Quantitative Genetics Validates Previous Genetic Variants and Identifies Novel Genetic Players Influencing Alzheimer's Disease Cerebrospinal Fluid Biomarkers

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Abstract. Cerebrospinal fluid (CSF) biomarkers have been extensively investigated in the Alzheimer's disease (AD) field, and are now being applied in clinical practice. CSF amyloid-beta ($A\beta_{1-42}$), total tau (t-tau), and phosphorylated tau (p-tau) reflect disease pathology, and may be used as quantitative traits for genetic analyses, fostering the identification of new genetic factors and the proposal of novel biological pathways of the disease. In patients, the concentration of CSF $A\beta_{1-42}$ is decreased due to the accumulation of $A\beta_{1-42}$ in amyloid plaques in the brain, while t-tau and p-tau levels are increased, indicating the extent of neuronal damage. To better understand the biological mechanisms underlying the regulation of AD biomarkers, and its relation to AD, we examined the association between 36 selected single nucleotide polymorphisms (SNPs) and AD biomarkers $A\beta_{1-42}$, t-tau, and p-tau in CSF in a cohort of 672 samples (571 AD patients and 101 controls) collected within ten European consortium centers.

Our results highlighted five genes, *APOE*, *LOC100129500*, *PVRL2*, *SNAR-I*, and *TOMM40*, previously described as main players in the regulation of CSF biomarkers levels, further reinforcing a role for these in AD pathogenesis. Three new AD susceptibility loci, *INPP5D*, *CD2AP*, and *CASS4*, showed specific association with CSF tau biomarkers. The identification of genes that specifically influence tau biomarkers point out to mechanisms, independent of amyloid processing, but in turn related to tau biology that may open new venues to be explored for AD treatment.

Keywords: Alzheimer's disease, cerebrospinal fluid biomarkers, endophenotypes, European multicenter study, quantitative trait loci

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia associated to aging and involving a complex interaction between genetic and environmental risk factors. The burden of AD dementia is substantial and diagnosis and treatment options remain limited, so the identification and validation of new biological pathways associated with pathology are needed. AD is characterized by the presence of extracellular $A\beta$ plaques and intracellular aggregates of hyperphosphorylated tau in the brain [1]. Cerebrospinal fluid (CSF) amyloid-beta 1–42 ($A\beta_{1-42}$), total tau (t-tau), and phosphorylated tau (p-tau) are established biomarkers for AD, and have been used as quantitative traits for genetic analyses. In patients with AD, the concentration of CSF $A\beta_{1-42}$ is decreased, reflecting the sequestration of $A\beta_{1-42}$ in amyloid plaques in the brain [2]. Conversely, t-tau and p-tau levels in CSF are increased [3] with CSF t-tau levels directly correlated with the number

of neurofibrillary tangles and the load of hyperphosphorylated tau present in the brain. Elevated CSF t-tau and p-tau levels are indicators of neuronal loss, and p-tau levels have been shown to predict cognitive decline and conversion to AD in subjects with mild cognitive impairment (MCI) [4]. Additionally, t-tau/$A\beta_{1-42}$ and p-tau/$A\beta_{1-42}$ ratios have been indicated to represent progression/conversion from MCI to AD [5].

AD has a strong genetic component, a portion of which is explained by the apolipoprotein E (*APOE*) $\varepsilon4$ allele and several other genes identified by candidate gene studies, genome-wide association studies (GWAS) and meta-analysis [6–10]. The top 10 AD risk variants identified, and listed in the Alz-Gene database, include A*POE*$_{\varepsilon2/3/4}$, *BIN1*, *CLU*, *ABCA7*, *CR1*, *PICALM*, *MS4A6A*, *CD33*, *MS4A4E*, and *CD2AP* (http://www.alzgene.org/). Further, 11 new AD variants with genome-wide significance (*p*-value $< 5 \times 10^{-8}$) were highlighted in 2013, in the largest meta-analysis performed to date with

74,046 samples [10]. Notably, most of these large well-powered genetic studies have been restricted to binary categories, such as clinical diagnosis. Quantitative traits, that provide higher power than regular case-control analyses, are now being increasingly used and have already successfully identified new genetic factors implicated in several diseases [11]. AD biomarkers that reflect disease pathology, namely CSF t-tau, p-tau, and $A\beta_{1-42}$ have in the last years started to be used as quantitative traits for genetic analyses [11–16]. This approach is of outmost importance since, besides a higher power to identify new genetic factors, it can provide novel underlying biological models of disease, associated with specific processes and pathways, ultimately pinpointing new potential therapeutic targets. It has been shown that genetic variants that increase risk for AD modify CSF $A\beta_{1-42}$ and tau levels. *APP, PSEN1, PSEN2,* and the common variants in *APOE* were previously found to have a genome-wide significant association with CSF $A\beta_{1-42}$ and tau levels in different studies [11, 14, 16, 17]. In 2013, the second largest GWAS performed to date ($n = 1269$), detected four independent genome-wide significant loci associated with CSF t-tau and p-tau, including, apart from *APOE, SNAR-I, GLIS3* and within the TREM gene cluster [11]. Recently, a new GWAS, from the same team, performed in an extended population ($n = 3,146$), resulted in five genome-wide significant loci, three repeating the results from the previous study (*APOE, SNAR-I,* and *GLIS3*) and two novel loci, associated again with p-tau (within *PCDH8* and *CTDP1*) [16]. Another GWAS in a smaller population ($n = 374$) detected four single nucleotide polymorphisms (SNPs) in the regions of the *APOE, LOC100129500, TOMM40,* and *EPC2* genes that reached genome-wide significance for associations with one or more CSF biomarkers [14]. Nonetheless, the majority of quantitative trait loci (QTL) studies published have been conducted in much smaller cohorts showing robust associations with SNPs surrounding *APOE* on chromosome 19, but failing to replicate most of the additional genome-wide associations. New studies, in different populations, are needed to consolidate the knowledge on the relation between these AD risk genes and AD biomarkers.

