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ORIGINAL INVESTIGATION

Linkage analysis for plasma amyloid beta levels in persons with hypertension implicates Aβ-40 levels to presenilin 2

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Abstract Plasma concentrations of Aβ40 and Aβ42 rise with age and are increased in people with mutations that cause early-onset Alzheimer's disease (AD). Amyloid beta (Aβ) plasma levels were successfully used as an (endo)phenotype for gene discovery using a linkage approach in families with dominant forms of disease. Here, we searched for loci involved in Aβ plasma levels in a series of non-demented patients with hypertension in the Erasmus Rucphen Family study. Aβ40 and Aβ42 levels were determined in 125 subjects with severe hypertension. All patients were genotyped with a 6,000 single nucleotide polymorphisms (SNPs) illumina array designed for linkage analysis. We conducted linkage analysis of plasma Aβ levels. None of the linkage analyses yielded genome-wide significant logarithm of odds (LOD) score over 3.3, but there was suggestive evidence for linkage (LOD $>$ 1.9) for two regions: 1q41 (LOD = 2.07) and 11q14.3 (LOD = 2.97), both for Aβ40. These regions were followed up with association analysis in the study subjects and in 320 subjects from a population-based cohort. For the Aβ40 region on chromosome 1, association of several SNPs was observed at the presenilin 2 gene (*PSEN2*) ($p = 2.58 \times 10^{-4}$ for rs6703170). On chromosome 11q14-21, we found some association ($p = 3.1 \times 10^{-3}$ for rs2514299). This linkage study of plasma concentrations of Aβ40 and Aβ42 yielded two suggestive regions, of which one points toward a known locus for familial AD.

Introduction

Together with neurofibrillary tangles, amyloid beta (Aβ) plaques in the brain are a pathological hallmark of

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Alzheimer's disease (AD) (Takashima 2009). The formation of the extracellular peptide Aβ results from the cleavage of the amyloid precursor protein (APP) by the enzymes β and γ secretase, as opposed to α and γ secretase (Murphy and LeVine 2010). Aβ is partially degraded within the brain and partly cleared from brain to plasma through the blood–brain barrier, a process in which several of the recently discovered Alzheimer risk genes are involved (Bettens et al. 2010). Excess production or poor clearance of amyloid beta may lead to neurotoxicity and formation of amyloid beta plaques. This is also true for truncated Aβ forms, Aβn-42 and Aβn40. The latter are more likely to be spliced into pathogenic forms. Aβn-42 accounts for 60 % of all Aβ species in pre-clinical AD stages (Sergeant et al. 2003). High plasma concentrations of Aβ are associated with an increased risk of AD (van Oijen et al. 2006) and there is revived interest in the use of this marker in clinical care, despite the fact that there may not be a causal relationship. Recent research has shown a decreased clearance of Aβ from the brain in those with mild cognitive dysfunction (Mawuenyega et al. 2010).

Aβ plasma levels were successfully used as an (endo)phenotype for gene discovery using a linkage approach in families with dominant forms of disease, leading to the discovery of presenilin 1 (*PSEN1*) (Ertekin-Taner et al. 2000). From a genetic perspective, it is of interest that the gene encoding the angiotensin converting enzyme (ACE) is not only consistently associated with AD, but also plays a role in the degradation of Aβ in the brain (Kehoe et al. 2009). Since hypertension in early life to middle age has now been well established as a risk factor for AD, and plasma Aβ levels are associated with hypertension (Lambert et al. 2011), we have conducted a genetic study aiming to identify genes involved in plasma Aβ levels in persons with hypertension.

Materials and methods

Study population

This study was embedded in the Erasmus Rucphen Family (ERF) study, a population-based study in a genetically isolated population. All approximately 3,000 participants in this study are living descendants of 22 couples who, at the end of the nineteenth century, had at least six children baptized in the community church. Extensive genealogy data are available from the year 1600 AD. Extensive data on the participants, including cardiovascular risk factors, family history, body composition, health habits, cognitive function, blood chemistry and genotyping, are available.

