-established determinant of Alzheimer's disease and cognitive function. We studied the age-specific effects of the APOE*ε4 allele on cognitive function in a series of 2208 related individuals from a family-based study conducted in an isolated population in the Southwest part of The Netherlands. The effect of the ε4 allele on cognitive function was evaluated using standard quantitative genetic analysis under a polygenic model, adjusted for cardiovascular risk factors. We found a significant association between the APOE*ε4 allele and reduced scores on the Adult Verbal Learning Test (AVLT) in persons aged 50 years and older (AVLT short-term memory P = 0.01, AVLT learning P = 0.001, AVLT delayed recall P = 0.01 and memory compound score P = 0.001). The effect of APOE*ε4 is most pronounced on learning ability, starting as early 40 years. The APOE*ε4 allele is also strongly associated to cholesterol levels and atherosclerosis. This association did not explain the effect of APOE on cognitive function. Our study suggests that APOE*ε4 is an important determinant of vascular and neurological pathology at late age.

Introduction

The epsilon4 allele of the apolipoprotein E gene (APOE*ε4) is the most important genetic risk factor for Alzheimer's disease (AD) [1]. Although its role in AD has long been known, recently a commercial genetic test for APOE*ε4 was launched to predict the risk of AD (http://www.labtestsonline.org) [2]. However, APOE*ε4 has also an established effect on lipid levels and through this on the risk and progression of atherosclerosis. Atherosclerosis and hypertension have been implicated in the AD and may partly explain the effects of APOE*ε4 on cognitive function [1]. If the effect of APOE*ε4 on neurodegeneration is in part caused by vascular pathology, this pathway opens the opportunity of clinical counseling of carriers by screening for vascular pathology. A crucial question in this respect is at which age pathology starts. It has been suggested that APOE*ε4 has clinically important effects on cognition in those who do not have signs or symptoms clinical AD. An extensive meta-analysis of all studies conducted in the period 1993-2004 showed evidence for a role of APOE*ε4 in cognitive function in non-demented people over 50 years [3]. APOE*ε4 was significantly related to reduced global cognitive functioning, episodic memory and executive function in a dose-dependent way, whereas no significant effects were seen for primary memory [3]. Although most studies focused on individuals aged 50 years and over, there is some evidence that with increasing age the effect of APOE*ε4 on cognition decreased. However, this trend was far from statistically significant in the meta-analysis. Animal studies provided significant evidence that apolipoprotein E has effects on early brain development [4], suggesting that APOE*ε4 may impact early cognitive reserve. For humans, the evidence supporting early effects of APOE on cognitive function is scarce and findings have been contradictory.

In the present study, we evaluated the effects of the APOE*ε4 allele on specific cognitive domains and vascular pathology over a wide age-range in a 3 generation family-based study. This design provides a powerful setting to address age specific effects of APOE*ε4 in a genetically and environmentally homogeneous background.

Description of the cognitive tests derived from the neuropsychological battery

Neuropsychological test	Cognitive domain	Task description	Score definition	Reference
Individual tests				
AVLT Short-term memory	Short-term memory	Recall immediately after presentation of 15 words	Number of correctly recalled words	Saan and Deelman 1986
AVLT Learning	Learning	Recall after 2nd to 5th presentation	Total number of correctly recalled words	Saan and Deelman 1986
AVLT Delay	Delayed recall	Recall after 30 minutes	Number of correctly recalled words	Saan and Deelman 1986
AVLT Recognition	Recognition	Recognize words from a list	Number of correctly recognized and rejected words	Saan and Deelman 1986
WAIS Verbal fluency	Semantic fluency and phonological fluency	Mention words fiting a frame (semantic & phonological)	Number of correctly mentioned words	Wechsler 2000
Trail-making test (TMT)	Cognitive flexibility	Connect numbers (A) and together with letters in accending order (B)	Ratio of time in seconds to complete part B over part A	Reitan 1955
Stroop	Susceptibility to interference	Read colors (card 2) which are wrongly named (card 3)	Ratio of time in seconds to complete the card 3 over card 2	Hammes 1978
WAIS Block design	Visuoconstructive abilites	Place blocks according to reference	Number of replicated blocks	Wechsler 2000
Compound scores				
Memory performance			Average of z-transformation of AVLT short, learning, delay, recognition	
Executive function			Average of z-transformation of WAIS verbal fluency, TMT, Stroop	
Overall cognitive function			Average of z-transformation of all tests	

AVLT: Auditory Verbal Learning Test
WAIS: Weschler Adult Intelligence Scale

Table 1

Materials and Methods

Study population

The Erasmus Rucphen Family (ERF) cohort, which is part of the Genetic Research in Isolated Population (GRIP) program, is a family-based study that includes inhabitants of a genetically isolated community in the south-western area of the Netherlands [5]. ERF aims to investigate the genetic origins of complex disorders and traits. The study population essentially consists of one extended family of descendants from 20 related couples that lived in the isolate between 1850 and 1900 and had at least 6 children. With relatively limited migration until the last few decades, the isolate now includes approximately 20,000 inhabitants. All data were collected between 2002 and 2005. The Medical Ethical Committee of the Erasmus Medical Center Rotterdam approved the study and informed consent was obtained from all participants.

Data collection

Participants underwent extensive medical and neuropsychological examinations at the ERF research centre. The examinations included the determination of cardiovascular risk factors, such as serum total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, systolic and diastolic blood pressure, and common carotid intima media thickness (IMT). Serum markers were determined using an automated enzymatic procedure (Boehringer Mannheim System). Blood pressure was measured twice on the right arm in a sitting position after at least five minutes rest, using an automated device (OMRON 711); the average of the two values was used for analysis. IMT was evaluated using ultrasonography according to previously applied protocols [6-8]. The outcome variable was defined as the mean IMT of the near and far wall of both common carotid arteries. The battery of neuropsychological tests included the Dutch version of the Auditory Verbal Learning Test (AVLT) [9], the Trail Making Test (TMT) [10], the Stroop colour-word test [11], the verbal fluency test [10] and the block design subtest of the Weschler Adult Intelligence Scale (WAIS) [12]. These tests were chosen to screen for cognitive deficits related to AD and other dementias [13] and cover different cognitive domains (Table 1). We assessed the general reading ability of the participants with the Dutch Adult Reading Test (DART) [14]. We also computed compound scores for memory performance, executive function and over-all cognitive function (Table 1), by averaging the z-transformed scores of several cognitive tests [15]. The z-scores were calculated based on the direction of the measurement of test performance. For tests where higher scores indicate better performance (AVLT and WAIS tests), $z = (x - \bar{x}) / sd$; otherwise (TMT and Stroop), $z = (\bar{x} - x) / sd$. In this way, higher compound scores indicate better performance.

Finally, the education level attained by the subjects within the Dutch educational system was determined according to eight ordinal categories from primary school to university [16].

Genotyping

Genomic DNA was extracted from whole blood samples using the salting out method [17]. Samples were genotyped for the APOE C112R (ε4 allele) and APOE R158C (ε2 allele) polymorphisms with a Taqman allelic discrimination Assay-By-Design (Applied Biosystems, Foster City, CA). The assays utilized 5 nanograms of genomic DNA and 2 microliter reaction volumes. The amplification and extension protocol included an initial activation step of 10 min at 95 degrees, which preceded 40 cycles of denaturation at 95 degrees for 15 seconds and annealing and extension at 50 degrees for 60 seconds. Allele-specific fluorescence was analysed on an ABI Prism 7900HT Sequence Detection System with SDS v 2.1 (Applied Biosystems, Foster City, CA).

Statistical analysis

A considerable proportion of participants failed to complete the TMT part B test (N = 171, 7.9%),

while some failed to complete the Stroop card III (N = 16, 0.7%) and WAIS block design tests (N = 64, 2.9%) within the time limit. We imputed their scores based on correlations between sex, age, and education level. We grouped the APOE genotypes based on the number of ε4 alleles in a dose-dependent manner [3], zero (ε2/ε2, ε2/ε3 and ε3/ε3 genotypes), one (ε2/ε4 and ε3/ε4 genotypes) and two copies (ε4/ε4 genotype). General characteristics of the study population among the genotypic groups were compared using the one-way ANOVA test for continuous variables and the chi-square test for dichotomous variables as implemented in SPSS V.11.0 (SPSS Inc. Chicago IL). The observed frequencies of the APOE genotypes were tested for deviations from Hardy-Weinberg equilibrium using the exact test for multiple alleles [18].

To evaluate the effect of the ε4 allele on cognitive functioning and adjust for family relationships, we performed the variable screening analysis under the polygenic model using the SOLAR software package version 4.1.0 [19]. SOLAR was chosen for its power in discriminating the genetic and environmental effects by utilizing all of the information that is provided by large, complex pedigrees. The effect of APOE genotype on cognitive tests was estimated by including APOE genotype (0, 1, or 2 number of ε4 alleles) as a covariate in the model, adjusted for other covariates including age, age-squared, sex, education, inbreeding, DART score, and cardiovascular risk factors (total cholesterol, triglycerides, IMT, and systolic and diastolic blood pressure). Inbreeding coefficients were computed based on all available genealogical information for the GRIP population (N = 107,091) using PEDIG software [20]. In addition, we investigated the interaction between APOE and age using a multiplicative model. Before SOLAR analyses, scores from the cognitive tests were normalized using a general rank-transformation [21].

To illustrate the age-specific effect of APOE on cognitive function, we smoothed the distribution of cross-sectional test scores across age, using locally weighted regression, or the LOESS smoother, implemented in the software package SigmaPlot version 8.02 [22].

Results

Information on both APOE genotype and cognitive tests is available for 2208 ERF participants in our study. We excluded 65 individuals who were illiterate, blind, deaf, retarded or who reported having a brain tumour, stroke or severe brain damage. The frequencies of APOE alleles were 4.8% for the ε2 allele, 74.1% for the ε3 allele and 21.1% for the ε4 allele. The allele and genotype distributions were in Hardy-Weinberg equilibrium (P = 0.64). There were no significant differences in age, sex, education level and blood pressure between APOE genotype groups (Table 2). Heterozygous and homozygous APOE*ε4 carriers had thicker IMT compared to non-carriers (P = 0.05, Table 2). Serum levels of total cholesterol (P = 1.75×10-7) and triglycerides (P = 4.80×10-5) significantly increased and serum HDL levels significantly decreased (P = 4.81×10-5) with an increasing number of APOE*ε4 alleles.

We studied the relationship between cardiovascular factors and cognitive function (Table 3). Serum levels of triglycerides were significantly associated with AVLT recognition (P = 0.04). There was significant and consistent evidence for association between IMT and multiple cognitive domains (AVLT learning, P = 0.01; AVLT recognition, P = 0.01; WAIS verbal fluency, P = 0.0001; memory compound score, P = 0.01; and over-all cognitive function compound score, P = 0.01). Systolic blood pressure was significantly associated with the Stroop test (P = 0.00001) and executive function compound score (P = 0.01). Adjustment for APOE status had little influence on the relationship between vascular risk factors and cognitive function.

Characteristics per APOE genotype

	APOE*4						
Characteristics	**0 (n=1342)**		**1 (n=699)**		**2 (n=102)**		**P-value**
Age (years)	49.0	14.9	49.3	14.4	48.8	13.7	0.85
Gender (% male)	42.9		43.9		46.2		0.76
Body mass index (kg/m2)	27.0	4.6	26.8	4.7	27.5	4.8	0.40
Education	3.24	0.05	3.08	0.07	3.00	0.18	0.07
IMT(mm)	0.81	0.21	0.84	0.21	0.83	0.17	**0.05**
Systolic blood pressure (mmHg/cm)	140.27	20.46	141.08	21.02	141.44	18.25	0.42
Diastoic blood pressure (mmHg/cm)	80.08	10.44	80.30	10.39	82.24	9.67	0.13
Fasting glucose (mmol/l)	4.62	1.03	4.56	0.87	4.62	0.88	0.35
Serum cholesterol (mmol/l)	5.49	1.07	5.70	1.10	5.87	1.22	**1.75E-07**
Serum Triglycerides (mmol/l)	1.30	0.75	1.41	0.82	1.62	0.95	**4.80E-05**
Serum HDL (mmol/l)	1.30	0.36	1.25	0.35	1.21	0.34	**4.81E-05**

Values presented are means and standard deviations or percentages

Table 2

Cardivasular factors and cognitive function

	Cholesterol		Triglycerides		HDL		IMT		SBP		DBP	
Cognitive domain	**beta**	**se**	**beta**	**se**	**beta**	**se**	**beta**	**se**	**beta**	**se**	**beta**	**se**
Individual tests												
AVLT Short-term memory	0.05	0.03	-0.03	0.04	0.17	0.10	-0.40	0.23	-0.002	0.001	-0.003	0.002
AVLT Learning	0.19	0.14	0.17	0.20	0.47	0.45	-3.52	1.09 **	-0.013	0.006	-0.008	0.007
AVLT Delay	0.08	0.05	0.11	0.07	0.07	0.16	-0.65	0.39	0.001	0.002	-0.002	0.003
AVLT Recognition	0.09	0.04	0.10	0.06 *	-0.13	0.14	-1.01	0.32 **	0.002	0.002	0.006	0.002
WAIS Verbal fluency	0.44	0.29	0.07	0.40	0.36	0.93	-10.03	2.26 ***	-0.045	0.013	-0.005	0.015
TMT	0.04	0.02	-0.01	0.03	-0.01	0.06	0.00	0.16	-0.001	0.001	-0.003	0.002
Stroop	0.00	0.01	0.00	0.01	-0.03	0.03	0.03	0.06	0.002	0.000 ****	0.000	0.001
WAIS Block design	0.12	0.23	-0.05	0.32	0.03	0.73	-1.94	1.76	-0.008	0.010	-0.013	0.012
Compound scores												
Memory performance	0.03	0.01	0.02	0.02	0.03	0.04	-0.32	0.10 **	0.000	0.001	0.000	0.001
Executive function	0.00	0.01	0.00	0.02	0.04	0.04	-0.21	0.09	-0.002	0.000 **	0.001	0.001
Overall cognitive function	0.01	0.01	0.01	0.01	0.03	0.03	-0.26	0.07 **	-0.001	0.000	0.000	0.000

se: standard errors
Except TMT and Stroop, higher absolute values indicate better performance
P values adjusted for age, sex, inbreeding, education, and family relationship
* P value < 0.05
** P value < 0.001

Table 3

Table 4 presents the effect of the APOE*ε4 allele on cognitive tests. There was a borderline significant association between APOE*ε4 and AVLT learning (P = 0.07), which became significant (P = 0.05) when adjusting for cardiovascular factors. Test scores generally showed a non-significant trend of poorer performance with an increasing number of ε4 alleles. Adjusting for cardiovascular factors had little influence on these results.

When studying cognitive function, there was significant evidence for interaction between APOE*ε4 and age. The interaction term of age and APOE*ε4 was significant for AVLT short-term memory (Pinteraction = 0.01), AVLT learning (Pinteraction = 0.05), and memory compound score (Pinteraction = 0.01), while for AVLT delayed recall (Pinteraction = 0.09) and AVLT recognition (Pinteraction = 0.07), the evidence was borderline significant.

Effect of APOE genotype on cognitive tests

Cognitive domain	APOE*E4							
	0 (n=1342)		1 (n=699)		2 (n=102)			
	mean	se	mean	se	mean	se	P1	P2
Individual tests								
AVLT Short-term memory	4.3	0.05	4.3	0.07	4.1	0.17	0.23	0.19
AVLT Learning	33.0	0.25	32.8	0.35	30.9	0.99	0.07	**0.05**
AVLT Delay	7.5	0.08	7.5	0.11	6.8	0.29	0.19	0.19
AVLT Recognition	27.8	0.06	27.8	0.09	27.7	0.20	0.68	0.96
WAIS Verbal fluency	61.5	0.51	61.0	0.71	60.8	1.83	0.80	0.98
TMT	2.7	0.03	2.7	0.04	2.7	0.10	0.64	0.64
Stroop	1.7	0.01	1.7	0.02	1.7	0.07	0.31	0.31
WAIS Block design	27.6	0.41	27.5	0.58	27.0	1.52	0.84	0.91
Compound scores								
Memory performance	0.00	0.02	-0.01	0.03	-0.16	0.08	0.14	0.15
Executive function	-0.01	0.02	-0.04	0.03	-0.02	0.08	0.71	0.92
Overall cognitive function	-0.01	0.02	-0.02	0.03	-0.10	0.07	0.23	0.31

se: standard errors

Except TMT and Stroop, higher absolute values indicate better performance

P1: adjusted for age, sex, inbreeding, education, DART, and family relationship

P2: additionally adjusted for total cholesterol, triglycerides, IMT, and systolic and diastolic blood presure

Table 4

When stratifying the data by age (Table 5), the ε4 allele was significantly associated with poorer memory performance in those over 50 years of age (AVLT short-term memory P = 0.01, AVLT learning P = 0.001, AVLT delayed recall P = 0.01 and memory compound score P = 0.001). Adjusting for cardiovascular factors had little influence on these effects (Table 5). In younger subjects (≤ 50 years of age), none of the tests were significantly associated to cognitive function (Table 5).

To illustrate the age-specific effect of APOE on memory performance, we plotted the smoothed distribution of test scores across age (Figure 1). Figure 1A shows that APOE has little influence on AVLT short-term memory. Only after the age of 65 years does some effect of the genotype

become apparent. APOE genotype seems to have the most pronounced effect on AVLT learning. The effect starts around age 40 years (Figure 1B). The effects on AVLT delayed recall and memory compound score are less pronounced, but there is a trend towards poorer cognitive performance with increasing number of APOE*ε4 alleles (Figure 1C and 1D).

Effect of APOE genotype on cognitive tests by age category

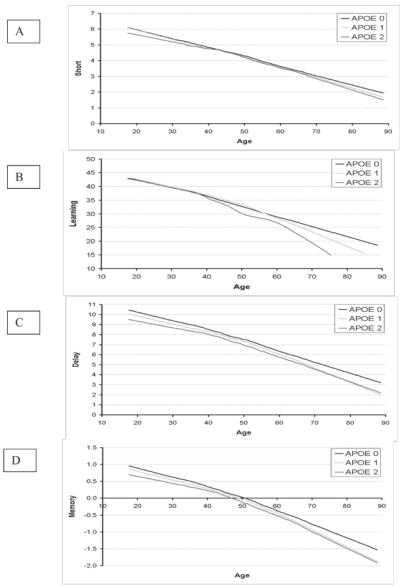
Cognitive domain	<=50 years								>50 years							
	0 (n=709)		1 (n=362)		2 (n=51)				0 (n=633)		1 (n=337)		2 (n=51)			
	mean	se	mean	se	mean	se	P1	P2	mean	se	mean	se	mean	se	P1	P2
Individual tests																
AVLT Short-term memory	4.99	0.06	5.00	0.09	4.84	0.23	0.64	0.59	3.55	0.06	3.46	0.08	3.30	0.20	**0.01**	**0.01**
AVLT Learning	37.22	0.30	37.62	0.40	35.47	1.33	0.72	0.89	28.34	0.32	27.63	0.45	26.28	1.15	**0.001**	**0.003**
AVLT Delay	8.66	0.10	8.87	0.14	8.18	0.40	0.63	0.99	6.12	0.11	6.09	0.15	5.38	0.33	**0.01**	**0.04**
AVLT Recognition	28.58	0.06	28.77	0.08	28.53	0.22	0.22	0.10	26.96	0.11	26.75	0.16	26.90	0.30	0.07	0.18
WAIS Verbal fluency	69.19	0.64	68.30	0.86	65.35	2.63	0.55	0.57	52.72	0.66	52.86	0.98	56.16	2.39	0.99	0.57
TMT	2.51	0.04	2.52	0.05	2.47	0.12	0.34	0.60	2.87	0.04	2.90	0.06	2.87	0.16	0.56	0.70
Stroop	1.59	0.01	1.59	0.01	1.66	0.12	0.99	0.47	1.88	0.02	1.91	0.03	1.82	0.05	0.45	0.24
WAIS Block design	35.65	0.56	35.14	0.82	33.47	2.35	0.97	0.73	18.70	0.37	19.23	0.52	20.44	1.42	0.55	0.36
Compound scores																
Memory performance	0.40	0.03	0.45	0.04	0.28	0.10	0.43	0.54	-0.44	0.03	-0.50	0.04	-0.61	0.10	**0.001**	**0.01**
Executive function	0.30	0.02	0.28	0.03	0.19	0.13	0.74	0.75	-0.35	0.03	-0.39	0.04	-0.23	0.09	0.66	0.77
Overall cognitive function	0.37	0.02	0.39	0.03	0.26	0.10	0.66	0.83	-0.44	0.02	-0.47	0.04	-0.47	0.07	**0.01**	0.07

se: standard errors
Except TMT and Stroop, higher absolute values indicate better performance
P1: adjusted for age, sex, inbreeding, education, DART, and family relationship
P2: additionally adjusted for total cholesterol, triglycerides, IMT, and systolic and diastolic blood presure

Table 5

Because a considerable number of people could not complete the TMT-B test (N = 141), we investigated the distribution of these missing scores. The E4 allele was significantly associated with the proportion of people who could not complete the TMT-B test (none ε4 = 5.4%, one ε4 = 8.5%, and two ε4 = 9.2%, P = 0.02). This effect was more pronounced in women (P = 0.004).

Age specific effect of the APOE E4 allele on memory performance



The distribution of test scores (y-axis) was smoothed across age (x-axis) using the LOESS smoother implemented in the software package SigmaPlot version 8.02. [22]. A: AVLT short-term memory; B: AVLT learning; C: AVLT delayed recall; D: memory compound score.

Figure 1

Discussion

In this study, we found a significant association between the APOE*ε4 allele and reduced memory performance in persons aged 50 years and older. This effect is independent of the effect of APOE on cardiovascular factors. In our analyses of cognitive function there is significant evidence for interaction between APOE*ε4 and age. The effect of APOE*ε4 increases significantly with age, particularly in terms of learning ability. As expected APOE*ε4 was strongly related to lipid levels and atherosclerosis, while serum levels of triglycerides, blood pressure and atherosclerosis were significantly associated to cognitive function. Additional adjustment for APOE status had little influence on the relationship between vascular risk factors and cognitive function.

The extensive meta-analysis of all studies conducted in the period 1993-2004 showed that APOE*ε4 was significantly related to reduced global cognitive functioning, episodic memory and executive function in a dose-dependent way, whereas no significant effects were seen for primary memory [3]. In contrast, in the present study we see a consistent and significant association to memory. Our findings are in agreement with a recent prospective, population-based study in 5804 subjects aged 70-80 years. That study showed that ε4 carriers had significantly poorer performance in immediate and delayed recall at baseline as well as greater decline during the

3.2 years of follow up [23]. The effect of APOE was less pronounced on attention and processing cognitive domains. Another study of 611 elderly clergymen showed that the APOE*ε4 allele had a pronounced influence on declines in episodic memory [24]. This study also used a compound score, including word list memory, recall, recognition, immediate and delayed recall, which is comparable to our compound score for memory. A number of smaller studies found a relation between APOE*ε4 and memory performance [25,26]. Furthermore, a family-based study of relatives of AD patients showed an effect of APOE*ε4 on memory in those not yet affected [27]. Finally, episodic memory loss is a key characteristic of AD [28-30] and several epidemiological studies found that measures of delayed recall and learning are predictive of the risk for developing dementia [31-33].

Most studies on APOE and cognition in humans have focused on the elderly. Animal studies, however, have demonstrated that apolipoprotein E has a role in early brain development [4]. In our study, the effect of APOE was not significant in people younger than 50 years of age. Of interest, APOE genotype showed some evidence for an early effect on learning ability. The effect starts in early middle age, at around 40 years.

Cardiovascular factors may potentially be an intermediate feature explaining part of the association between APOE and cognitive function. As expected, we observed a strong association between APOE and serum levels of total cholesterol, triglycerides, and HDL. Systolic blood pressure and the presence of atherosclerosis as measure by IMT were significantly and consistently associated with multiple cognitive domains. Although the relationship between blood pressure and Alzheimer's disease is only observed in prospective studies[34,35], also other studies have found a strong relationship between cognitive function to blood pressure [36] and atherosclerosis [37,38]. In line with the studies on Alzheimer's disease that suggest the effect of APOE on the risk of disease is determined primarily by the effect on lipid metabolism with the brain, the association between APOE and memory performance remained significant after adjusting for serum levels of total cholesterol and triglycerides, IMT, and systolic and diastolic blood pressure. Also other estimates for the relation between APOE*ε4 and cognitive function did not change when adjusting for vascular pathology. This indicates that the effect of APOE on cognitive functioning is not likely determined by the effect of APOE on cardiovascular factors. At the same time, the additional adjustment for APOE status had little influence on the relationship between vascular risk factors and cognitive function. The finding implies that measuring APOE will not be clinically relevant for preventive strategies targeting the relationship between vascular risk factors and cognitive function.

In summary, APOE*ε4 is associated with poorer memory performance in older people. The effect of APOE*ε4 increases significantly with age and is independent of vascular pathology. The effect of APOE*ε4 on learning ability starts as early as the age of 40 years. In light of the commercial test recently made available for APOE genotyping, our findings suggest that those who take

the test should be informed not only about the risk of AD but also about the effect of APOE genotype on cognitive function and vascular pathology. Whether or not the test is clinically useful remains to be determined in further studies [2]. Our findings clearly show that independent of the test outcome, management of vascular problems will be crucial for maintenance of cognitive function.

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04 Genome-wide linkage screen of cognitive function identifies susceptible chromosomal regions

Abstract

Cognitive function is a complex trait which involves multiple environmental and genetic factors. To localize genes involved in cognitive functioning, we conducted genome-wide linkage analyses in a large family from a genetically isolated population. A broad range of cognitive test measurements were available for 2882 participants. We performed non-parametric linkage analysis in participants with low cognitive test scores, defined as a score in the lowest 10% of the distribution. Genome-wide significant and suggestive thresholds for linkage were estimated empirically using simulation study. Fine-mapping was performed in significant linkage regions comparing participants with low cognitive scores to those with high cognitive scores in the same population. Replication analyses were performed in an independent study, the Rotterdam Study, which is an ongoing population-based cohort study with comparable cognitive estimates. We found significant linkage (LOD > 3.78) of cognition was found to chromosomes 1p13.1, 12q24.33, 19q13.43, 20p13, 21q22.13 and 21q22.3. Fine-mapping showed significant associations to chromosome 1 (p-value=0.03) and 21 (p-value=0.01) after multiple testing correction. The region on 21q22.13 was replicated in the Rotterdam Study (nominal p-value 0.003). The fine-mapping and replication results pointed to variants within the potassium inwardly-rectifying channel, subfamily J, member 6 gene (KCNJ6).

Introduction

Cognitive function is a broad concept referring to multiple cognitive domains, among which memory, language, executive function and visuospatial ability. Impairment of cognitive function is seen in patients with various diseases including dementia, bipolar disorder, schizophrenia and attention deficit hyperactivity disorder (ADHD) [1-3].The heritability estimates for cognitive function vary between 20-79% [4,5], making cognition a potential endophenotype for the various neuropsychiatric outcomes [6,7].The genes implicated in cognitive function are poorly understood. Many candidate genes have been studied [8,9], and the most consistently reported genetic factor affecting cognition is apolipoprotein E (APOE), the major genetic determinant of early- and late-onset Alzheimer's disease [10,11]. The effect of APOE on cognitive function is small, especially compared to its effect in Alzheimer's disease [12]. In search for unknown genes, both genome wide association studies as well as linkage studies have been conducted. The three genome wide association studies that have so far been conducted, were relatively small including 333 up to 700 persons in the discovery set and identified three genes: the sortilin-related receptor gene (SORL1) in relation to abstract reasoning[13], the WW and C2 domain containing 1 gene (KIBRA) in relation to delayed recall[14] and the sodium channel, voltage-gated, type I, alpha subunit gene (SCN1A) in relation to short-term memory[15]. In addition, several linkage studies have been conducted aiming to identify genes with a relatively large effect on cognition. There is evidence for linkage of memory to chromosomes 4 and 12, mental flexibility to chromosomes 5 and 11, IQ to chromosomes 2 and 6, motor timing to chromosomes 2 and 13, processing speed to chromosome 14, and reading ability to chromosome 18

[13,16-23] (Table 1). In contrast to dementia oriented studies of APOE, the linkage studies conducted were predominantly using cognitive function as an endophenotype for psychiatric outcomes including schizophrenia, alcohol dependence and ADHD. Two general issues of linkage studies is that the power of linkage analysis is low, requiring large sample sizes and that the regions of interest that are identified are large and single genes cannot be pinpointed. To overcome the first issue, we embedded our study within a large family-based program and to overcome the second issue, we combined our linkage studies with an association analysis in the area under the peak, allowing us to narrow down the region of interest [24].

Previous linkage findings on cognitive traits (LOD >= 2.5) in chronological order

First author	Year	Family	Trait	Chromosome	LOD
Paunio	2004	Schizophrenia	Verbal learning, memory	4q21	3.8
Posthuma	2005	General population	Performance IQ	2q24.1-31.1	4.4
			Verbal and full scale IQ	6p25.3-22.3	3.2
Buyske	2006	Alcohol dependence	Digit Span Test	11q25	3.1
			Digit Symbol Substitution Task	14q11	6.0
			Digit Symbol Substitution Task	14q24.2	3.9
Dick	2006	Alcohol dependence	Full scale IQ	6p	3.3
Luciano	2006	General population	Cambridge Contextual Reading Test, Performance IQ	2q24-31	4.2, 3.7
		Adolescent twins	Arithmetic—verbal subtest	6p	3.3
			Schonell reading test	14q13-21	3.2
			Arithmetic—verbal subtest	21q22	3.0
Singer	2006	Adult twins	Prospective memory	12q22	2.8
Seshadri	2007	General population	Reading Test, Native intelligence	18p11	5.1
Almasy	2008	Schizophrenia	Abstraction, mental flexibility	5q	3.4
Rommelse	2008	ADHD	Motor Timing	2q21.1	3.9
			Digit span	13q12.11	4.0

Table 1

Here, we report the findings of a linkage analysis of various cognitive traits. The analyses were conducted in a large family-based study, the Erasmus Rucphen Family study (ERF) including 2882 persons derived from a genetically isolated population in the Netherlands. This population was not selected for any disease and participants were tested with an extensive neuropsychological test battery with exclusion of prevalent dementia. Since we are targeting genes with a major effect, we selected persons from the extremes of the trait distribution for the linkage analysis. For the fine-mapping of the region we used dense genotyping in the regions under linkage peak in ERF and replicated the findings in a large outbred, population-based cohort, the Rotterdam Study.

Materials and Methods

Study population

The study was conducted in a genetically isolated population in the South-West of the Netherlands. Participants were part of an ongoing family-based cohort study, the ERF-study, which is embedded in a program aiming to identify genetic risk factors of complex diseases. Participants are all descendents of a limited number of founders living in the 19th century. Extensive genealogical data is available for this population [25,26]. The study protocol included venous puncture for DNA isolation and chemistry, cognitive evaluation, cardiovascular examination, eye assessments and body composition measurements. All participants gave informed consent and the study was approved by the medical ethics committee at Erasmus MC University Medical Center. For the current study, participants with a history of cerebrovascular accidents, dementia, brain tumors or other conditions that could have influenced reliable neuropsychological assessment were excluded from analysis (N=80). Cognitive test data was available for 2882 participants.

Cognitive evaluation

A 50-minute test battery was used including tests that were applicable over a wide age-range and for which a Dutch validated version was available [27,28]. General cognitive ability was tested using the Dutch Adult Reading Test (DART), which measures the number of correctly pronounced words. Memory was tested with a word learning test (after Rey's Auditory Verbal Learning test) [27] consisting of five exposure trials measuring immediate memory, learning, delayed recall and recognition. Immediate memory was defined as the total number of words recalled at the first trial, learning was defined as the total number of correctly recalled words in trial 2 to 5, delayed recall was defined as the number of correctly recalled words after 20 minutes delay and recognition was defined as the sum of the number of correctly recognized and correctly rejected words. Executive function was assessed with the time-demanding Trail Making Test (part A and B), the Stroop Colour and Word Test (card I, II and III), and with the verbal fluency test. The first two tests are time-demanding tasks, in which subjects have to connect numbers and letters (TMT) or name correct words and colours (Stroop) as quickly as possible. Verbal fluency was assessed with an animal naming and letter naming subtask, in which subjects had to name as many items as possible of the same category with a one minute time limit. Visuospatial ability (VSA) was assessed with the WAIS III - Block Design Test. The test consisted of reproducing two-dimensional patterns using cubes that have red, white, and half-red-half-white faces. The total number of correctly reproduced blocks within a time limit was used as a score.

For analyses, we used the ratios of TMT-B and TMT-A, and of the Stroop Color-Word card III and card II. People with missing test scores on TMT-B due to exceeding the time limit or misunderstanding of test instructions, were given the lowest score of 300 seconds.

To derive more general measures of cognition for analyses, we also computed three composite scores.

These scores were based on z-score transformations of test values [29]. A memory composite score (zmem) was derived by taking the average of z-scores for immediate memory, learning, delayed recall and recognition. An executive composite score (zexec) was derived from average of z-scores for verbal fluency, stroop-ratio and TMT-ratio. Finally, a global cognitive function score (zglob) was computed by taking the average of z-scores for all tests, but the DART.
Participants were asked to report their highest level of education. Education was categorized in 8 categories: 1. primary education; 2. primary education plus a higher not completed education; 3. lower vocational education; 4. lower secondary education; 5. intermediate vocational education; 6. higher secondary education; 7. higher vocational education; 8. university training. Years of education varied from 6 years in the lowest category to at least 16 years in the highest.

Statistical analysis
General descriptive statistics were performed with SPSS for Windows (version 15.0) using logistic regression or Chi2-statistics for the comparison of cases and controls.

Linkage analysis
We performed linkage analysis of affected only, for which the cases were defined as individuals with the lowest 10% of the distribution of the residuals from the regression of the cognitive scores onto age, sex and education.
All study subjects were part of one large pedigree containing 23612 individuals spanning 18 generations. For analysis, we constructed smaller subpedigrees with a maximum bit size of 18 using the software PEDCUT [30] because of the linkage software restraints.
Samples were genotyped on the Illumina HumanHap 6k Beadchip linkage panel. Markers with a minor allele frequency greater than 5% and callrate higher than 95% were used in the analyses. Genotyping errors were checked with MERLIN and PEDCHECK [31,32]. Markers showing high Mendelian inconsistency rates were excluded from analysis. In case of sporadic errors, inconsistent variants were set to missing. For analyses, there were 5250 autosomal SNPs available. The linkage analysis included 233 to 260 cases depending on the cognitive trait that was analyzed.
Before running linkage analysis, the data was reformatted with the software MEGA2 to derive the correct input format [33]. We performed genome-wide nonparametric linkage analysis in MERLIN [31] using a pair-wise approach of estimating IBD allele sharing [34-36].
Thresholds for genome-wide significant and suggestive findings were estimated by performing 500 genome-wide simulations on the global cognitive trait (zglob) [37]. For these simulations, we used the complete pedigree. The typed marker set was used for simulation of the number of markers and intermarker distances using GENEDROP. Simulation linkage

analyses were done using the same files containing allele frequencies, pedigrees and genetic model as were used in the original linkage analysis. Per simulation, the highest log of odds (LOD) was extracted and combining the 500 simulations resulted in a LOD score of 3.79 corresponding to a genome-wide type 1 error rate of 5% (significant threshold) and of 2.72 corresponding to a type 1 error rate of 50% (suggestive threshold).

Association analysis

Significant regions were further studied in a denser genotype set. We selected SNPs within a region around the highest LOD-score minus 1 LOD. A random sample of the population was genotyped using the Illumina HumanHap 320 K chip. We performed logistic regression using the extremes of the distribution to define cases and controls. Cases were defined as described above; controls were participants within the highest 50% of the age, sex and education-adjusted trait. Family-relationship was taken into account by adjusting the p-values using genomic control [38]. The genomic control inflation factors ranged from 0.98 for immediate memory to 1.86 for DART with a mean of 1.17. Analyses were performed in GenABEL(R-library) [39] using the GRAMMAR method [40]. We corrected for multiple testing by performing 10,000 permutations.