The main goal of the present study was to further clarify the association of specific genetic variants with AD biomarkers. With this aim, we used a quantitative traits genetics approach including 36 top SNPs, selected from previous studies on CSF biomarkers and AD genetic variants, for association with CSF

levels of t-tau, p-tau, and $A\beta_{1-42}$. Here, we used an independent European multicenter cohort, the third largest to date with 672 samples, using similar methodologies between centers, and have successfully contributed to decipher previous inconsistencies between AD genetic variants and AD CSF biomarkers, and further pinpoint pathways and biological mechanisms underlying AD.

METHODS

Study population

This multicenter study was performed within the BIOMARKAPD consortium (http://www.neuro degenerationresearch.eu/initiatives/annual-calls-for-proposals/closed-calls/biomarkers-transnational-call/results-of-biomarker-call/biomarkapd/), gather ing 10 research centers from eight European countries. The initial dataset used comprised 700 samples from 595 AD patients and 105 controls (including healthy and subjective memory complaints subjects) (Table 1) coming from: Instituto de Medicina Molecular, Faculty of Medicine, University of Lisbon, Portugal; Institute of Clinical Medicine and Institute of Biomedicine, University of Eastern Finland, Kuopio, Finland; CHUC - Centro Hospitalar e Universitário de Coimbra, Portugal; Memory Unit, Neurology Department and Sant Pau Biomedical Research Institute, Hospital de la Santa Creu i Sant Pau, Autonomous University of Barcelona, Barcelona, Spain, and Centro de Investigación Biomédica en Red en enfermedades Neurodegener-ativas, CIBERNED, Madrid, Spain; MAC Memory Center and Molecular Markers Laboratory, IRCCS Cento S. Giovanni di Dio Fatebenefratelli, Brescia, Italy; Department of Neuroscience, Psychology, Drug Research and Child Health, University of Florence, Italy; Radboud University Medical Centre, Radboud Alzheimer Center, Nijmegen, The Netherlands; Department Geriatric Psychiatry (CIMH), Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany; Danish Dementia Research Centre, Department of Neurology, Rigshospitalet, University of Copenhagen, Denmark; 1st Department of Neurology, Aristotle University of Thessaloniki, Makedonia, Greek Alzheimer Association and Greek Association of Alzheimer's Disease and Related Disorders "Alzheimer Hellas", Greece.

Patients were evaluated by neurologists with long-standing expertise in dementia, and all were subjected

Table 1
Summary of sample characteristics for the Center(s) in each country and for all Centers

	Denmark	Finland	Germany	Greece	Italy	Portugal	Spain	The Netherlands	All Centers
All samples									
N	29	237	49	25	79	118	92	43	672
Age (y); mean ± SD	67 ± 8	72 ± 7	71 ± 9	75 ± 8	69 ± 9	64 ± 9	67 ± 10	72 ± 8	69 ± 9
APOE ε4+(%)	45	69	35	24	50	43	42	63	53
Male (%)	52	34	41	52	33	32	38	40	36
p-tau level, pg/ml	60 ± 31	80 ± 34	81 ± 48	53 ± 22	74 ± 33	74 ± 41	69 ± 35	99 ± 41	76 ± 39
t-tau level, pg/ml	350 ± 191	501 ± 261	397 ± 300	348 ± 230	524 ± 283	583 ± 337	508 ± 364	648 ± 383	480 ± 265
$A\beta_{1-42}$ level, pg/ml	499 ± 348	490 ± 197	717 ± 244	352 ± 172	427 ± 198	474 ± 198	516 ± 250	450 ± 167	488 ± 220
Controls									
N	11	29	18	–	11	–	32	–	101
Age (y); mean ± SD	65 ± 10	67 ± 7	66 ± 9	–	66 ± 10	–	60 ± 9	–	65 ± 9
APOE ε4+(%)	27	17	28	–	27	–	22	–	23
Male (%)	55	34	61	–	27	–	31	–	40
p-tau level, pg/ml	49 ± 21	59 ± 17	41 ± 11	–	45 ± 12	–	41 ± 14	–	48 ± 17
t-tau level, pg/ml	172 ± 102	274 ± 103	169 ± 64	–	212 ± 80	–	236 ± 86	–	263 ± 99
$A\beta$1–42 level, pg/ml	911 ± 161	803 ± 158	786 ± 238	–	688 ± 161	–	760 ± 182	–	791 ± 184
Alzheimer's disease									
N	18	208	31	25	68	118	60	43	571
Age (y); mean ± SD	68 ± 5	73 ± 7	74 ± 8	75 ± 8	70 ± 8	63 ± 9	71 ± 8	72 ± 8	70 ± 9
APOE ε4+(%)	56	76	39	24	53	43	53	63	58
Male (%)	50	34	29	52	34	32	42	40	36
p-tau level, pg/ml	66 ± 34	84 ± 35	97 ± 47	53 ± 28	79 ± 33	74 ± 41	84 ± 34	99 ± 41	81 ± 40
t-tau level, pg/ml	374 ± 223	545 ± 260	492 ± 308	348 ± 230	575 ± 271	583 ± 337	644 ± 373	648 ± 383	525 ± 263
$A\beta_{1-42}$ level, pg/ml	247 ± 109	446 ± 159	673 ± 238	352 ± 172	385 ± 169	474 ± 198	386 ± 171	450 ± 167	433 ± 178