For this study, hypertensive subjects aged 55–75 years who did not have a history of stroke or dementia were selected from the study population. Hypertension was defined as a systolic blood pressure of $\geq$160 mmHg, a diastolic blood pressure of $\geq$100 mmHg and/or the use of antihypertensive medication. We chose to limit ourselves to subjects with this high threshold for hypertension to get the maximally informative subjects out of our cohort. Shah et al. (2012) provide a good insight into the interaction between amyloid beta and hypertension in the risk for AD and vascular brain changes. Of the 261 eligible individuals invited for this study, 135 agreed to participate. The participants and non-participants were similar with respect to cardiovascular risk factors, but the participants had a slightly higher duration of education (9 years in participants as opposed to 7.5 years in non-participants). All participants gave informed consent. The study was approved of by the Erasmus University Medical Center Medical Ethics Committee.

A replication sample was obtained from the Rotterdam Study (RS), a population-based cohort from the Rotterdam region (Hofman et al. 2007). There were 320 individuals available who met the same inclusion criteria as our discovery subjects (age 55–75 years, hypertensive and free from stroke and dementia) and had genotype information and amyloid beta measurements available.

Aβ measurements

Non-fasting blood samples were obtained in EDTA tubes and immediately cooled on ice. Plasma was extracted and stored at −80 °C. Plasma Aβ concentrations were measured with a fluorimetric bead-based immunoassay using xMAP® technology (Innogenetics®). Aβ40, Aβ42, and the truncated forms Aβn-42 and Aβn-40 were measured. From these measurements, we also calculated the ratios for Aβ40/Aβ42 and Aβ42/Aβn-42 (Hansson et al. 2010; Lambert et al. 2009).

Genotyping

Genotyping and pedigree data were available for 125 of the 129 subjects with full phenotype data. For all participants, genomic DNA was extracted from peripheral venous blood utilizing the salting out method (Miller et al. 1988). For genome-wide linkage analysis, genotyping was performed using the Illumina 6K linkage panel. Of the 6,000 single nucleotide polymorphisms (SNPs) on the array, 5,250 were used for analysis after quality control and excluding X-chromosomal SNPs. The genotyping was performed at the Centre National de Génotypage in France according to the manufacturer's protocol.

For association analysis, we used SNPs from dense genotyping platforms that included Illumina 318K, Illumina 370K, Illumina 610K and Affymetrix 250K, which

were merged as previously described (Demirkan et al. 2011). Genotyping on these platforms was performed according to the described protocols. Additionally, the array data were used to impute genotypes (using MACH version 1.0.16) (Li et al. 2009, 2010) to the HapMap rel. 22 CEU panel for meta-analysis. The genome-wide imputed dataset consisted of approximately 2.5 million SNPs.

For the replication cohort, we extracted genotype data for the regions of interest from the imputed genotype dataset. This dataset consists of approximately 2.5 million SNPs, genotyped on the Illumina HumanHap550-Duo BeadChip® and imputed with MACH.

Statistical analysis

Statistical analysis of quantitative traits was performed using PASW Statistics version 17.0 (SPSS Inc.) for Windows. The Aβ measurements were normally distributed, but kurtosis was increased for all distributions (from 1.98 to 3.29). Inverse-normal transformation of ranks was applied to all individual traits for linkage analysis, and ln transformation was used for the association analyses for the ratios as these were not normally distributed. The rank transformation was performed using the GenABEL package for R (Aulchenko et al. 2007). The family-based design of the ERF study makes it possible to evaluate the heritability of Aβ levels (Aβ40, Aβ42 and truncated forms Aβn40 and Aβn-42) using the pedigrees in the SOLAR program (Almasy and Blangero 1998).

Linkage

We performed linkage analysis for the different amyloid beta subtypes as defined above with age and sex as covariates, using the variance components method as implemented in Merlin (Abecasis et al. 2002). For computational reasons (software limitations), the study population was divided into 34 sub-pedigrees each consisting of two to seven subjects. A significant linkage result was defined as an LOD peak of $\geq$3.3, and a suggestive linkage result as an LOD peak of $\geq$1.9 (Lander and Kruglyak 1995). Additionally, we investigated all the remaining regions with an LOD peak $\geq$1.5. Linkage regions were defined as the region bounded by the maximum LOD score minus 1.