Replication

We sought replication in the Rotterdam Study, which includes participants from a population-based cohort situated in a rural area of Rotterdam, The Netherlands [41]. The study includes 7983 elderly Caucasians aged 55 years or older who were invited for extensive examinations at baseline and three follow-up rounds. The study was approved by the local Medical Ethics Committee and all participants gave informed consent. Neuropsychological tests included the word learning test, the Stroop Color and Word Test, Verbal fluency (animal naming) and the Letter Digit Substitution Task. We computed composite scores for memory by using the z-scores of immediate memory and delayed recall. For executive, we used the z-scores of Stroop-ratio and verbal fluency test, and we used all tests for the estimation of the global cognition composite score (zglob). Cases and controls were defined similarly to the discovery study. In the current study, we did not include people who were demented or had a stroke prior to the neuropsychological testing. Genotyping was done on the Illumina Infinium HumanHap 550-schip v3.0 and the genotype data was used to impute to 2.5 million non-monomorphic, autosomal single nucleotide polymorphisms (SNPs) using release 22 HapMap CEU population as a reference. For the analyses, we selected SNPs within the linkage peak as described above. Analyses were performed in GenABEL (R-library) [39] using logistic regression models. To adjust for multiple testing, a Bonferroni corrected p-value <0.05 was considered significant.

Results

Baseline characteristics of the cases and controls are shown in Table 2. The descriptives of the specific cognitive tests can be found in the Supplementary Material (Supplementary Table 1). The mean age of the cases ranged from 48 to 51 years in the ERF Study and from 57 to 63 years in the Rotterdam Study.

Baseline characteristics of the study population by composite scores cognitive function

	Linkage			Association											
				Family-based study						Population-based study					
	zglob	zmem	zexec	zglob		zmem		zexec		zglob		zmem		zexec	
	Case	Case	Case	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control
N	251	248	233	215	1101	224	1115	204	1171	191	955	315	1576	205	1026
Gender (% female)	59.4%	57.3%	58.4%	59.1%	57.1%	55.8%	57.0%	58.8%	56.6%	60.2%	57.9%	57.6%	58.4%	56.2%	57.5%
Age (mean (sd))	49.2 (17.6)	48.9 (19.1)	50.9 (16.6)	50.2 (17.8)	48.2 (13.7)	51.2 (16.7)	48.8 (13.8)	51.2(16.7)	49.5 (13.4)	63.4 (5.9)	63.7 (5.5)	62.9 (5.6)	63.7 (5.6)	66.2 (6.8)	65.4 (6.5)
Education (% of only primary education)	34.3%	36.3%	35.6%	34.0%	28.8%	34.8%	29.4%	37.3%	30.5%	15.2%	11.9%	13.7%	12.7%	17.8%	15.2%

Cases are defined as the lowest 10 % of the residuals of the trait regressed on sex, age and education. Controls are defined as highest 50% of the residuals.
** significant differences between cases and controls (p<0.01)
† use of antidepressive medication, HADS-D score and APOE ε4-carriership were known for less persons than were included in the genetic analysis.
zmem: composite score for memory; zexec: composite score for executive function; zglob: composite score for global cognition.

Table 2

Genome-wide significant results of linkage analysis in the ERF Study (LOD>3.78)

Chromosome	Variant	Physical Position	cM*	Trait	Domain	Non-parametric model	Gene	Candidate genes in region*
						LOD		
1p13.1	rs1555793	117358754	135 – 142	Stroop	Executive	**4.21**	IGSF2	PTGFRN, NGF
12q24.33	rs2270928	132238673	170 – 173	Zglob	Global	**6.36**	ZNF10	
				Zmem	Memory	**5.70**		
				WLTD	Memory	**4.89**		
				TMT	Executive	**4.37**		
				Recognition	Memory	**4.09**		
19q13.43	rs893186	63660991	110 – 111	DART	Global	**5.26**	ZNF324B	SLC27A5, HNG
				TMT	Executive	**4.09**		
				Fluency	Executive	**3.99**		
				Recognition	Memory	**3.90**		
20p13	rs751596	99330	0 – 4	VSA	Visuospatial	**4.10**	intergenic	TRIB3
				WLTI	Memory	**3.80**		
21q22.13	rs2835629	37443530	43 – 49	VSA	Visuospatial	**4.26**	TTC3	DYRK1A
				Zglob	Global	**4.14**		KCNJ6
				Stroop	Executive	**3.92**		
21q22.3	rs2256207	46886508	70 – 79	VSA	Visuospatial	**4.52**	PRMT2	S100B
				WF	Executive	**3.78**		PCNT

LOD scores in bold pass the significance threshold.
* boudaries of the linkage peak defined as the highest LOD +/- 1LOD.
Stroop: stroop-ratio; zglob: composite score for global cognition; zmem: composite score for memory; WLTD: AVLT delayed recall; TMT: trail making test ratio; DART: Dutch Adult R
VSA: Block Design Test; WLTI: AVLT immediate recall; WF: verbal fluency

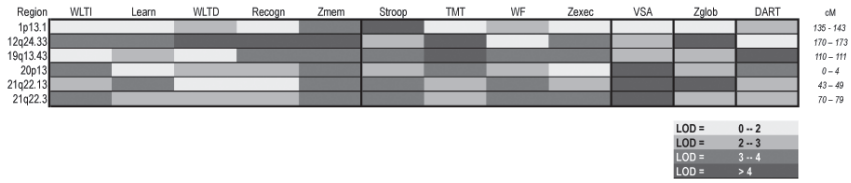
Table 3

Table 3 shows the significant linkage regions with LOD scores greater than 3.79. Evidence for linkage to cognition was found on chromosomes 1p13.1, 12q24.33, 19q13.43, 20p13, 21q22.13 and 21q22.3. An overview of LOD scores per trait for these six regions is shown in Figure 1. The figure shows that the regions on chromosome 12, 19, 20 and 21 are significantly linked to at least 2 different cognitive tests. Table 4 gives the results of the fine-mapping analysis of the significant linkage regions in ERF, while Figure 2 shows the replication findings in the Rotterdam Study.

The most consistent findings of linkage and association was found on chromosome 21, where linkage was found to VSA, global cognition (zglob), Stroop and verbal fluency. The two regions showed

significant evidence for association when adjusting for multiple testing by permutation testing (see Table 4). In the first region (21q22.13), significant association was seen with global cognition (zglob) and executive function (zexec, fluency) to variants within the potassium-inwardly-rectifying-channel-subfamily-J-member-6 gene (KCNJ6). This region was replicated in the outbred population (Rotterdam Study), where a variant (rs2836034) within KCNJ6 was significantly associated with memory (zmem) after Bonferonni correction (p=0.04; see Figure 2). In the second region on chromosome 21 (21q22.3), immediate memory (p=0.04), delayed recall (p=0.01) and recognition (p=0.04) were significantly associated to variants within the protein-arginine-methyltransferase-2 gene (PRMT2) and the minichromosome-maintenance-complex-component-3-associated-protein gene (MCM3AP). The highest LOD scores in the region for these traits were 3.07, 2.59 and 2.10 respectively. This region was not replicated in the Rotterdam study.

Heatplot depicting the highest LOD score per trait and region in the ERF Study



WLTI: AVLT immediate recall; Learn: word learning test trial 2-5; WLTD: AVLT delayed recall;
Recogn: AVLT recognition; zmem: composite score for memory; Stroop: stroop-ratio; TMT: trail making test ratio;
WF: verbal fluency; zexec: composite score for executive function; VSA: Block Design Test; zglob: composite score for global cognition;
DART: Dutch Adult Reading Test.

Figure 1

The peak under the LOD score on chromosome 1p13.1 included an interesting candidate gene, the nerve-growth-factor-beta gene (NGF) and significant association was seen with multiple cognitive traits after correction for multiple testing in the ERF Study. Although the findings were consistent when replicating the association in the Rotterdam Study for two variants (rs1555793 and rs10801929) in the sense that the lowest p-values were found for the same cognitive tests as in ERF (stroop nominal p=0.06 and zexec nominal p=0.03). However, these SNPs were not significant after Bonferroni correction.

The chromosome 12q24.33 region, was found to be significantly linked to memory (delayed recall, recognition and zmem), executive function (TMT) and global cognition (zglob) and fine-mapping pointed to the fibrosin-like-1 gene (FBRSL1) for delayed recall (rs2323982) and to an intergenic region between the pseudogene LOC647503 and the zinc-finger-protein-605 gene (ZNF605) for Stroop (rs1278607). In the replication sample, the smallest p-value for rs2323982 was also found with delayed recall, but was not significant (nominal p-value of 0.06).

At chromosome 19q13.43, we could not pinpoint a significant region with association. There was some evidence for association of memory to the zinc-finger-and-SCAN-domain-containing-18 gene (ZSCAN18), which was not significant after adjusting for multiple testing. In the Rotterdam Study we observed significant associations between executive function (stroop) and rs1051827 located in the zinc-finger-protein-606 gene (ZNF606), at 100 kb distance from ZSCAN18 (nominal p=0.001).

Results of the association analysis under the significant linkage peaks (highest LOD +/- 1 LOD) in the ERF Study

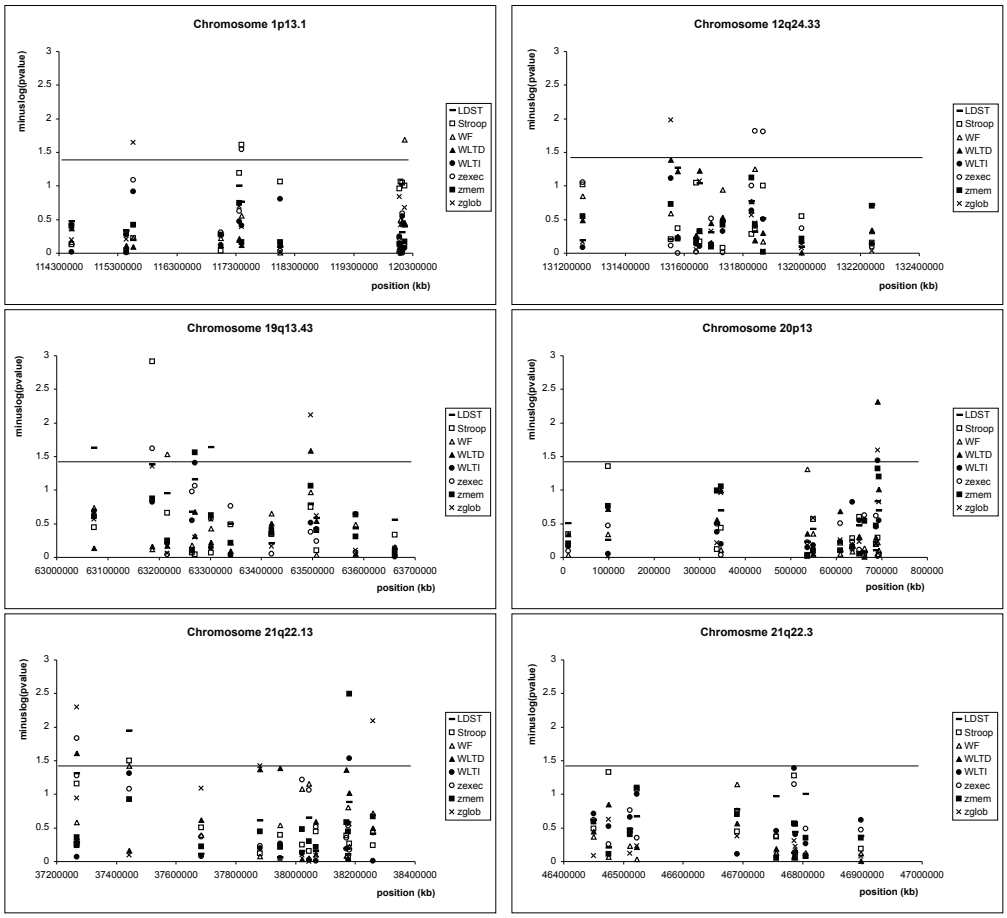
Chromosome	Best Variant	Physical position	MAF	Trait	Domain	Nominal p-value	P -value†	Gene	Flanking genes
1p13.1	rs6691374	115444341	0.188	Stroop‡	Executive	5.34E-03	**0.033**	intergenic	TSPAN2, NGF
	rs1146179	115560916	0.218	TMT	Executive	5.65E-04	**0.010**	intergenic	TSPAN2, NGF
	rs699718	117047372	0.156	DART	Global	1.94E-04	**0.023**	intergenic	GAPDHL9, IGSF3
	rs6657718	114517886	0.156	Fluency	Executive	1.64E-03	**0.023**	intergenic	SYT6, MRP63P1
	rs10801929	117395987	0.329	Zexec	Executive	3.08E-03	**0.044**	intergenic	IGSF2, TTF2
	rs2582783	120126518	0.338	WLTD	Memory	2.45E-03	**0.043**	intergenic	REG4, HMGCS2
				Recogn		1.17E-03	**0.032**		
				Zmem		1.22E-03	**0.027**		
	rs10494201	118053780	0.315	VSA	Visuospatial	7.84E-04	**0.009**	intergenic	FAM46C, LOC100131261
12q24.33	rs10781655	131554987	0.384	Zglob‡	Global	0.02	0.223	MUC8	
			0.384	Zmem‡	Memory	3.51E-03	0.061	MUC8	
	rs2323982	131652594	0.324	WLTD‡	Memory	9.60E-04	**0.010**	FBRSL1	
	rs11246991	131254068	0.138	TMT‡	Executive	0.01	0.329	GALNT9	
	rs1132375	131841610	0.430	Recognition‡	Memory	0.10	0.918	ANKLE2	
	rs1882297	131579207	0.282	WLTI	Memory	6.29E-03	0.083	FBRSL1	
	rs1574157	131640365	0.037	Zexec	Executive	0.02	0.245	FBRSL1	
	rs1278607	132000536	0.448	Stroop	Executive	2.43E-03	**0.038**	intergenic	LOC647503, ZNF605
19q13.43	rs260470	63496625	0.267	DART‡	Global	0.02	0.367	ZNF8	
	rs260423	63420161	0.098	TMT‡	Executive	0.01	0.261	ZNF274	
	rs3915790	63507665	0.198	Fluency‡	Executive	0.04	0.729	intergenic	ZNF8, ZSCAN22
	rs8100801	63583872	0.049	Recognition‡	Memory	0.03	0.701	ZNF837	
	rs3810126	63302066	0.272	Zmem	Memory	7.97E-03	0.239	ZSCAN18	
20p13	rs3746807	693963	0.126	VSA‡	Visuospatial	3.20E-03	0.185	C20orf54	
	rs1418258	11799	0.398	WLTI‡	Memory	1.81E-03	**0.019**	intergenic	DEFB125
	rs6085394	608793	0.245	DART	Global	1.82E-03	0.070	intergenic	SCRT2, C20orf54
21q22.13	rs1893654	37267597	0.489	VSA‡	Visuospatial	1.08E-03	0.920	HLCS	
	rs1892682	38169935	0.256	Zglob‡	Global	6.17E-03	**0.011**	KCNJ6	
				Zexec	Executive	3.88E-04	**0.008**		
	rs2835872	37949142	0.284	Stroop‡	Executive	6.24E-03	0.268	KCNJ6	
	rs2835933	38045624	0.266	Fluency	Executive	2.51E-04	**0.025**	KCNJ6	
21q22.3	rs2298694	46474790	0.041	VSA‡	Visuospatial	0.02	0.432	LSS	
	rs2070429	46786139	0.350	Fluency‡	Executive	0.04	0.534	DIP2A	
	rs2839376	46897344	0.082	WLTI	Memory	1.27E-03	**0.041**	PRMT2	
	rs2839182	46510580	0.131	WLTD	Memory	4.80E-04	**0.013**	MCM3AP	
	rs2839193	46522771	0.212	Recogn	Memory	8.91E-04	**0.038**	MCM3AP	
	rs2075906	46449972	0.129	Zexec	Executive	4.48E-03	0.082	LSS	

Results are shown for the traits presented in Table 3 and the significant traits within the region. Significant results are shown in bold. † p-value after 10.000 permutations.
‡ cognitive tests that were found in the linkage analysis as shown in Table 3.
WLTI: AVLT immediate recall; Learn: word learning test trial 2-5; WLTD: AVLT delayed recall; Recogn: AVLT recognition; zmem: composite score for memory; Stroop: stroop-ratio; TMT: trail making test ratio; WF: verbal fluency; zexec: composite score for executive function; VSA: Block Design Test; zglob: composite score for global cognition; DART: Dutch Adult Reading Test.

Table 4

Finally, at chromosome 20p13 fine-mapping showed significant association with immediate memory to a variant at the terminal end of the chromosome near the defensin beta 125 gene (DEFB125) (p after adjustment for multiple testing=0.02). However, the Rotterdam study did not confirm this, although nominal significance was found with delayed recall and immediate memory and a variant at 680 kb distance (nominal p=0.005 and 0.04).

Regional plot for associations in the linkage regions in the Rotterdam Study



Chromosomal positions are depicted on the x-axis; the minus logarithms of nominal p-values are depicted on the y-axis.
The horizontal line represent the significance threshold (p<0.05). The red dots represent the p-values that were significant after Bonferonni correction.
Open symbols represent memory related cognitive tests; filled symbols represent executive function related cognitive tests.

Figure 2

Discussion

Our linkage analysis of cognitive function in the ERF Study yielded a total of 6 genome-wide significant regions. Significant linkage of cognitive tests was found to chromosomes 1p13.1, 12q24.33, 19q13.43, 20p13, 21q22.13 and 21q22.3. The strongest evidence for a genetic determinant of cognitive function in our study is found for chromosome 21q22.13. The two regions on chromosome 21 are implicated in the so-called Down's syndrome critical region, which is an intensively studied region within the human genome and is recognized to be crucial for Alzheimer's disease as the amyloid-precursor-protein (APP) gene is in the region , however, APP is located 3 Mb from our linkage peak [42-44]. After fine-mapping of these regions, the region at chromosome 21q22.13 was replicated in an independent population-based sample from an outbred population. Earlier, Luciano et al. found evidence for linkage of verbal IQ to the 21q22 region [19]. The variant within the highest LOD score on chromosome 21q22.13 lies in an intron of the tetratricopeptide-repeat-domain-3 (TTC3) gene. This gene is involved in protein metabolism and may play a role in neuronal development [45], but associations with cognition or related phenotypes have not been described so far. Other candidates in the linkage peak are the KCNJ6 and dual-specificity-tyrosine-phosphorylation-regulated-kinase-1A (DYRK1A) genes. Combining the linkage result with that of association (fine-mapping and replication), the most convincing evidence concerns the KCNJ6 gene for which 1 intronic variant was associated to executive function and global cognition in ERF and to memory in the Rotterdam study. The KCNJ6 gene is expressed in the brain and may play a role in long term potentiation, which is thought to be one of the cellular mechanisms involved in learning and memory [46]. A recent genome-wide association study suggests that this gene is associated with bipolar schizoaffective disorder [47]. The DYRK1A gene was previously associated with the personality trait of conscientiousness in a genome-wide association study [48]. Levels of DYRK1A mRNA were elevated in brains of AD patients [49] and the gene has been related to learning and memory deficits in Down syndrome mouse models [50,51].

The other regions which we found in ERF were not replicated in the Rotterdam Study, however, they could be of interest, because each contains promising candidate genes. One explanation why we could not replicate the findings in the population-based study, could be the different characteristics of a family-based study and population-based study. Family-based linkage studies are mainly designed to find rare variants associated with the disease, while association studies are more powerful in finding common variants [25].

Chromosome 21q22.3 contains the S-100-calcium-binding-protein-beta-chain gene (S100B), which was associated to cognitive ability previously [52,53]. The gene is located less than 0.4 Mb to the most significant variant in the fine-mapping analysis. The region on chromosome 1 was linked to Alzheimer's disease in a large linkage meta-analysis [54] and there are multiple genes of interest in this region. The most significant variant in our linkage analysis lies in an intron of the immunoglobulin-superfamily-member-2 gene (IGSF2), a gene that is involved in the inhibition of T-cell proliferation.

Although this is an interesting candidate, there may be other neighboring genes explaining our findings. The nerve-growth-factor-beta-peptide gene (NGF), is involved in the regulation of neuronal growth and differentiation and has been associated with AD and personality traits [55]. Our fine-mapping analysis shows some significance of executive function to variants near IGSF2 and NGF. The chromosome 12 region which showed significant linkage in our analysis is adjacent to the region which was found to be suggestively linked to prospective memory in a previous study [23]. In the fine-mapping of this region, an intronic SNP within the fibrosin-like-1 (FBRSL1) gene was associated to delayed recall. This gene is expressed in the brain, but its function is largely unknown, asking for further research. We could not confirm the region on chromosome 19q13.43 with association. Chromosome 20p13, which we found linked to visual spatial ability and memory contains an obvious candidate gene, the prion-protein gene (PRNP), which is associated with multiple cognitive traits in the general population [56-58]. The highest LOD-score in the current study was found at 4.5 Mb distance from PRNP.

We confirmed some interesting chromosomal regions previously found in related phenotypes and found possible new regions. Interestingly, most regions were related to multiple cognitive domains, which fits the 'generalist gene' hypothesis indicating that there are pleiotrophic pathways involved in cognitive function [5]. There are some issues that need to be discussed. First, 3 of the 6 linkage peaks were located at the terminal ends of the chromosomes, which may have lead to inflation of the estimates, because there are no neighbouring markers. However, there were multiple markers contributing to the peaks leading us to believe that the height of the peak was not due to a single marker estimate. Second, although we used a linkage panel, the markers were relatively dense compared to STR marker sets, which may have caused inflation of peaks due to LD between markers. This may partly explain our exceptionally high peaks. Therefore, our results should be interpreted in light of the simulation based thresholds for significant and suggestive findings as shown in this paper. It is of interest that the variants identified with our association analyses (fine-mapping and replication) were intronic variants of KCNJ6 that were significant after adjustment for multiple testing, which may imply that they are not directly involved in protein transcription.

The strengths of the study are the cohort design of the discovery as well as the replication sample, and the fact that study subjects were not selected on the trait of interest. Moreover, the population is derived from a young genetic isolate, which increases the study power because of the smaller gene pool. Yet, the findings can be extended to the general population, because the genetic makeup does not differ largely from the general population [25].

In summary, our study demonstrates evidence for significant linkage of chromosomes 1p13.1, 12q24.33, 19q13.43, 20p13, 21q22.13 and 21q22.3 to cognitive function. The region 21q22.13 was replicated in an independent sample and is most likely explained by the KCNL6 gene.

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05 Genome-wide association study of cognitive executive functions: Meta-analysis of the CHARGE consortium

Abstract

Introduction

Executive function is an important part of cognitive function. To explore common variants that contribute to the normal variation in cognition, we conducted genome-wide association analyses in the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium, which includes large prospective population-based cohorts.

Materials and Methods

Neuropsychological testing was available for 13 cohorts. For executive function and processing speed tasks, we included the Trail Making Test (TMT) parts A and B and the Stroop Color and Word Test in the analyses. All the individual studies used their genotyped data to impute to 2.5 million single nucleotide polymorphisms (SNPs). The analyses were performed in Caucasians older than 45 years who were free of dementia and clinical stroke at times of cognitive testing. Each study applied an additive genetic model using linear regression models adjusting for age and sex. Meta-analyses were performed for TMT-A, TMT-B, TMT-B minus TMT-A using fixed-effects models and on Stroop using sample-sized weighted models.

Results

The meta-analysis included 5,477 participants for the TMT-A, 6,212 for TMT-B and TMT-B minus TMT-A and 7,777 for the Stroop. The most significant association was found with TMT-B and a SNP on chromosome 18. This SNP was just above the genome-wide significant threshold with a p-value of 6.95*10-8. When comparing our findings of the GWAS on cognitive function to the GWAS of AD and schizophrenia published earlier, we identified two other genes of interest: STXBP6 and PCDH9. However, these SNPs did not reach genome wide significance in the present analysis nor in those of the disease outcomes.

Conclusions

In conclusion, we found compelling evidence for a region on chromosome 18 that is involved in TMT-B and preliminary evidence for STXBP6 and PCDH9. However, our findings await replication which is at present ongoing.

Introduction

Cognitive function is a broad concept referring to multiple cognitive domains, among which are memory, language, executive function and visuospatial ability. Normal cognitive ability is an important determinant of quality of life. Impairment of these functions is seen in patients with various diseases including dementia, bipolar disorder, schizophrenia and attention deficit hyperactivity disorder (ADHD) [1-3]. Executive function is one of the major processes of the frontal lobe, and includes a range of tasks, among which are response inhibition, attention, cognitive flexibility and planning [4]. Genetic factors account for over 20% of the variability in executive function traits [5-9]. Finding susceptibility genes for cognitive functioning may provide insight into the normal variation in executive function, but

may also increase the knowledge of diseases that are associated with cognitive impairment.
Although various genes have been identified, consistency is yet to be found, which is due to lack of replication and meta-analyses [10]. Of candidate genes, the apolipoprotein E (APOE) gene seems the most promising genetic risk factor for executive function and memory [11]. Linkage regions that were previously found for executive function tasks were located on chromosomes 2q, 5q, 11q, 13q and 14q [12-14]. To our knowledge there are currently 4 genome-wide association studies on cognitive traits in adults [15-18]. In these studies associations were found of memory with the calsynthenin-2 (CLSTN2) gene on chromosome 3, with the WW-and-C2-domain-containing-1 (KIBRA) gene on chromosome 5 and with the sodium-channel-voltage-gated-type I-alpha subunit (SCN1A) gene on chromosome 2. Abstract reasoning was associated with the sortilin-related-receptor (SORL1) gene located on chromosome 11. However, no genes were identified for executive function by GWAs. Furthermore, the studies of memory and abstract reasoning were small and liberal significance thresholds were used.
To explore common variants that contribute to the normal variation in executive function, we performed a large-scale meta-analysis combining GWAS from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium, including 12 prospective population-based cohorts. We present results from four meta-analyses.

Materials and Methods

Consortium

The Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium includes large prospective population-based cohorts [19]. Each cohort has extensive phenotypic information on various traits and genome-wide data available. Guidelines for collaboration were approved and phenotype-specific workgroups agreed on phenotype harmonization, the selection of covariates and analytic plans for each study and for the overall meta-analyses. Each study obtained approval from Institutional Review Boards. All participants gave written consent for study participation and use of DNA for genetic research.

Setting

Details of cohort selection, risk factor assessment and cognitive testing in the 5 cohorts included in the current study have been described previously and included in the supplementary data. Briefly, the Aging Gene-Environment Susceptibility – Reykjavik Study (AGES) included 30,795 persons born between 1907 and 1937 who lived in Reykjavik at the 1967 baseline examination. Re-examination of surviving members of the cohort was initiated in 2002 as part of the AGES-Reykjavik Study (N=2300) [20]. The Atherosclerosis Risk in Communities Study (ARIC) included 15792 persons of 45 to 64 years of age from 4 US communities between 1987 to1989. The study included blacks as well as whites (N=11478) [21]. The Austrian Stroke Prevention Study (ASPS) included 2007 persons living

in Graz, Austria. An extended diagnostic work-up was performed in a subset of age 45 to 85 years between 1991 and 1994 and between 1999 and 2003 [22,23]. The Cardiovascular Health Study (CHS) enrolled persons aged 65 years and older from 4 US communities. Baseline examination was either in 1989 to 1999 or 1992 to 1993 (N=5888, including 4925 whites) [24]. The Erasmus Rucphen Family study (ERF) enrolled 3000 persons from a genetically isolated population located in the South West of the Netherlands. Baseline examination was performed between 2002 and 2005 [25]. The Framingham Heart Study (FHS) included 3 generation of participants from the US. Cognitive testing data was collected between 1999 and 2004 for survivors from the Original cohort (N=5209) that has been followed since 1948 and the offspring cohort since 1971 (N=5124) [26,27]. The Rotterdam Study is a population-based cohort study among inhabitants of a district of Rotterdam (Ommoord), The Netherlands. In 1990-1993, 7,983 persons participated and were re-examined every 3 to 4 years [28].

Cognitive testing

The study program of each study included neuropsychological testing. We focused on the Trail Making Test parts A and B (TMT). The TMT is a time-demanding task in which participants are asked to connect a randomly placed series of letters and numbers as quickly as possible. In TMT-A only the numbers have to be connected, in TMT-B numbers and letters have to be connected alternately (from 1 to A, to 2 to B etc). The score was defined as the time in seconds to complete the task. Participants who passed the maximum test time for TMT-B were given the maximum time score of 300 seconds. There were no participants who timed out on TMT-A. For analysis, we used time in seconds on TMT-A and TMT-B. Additionally, we used the time difference between TMT-B and -A (TMT-BminusA). The times were transformed by taking the natural logarithm. The Stroop Color and Word Test is also a time demanding task consisting of three cards. In card I, participants have to name the right words as quickly as possible, in card II they have to name the right colors as quickly as possible and in card III they have to name the colors in which the words are printed as quickly as possible. On this card the meaning of the word is different than the color the word is printed in (e.g. blue is written whereas the word is printed in red). ARIC recorded number of words; AGES, ASPS, ERF and RS recorded time in seconds. For analysis, we used the time difference between card III and II (Stroop interference).

Genotyping

The consortium was formed after the individual studies had finalized their GWAS platforms, and the studies included used different platforms: the Illumina HumanCNV370-Duo BeadChip® for AGES and CHS; the Affymetrix GeneChip SNP Array 6.0® for ARIC; the Illumina Human610-Quad BeadChip® for ASPS; the Affymetrix GeneChip Human Mapping 500K Array Set® and 50K Human Gene Focused Panel® for FHS; a combination of the Illumina HumanHap 320K array, Illumina HumanHap 6k Beadchip array, the Illumina Human 370K-Duo SNP array and the Affymetrix GeneChip® Human

Mapping 250K Nsp Array for ERF; and the Illumina HumanHap550-Duo BeadChip® for the Rotterdam Study.

All studies included used their genotyped data to impute to 2.5 million non-monomorphic, autosomal single nucleotide polymorphisms (SNPs) using release 22 HapMap CEU population as a reference. As detailed previously [29], cohort-specific quality controls included filters for call rate, heterozygosity, and number of Mendelian errors per individual. SNP-specific quality controls included filters for call rate, minor allele frequency, Hardy-Weinberg equilibrium, and differential missingness by outcome or genotype (mishap test in PLINK, http://pngu.mgh.harvard.edu/purcell/plink/). The set of genotyped input SNPs used for imputation in each study was selected based on their highest quality GWA data. We used a call rate >95% in ARIC, >97% in CHS and FHS, and >98% in AGES-Reykjavik, ASPS, ERF and Rotterdam; a minor allele frequency >0.01 in each study; a Hardy-Weinberg p>1x10-5 in ARIC and CHS and p>1x10-6 in AGES-Reykjavik, ASPS, ERF, FHS and Rotterdam; a test of differential missingness by the "mishap" test in PLINK p>1x10-9 in each study except ERF and ARIC. We used either the Markov Chain Haplotyping (MaCH) package (http://www.sph.umich.edu/csg/abecasis/MACH, version 1.0.15 or 1.0.16 software) for AGES-Reykjavik, ARIC, ASPS, ERF, FHS and Rotterdam for imputation to the plus strand of NCBI build 36, HapMap release #22, or BIMBAM15 programs for CHS for imputation to the plus strand of NCBI build 35. For each imputed SNP informativity of imputation was estimated as the ratio of the empirically observed dosage variance to the expected binomial dosage variance.

Study population

Participants were eligible for the discovery cohort if they did not have prevalent dementia, clinical stroke and were Caucasian. Participants were excluded when consent was declined or genotyping failed. Only participants aged 45 and older were included.

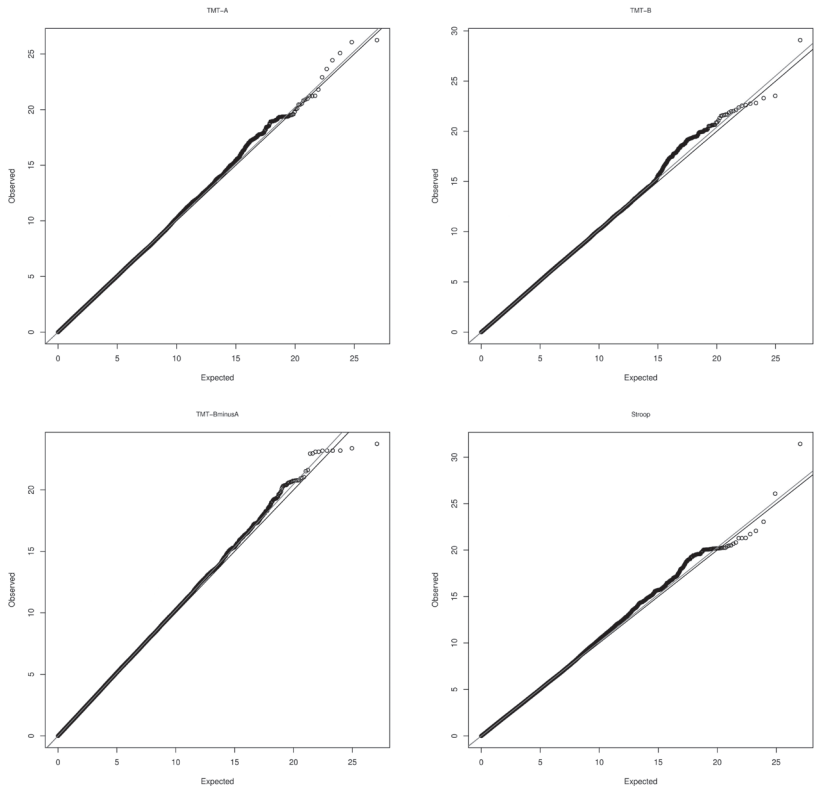
Statistical analysis within studies

An additive genetic model was used in each study, fitting a 1-degree-of-freedom test relating genotype dosage (0, 1 or 2 copies of the minor allele) to cognitive tests. Linear regression models were used adjusting for sex and age and secondary adjusting for age, sex and education. ARIC and CHS additionally adjusted for study site; FHS and ERF the additionally adjusted for family structure. We did not adjust for additional covariates to avoid adjusting for variables that might be involved in the causal pathway. Education was divided into 4 categories corresponding to the years and level of education. Category 0 corresponded to less than high school (0-11 years); category 1 corresponded to a high school degree (12 years); category 2 corresponded to more than 12 years of education, but not a college degree and category 3 corresponded to college degree and above.

Studies were screened for latent population substructure, including cryptic relatedness, using suitable programs: EIGENSTRAT in ARIC, FHS and AGES [30,31], an IBD matrix in ASPS and Rotterdam [32], and using principal component analysis in CHS. When appropriate, components related to the cognitive phenotype under study were included as covariates in the linear regression. In ERF, analyses were performed in GenABEL (R-library) [33], using the mmscore method, with a kinship matrix that was estimated from the genotype data to adjust for relatedness of the population [34].

We studied quantile-quantile (Q-Q) plots to ensure that the p-value distributions in each of the cohorts conformed to a null distribution at all but the extreme tail. We also calculated the genomic inflation factor lambda, which measures over-dispersion of test-statistics from association tests indicating population stratification and can be used to apply genomic control [33]. The lambda for TMT was less than 1.02 for all of the studies.

Quantile-quantile plots of TMT-A, TMT-B, TMT-BminusA and Stroop results



Q-Q plot of the inverse variance meta-analyses, it shows the distribution of the observed test statistic (negative log of p-values, on the y-axis) plotted against the distribution of test statistic expected under the null-hypothesis (on the x-axis).

Figure 1

Meta-analysis

After quality control and filtering within each study, AGES had either genotyped or imputed data for 2,532,729, ARIC for 2,543,887 SNPS, ASPS for 2,543,887 SNPs, CHS for 2,531,169 SNPs, FHS for 2,540,223 SNPs, the Rotterdam study for 2,543,887 SNPs and ERF for 2,543,887 SNPs. We restricted our meta-analysis to autosomal SNPs that were common to all studies and had a minor allele frequency >0.01 and an imputation quality >0.3.