Age at the lumbar puncture in years with the mean and the standard deviation; percentage of APOE ε4 + allele carriers; percentage of males. For each biomarker the mean in pg/ml with the standard deviation is shown.

to clinical history and neurological examination. Diagnosis of AD was made in accordance to the guidelines of the National Institute of Neurological Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) and of the National Institute on Aging-Alzheimer's Association [18, 19]. Demographic and clinical characteristics of the study participants were obtained by medical interview at the time of CSF and blood sampling as well as inspection of medical records. The Mann-Whitney Rank test was applied for comparison of age differences between cases and control subjects, and the Fisher exact test was used to evaluate difference of proportions in gender between cases and control subjects. Cases are significantly older than control subjects ($p < 0.001$) and there was a significant difference in the distribution of gender between cases and control subjects ($p = 0.001$), hence all statistical analyses were adjusted for age and gender.

The BIOMARKAPD project was approved by the ethical committees of the participating centers, and all participants or their legal representatives signed a written informed consent form in accordance with the Declaration of Helsinki.

CSF $A\beta_{1-42}$, t-tau, and p-tau quantification

Quantification of biomarkers was done locally in each center. To reduce any possible heterogeneity, CSF levels of $A\beta_{1-42}$, t-tau, and p-tau were measured using the same type of platform (regular ELISA) and the same commercially available enzyme-linked immunosorbent assays INNOTEST® β-amyloid (1–42), Innotest hTau Ag, and Innotest Phospho-tau (181P) (Innogenetics, Ghent, Belgium) at all centers. Biomarkers quantification was performed according to manufacturer's instructions by experienced laboratory technicians. Before any analysis, raw values of CSF levels were lof$_{10}$-transformed to approximate a normal distribution.

To reduce the potential influence of extreme outliers, GraphPad Pris 6® (GraphPad Software Inc., La Jolla, CA) software was used to identify the CSF quantification outliers by ROUT method and 20 extreme outliers were removed from the analysis. PASW Statistics 21.0® (SPSS Inc.) software was used to perform the univariate analysis of variance of CSF biochemical data, adjusted for age and gender (ANCOVA).

SNP selection

Individual samples were genotyped for the most meaningful SNPs, previously found to be associated with AD and/or with CSF $A\beta_{1-42}$, p-tau and t-tau biomarkers. SNPs were selected according the following criteria: 1) the top SNPs found to be associated with AD: The top 10 SNPs in AlzGene (an online database providing meta-analysis of published AD and genetic association studies, for AlzGene top results criteria, see [20]); and the top 4 SNPs from the largest and most recent AD meta-analysis carried out so far, with 74,046 individuals (reaching GWAS significance in the combined discovery and replication dataset [10]) (Supplementary Table 1). 2) The top SNPs found to be associated with CSF $A\beta_{1-42}$, t-tau, and/or p-tau biomarkers in two of the largest GWAS performed to date: The 17 SNPs highlighted in the study of Cruchaga et al., a GWAS for CSF t-tau and p-tau levels [11]; and the 9 SNPs highlighted in Kim et al., a GWAS study of the CSF biomarkers $A\beta_{1-42}$, t-tau, p-tau, p-tau/$A\beta_{1-42}$, and t-tau/$A\beta_{1-42}$ [14]. Following this prioritization approach—selection of the most significantly associated SNPS with AD and/or CSF biomarkers, from major studies (larger datasets, more representative and statistically significant)—40 SNPs were selected (14 SNPs found associated to AD and 26 SNPs associated to AD CSF biomarkers). Since two SNPs were common between two studies (rs429358 from Kim et al. [14] and AlzGene [20]; and rs2075650 from Cruchaga et al. [11] and Kim et al. [14]), the final number of selected SNPs was 38. Individual samples were genotyped for these 38 SNPs (Supplementary Table 1).

Genotyping

DNA was extracted using QIAamp DNA Blood Maxi kits (Qiagen, Germantown, MD, USA) or an available salting out procedure whenever appropriate and diluted in Tris–EDTA (TE) buffer. The concentrations of extracted DNA were determined by Nanodrop (NanoDrop Technologies, Wilmington, DE).

A total of 38 selected SNPs were genotyped using the Sequenom's iPlex assay (Sequenom, San Diego, CA, USA) and the Sequenom MassArray K2 platform according to the manufacturer's protocol. Samples from nine centers were genotyped by the Lisbon Centre (480 samples; at the Genomics Unit of the Instituto Gulbenkian de Ciência). AD samples from Kuopio Centre were genotyped locally (220 samples).

Extensive quality control was performed using eight HapMap (http://hapmap.ncbi.nlm.nih.gov/) controls of diverse ethnicity, Hardy-Weinberg equilibrium (HWE) with $p < 0.001$, and a minimum of 95% call rate for each SNP. Genotype determinations were performed blinded to affection status. Two SNPs did not meet the quality control criteria (rs12972970 and rs59860681) and were excluded. Twenty-eight samples (24 patients and 4 controls) with <90% call rate or corresponding to duplicates were excluded from analysis.