Fine mapping with association analysis

With the same traits and covariates, we performed association analysis with a polygenic model using GenABEL package for R (Aulchenko et al. 2007). The SNPs for the regions under the linkage peaks were taken from the dense panel of 700,000 SNPs. Association analysis was also performed for the SNPs of interest in the Rotterdam Study.

A meta-analysis of the results from the two studies for the regions under the peak was performed using the Metal software package for meta-analysis (Willer et al. 2010). In this meta-analysis, we used the 2.5 million imputed SNP dataset for ERF.

eQTL analyses

Information on the power of the identified SNP as expression quantitative trait loci (eQTL), i.e., the association of the SNPs found in our analyses to gene expression, was extracted from the mRNA by the SNP browser (MRBS) by Liang et al. (Dixon et al. 2007). (www.sph.umich.edu/csg/liang/asthma). We applied the genome-wide significance threshold for eQTLs defined by the authors of this database: an LOD of 6.076, corresponding to a p value of 1.2×10^{-7}. We also extracted data on eQTL associations from the ScanDB database (www.scandb.org), which is based on the eQTL analyses by Zhang et al. (2008).

Results

Descriptives

Table 1 provides a description of the study population. Of the 135 subjects, 6 were excluded from analysis due to unsuccessful phenotype collection and 4 more because of insufficient genotype or pedigree data. Plasma Aβ40 was significantly correlated with BMI ($p = 0.007$).

We assessed the role of possible confounding factors by performing linear regression analyses on Aβ values with and without suspected confounders. We found no evidence for confounding from either any medication acting on the RAS system, for Aβ40 ($p = 0.683$) or Aβ42 ($p = 0.481$), or for diabetes (p values: in Aβ40, diabetes status = 0.681, HbA1C = 0.588; in Aβ42, diabetes = 0.067, HbA1C = 0.133).

We also assessed partial correlations between Aβ levels and cognitive functioning in a neuropsychological test battery including Stroop, Trailmaking test, Block Design, 15 word memory test, and word fluency. There was a nominally significant ($p = 0.005$, we ran 11 correlations for 2 Aβ measurements) partial correlation, corrected for age, sex and eduction, between Aβ42 levels and the Z score for word fluency, but not for any other individual or composite measure to Aβ42 or Aβ40.

Heritability

First, we estimated the heritabilities of Aβ levels (Aβ40, Aβ42 and truncated forms Aβn40 and Aβn-42) using the

Table 1 Baseline characteristics of 125 participants with Aβ measurements

Characteristics	Mean (SD)
Age	64.42 (4.57)
Female (*n*, %)	65 (52)
Only primary education (*n*, %)	42 (34)
Systolic blood pressure	146 (18)
Diastolic blood pressure	84 (10)
APOE e4 carriers (*n*, %)	50 (40)[a]
Current smoker (*n*, %)	36 (29)
Body mass index	29.1 (4.4)
Total cholesterol	5.1 (1.12)
HbA1c	5.84 (0.59)
Aβ40 (pg/ml)	179 (39)
Aβ42 (pg/ml)	41 (14)
Aβn40 (pg/ml)	176 (36)
Aβn-42 (pg/ml)	28 (7)
Aβ42/Aβ40	0.23 (0.07)
Aβ42/Aβn-42	1.55 (0.46)

[a] 38 subjects (30 %) carried 1 *APOE* e4 allele and 12 subjects (10 %) carried two *APOE* e4 alleles

full ERF pedigree. We estimated a heritability of 0.23 ($p = 0.19$), 0.30 ($p = 0.20$), 0.12 ($p = 0.31$) and 0.55 ($p = 0.07$) for Aβ40, Aβ42, Aβn40 and Aβn-42, respectively. None of the heritabilities were significant. However, in a rank-transformed analysis to adjust for non-normality in pedigree fragments, the heritability estimates for Aβ40 ($p = 0.04$) and Aβ42 ($p = 0.046$) were significant.