Baseline characteristics by study population

Cohort		TMT-A	TMT-B	TMT-BminusA	Stroop interference
		seconds	seconds	seconds	seconds
AGES	N	-	-	-	2644
	Age	-	-	-	75.9 (5.33)
	Gender (% female)	-	-	-	58.9
	Test score	-	-	-	41.7 (21.5)
ARIC	N	438	436	436	430
	Age	72.6 (4.2)	72.6 (4.2)	72.6 (4.2)	72.5 (4.2)
	Gender (% female)	59.8	59.6	59.6	60.0
	Test score †	41.6 (17.6)	114.6 (49.6)	73.6 (43.1)	-29.9 (9.5) †
ASPS	N	-	830	-	261
	Age	-	65.2 (8.0)	-	65.1 (7.5)
	Gender (% female)	-	56.9	-	57.1
	Test score	-	128.0 (60.2)	-	47.2 (22.7)
CHS	N	1249	1249	1249	-
	Age	79.4 (3.8)	79.4 (3.8)	79.4 (3.8)	-
	Gender (% female)	61.0%	61.0%	61.0%	-
	Test score	52.3 (22.0)	141.5 (62.5)	89.2 (53.3)	-
ERF	N	1267	1255	1255	1238
	Age	58.7 (8.9)	58.7 (8.8)	58.7 (8.8)	58.6 (8.8)
	Gender (% female)	54.8	54.7	54.7	55.5
	Test score	45.9 (20.8)	139.5 (78.0)	94.0 (67.2)	54.2 (33.0)
FHS	N	2475	2440	2440	-
	Age	64.7 (11.9)	64.6 (10.9)	64.6 (10.9)	-
	Gender (% female)	54.8	54.7	54.7	-
	Test score	35.7 (20.2)	92.0 (55.6)	60.0 (46.2)	-
RS	N	-	-	-	3204
	Age	-	-	-	64.4 (6.7)
	Gender (% female)	-	-	-	57.9
	Test score	-	-	-	32.1 (18.9)

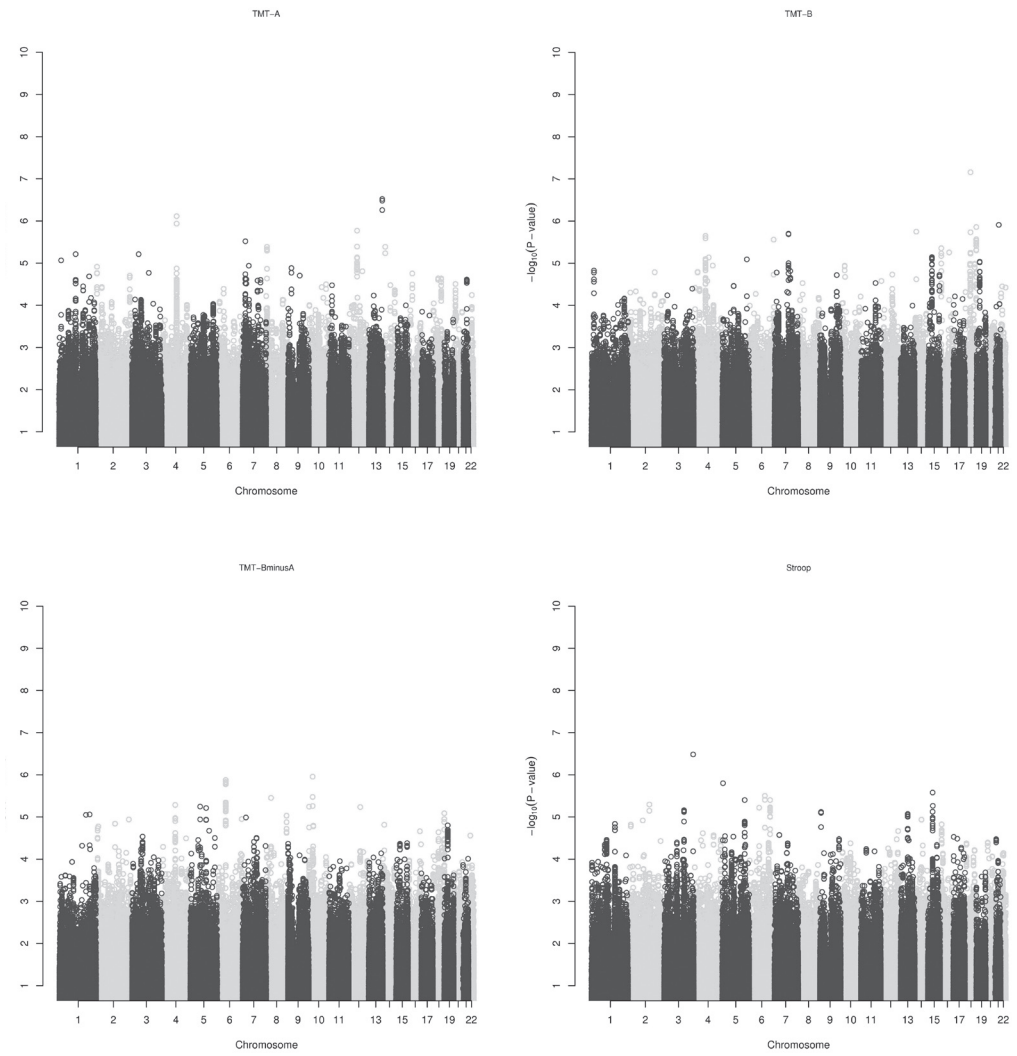
†: score in number of words

Table 1

We used an inverse-variance meta-analysis as our primary method after applying genomic control within each individual study. Beta estimates were weighted by their inverse variance and a combined estimate was obtained by summing the weighted betas and dividing by the summed weights. Hence results for SNPs imputed with low certainty were down-weighted because the low informativity of imputation ensures a large variance. In contrast, studies with large sample sizes and with directly genotyped or well-imputed SNPs had a greater effect on the meta-analyses p-value because of small variances. In a secondary analysis we used an effective sample size weighted meta-analysis technique after applying genomic control within each individual study. For each SNP the z-statistic was weighted by the effective sample size (product of the sample size and the ratio of the empirically observed dosage variance to the expected binomial dosage variance for imputed SNPs). A combined estimate was obtained by summing the weighted z-statistics and dividing by the summed weights. Hence results for SNPs imputed with low certainty were down-weighted. In contrast, studies with

large sample sizes and with directly genotyped or well-imputed SNPs had a greater effect on the meta-analyses p-value. We undertook the meta-analysis using the METAL software by Abecasis and Willer in 2007.

Genome-wide plots of TMT-A, TMT-B, TMT-BminusA and Stroop results



Minus log p-values (x-axis) are plotted against their genomic position (y-axis). The plot is based the fixed-effects meta-analysis.

Figure 2

We estimated the genomic inflation factor lambda after meta-analysis. The estimate of lambda was 1.010 for TMT-A, 1.022 for TMT-B and 1.024 for TMT-BminusA indicating no significant inflation of p-values. The quantile-quantile (Q-Q) plots of our inverse variance meta-analyses results for the 3 tests show the distribution of the observed test statistic (negative log of p-values, on the y-axis) plotted against the distribution of test statistic expected under the null-hypothesis (on the x-axis) (Supplementary Figures 1).

A p-value ≤ 5.0*10-8 was considered genome-wide significant [36] and a p-values between 5.0*10-8 and 1.0*10-5 were considered highly suggestive associations. P-values <1.0*10-3 were considered suggestive associations.

We additionally studied overlap between our suggestive associations and previously reported genome-wide data from studies in cognitive function and in diseases for which cognition may be an endophenotype. We used three published meta-analyses with publicly available data on various cognitive tests [18], Alzheimer's disease (AD) [37] and schizophrenia (SCZ) [38].

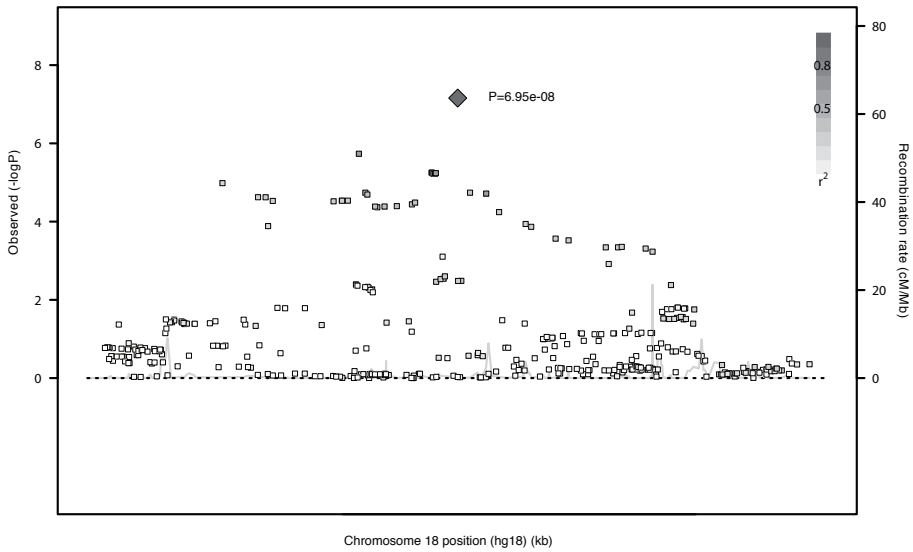
Results

Baseline characteristics of the study populations are depicted in Table 1. The mean age of the study population was 68.3 years and 57.6% were women. The mean test score on TMT-A was 43.9 seconds among the 5,477 participants that were included in the meta-analysis. The mean test score of the 6,212 participants included in the TMT-B analysis was 123.1 seconds and the mean time difference on TMT-BminusA was 79.2 seconds among 5,377 participants. For Stroop interference, the mean test score for the cohorts using time in seconds was 43.8 seconds, the mean test score for ARIC was -29.9 words.

Figure 2 shows the genome-wide plots for each trait, depicting the p-values by their genomic position. Highly suggestive loci with a p-value smaller than 10-5 are presented in Supplementary Tables 1 to 4. The most significant finding was seen for TMT-B for a SNP on chromosome 18. This SNP was just above the genome-wide significant threshold with a p-value of 6.95*10-8. A detailed plot for all SNPs within a region of 250kb is shown in Figure 3, which shows that there are multiple SNPs in the region in linkage disequilibrium with this SNP. All cohorts show similar effect sizes and direction of effect for this SNP (Figure 4). The p-value for this SNP is 0.09 for TMT-A and of 3.35*10-5 for TMT-BminusA. The SNP is intergenic, and located in between two interesting genes.

The lowest p-value for TMT-A was 3.35*10-7 for a SNP on chromosome 13. The second ranked SNP with a p-value of 7.69*10-7 was located in a region that was also associated with TMT-B (p-value 7.30*10-6). For TMT-BminusA, the lowest p-value was 1.11*10-6 for a SNP on chromosome 10. Finally, on chromosomes 18 the same SNP was associated with TMT-B and TMT-BminusA (p-value 1.39*10-6 and 8.19*10-6, respectively). Stroop interference was associated to two regions on chromosome 3. The first ranked SNP was associated with a p-value of 2.38*10-7, which is rare variant (MAF=0.03).

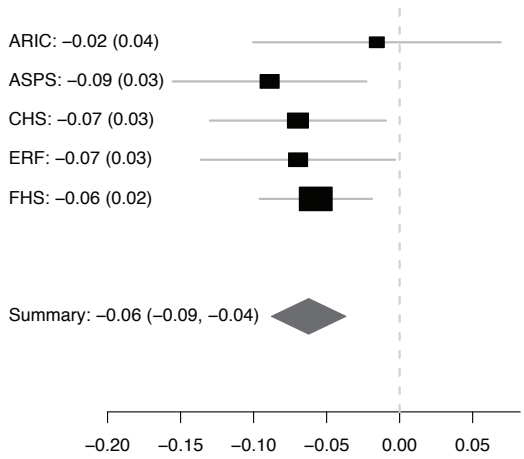
Regional plot for associations in the region around the top hit (+/- 250kb)



All SNP are plotted with their meta-analysis probability values against their genomic position. The color of the triangles represents the linkage disequilibrium between SNP. Light blue line represents estimated recombination rates.

Figure 3

Forest plot of the results per cohort for the top hit – TMT-B



The values on the x-axis represent the betas.

Figure 4

Next, we performed a data mining study comparing our findings to the GWAS findings of the largest studies on AD and schizophrenia [35,36]. At the suggestive threshold of 10-3, 2731 SNPs were associated with TMT-A, 2790 SNPs with TMT-B, 2761 with TMT-BminusA and 2980 SNPs with Stroop. When comparing our suggestive associations (p<10-3) with the disease based genome-wide studies (Table 2), we did not see an overlap for the top SNPs identified for TMT-A, TMT-B, TMT-BminusA or Stroop. However, one putative AD gene, the sortilin-related-receptor 1 gene (SORL1) did show some overlap with Stroop. There are two additional interesting findings. First, we found one region with overlapping SNPs for both TMT-B and TMT-BminusA. This region on chromosome 14 overlapped with the findings in Alzheimer's disease [35] and contains the neuro-oncological ventral antigen 1 (NOVA1) and the syntaxin binding protein 6 (STXBP6) genes. Second, the region identified in the top ranking of the Stroop data, which concerned SNP within the protocadherin 9 gene (PCDH9) (p=8.49*10-6; supplementary table 4), was identified also in the AD GWAS, although a different SNP was reported.

Overlap of the GWAS findings for TMT-A, TMT-B, TMT-BminusA and Stroop interference with GWAS of related outcomes

SNP	Chrom	Effect	SE	MAF	Our p-value	Direction	Gene	Flanking genes	AD	SCZ
TMT-A										
SNPa	8	-0.0298	0.0088	0.1980	7.68E-04	----	C8orf79			1.76E-04
SNPb	15	0.0268	0.008	0.2521	8.68E-04	++++		BNC1, SH3GL3	1.79E-04	
SNPc	19	-0.0293	0.0085	0.2658	5.97E-04	---+	ZNF610		5.28E-04	
TMT-B										
SNPd	1	-0.0448	0.0136	0.36	9.55E-04	---+-	DAB1			8.20E-04
SNPe	4	-0.0358	0.0106	0.16	7.15E-04	-+---	intergenic	BOD1L, AC095052.1		8.67E-04
SNPf *	6	0.0427	0.0113	0.17	1.56E-04	+++++	intergenic	HLA-region	9.32E-04	
SNPg *	14	0.029	0.0083	0.46	4.83E-04	+++++	intergenic	STXBP6, NOVA1	9.43E-04	
TMT-BminusA										
SNPh	3	-0.0519	0.0139	0.38	1.90E-04	----	CACNA2D3			2.35E-04
SNPi	10	-0.0497	0.0148	0.23	8.12E-04	-+--	LIPA			3.71E-04
SNPj	14	-0.0462	0.0133	0.47	5.39E-04	----	intergenic	STXBP6, NOVA1	9.51E-05 8.22E-04 (apoe neg)	
SNPk *	18	-0.0523	0.0133	0.29	8.70E-05	----	intergenic	C18orf22, ADNP2	5.86E-04 (apoe neg)	
SNPl *	18	0.0412	0.0124	0.42	9.18E-04	++++	DCC			6.44E-05
Stroop †		**Weight**	**Z-score**							
SNPm	6	7435.37	3.464	0.3311	5.33E-04	+++++	JARID2			0.03
SNPn	8	7521.56	-3.305	0.2080	9.50E-04	-----	C8orf79			1.76E-04
SNPo	11	7573.78	3.401	0.0252	6.71E-04	+++++	SORL1		9.70E-03	
SNPp	13	7732.09	-3.916	0.3469	9.02E-05	-+---	PCDH9		5.74E-04 (apoe neg)	
SNPq	15	7720.07	-3.352	0.1405	8.01E-04	-----	intergenic	CA12, USP3	6.80E-04 (apoe neg)	
SNPr	15	6967.1	3.512	0.2833	4.44E-04	++-++	intergenic	KLHL25, AGBL1		3.43E-04

P-values are on the fixed effects meta-analysis adjusted for age and sex. † P-values are based on the sample-size weighted meta-analysis of adjusted for age and sex. Highlighted SNPs overlapped between our traits. * More than one SNP showed overlap.

Table 2

Discussion

The current study is a large meta-analysis of genome-wide associations studies performed in 7 cohorts including over 6000 subjects. Although not fulfilling the criteria for genome-wide significance, by far the most interesting region is the chromosome 18 region that shows association to TMT-B. We found one SNP approaching genome wide significance in the analysis of TMT-B. The SNP is located between two interesting genes. Unfortunately, none of the other tests showed p-values that were

close to significance. At this stage, our study is awaiting replication for the top ranked SNPs. When considering the highest ranked SNPs based on p-values, however, we find little overlap between the SNPs associated with the various cognitive tests. The lack of overlap for genes involved in TMT-A, in TMT-B and Stroop may be explained by the fact that TMT-A is an outcome related to processing speed while TMT-B and Stroop are outcomes related to executive function. There may be several explanations for the lack of overlap between the findings for TMT-B and Stroop. We may have lacked statistical power to identify true positive SNPs and the top SNPs may primarily represent noise. Alternatively, the overlap in brain function as measured by TMT-B and Stroop may be limited. While all the measures were timed and likely share a common processing speed factor, the executive factors underlying TMT-B and Stroop likely reflect diverse higher order factors. If this is the case, one may expect different genes to be involved in different tests.

Despite the low power, we still see an overlap of our GWAS with previous GWAS on AD and schizophrenia, for which cognitive functioning is an endophenotype. It is of interest that not the apolipoprotein E gene (APOE) but rather SORL1 pops up in both cognitive function and AD analyses. SORL1 as been identified as a gene that may be involved in AD [39,40]. When comparing our findings to those of the 4 earlier genome-wide association studies on cognitive traits in adults [15-18], we only see an overlap with the relation of SORL1 to abstract reasoning. The gene of most interest in the comparative analysis of our findings to those in AD and Schizophrenia is STXBP6 [37]. This gene forms non-fusogenic complexes with the synaptosomal-associated-protein-25kDa (SNAP25) and syntaxin-1A (STX1A) genes and may thereby modulate the formation of functional SNARE complexes and may be involved in exocytosis [41]. SNAP25 has been associated both to schizophrenia and Attention Deficit Hyperactivity Disorder (ADHD) [42,43]. Further, a region was identified in the top of the GWAs of Stroop data (PCDH9), which overlapped with the AD GWAS. PCDH9 belongs to the protocadherin gene family, a subfamily of the cadherin superfamily [44]. The gene encodes a cadherin-related neuronal receptor that localizes to synaptic junctions and is putatively involved in specific neuronal connections and signal transduction. Protocadherin 9 cadherin-related neuronal receptor, predominantly expressed in brain, localizes to synaptic junctions and is involved in specific neuronal connections and signal transduction. The gene is also expressed in other tissues in which it exerts a developmentally regulated expression pattern.

In conclusion, we found compelling evidence for a region on chromosome 18 that is involved in TMT-B. When comparing our findings of the GWAS on cognitive function to the GWAS of AD and schizophrenia we identified to other genes of interest: STXBP6 and PCDH9. For these two genes the evidence for association is very preliminary as these SNPs did not reach genome wide significance in the present analysis nor in those of the disease outcomes. Our findings await replication studies that are presently ongoing.

TMT-A: Listing of all SNPs with a p-value < 10^{-5}

SNP	Chrom	Effect	SE	MAF	P-value	Direction	Gene	Flanking genes	Number of SNPs <10^{-5}
Adusted for age+sex									
SNP1	13	0.0483	0.0094	0.1676	3.01E-07	++++	MYO16		2
SNP2	4	-0.0379	0.0077	0.71	7.69E-07	----	ADH5		1
SNP3	12	0.0463	0.0097	0.8324	1.70E-06	+-++	OR6C70		5
SNP4	7	-0.0341	0.0073	0.3707	3.03E-06	----	AC006397.1		
SNP5	14	-0.0537	0.0117	0.818	4.10E-06	----	intergenic	SNX6, RPL23AP8, CFL2	1
SNP6	8	0.0338	0.0073	0.4737	4.12E-06	++++	intergenic	LOC392180, RPL23AP54	3
SNP7	1	0.033	0.0073	0.5565	6.12E-06	++++	KCND3		0
SNP8	3	0.039	0.0086	0.2207	6.13E-06	++++	TGM4		0
SNP9	1	-0.0346	0.0078	0.5545	8.60E-06	----	intergenic	KIAA1026, PRDM2	

P-values are based on the fixed effects meta-analysis of CHS, FHS, ERF, and ARIC.
]Number of SNPs refers to additional SNPs within 250kb of the top SNP.

Supplementary table 1

TMT-B: Listing of all SNPs with a p-value < 10^{-5}

SNP	Chrom	Effect	SE	MAF	P-value	Direction	Gene	Flanking genes	Number of SNPs <10^{-5}
Adusted for age+sex									
SNP1	18	0.0725	0.0134	0.1137	6.95E-08	+++++	intergenic	PIK3C3, RIT2	6
SNP2	21	0.0378	0.0078	0.4460	1.23E-06	+++++	intergenic	CLIC6, RUNX1	0
SNP3	18	-0.0573	0.0119	0.1127	1.39E-06	-----	ADNP2		8
SNP4	4	0.0459	0.0097	0.2094	2.25E-06	+++++	SEPT11		5
SNP5	7	0.0385	0.0081	0.3615	1.99E-06	+++++	intergenic	BET1, Col1A2	1
SNP6	4	0.0869	0.0194	0.0459	7.30E-06	+++++	ADH4		0
SNP7	6	0.0572	0.0122	0.2159	2.76E-06	+++++	intergenic	LOC728275, LOC728316	0
SNP8	16	0.0615	0.0134	0.0899	4.41E-06	+++++	intergenic	A2BP1, LOC283953	2
SNP9	16	0.0589	0.013	0.1163	5.52E-06	+++++	intergenic	CDH8	1
SNP10	15	0.035	0.0078	0.3980	7.13E-06	+++++	intergenic	SQRDL, SLC24A5	8
SNP11	19	-0.0363	0.0082	0.3403	9.18E-06	-----	intergenic	AC022145.1, ZNF730	1

P-values are based on the fixed effects meta-analysis of ASPS, ARIC, ERF, CHS, and FHS.
Number of SNPs refers to additional SNPs within 250kb of the top SNP.

Supplementary table 2

TMT-BminusA: Listing of all SNPs with a p-value < 10^{-5}

SNP	Chrom	Effect	SE	MAF	P-value	Direction	Gene	Flanking genes	Number of SNPs <10^{-5}
Adusted for age+sex									
SNP1	10	0.1587	0.0326	0.0532	1.11E-06	++++	intergenic	GPR158, MYO3A	2
SNP2	6	-0.0761	0.0157	0.1946	1.33E-06	----	PRIM2	BAG2	24
SNP3	8	-0.1512	0.0326	0.0437	3.52E-06	----	NRG1		0
SNP4	12	-0.1224	0.027	0.0758	5.82E-06	----	intergenic	E2F7, NAV3	0
SNP5	18	-0.0872	0.0196	0.1115	8.19E-06	----	ADNP2		0
SNP6	4	0.0709	0.0156	0.1891	5.19E-06	++++	HERC3		0
SNP7	5	0.0596	0.0131	0.3743	5.67E-06	++++	MAST4		0
SNP8	5	-0.0974	0.0215	0.1039	6.12E-06	----	intergenic	LOC100289569, EFNA5	1
SNP9	1	0.0874	0.0197	0.1630	8.71E-06	++++	NUAK2		

P-values are based on the fixed effects meta-analysis of ARIC, ERF, CHS and FHS adjusted for age and sex.
Number of SNPs refers to additional SNPs within 250kb of the top SNP.

Supplementary table 3

Stroop interference: Listing of all SNPs with a p-value < 10^{-5}

SNP	Chrom	Weight	Z-score	MAF	P-value	Direction	Gene	Nearby*	Number of SNPs <10^{-5}
SNP1	3	7184.95	5.106	0.0307	2.38E-07	++-+++	ATP13A5		0
SNP2	15	7417.36	-4.698	0.4470	2.63E-06	-----	TMOD2		2
SNP3	6	5869.31	4.661	0.094	3.15E-06	+++++	intergenic	POPDC3, PREP	1
SNP4	5	7440.17	4.614	0.1168	3.96E-06	+-+++	GPX3		0
SNP5	6	7333.42	-4.613	0.2180	3.96E-06	-----	intergenic	LOC100129554, RPS3AP24	9
SNP6	6	6560.34	4.526	0.4701	6.03E-06	+++++	intergenic	NUFIP1P, RNU7-66P	0
SNP7	3	7745.52	4.497	0.2397	6.91E-06	++++++	CPNE4		13
SNP8	9	7416.22	-4.476	0.040	7.59E-06	-----	intergenic	LOC392285 (RPL4P5), C9orf123	1
SNP9	13	7729.08	-4.452	0.3639	8.49E-06	-----	PCDH9		11

P-values are based on the sample-size weighted meta-analysis of AGES, ARIC, ASPS, ERF, RS (N=7,777) adjusted for age and sex.
*: nearest gene to snp; number of SNPs refers to additional SNPs within 250kb of the top SNP. SNPs highlighted overlap between the two models.

Supplementary table 4

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Part III

Genetic Determinants of Alzheimer's Disease

06 Cathepsin D gene and the risk of Alzheimer's disease: A population-based study and meta-analysis

Abstract

Cathepsin D (CTSD) is a gene involved in amyloid precursor protein processing and is considered a candidate for Alzheimer's disease (AD). The aim of the current study was to examine if variation in CTSD increases the risk of AD. We performed a candidate-gene analysis in a population-based cohort-study (N=7983), and estimated the effect of CTSD on the risk of AD. Additionally, a large meta-analysis was performed incorporating our data and previously published data. The T-allele of CTSD rs17571 was associated with an increased risk of AD (p-value 0.007) in the Rotterdam Study. This association was predominantly found in APOE ε4 noncarriers. A meta-analysis of previously published data showed a significantly increased risk of AD in carriers of the T-allele of rs17571 (OR 1.22, 95% CI 1.03-1.44), irrespective of APOE ε4 carrier status. This study adds to the evidence that CTSD increases the risk of AD, although the effect size is moderate.

Introduction

Neurodegenerative diseases like Alzheimer's disease (AD) are highly prevalent diseases in the elderly and a major burden on society [1,2]. Neuropathologically, AD is characterized by neuritic plaques (amyloid) and neurofibrillary tangles (hyperphosphorylated tau protein) [3,4]. Progress has been made in understanding the etiology of AD, but the exact pathogenesis has yet to be determined. Alzheimer's disease is most likely caused by various interacting environmental and genetic risk factors [5]. The best known and most consistently reported genetic risk factor is apolipoprotein E (APOE), which is involved in about 50% of late-onset AD cases [6,7]. Other established genes in AD, such as the beta amyloid precursor protein gene (APP), and the presenilin 1 and 2 genes (PSEN1 and PSEN2) [8,9], are mainly implicated in early-onset AD [10]. Although various other genes have been reported in AD, most of them lack sufficient replication. There is particular interest in genes coding for proteins involved in the cleavage of amyloid precursor protein or clearance of beta-amyloid [11-13]. One such protein is cathepsin D, a lysosomal enzyme found in neuritic plaques [14,15]. This protein has secretase activity, and presumably, a role in the processing of tau protein, APP, amyloid beta and apolipoprotein E [16-19]. A polymorphism in exon two of the gene results in a C- to T-transition, resulting in an alanine to valine substitution in the protein. This polymorphism is associated with AD, although reports are inconsistent and a meta-analysis failed to show an association [20]. An explanation for this inconsistency might be that most previous studies used prevalent cases of AD, usually in a clinic-based setting, which might result in biases due to selective case-ascertainment, low-response rates and, possibly, case-fatalities.

The aim of the current study was to examine if polymorphisms in CTSD increase the risk of incident AD in a population-based setting. Additionally, we studied a set of dense SNPs covering a 150kb region flanking CTSD to evaluate the presence of other genetic variants that might be associated with AD. Finally, we performed a meta-analysis pooling our results with previously published data.

Materials and Methods

Study population

The current study was performed in the Rotterdam Study, which is an ongoing population-based study of 7983 elderly Caucasians (55 years and older). The study aims to find determinants of chronic disease in the elderly [21]. At baseline (1990-1993) and three follow-up rounds (1993-1994, 1997-1999, 2002-2004) participants were invited for extensive examinations. The study was approved by the Medical Ethics Committee at ErasmusMC, and all participants gave written informed consent. Only people who were not demented at baseline were included in the current study (N=6886).

Genotyping

Genomic DNA was extracted from whole blood samples according to standard methods [22]. The samples were genotyped for polymorphisms in APOE and CTSD with Taqman allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA). All measurements were performed in accordance with the manufacturer's protocols and primer and probe sequences for the SNPs are available from the manufacturer. Details on APOE have been published previously [23], and for CTSD, 3 SNPs were typed (rs2292963, rs2292962, rs17571). These SNPs as tagging SNPs covering the gene were selected based on the blocks that were seen in the CEU population available from the HapMap database (release 2005). We selected the functional variant and 2 SNPs with a minor allele frequency ≥ 0.05 (http://www.hapmap.org) [24] [25]. All genotypes were in Hardy-Weinberg equilibrium.

To test whether additional SNPs in the region were associated with AD, we also used data from a genome-wide screen. This screen was part of a large project on the genetics of complex diseases: samples were typed on version 3 of the Illumina-Infinium-II HumanHap550SNP array [26]. We utilized SNPs in a 150kb region centered on the CTSD gene.

The mismatch between the two platforms was previously tested for 24 other SNPs and corresponded to 0.3% (range 0.2-0.6%). The cross platform agreement for rs17571 in the current study was 99.7%. Only samples of good quality DNA were genotyped, and after genotypic quality control 6112 individuals (493 with incident AD) were available for analysis.

Ascertainment of incident AD

Incident AD was diagnosed with a three-step protocol [27]. During the visits to the research centre, all individuals were screened with two cognitive tests (Mini Mental State Examination (MMSE) and Geriatric Mental State schedule (GMS)). Further testing with the Cambridge examination for mental disorders of the elderly (Camdex) was performed in individuals with scores on the MMSE < 26 or on the GMS > 0. When dementia was suspected and additional testing was required for the diagnosis, individuals were examined by a neuropsychologist. Additionally, imaging data was

used when available. A team consisting of a neurologist, a neuropsychologist and a research physician finally ascertained the diagnosis of AD according to internationally accepted criteria. In addition to the visits to the research center, the population was continuously monitored for incident AD through the medical records of general practitioners and the Regional Institute for Outpatient Mental Health Care. There were no cases with a family-history suggestive of autosomal dominant AD. Follow-up was complete through January 1 2005, during which 493 persons developed AD.

Statistical analyses

General descriptive statistics were performed with T-tests for normally distributed variables and Chi-square statistics for categorical or dichotomous variables (version 15.0 of SPSS). To test the associations between the polymorphisms and AD, we used Chi-square statistics and logistic regression models adjusted for age and sex. Additionally, we used Cox regression models to incorporate time-to-event. To further explore the association, we repeated the analyses stratified by APOE ε4 carrier status. A p-value <0.05, adjusted for multiple testing, was considered significant.

A meta-analysis was done for CTSD rs17571 using rmeta (R package). To find previously published studies, we searched PubMed using the key-words: CTSD, Alzheimer's disease, gene and association. Additionally, we checked the reference lists of these papers and searched the AlzGene database (http://www.alzgene.org) [28]. Meta-analysis was conducted on data from previously published studies (from 1999 to January 1st 2009) both overall and stratified by APOE ε4 carrier status. For the meta-analyses, random effects models were applied.

Baseline characteristics of the study population (N=6112)

	Controls	Cases	p-value
N	5619	493	
Sex (% women)	3242 (58%)	366 (74%)	<0.01
Age	68.2	76.3	<0.01
Mean follow-up	9.6	6.4	<0.01
APOE ε4 carriers (%)	1423 (26%)	203 (43%)	<0.01

Table 1

Results

There were 493 incident AD cases in this population of 6112 individuals with a mean age of onset of 82.8 (±6.9) years. The baseline characteristics of the study population are shown in Table 1. Cases were older and were more often women and carriers of the APOE ε4 allele than control subjects.

Table 2 shows the genotype frequencies for the three tested SNPs. Carriers of the T-allele of rs17571 had a higher risk of AD (OR 1.36, 95% CI 1.09-1.71). This effect was equally strong in the Cox analysis (HR 1.34, 95% CI 1.08-1.64). The two other SNPs were not associated with a

higher risk of AD. Next, the population was stratified by APOE ε4 carrier status (Table 3). The association of rs17517 with AD was predominantly found in APOE ε4 noncarriers (adjusted HR 1.42, 95% CI 1.09-1.87). No association was found in APOE ε4 carriers.

Additionally, we studied a set of dense SNPs in a 150kb region flanking CTSD to test whether other genetic variants explained the association of rs17571 with AD. The results are shown in Figure 1. Rs17571 and rs2292963 were the only SNPs within the gene, and rs2292962 was not present on the array. The 85 other SNPs in the flanking region were neither in strong LD with these two SNPs nor significantly associated with AD. When ranking the p-values of the whole genome-wide association study, rs17571 was ranked at the 5115th position, which is within the top 10% of the results.