Quantitative trait loci

A total of 36 SNPs, that overpassed quality control, were used in the QTL analysis. The final dataset consisted of 672 subjects comprising 571 patients and 101 controls. The QTL analysis was performed to assess the main effect of each tested SNP on CSF biomarkers levels ($A\beta_{1-42}$, t-tau, p-tau, t-tau/$A\beta_{1-42}$ and p-tau/$A\beta_{1-42}$). At each SNP, CSF biomarkers levels were regressed onto genotype counts in a regression model that included affection status, gender, age, and collection site as covariates. A second stage of QTL analysis was performed which included the *APOE* genotype as a fifth covariate. $A\beta_{1-42}$, t-tau, p-tau, t-tau/$A\beta_{1-42}$, and p-tau/$A\beta_{1-42}$ were $\log_{10}$-transformed to approximate a normal distribution before QTL analysis. SNPassoc® v.1.4-9 package [21] implemented in the R freeware (http://cran.r-project.org/) was used for logistic regression analysis. Results were considered significant below the conventional level of 0.05. Bonferroni corrections for multiple tests were carried out to exclude type I errors (the significance level for 36 tests is set at p-value $< 1.39 \times 10^{-3}$).

RESULTS

Variability in CSF $A\beta_{1-42}$, t-tau, and p-tau levels and association to common variants

We evaluated a final set of 36 selected SNPs for association with the CSF levels of $A\beta_{1-42}$, t-tau and p-tau in a large population of 672 unrelated European individuals. In this study, we followed a standardized quantification methodology to measure the CSF levels of $A\beta_{1-42}$, t-tau and p-tau at the 10 participating centers and performed stringent quality control (QC) in both the genotype and the phenotype data

previously to any analysis. While there are differences in the absolute levels of the biomarker measurements between the different centers, reflecting differences within the parameters reported by the analytical protocol QC, the CSF $A\beta_{1-42}$, t-tau, and p-tau levels show similar characteristics (Table 1). CSF $A\beta_{1-42}$, t-tau, and p-tau levels are normally distributed after log transformation and show a 6- to 16-fold difference between AD patients and control samples (in the centers that contained both types of samples). As expected we obtained lower values of $A\beta_{1-42}$ and higher values of t-tau and p-tau levels in AD patients as compared to controls, either at each center or when looking at the total values for all the centers (Table 1).

Loci associated with CSF $A\beta_{1-42}$ levels

Ten SNPs in the regions of *APOE, TOMM40, PVRL2,* and *LOC100129500*, in chromosome 19, reached significance after Bonferroni adjustment (corrected $p < 1.39 \times 10^{-3}$) (Table 2). The strongest associations were found within the *APOE* gene, as expected, for rs769449 ($p = 2.57 \times 10^{-14}$) and rs429358 ($p = 2.03 \times 10^{-17}$). rs429358, that defines $\varepsilon4$ allele, had the most significant association with CSF $A\beta_{1-42}$, t-tau, and p-tau levels and also with the t-tau/$A\beta_{1-42}$ and p-tau/$A\beta_{1-42}$ ratios (Fig. 1). Also, several variants in *TOMM40* and *PVRL2* reached highly significant p values ($p < 1 \times 10^{-08}$) (Table 2, Fig. 1). All these SNPs were found to be associated with lower levels of $A\beta_{1-42}$, suggestive of a contribution to an increased risk for AD. Nevertheless, when the *APOE* genotype was included in the model as covariate, the association for these SNPs became non-significant, confirming the strong influence of *APOE* on the results (Table 3).

Loci associated with CSF t-tau and p-tau levels

Sixteen SNPs in the regions of *APOE, TOMM40, PVRL2, INPP5D, SNAR-I, CD2AP, GLIS3, LOC100129500*, and *CASS4* were associated with t-tau and/or p-tau levels ($p < 0.05$) (Table 2). One to four SNPs in several loci showed highly significant p values ($p < 1 \times 10^{-05}$), particularly in chromosome 19 with the strongest association observed for *APOE* (rs429358, $p = 1.40 \times 10^{-11}$ and $p = 3.18 \times 10^{-8}$, association to t-tau and p-tau levels, respectively) (Table 2, Fig. 1). Similarly to CSF $A\beta_{1}^{-}{}_{42}$ levels, the strongest associations for CSF t-tau and p-tau, after *APOE*, were found in

four SNPs in *TOMM40* and two SNPs in *PVRL2* regions ($9.10 \times 10^{-07} \le p \le 3.21 \times 10^{-4}$), located on chromosome 19 (Table 2, Fig. 1), all overpassing Bonferroni correction. All these SNPs were associated with higher levels of t-tau and p-tau, suggestive of an increased risk for AD. Notwithstanding, when we adjusted the analysis for *APOE* genotype (Table 3), most of the associations became non-significant implying again a strong influence of *APOE* on these results.

Interestingly, four SNPs outside the *APOE* region, in *INPP5D, SNAR-I, CD2AP,* and *CASS4* genes, were associated with CSF t-tau, p-tau, or both ($p < 0.05$) (Table 2), remaining significant (except CD2AP) after adjustment for *APOE* genotype (Table 3). Remarkably, these SNPs were not associated with $A\beta_{1-42}$ levels. While the two SNPs in *SNAR-I* were associated with higher levels of t-tau and p-tau (indicative of an increased risk for AD), SNPs in *INPP5D* and *CASS4* were associated with lower levels of t-tau and/or p-tau suggestive of a protective role in AD.