Linkage analysis

None of the linkage analyses yielded genome-wide significant log odd (LOD) scores over 3.3, but there was suggestive linkage (LOD > 1.9) to Aβ40 in two regions: 1q41 (LOD = 2.07) and 11q14.3 (LOD = 2.97) (Table 2). The other individual traits had maximum LODs between 1.5 and 1.9: 2p22.2 (LOD = 1.86) for Aβn40, 15q13.3 (LOD = 1.63) and 15q26.1 (LOD = 1.7) for Aβ42, and 1q31.1 (LOD 1.6) and 17q25.1 (LOD = 1.61) for Aβn-42. For the ratios, we found no evidence for linkage. Online resource 1 shows the genome-wide linkage plots for all traits and zooms in on the regions with LOD > 1.9. Although the Aβ40 and Aβ42 levels are highly correlated (partial correlation = 0.433, $p < 0.001$), there is no overlap in linkage peaks between the two. In the chromosome 1 region for Aβ40, the maximum LOD for Aβ42 was 0.35. In the chromosome 11 region, there was no evidence for any linkage for Aβ42 (LOD = 0). Also vice versa, the chr15 regions linked to Aβ42 showed no evidence for linkage to Aβ40 (LOD max 0.05).

Fig. 1 **a** Local linkage and association plots for the Aβ40 region on chromosome 1. This figure shows the linkage and association results for the linkage region defined on chromosome 1. In *A*, a detail of the linkage plot is shown. *B* and *C*, respectively, show the association results under the peak from the imputed datasets for ERF and RS. *D* Gives the meta-analysis results for the two. A consistent signal can be seen in the *PSEN2* region at 2.25×10^8 bp. **b** Local linkage and association plots for the Aβ40 region on chromosome 11. This figure gives the linkage and association results for the linkage region defined on chromosome 11. In *A*, a detail of the linkage plot is shown. *B*, *C*, respectively, show the association results under the peak from the imputed datasets for ERF and RS. *D* Gives the meta-analysis results for the two. There is no consistent signal across the two cohorts within the region on chromosome 11 ▶

Association and eQTL analyses

We further explored the suggestive regions on chromosome 1 and 11 using the dense SNP genotypes from the microarrays. There were 1,216 directly genotyped SNPs available for association analysis in the region identified on chromosome 1, and 562 SNPs for the region on chromosome 11. For the meta-analysis in the imputed SNP sets, there were 14986 SNPs for the region on chromosome 1, and 3981 SNPs for the region on chromosome 11. Figure 1a, b shows the local linkage and association plots for these regions. Results for the top hits in the directly genotyped dataset are provided in Online Resource 2.

For the Aβ40 region on chromosome 1, nominal association was observed for an SNP lying 82 kb upstream from the presenilin 2 gene (*PSEN2*) (rs6703170: $p = 2.58 \times 10^{-4}$) (Online Resource 2). In the meta-analysis of ERF and the Rotterdam Study, rs6697254 had the lowest *p* value, but the allele frequency was low (0.0057) making the finding unreliable. Rs12409752 had the lowest *p* value with a common risk allele (MAF 0.27, $p = 1.1 \times 10^{-4}$) which is an intergenic SNP between *ITPKB* and *PSEN2*. There was a large block of *PSEN2* intronic SNPs spanning from 225130294 to 225149349 kb (NCBI build 36.3), which were associated with Aβ40. When considering eQTLs, several SNPs were associated (lowest $p = 2.40 \times 10^{-10}$ for rs2236914) with *PSEN2* expression levels. The meta-analysis results and eQTL results are given in Online Resource 3.