Association of *CTSD* with AD

SNP	Physical position	Controls					Cases					p-value	OR (95% CI)
rs2292963	1732908	**CC**	**TC**	**TT**	**C-allele**	**T-allele**	**CC**	**TC**	**TT**	**C-allele**	**T-allele**		
		3343	1903	269	0.78	0.22	293	161	36	0.76	0.24	0.24	1.10 (0.94-1.28)
												†0.35	1.08 (0.92-1.27)
rs2292962	1734897	**GG**	**AG**	**AA**	**G-allele**	**A-allele**	**GG**	**AG**	**AA**	**G-allele**	**A-allele**		
		3998	1292	89	0.86	0.14	371	101	8	0.88	0.12	0.20	0.88 (0.72-1.07)
												†0.16	0.86 (0.69-1.06)
rs17571	1739170	**CC**	**TC**	**TT**	**C-allele**	**T-allele**	**CC**	**TC**	**TT**	**C-allele**	**T-allele**		
		4628	735	32	0.93	0.07	386	81	6	0.90	0.10	0.007	1.36 (1.09-1.71)
												†0.008	1.38 (1.09-1.75)

†: adjusted for age and sex

Table 2

Association of *CTSD* with AD, stratified by APOE ε4 carrier status

	APOE ε4 carriers								APOE ε4 noncarriers							
	Controls			Cases			p	OR (95% CI)	Controls			Cases			p	OR (95% CI)
rs2292963	**CC**	**TC**	**TT**	**CC**	**TC**	**TT**			**CC**	**TC**	**TT**	**CC**	**TC**	**TT**		
	847	490	54	122	69	11	0.64	1.06 (0.83-1.36)	2394	1330	206	160	82	24	0.22	1.14 (0.93-1.39)
							0.91†	1.02 (0.78-1.32)							0.24†	1.131 (0.92-1.40)
rs2292962	**GG**	**AG**	**AA**	**GG**	**AG**	**AA**			**GG**	**AG**	**AA**	**GG**	**AG**	**AA**		
	991	347	19	147	45	5	0.90	0.98 (0.72-1.33)	2862	900	68	207	51	3	0.09	0.78 (0.59-1.04)
							0.84†	0.97 (0.70-1.34)							0.06†	0.75 (0.55-1.01)
rs17571	**CC**	**TC**	**TT**	**CC**	**TC**	**TT**			**CC**	**TC**	**TT**	**CC**	**TC**	**TT**		
	1152	195	5	161	30	3	0.24	1.25 (0.86-1.81)	3314	509	27	207	48	3	0.009	1.48 (1.11-1.99)
							0.29†	1.24 (0.84-1.83)							0.008†	1.52 (1.12-2.07)

†: adjusted for age and sex

Table 3

Finally, we performed a large meta-analysis pooling our data with previously published data. For this analysis, 26 studies reporting on the association of CTSD with AD in Caucasians were identified [13,29-51]. Eight of these [13,31-37] were not analyzed, because they overlapped with other published studies or sufficient data was not provided in the published paper. The latter concerned three publications, of which one found an association between the T-allele of rs17571 and AD, one did not find an association and one did not report this outcome. Additionally, one study was dropped because the control genotypes were out of Hardy-Weinberg Equilibrium (HWE) [44]. An overview of eligible studies is given in Supplementary Table 1. The other 17 studies included a total of 3798 AD cases and 3865 controls. Thirteen included AD patients

diagnosed according to the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria, one study used the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria to identify cases, and one study analyzed autopsy confirmed cases only. Most studies included sporadic late-onset cases, however, there were studies with combinations of early-onset, late-onset, sporadic and familial cases. There was one study, which included only non-familiar early-onset cases [32]. Controls did not have a diagnosis of Alzheimer's disease, and were selected either from a population-based or clinical sample. The T-allele frequency ranged from 0.05 to 0.11 in controls and from 0.05 to 0.14 in cases. The summary odds ratio of the meta-analysis was 1.22 (95% CI 1.03-1.44). There was significant between-study heterogeneity (p=0.002). When the original study was excluded, the pooled odds ratio dropped to 1.17 (95% CI 0.99-1.37), which was borderline significant (p=0.06). When excluding both Emahazion et al. and the original report, the between-study heterogeneity disappeared (p=0.09) and the summary odds ratio became 1.21 (95% CI 1.05-1.40). To explore how evidence accumulated over time, we performed a cumulative meta-analysis, which is shown in Figure 2. The results show a stable

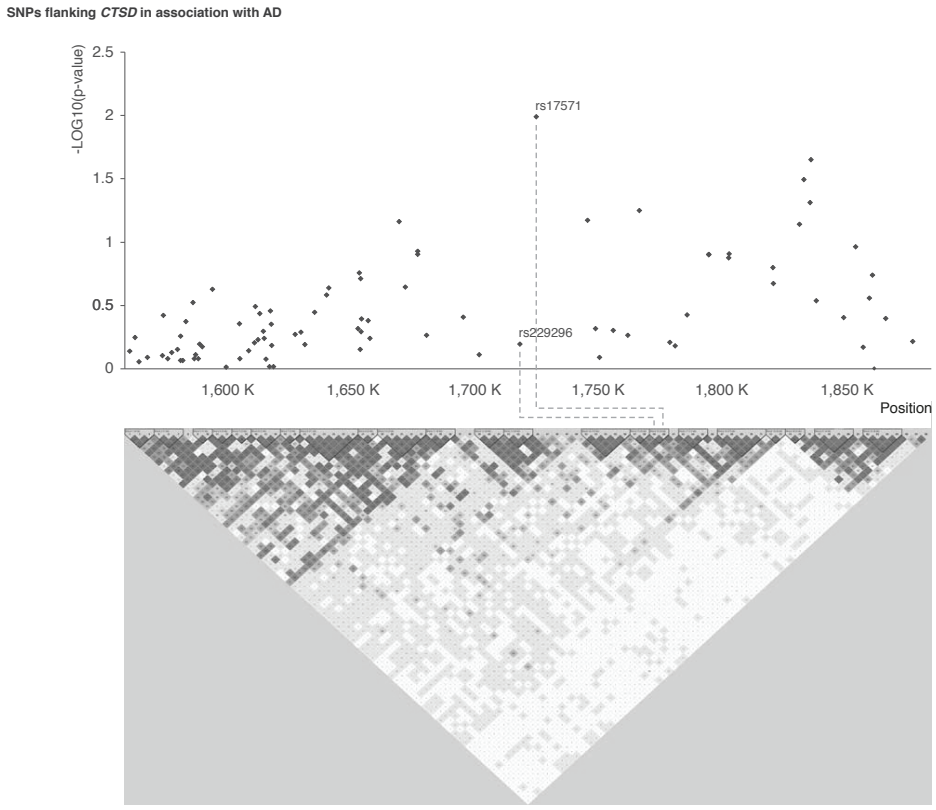
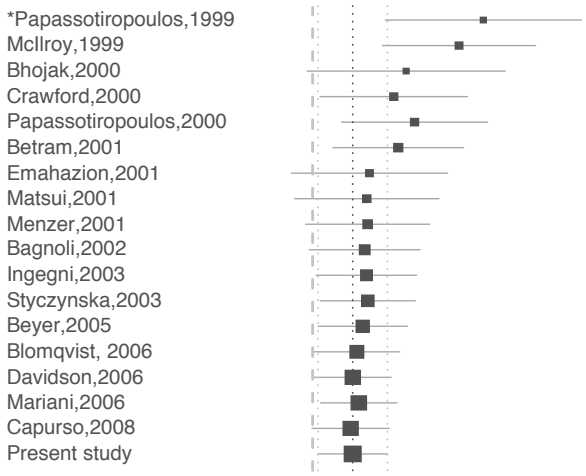


Figure 1

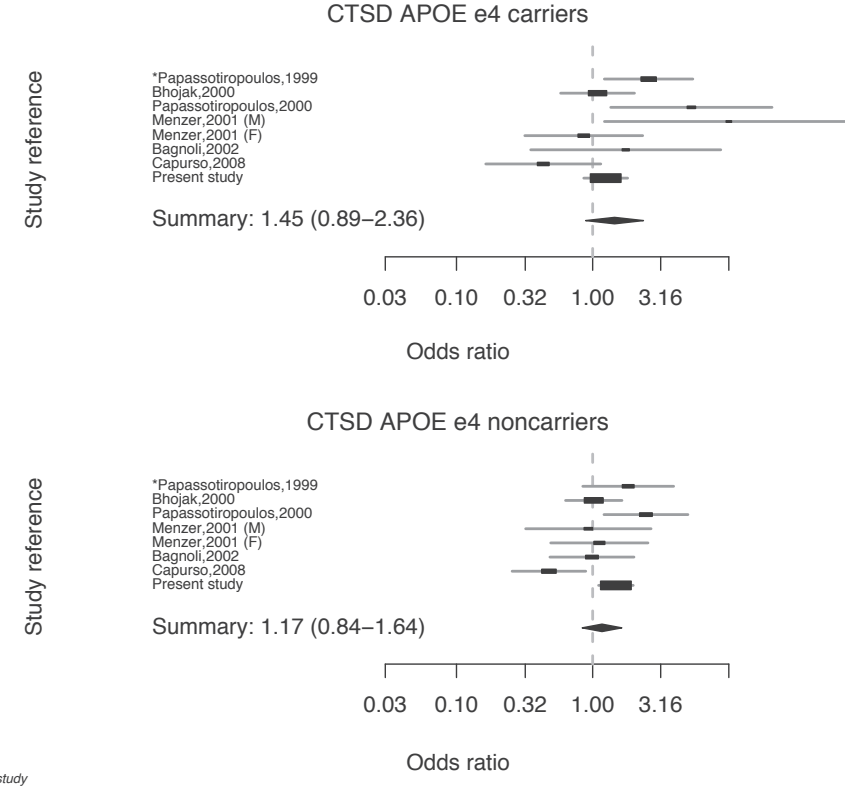
Cumulative meta-analysis of *CTSD* rs17571 in AD



**: original study*

Figure 2

meta-analysis of CTSD rs17571 in AD stratified by APOE ε4 carrier status



**: original study*

Figure 3

summary odds ratio of 1.2 since 2006. When we analyze the data without the present study, the summary odds ratio is 1.21 with a 95% CI ranging from 1.00 to 1.46 (p-value of 0.053). The meta-analysis shown on the Alzgene database (http://www.alzgene.org) presents an OR of 1.17 (95% CI 0.98-1.41) in Caucasians. The main explanation for the difference with our results is the exclusion of the study in which the control genotypes were not in HWE.

For the APOE ε4 stratified analyzes, 6 studies were available [30,38,40,47-49] with a total of 1446 cases and 1699 controls. The results of this meta-analysis are shown in Figure 3. The pooled odds ratio was greater in APOE ε4 carriers than noncarriers, but non-significant.

Discussion

In this population-based cohort study and meta-analysis, we found that rs17571 in CTSD was associated with incident AD. This association was more pronounced in APOE ε4 noncarriers, however, a stratified meta-analysis did not confirm this finding. When ranking the p-values of the GWAS, rs17571 ranked 5115th, which is within the top 10%.

Evidence for association of CTSD with AD, thus far, has been very inconsistent. Most studies failed to replicate the original study, with one even showing a significant opposite effect [32] and only a few replicating the original finding [43,49]. Also, meta-analyses thus far did not find a significant association (http://www.alzgene.org) [20,52]. Pooling our longitudinal data with previously published data strengthens the evidence for an association of CTSD with AD. The meta-analysis clearly shows an association in Caucasians, even in a heterogeneous group of early-onset, late-onset, sporadic and familial cases. The data reported in previous studies do not allow us to perform separate analyses in sporadic late-onset AD cases, which would be of interest for further research. Although differences in genotype distribution between early-onset and late-onset cases were not found in a previous study [41], excluding the study of Emahazion et al., which included only early-onset AD cases, reduced between-study heterogeneity. The between-study heterogeneity disappeared when additionally excluding the original study. With the accumulation of data over time we saw a stabilizing odds ratio and a narrowing of the 95% confidence level boundaries.

There were no genome-wide association studies (GWAS) with publicly accessible data that included the SNP of interest. These studies typed two SNPs in LD with rs17571, but these were not associated with AD (rs7938305: p=0.26, OR 0.86; rs17834326: p=0.54, OR 0.97) [53,54]. We decided not to include these in the meta-analysis, because we intended to analyze the functional SNP for the candidate gene analysis only.

Most studies do not find differences between the effect of CTSD in APOE ε4 carriers and noncarriers or a significant interaction between these two genes [29,30,41,42]. Although the results in our study show the effect of the T-allele of rs17571 predominantly in APOE ε4 noncarriers, the meta-analysis does not confirm this finding. In fact, the meta-analysis showed a more pronounced effect in ε4 carriers, however, the difference was not significant.

This finding is in line with the suggestion that in AD, CTSD influences APOE independent pathways [55]. CTSD encodes a protein found in senile plaques and CSF [14,18,56]. The mRNA is upregulated in neurons in brains of AD patients, and the immunoreactivity is greater than in normal brains [14,15]. The CTSD protein is able to cleave APP [57] and tau protein [16], although the enzyme seems not to be essential for APP processing [58]. Animal studies show that mutations in CTSD causing dysfunction of the protein, result in loss of neurons in the brain and neurodegeneration [59]. In humans, the biological implication of the polymorphism rs17571 is not clear, although multiple biological associations have been found.

None of the published GWAS, including our own, showed genome-wide evidence for a role of CTSD in AD, however, rs17571 reached the top 10% of our GWA findings. Most GWAS are underpowered to find genes with small effects. The relatively high ranking is in accordance with simulations of GWAS by Zaykin et al.[60].

The strength of the current study is the inclusion of a large population-based cohort study, which has several advantages over clinic-based case-control studies. The main advantages are reduction in biases due to selective case-ascertainment, defining the control group, and low-response rates. Furthermore, a major advantage is the use of incidence instead of prevalence cases, reducing prevalence-incidence bias that may occur when genetic variants are determining mortality. A limitation of the study could be misclassification of cases, but this misclassification would have occurred randomly and would thus not have lead to an overestimation of the effect size. Finally, the population-based design limits the number of cases available.

In conclusion, although the effect of CTSD on the risk of AD is small (OR 1.2), it might be important as one of multiple genetic risk factors adding up to a higher risk of AD. Our findings add to the evidence of an association with AD, and show that the odds ratio stabilized over time.

Overview of studies eligible for the meta-analysis

First author	Year	Included in meta-analysis		Controls	AD	Control		Case		Reference
				N	N	T-allele frequency	C-allele frequency	C-allele frequency	T-allele frequency	
***Papassotiropoulos**	1999	yes		351	102	0.07	0.93	0.86	0.14	(Papassotiropoulos et al., 1999)
McIlroy	1999	yes		187	183	0.05	0.95	0.92	0.08	(McIlroy et al., 1999)
Bhojak	2000	yes		316	531	0.09	0.91	0.90	0.10	(Bhojak et al., 2000)
Crawford	2000	yes		120	210	0.08	0.92	0.90	0.10	(Crawford et al., 2000)
Papassotiropoulos	2000 (b)	yes		184	127	0.05	0.95	0.88	0.12	(Papassotiropoulos et al., 2000b)
Bertram	2001	yes		182	200	0.09	0.91	0.91	0.09	(Bertram et al., 2001)
Emahazion	2001 (I)	yes		149	120	0.11	0.89	0.95	0.05	(Emahazion et al., 2001)
Matsui	2001	yes		50	69	0.08	0.92	0.93	0.07	(Matsui et al., 2001)
Menzer	2001	yes		302	324	0.06	0.94	0.92	0.08	(Menzer et al., 2001)
Bagnoli	2002	yes		126	197	0.11	0.89	0.88	0.12	(Bagnoli et al., 2002)
Ingegni	2003	yes		120	142	0.10	0.90	0.87	0.13	(Ingegni et al., 2003)
Styczynska	2003	yes		100	100	0.05	0.96	0.94	0.07	(Styczynska et al., 2003)
Beyer	2005	yes		181	206	0.08	0.92	0.92	0.08	(Beyer et al., 2005)
Blomqvist	2006	yes		173	385	0.06	0.94	0.94	0.06	(Blomqvist et al., 2006)
Davidson	2006	yes		767	560	0.08	0.92	0.92	0.08	(Davidson et al., 2006)
Mariani	2006	yes		136	100	0.07	0.93	0.86	0.14	(Mariani et al., 2006)
Capurso	2008	yes		421	242	0.08	0.92	0.94	0.06	(Capurso et al., 2008)
Blomqvist	2006	no	overlap Emahazion et al. 2001	-	-	-	-	-	-	(Blomqvist et al., 2006)
Capurso	2005	no	overlap Capurso et al. 2008	218	168	-	-	-	-	(Capurso et al., 2005)
Corder	2006	no	no data provided	120	180	-	-	-	-	(Corder et al., 2006)
Emahazion	2001 (II)	no	no data provided	176	116	-	-	-	-	(Emahazion et al., 2001)
Kolsch	2004	no	overlap Papassotiropoulos et al. 1999	-	-	-	-	-	-	(Kolsch et al., 2004)
Papassotiropoulos	2000 (a)	no	overlap Papassotiropoulos et al. 1999&2000	-	-	-	-	-	-	(Papassotiropoulos et al., 2000a)
Papassotiropoulos	2002	no	no data provided	24	41	-	-	-	-	(Papassotiropoulos et al., 2002)
Prince	2001	no	overlap Blomqvist et al. 2005	-	-	-	-	-	-	(Prince et al., 2001)
Mateo	2002	no	deviation from HWE in control group	346	311	0.10	0.90	0.91	0.09	(Mateo et al., 2002)

*Original report; N: number; allele frequencies are reported for rs17571

Supplementary Table

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07 Transferrin and HFE genes interact in Alzheimer's disease risk: the Epistasis Projects

Abstract

Iron overload may contribute to the risk of Alzheimer's disease (AD). In the Epistasis Project, with 1757 cases of AD and 6295 controls, we studied four variants in two genes of iron metabolism: haemochromatosis (HFE) C282Y and H63D, and transferrin (TF) C2 and
-2G/A. We replicated the reported interaction between HFE 282Y and TF C2 in the risk of AD: synergy factor = 1.75 (95% confidence interval: 1.1-2.8, p = 0.02) in Northern Europeans. The synergy factor was 3.1 (1.4-6.9, 0.007) in subjects with the APOEε4 allele. We found another interaction, between HFE 63HH and TF -2AA, markedly modified by age. Both interactions were found mainly or only in Northern Europeans. The interaction between HFE 282Y and TF C2 has now been replicated twice, in altogether 2313 cases of AD and 7065 controls, and has also been associated with increased iron load. We therefore suggest that iron overload may be a causative factor in the development of AD. Treatment for iron overload might thus be protective in some cases.

Introduction

Brain iron in ageing and in Alzheimer's disease

Since the 1950s, iron has been reported to increase with age in the brain [1] and has been proposed a role in Alzheimer's disease (AD) [2]. Although a more rapid rise in brain levels of non-haem iron is seen in a person's first 30 years [1], levels of iron and of iron-related proteins continue to rise with normal ageing in many, but not all brain regions [3-9]. Disturbances of iron metabolism have been widely reported in AD brain (below) and also in autopsy brain tissue and CSF and in mild cognitive impairment (MCI) [10,11]. Raised iron levels have been commonly found in AD, e.g. in the basal ganglia [in vivo: [12,13]; autopsy: [14]], in the hippocampus [in vivo: [13,15]; autopsy: [16,17]] and in the neocortex [autopsy: [14,17,18]]. Iron has been located in postmortem tissue in and around senile plaques and neurofibrillary tangles [19-22]. There have been varying reports concerning a possible cellular imbalance of the iron:ferritin ratio [4,7,18,23] and also on the levels and activity of the important ferroxidase, ceruloplasmin [24-26] in AD brain. Altered patterns of transferrin expression have been reported in AD brain [4,14]. Increased expression of lactoferrin has been found both in neurones and glia in numerous regions of AD brain [27]. This may suggest increased use of non-transferrin routes of cellular iron uptake, e.g. via lactoferrin.

Excess iron and oxidative stress

Higher brain iron levels have been correlated with greater cognitive impairment in AD [13,28]. It has been proposed that excess iron in AD leads to oxidative damage [29-32]. Such damage to lipids [33-36], to proteins [37-39] to DNA [40] and to RNA [41] has been found in AD, including early AD, and in MCI. RNA oxidation can lead to reduced rates of protein synthesis, which have been reported in MCI and AD brain tissue [42]. Markers of lipid peroxidation have also been found in the CSF of MCI patients, and in MCI patients who subsequently converted to AD [43,44]. In short, oxidative stress

is seen early in AD, indeed in the pre-clinical stage, and provokes an antioxidant response [45]. Moreover, markers of lipid peroxidation in ventricular fluid have been correlated with cortical atrophy, reduced brain weight and severity of AD [46]. Excess iron may also contribute to neurodegeneration via, for instance, the metabolism and toxicity of β-amyloid [29,47-51], the loss of calcium homeostasis [52] and the degradation of a subset of microglia [53].

Transferrin and HFE in AD

Two genes of iron metabolism have been much studied in AD: transferrin (TF) and its C2 variant (rs1049296), and the haemochromatosis gene (HFE) and its C282Y (rs1800562) and H63D (rs1799945) variants. These HFE variants affect blood iron status, with the rare 282Y homozygotes (frequency < 1%) having the highest transferrin saturation [54]. Since 1993 when an association of TF C2 with AD was first proposed [55], there have been altogether 14 independent studies of that association. Although most studies have been negative, the AlzGene meta-analysis of the allele [56] (http://www.alzforum.org/res/com/gen/alzgene/) currently shows a significant, though low, odds ratio of AD: 1.2 (95% confidence interval: 1.04-1.3) (10 December 2009), with a similar pattern in Caucasians and East Asians. Also, a large family study [57] supported the association. There have been 13 independent, association studies of HFE, with mixed results. The AlzGene meta-analysis of the 282Y allele currently gives an odds ratio of AD of 1.04 (0.9-1.2). However, three studies [58-60] have proposed that the 63D allele may be associated with lower onset age of AD, possibly in interaction with apolipoprotein ε4 (APOE ε4). Also, the Oxford Project to Investigate Memory and ageing (OPTIMA) reported an interaction between TF C2 and HFE 282Y in the risk of AD [61]. This interaction was recently replicated [62] in a large collaborative study. Further, our preliminary examination of data from the Rotterdam Study revealed another potential interaction in the risk of AD. That was between HFE H63D and a single nucleotide polymorphism (SNP) just two base-pairs upstream of the start codon of TF (rs1130459, -2G/A) (data not shown). We therefore examined four SNPs, HFE C282Y, HFE H63D, TF C2 and TF -2G/A (Table 1), in the Epistasis Project. This project is a collaboration of seven AD research groups, contributing DNA samples from 1757 cases of AD and 6295 controls [63].

Methods

Study population

The Epistasis Project aims primarily to replicate genetic interactions that have been reported to affect the risk of AD. Sample-sets were drawn from narrow geographical regions with relatively homogeneous, Caucasian populations, by seven AD research groups: Bonn, Bristol, Nottingham, OPTIMA, Oviedo, Rotterdam and Santander. All AD cases were diagnosed "definite" or "probable" by CERAD or NINCDS-ADRDA criteria. Full details are given in our previous paper [63].

Genotyping

Genotyping for the six centres other than Rotterdam (below) was performed at the Wellcome Trust Sanger Institute, using the iPLEX Gold assay (Sequenom Inc.). Whole genome amplified DNA was used for 82% of samples; genomic DNA was used for the 18% of samples that were not suitable for whole genome amplification. A Sequenom iPLEX, designed for quality control purposes, was used to assess genotype concordance between genomic and whole genome amplified DNA for 168 individuals. Assays for all SNPs were designed using the eXTEND suite and MassARRAY Assay Design software version 3.1 (Sequenom Inc.). Samples were amplified in multiplexed PCR reactions before allele specific extension. Allelic discrimination was obtained by analysis with a MassARRAY Analyzer Compact mass spectrometer. Genotypes were automatically assigned and manually confirmed using MassArray TyperAnalyzer software version 4.0 (Sequenom Inc.). Gender markers were included in all iPLEX assays as a quality control metric for confirmation of plate/sample identity. Genotyping of rs1130459 was carried out using KASPar technology by KBioscience (http://www.kbioscience.co.uk). Repeat genotyping of rs1049296 was performed using TaqMan technology according to standard conditions (C_7505275_10; Applied Biosystems, Foster City, CA, U.S.A.).

Genotyping in the Rotterdam cohort was done on Version 3 Illumina-Infinium-II HumanHap550 SNP array (Illumina, San Diego, USA) and additionally, SNPs were imputed using MACH software (http://www.sph.umich.edu/csg/abecasis/MACH/) with HapMap CEU Release 22 as a reference [64]. The reliability of imputation was estimated for each imputed SNP with the ratio of expected and observed dosage variance (O/E ratio). Only samples with high-quality extracted DNA were genotyped; 5974 were available with good quality genotyping data; 5502 of these had reliable phenotypes. For this study, rs1800562 and rs1049296 were genotyped, and rs1799945 and rs1130459 were imputed.

Statistical analysis

We assessed associations with logistic regression models and synergy factor analysis [65], controlling for age, gender, study centre and the ε4 allele of apolipoprotein (APOEε4), using R Version 2.6.1 (R Foundation for Statistical Computing, Vienna, Austria). Heterogeneity between centers was controlled by fitting a fixed effect corresponding to contrasts between the baseline centre and the six other centers (having compared models with fixed- and random-effect terms in centre, goodness of fit was measured using Akaike's Information Criterion, which favored using fixed effects only). Over-dispersion was controlled by fitting generalized linear models with a quasi-binomial family with logit link. Where the overall synergy factor was significant at $p < 0.05$, the seven individual centers and the two geographical regions, North Europe and North Spain, were also examined. Power calculations were based on the synergy factor values. Comparisons of allelic frequencies between North Spain and North Europe were by Fisher's exact test. Linear regression models were used to compare onset ages. Linkage disequilibrium data were estimated using the R genetics library (http://cran.r-project.

org/web/packages/genetics/ index.html). All tests of significance and power calculations were two-sided.

Results

Hardy-Weinberg analysis, allelic frequencies and linkage disequilibrium

Hardy-Weinberg analysis was performed for the four SNPs of Table 1 in both cases and controls of the Rotterdam samples, genotyped by Rotterdam, and of the samples from the other six centres, genotyped by the Sanger Institute. In one of these 16 analyses, i.e. of rs1049296 in controls from the six centres, the genotypes were not in Hardy-Weinberg equilibrium. These samples were therefore retyped by a different method. The two methods, Sequenom and TaqMan, were in 99.6% agreement in the 2317 samples and neither were in Hardy-Weinberg equilibrium in controls (p = 0.01). There are three reported, overlapping copy number variations, each between 1.3 and 1.7 kb long, all just over 6 kb downstream of TF and just over 9kb from rs1049296 (TF C2) (Database of Genomic Variants: http://projects.tcag.ca/variation/).

Studied SNPs

Gene	SNP	Minor allele frequency in controls			Linkage disequilibrium in controls			
		North Europe	North Spain	Difference: p	North Europe D'	North Europe r^2	North Spain D'	North Spain r^2
HFE	rs1800562 C282Y	6.3% (Y)	3.45% (Y)	**0.0003**	0.992	0.011	0.992	0.011
	rs1799945 H63D	14.5% (D)	24.0% (D)	**$4.5x10^{-16}$**				
TF	rs1049296 C2 = P589S	16.4% (S)	17.65% (S)	0.34	0.602	0.080	0.338	0.026
	rs1130459 -2G/A	47.2% (A)	48.0% (A)	0.64				

SNP = single nucleotide polymorphism; *HFE* = the haemochromatosis gene; *TF* = the transferrin gene; *D'* = ratio of observed linkage disequilibrium to maximum possible linkage disequilibrium; *r* = correlation coefficient.

Table 1

Table 1 gives the allelic frequencies and structure of linkage disequilibrium (LD) of the two pairs of SNPs in controls. The allelic frequencies of HFE were strikingly different between North Europe and North Spain (Supplementary Table 1), but the frequencies were rather similar for TF. In contrast, the LD structure of HFE was nearly identical in the two regions, but differed somewhat for TF: D' = 0.6 versus 0.3 (Table 1). Supplementary Table 2 shows the allele frequencies by country, which were largely consistent in North Europe.

Odds ratios of Alzheimer's disease for the four SNPs, i.e. the main effects

Gene	Genotype	Numbers				Adjusted[a] odds ratios (95% CI, *p*)		
		North Europe		North Spain		All	North Europe	North Spain
		Controls	AD cases	Controls	AD cases			
HFE	282YY+CY	667	117	30	38	1.0 (0.8-1.2, 0.8)	0.9 (0.7-1.2, 0.55)	1.3 (0.7-2.15, 0.4)
	282CC	4830	945	428	428			
	63DD+HD	1478	281	193	191	1.0 (0.8-1.1, 0.6)	0.9 (0.8-1.1, 0.4)	1.1 (0.8-1.4, 0.7)
	63HH	4016	774	267	269			
TF	589SS+PS	1647	333	139	163	1.1 (0.96-1.3, 0.2)	1.0 (0.9-1.2, 0.7)	1.2 (0.9-1.7, 0.2)
	589PP	3847	724	317	296			
	-2AA+GA	1213	211	100	141	1.1 (0.97-1.3, 0.1)	1.0 (0.8-1.2, 0.9)	**1.6 (1.1-2.2, 0.01)**
	-2GG	4258	716	348	297			

SNP = single nucleotide polymorphism; *HFE* = the haemochromatosis gene; *TF* = the transferrin gene; AD = Alzheimer's disease; CI = confidence interval.
[a]All analyses controlled for centre, age, gender and genotype of apolipoprotein ε4.

Table 2

Main effects: AD risk and onset age

We found no significant main effects on AD risk of three of the four SNPs (Table 2). But the genotype, TF -2AA+GA, was associated with risk of AD only in North Spain: odds ratio = 1.6 (95% confidence interval: 1.1-2.2, p = 0.01). Although we found an apparent association of the HFE 63D allele with lower onset age of AD (p = 0.0008) in unadjusted analysis, we did not replicate the association on controlling for centre, age, gender and APOEε4 (p = 0.36). In the five centres with data on onset ages (Bonn, Bristol, OPTIMA, Santander, Rotterdam: n = 1249), the median onset age was 74.0 years (interquartile range: 68.0-80.0, n = 389) in patients with HFE 63D, while it was 76.0 years (69.0-82.6, 860) in HFE 63H homozygotes. There was no interaction between HFE H63D and APOEε4 in onset age.

Interactions between *HFE* and *TF* SNPs in the risk of Alzheimer's disease

Interaction	Dataset	Numbers		Power[a]	Adjusted[b] *SF* (95% CI, *p*)
		Controls	AD		
HFE 282Y+	All	6227	1672	43%	1.4 (0.9-2.2, 0.15)
× *TF* C2+	North Europe	5723	1152	34%	**1.75 (1.1-2.8, 0.02)**
	North Spain	504	520	17%	0.5 (0.2-1.8, 0.3)
HFE 63HH	All	6206	1496	76%	**1.5 (1.1-2.1, 0.02)**
× *TF* -2AA	North Europe	5706	1008	58%	**2.0 (1.3-3.05, 0.002)**
	North Spain	500	488	40%	1.3 (0.7-2.55, 0.4)

SNP = single nucleotide polymorphism; *HFE* = the haemochromatosis gene; *TF* = the transferrin gene; *SF* = synergy factor; CI = confidence interval; Y+ and C2+ group the genotypes, YY+CY and C2/C2+C2/-, respectively.
[a]Power to detect an *SF* of 1.4 (first interaction) or of 1.5 (second interaction) at *p* = 0.05.
[b]All analyses controlled for centre, age, gender and genotype of apolipoprotein ε4.

Table 3

Interactions

We also replicated both interactions in the risk of AD, i.e. the interaction between HFE 282Y and TF C2 and that between HFE 63HH and TF -2AA, but only in Northern Europeans (Table 3): synergy factors = 1.75 (95% confidence interval: 1.1-2.8, p = 0.02) for HFE 282Y × TF C2 and 2.0 (1.3-3.05, 0.002) for HFE 63HH × TF -2AA. Neither result was due to any distortion in control frequencies (data not shown). In view of reported sex differences in iron status and related genetics [66], we also examined these interactions by sex. We found no significant differences between men and women in either interaction (data not shown). In the OPTIMA report [61], we had suggested that there might be a further interaction between HFE 282Y, TF C2 and APOEε4. Here we found that the interaction between these two iron-related SNPs only occurred in subjects with APOEε4, where the synergy factor in North Europeans was 3.1 (1.4-6.9, 0.007), but not in APOEε4 negatives, where the synergy factor was 1.05 (0.55-2.0, 0.87). However, there were no significant interactions between APOEε4 and either SNP or both together.

Effect on the interaction between *HFE* 63HH and *TF* -2AA of stratification by age ± 80 years

Age	*TF* -2G/A genotype	Adjusted* *OR*: *HFE* 63HH vs 63D-positive (95% CI, *p*)		Adjusted[a] *SF*: *HFE* 63HH × *TF* -2AA (95% CI, *p*)	
		North Europe	North Spain	North Europe	North Spain
< 80 years	-2AA	1.55 (0.85-2.9, 0.2)	0.9 (0.4-1.8, 0.7)	**2.2 (1.1-4.4, 0.03)**	1.0 (0.4-2.3, 1.0)
	-2G-positive	0.8 (0.55-1.1, 0.2)	0.9 (0.5-1.4, 0.5)		
> 80 years	-2AA	**2.2 (1.2-4.0, 0.01)**	3.1 (0.9-11.2, 0.08)	**2.2 (1.1-4.2, 0.02)**	2.9 (0.8-10.8, 0.1)
	-2G-positive	1.0 (0.8-1.3, 0.95)	1.1 (0.6-2.0, 0.85)		

HFE = the haemochromatosis gene; *TF* = the transferrin gene; *OR* = odds ratio; CI = confidence interval; *SF* = synergy factor; age is at death or last examination (an age of 80 years is equivalent to an onset age of 75 years in our dataset). Numbers of cases and controls are given in Supplementary Table 3. [a]All analyses controlled for centre, age, gender and genotype of apolipoprotein ε4.

Table 4

Studied SNPs, by genotype

Gene	SNP	Subjects	Numbers, by genotype			Totals
HFE	rs1800562 C282Y	Controls	AA: 23	GA: 716	GG: 5532	6271
		AD	7	166	1556	1729
	rs1799945 H63D	Controls	GG: 161	CG: 1589	CC: 4512	6262
		AD	61	464	1178	1703
TF	rs1049296 C2 = P589S	Controls	TT: 196	CT: 1671	CC: 4374	6241
		AD	64	482	1136	1682
	rs1130459 -2G/A	Controls	GG: 1711	GA: 3137	AA: 1373	6221
		AD	397	735	386	1518

SNP = single nucleotide polymorphism; *HFE* = the haemochromatosis gene; *TF* = the transferrin gene

Supplementary Table 1

Note that the second interaction above suggests that HFE 63HH is a risk factor for AD, depending on the TF -2G/A genotype, whereas in apparent contradiction, age data suggest that HFE 63HH might be associated with higher onset age. We therefore examined this genetic interaction by age. There was a clear interaction in our overall dataset between HFE 63HH and age at death or last examination as a continuous variable ($p < 0.00001$) and between that genotype and age ± 80 years [synergy factor = 1.9 (1.45-2.6, 0.00001)], indicating increased risk associated with that genotype in the older subset. However, Table 4 shows that the interaction between HFE 63HH and TF -2AA applied equally to both age subsets in Northern Europeans. The Northern Spanish dataset lacked power in this subset analysis. However, it was consistent with North Europe in the older subset, but showed no effect in the younger subset (Table 4). Stratification by the median age of controls, 76.88 years instead of 80 years, produced similar results, except that the synergy factor for Northern Europeans in the younger subset dropped below significance: 2.25 (0.97-5.2, 0.06).

Control allele frequencies by country

Gene	SNP	Minor allele frequency in controls			
		Britain	The Netherlands	Germany	Spain
HFE	rs1800562 C282Y	51/816 = 6.25% (Y)	652/10222 = 6.4% (Y)	23/462 = 5.0% (Y)	36/1042 = 3.45% (Y)
	rs1799945 H63D	134/802 = 16.7% (D)	1472/10222 = 14.4% (D)	56/462 = 12.1% (D)	249/1038 = 24.0% (D)
TF	rs1049296 C2 = P589S	125/800 = 15.6% (S)	1688/10220 = 16.5% (S)	70/442 = 15.8% (S)	180/1020 = 17.65% (S)
	rs1130459 -2G/A	358/746 = 48.0% (A)	4829/10220 = 47.25% (A)	209/462 = 45.2% (A)	487/1014 = 48.0% (A)

SNP = single nucleotide polymorphism; *HFE* = the haemochromatosis gene; *TF* = the transferrin gene

Supplementary Table 2

Supplementary Table 3. Effect on the interaction between *HFE* 63HH and *TF* -2AA of stratification by age ± 80 years

Age	*TF* -2G/A genotype	Numbers				Adjusted* *OR*: *HFE* 63HH vs 63D-positive (95% CI, *p*)		Adjusted* *SF*: *HFE* 63HH × *TF* -2AA (95% CI, *p*)	
		North Europe		North Spain					
		Controls	AD	Controls	AD	North Europe	North Spain	North Europe	North Spain
< 80 years	-2AA	804	112	59	109	1.55 (0.85-2.9, 0.2)	0.9 (0.4-1.8, 0.7)	**2.2 (1.1-4.4, 0.03)**	1.0 (0.4-2.3, 1.0)
	-2G-positive	2843	342	200	227	0.8 (0.55-1.1, 0.2)	0.9 (0.5-1.4, 0.5)		
> 80 years	-2AA	457	115	53	50	**2.2 (1.2-4.0, 0.01)**	3.1 (0.9-11.2, 0.08)	**2.2 (1.1-4.2, 0.02)**	2.9 (0.8-10.8, 0.1)
	-2G-positive	1610	447	194	116	1.0 (0.8-1.3, 0.95)	1.1 (0.6-2.0, 0.85)		

HFE = the haemochromatosis gene; *TF* = the transferrin gene; *OR* = odds ratio; CI = confidence interval; *SF* = synergy factor; age is at death or last examination (an age of 80 years is equivalent to an onset age of 75 years in our dataset).
*All analyses controlled for centre, age, gender and genotype of apolipoprotein ε4.

Supplementary Table 3

Supplementary Table 4 shows how the association with AD of each risk factor (genotype) is changed by the presence of the interacting factor. For instance, the association with AD of HFE 63HH is changed from an odds ratio of 0.9 (0.75-1.1, 0.4) to an odds ratio of 1.8 (1.2-2.6, 0.004) by the presence of TF -2AA.

Supplementary Table 4. Odds ratios of Alzheimer's disease for interacting genotypes, stratified by each other, in Northern Europeans

Odds ratio of AD for:-	In the subset:-	Numbers		Adjusted* odds ratios of AD (95%CI, *p*)
		Controls	AD cases	
HFE 282Y+	*TF* C2-	Y+ 500	Y+ 71	0.8 (0.6-1.04, 0.08)
		Y- 3514	Y- 725	
	TF C2+	Y+ 199	Y+ 49	1.3 (0.9-2.0, 0.2)
		Y- 1510	Y- 307	
TF C2+	*HFE* 282Y-	C2+ 1510	C2+ 307	1.0 (0.8-1.2, 0.8)
		C2- 3514	C2- 725	
	HFE 282Y+	C2+ 199	C2+ 49	**1.8 (1.1-2.9, 0.015)**
		C2- 500	C2- 71	
HFE 63HH	*TF* -2G+	HH 3279	HH 570	0.9 (0.75-1.1, 0.4)
		D+ 1167	D+ 213	
	TF -2AA	HH 906	HH 173	**1.8 (1.2-2.6, 0.004)**
		D+ 354	D+ 52	
TF -2AA	*HFE* 63D+	AA 354	AA 52	**0.6 (0.4-0.9, 0.01)**
		G+ 1167	G+ 213	
	HFE 63HH	AA 906	AA 173	**1.25 (1.02-1.5, 0.035)**
		G+ 3279	G+ 570	

HFE = the haemochromatosis gene; *TF* = the transferrin gene; CI = confidence interval; Y+, C2+, G+ and D+ group the genotypes, YY+CY, C2/C2+C2/-, GG + GA and DD + DH, respectively.
*All analyses controlled for centre, age, gender and genotype of apolipoprotein ε4.