SNP association with t-tau/$A\beta_{1-42}$ and p-tau/$A\beta_{1-42}$ levels

In addition to $A\beta_{1-42}$, t-tau and p-tau levels, the ratios t-tau/$A\beta_{1-42}$ and p-tau/$A\beta_{1-42}$ have been used to effectively distinguish patients with AD from controls. Twelve SNPs in *APOE, TOMM40, PVRL2, LOC100129500,* and *SNAR-I*, were found associated with t-tau/$A\beta_{1-42}$ and/or p-tau/$A\beta_{1-42}$ ratios ($p < 0.05$) (Table 2). Also, in this case, the strongest associations were with the two SNPs in *APOE* (rs769449 and rs429358; $1.85 \times 10^{-19} \le p \le 1.29 \times 10^{-13}$), just followed by the high associations in *TOMM40* and *PVRL2* with $p < 1 \times 10^{-08}$ (all overpassing Bonferroni correction), once again reinforcing the importance of these regions in the determination of the levels of AD biomarkers. The association with higher CSF t-tau/$A\beta_{1-42}$ and/or p-tau/$A\beta_{1-42}$ ratios further suggests its contribution to an increased risk in AD. All associations but for rs429358 became non-significant when *APOE* genotype was used as covariate in the analysis, further indicating a strong *APOE* dependent effect on the levels of the several biomarkers studied.

DISCUSSION

AD diagnosis has substantially improved over the past decade with CSF biomarkers acquiring an

Table 2

Significant SNPs for $A\beta_{1-42}$, t-tau, and p-tau levels, and with t-tau/$A\beta_{1-42}$ and p-tau/$A\beta_{1-42}$ ratios (adjusted for affection status, age, gender, and collection site). Significance after Bonferroni adjustment (corrected $p < 1.39 \times 10^{-3}$)

Chr	dbSNP	MAF	Gene	Reason for SNP Selection	Model	SNP Type/Location	$A\beta_{1-42}$	t-tau	p-tau	t-tau/$A\beta_{1-42}$	p-tau/$A\beta_{1-42}$
2	rs35349669	0.435	*INPP5*	AD assoc.*	B	Intron	0.6229	$\mathbf{3.84 \times 10^{-02}}$ ↓	0.4122	0.0596	0.2959
3	rs1316356	0.396	*SNAR-I*	CSF assoc.*	A	Intergenic	0.7636	$\mathbf{1.77 \times 10^{-03}}$ ↑	$\mathbf{3.26 \times 10^{-03}}$ ↑	$\mathbf{4.98 \times 10^{-02}}$ ↑	0.1176
3	rs9877502	0.399	*SNAR-I*	CSF assoc.*	A	Intergenic	0.9000	$\mathbf{2.49 \times 10^{-03}}$ ↑	$\mathbf{5.48 \times 10^{-03}}$ ↑	$\mathbf{4.48 \times 10^{-02}}$ ↑	0.10848
6	rs9349407	0.280	*CD2AP*	AD assoc.*	C	Intron	0.6589	$\mathbf{4.77 \times 10^{-02}}$ ↑	0.1488	0.2051	0.385
9	rs514716	0.151	*GLIS3*	CSF assoc.*	A	Intron	0.8136	$\mathbf{4.88 \times 10^{-02}}$ ↑	0.0729	0.0996	0.24973
19	rs12972156	0.251	*PVRL2*	CSF assoc.*	C	Intron	$\mathbf{2.25 \times 10^{-09}}$ ↓	$\mathbf{8.33 \times 10^{-05}}$ ↑	$\mathbf{3.21 \times 10^{-04}}$ ↑	$\mathbf{1.27 \times 10^{-08}}$ ↑	$\mathbf{5.27 \times 10^{-09}}$ ↑
19	rs34342646	0.270	*PVRL2*	CSF assoc.*	C	Intron	$\mathbf{1.01 \times 10^{-08}}$ ↓	$\mathbf{6.93 \times 10^{-06}}$ ↑	$\mathbf{2.53 \times 10^{-05}}$ ↑	$\mathbf{2.05 \times 10^{-09}}$ ↑	$\mathbf{6.83 \times 10^{-10}}$ ↑
19	rs71352238	0.269	*TOMM40*	CSF assoc.*	C	Intergenic	$\mathbf{7.88 \times 10^{-09}}$ ↓	$\mathbf{9.10 \times 10^{-07}}$ ↑	$\mathbf{4.01 \times 10^{-06}}$ ↑	$\mathbf{2.62 \times 10^{-10}}$ ↑	$\mathbf{1.02 \times 10^{-10}}$ ↑
19	rs157580	0.264	*TOMM40*	CSF assoc.*	C	Intron	$\mathbf{2.96 \times 10^{-04}}$ ↑	$\mathbf{4.76 \times 10^{-02}}$	0.0713	$\mathbf{2.13 \times 10^{-03}}$ ↓	$\mathbf{1.22 \times 10^{-03}}$ ↓
19	rs2075650	0.263	*TOMM40*	CSF assoc.*	C	Intron	$\mathbf{1.05 \times 10^{-09}}$ ↓	$\mathbf{2.31 \times 10^{-06}}$ ↑	$\mathbf{1.40 \times 10^{-05}}$ ↑	$\mathbf{2.10 \times 10^{-10}}$ ↑	$\mathbf{9.66 \times 10^{-11}}$ ↑
19	rs34404554	0.264	*TOMM40*	CSF assoc.*	C	Intron	$\mathbf{1.57 \times 10^{-09}}$ ↓	$\mathbf{2.73 \times 10^{-06}}$ ↑	$\mathbf{1.37 \times 10^{-05}}$ ↑	$\mathbf{2.88 \times 10^{-10}}$ ↑	$\mathbf{1.12 \times 10^{-10}}$ ↑
19	rs11556505	0.264	*TOMM40*	CSF assoc.*	C	Synonymous	$\mathbf{1.71 \times 10^{-09}}$ ↓	$\mathbf{2.50 \times 10^{-06}}$ ↑	$\mathbf{1.31 \times 10^{-05}}$ ↑	$\mathbf{2.78 \times 10^{-10}}$ ↑	$\mathbf{1.01 \times 10^{-10}}$ ↑
19	rs769449	0.295	*APOE*	CSF assoc.*	C	Intron	$\mathbf{2.57 \times 10^{-14}}$ ↓	$\mathbf{2.82 \times 10^{-09}}$ ↑	$\mathbf{4.11 \times 10^{-06}}$ ↑	$\mathbf{1.92 \times 10^{-15}}$ ↑	$\mathbf{1.29 \times 10^{-13}}$ ↑
19	rs429358	0.340	*APOE*	AD/CSF assoc.*	C	Intergenic	$\mathbf{2.03 \times 10^{-17}}$ ↓	$\mathbf{1.40 \times 10^{-11}}$ ↑	$\mathbf{3.18 \times 10^{-08}}$ ↑	$\mathbf{1.85 \times 10^{-19}}$ ↑	$\mathbf{5.89 \times 10^{-18}}$ ↑
19	rs439401	0.282	*LOC100129500*	CSF assoc.*	C	Intron	$\mathbf{1.43 \times 10^{-04}}$ ↑	$\mathbf{3.82 \times 10^{-02}}$ ↓	0.3982	$\mathbf{1.18 \times 10^{-03}}$ ↓	$\mathbf{5.47 \times 10^{-03}}$ ↓
20	rs7274581	0.060	*CASS4*	AD assoc.*	A	Intron	0.9363	$\mathbf{4.79 \times 10^{-02}}$ ↓	$\mathbf{1.01 \times 10^{-02}}$ ↓	0.1887	0.1184