When analyzing the chromosome 11 region in more detail, there were no strong association results. The SNPs showing some association in ERF (top hit rs2514299, $p = 3.2 \times 10^{-3}$) (Online Resource 2) did not replicate in the RS. The two SNPs most strongly associated with Aβ40 levels emerging from a meta-analysis of RS and ERF were rs947937 ($p = 7.3 \times 10^{-5}$) and rs947935 ($p = 9.6 \times$

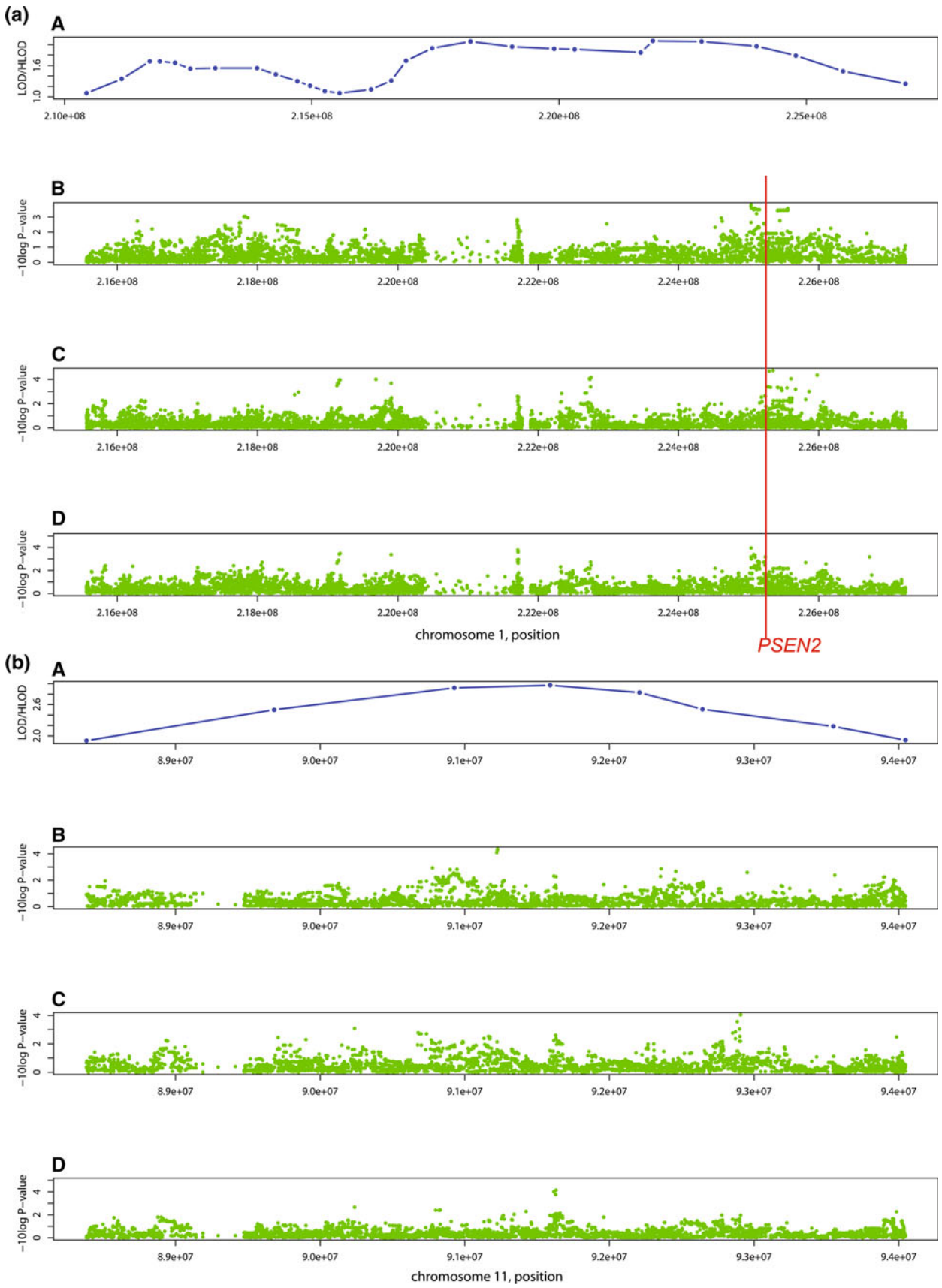
(a)
A
LOD/HLOD
1.0
1.6
2.10e+08
2.15e+08
2.20e+08
2.25e+08
B
−10log P−value
0
1
2
3
2.16e+08
2.18e+08
2.20e+08
2.22e+08
2.24e+08
2.26e+08
C
−10log P−value
0
2
4
2.16e+08
2.18e+08
2.20e+08
2.22e+08
2.24e+08
2.26e+08
D
−10log P−value
0
2
4
2.16e+08
2.18e+08
2.20e+08
2.22e+08
2.24e+08
2.26e+08
chromosome 1, position
PSEN2
(b)
A
LOD/HLOD
2.0
2.6
8.9e+07
9.0e+07
9.1e+07
9.2e+07
9.3e+07
9.4e+07
B
−10log P−value
0
2
4
8.9e+07
9.0e+07
9.1e+07
9.2e+07
9.3e+07
9.4e+07
C
−10log P−value
0
2
4
8.9e+07
9.0e+07
9.1e+07
9.2e+07
9.3e+07
9.4e+07
D
−10log P−value
0
2
4
8.9e+07
9.0e+07
9.1e+07
9.2e+07
9.3e+07
9.4e+07
chromosome 11, position