Supplementary Table 4

Discussion

The interactions

Our results reveal a complex three-way interaction between HFE 63HH, TF -2AA and age (Table 4). This explains the paradox that HFE 63HH is a risk factor for AD, contingent on TF -2G/A genotype, yet may also be associated with a higher onset age of AD. The explanation is that the risk effect of HFE 63HH only applies to older people, possibly through additional interactions with survival-promoting factors. Nevertheless, we consider that this interaction should remain tentative until replicated in another large study.

In contrast, the interaction between HFE 282Y and TF C2 has now been replicated in two large, independent studies: Kauwe et al [62] and the present study. Kauwe et al used 1161 cases and 1342 controls, and they reported a combined synergy factor of 2.4 (1.4-4.2, 0.002), controlling for centre, age, gender and APOEε4 (as we did). The discordant results of association studies of these two SNPs when examined individually [see the AlzGene meta-analyses [56] may be partly due to this interaction. For instance, in our Northern European dataset, TF C2 was only associated with AD risk in the presence of HFE 282Y [odds ratio = 1.8 (1.1-2.9, 0.015)], whereas it had no effect in the latter's absence [odds ratio = 1.0 (0.8-1.2, 0.8)] (Supplementary Table 4). It is noticeable that nothing was obtained from the examination of the main effects of HFE 282Y and TF C2 (Table 2). But an important result has been derived from the study of their interaction. This interaction is so far the only example of epistasis in AD to have been consistently replicated in such numbers: altogether 2313 cases and 7065 controls.

Both interactions were found only or mainly in Northern Europeans (Tables 3 and 4). The samples used by Kauwe et al were also mainly Northern European. We found differences between North Europe and North Spain both in allelic frequencies and in LD structure (Table 1). There are several examples of differences between North and South Europe in genetic studies [67-69]. In our meta-analysis of the indel in the angiotensin 1-converting enzyme in AD [69], we were only able to remove the marked heterogeneity in our analyses by geographic stratification. This revealed clear differences between North and South Europe. Also in iron metabolism, a higher proportion of haemochromatosis patients are HFE 282Y homozygotes in North than in South Europe [67]. Other, as yet unknown, genes may modify the associations with AD of our studied SNPs in the Northern Spanish. However, we cannot rule out either interaction in that population, in view of the relatively low power in that subset (Table 3).

Although there is some LD between the two SNPs in HFE and also between the two in TF (Table 1), neither interaction is due to the effects of such LD. We cannot rule out that either interaction is due to LD with other polymorphisms. However, we suggest that each interaction may independently contribute to AD risk, by separate mechanisms, both of which lead to iron overload. There are a number of potential mechanisms, which we outline in the next section.

The four SNPs: potential mechanisms of the associations with AD

The HFE protein regulates iron metabolism in at least three ways. First, the protein has been shown to bind transferrin receptor 1 (TfR1) [70], thereby reducing the affinity of TfR1 for transferrin and decreasing cellular iron uptake. Second, HFE has been reported to lower cellular iron levels without binding TfR1 [71]. Third, HFE inhibits iron export from various cells, including macrophages [72-74]. The most likely mechanism of the association of the 282Y variant with AD may be the loss of the first function of HFE above. That is because the variant fails to reach the cell surface and thus to bind TfR1 [70,75]. Hence it leaves transferrin free to bind TfR1 with high affinity, leading to increased cellular uptake of iron. HFE 282Y may also be defective in the third function above [72]. However, that function may be the mechanism of the association of the H63D polymorphism with AD, assuming that HFE inhibits iron export from microglia, as it does from macrophages [72,73]. A subset of microglia store excess brain iron in ferritin [53]. This role may lead to their dystrophy [53,76] where brain iron accumulation is excessive, as in ageing and in AD (Introduction). The proportion of ferritin-positive microglia increases with ageing and further increases in AD [53]. However, the 63D variant [73] and possibly also 282Y [72] have lost the ability to block iron export, which may result in greater release of iron from microglia. H63D may also affect the first function of HFE (above): although the variant binds TfR1, it is reported to have little influence on the affinity of TfR1 for transferrin [70]. Other potential mechanisms associated with H63D include the promotion of glutamate toxicity [77] and of tau phosphorylation [78]. Oxidative stress-related mechanisms of these two variants are consistent with three findings of a Rotterdam study [79]. First, that both variants were associated with higher

serum levels of the antioxidant, bilirubin; second, that serum bilirubin correlated with iron load; and third, that high serum bilirubin was associated with reduced mortality in 282Y heterozygotes and 63D homozygotes. The interaction between HFE 282Y and TF C2 may be modified by APOEε4 (3.3 above), which itself has been associated with oxidative stress [80-84] and with greater vulnerability to Fe2+ ions [85].

The C2 variant of transferrin has also been proposed to increase the risk of oxidative stress [86], but the mechanisms remain unresolved. In vitro studies [87,88] have shown no differences in the iron-binding properties of the variant. Its changed glycosylation pattern [89] could be relevant, however. Glycosylation patterns are altered in CSF in AD [90], notably of transferring [91], which also has a higher oxidation index in AD plasma [92]. Although such partial changes in the glycosylation of transferrin do not affect its receptor binding [93,94], they may speed its degradation [89]. Changes in the glycosylation patterns of transferrin have been reported in various diseases, e.g. rheumatoid arthritis [95]. Total iron-binding capacity in blood may be marginally lower in C2 homozygotes [96-98], although the difference was only significant in one study [96]. In an OPTIMA study [99] with a subset of subjects from this study, transferrin saturation was higher in non-demented elderly with both HFE 282Y and TF C2, although neither variant alone had any effect. This increased transferrin saturation was due rather to raised serum iron in bi-carriers of these variants, than to lower iron-binding capacity. Iron load did not differ by genetic combination in AD [99]. The C2 variant may also have a role in β-amyloid metabolism [100,101]. The function of the -2G/A variant of TF has not yet been studied. However, given its position between the start site and the promoter of TF, very close to the former, it may well affect the expression of the gene.

Conclusions

We suggest that the combination, HFE 282Y with TF C2, and possibly also HFE 63HH with TF -2AA, may contribute to iron overload and thus to oxidative stress in the pre-clinical phase of AD. The effect of the former combination is influenced by APOEε4 and that of the latter combination depends on age. These interactions may partly explain the discordant results of previous studies. There are various potential mechanisms to obtain these effects, but these mechanisms remain unproven. Further study should include the adequately-powered examination of brain iron levels in subjects with these genetic combinations, in AD and particularly in MCI and elderly controls. Excess brain iron has also been found in other neurodegenerative conditions, such as Parkinson's disease [102,103]. It may be of interest to examine these genetic interactions in those conditions as well, and also in relation to cognitive performance in elderly controls, provided sufficient power is available. To have even 50% power to replicate the interaction between HFE 282Y and TF C2 at $p = 0.05$ in a Northern European sample, i.e. with control allelic frequencies similar to those in Table 1, would require 2400 cases and 2400 controls. It would require an even larger dataset in other populations, which have

still lower frequencies of HFE 282Y. The interaction between HFE 63HH and TF -2AA would require 1025 cases and 1025 controls to have 50% power. However, the former interaction has now been replicated twice independently, in samples totalling 2313 cases and 7065 controls, i.e. in Kauwe et al [62] and in the Northern Europeans of this study. Risk factors for AD may act many years before disease onset, as with high blood pressure [104,105] and with high serum cholesterol [106]. In the OPTIMA study cited above [99], the 282Y/C2 combination was also associated with higher iron load in non-demented elderly. We may therefore conclude that iron overload can be one of the causative factors in the development of AD. In our controls, 3.5% of Northern Europeans have the 282Y/C2 combination and 15.9% have the 63HH/-2AA combination (but the latter interaction needs further replication). Altogether, 18.1% have one or other combination. We suggest that treatment for iron overload, e.g. venesection [107] or iron chelation [108,109] might benefit elderly people with these genetic combinations and needs to be explored further.

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Part IV

Genetic determinants of age-related brain pathology in hypertensive patients

08 Genetic risk factors for cerebral small vessel disease in hypertensive patients from in a genetically isolated population

Abstract

Background

Asymptomatic cerebral lesions on MRI such as white matter lesions (WML), lacunes and microbleeds are commonly seen in the elderly. We examined the role of a series of candidate genes involved in blood pressure regulation and amyloid metabolism.

Materials & Methods

The study was embedded in a family-based cohort sampled from a Dutch genetically isolated population. We selected individuals between 55 and 75 years of age with hypertension (N=129). Volumes of WML and presence of lacunes and microbleeds were assessed with MRI. We studied 3 genes involved in blood pressure regulation (Angiotensin, Angiotensin II type 1 Receptor, alpha-Adducin), and 2 genes involved in the amyloid pathway (Apolipoprotein E (APOE) and sortilin-related receptor gene (SORL1)).

Results

All participants had WML (median volume, 3.1 mL, interquartile range, 1.5 – 6.5 mL), lacunar infarcts were present in 15.5% and microbleeds in 23.3%. Homozygosity for the APOE ε4 allele was associated with lacunes (OR, 4.8; 95% CI, 1.2-19.3). Individuals carrying two copies of the variant allele of 4 SNPs located at the 3'-end of SORL1 (rs1699102, rs3824968, rs2282649, rs1010159), had significantly more often microbleeds (highest odds ratio, 6.87; 95% CI, 1.78-26.44).

Conclusion

The association of SORL1 with microbleeds suggests that the amyloid cascade is involved in the etiology of microbleeds in populations with hypertension.

Introduction

Asymptomatic cerebral lesions, such as white matter lesions (WML), lacunes, and microbleeds are common in the elderly, and associated with stroke and cognitive impairment [1-4]. The mechanisms underlying the pathogenesis of these lesions are largely unknown. Several studies, however, suggest that changes in blood vessels in the brain lead to ischemic damage, causing WML and lacunes, and that leakage of red blood cells might lead to hemosiderin depositions or microbleeds [5,6]. Damage to vessels is most likely a consequence of hypertension and atherosclerosis, but there is also data suggesting that lesions are related to amyloid angiopathy [7].

Since hypertension is a risk factor for cerebral small vessel disease (CSVD), various genetic studies targeted genes that are involved in blood pressure regulation. Most widely studied are the alfa-adducin gene (ADD1), which increases renal sodium absorption, and the angiotensin (AGT) and angiotensin II type 1 receptor (AGTR1) genes, which play a role in the renin-angiotensin system [8].

The ADD1-Gly460Trp, AGT-M235T and AGTR1-C573T polymorphisms are associated with increased risk of atherosclerosis and CSVD [9-11]. Also genes involved in the amyloidogenic pathway have been implicated in CSVD. Two identified genes are the apolipoprotein E (APOE) and sortilin-related receptor (SORL1) genes. Although the exact function of APOE in the brain is not fully unraveled yet, this gene is thought to aid beta-amyloid clearance [12]. Carriers of the APOE ε4 allele have an increased risk of WML and microbleeds [13,14]. SORL1 codes for a neuronal apolipoprotein receptor, and is thought to regulate processing of the amyloid precursor protein in the brain [15]. Multiple SNPs within SORL1 are associated with Alzheimer's disease (AD) and cognitive impairment, and recently also with cerebral atrophy and cerebrovascular disease [16,17]. We selected the key SNPs from the original report for the current study [16].

The aim of the study was to examine the role of genes involved in blood pressure regulation and amyloid processing in the development of WML, lacunes, microbleeds and cognitive impairment.

Materials and Methods

The study was embedded in a population-based study in a genetically isolated population (Erasmus Rucphen Family (ERF)-Study). Considering hypertension as major risk factor for CSVD, we selected participants aged 55 to 75 years with hypertension to ensure a high prevalence of pathology. Hypertension was defined as systolic blood pressure ≥160 and/or diastolic blood pressure ≥100 and/or use of antihypertensive medication. Persons with a history of stroke or dementia or with MRI-contraindications were excluded. A random subset of 261 was invited out of 330 individuals who were eligible for the study; 135 agreed to participate. These had higher levels of education than nonparticipants (p=0.01). All participants gave informed consent and the study was approved by the Medical Ethics Committee.

Brain imaging was done on a 1.5-T MRI scanner (Signa Excite II, General Electric Healthcare, Milwaukee, WI, USA) with use of previously described protocols.[18] Volumes of WML were obtained by an automatic brain-tissue segmentation method.[19] To take into account differences in headsize, these volumes were analyzed as percentage of intracranial volume. Lacunes and microbleeds were rated by two trained reviewers; when there was no consensus a neuroradiologist decided. Previous agreement rates in our group with the same reviewers and neuroradiologist were good (κ=0.87 (intraobserver) and κ=0.85 (interobserver)).[14] MRI-scans could not be acquired in 4 participants because of physical constraints and 2 persons were excluded from analyses (both had a large incidentally discovered brain tumor). In total, complete information was available for 129 individuals.

Cognitive function was assessed with a word learning test, the Trail Making Test (TMT), the Stroop Color and Word (CW-) test, verbal fluency tests, and the block-design subtest of WAISIII.[20] From the word learning test, we derived four scores: working memory, learning, delayed recall, and recognition. From the TMT, a ratio score was computed: time on part B divided by time on part A. This was also done for the Stroop CW-test (time on card III divided by time on card II).

Blood pressure was measured by a mercury-based sphygmomanometer. Blood was taken for estimation of levels of total cholesterol, high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c).[20] Samples were genotyped for ADD1-Gly460Trp, AGT-M235T, AGTR1-C573T, APOE (rs429358, rs7412), and SORL1 (rs668387, rs689021, rs641120, rs1699102, rs3824968, rs2282649, rs1010159) with Taqman allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA).[9,11,20]

To analyze the association of genotypes with WML volume, lacunes and microbleeds, regression models were used. White matter lesion volume was transformed by taking the natural logarithm. Haplotypes were derived with the software package SimWalk2 [21]; these haplotypes were considered as a fixed factor in a general linear model. Student's T-test and Chi-square statistics were used to perform general descriptive statistics (version 15.0 of SPSS). To compare baseline characteristics, we defined CSVD as a WML volume greater than the 75th percentile, or presence of lacunes or microbleeds. Analyses were adjusted for age and sex, and p-values were adjusted for family relationship. We corrected for multiple testing by performing 10.000 permutations.

Results

The mean age of the participants was 64.5 (± 4.6) years and 52.7% were female. In this hypertensive population, WML were seen in all participants (median, 3.11 mL; interquartile range, 1.51-6.50 mL). Lacunes were present in 20 individuals (15.5%), and microbleeds in 30 individuals (23.3%) with lobar location in 63%. Those with WML greater than the 75th percentile more often had lacunes (p<0.001) and microbleeds (p=0.003). Age was associated with larger WML volumes (p<0.001) and presence of microbleeds (p=0.02). Other associations between CSVD and cardiovascular risk factors were not observed (Table 1).

Individuals with CVSD versus individuals without CVSD

	CSVD -	CSVD +	p-value
Number	77	52	
Female %	51.9	53.8	0.49
Age	63.3 (4.5)	66.3 (4.1)	**<0.001**
Systolic blood pressure (mmHg)	143.8 (18.6)	149.5 (17.3)	0.08
Diastolic blood pressure (mmHg)	83.2 (10.0)	84.5 (9.2)	0.45
BMI	29.5 (4.7)	28.6 (3.6)	0.29
Cholesterol (mmol/L)	5.2 (1.1)	5.2 (1.2)	0.89
HDL-C (mmol/L)	1.5 (0.3)	1.3 (0.3)	0.32
LDL-C (mmol/L)	3.4 (1.0)	3.3 (1.0)	0.65
Creatinine (mmol/L)	73.3 (20.3)	72.5 (17.9)	0.88
Diabetes (%)	15.6	15.4	0.98
Current smokers (%)	71.4	73.1	0.50
Use of alcohol (%)	62.3	67.3	0.58
Depressive symptoms† (%)	16.9	19.2	0.82
Education (% with primary education)	32.5	36.5	0.71

Values are presented as mean (±sd). CVSD: Cerebral small vessel disease, defined as wml ≥ P75 or lacune or cerebral microbleeds. BMI: Body Mass Index † defined as a score on CES-D ≥ 16 and/or a score of ≥ 11 on HADS-D and/or use of antidepressants

Table 1

The results of the association analyses between the polymorphisms and WML volume, lacunes and microbleeds are shown in Table 2. Genotype frequencies of ADD1, AGT and AGTR1 were not associated with WML volume, lacunes or microbleeds. Individuals carrying two copies of the APOE ε4 allele had more WML, lacunes and microbleeds than those without the ε4 allele; however, the effects were small. This difference was significant for lacunes (p=0.04).

The sortilin-related receptor gene was consistently associated with the presence of microbleeds. Individuals carrying two copies of the variant allele of the 4 SNPs located at the 3'-end of the gene (rs1699102, rs3824968, rs2282649, rs1010159) more often had microbleeds (highest odds ratio, 6.87; 95% CI, 1.78-26.44). In this hypertensive population, these SNPs were also associated with cognitive function. The SNPs rs1699102 (p=0.001), rs3824968 (p=0.004), rs2282649 (p=0.001), and rs1010159 (p=0.002) were all associated with TMT-ratio. The haplotype analysis of the SORL1 SNPs also showed association with microbleeds (p=0.05), most significantly when the haplotypes were based on rs1699102, rs3824968, rs2282649 and rs1010159 only (p=0.03). None of the SORL1 SNPs showed evidence for association with WML or lacunes. Genotype frequencies for the related SNPs were similar for participants and nonparticipants.

Association of *AGT*, *AGTR1*, *ADD1*, *APOE* and *SORL1* with WML, microbleeds and lacunar infarcts

Gene	Genotype	WML‡			Lacunar infarcts				Microbleeds			
		N	Mean difference (95% CI)	P	Absent	Present	OR (95% CI)	P	Absent	Present	OR (95% CI)	P
AGT	MM	33	ref		28	5	ref		26	7	ref	
	MT	64	-0.32 (-0.73 – 0.08)	0.12	52	12	1.32 (0.40-4.37)	0.65	48	16	1.37 (0.47-3.97)	0.57
	TT	23	-0.25 (-0.76 – 0.26)	0.33	21	2	0.57 (0.10-3.34)	0.53	18	5	1.19 (0.31-4.57)	0.80
AGTR1	TT	24	ref		20	4	ref		20	4	ref	
	CT	53	-0.10 (-0.57 – 0.36)	0.66	46	7	0.71 (0.18-2.78)	0.62	38	15	1.94 (0.55-6.86)	0.30
	CC	42	0.05 (-0.44 – 0.54)	0.84	35	7	1.19 (0.29-4.82)	0.81	33	9	1.60 (0.41-6.20)	0.50
ADD1	GG	77	ref		63	14	ref		54	23	ref	
	GT/TT	46	-0.22 (-0.56 – 0.12)	0.21	41	5	0.55 (0.18-1.66)	0.29	39	7	0.41 (0.16-1.07)	0.07
APOE	0 copies ε4	78	ref		66	12	ref		61	17	ref	
	1 copy ε4	39	-0.01 (-0.37 – 0.36)	0.97	36	3	0.39 (0.10-1.52)	0.18	30	9	0.96 (0.37-2.48)	0.94
	2 copy ε4	12	0.31 (-0.27 – 0.88)	0.29	7	5	4.77 (1.18-19.32)	**0.04**	8	4	1.99 (0.50-7.94)	0.33
SORL1 rs668387	CC	30	ref		26	4	ref		22	8	ref	
	CT	62	-0.04 (-0.45 – 0.38)	0.86	52	10	1.15 (0.32-4.11)	0.83	52	10	0.46 (0.15-1.36)	0.16
	TT	31	-0.11 (-0.58 – 0.37)	0.66	25	6	1.32 (0.32-5.42)	0.70	20	11	1.28 (0.41.4.00)	0.67
SORL1 rs689021	GG	26	ref		22	4	ref		19	7	ref	
	AG	63	-0.07 (-0.51 – 0.36)	0.74	54	9	0.85 (0.23-3.10)	0.80	52	11	0.50 (0.16-1.54)	0.23
	AA	31	-0.18 (-0.68 – 0.32)	0.47	25	6	1.12 (0.27-4.64)	0.88	21	10	1.06 (0.32-3.51)	0.92
SORL1 rs641120	CC	29	ref		25	4	ref		21	8	ref	
	CT	61	-0.07 (-0.49 – 0.35)	0.74	52	9	0.99 (0.27-3.59)	0.99	51	10	0.44 (0.15-1.32)	0.14
	TT	31	-0.18 (-0.66 – 0.30)	0.47	25	6	1.26 (0.31-5.18)	0.73	21	10	1.01 (0.32-3.22)	0.99
SORL1 rs1699102	TT	49	ref		40	9	ref		42	7	ref	
	CT	55	0.15 (-0.22 – 0.51)	0.43	48	7	0.65 (0.22-1.93)	0.34	43	12	1.76 (0.61-5.07)	0.30
	CC	17	0.15 (-0.37 – 0.68)	0.56	15	2	0.60 (0.11-3.20)	0.55	9	8	6.81 (1.79-25.97)	**0.005**
SORL1 rs3824968	TT	51	ref		41	10	ref		42	9	ref	
	AT	53	0.06 (-0.30 – 0.41)	0.75	47	6	0.50 (0.16-1.52)	0.22	42	11	1.19 (0.43-3.30)	0.74
	AA	15	0.28 (-0.25 – 0.81)	0.30	13	2	0.74 (0.14-3.92)	0.72	8	7	5.90 (1.54-22.70)	**0.01**
SORL1 rs2282649	CC	56	ref		47	9	ref		47	9	ref	
	CT	49	0.13 (-0.24 – 0.49)	0.49	42	7	0.88 (0.30-2.63)	0.82	38	11	1.60 (0.58-4.46)	0.37
	TT	15	0.32 (-0.22 – 0.87)	0.24	13	2	0.96 (0.18-5.15)	0.96	8	7	6.87 (1.78-26.44)	**0.005**
SORL1 rs1010159	TT	52	ref		43	9	ref		44	8	ref	
	CT	57	0.30 (-0.05 – 0.65)	0.10	48	9	0.89 (0.32-2.49)	0.83	42	15	2.05 (0.76-5.49)	0.16
	CC	20	0.21 (-0.28 – 0.69)	0.40	18	2	0.65 (0.12-3.38)	0.61	13	7	4.17 (1.18-14.70)	**0.03**

WML: white matter lesion volume, P: p-value adjusted for age, sex and family-relationship. ref: reference genotype. ‡ natural log transformed variable

Table 2

Discussion

This study shows that two genes involved in the amyloidogenic pathway and previously described in AD, APOE and SORL1, were associated with CSVD. The presence of two APOE-ε4 alleles was associated with lacunes. Given the role of apolipoprotein in the brain, this finding suggests involvement of beta-amyloid clearance in the pathogenesis of lacunes.[22] Alternatively, the association in this hypertensive population might be explained by the effect of APOE on cardiovascular factors.[20]

Our observation that SORL1 was associated with microbleeds has not been previously reported. This gene regulates APP processing and SORL1 deficiency leads to increased levels of beta-amyloid and enhances amyloid pathology in the brain.[16] In our study, microbleeds were predominantly located in lobar brain regions, implying that most of the lesions resulted from amyloid angiopathy.[14] In amyloid angiopathy-related AD, the majority of plaques is centered on vessel walls or in the immediate perivascular regions.[23] The relationship between SORL1 and microbleeds is of interest, because of increasing evidence that beta-amyloid plays a role in neurodegeneration through perivascular interstitial fluid drainage.[24] Through its role in microbleeds, SORL1, may link to dementia.[1] In line with this hypothesis, we found that the SNPs associated with microbleeds were marginally associated with cognitive function. This indirect role of SORL1 in plaque formation may also explain why the effects of the gene are modest in AD (http://www.Alzgene.org).

The genes involved in blood pressure regulation were not associated with CVSD. Earlier findings have been inconsistent.[9,11,25] The lack of an association in our study might be due to the fact that all participants were hypertensive, and that the blood pressure ranges were small. Indeed, blood pressure itself was not significantly associated with lesions.

The strength of our study is the population-based design, performed in a homogenous group of middle-aged individuals with hypertension derived from a genetically isolated population, which increases statistical power of genetic association studies. The size of the study is limited, however, and allows mainly detecting genetic variants with large effects. The association of SORL1 with microbleeds therefore remains to be confirmed. A question that remains to be answered in larger populations is whether hypertension interacts with SORL1 in the association with microbleeds, which might possibly be an alternative interpretation of our results.

The observed association of SORL1 with microbleeds in our study suggests that the amyloid cascade is involved in the etiology of microbleeds in populations with hypertension.

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09 The relation of the sortilin-related receptor gene to hippocampal volume and plasma amyloid beta levels in a family-based study of hypertensive patients

Abstract

Introduction

The sortilin-related receptor gene (SORL1) is an interesting candidate involved in Alzheimer's disease (AD). SORL1 consists of two functional domains, one functions in the cholesterol pathway and the other in the APP processing pathway. The gene has been associated to AD and cerebrovascular disease. We studied the relationship of SORL1 with hippocampal volume and plasma Aβ levels.

Materials & Methods

The study was embedded in a family-based cohort sampled from a Dutch genetically isolated population. We selected individuals between 55 and 75 years of age with hypertension (N=128). Volumes of the hippocampi were assessed with MRI and non-fasting blood sample were taken to assess plasma Aβ levels. We studied the effect of 7 variants within SORL1 that were previously reported in AD, by performing the variable screening analysis under the polygenic model and haplotype analyses.

Results

Three variants located near the 3'-end of SORL1 were significantly associated to hippocampal volume. The 3-SNP haplotypes for rs1699102, rs3824968 and rs2282649 (CAT) and for rs3824968, rs2282649 and rs1010159 (ATC) were associated to higher hippocampal volumes on MRI. We did not find significant associations of single variants with plasma Aβ levels.

Conclusion

Variants near the 3'end of SORL1 are associated to hippocampal volume. Our findings need replication in larger cohorts.

Introduction

Neurodegenerative diseases like Alzheimer's disease (AD) are highly prevalent diseases in the elderly and a major burden on society [1,2]. Neuropathologically, AD is characterized by neuritic plaques and neurofibrillary tangles [3,4]. Neuritic plaques contain amyloid beta (Aβ) proteins, which are formed after proteolytic processing of the amyloid precursor protein (APP). Aβ proteins are present in full-length species (β40 and β42) and in shorter amino-terminal truncated species (βn40 and βn42). The latter accounts for 60% of all Aβ species in pre-clinical AD stages [5]. Aβ pathology may also be present in brains of cognitively healthy elderly at postmortem and can visualized with PIB-PET during life [6,7]. The deposition and clearance of Aβ in the brain is related to Aβ levels in plasma, but the mechanism is not fully understood [8]. Both increased and decreased risks as well as no effects have been reported for Aβ40 and Aβ42 [9-14].

Another early biomarker of AD is hippocampal atrophy [15,16]. Although the hippocampus has a low Aβ load in non-AD brains [17], the correlation of hippocampal atrophy on MRI with Aβ CSF levels and with 11C-PIB uptake in PET studies of AD patients and healthy controls is high [18-20].

The heritability of plasma Aβ levels and medial temporal lobe atrophy have both been estimated

around 60%, since no major genes have been identified yet, it is of interest to find genetic risk factors that influence the variability of these traits [21-25]. Previous studies have suggested overlap between genes affecting brain volumes and those affecting blood pressure [26], making it interesting to study genetic susceptibility in hypertensive patients.

In the current study, we focused on the sortilin-related receptor gene (SORL1), which is involved in the APP pathway. The SORL1 protein may act as a sorting receptor for APP [27,28] and variants in the SORL1 gene are associated to AD although the effect size is small and evidence is unstable [29-31]. We have recently found association of variants in SORL1 with cerebral microbleeds [32]. In addition to a role in APP processing, the SORL1 protein is related to low-density lipoprotein receptors and may be involved in atherosclerotic processes [33], which was also suggested by associations of variants in SORL1 with cerebrovascular disease and cerebral atrophy reported by a recent study [34]. The aim of the current study was to examine whether SORL1 was related to plasma Aβ levels and hippocampal atrophy on MRI. We studied this in a hypertensive subset of the Erasmus Rucphen Family (ERF) study, which is a family-based study in a genetically isolated population.

Materials and Methods

Study population

The study was embedded in a population-based study in a genetically isolated population in the Netherlands: the Erasmus Rucphen Family (ERF)-Study. Participants are all descendents of a limited number of founders living in the 19th century. Extensive genealogical data is available to the year 1600 [35,36]. The current study was designed to find genetic risk factors for cerebral small vessel disease (CSVD). Considering hypertension as major risk factor for CSVD, we selected participants aged 55 to 75 years with hypertension to ensure a high prevalence of pathology. Hypertension was defined as systolic blood pressure ≥160 and/or diastolic blood pressure ≥100 and/or use of antihypertensive medication. Persons with a history of stroke or dementia or with MRI-contraindications were excluded. A random subset of 261 was invited out of 330 individuals who were eligible for the study; 135 agreed to participate. The distribution of cardiovascular risk factors was similar among participants and non-participants. There was a small difference in completed years of education, which was higher for participants who had a mean level of 7.5 years compared to 9 years in non-participants (p=0.02). All participants gave informed consent and the study was approved by the Medical Ethics Committee at Erasmus MC University Medical Center.

Brain imaging

Brain imaging was done on a 1.5-T MRI scanner (Signa Excite II, General Electric Healthcare, Milwaukee, WI, USA) with use of a previously described protocol [37]. Volumes of the hippocampi, normal white matter (WM), white matter lesions (WML), grey matter (GM) and cerebrospinal fluid (CSF) were obtained by an automatic brain-tissue segmentation method which which was validated

previously [38,39]. The hippocampal formation included CA1 to CA4, the gyrus dentatus, and the subiculum. Volumes of the left and right hippocampus were measured with an automatic segmentation method based on intensity and regularity energy models. All samples were visually checked and when necessary manually segmented with specific software (http://www.bic.mni.mcgill.ca/software/) on coronal slides with continuous reference to all orientations. For analyses, the volumes of the left and right hippocampus were summed. To take into account differences in headsize, hippocampal volumes were analyzed as percentage of intracranial volume, which was defined as the total volume of WML, WM, GM and CSF. MRI-scans could not be acquired in 4 participants because of physical constraints and 2 persons were excluded from analyses (both had a large incidentally discovered brain tumor).

Aβ measurements

Non-fasting blood samples were obtained, which were immediately cooled on ice. From the samples plasma was extracted and stored at -80 ºC. Plasma Aβ concentrations were measured using a fluorimetric bead-based immunoassay using xMAP® technology (Innogenetics®). We obtained Aβ40, Aβ42, and the truncated forms Aβn40 and Aβn42. For analyses, we also used Aβ42/Aβ40 ratio. In total, complete information was available for 128 individuals.

Covariates

Body weight and height were measured in centimeters and the body-mass index (BMI) was calculated from these measurements. Blood was taken for estimation of levels of total cholesterol, high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c) and creatinine in serum (Roche®). Glycosylated hemoglobin (HbA1c) was measured from full blood obtained in vacucontainers containing sodium citrate (Bio-Rad®) and analyzed using High-Performance Liquid Chromatography.

Genotyping

Genomic DNA was extracted from whole blood samples using the salting out method [40]. Samples were genotyped for APOE (rs429358, rs7412), and SORL1 (rs668387, rs689021, rs641120, rs1699102, rs3824968, rs2282649, rs1010159) with Taqman allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA) [41].

The SNPs within SORL1 were chosen, because we considered them as the key SNPs from the original report [31].

Statistical analyses

General descriptive statistics were estimated with one-way ANOVA and Chi-square statistics as implemented in the software SPSS (version 15.0). The observed frequencies of the genotypes were tested for deviations from Hardy-Weinberg equilibrium (HWE) by exact test. All genotypes were in HWE ($p>0.05$).

We first estimated whether plasma Aβ levels were associated to hippocampal volume on MRI with linear regression models. Second, to analyze the association of the genotypes with hippocampal volume and plasma Aβ levels, we performed the variable screening analysis under the polygenic model using the SOLAR software version 4.1.0 [42]. This software allows adjusting for family-relationships taking into account the pedigree structure. To reduce computational time, the large pedigree was cut into smaller subpedigrees of 18 bitsize before analysis [43]. One person could not be linked to these subpedigrees. The analyses in SOLAR were adjusted for age, sex and inbreeding coefficient, which was estimated from the genealogical data of the complete pedigree of the population using the software PEDIG [44]. The genotypes were entered in the model as a covariate with the reference genotype group coded as 0, the heterozygote genotypes coded as 1 and the other genotype group coded as 2. For APOE, 0 was used for the ε2/ε2, ε2/ε3 and ε3/ε3 genotypes, 1 for the ε2/ε4 and ε3/ε4 genotypes and 2 for the ε4/ε4 genotype.

Haplotypes for SORL1 were derived with the software package SimWalk2 [45]. Haplotypes with a frequency >5% were implemented as binary factors in the models and analyzed in SOLAR. To adjust for multiple testing we used Bonferroni correction adjusting for 7 independent tests.

Results

Baseline characteristics of the study population are shown in Table 1. The mean age was 64.6 years and there were slightly more women than men. Hippocampal volume was not significantly associated to plasma levels of Aβ.

Descriptive characteristics of the study population

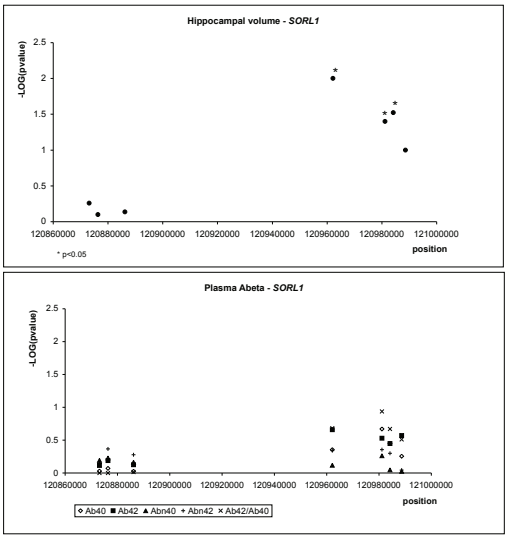
N	128
Sex (% women)	52.3%
Age	64.6 (4.5)
BMI	29.2 (4.3)
HDL-c (mmol/L)	1.3 (0.3)
Creatinine (µmol/L	73.0 (19.4)
HbA1c (%)	5.8 (0.6)
White matter lesion volume*	0.49 (0.56)
APOE E4 carriers (%)	39.1%

BMI: body-mass index, APOE: Apolipoprotein E gene.
**percentage of intracranial volume*

Table 1

The SORL1 gene was significantly associated with hippocampal volume (Table 2). Single SNP analyses showed that subjects with the minor genotype of 3 SNPs located near the 3'-end of SORL1 had significantly higher hippocampal volumes than subjects with the major genotype (Figure). Additional adjustment for the APOE genotype resulted in greater effect size and more significant p-values for these associations.

Regional plots depicting the associations of SORL1 with hippocampal volume (first) and Aβ levels (second)



The x-axis depicts the physical position on the chromosome (kb); the y-axis depicts the minus log p-values.

Figure 1

Haplotype analyses revealed significant associations of the 3-SNP haplotypes for rs1699102, rs3824968 and rs2282649 (CAT) and for rs3824968, rs2282649 and rs1010159 (ATC) with higher hippocampal volumes (Table 3). These haplotypes were the second most frequent in our population (18.5% and 19.3% respectively). The p-value of the CAT-haplotype remained significant after adjustment for multiple testing. Additional adjustment for APOE genotype did not change these results. The haplotypes consisting of the 3 SNPs located at the 5'-end of SORL1 (rs668387, rs689021 and rs641120) were not significantly associated.

The SORL1 SNPs were not significantly associated with plasma Aβ levels (Table 4 and Figure). The results were similar when adjusting these analyses additionally for creatinine, high-density lipoprotein, HbA1c, BMI and white matter lesion volume (data not shown).