Chr., chromosome; dbSNP, single nucleotide polymorphism; MAF, minor allele frequency; nb, number; Alleles (minor/major); *INPP5D*, Inositol Polyphosphate-5-Phosphatase D; *SNAR-I*, small ILF3/NF90-associated RNA I; *CD2AP*, CD2 Associated protein; *GLIS3*, GLIS Family Zinc Finger 3; *PVRL2*, poliovirus receptor-related 2 (herpesvirus entry mediator B); *TOMM40*, translocase of outer mitochondrial membrane 40 homolog (yeast); *APOE*, apolipoprotein E; *LOC100129500*, hypothetical LOC100129500; *CASS4*, Cas Scaffolding Protein Family Member 4; AD assoc. *, top SNP previously found associated with AD; CSF assoc.*, top SNP previously found associated with CSF biomarkers ($A\beta_{1-42}$, t-tau, and/or p-tau); AD/CSF assoc.*, top SNP previously found associated with AD and with CSF biomarkers ($A\beta_{1-42}$, t-tau, and/or p-tau); ↑, association with higher levels of CSF biomarker; ↓, association with lower levels of CSF biomarker. Significance p-value ($p < 0.05$) in bold. Analysis adjusted for affection status, age, gender, and collection site. Model: [A]: Dominant model; [B]: Recessive model; [C]: log-Additive model.

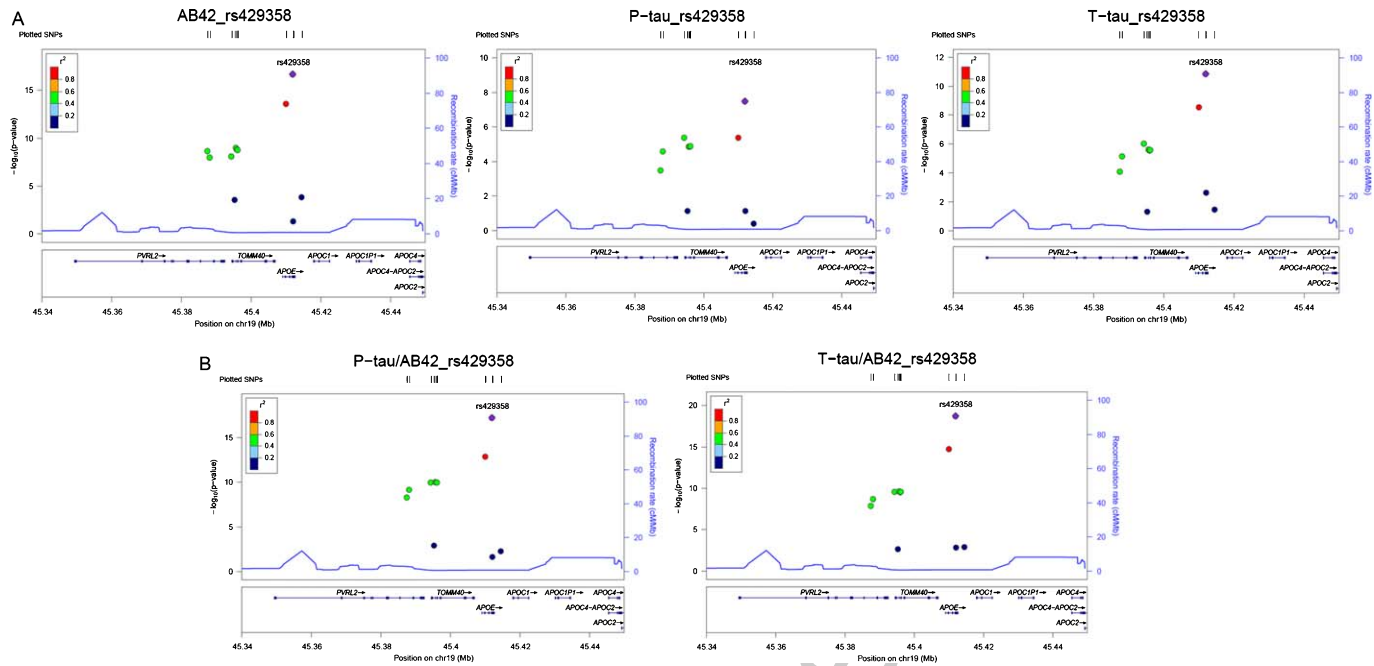Fig. 1. Regional plots for associations with CSF t-tau, p-tau, $A\beta_{1-42}$ and with ratios p-tau/$A\beta_{1-42}$ and t-tau/$A\beta_{42}$. A) Plots are centered on the most significant SNP, rs429358 within *APOE*, along with the combined-analysis results for SNPs in the region surrounding it (typically ± 0.1 Mb). B) Plots are centered on the most significant SNP, rs429358 within *APOE*, along with the combined-analysis results for SNPs in the region surrounding it (typically ± 0.1 Mb). Symbols are colored according to the LD of the SNP with the top SNP. The light blue line represents the estimated recombination rate marked on the right-hand *y*-axis of each regional plot. Gene annotations are shown as dark blue lines (http://locuszoom.sph.umich.edu/locuszoom/). See also Supplementary Table 1.