Table 2 Identified linkage regions for the different measurements

Chromosome	SNP with highest LOD	Physical position	Total region LOD-1 (cM)	Trait	LOD
1	rs11584610	221898214	228.695–237.703	Aβ40	2.07
1	rs11584662	184847447	187.881–230.45	Aβn-42	1.60
2	rs2691123	37070531	55.057–68.632	Aβn40	1.86
11	rs10830888	91591095	96.234–99.186	Aβ40	2.97
15	rs1399073	31331558	21.917–38.757	Aβ42	1.63
15	rs6497019	91428643	96.069–117.909	Aβ42	1.70
17	rs11869620	70326171	99.289–115.245	Aβn-42	1.61

10^{-5}) (Online Resource 3). eQTL analysis in this region showed no convincing results.

Discussion

In this family-based study, we found the highest heritability for Aβ42 and Aβn-42. This heritability is lower than that found in an extended family affected with late-onset Alzheimer's disease (LOAD) (Ertekin-Taner et al. 2001). There was no genome-wide significant linkage of Aβ levels as none of the regions reached an LOD score of 3.3 or higher, although the region on chromosome 11 approached this genome-wide significance level at an LOD score of 2.97. In total, two regions showed suggestive linkage with an LOD of >1.9. Of these two regions, the chromosome 11 region showed the highest LOD score. In this region, the ERF and RS association analyses were not consistent. In the second region on chromosome 1 with an LOD score of 2.07, *PSEN2* is the most remarkable gene, showing evidence both for association and an effect on expression levels of PSEN2.

On comparing our data with the literature, there are a number of remarkable findings. First, the *PSEN2* gene is a known causative gene for some cases of familial AD (Van Broeckhoven 1995). *PSEN2* was identified due to its homology to *PSEN1* (Rogaev et al. 1995). Its penetrance is lower than that of *PSEN1*. In its turn, *PSEN1* was identified in a study using Aβ plasma levels as an (endo)phenotype for gene discovery using a linkage approach in families with dominant forms of AD (St George-Hyslop et al. 1992). The presenilins are the proteases in the gamma secretase complex in the cell membrane responsible for the cleavage of APP into amyloid beta. Additionally, the presenilin 2 protein has been proven to downregulate cytokine-induced inflammatory responses in the brain, which can lead to neurodegeneration (Jayadev et al. 2010). On comparing the meta-analysis association results with the eQTL databases, several top SNPs showed significant association with *PSEN2* expression levels, i.e., were eQTLs for this gene. This supports our theory that it is a variant in, or in the regulatory region of, *PSEN2* causing this signal and pinpoints familial AD gene *PSEN2* as playing a possible role in the multifactorial LOAD pathogenesis. Our study connects a common variant near *PSEN2* to Aβ metabolism relatively early in life.