Association of *SORL1* with hippocampal volume

Gene variant	Genotype	Hippocampal volume‡ N		Mean (SD)	P1	P2
SORL1 rs668387	CC	36	CC	0.52 (0.03)	0.55	0.58
	CT	60	CT	0.53 (0.06)		
	TT	31	TT	0.51 (0.06)		
SORL1 rs689021	GG	34	GG	0.51 (0.05)	0.80	0.83
	AG	62	AG	0.53 (0.05)		
	AA	31	AA	0.51 (0.06)		
SORL1 rs641120	CC	36	CC	0.51 (0.05)	0.73	0.76
	CT	60	CT	0.53 (0.05)		
	TT	31	TT	0.51 (0.06)		
SORL1 rs1699102	TT	55	TT	0.51 (0.05)	**0.01**	**0.01**
	CT	55	CT	0.52 (0.05)		
	CC	17	CC	0.54 (0.06)		
SORL1 rs3824968	TT	59	TT	0.51 (0.05)	**0.04**	**0.03**
	AT	53	AT	0.52 (0.06)		
	AA	15	AA	0.55 (0.03)		
SORL1 rs2282649	CC	63	CC	0.51 (0.05)	**0.03**	**0.02**
	CT	49	CT	0.52 (0.06)		
	TT	15	TT	0.55 (0.03)		
SORL1 rs1010159	TT	51	TT	0.51 (0.04)	0.10	0.07
	CT	56	CT	0.52 (0.07)		
	CC	20	CC	0.54 (0.04)		

P1: p-value adjusted for age, sex and family-relationship. P2: P1 additionally adjusted for APOE genotype.
‡ percentage of intracranial volume

Table 2

Association *SORL1* haplotypes gene with hippocampal volume‡

SNPs	Haplotype	Frequency % (N)	P*	Effect	P**
rs668387- rs689021- rs641120	CGC	37.8 (96)	0.61	-	0.63
	CAT	33.5 (85)	0.90	+	
	TAT	7.9 (20)	0.53	+	
	TGC	7.5 (15)	0.73	+	
rs1699102-rs3824968- rs2282649	TTC	53.5 (136)	0.21	-	0.16
	CAT	18.5 (47)	**0.005**	+	
	CAC	6.3 (16)	0.43	-	
	CTT	5.1 (13)	0.60	+	
	CTC	5.1 (13)	0.58	-	
rs3824968- rs2282649- rs1010159	TCT	50.4 (128)	0.10	-	0.25
	ATC	19.3 (49)	**0.05**	+	
	TCC	8.3 (21)	0.53	+	
	TTC	5.9 (15)	0.60	+	
	ACT	5.9 (15)	0.74	**+**	

*P: p-value adjusted for age, sex and family-relationship. ‡ percentage of intracranial volume. *p-value for haplotype; **p-value for region. Effect: direction of effect. The + sign refers to increased hippocampal volumes and the – sign to decreased hippocampal volumes.*

Table 3

Association of *SORL1* with plasma Aβ levels

Gene variant	Genotype	Aβ40 (pg/mL) N	Mean (SD)	P	Aβ42 (pg/mL) N	Mean (SD)	P	Aβn40 (pg/mL) N	Mean (SD)	P	Aβn42 (pg/mL) N	Mean (SD)	P	Aβ42/Ab40 N	Mean (SD)	P
SORL1 rs668387	CC	34	172.24 (43.60)	0.94	35	39.15 (14.75)	0.77	35	168.74 (39.21)	0.64	33	27.33 (6.14)	0.71	34	0.24 (0.08)	0.46
	CT	61	182.59 (43.07)		61	41.83 (11.97)		61	178.48 (40.71)		56	28.78 (7.80)		61	0.24 (0.07)	
	TT	31	175.15 (27.93)		31	43.11 (14.74)		31	174.31 (29.25)		30	26.76 (4.82)		31	0.25 (0.07)	
SORL1 rs689021	GG	32	175.12 (39.07)	0.85	33	41.59 (16.27)	0.65	33	175.63 (30.76)	0.59	33	28.13 (6.03)	0.43	32	0.25 (0.07)	0.99
	AG	63	182.50 (43.91)		63	41.33 (12.12)		63	176.95 (43.34)		57	28.43 (7.81)		63	0.24 (0.08)	
	AA	31	171.71 (31.88)		31	41.36 (13.17)		31	169.46 (32.43)		29	26.46 (4.86)		31	0.24 (0.06)	
SORL1 rs641120	CC	34	173.53 (40.92)	0.94	35	41.11 (16.33)	0.75	35	172.47 (36.82)	0.69	34	27.90 (6.09)	0.53	34	0.25 (0.09)	0.97
	CT	61	183.62 (42.92)		61	41.60 (11.91)		61	178.81 (40.79)		56	28.58 (7.80)		61	0.23 (0.07)	
	TT	31	171.71 (31.88)		31	41.36 (13.17)		31	169.46 (32.43)		29	26.46 (4.86)		31	0.24 (0.06)	
SORL1 rs1699102	TT	55	174.53 (40.52)	0.44	56	41.69 (14.38)	0.22	56	169.50 (38.34)	0.76	53	28.64 (6.72)	0.45	55	0.25 (0.07)	0.21
	CT	54	184.19 (37.86)		54	41.83 (13.40)		54	183.52 (37.21)		50	27.16 (6.45)		54	0.23 (0.09)	
	CC	17	169.34 (44.34)		17	39.13 (10.59)		17	164.42 (33.31)		16	27.51 (7.63)		17	0.24 (0.04)	
SORL1 rs3824968	TT	59	173.28 (40.10)	0.21	60	41.86 (14.24)	0.30	60	169.84 (38.12)	0.55	57	28.50 (6.47)	0.44	59	0.25 (0.06)	0.12
	AT	53	184.54 (38.27)		53	41.33 (13.27)		53	182.39 (37.24)		49	27.22 (6.69)		53	0.23 (0.09)	
	AA	14	172.81 (45.37)		14	39.76 (11.17)		14	167.16 (35.40)		13	27.55 (8.07)		14	0.24 (0.04)	
SORL1 rs2282649	CC	63	175.97 (42.13)	0.35	64	41.63 (14.10)	0.36	64	173.09 (38.96)	0.89	60	28.39 (6.38)	0.50	63	0.25 (0.07)	0.21
	CT	49	182.01 (35.91)		49	41.58 (13.38)		49	179.16 (36.98)		46	27.27 (6.84)		49	0.23 (0.09)	
	TT	14	172.81 (45.37)		14	39.76 (11.17)		14	167.16 (35.40)		13	27.55 (8.07)		15	0.24 (0.04)	
SORL1 rs1010159	TT	52	175.92 (43.42)	0.55	52	41.75 (12.71)	0.27	52	170.69 (39.69)	0.95	48	27.88 (6.70)	0.90	52	0.24 (0.07)	0.31
	CT	56	181.93 (36.84)		56	42.55 (14.05)		56	181.60 (37.46)		53	27.94 (6.70)		56	0.24 (0.09)	
	CC	18	171.57 (40.20)		19	37.09 (13.46)		19	165.87 (30.75)		18	27.61 (7.17)		18	0.23 (0.04)	

P: p-value adjusted for age, sex and family-relationship. SD: standard deviation

Table 4

Discussion

The present study shows that variants in SORL1 are associated to hippocampal volume with a protective effect of the AT-haplotype consisting of rs3824968 and rs2282649. This is in line with the results of a previous study reporting associations of SORL1 with cerebrovascular disease and brain atrophy [34]. Although not significant in our study, Cuenco et al. also found that lower hippocampal volumes were associated with the TC-haplotypes of these SNPs. Since neuronal loss in AD can be seen as hippocampal atrophy on MRI, these SORL1 variants may exert their effect on the processing of APP [46,47], however, the cross-sectional design of the current study limits the interpretation of underlying pathophysiological mechanisms. The haplotypes constructed from the SNPs at the 5'-end of SORL1 were not significantly associated to hippocampal volume, but the direction of effect was similar to the direction seen in AD, in which the CGC-haplotype was associated to an increased risk and the TAT-haplotype to a decreased risk of AD [31]. The opposite effects of these haplotypes on the risk of cerebrovascular disease that were found in a previous study [34] may suggest that one of the functional domains in SORL1 could be involved in the cholesterol pathway and the other in the APP processing pathway [48]. This may explain the heterogeneous findings in AD and MRI-traits (http://www.Alzgene.org) [30,34].

Our study did not find an association of SORL1 with plasma Aβ. One explanation could be that plasma Aβ, although linked to brain Aβ may not accurately reflect brain Aβ pathology. Studies measuring Aβ levels in CSF or postmortem studies may provide more accurate measurements of brain Aβ pathology. Also, the effect of SORL1 on AD pathology may be contributable to other mechanisms in the APP-pathway than Aβ generation. Plasma Aβ may be an indicator of vascular damage, rather than neurodegeneration, since it has been associated to white matter lesions and cerebral small vessel disease [49,50]. Increased levels of plasma Aβ could be a cause of microvascular damage, but could also be a consequence if damage to small vessels results in leakage of Aβ from the brain to the circulation. The effect of SORL1 on CSF Aβ levels warrants further study and may elucidate the role of SORL1 and Aβ in cerebrovascular and neurodegenerative disease processes.

Our observation of a strong association of SORL1 with hippocampal volume in hypertensive patients may reflect a possible interaction of SORL1 with hypertension, which is also supported by previous findings of a stronger effect of SORL1 with AD in patients with cerebrovascular disease [34].The pathophysiology of AD is likely multifactorial with vascular as well as neurodegenerative factors influencing the disease process [51]. Both cerebrovascular disease and hippocampal atrophy are associated with an increased risk of dementia and cognitive dysfunction [52-55]. This may be due to interaction between vascular and neurodegenerative processes in their effect on dementia [56], as has been demonstrated by increased white matter lesion volumes in patients with greater hippocampal atrophy [57].

The strength of our study is that it was performed in a homogenous group of middle-aged individuals with hypertension derived from a genetically isolated population, which increases statistical power of genetic association studies. The size of the study is limited, however, and allows mainly detecting genetic variants with large effects. A question that remains to be answered in larger populations is whether hypertension interacts with SORL1.

To summarize, variants near the 3'end of SORL1 are associated to hippocampal volume on MRI. Our findings need replication in larger cohorts.

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10 Polymorphisms of the renin-angiotensin system and alfa-adducin in associate to circulating amyloid beta levels

Abstract

Background

The renin-angiotensin system (RAS) has been implicated in Alzheimer's disease (AD) and degradation of amyloid beta (Aβ). Genetic studies focused on the angiotensin converting enzyme gene (ACE). Less is known for the other genes implicated in the RAS system. We studied the association of three commonly studied genetic variants with circulating Aβ.

Materials & Methods

The study was embedded in a family-based cohort sampled from a Dutch genetically isolated population. We selected individuals between 55 and 75 years of age with hypertension (N=128). Non-fasting blood sample were taken to assess plasma Aβ levels using xMAP® technology. We studied the effect of 3 variants within the angiotensin, angiotensin II type 1 receptor and adducin genes (AGT-M235T, AGTR1-C573T and ADD1-Gly460Trp) that were previously reported in cerebrovascular and cardiovascular disease.

Results

The AGT-M235T TT-genotype was significantly associated with higher levels of plasma Aβ42 (p=0.008) and truncated Aβn42 (p=0.02). The association to Aβ42 remained significant after adjusting for potential confounders and multiple testing. Persons with the variant genotype of ADD1-Gly460Trp tended to have lower levels of Aβn40 than persons with the wild type genotype (p=0.02), but this association did not remain significant after adjustment for confounders and multiple testing. No associations were found between plasma Aβ and AGTR1-C573T.

Conclusion

Our study shows that the AGT-M235T polymorphism is associated to plasma Aβ levels in hypertensive patients.

Introduction

Alzheimer's disease (AD) is characterized by neuritic plaques of which amyloid beta (Aβ) is the main component [1-3]. Aβ is present in full-length species (Aβ40 and Aβ42) and in shorter amino-terminal truncated species (Aβn40 and Aβn42). The latter concerns proteins which may predominantly be spliced to pathogenic forms and account for 60% of all Aβ species in pre-clinical AD stages [4]. Aβ pathology is also found in brains of cognitively healthy elderly at postmortem examination and visualized during life using PET studies [5,6]. The deposition and clearance of Aβ in the brain is related to Aβ levels in plasma, but the mechanism is not fully understood [7].

The renin-angiotensin system (RAS) is mainly involved in the regulation of blood pressure and salt homeostasis, but has also been implicated AD [8]. In AD brains, RAS is activated [9] and receptors for angiotensin II are present in brain tissue [10]. The gene encoding the angiotensin converting enzyme (ACE), is one of the most studied genes involved in the RAS system and has consistently been associated with AD [11,12]. There is experimental evidence for a role of ACE in the degradation of

amyloid beta (Aβ) in the brain [13,14] and inhibition of ACE activity results in increased Aβ deposition in the brain [15].

Far less is known for the other genes implicated in the RAS system in relation to AD pathology. The main other genes that have been studied are the angiotensin (AGT) and angiotensin II type 1 receptor (AGTR1) genes. These genes play a role in RAS by affecting the protein translation [16,17] and specific polymorphisms (AGT-M235T and AGTR1-C573T) have been associated to increased risk of cardiovascular and cerebrovascular disease [18-23]. Another gene that could be of interest given its strong link to RAS is the alfa-adducin gene (ADD1). ADD1 has been associated to increased renal sodium uptake [24]. Alfa-adducin is highly expressed in brain regions, including the hippocampus [25]. Different expression of the adducin subunits (α, β and γ) has been related to impaired learning, impaired motor function and synaptic plasticity in the hippocampus of mice [26]. We have previously shown a relationship of the ADD1-Gly460Trp variant and ischemic brain changes, mainly in hypertensive patients [27]. The reason why RAS genes interact with hypertension is far from clear. Hypertension early in life has been associated to AD at old age [28,29]. This may suggest that hypertensive patients are at increased risk of early AD pathology, despite the fact that they do not show symptoms of AD or cognitive decline[30]. Early amyloid pathology, however, may induce increased levels of Aβ in the blood.

To follow-up our work and that of others on the role of RAS genes other than ACE in Aβ pathology, we conducted a study relating these genes to plasma Aβ levels in individuals with hypertension. In the current study, we were interested in the role of Aβ to genes that we earlier implicated in MRI pathology: AGT-M235T, AGTR1-C573T and ADD1-Gly460Trp. We studied the association of these variants with plasma Aβ levels in individuals with hypertension from the Erasmus Rucphen Family (ERF) study, which is a family-based study in a genetically isolated population.

Materials and Methods

Study population

The study was conducted in a family-based study in a genetically isolated population in the Netherlands: the Erasmus Rucphen Family (ERF) study. Participants are all descendents of a limited number of founders and extensive genealogical data is available [31,32]. The current study was designed to find genetic risk factors for cerebral small vessel disease and included participants aged 55 to 75 years with hypertension. Hypertension was defined as systolic blood pressure ≥160 and/or diastolic blood pressure ≥100 and/or use of antihypertensive medication. Persons with a history of stroke or dementia or with MRI-contraindications were excluded. Two-hundred sixty one persons were invited; 135 agreed to participate. The distribution of cardiovascular risk factors was similar among participants and non-participants. There was a small difference in completed years of education, which was higher for participants who had a mean level of 7.5 years compared to 9 years in non-participants (p=0.02). All participants gave informed consent and the study was approved by

the Medical Ethics Committee at Erasmus MC University Medical Centre.

Aβ measurements

Non-fasting blood samples were obtained during the visit at the research center, following standardized protocols. Samples were immediately cooled on ice. Plasma was extracted within the same day and stored at -80 ºC. Plasma Aβ concentrations were measured using a fluorimetric bead-based immunoassay using xMAP® technology (Innogenetics®) using the manufacturers protocols. We obtained Aβ40, Aβ42, and the truncated forms Aβn40 and Aβn42. For analyses, we also used Aβ42/Aβ40 ratio.

Covariates

We evaluated the association of Aβ levels to covariates that were reported in earlier studies [33,34]. Body weight (kilograms) and height (centimeters) were measured and the body-mass index (BMI) was calculated from these measurements. Blood pressure was measured twice in sitting position by one physician using a sphygmomanometer. Blood was taken for the assessment of levels of high-density lipoprotein cholesterol (HDL-c) and creatinine in serum (Roche®) [35]. Glycosylated hemoglobin (HbA1c) was measured from full blood obtained in vacucontainers containing sodium citrate (Bio-Rad®) and analyzed using High-Performance Liquid Chromatography.

Genotyping

Genomic DNA was extracted from whole blood samples using the salting out method [36]. Samples were genotyped for ADD1-Gly460Trp, AGT-M235T, AGTR1-C573T, with Taqman allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA) [19,27]. Samples were also genotyped for Apolipoprotein E (APOE) (rs429358, rs7412) to assess ε4 carrier status. In total, complete information was available for 128 individuals.

The observed frequencies of the genotypes were tested for deviations from Hardy-Weinberg equilibrium using the exact test for multiple alleles [37]. For all genetic variants, the allele and genotype distribution were in Hardy-Weinberg equilibrium ($p>0.05$).

Statistical analyses

General descriptive statistics were estimated with one-way ANOVA and Chi-square statistics as implemented in the software SPSS (version 15.0). Correlation coefficients between Aβ levels and covariates were assessed using SPSS. To analyze the association of the genotypes with and plasma Aβ levels, we performed the variable screening analysis under the polygenic model using the SOLAR software version 4.1.0 [38]. This software allows adjusting for family-relationships taking into account the pedigree structure. To reduce computational time, the large pedigree was cut into smaller subpedigrees of 18 bitsize before analysis [39]. One person could not be linked to these

subpedigrees, therefore we could analyze 128 persons. The analyses in SOLAR were adjusted for age, sex and inbreeding coefficient, which was estimated from the genealogical data of the complete pedigree of the population using the software PEDIG [40]. In a second model, we adjusted additionally for creatinine, HDL-c, HbA1c and BMI. The genotypes were entered in the model as a covariate with the reference genotype group coded as 0, the heterozygote genotypes coded as 1 and the rare homogeneous genotype group coded as 2.

Descriptive characteristics of the study population

N	128
Sex (% women)	52.3%
Age	64.6 (4.5)
BMI	29.2 (4.3)
HbA1c (%)	5.8 (0.6)
Creatinine (µmol/L	73.0 (19.4)
HDL-c (mmol/L)	1.3 (0.3)
APOE ε4	39.1%

BMI: body-mass index. SBP: systolic blood pressure; DBP: diastolic blood pressure; HbA1c: Glycosylated hemoglobin; HDL-c: high-density lipoprotein cholesterol; APOE ε4: carriers of the apolipoprotein ε4 allele. Values represent percentages or mean (standard deviation).

Table 1

Correlation between plasma Aβ levels and covariates

	Aβ40	Aβn40	Aβ42	Aβn42	Aβ42/Aβ40	Sex	Age	BMI	HbA1C	Creat	HDL-C (mmol/L)	*APOE*
Aβ40	1	0.77***	0.40***	0.23**	-0.38***	0.01	-0.02	0.24*	0.05	0.08	-0.18*	0.13
Aβn40		1	0.32***	0.38***	-0.31***	0.05	-0.01	0.20	0.16	0.15	-0.18*	0.10
Aβ42			1	0.35***	0.66***	-0.14	-0.01	0.10	0.12	0.04	0.04	-0.13
Aβn42				1	0.22**	-0.09	-0.02	0.04	0.05	0.10	0.03	-0.10
Aβ42/Aβ40					1	-0.22*	0.04	-0.11	0.07	0.02	0.19*	-0.21*

Significant correlations are depicted in bold. Significant correlations are depicted in bold. * p<0.05; ** p<0.01; ***p<0.001.
BMI: body-mass index; HbA1c: Glycosylated hemoglobin; Creat: Creatinine; HDL-c: high-density lipoprotein cholesterol; LDL-c: high-density lipoprotein cholesterol; *APOE*: Apolipoprotein E gene defined as having 0, 1 or 2 copies of the ε4 allele.

Table 2

Results

Table 1 shows the baseline characteristics of the study population. The mean age was 64.6 years and 52.3% were women. Correlations between the different Aβ measurements and covariates are given in Table 2. Of the covariates, sex and HDL-c were significantly associated to the Aβ42/Aβ40 ratio. HDL-c and BMI were related to Aβ40 and Aβn40. APOE was correlated to the Aβ42/Aβ40 ratio (R=-0.21; p=0.03), but not to single Aβ measurements. As expected, the different Aβ proteins were highly correlated (all p-values <10-5) and there was a strong correlation between Aβ42 and the Aβ42/Aβ40 ratio (R=0.66; p=3.03*10-17).

Table 3 shows mean Aβ levels per genotype group for each gene. AGT-235T was significantly associated with higher levels of Aβ42 (p=0.008) and Aβn42 (p=0.02). Individuals with the MM-genotype had mean Aβ42 plasma levels of 39.3 pg/mL compared to 48.0 pg/mL in individuals with the TT-genotype. For Aβn42 levels, a mean of 25.9 pg/mL was seen in individuals with the MM

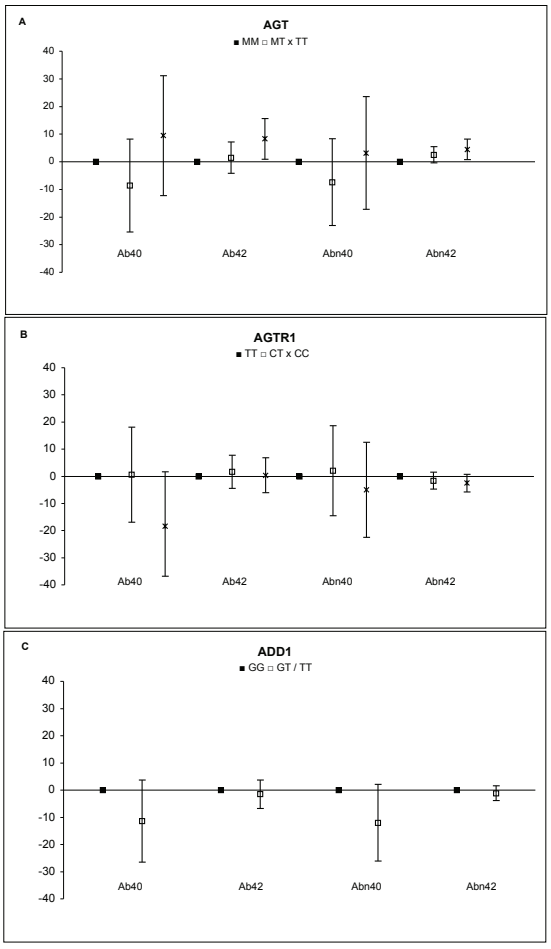
Association of *AGT*, *AGTR1*, and *ADD1* plasma abeta levels

Gene	Genotype	Aβ40			Aβ42			Aβn40			Aβn42			Aβ42/Aβ40		
		N	Mean (SD)	P	N	Mean (SD)	P	N	Mean (SD)	P	N	Mean (SD)	P	N	Mean (SD)	P
AGT	MM	40	179.0 (36.7)	0.57	41	39.3 (14.2)	0.008	41	176.6 (31.8)	0.98	39	25.9 (5.9)	0.02	40	0.23 (0.08)	0.15
	MT	63	171.6 (35.6)		63	40.4 (11.7)		63	169.9 (37.5)		58	28.3 (6.1)		63	0.24 (0.07)	
	TT	23	193.6 (52.6)		23	48.1 (14.9)		23	185.0 (46.7)		22	30.2 (8.7)		23	0.25 (0.07)	
AGTR1	TT	32	182.9 (35.3)	0.17	33	40.2 (15.1)	0.82	33	174.8 (29.8)	0.99	30	29.3 (6.1)	0.15	32	0.23 (0.08)	0.18
	CT	52	184.4 (42.2)		52	41.9 (13.4)		52	178.7 (41.3)		50	27.6 (6.8)		52	0.23 (0.06)	
	CC	42	166.3 (39.0)		42	41.7 (12.4)		42	169.9 (39.4)		39	27.2 (7.1)		42	0.26 (0.08)	
ADD1	GG	81	182.9 (41.7)	0.06	82	42.1 (14.2)	0.64	82	179.8 (38.3)	0.06	78	28.4 (6.8)	0.31	81	0.24 (0.08)	0.49
	GT/TT	45	169.1 (35.4)		45	40.1 (11.9)		45	165.6 (35.3)		41	26.9 (6.5)		45	0.24 (0.06)	

P: p-value adjusted for age, sex and family-relationship. Values are in pg/mL.

Table 3

Associations of AGT, AGTR1, and ADD1 with plasma Aβ levels, adjusted for covariates



Genotypes for AGT-M235T (A), AGTR1-C573T (B) and ADD1-Gly460Trp (C) are depicted on the x-axis. Values on the y-axis represent the differences in the mean plasma Aβ levels compared to the reference genotype with 95% confidence intervals. The differences were adjusted for age, sex, family-relationship, creatinine, high-density lipoprotein cholesterol, glycosylated hemoglobin and body mass index.

Figure 1

genotype and 30.2 pg/mL in those with the TT-genotype. ADD1 was associated with Aβn40 (p=0.02). Individuals with the GT/TT genotype had significantly lower levels of Aβn40 (165.6 pg/mL) than persons with the GG genotype (179.8 pg/mL). No significant association was seen to the Aβ42/Aβ40 ratio for any of the genes.

The Figure shows the differences in the mean Aβ levels compared to the reference genotype, adjusting for additional covariates. The findings remained significant for the relation of AGT with Aβ42 and Aβn42. Regarding the Aβ40 levels and the Aβ42/Aβ40 ratio there was no significant trend (A). After adjusting, no association was seen between AGTR1 and plasma Aβ (B) and the association of ADD1 with Aβn40 became borderline significant (p-value 0.06) (C).

Discussion

The present study shows that individuals with the AGT-M235T TT-genotype have significantly higher levels of plasma Aβ42 and Aβn42. There was also some evidence, although not significant after adjusting, that individuals with GT/TT genotypes of the ADD1-Gly460Trp variant have lower levels of Aβn40, but there was no association between AGTR1 and Aβ. APOE was correlated to a lower Aβ42/Aβ40 ratio.

Before interpreting the findings, a few methodological issues need to be addressed. First, we did not do a formal Bonferroni correction of the threshold p-value, because the Aβ outcomes were all related (Table 2). Correcting the p-value for the number of association analyses (Table 3; N=15) would therefore have been a too conservative approach. Alternatively, when using for example the 3 genes and 2 Aβ outcomes (Aβ40 and Aβ42), the p-value observed for AGT would be below the 0.0083 threshold that is then obtained.

Second, the sample size of the current population was relatively small. In this respect our finding that APOE was correlated to a lower Aβ42/Aβ40 ratio is important, serving as a proof of principle. However, also the allele distributions are important when considering the power of the study. Because some genotypes were less frequent than APOE ε4, the small sample size may explain that the major differences in mean Aβ levels between the genotype groups of AGTR1 and ADD1 that were observed were not significant. For example, large differences in the mean Aβ40 levels were observed for AGT and ADD1 (Figure 1) and for ADD1 large differences were observed for Aβn40, but none of the differences reached significance. Because these relationships do have biological plausibility, replication of our findings in larger datasets is warranted.

It is also important to realize that the associations of plasma Aβ levels with dementia have been inconsistent. Increased risk has been reported for high plasma levels of both Aβ40 and Aβ42, but also decreased risk for high levels of Aβ42 have been found [33,34,41-45]. An increased risk with higher levels of plasma Aβ42 has also been reported for cognitive decline [43]. One of the explanations for these discrepancies may be that plasma Aβ levels increase with aging, but not necessarily with disease progression [44]. It is thought that with disease progression Aβ42 is increasingly deposited in the brain and levels are consequently lower in plasma. In a young population with an increased

risk of AD pathology like ours, higher levels of Aβ42 could then be expected in persons carrying risk genotypes. The age of the study population and timing of Aβ measurements may be important contributors to the inconsistencies in literature.

From a biological perspective it is of interest that we found higher levels of plasma Aβ42 in persons with the TT-genotype of AGT-M235T. This genotype was previously described to increase the risk of cerebrovascular small vessel disease [23]. Previous studies have also reported a role in large vessel disease, but a recent meta-analysis did not confirm these findings [46]. This may imply that AGT exerts its effects via an independent brain RAS system [47] and not through increased levels of AGT II affecting atherogenic processes [48]. Not many groups have studied this gene in relation to dementia, and the findings are not convincing [49,50] with possibly an increased risk in patients with the AGT-M235T TT-genotype [51]. The RAS proteins have been implicated in AD pathology, because receptors for angiotensin II are present in brain tissue and RAS is activated in AD brains [9,10]. Along with cerebrovascular effects, RAS could thus have a direct effect on brain pathology and possibly on Aβ. Indirect evidence is given by the association of ACE, another gene involved in this system, with AD [11,12] and the role of ACE in the degradation of Aβ in the brain [13,14]. It has been shown that inhibition of ACE activity results in increased Aβ deposition in the brain [15]. Like RAS, alfa-adducin also plays a role in the brain [25,26]. Our results are suggestive for an association of the variant allele of ADD1-Gly460Trp to lower levels of Aβ40, but these findings should be interpreted with caution as they were not significant when adjusting for multiple testing using Bonferroni correction. Whereas it is an interesting candidate for AD pathology [26], the evidence was found in animal studies and no human studies have been conducted to our knowledge. The role of ADD1 in AD amyloid pathology is not known and as mentioned, inconsistent associations have been reported between plasma Aβ40 and dementia. Our findings therefore warrant further study.

Alternative explanations of our findings are possible. Aβ may have a direct effect on brain vasculature and vascular damage may have contributed to our findings, since the studied variants are all implicated in atherogenic effects [52]. Associations have been reported for Aβ with cerebral small vessel disease [53,54] and vascular dysfunction has been suggested in AD pathology [55]. On the other hand, damage to cerebral small vessels due to other processes may result in leakage of Aβ causing higher levels of circulating Aβ. We found moderate correlations between plasma Aβ and cardiovascular factors, which have been shown to be highly correlated to cerebral small vessel disease, and adjusting for these factors did not affect our main finding. The studied variants and Aβ are also both correlated to renal function, however, adjusting for creatinine as a measure for kidney function did not alter our findings.

The strength of our study is the family-based design, performed in a homogenous group of middle-aged individuals with hypertension derived from a genetically isolated population, which increases statistical power of genetic association studies. The size of the study is limited, however, which

increases the chance of false-positive and -negative findings. In sum, we found a consistent association of the AGT-M235T TT-genotype to plasma Aβ42 and Aβn42. A question that remains to be answered in longitudinal data is the role of AGT and ADD1 in midlife plasma Aβ levels and the effect of changing Aβ levels on AD pathology.

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Part V

General discussion and summary

11 Findings of this thesis

In this thesis I describe my research on genetic determinants of cognitive function and age-related brain changes. I have used outcomes that are highly heritable as endophenotypes for my studies of Alzheimer's disease, including cognitive function, Aβ plasma levels and age-related brain changes as visible on magnetic resonance imaging (MRI). Different study-designs were chosen to investigate our research questions including candidate gene studies, genome wide linkage analysis and genome wide association studies. In the following chapter, I will discuss the main findings of this thesis.

One of the most extensively studied candidate gene in Alzheimer's disease is the apolipoprotein E gene (APOE) [1-3]. The ε4 allele of this gene is a well-established determinant of AD with a large effect on disease risk. Based on the hypothesis that cognitive function may be a relevant endophenotype for AD, we studied the relation between APOE and cognitive function in **chapter 3**. We found that the APOE*ε4 allele was significantly associated with lower test scores on the Adult Verbal Learning Test in individuals older than 50 years of age. This effect of APOE*ε4 was independent of the effect of APOE*ε4 on vascular risk factors and most pronounced on learning ability. Similar to the findings of others [4], we found that the APOE*ε4 allele has an effect on cognitive function, but that in contrast to AD the effect is relatively small. We focused our gene discovery studies on cognitive function, since this outcome showed the most consistent association to APOE [4] and may therefore be the most promising endophenotype.

To explore new susceptibility regions for cognitive functioning without prior assumptions of pathways involved, we conducted a hypothesis-free genome-wide search on a range of cognitive tests. In **chapter 4** we present the findings of a non-parametric linkage analyses in the Erasmus Rucphen Family (ERF) Study, which is a family-based study in a genetically isolated population. Since we were targeting genes with a major effect, we selected individuals from the lower extremes of the trait distribution for the linkage analysis. Thresholds for significant and suggestive linkage were estimated by a simulation study. Significant linkage (LOD > 3.78) to cognitive functioning was found on chromosomes 1p13.1, 12q24.33, 19q13.43, 20p13, 21q22.13 and 21q22.3. For the fine-mapping of the region, we used dense genotyping in the regions under the linkage peak in ERF and replicated these findings in a large outbred, population-based cohort, the Rotterdam Study (RS) [5]. Fine-mapping showed significant associations to chromosome 1 (p-value=0.03) and 21 (p-value=0.01) after correction for multiple testing, and association with the latter region on 21q22.13 was replicated in the Rotterdam Study (nominal p-value 0.003). Both fine-mapping and replication pointed to variants within the potassium inwardly-rectifying channel, subfamily J, member 6 gene (KCNJ6).

Whereas linkage analysis in the extremes of the distribution specifically targets variants with larger effects, we conducted a genome-wide association study of cognitive function as a continuous outcome in search of common variants with small effects. In **chapter 5** we describe a meta-analysis of different

genome-wide association studies performed in the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium. This consortium includes large prospective population-based cohorts. Neuropsychological testing was available for 13 cohorts. In this thesis, we focussed on executive function and processing speed tasks including the Trail Making Test (TMT) parts A and B and the Stroop Color and Word Test in the analyses. All individual studies used their genotyped data to impute to 2.5 million single nucleotide polymorphisms (SNPs). The analyses were performed in Caucasians older than 45 years who were free of dementia and clinical stroke at times of cognitive testing. The most significant finding was found with TMT-B and a SNP on chromosome 18. This SNP was just above the genome-wide significant threshold with a p-value of 6.95*10-8 and located between two plausible candidate genes. We further conducted an exploratory analysis in which we searched for overlap between our findings and the genome-wide association analyses published for AD and schizophrenia. Overlap with previous genome-wide association studies was found for multiple other SNPs with a p-value smaller than 1.0*10-3, of which the sortilin-related-receptor-1 (SORL1), the syntaxin-binding-protein-6 (STXBP6) and the protocadherin-9 (PCDH9) genes are the most interesting genes. The genes in the regions that we identified in this study may provide further insights into the pathways involved in the normal variation of cognition. Our findings, however, await replication, which is currently ongoing.

A preliminary comparison between the findings of the genome-wide linkage and association analysis suggests no overlap in genes, which may be expected in light of the mechanisms underlying the methods. Linkage is designed to target rare variants with large effects and association on the other hand is designed to find common variants with moderate effects.
Of interest is also that we did not find evidence for a role of APOE, or the recently discovered AD genes, PICALM, CRI and CLU [11,12] in cognitive function in our genome-wide association analyses. This finding reveals, again as expected, that findings on endophenotypes cannot be translated 1:1 to the disease of interest. Another issue to realize is that tests assess different aspects of cognitive function. Indeed we found that APOE was associated to the Adult Verbal Learning Test in chapter 3 but non-significantly to TMT-A, B or Stroop.

Having studied cognitive function as endophenotype, we further studied age-related brain changes as a second group of endophenotypes. We considered plasma Aβ levels as biomarkers for the presence of senile plaques and amyloid angiopathy, and asymptomatic brain lesions on MRI as age-related brain changes. We have focused on lacunar infarcts, white matter lesions (WML), microbleeds and hippocampal atrophy. All are associated with hypertension, stroke, dementia and cognitive impairment [13-18], and are also found in healthy elderly.

We examined the role of candidate genes involved in blood pressure regulation and in amyloid metabolism. We studied APOE, the renin-angiotensin system (RAS) related genes (Angiotensin, Angiotensin II type 1 Receptor, alpha-Adducin) and the sortilin-related receptor (SORL1) gene.
RAS genes are involved in the regulation of blood pressure and salt homeostasis and the RAS proteins have also been implicated in Alzheimer's disease [19]. Receptors for angiotensin II are present in brain tissue [20] and an increased activation of RAS is seen in AD brains [21]. As already mentioned, APOE has consistently been associated with AD and there is increasing evidence that also SORL1 is associated with AD [4,22,23]. SORL1 consists of two functional regions, one functioning in the cholesterol pathway and the other in the APP processing pathway [24,25]. Interestingly, the gene has also been associated to cerebrovascular disease in a previous study [26] and also emerged in our comparative analysis in the genome-wide association study (**chapter 5**).