Table 3

Significant SNPs for $A\beta_{1-42}$, t-tau, and p-tau levels, and with t-tau/$A\beta_{1-42}$ and p-tau/$A\beta_{1-42}$ ratios (adjusted for affection status, age, gender, collection site, and *APOE* genotype). Significance after Bonferroni adjustment (corrected $p < 1.39 \times 10^{-3}$)

Chr	dbSNP	MAF	Gene	Reason for SNP Selection	Model	Location/SNP type	$A\beta_{1-42}$	t-tau	p-tau	t-tau/$A\beta_{1-42}$	p-tau/$A\beta_{1-42}$
2	rs35349669	0.435	*INPP5D*	AD assoc.*	B	Intron	0.6483	**3.20×10^{-02}** ↓	0.3958	0.0523	0.3095
3	rs1316356	0.396	*SNAR-I*	CSF assoc.*	A	Intergenic	0.4082	**7.17×10^{-03}** ↑	**1.01×10^{-02}** ↑	0.1637	0.3208
3	rs9877502	0.399	*SNAR-I*	CSF assoc.*	A	Intergenic	0.4645	**1.16×10^{-02}** ↑	**1.97×10^{-02}** ↑	0.1745	0.3379
6	rs9349407	0.280	*CD2AP*	AD assoc.*	C	Intron	0.8520	0.0606	0.1620	0.2524	0.4068
9	rs514716	0.151	*GLIS3*	CSF assoc.*	A	Intron	0.9809	0.0787	0.1011	0.1844	0.3812
19	rs12972156	0.251	*PVRL2*	CSF assoc.*	C	Intron	0.6220	0.7631	0.3913	0.9225	0.4180
19	rs34342646	0.270	*PVRL2*	CSF assoc.*	C	Intron	0.8508	0.6813	0.1105	0.7326	0.2041
19	rs71352238	0.269	*TOMM40*	CSF assoc.*	C	Intergenic	0.8662	0.3599	**3.62×10^{-02}** ↑	0.4839	0.1123
19	rs157580	0.264	*TOMM40*	CSF assoc.*	C	Intron	0.6392	0.7307	0.8672	0.9011	0.7270
19	rs2075650	0.263	*TOMM40*	CSF assoc.*	C	Intron	0.6791	0.6103	0.0977	0.6009	0.1596
19	rs34404554	0.264	*TOMM40*	CSF assoc.*	C	Intron	0.7389	0.6585	0.0992	0.6691	0.1778
19	rs11556505	0.264	*TOMM40*	CSF assoc.*	C	Synonymous	0.7934	0.6599	0.0964	0.6985	0.1735
19	rs769449	0.295	*APOE*	CSF assoc.*	C	Intron	0.3706	0.2731	0.1632	0,2556	0.1987
19	rs429358	0.340	*APOE*	AD/CSF assoc.*	C	Intergenic	0.0877	**8.85×10^{-03}** ↑	**3.42×10^{-05}** ↑	**8.27×10^{-03}** ↑	**2.40×10^{-04}** ↑
19	rs439401	0.282	*LOC100129500*	CSF assoc.*	C	Intron	0.6585	0.6680	0.7921	0.7731	0.5624
20	rs7274581	0.060	*CASS4*	AD assoc.*	A	Intron	0.9953	**3.43×10^{-02}** ↓	**5.48×10^{-03}** ↓	0.1251	0.0873

Chr., chromosome; dbSNP, single nucleotide polymorphism; MAF, minor allele frequency; nb, number; Alleles (minor/major); *INPP5D*, Inositol Polyphosphate-5-Phosphatase D; *SNAR-I*, small ILF3/NF90-associated RNA I; *CD2AP*, CD2 Associated protein; *GLIS3*, GLIS Family Zinc Finger 3; *PVRL2*, poliovirus receptor-related 2 (herpesvirus entry mediator B); *TOMM40*, translocase of outer mitochondrial membrane 40 homolog (yeast); *APOE*, apolipoprotein E; *LOC100129500*, hypothetical LOC100129500; *CASS4*, Cas Scaffolding Protein Family Member 4; AD assoc.*, top SNP previously found associated with AD; CSF assoc.*, top SNP previously found associated with CSF biomarkers ($A\beta_{1-42}$, t-tau, and/or p-tau); AD/CSF assoc.*, top SNP previously found associated with AD and with CSF biomarkers ($A\beta_{1-42}$, t-tau, and/or p-tau); ↑, association with higher levels of CSF biomarker; ↓, association with lower levels of CSF biomarker. Significance *p*-value ($p < 0.05$) in bold. Analysis adjusted for affection status, age, gender, collection site, and *APOE* genotype. Model:[A]: Dominant model;[B]: Recessive model;[C]: log-Additive model.

important role in the clinical practice, where these have been increasingly used, improving early and differential diagnosis of AD [22].