Second, it is interesting that we found a positive association of *PSEN2* with Aβ40, while there was no evidence of any linkage or association with Aβ42 (best LOD for the region is 0.32). These findings support several recent reports that indicate that Aβ40 is a determinant at least as important as Aβ42, although whether its effect is protective or risk increasing is currently being debated (Kumar-Singh et al. 2006; van Oijen et al. 2006). Animal experiments have shown that all tested familial *PSEN* and *APP* mutations resulted in decreased Aβ40 production with an accumulation of APP C-terminal fragments, a sign of decreased PSEN activity, but only some mutations including the *PSEN2* N141I (Volga German mutation) affected Aβ42 levels (Kumar-Singh et al. 2006). Our method does not allow us to pinpoint a specific mutation in the gene or its promoter regions, but it is possible that the variant underlying our signal selectively affects Aβ40 levels. We did not see evidence for association with the Aβ42/Aβ40 ratio.

The region on chromosome 11 is of particular interest for two reasons. First, the LOD score in this region approaches genome-wide significance. Second, this region has been associated with various neuropsychiatric disease, including autism (Anderson et al. 2009) and schizophrenia (Gurling et al. 2001; Petit et al. 1999) and it is close to the region 11q25 previously found associated with depressive disorder (Schol-Gelok et al. 2010), schizophrenia (Vorstman et al. 2006) and late-onset Alzheimer's disease (Liu et al. 2007). Given the evidence of co-occurrence of depression and Alzheimer's disease (Aznar and Knudsen 2011), this region is of particular interest from a clinical perspective. Within this linkage region, we cannot clearly identify a likely candidate gene based on the association or eQTL analyses.

There was no evidence of linkage with the *APOE* region on chromosome 19. *APOE* is the best-established risk gene

for sporadic Alzheimer's disease, plasma ApoE levels have been associated with amyloid beta burden in the brain, and ApoE is believed to play a role in the clearance of Aβ from the brain (Bettens et al. 2010; Thambisetty et al. 2010). A lack of power in the current study may be a possible explanation for this finding. Another explanation is that plasma Aβ42 and Aβ40 levels show no correlation to *APOE*. Only the Aβ42/Aβ40 ratio was correlated to *APOE* E4 carrier status in our sample ($r^2 = -21$; $p = 0.023$). Finally, compared to association, linkage analysis may not be as powerful an approach to identify a common susceptibility gene such as *APOE*. The strongest association signal within 1 Mb of the *APOE* gene was seen for rs1661197 ($p = 0.001$) with Aβ42. This SNP is located 312 kb away from the gene.

The main limitation of this linkage project is its limited sample size, due to the narrow inclusion criteria for this study and financial and logistics issues. Also, plasma amyloid beta is a rough estimate of the Aβ load in the brain and little is known about the variations and the changes of clearance from brain to plasma over time. There is no circadian variation in plasma Aβ levels (Lachno et al. 2009). However, our heritability studies show that Aβ42 and Aβ40 plasma levels are stable enough to yield significant evidence for familial clustering of increased Aβ levels at early age before the onset of dementia. Several studies have shown its value as an easily obtainable biomarker for risk of AD (Ertekin-Taner et al. 2008; van Oijen et al. 2006), although there are also negative studies (Hansson et al. 2010; Kester et al. 2010). It is the best method available for population-based research, as it is ethically not feasible to perform lumbar punctures on healthy volunteers at a large scale in a population-based setting.

Plasma amyloid beta 40 levels in healthy middle-aged subjects are associated with the locus containing the *PSEN2* gene associated with early-onset Alzheimer's disease, and with a locus on chromosome 11. Our findings support the involvement of these regions in the development of sporadic late-onset Alzheimer's disease. Additionally, the identification of a known gene involved in the plasma Aβ levels in this hypothesis-free experiment can be considered a sign of robustness for this method in our inbred population. Lastly, our eQTL analysis underlines the new associations of interest in interpreting the results of an association analysis. Next-generation sequencing and expression analyses will hopefully allow us to investigate the linkage peaks identified in much more detail in the future.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement All research described in this paper was carried out in compliance with the laws and customs for scientific research in the Netherlands. The Erasmus University Medical Center Medical Ethics Committee approved both the Erasmus Rucphen Family Study and the Rotterdam Study.

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