First, we studied all five variants in relation to the MRI endophenotypes: volumes of WML and presence of lacunes and microbleeds in a subgroup of the ERF study aged 55 and 75 years with hypertension (**chapter 8**). WML was present in variable severity in all participants, whereas lacunar infarcts were present in 15.5% and microbleeds in 23.3%. Homozygosity for the APOE ε4 allele was associated with lacunes (OR, 4.8; 95% CI, 1.2-19.3). Individuals carrying two copies of the variant allele of 4 SNPs located at the 3'-end of SORL1 (rs1699102, rs3824968, rs2282649, rs1010159), had an increased risk of microbleeds (highest odds ratio, 6.87; 95% CI, 1.78-26.44), which is suggestive for the hypothesis that the amyloid cascade is involved in the etiology of microbleeds in populations with hypertension.

Second, in **chapter 9** we studied SORL1 in relation to hippocampal volume and plasma Aβ levels in the same subgroup of the ERF study. Hippocampal volumes were quantitatively measured on MRI and plasma Aβ levels were determined in non-fasting blood samples. We studied the effect of 7 variants within SORL1 that were previously reported in AD. Three variants located near the 3'-end of SORL1 were significantly associated to hippocampal volume. The 3-SNP haplotypes for rs1699102, rs3824968 and rs2282649 (CAT) and for rs3824968, rs2282649 and rs1010159 (ATC) were associated to higher hippocampal volumes when adjusting for multiple testing. We did not find significant associations of single variants with plasma Aβ levels.

Third, we studied the association of the three variants within the angiotensin, angiotensin II type 1 receptor and adducin genes (AGT-M235T, AGTR1-C573T and ADD1-Gly460Trp) in the same middle-aged hypertensive subset of ERF. Variants in these genes were previously reported in cerebro- and cardiovascular disease in relation to circulating levels of plasma Aβ (**chapter 10**). The AGT-M235T TT-genotype was significantly associated with higher levels of plasma Aβ42 (p=0.008) and truncated Aβn42 (p=0.02). The association to Aβ42 remained significant after adjusting for potential

confounders and multiple testing. No significant associations were found between AGTR1-C573T or ADD1-Gly460Trp and plasma Aβ.

Taken together, the most interesting finding of our studies may be the associations that were found for SORL1 in various study designs. Our candidate gene analyses showed association of SORL1 with cognition as well as microbleeds and hippocampal volume. SORL1 also emerged in our genome-wide association meta-analyses of cognitive function. A word of caution is, however, needed: our candidate gene studies were performed in a small sample size and were restricted to hypertensive individuals. These findings therefore need replication in larger cohorts in the general population.

Finally, we conducted two candidate gene studies in Alzheimer's disease to elucidate the role of two interesting pathways. Iron overload may contribute to the risk of Alzheimer's disease. We earlier have studied the genes implicated in hemochromatosis in relation to AD [27]. We found an effect of the hemochromatosis gene (HFE) on the age of onset of AD. The HFE-63D mutation was related to an earlier onset in APOE*ε4 carriers, but not to the disease risk. Other groups reported evidence in other variants in hemochromatosis genes HFE-C282Y and -H63D, and transferrin (TF) [22]. In the Epistasis Project, with 1757 AD cases and 6295 controls, we studied four variants in two genes of iron metabolism: HFE-C282Y and -H63D, and TF-C2 and -2G/A (**chapter 7**). We replicated the interactive effect between HFE-282Y and TF-C2 on the risk of AD in Northern Europeans. We also found an interaction between HFE-63HH and TF-2AA, which was markedly modified by age. The interaction between HFE-282Y and TF-C2 has now been replicated twice, in a total of 2313 cases of AD and 7065 control. There are a number of limitations of this study that hamper firm conclusions.

First, both interactions were found mainly or only in Northern Europeans. In fact, there was an absence of a relation between HFE and AD in a Northern Spanish population. From a statistical perspective, the exclusion of the Spanish data is problematic. Although the allele frequencies in Northern Spain differed from those in the Northern Europeans, this does not imply that the relation to AD should be different. A second problem is that although we pooled the data, the numbers are small and as a consequence the study power is low, making the analysis susceptible to false positive findings.

We also studied the Cathepsin D gene (CTSD) in relation to AD (**chapter 6**). CTSD is involved in amyloid precursor protein processing and is therefore considered a candidate for AD. We performed a candidate-gene analysis in the Rotterdam Study, which is a population-based cohort-study (N=7983) and estimated the effect of CTSD variants on the risk of AD. Additionally, we performed a large meta-analysis incorporating our data and previously published data. The T-allele of CTSD rs17571 was associated with an increased risk of AD (p-value 0.007) in the Rotterdam Study. This association was predominantly found in APOE ε4 noncarriers. A meta-analysis of previously published data showed

a significantly increased risk of AD in carriers of the T-allele of rs17571 (OR 1.22, 95% CI 1.03-1.44), irrespective of APOE ε4 carrier status.

Besides these genetic studies, in **chapter 2** we also performed a classical epidemiological study in which we studied a combination of cardiovascular risk factors as composed in the metabolic syndrome (MetS) in relation to cognition. While type 2 diabetes is known to be associated with poorer cognitive performance [28,29], fewer studies have reported on the association of MetS and contributing factors, such as insulin-resistance (HOMA-IR), low adiponectin-, and high C-reactive protein (CRP)- levels [30,31]. We studied whether these factors are related to cognitive function and which of the MetS components are independently associated. Also this study was performed in the ERF study where extensive data on physical examination, biomedical measurements and neuropsychological testing were available. Linear regression models were used to determine the association between MetS, HOMA-IR, adiponectin levels, CRP, and cognitive test scores. We found that predominantly women with MetS and high HOMA-IR had lower scores on executive function tests (p=0.03 and p=0.009). The most consistent individual component of MetS, contributing to the association with executive test scores was systolic blood pressure. We interpret these results with caution, however, since the design was cross-sectional and with very strict multiple testing adjustment using Bonferroni would result in only borderline significant p-values. Longitudinal studies will be needed to gain insight in the causality of our reported findings and may result in more conclusive findings.

A huge challenge in genetic and epidemiological research, especially in the candidate gene studies and an exploratory study such as we conducted for MetS, is how to improve the quality and validation of candidate genes in AD. This will be discussed in the next chapter.

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12 Grading the credibility of genetic associations in Alzheimer's disease using the Venice criteria: practiccal considerations following from the Alzgene database

Abstract

Background

The epidemiological credibility of genetic associations in Alzheimer's disease (AD) in the 'top list' of the Alzgene database is graded using the so-called Venice criteria. We aimed to evaluate the robustness of these criteria

Materials and Methods

Hypothetical results from simulated studies were added to the meta-analyses of these associations that were graded with strong credibility or with moderate or weak credibility due to inconsistency of replication (high between-study heterogeneity (I2)) or low summary odds ratio ($0.87 < OR < 1.15$). Robustness was quantified as the sample size needed to change the grading.

Results

For 5 out of 8 associations graded with strong credibility, the grade changed to weak credibility because of small summary OR after the addition of studies with effects similar to the lowest/highest published OR and sample sizes ranging from 80 to 2000 . For 4 out of 8, new studies could introduce large I2 when their sample sizes were 400 to 1600. These associations ended up with a small summary OR and one became non-significant. Two out of four associations graded with moderate and weak evidence because of I2 >25%, could not become strong evidence because of one outlier in each analysis. Finally, associations with weak credibility due to small OR only became non-significant when new studies with no effect had sample sizes ranging over 3400 to 6600.

Conclusion

The Venice criteria are very helpful criteria to grade the credibility of genetic associations, but its practical usefulness may be limited due to outliers and small effects. Further guidance is needed on how to deal with these situations.

Introduction

Unraveling the genetic basis of Alzheimer's Disease (AD) has proven a major challenge. After the discovery of the three genes that cause Mendelian forms of the disease (APP, PSEN1 and PSEN2) and the major susceptibility gene Apolipoprotein E (APOE) there have been no further major breakthroughs. Also, the first large-scale genome-wide association studies (GWAS) did not identify loci other than APOE [1-4], but recent large consortia meta-analyses discovered several new loci [5,6], including CR1 on chromosome 1, CLU on chromosome 8 and PICALM on chromosome 11.

One of the reasons for the absence of gene discoveries is that many genetic association studies in AD were too small to detect low-risk susceptibility variants. Meta-analyses combining the results of multiple small studies are therefore more powerful and necessary approaches for the identification

of AD susceptibility genes. To facilitate these meta-analyses, the online Alzgene database was constructed (www.alzgene.org). This database currently includes 1,236 studies covering almost 600 genes and 2,400 polymorphisms (accessed 1th December 2009) [7].

It is likely that not all genetic associations reported are true associations, because some meta-analyses were rather small in sample size and some associations were only borderline significant. Distinguishing true from false positive findings is one of the greatest challenges in genetic epidemiology [8,9]. Recently, new guidelines were developed by a consensus workshop of the Human Genome Epidemiology Network for grading the epidemiological strength of cumulative evidence on genetic associations [10]. These so-called Venice criteria have recently been implemented in the Alzgene database to grade the credibility of epidemiological evidence for the genetic associations. Alzgene reports a 'top list' of 35 significant genetic associations (Table 1).

The Venice criteria value genetic associations based on 1. the amount of evidence; 2. consistency of replication and 3. protection from bias. Significant meta-analyses in large populations with little heterogeneity between the results of the individual studies and without evidence for bias are considered strong evidence for genetic association, whereas any violations to these three criteria result in moderate or weak evidence.

The Venice criteria are very useful to highlight quality differences between meta-analyses, but they may face limitations when applied too strictly. First, the criteria suggest that genetic associations with moderate or weak evidence can only be valued higher and that genetic associations with strong evidence cannot change to moderate or weak evidence after updating the analyses with newer studies. Second, according to the criteria genetic associations with small effect sizes (0.87< OR <1.15) are by definition graded as weak evidence because bias cannot be ruled out, except when they are investigated in consortia, which are considered protected from bias [11]. It is clear that a single study with a small OR has limited credibility, but when multiple large studies show the same small OR there may be less reason to consider these hampered by bias. Third, the criteria provide no guidance on how to handle outlying studies and subgroup analyses. The latter is particularly relevant with regard to genetic heterogeneity which may show genetic associations in some ethnic groups but not in an overall analysis. The aim of our study was to investigate the practical implications of these limitations. We evaluated the robustness of the Venice grades of genetic associations in AD by adding hypothetical results of simulated studies to the meta-analyses. The research questions that were addressed are: 1. what sample size is needed to change a grade of strong evidence to moderate evidence due to increased heterogeneity or to weak evidence due to small OR?; 2. what sample size is needed to change associations with weak evidence into non-significant associations?; 3. to what extent are grades of moderate or weak evidence due to high heterogeneity determined by the effect of a single study?

Materials and Methods

Venice criteria

The Venice criteria grade the strength of the epidemiological evidence for genetic associations taking account of the amount of evidence, consistency of replication and protection from bias [10]. For amount of evidence, associations receive an A when the combined number of cases and controls in the minor genetic group exceeds 1000, a B when the number is between 100 and 1000, and a C when it is below 100. For consistency of replication, the degree of between study heterogeneity in study results (I2) is considered. Point estimates of I2 below 25% receive an A, I2 between 25% and 50% receive a B and I2 exceeding 50% receive a C. A C is also given to non-significant associations. For protection from bias, the guidelines propose to consider potential sources of bias at the level of individual studies including errors in phenotypes, genotypes and confounding, and at the level of meta-analysis including publication and other selective reporting biases [10]. Associations receive an A when bias is not likely to affect the presence of the association, a B when there is no demonstrable bias, but important information is missing for its appraisal and a C when there is demonstrable clear or potential bias that had invalidated the association. Meta-analyses also receive an A when the OR deviates more than 1.15-fold from the null (>1.15 or <0.87). Strong epidemiological evidence for significant association was given to the meta-analyses that received three A's, moderate evidence to those that received any B, but not any C, and weak to those that received a C in any of the three criteria.

Alzgene database

The Alzgene database is a publicly available database of published genetic association studies in AD [7]. The studies are identified through systematic literature searches, which are continuously updated. The database includes studies that are published in English in peer-reviewed journals. Meta-analyses are conducted for associations that have been investigated in at least four independent samples. All genetic associations with a significant summary OR for at least one polymorphism are presented in a 'top list'. The epidemiological credibility of all loci in the top list is graded using the Venice criteria. The criteria are implemented as described above with the amount of evidence defined as the number of minor alleles.

Analyses

We performed meta-analyses on loci presented in the Alzgene's 'top list' (Table 1), that were graded with strong and moderate epidemiological credibility and on associations with weak epidemiological credibility due to high I2 or a small OR. We used data available from the Alzgene database [7], but excluded studies in which control genotypes were out of Hardy-Weinberg Equilibrium (HWE). To test the robustness of these grades, we updated the meta-analyses with results of simulated studies. Robustness was quantified as the sample needed to change the reported grades, which could be a

result of an increased I2, a smaller OR below the threshold of 1.15 or a non-significant summary OR. When the required sample size is large, substantial evidence is needed to change the credibility of the associations.

The meta-analyses were performed in R using the Rmeta library (www.r-project.org) using random effect models. The degree of heterogeneity between studies was assessed using the I2 statistic [12]. Fixed effects meta-analyses were used in the analysis of APOE with simulated data and in the meta-analysis with simulated data introducing high I2 in order to prevent adjustment of induced heterogeneity.

For the simulated data, we assumed that the allele frequencies of the controls were equal to the overall allele frequency in the meta-analysis from the Alzgene database. The allele frequencies in cases were calculated from the frequencies in controls and the OR, assuming HWE and a 50:50 case/control ratio. The betas were calculated by taking the natural logarithm of the OR and the standard errors were calculated by dividing the beta by the square root of the chisquare, which in turn was estimated from a contingency table of the observed and expected allele frequencies in cases and controls. Beta and standard error were then added to the meta-analysis. This was repeated with increasing sample sizes until an effect was seen on the summary OR or I2.

First, we addressed the robustness of associations graded with strong evidence by investigating the sample size needed to change this grade to moderate due to increased I2 or to weak due to small OR. To study the sample size needed to increase I2 >25%, we examined two scenarios in which we added a study with an effect similar to the lowest and highest OR observed in published studies. To study the sample size needed to change the summary OR towards the null, defined by the Venice criteria as $0.87 < OR < 1.15$ [10] or to non-significance, we added the same hypothetical studies as mentioned above. Additionally, we investigated a scenario where we added a hypothetical study that showed no effect (OR=1).

Next, we addressed the robustness of associations with moderate and weak evidence by investigating the sample size needed to reduce I2 or to get a non-significant summary OR.

For I2, we studied the addition of simulated data with an effect similar to the current published summary OR. We investigated two scenarios, adding a single study and adding multiple studies with a sample size of 2000. For an effect on the summary OR, we examined whether the small effect size remained significant using two scenarios in which we added a study with no effect and a study with an effect similar to the lowest/highest OR observed in published studies.

Finally, we studied whether high I2 in associations with moderate and weak evidence were due to the effect of one single study by removing one study at a time.

Top findings of genetic associations with Alzheimer's disease listed at Alzgene.org

#	Gene	Ethnicity	Polymorphism	N minor (Grade)	I2 (Grade)	Bias Reason (Grade)	Overall Grade	OR	Number of studies
1	**APOE (ε2/3/4)**	Caucasian	apoe ε2/3/4	3525 (A)	0 (A)	(A)	Strong	3.81 (3.38-4.29)	28
2	**CLU**	All	rs11136000	20271 (A)	0 (A)	(A)	Strong	0.85 (0.82-0.89)	8
3	**PICALM**	All	rs541458	13335 (A)	0 (A)	(A)	Strong	0.87 (0.83-0.91)	6
4	**TNK1**	All	rs1554948	5343 (A)	9 (A)	(A)	Strong	0.86 (0.80-0.93)	6
5	**ACE**	Caucasian	rs1800764	1371 (A)	0 (A)	(A)	Strong	0.79 (0.68-0.92)	4
6	**TFAM**	All	rs2306604	1604 (A)	0 (A)	(A)	Strong	0.82 (0.72-0.94)	5
7	**CST3**	Caucasian	rs1064039	1203 (A)	4 (A)	(A)	Strong	1.16 (1.00-1.13)	8
8	**IL1B**	Caucasian	rs1143634	1206 (A)	0 (A)	(A)	Strong	1.18 (1.04-1.39)	5
9	**CR1**	All	rs6656401	6653 (A)	44 (B)	(A)	Moderate	1.19 (1.09-1.28)	7
10	hCG2039140	All	rs1903908	768 (B)	0 (A)	(A)	Moderate	1.23 (1.06-1.44)	4
11	SORL1	Caucasian	rs12285364	680 (B)	7 (A)	(A)	Moderate	1.26 (1.06-1.49)	9
12	CHRNB2	All	rs4845378	227 (B)	0 (A)	(A)	Moderate	0.67 (0.50-0.90)	4
13	SORCS1	All	rs600879	567 (B)	0 (A)	(A)	Moderate	1.24 (1.04-1.48)	4
14	DAPK1	All	rs4878104	4219 (A)	0 (A)	Low OR, Regr (C)	Weak	0.88 (0.82-0.95)	7
15	PRNP	Caucasian	rs1799990	3521 (A)	7 (A)	Low OR, HWE (C)	Weak	0.91 (0.83-0.99)	10
16	**MTHFR**	All	rs1801133	8120 (A)	22 (A)	Low OR (C)	Weak	1.13 (1.04-1.24)	25
17	**GAB2**	Caucasian	rs10793294	1711 (A)	78 (C)	(A)	Weak	0.69 (0.54-0.88)	5
18	**LOC651924**	All	rs6907175	5072 (A)	3 (A)	Low OR (C)	Weak	0.89 (0.82-0.96)	6
19	**GWA_14q32.13**	All	rs11622883	5031 (A)	35 (B)	Low OR (C)	Weak	0.88 (0.80-0.97)	6
20	**BDNF**	Caucasian	rs6265	4145 (A)	0 (A)	Low OR (C)	Weak	1.09 (1.02-1.17)	16
21	NEDD9	All	rs760678	5336 (A)	39 (B)	Low OR, Regr (C)	Weak	0.89 (0.81-0.97)	8
22	CH25H	All	rs13500	729 (B)	65 (C)	Regr (C)	Weak	1.44 (1.08-1.93)	7
23	**IL1A**	Caucasian	rs1800587	4749 (A)	32 (B)	Low OR (C)	Weak	1.09 (1.00-1.19)	18
24	TF	All	rs1049296	2824 (A)	30 (B)	Regr (C)	Weak	1.18 (1.04-1.33)	14
25	LOC439999	All	rs498055	5288 (A)	49 (B)	F, HWE (C)	Weak	1.15 (1.03-1.29)	7
26	CALHM1	Caucasian	rs2986017	4523 (A)	68 (C)	F (C)	Weak	1.18 (1.03-1.35)	10
27	TNF	All	rs4647198	1515 (A)	0 (A)	F, HWE (C)	Weak	1.35 (1.04-1.76)	4
28	PGBD1	All	rs3800324	550 (B)	0 (A)	Regr, F (C)	Weak	1.21 (1.02-1.24)	7
29	THRA	All	rs939348	3248 (A)	0 (A)	Low OR, Regr (C)	Weak	1.10 (1.01-1.19)	6
30	ENTPD7	All	rs911541	2241 (A)	1 (A)	Low OR, F (C)	Weak	1.10 (1.01-1.21)	4
31	IL33	All	rs11792633	5896 (A)	63 (C)	F (C)	Weak	0.84 (0.72-0.99)	4
32	GAPDHS	All	rs4806173	2623 (A)	51 (C)	F (C)	Weak	0.87 (0.75-1.00)	4
33	OTC	All	rs5963409	375 (B)	18 (A)	F (C)	Weak	X-chromosomal	10
34	GALP	All	rs3745833	4132 (A)	62 (C)	Low OR, Regr (C)	Weak	1.13 (1.00-1.29)	6
35	PSEN1	All	rs165932	13867 (A)	54 (C)	Low OR, HWE (C)	Weak	0.92 (0.86-1.00)	43

Derived from www.alzgene.org; accessed 1st Dec 2009. In bold: the associations that were studied in the current study.
Grade: based on Venice criteria [10]. OR: odds ratio, Regr: modified regression test to assess positive publication bias, HWE: deviations from Hardy-Weinberg Equilibrium, F: exclusion of firs
study diminishes the association

Table1

Results

Descriptives

Of the 35 significant associations presented in Table 1, eight were graded with strong evidence for association, five with moderate evidence and 22 with weak evidence for association. The 8 associations with strong evidence included 3 loci identified in GWAS and 5 loci identified in meta-analyses of candidate gene studies. Figure 1A and 1B show that the effect sizes of the polymorphisms that were graded with strong evidence remained unchanged when the number of minor alleles was higher than 1,000. With the exception of APOE, most ORs were close to the 1.15 threshold specified in the Venice criteria.

There were only two reasons why genes were graded with moderate evidence. Four out of five genetic associations had insufficient amount of data and the fifth, CR1, had high I2. Of the 22 associations that were graded with weak evidence, three did not have a sufficient amount of data, and 19 had high I2, small OR or presence of biases. The associations graded with weak evidence due to HWE deviations, TNF, LOC439999, PRNP and PSEN1 were no longer significant after the studies in which controls were out of HWE were removed from the meta-analysis. Five associations were graded with

weak evidence only because of high I2 or small OR. Figure 2 shows that the cumulative OR of CR1 did not change in time, but the cumulative OR of GAB2 did. The cumulative ORs for LOC651924, GWA_14q32.13, BDNF and Il1A fluctuated around a stable value (0.89 and 1.09) after the number of minor alleles reached 2500.

Sample sizes needed to obtain an I^2 higher than 25% for loci that are graded with strong evidence of association

Gene	Range of published OR	When OR=lowest* Sample size	When OR=highest* Sample size
CLU	0.76-0.93	4400	10 000
PICALM	0.83-0.95	>1 000 000	6000
TNK1	0.76-1.05	1,600	520
TFAM	0.60-1.00	400	1600
ACE	0.74-0.84	>1 000 000	>1 000 000
CST3	0.88-1.60	620	500
IL1B	1.09-1.64	>1 000 000	560

OR range: minimum and maximum odds ratio available from published studies included

Table 2

Robustness of strong evidence

Table 2 shows that for ACE it was impossible to change its strong evidence to moderate evidence because of increased I2, because I2 could not increase over 25%. For PICALM and IL1B it was only possible when the study had the same effect as the published outlier and for CLU it was only possible with relatively large sample sizes. These findings did not change when we added multiple studies with moderate sample sizes (N=2000 each) instead of one single large study (data not shown).For TNK1, TFAM and CST3 even small sample sizes could increase I2 and change the grading from strong to moderate. In addition, the new summary OR became small (0.87 < OR <1.15) and for CST3 non-significant.

Samples size needed to obtain a summary OR lower than 1.15 or non-significant result for loci that are graded with strong evidence of association

	Published			*Small OR*		*Non-significance*
Gene	OR	95% CI	OR range	When OR=1 Sample size	When OR=lowest/highest* Sample size	When OR=lowest/highest* Sample size
APOE4	3.83	3.30-4.45	2.17-9.93	39000	Not Possible	Not Possible
CLU	0.86	0.82-0.89	0.76-0.93	4000	9000	Not Possible
PICALM	0.87	0.83-0.91	0.83-0.95	600	2000	Not Possible
TNK1	0.86	0.80-0.93	0.76-1.05	800	600	6000
TFAM	0.82	0.72-0.94	0.60-1.00	1000	1000	2500
ACE	0.79	0.68-0.92	0.74-0.84	1200	Not Possible	Not Possible
CST3	1.15	1.01-1.33	0.88-1.60	160	80	150
IL1B	1.19	1.04-1.36	1.09-1.64	600	1500	Not Possible

OR: summary odds ratio of meta-analysis; 95% CI: 95% confidence interval; OR range: minimum and maximum odds ratio from published studies included in the meta-analyses listed in Table 1. Sample size: number of cases and controls (50:50);
* OR of the added hypothetical studies was the lowest/highest of the range of published OR.

Table 3

Table 3 shows that a small study could lower the summary OR below the threshold of 1.15, except for APOE, CLU and ACE. For APOE, only a very large study with no effect could lower the summary OR below the threshold, while for CLU and ACE a smaller study could. Yet for all three genes, an OR of 1 is outside the range of published ORs. For more realistic OR values, it was impossible to lower the summary OR of APOE and ACE and required a large study for CLU. For the other 5 genes, studies with 80 to 2000 participants were sufficient to lower the summary OR and change the grading from strong to weak. Three associations, TNK1, TFAM and CST3, became non-significant.

Robustness of moderate and weak evidence

Of the studies that were graded with moderate or weak evidence because of high I2, for 2 associations (CR1 and GAB2), it was impossible to reduce I2 below 25% with the addition of one study, but I2 could be reduced by the addition of 8 studies each with a sample size of 2000 (Table 4). For the other 2, GWA14q32.13 and IL1A, small sample sizes of only 200 participants were sufficient to reduce I2. Table 5 shows that for LOC651924, GWA_14q32.13, BDNF, IL1A and MTHFR, a sample size between 3400 and 6600 was needed to obtain a non-significant meta-analysis result after adding a study in which OR=1. A non-significant meta-analysis could be obtained at smaller sample sizes with the addition of studies with an effect size similar to the published ORs, however, for BDNF and IL1A these ORs were major outliers.

Sample sizes needed to obtain an I^2 lower than 25% for loci that are graded with moderate or weak evidence because of high I^2

	Published			When adding one study*	When adding multiple studies (N=2000)*
Gene	OR	95% CI	OR Range	Sample size	Studies added
CR1	1.18	1.07-1.29	0.99-1.29	>1 000 000	8
GAB2	0.64	0.47-0.86	0.51-0.91	>1 000 000	8
GWA14q32.13	0.89	0.80-0.97	0.76-1.00	200	Not relevant
IL1A	1.09	1.02-1.18	0.71-2.33	200	Not relevant

OR: summary odds ratio of meta-analysis; Sample size: number of cases and controls (50:50);
* OR of the added hypothetical studies was the summary published OR

Table 4

Sample size needed to obtain a non-significant OR for loci that are graded with weak evidence because of small effect size

	Published			When OR=1	When OR=lowest/highest*
Gene	OR	95% CI	OR Range	Sample size	Sample size
LOC651924	0.89	0.82-0.96	0.78-0.99	6000	6200
GWA_14q32.13	0.89	0.80-0.97	0.76-1.00	5600	5600
BDNF	1.09	1.02-1.17	0.66-1.90	6200	400
IL1A	1.09	1.02-1.18	0.71-2.33	3400	200
MTHFR	1.12	1.03-1.22	0.88-1.97	6600	1200

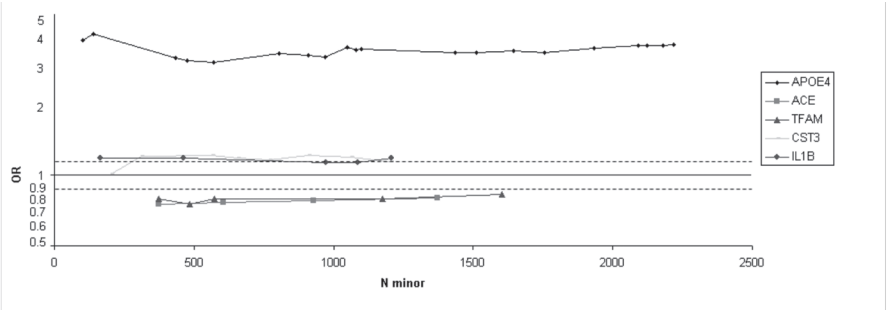
OR: summary odds ratio of meta-analysis; 95% CI: 95% confidence interval; OR range: minimum and maximum odds ratio available from published data on Alzgene Sample size: number of cases and controls (50:50).
* OR of the added hypothetical studies was the lowest/highest of the range of published OR.

Table 5

Finally, we investigated whether high I of associations with moderate or weak grades could be attributed to one single study. For CR1 and GAB2, the high I2 was indeed due to one contributing study, which showed an opposite direction of effect. Excluding the respective study from the meta-analysis reduced the heterogeneity from 48% to 0% for CR1 and from 78% to 0% for GAB2. The

summary OR also became stronger with less wide 95% confidence intervals. For the two other associations that were graded with weak evidence, the grading did not change when only one study was removed at the time.

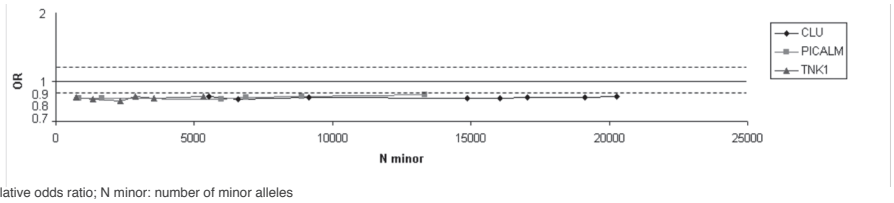
Cumulative association of loci with strong evidence for association with AD from candidate gene studies



OR: cumulative odds ratio; N minor: number of minor alleles

Figure 1a

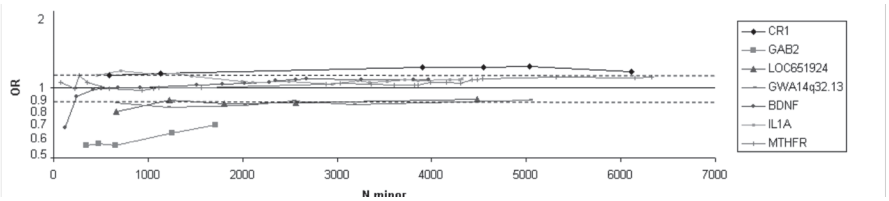
Cumulative association of loci with strong evidence for association with AD identified in genome wide association studies



OR: cumulative odds ratio; N minor: number of minor alleles

Figure 1b

Cumulative association of loci with moderate or weak evidence for association with AD



OR: cumulative odds ratio; N minor: number of minor alleles

Figure 2

Discussion

The Venice criteria are very useful in valuing genetic associations with AD. By assessing the sample size, heterogeneity and the presence of bias, the criteria identify potential short-comings in the data synthesis that have to be solved before one can rely on the associations found. However, our data show that there are some concerns and limitations. First, genetic associations that are graded with strong evidence can change to weak evidence when the analyses are updated with hypothetical, but realistic new data when the summary OR becomes smaller than the threshold of 1.15. Second, a number of genetic associations with small significant effect size seem robust, but would still be graded with weak evidence because of low OR. Third, for some genetic associations, the weak or moderate grade is determined by the effect of a single study with outlying effect.

The Venice criteria grade the strength of the epidemiological evidence for genetic associations taking account of the amount of evidence, consistency of replication and protection from bias [10]. The definition of the amount of evidence as defined in the criteria as the number in the minor genetic group could be specified more clearly. The Alzgene database is currently using the number in the minor allele group, which is obviously twice the size of the minor genotype group, which may as well be considered as the minor genetic group. The sample size is, however, a measurable estimate of the amount of evidence and the only debate could be the sample size that is used as a cut off, since it appears from our study and recent genome-wide association studies that larger sample sizes than 1000 are needed to find a robust estimate.

The consistency of replication as depicted in the I2, is also a measurable estimate of evidence. Our data show, however, that the associations with strong evidence can change to moderate evidence because the addition of future studies can result in an increased I2. Especially for associations that do not include large sample sizes (TNK1, TFAM and CST3) realistic sample sizes of only hundreds can cause an increased I2. Moreover, just one study can influence I2 and when removed associations graded with moderate and weak evidence can change to strong evidence, as is seen for CR1 and GAB2 in our analysis. Also, the grades can change with the addition of future studies causing I2 to decrease below 25%, which is what one could expect. Studies with sample sizes of only 200 could result in strong evidence for GWA14q32.13 and IL1A. Although our data show that a threshold of 25% is reasonable in real data, the application of a between-study heterogeneity threshold may be misleading. It should of course be taken into account when meta-analyzing genetic associations [13,14], but it may also reveal inconsistencies in study-designs rather than inconsistencies of genetic associations. We argue that when heterogeneity is present, finding genuine heterogeneity is very important, but using heterogeneity as a sole argument in grading genetic associations may not be sufficient [15]. Moreover, even in the presence of heterogeneity, if the direction of effect is the same among studies and only the effect sizes are different, the results may point to a true association. At least the latter will not affect the overall conclusion. Another argument is that most tests for heterogeneity lack power when sample sizes are small, resulting in relatively high estimates in large studies and

are therefore hard to interpret reliably [16,17]. It has been suggested by others to include confidence intervals of the heterogeneity measurement to better evaluate meta-analyses and including such boundaries in the criteria would be of additional value [12].

In addition to considering potential sources of bias on the level of individual studies and meta-analyses, the criteria also propose the use of an OR threshold. The underlying idea being that studies with small effects are more likely biased. This criterion results in weak grading for all genetic associations with an OR smaller than 1.15 (or greater than 0.87). Our results show (below), however, that a number of these associations are robust. Strong evidence is currently given to 8 genetic associations with AD. While the published OR-ranges of the majority of strong graded associations are close to 1, future studies can affect the grading. In the current study, 5 associations are graded with weak evidence after the addition of new data and 3 associations even become non-significant. Only for APOE, ACE, and CLU large (unrealistic) samples sizes are required. Weak evidence because of a small OR is given to 5 associations with AD, but our data show that the associations will only become non-significant with the addition of either very large sample sizes or studies with an unrealistic OR. Therefore these associations may be true findings, underlining the importance to consider genetic associations with small effects. Moreover, genes with small effects are expected in light of the results from recent GWAS [18].

A valuable addition to published meta-analyses would be performing cumulative meta-analyses and plotting the results can be a useful application [17]. One could expect that with time evidence will accumulate leading to a stabilizing either significant or non-significant association. There is no easy solution for tackling the robustness of genetic associations, but initiating large consortia or meta-analyze published data as we have shown, seem to be the most promising methods [11].

We limited ourselves to the Alzgene database and genes that were published in the top list at the 1st of December 2009 and meanwhile new associations have emerged. During the preparation of this manuscript there was an update of the Alzgene database. The new top list, however, reassures our conclusions rather than compromising them. In the top list there are 4 new associations graded with strong evidence. Three are new (SORL1, IL8, LDLR) and the other is GWA_14q32.13, which moved up the list due to exclusion of one single study resulting in a greater summary OR (0.84 instead of 0.89). The 3 new genes graded with strong evidence are small studies or show only a large OR in a specific ethnic group (SORL1), and these will likely change with the addition of future data. Two associations dropped from strong to weak evidence, just like we predicted. IL1B dropped because of the addition of one non-significant study (N=465, OR=0.98), resulting in a non-significant OR when excluding the studies out of HWE. TFAM dropped because of the addition of one non-significant study with an OR beyond the published OR range so far (N=485, OR=1.15), causing increased I2 and first study bias.

To summarize, the Venice criteria are useful criteria for the appreciation of meta-analyses of genetic associations. However, clarification is needed on how to handle the grading in the presence of outliers

and when genetic variants have small effects. Our study evaluates the Venice criteria specifically in AD, and although many findings could be extrapolated to other diseases, the criteria should be evaluated in many field synopses. With the emerging of new data, associations should be re- and meta-analyzed and guidelines should be re-evaluated [17].

In conclusion, associations with strong credibility could change with the addition of new future data and some associations with weak credibility due to small OR may have sufficient amount of evidence. The practical usefulness of the interim Venice criteria may therefore be limited and further guidance is needed on how to deal with these situations.

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13 Future research

Genetic epidemiologic research in neuroscience has many challenges and there are also many opportunities for research in the genomics era. A first step of research is genome-wide association study, which are present ongoing for many neurological diseases including stroke [1,2], Alzheimer's disease (AD) [3,4], frontal lobe dementia [5] and even for rare outcomes such as Creutzfeldt-Jakob disease [6]. Although these studies of stroke and AD have been successful and have revealed three new genes, the heritability of the disease is far from explained completely. The recent identified genes involved in AD, PICALM, CLU and CR1 contribute little to the discrimination of persons who will and will not develop disease (unpublished data). Age, sex and the apolipoprotein E (APOE) gene remain the main predictors for developing AD.

How to take this research further? There are ample opportunities for finding new genes by enlarging the data sets studied in terms of the number of patients included as well as the number of markers studied. The experience in other outcomes such as lipid levels and blood pressure is that enlarging the study sample still allows the identification of new genes with small effects. Also, preliminary studies show some improvement in findings using the data of the 1000 genomes as a base population for imputations instead of HAPMAP. The variation captured with HAPMAP is limited, because it is based on 60 individuals. Considering the successes of genome-wide association studies of quantitative outcomes (e.g. blood pressure), such studies have the potential to become more successful than those of binary outcomes (e.g. hypertension). Similarly, there are opportunities for genome-wide association of endophenotypes as cognitive functioning and brain imaging data such as hippocampal volumes, generalized brain atrophy and microbleeds.

In the near future, an important trend in neurogenetic research will be the formation of large consortia with samples sizes of thousands of participants. An important question that remains to be addressed is the genetic architecture underlying diseases such as AD and related disorders. Rare mutations have been implicated in the amyloid precursor protein (APP), and presenilin 1 (PSEN1) and 2 (PSEN2) genes in familial forms of AD. Further in sporadic AD, APOE is a common variant with a large effect while PICALM, CLU and CRI are common variants with small effects. The GWA studies conducted to date do not show evidence for common variants with effects comparable to that of APOE [3,4], whereas these studies had sufficient statistical power to reveal those. However, there are two types of variants for which the present studies were underpowered: 1. variants with small effect, similar or smaller than PICALM, CLU and CR1, and 2. rare variants with large effects. A first step that should be taken in the near future is to examine the evidence for a polygenic form of inheritance in which there are a large number of variants with very small effect covering the full genome. Recently, a new method to evaluate polygenic effects has been developed and applied successfully in schizophrenia and bipolar disorder [7].

High throughput sequencing offers opportunities to evaluate the presence of rare variants with large effects. The cost of high throughput sequencing is and will go down allowing the sequencing of larger groups of patients. However, data analysis is still a challenge. Based on our findings, the first regions of interest will be the sortilin-related receptor 1 gene (SORL1). Evidence is increasing for a role in Alzheimer's disease and cerebrovascular disease [8-10]. However, there are other regions for which there is consistent evidence for linkage in families and subsequent association that remain to be elucidated by deep sequencing including chromosome 10, chromosome 1 and chromosome 3 [11-14]. The latter region is identified in our own study. One may even argue that the chromosome 19 region, around APOE, requires further sequencing, as we and others have identified signals in genome wide association studies independent of APOE (unpublished data). Sequencing of the whole genome would also be of interest to target rare variants. The highest chances of success are probably to be expected in younger cohorts and family data. Studying rare variants would add to the field and may unravel new pathways involved in the disease. It goes without saying that it is of great importance to follow-up on the genome-wide results. Not only by performing replication studies and deep sequencing, but also by performing functional studies. Animal studies, for example can add much knowledge to the expression and function of newly discovered variants.

In future neuroscience research, genetic epidemiology has to explore whether alternative genetic mechanisms explain part of the missing heritability. Are there common or rare structural variants that explain part of the missing heritability? These have been implicated in various psychiatric outcomes and APP duplication has been seen in AD [15]. Another important question is whether there are epigenetic effects or post-translational modifications that are relevant for these disorders. Last but not least, the evidence for gene-interactions is to be explored. So far, no convincing evidence for gene-gene interaction has been found. Although technically it is possible to study interactions across the whole genome, there are still major computational problems to be resolved.

Gene-environment interactions have been Holy Grail in the field of genetic epidemiology. Large scale gene-environment interactions are within the scope of genetic epidemiological research. Although susceptible to false-positives, statistical approaches using robust standard errors have been developed and have enabled gene-interaction studies. However, in recent years there has been little progress in finding environmental risk factors that are consistently implicated in AD and related disorders. A point of consideration is how to develop the field of epidemiology to find consistent environmental risk factors. In the last few years, genetic epidemiology has been involved in genome-wide association studies. Large consortia were formed to improve the power to pinpoint SNPs that were replicated across different studies. To move the field of epidemiological research further, such large consortia should also be formed to target classical environmental risk factors. These consortia are thus not only needed to find new genetic risk factors, they are also needed to find consistent

evidence for environmental risk factors. Needless to say that there is an urge to find risk factors that can be used for early prevention of a devastating disease like Alzheimer's disease. The large number of non-replicable findings in epidemiology, however, has raised questions whether epidemiology is facing its limits. The lesson to be learned from genomic research: 1) target lower p-values as they have positive predictive values; 2) replicate findings before publication; 3) form large consortia to be able to meet 1 and 2.

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14 Nederlandse samenvatting en conclusies

Dit proefschrift beschrijft ons onderzoek naar genetische determinanten van cognitieve functie en aan veroudering gerelateerde hersenenveranderingen. Voor dit onderzoek hebben we gebruikt gemaakt van uitkomsten die in hoge mate erfelijk zijn en deze gebruikt als endofenotypes voor de studie van de ziekte van Alzheimer, met inbegrip van cognitieve functies, Aβ plasma waarden en aan veroudering gerelateerde hersenenveranderingen die zichtbaar zijn op magnetische resonantie beeldvorming (MRI) van de hersenen. Verschillende studie ontwerpen werden gekozen om onze onderzoeksvragen te benaderen, zoals kandidaat-gen, genoom-wijd linkage en genoom-wijd associatie onderzoek.

Eén van de meest bestudeerde kandidaat-genen in de ziekte van Alzheimer (AD) is het Apolipoproteïne E gen (APOE) [1-3]. Het ε4 allel van dit gen is een bekend en consistent gerapporteerde risicofactor voor AD. Gebaseerd op de hypothese dat cognitieve functies relevante endofenotypes zijn voor AD, bestudeerden we de relatie tussen APOE en cognitieve functies in **hoofdstuk 3**. We vonden dat het APOE*ε4 allel significant geassocieerd was met lagere testscores op de Adult Verbal Learning Test bij personen ouder dan 50 jaar. Dit effect van APOE*ε4 was onafhankelijk van het effect van APOE*ε4 op vasculaire risicofactoren en het meest uitgesproken op het leervermogen. Vergelijkbaar met de bevindingen van anderen [4], vonden we dat het APOE*ε4 allel een effect heeft op het cognitief functioneren, maar dat dit effect in tegenstelling tot AD relatief klein is. We hebben ons in de studies naar het ontdekken van nieuwe genen gericht op cognitieve functies, omdat deze uitkomsten de meest consistente associaties toonden met APOE [4], wat zou kunnen betekenen dat cognitief functioneren het meest belovende endofenotype is.

Om nieuwe genetische regio's voor cognitieve functioneren te onderzoeken zonder voorafgaande aannames van de onderliggende pathofysiologie hebben we een hypothese-vrije genoom-wijde studie verricht op verschillende cognitieve testen. In **hoofdstuk 4** presenteren we de bevindingen van een genoom-wijde linkage analyse in de Erasmus Rucphen Family (ERF) Studie, dat een familie-studie is in een genetisch geïsoleerde populatie. Aangezien we geïnteresseerd waren in genen met een groot effect, hebben we alleen personen met lage cognitieve scores geincludeerd deze linkage analyse. Afkapwaarden voor statistische significantie en suggestie werden bepaald met simulatie studies. Een significant resultaat (LOD> 3,78) werd gevonden met regio's op chromosoom 1p13.1, 12q24.33, 19q13.43, 20p13, 21q22.13 en 21q22.3. Voor het verder definiëren van genen in de regio's, analyseerden we de regio's onder de piek met dichtere genotypering en probeerden we deze bevindingen in een groot populatie cohort, de Rotterdam Studie, te repliceren [5]. Deze analyses resulteerden in significante associaties op chromosoom 1 (p-waarde = 0,03) en 21 (p-waarde = 0,01) in ERF en de laatstgenoemde regio op 21q22.13 werd gerepliceerd in de Rotterdam Studie (nominale p -waarde 0,003). Deze regio bevat het kalium kanaal familie J 6 gen (KCNJ6).

Linkage analyse in de laagste cognitieve scores is vooral gericht op het vinden van zeldzame genetische varianten met een groot effect. Om vaker voorkomende genetische varianten met een klein effect te vinden, verrichten we een genoom-wijde associatie studie op iedereen met cognitieve functie scores en daarbij gebruikten we deze scores als continue variabelen. In **hoofdstuk 5** beschrijven we een meta-analyse van verschillende genoom-wijde associatie studies uitgevoerd in de cohorten voor het Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium. Dit consortium bevat grote prospectieve bevolkings-cohorten. Neuropsychologische testen waren beschikbaar voor 13 cohorten. In dit proefschrift, hebben we ons gericht op executieve functies en snelheidstaken: de Trail Making Test (TMT) deel A en B en de Stroop Kleur-en Woord-Test. De analyses werden uitgevoerd in blanke deelnemers ouder dan 45 jaar, die geen dementie hadden en geen tekenen van herseninfarcten of –bloedingen ten tijde van de cognitieve testen. De belangrijkste bevinding werd gevonden met TMT-B en een SNP op chromosoom 18. Deze SNP had een p-waarde net boven de genoom-wijde significante afkapwaarde met een p-waarde van 6,95*10-8. Deze SNP is gelegen tussen twee plausibele kandidaat-genen. Wij hebben daarna een verkennende analyse uitgevoerd waarin we gezocht hebben naar overlap tussen onze bevindingen en andere genoom-wijde associatie analyses gepubliceerd voor AD en schizofrenie. Overlap met vorige genoom-wijde studies werd gevonden voor meerdere andere SNPs met een p-waarde kleiner dan 1,0*10-3, waarvan de sortilin-related-receptor-1 (SORL1), de syntaxin-bindend-eiwit-6 (STXBP6) en de protocadherine-9 (PCDH9) genen het meest interessant zijn. De genen in de regio's die in onze studies werden gevonden, kunnen nieuwe inzichten geven in de mechanismen onderliggend aan de normale variatie in het cognitief functioneren. Onze bevindingen moeten echter gerepliceerd worden, wat momenteel gaande is.

Een eerste vergelijking tussen de bevindingen van de genoom-wijde linkage en associatie studies toont geen overlap in genen. Overlap zou men ook niet mogen verwachten, omdat de mechanismen die ten grondslag liggen aan de beide methoden verschillen. Linkage is vooral bedoeld om zeldzame varianten met groot effect te vinden en associatie is vooral geschikt om veel voorkomende varianten te vinden met klein of matig effect.
Van belang is ook dat we in onze genoom-wijde associatie studie geen bewijs vinden voor een rol van APOE, of voor de recent ontdekte AD genen, PICALM, CR1 en CLU [11,12] in cognitieve functie. Deze bevinding toont, dat de resultaten van analyses op endofenotypes niet één op één kan worden vertaald tot de ziekte. Ook is het van belang te vermelden dat cognitieve testen verschillende aspecten van het cognitief functioneren beoordelen. Zo vonden we inderdaad dat APOE geassocieerd was met de Adult Verbal Learning Test in hoofdstuk 3, maar niet-significant geassocieerd was met TMT-A, TMT-B of Stroop.

Na het onderzoeken van cognitieve functie als endophenotype, bestudeerden we de aan veroudering gerelateerde hersenveranderingen als een tweede groep van endofenotypen. We hebben plasma Aβ waarden gebruikt als biomarkers voor de aanwezigheid van seniele plaques en amyloïd angiopathie, en asymptomatische laesies die zichtbaar zijn op MRI van de hersenen als leeftijd-gerelateerde hersenenveranderingen. We hebben ons in dit gedeelte van het onderzoek gericht op witte stof laesies (WML), lacunair infarcten, microbloedingen en hippocampus atrofie. Al deze parameters zijn geassocieerd met een hoge bloeddruk, beroerte, dementie en cognitieve stoornissen [13-18], maar worden ook gevonden in gezonde ouderen.

We onderzochten de rol van enkele kandidaat-genen die betrokken zijn bij de regulatie van bloeddruk en amyloid metabolisme. We bestudeerden het APOE gen, genen gerelateerd aan het renine-angiotensine systeem (RAS) (Angiotensine, angiotensine II receptor type 1, alfa-Adducin) en het sortilin-gerelateerde receptor gen (SORL1).
Het is bekend dat RAS-genen betrokken zijn bij de regulatie van de bloeddruk en zout-homeostase en dat RAS-eiwitten betrokken kunnen zijn bij AD [19]. Receptoren voor angiotensine II zijn aanwezig in de hersenen [20] en een verhoogde activatie van RAS wordt gezien in de hersenen van AD patiënten [21]. Zoals boven genoemd, is APOE consequent in verband gebracht met AD en er zijn steeds meer aanwijzingen dat ook SORL1 een rol speelt in AD [4,22,23]. SORL1 bestaat uit twee functionele regio's, één functionerend in het cholesterol metabolisme en de andere in de verwerking van het amyloid voorloper eiwit (APP) [24,25]. Dit gen is recent tevens in verband gebracht met cerebrovasculaire ziekte [26] en opvallend genoeg vinden we SORL1 ook in onze verkennende vergelijkende analyses van de genoom-wijde associatie studies.

We bestudeerden eerst alle vijf de varianten in relatie tot de volgende MRI endofenotypes: WML, lacunaire infarcten en microbloedingen. We onderzochten dit in de ERF-studie in een subgroep van 55 tot 75 jaar met hypertensie (**hoofdstuk 8**). Alle deelnemers hadden enige mate van WML, terwijl lacunair infarcten aanwezig waren in 15,5% en microbloedingen in 23,3% van de deelnemers. Homozygositeit voor het APOE ε4 allel werd geassocieerd met lacunaire infarcten (OR, 4.8; 95% CI, 1.2-19.3). Individuen met twee kopieën van het variant allel van 4 SNPs gelegen aan het 3'-einde van SORL1 (rs1699102, rs3824968, rs2282649, rs1010159), hadden een verhoogd risico op microbloedingen (hoogste odds ratio, 6.87; 95% CI, 1.78-26.44). Dit zou de hypothese ondersteunen dat de amyloid cascade betrokken is bij de etiologie van microbloedingen in populaties met hypertensie.

Ten tweede bestudeerden we de relatie tussen het SORL1 gen en hippocampus volume en plasma Aβ waarden in **hoofdstuk 9**. Hiervoor gebruikten we dezelfde subgroep van de ERF-studie. Hippocampus volumes werden kwantitatief gemeten met MRI en plasma Aβ waarden werden bepaald

in niet-nuchtere bloedmonsters. We bestudeerden het effect van dezelfde 7 varianten binnen SORL1, waarvan eerder een relatie met AD was aangetoond. Drie varianten gelegen nabij het 3'-einde van SORL1 waren significant geassocieerd met hippocampus volume. De 3-SNP haplotypen voor rs1699102, rs3824968 en rs2282649 (CAT) en voor rs3824968, rs2282649 en rs1010159 (ATC) waren geassocieerd met grotere hippocampus volumes. We vonden geen significante associaties van deze varianten met plasma Aβ waarden.

Ten derde bestudeerden we de associatie van drie varianten binnen de angiotensine, angiotensine II type 1 receptor en adducin genen (AGT-M235T, AGTR1-C573T en ADD 1-Gly460Trp) in relatie tot Aβ plasma waarden in dezelfde hypertensieve subgroep van de ERF-studie (**hoofdstuk 10**). Deze varianten werden in eerdere onderzoeken in verband gebracht met vasculaire ziekten van hart en hersenen. Het AGT-M235T TT-genotype was significant geassocieerd met hogere waarden van plasma Aβ42 ($p = 0,008$) en van getrunceerd Aβn42 ($p = 0,02$). De associatie met Aβ42 bleef significant na correctie voor potentiële confounders en het toepassen van meerdere testen. We vonden geen significante associaties tussen AGTR1-C573T of ADD 1-Gly460Trp en plasma Aβ.

Samenvattend is de meest interessante bevinding van de bovengenoemde studies de associaties met SORL1 die werden gevonden in diverse studie-ontwerpen. Onze kandidaat-gen analyses toonden associatie van SORL1 met cognitieve functies evenals met microbloedingen en hippocampus volume. SORL1 kwam ook naar voren in onze genoom-wijde associatie meta-analyses van de cognitieve functies. Enige terughoudendheid in de interpretatie van onze resultaten is echter nodig: onze kandidaat-gen studies werden uitgevoerd in een kleine steekproef en waren beperkt tot hypertensieve personen. Onze bevindingen moeten dus gerepliceerd worden en dienen onderzocht te worden in grotere cohorten in de algemene bevolking.

In het derde gedeelte van dit proefschrift bespreken we twee kandidaat-gen studies in AD. Met deze studies wilden we de rol van twee interessante pathofysiologische mechanismen nader onderzoeken. Eén van de mechanismen is het ijzer-metabolisme, waarvan bekend is dat ijzerstapeling kan bijdragen aan het risico op AD. Onze onderzoeksafdeling heeft eerder al de relatie tussen hemochromatose-genen en AD onderzocht. In deze studies werd een effect van het hemochromatose gen (HFE) op de leeftijd waarop AD zich openbaart gevonden [27]. De HFE-63D mutatie was geassocieerd met het op jongere leeftijd ontstaan van AD in mensen die het APOE*ε4 allel bij zich dragen, maar deze mutatie was niet geassocieerd met een hoger risico op AD. Andere studie-groepen vonden associaties tussen AD en andere varianten in de hemochromatose genen HFE-C282Y en -H63D, en in het transferrine gen (TF) [22]. In het Epistasis Project, waarin 1757 AD patienten en 6295 controles zijn geïncludeerd, bestudeerden we vier varianten in twee genen die een rol spelen in het ijzer-metabolisme: HFE-C282Y, HFE -H63D, TF-C2 en TF-2G/A (**hoofdstuk 7**). Wij repliceerden

de interactieve werking tussen HFE-282Y en TF-C2 op het risico op AD in Noord-Europeanen. We vonden ook een interactie tussen HFE-63HH en TF-2AA, die aanzienlijk werd beïnvloed door de leeftijd. De interactie tussen HFE-282Y en TF-C2 is nu twee keer gerepliceerd in een totaal aantal van 2313 AD patiënten en 7065 gezonde controles.
Er zijn desondanks beperkingen in de interpretatie van de resultaten van deze studie, die het trekken van harde conclusies belemmeren. Ten eerste werden beide interacties voornamelijk in Noord-Europeanen gevonden en werd er geen relatie tussen HFE en AD gevonden in een Noord-Spaanse populatie. Vanuit statistisch oogpunt is de uitsluiting van de Spaanse gegevens problematisch. Hoewel de allel-frequenties in Noord-Spanje verschilden van die in de Noord-Europeanen, betekent dit niet dat de relatie met AD anders zou zijn. Een tweede probleem is dat ondanks het samenvoegen van de gegevens, de aantallen klein zijn en als gevolg daarvan de statistische power van deze studie laag is, waardoor de analyse gevoelig was voor vals-positieve bevindingen.

De tweede kandidaat-gen studie, die we verrichtten met betrekking tot AD, betrof het Cathepsine D gen (CTSD) (**hoofdstuk 6**). CTSD is betrokken bij het verwerken van het amyloid voorloper eiwit (APP) en is daarom een interessant kandidaat-gen voor AD. We onderzochten CTSD in relatie tot AD in de Rotterdam Studie, dat een populatie-gebaseerde cohort-studie is (N = 7983). Daarnaast deden we een grote meta-analyse waarin onze resultaten werden samengevoegd met resultaten van eerder gepubliceerde gegevens. In de Rotterdam Studie vonden we een verhoogd risico op AD in deelnemers die het T-allel van CTSD rs17571 droegen (p-waarde 0,007). Deze associatie werd voornamelijk gevonden in mensen die niet het APOE*ε4 allel hadden. De meta-analyse toonde eveneens een significant verhoogd risico op AD bij dragers van het T-allel van rs17571 (OR 1,22, 95% CI 1.03-1.44), maar dit risico bleek onafhankelijk van de APOE*ε4 status.

Naast bovengenoemde genetische studies, hebben we ook een klassieke epidemiologische studie verricht in **hoofdstuk 2** waarin we een combinatie van cardiovasculaire risicofactoren, samengesteld in het metabool syndroom (MetS) onderzochten met betrekking tot cognitieve functies. Diabetes type 2 is een bekende risicofactor voor slechtere cognitieve prestaties [28,29], maar er zijn minder studies verschenen over de associatie van MetS en bijdragende factoren, zoals insuline-resistentie (HOMA-IR), laag adiponectin, en hoge C-reactief proteïne (CRP) waarden in het bloed [30,31]. We onderzochten of deze factoren gerelateerd zijn aan cognitieve functies en ook welke MetS componenten onafhankelijk geassocieerd zijn. Voor deze studie gebruikten we de ERF-populatie waarin uitgebreide gegevens ten aanzien van lichamelijk onderzoek, biomedische bepalingen en neuropsychologische testen beschikbaar zijn. We vonden dat overwegend vrouwen met MetS en hoge HOMA-IR lagere scores op executieve cognitieve testen hadden (p = 0,03 en p = 0,009). De meest consistente individuele component van het MetS, was de systolische bloeddruk. We interpreteerden

deze resultaten met voorzichtigheid, aangezien het een cross-sectionele studie betrof en slechts borderline significante p-waarden werden gevonden na correctie voor het gebruik van meerdere testen. Longitudinale studies zullen nodig zijn om beter inzicht te krijgen in de causaliteit van onze bevindingen en zullen moeten bijdragen aan meer definitieve conclusies.

Een grote uitdaging in genetisch epidemiologisch onderzoek is de validering van kandidaat-genen in AD te verbeteren. Er worden jaarlijks vele nieuwe genetische associaties gerapporteerd, maar vrijwel geen kunnen worden gerepliceerd. In **hoofdstuk 12** hebben we gesproken over de toepassing van de zogenaamde *Venice*-criteria in de validering van genetische associaties in AD. The *Venice*-criteria evalueren de kwaliteit van de sterkte van het epidemiologische bewijs voor genetische associaties aan de hand van 3 punten: de hoeveelheid data, de consistentie van replicatie en mate van bias. Hoewel de eerste twee punten robuust bleken te zijn, was de laatste moeilijker te objectiveren, vooral wanneer milde uitschieters een rol spelen en wanneer de associaties een klein effect hebben. Daarom verwachten we dat de toevoeging van nieuwe toekomstige studies de huidige indeling van de meest sterk bewezen genetische associaties beïnvloeden en slechts enkele associaties robuust zullen blijken. The *Venice*-criteria vereisen verdere verfijningen voor de praktische toepassing in genetisch epidemiologisch onderzoek.

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Ik hoop van harte dat er nieuwe data in ERF gegenereerd zullen worden.

Daarnaast mijn andere promotor en mede-initiatiefnemer van de ERF-studie, prof. dr. B.A. Oostra; beste Ben, jij was de man van de grote lijnen en soms bijna vaderlijke bezorgdheid. Het advies om toch vooral op vakantie te gaan in de afrondende fase heb ik met dank ter harte genomen. Ik heb het erg gewaardeerd dat je de stabiele factor was binnen mijn promotietraject.

Natuurlijk wil ik ook mijn co-promotor, dr. J.C. van Swieten hier bedanken; beste John, zonder jouw inmenging was ik nooit betrokken geraakt bij dit promotieonderzoek. Jij zorgde voor de link tussen de kliniek en genetische epidemiologie. Jouw input in de stukken was onmisbaar en ik heb het erg gewaardeerd dat ik altijd bij je terecht kon.

Zonder de leden van de kleine en grote commissie had deze dag niet plaats kunnen vinden. Dr. Bonifati, Dr. Biessels en Prof. Dr. Boomsma, ik wil jullie heel hartelijk danken voor het op zo'n korte termijn lezen en beoordelen van het manuscript.
Ook de leden van de grote commissie wil ik danken voor hun tijd om te opponeren. Prof. dr. Koudstaal, dank, we zullen elkaar nog veel spreken de komende jaren, ik verheug me erop. Dr. de Leeuw, als één van mijn vroegere voorgangers, vind ik het een bijzondere eer dat je tijd hebt genomen in de commissie zitting te nemen.

Het vervolg onderzoek in ERF was nooit gelukt zonder de hulp van Prof. Dr. M.M.B. Breteler en Dr. A. van der Lugt.
Beste Monique, de eerste aanzet van het onderzoek is dankzij jou tot stand gekomen. We hebben

een aantal interessante papers geschreven mede door de toegang die ik had tot de ERGO data. Ik bewonder je gedrevenheid en betrokkenheid bij mijn project.

Beste Aad, dankzij jou inmenging konden we gelukkig doorgaan met het inplannen van deelnemers en liep de inclusie geen vertraging op. Je hebt me veel geleerd door het samen beoordelen van de scans. Ik heb je feedback op de manuscripten en enthousiasme voor het onderzoek erg gewaardeerd.

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Petra, jij hebt heel wat deelnemers heen en weer gereden tussen Brabant en Rotterdam. 's Ochtends vroeg al op pad en na het file-leed ging je met frisse moed met de deelnemers aan de slag. Ik was blij dat jij het platte Brabants goed onder de knie hebt, want wat mijn Groningse achtergrond kon ik het vaak niet verstaan.

Dr. M.W. Vernooij en dr. M.A. Ikram hebben geholpen alle scans te beoordelen. Beste Meike, dank voor de wijze lessen voor het opstarten van het onderzoek en het leren lezen van de scans op microbleeds en lacunaire infarcten.
Beste Arfan, we hebben heel wat analyses besproken en veel discussies gehad. Ik heb deze discussies altijd erg leuk gevonden en was blij dat ik vaak snel bij je terecht kon. Zeker aan het eind, omdat je er voor gezorgd hebt dat de replicatie data er op tijd waren. Ik zie je vast nog veel in de kliniek.

De andere co-auteurs van de manuscripten wil ik hier ook bedanken.
Beste Carola, Tom, Wiro, Henri, Renske, Fedde, Cecile en Peter: dank voor jullie input in de manuscripten!
Peter, je prachtige tekeningen staan nog steeds op het bord. Dank voor de gesprekjes tijdens je werk in Rotterdam. Fedde, veel succes met het afronden van je proefschrift!
Cecile, jij was natuurlijk meer dan een co-auteur. Ik heb ontzettend veel van je geleerd, dank voor je geduld en uitleg!

Met veel plezier heb ik de afgelopen jaren gewerkt op de 22e. Dankzij de hulp van mijn collega's werden zelfs de ingewikkeldste analyses een uitdaging en werden de dalen minder diep.
Yurii thanks for explaining the methodological parts and your input in the analyses.
Annelous, er is een hoop veranderd sinds we samen een kamer deelden. Ik heb veel van je geleerd

en ik ben blij dat ik in het begin met jou een kamer deelde. Je hebt me leren incasseren en relativeren. Dat je een olifant niet in één keer op kunt eten, maar beetje bij beetje, stukje voor stukje, herhaal ik nog vaak voor mezelf.
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Fan, thanks for all the patience in explaining the software and your nice companionship. How are the Dutch lessons going?
Aaron, thanks for all the help and the nice chats, especially the ones on Friday!
Maksim, it was nice working with you. I like your sense of humor.
Linda, het manuscript komt tot een eind! Veel succes met de rest van je werk.
Ayse, I enjoyed the dinners with you and Najaf, finally some girl talk.
Sophie, goed dat je de CJD registratie onder je hoede hebt genomen. Veel succes met het afronden van je master.
Zonder de volgende mensen zouden er geen genotypes bepaald zijn: Jeannette,
Andy, Bernadette, Sue Ellen en Andrea dank voor jullie hulp in het lab, ook als ik pas laat met samples terug kwam. Jeannette, je weet gelukkig altijd alles terug te vinden. Ik vond het een erg prettige samenwerking!

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Jeannette jouw hulp met het verzamelen van alle hoofdstukken en samenbinden van de laatste versies was onmisbaar, heel veel dank hiervoor.

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Liesbeth en Danielle, ik vond het erg leuk dat jullie mijn kamergenootjes zijn geworden. Onze trajecten verschillen niet zo veel en ik heb veel gehad aan jullie luisterend oor. Liesbeth, voor jou zit het er bijna op, veel succes volgende maand. Danielle, veel plezier op je bruiloft volgende maand. Martje, heel veel succes.

I also would like to thank the international collaborators.
Tatiana, Irina, Anatoly: thanks for all the work and help. It was nice meeting you guys in person in Rotterdam.
Donald: thank you for the fruitfull collaboration.
All the consortia in CHARGE: thank you for your trust in me, so I could do the meta-analyses. Dear Tom, Stephanie and Jan, it was nice working with you and hopefully we will continue collaborating in the near future.

Co-dames: hopelijk volgen er nog vele dinertjes! Jullie geven me een hoop energie.
Sun-D: tijden veranderen, wat een verschil met twaalf jaar geleden! Helemaal geweldig dat jullie vrij konden nemen om er vandaag bij te zijn. Straks is er meer tijd om weer af te spreken, ik heb er zin in.
Riek en Marijke, jullie blijven in mijn gedachten, ik wou dat ik op jullie promoties had kunnen zijn.
Berit, Christine, Groningen-dames en natuurlijk SHOT! dank voor jullie interesse en geduld, hopelijk kan ik me nu weer vaker bij jullie aansluiten, want ik heb een veel verhalen en gezelligheid gemist.

Dear paranimphs, many thanks for being here today!
Najaf, I enjoyed sharing the office with you. I am happy that we met and we get along so well. I miss the coffees in the morning and the chats. Hopefully, we will find more time to drink coffee and have dinner together!

Renate, super dat mijn paranimf bent vandaag. Ik ben erg blij dat je altijd in de buurt bent en gelukkig wil je meestal wel naar me luisteren, al is het de zoveelste keer hetzelfde probleem. Ik waardeer je professionele nuchtere advies. Zoals je weet kan ik me aan het begin van de week al verheugen op het vrijdagse biertje. We hebben er nog een hoop uit te proberen, want we zijn nog niet door het assortiment heen.

Sjef, Elly, Inge, Ton: dank voor de interesse in mijn onderzoek en de hulp in ons nieuwe huis!

Pa en ma, jullie weten het wel: onmisbare stabiliteit. Het wordt tijd dat ik weer eens jullie kant op kom in plaats van andersom.

Sander, thanks! Zonder jou was het boekje er niet geweest in de vorm zoals die nu is. Ik ben erg blij met het resultaat, dankzij jouw perfectionisme is het erg mooi geworden. Ik vind het geweldig dat jij en Linda naar Nederland zijn gekomen deze week. Al woon je straks ook een eindje weg, misschien zullen we elkaar wat vaker zien. Ik mis de wandelingetjes samen met jou door de stad.

Bas, met jou samen komt alles goed. We hebben drukke tijden gehad, maar samen kunnen we de wereld aan. Jij had jouw project en ik het mijne, de volgende doen we samen. Ik verheug me eerst eens op een lekker weekendje niet klussen!

Curriculum Vitae

Maaike Schuur was born in Annen, the Netherlands on the 11th of January 1979. After graduating from high school in 1997 at het Maartens College in Haren (Gymnasium), she moved to Groningen to study pharmacy at the University of Groningen. After two years she moved to Rotterdam to start her medical training at Erasmus University.

During her medical training she worked as a student assistant at the Department of plastical surgery at Eramus Medical Center (head of department prof. dr. S.E.R. Hovius) and was student representative in the education committee at Erasmus Medical Center.

In 2003 she was enrolled in a graduation project to study the additional value of follow-up surveillance imaging in children treated for brain tumors at the Department of Children's Oncology at Erasmus Medical Center under the supervision of dr. R.E. Reddingius, dr. C. Catsman-Berrevoets and dr. M.H. Lequin.

After her medical exam in 2005, she worked at the department of Neurology at Erasmus Medical Center (head: prof. dr. P.A.E. Sillevis Smitt). During her clinical work she decided to start a PhD project on the genetic epidemiology of cognitive function and age-related brain changes at the Genetic Epidemiology Unit of the Department of Epidemiology at Erasmus Medical Center under supervision of prof. dr. C.M. van Duijn, prof. dr. B.A. Oostra and dr. J.C. van Swieten, which she started in 2006. In 2008 she graduated from Nihes Master of Science program on Genetic Epidemiology. During her PhD project she was a member of the PhD-committtee representing PhD-students enrolled in the Nihes graduation program. She was also involved in the Dutch registration of patients with Creutzfeldt Jakob Disease.

In March 2010 she started her specialty training in Neurology at the Department of Neurology at Erasmus MC Medical Center Rotterdam.

List of Publications

Schuur M, Lequin M.H., Catsman-Berrevoets C.E., Graaf de N., Reddingius R.E. The surplus value of surveillance neuro-imaging in the follow-up of children with a primary brain tumor. Pediatric Blood and Cancer, Vol 45 (4), October 2005, abstract.

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M. Schuur, J.C. van Swieten, S. Schol-Gelok, M.A. Ikram, M.W. Vernooij, F. Liu1, A. Isaacs, R. de Boer, I. de Koning, W.J. Niessen, H. Vrooman, B.A. Oostra, A. van der Lugt, M.M.B. Breteler, C.M. van Duijn. Genetic risk factors for cerebral small vessel disease in hypertensive patients from a genetically isolated population. Journal of Neurology, Neurosurgery and Psychiatry, In Press.

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Schuur M, Henneman P, van Swieten JC , M.C. Zillikens, de Koning I, Janssens ACJW, Witteman JCM, Aulchenko YS, Frants RR, Oostra BA,Willems van Dijk K, van Duijn CM. Insulin-resistance and the metabolic syndrome are related to executive function in women in a large family-based study. European Journal of Epidemiology, In press.

Portfolio Summary

Erasmus MC
Universitair Medisch Centrum Rotterdam

Summary of PhD training and teaching

Name PhD student: Maaike Schuur Erasmus MC Departments: Epidemiology & Neurology Research School: Nihes	PhD period: Aug 2006- Febr 2010 Promotores: prof. dr. C.M. van Duijn, prof. dr. B.A. Oostra Supervisor: dr. J.C. van Swieten

1. PhD training

	Year	Workload (ECTS)
General courses		
- Biomedical English Writing and Communication	2008	2
Specific courses (e.g. Research school, Medical Training)	2006-2008	35
- Master in Genetic Epidemiology (Nihes)		
Seminars and workshops		
- Weekly scientific seminars Dept. of Epidemiology	2006-2010	5
Presentations		
- Genetic Epidemiology Unit of Dept. of Epidemiology: *Evidence of genetic associations in Alzheimer's disease - Considerations in using the Venice criteria"*	2009	1
- Genetic Epidemiology Unit of Dept. of Epidemiology: *"Cathepsin D gene and the risk of Alzheimer disease: a meta-analysis"*;	2009	1
- Genetic Epidemiology Unit of Dept. of Epidemiology: *"Genome-wide association study on cognitive traits"*	2008	1
- Genetic Epidemiology Unit of Dept. of Epidemiology: *"Genetic and epidemiological study on cognitive traits"*	2008	1
- Dept. of Epidemiology: *"Genome-wide association study on cognitive traits"*	2008	1
- Dept. of Neurology: "Onderzoek naar risicofactoren voor cognitieve stoornissen"	2007	1
- Genetic Epidemiology Unit of Dept. of Epidemiology: *"Association of KIBRA gene with cognitive function";*	2007	1
- Genetic Epidemiology Unit of Dept. of Epidemiology: *"Genetic susceptibility of white matter lesions"*	2007	1
(Inter)national conferences		
- NCHA-meeting Delft: oral presentation: "*Genome-wide association study of cognitive executive functions: meta-analysis of CHARGE-consortium"*	2010	1
- CHARGE-meeting Washington DC: *"Preliminary results of genome-wide meta-analysis on executive function"*	2009	1
- VasCog conference Singapore: poster *"Genetic risk factors for cerebral small vessel disease in a genetically isolated population"*	2009	1

- VasCog conference Singapore: poster *"Cognitive function is impaired in people with metabolic syndrome"*	2009	1
- AD/PD conference Prague: poster "*Cognitive function is impaired in people with metabolic syndrome"*	2009	1
- Theraprion meeting Milan	2008	0.5
- Meeting of European CJD surveillance network Riga	2008	0.5
- Meeting of European CJD surveillance network Hameln	2007	0.5
- ISAO meeting Antwerpen	2006	0.5
Other		
- Coordinator of Dutch Registry of Creutzfeldt Jakob Disease	2006-2009 2006-2009	10 5
- PhD member in PhD-committee	2006-2009	5
- PhD member in meetings of the Departmental Staff of the Dept. of Epidemiology	2007-2009	5
- Research physician in the ERGO research center	2006-2007	1
- Organisation of scientific meetings of the Genetic Epidemiology Unit of the Dept. of Epidemiology		
2. Teaching		
Supervising practicals and excursions, Tutoring		
- Principles of Genetic Epidemiology (Nihes)	2008, 2009	20
Supervising Master's theses		
- Genome-wide linkage screen on cognitive traits	2009	10
- Genetic study of familial Creutzfeldt Jakob Disease	2009	10
Other		
- Review of various papers for international journals	2008-2010	5

Rotterdam, May 12th 20 | 0

Maaike Schuur