Our study intends to strengthen the main findings in AD CSF biomarkers quantitative genetics, clarifying previous inconsistencies and reinforcing the implication of specific underpinning mechanisms in AD. Here, we disclose novel associations between AD genetic risk variants and CSF biomarkers that can contribute to mechanistic insights for AD pathogenesis and also provide valuable information for newly potential underlying biological mechanisms.

Our top findings for significant association were verified within nine genes: *APOE, TOMM40, LOC100129500, PVRL2, SNAR-I, GLIS3, CASS4, INPP5D,* and *CD2AP*.

APOE, located at chromosome 19q13.2 is the most well characterized genetic risk factor for AD and is highly expressed in liver and brain playing an important role in mobilization and redistribution of cholesterol [23]. It is known that *APOE* binds to Aβ influencing the clearance of soluble Aβ and Aβ aggregation [14, 24], which explains a large proportion of the variance in Aβ levels, with no other locus showing similarly large effects. More recently evidence started to accumulate related to the influence of *APOE* on tau pathology by an Aβ-independent mechanism [11, 14, 16]. In our findings, rs429358 (which defines the ε4 allele) is strongly correlated with the CSF biomarkers, $A\beta_{1-42}$, t-tau, and p-tau and also with the ratios (t-tau/$A\beta_{1-42}$ and p-tau/$A\beta_{1-42}$) along with rs769449, both associated with lower CSF $A\beta_{1-42}$ and higher levels of tau, suggestive of an increased risk for the disorder. In turn, rs7412 (which defines ε2 allele) is not associated with any CSF biomarker (data not shown). These results replicate the findings of Cruchaga et al. and of Deming et al., both reported rs769449 as the most significantly associated *APOE* SNP being highly associated with CSF $A\beta_{1-42}$, t-tau, and p-tau markers [11, 16]. Additionally, it replicates the significant association of rs429538 in the GWAS from the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort, which investigated the influence of genetic variation on CSF biomarkers in 374 non-Hispanic Caucasian participants, showing that rs429538 is associated with CSF $A\beta_{1-42}$, p-tau, p-tau/$A\beta_{1-42}$ and t-tau/$A\beta_{1-42}$ ratio ($p < 1 \times 10^{-6}$ in all biomarkers) [14].

Apart of *APOE*, the strongest associations were shown with *TOMM40* and *PVRL2*. *TOMM40*, a nearby gene (positioned about 15 kb upstream) of *APOE*, is a transporter of proteins across the mitochondrial membrane, and a sortilin-related receptor, which functions to partition amyloid-β protein precursor (AβPP) away from β-secretase and γ-secretase. In our findings, *TOMM40* variants were significantly associated with lower CSF $A\beta_{1-42}$ levels and higher tau levels, as well as with higher ratios of p-tau/$A\beta_{1-42}$ and t-tau/$A\beta_{1-42}$, suggesting a link between these SNPs and AD risk. The main findings in this gene are also consistent with the report by Cruchaga et al. and Kim et al. showing TOMM40 as a region correlated with CSF $A\beta_{1-42}$ and t-tau levels, and with the ratios p-tau$_{181}$/$A\beta_{1-42}$ and t-tau/$A\beta_{1-42}$ [11, 14]. *TOMM40* has been recognized as a genetic risk factor for AD [25], namely through the association of the intronic SNP rs2075650, which in turn is known to be in tight linkage disequilibrium with the APOE locus [26]. More recently, a study from Zeitlow et al. supported the hypothesis that deregulation of *TOMM40* expression alters mitochondrial function, leading to pathophysiological consequences, including neurological defects [27]. Their data suggests that high expression levels of *TOMM40* may be protective of mitochondrial function and could eventually be an interesting target for therapeutic intervention in AD [27]. The gene *PVRL2*, encodes the poliovirus receptor 2, a member of the immunoglobulin superfamily expressed in diverse cell tissues, including neurons and is recognized to be a risk factor contributing to AD pathogenesis [11]. In our study, both rs12972156 and rs34342646 were the most significantly associated with lower CSF $A\beta_{1-42}$ and higher t-tau levels, suggestive of an influence on increased risk for AD and replicating the GWAS results of Cruchaga et al. [11].

LOC100129500 is located in a region which overlaps *APOE* and *APOC1* and has been associated with AD [28]. From the tested SNPs, rs439401 showed an association with higher CSF $A\beta_{1-42}$ levels and with lower t-tau levels, replicating the associations found by Kim et al., in the recent GWAS of CSF biomarkers ($p < 1 \times 10^{-6}$) [14].

SNAR-I is highly expressed in brain and is involved in neuronal synaptogenesis [11]. The SNPs rs1316356 and rs9877502 were associated with higher CSF t-tau and p-tau levels, but not with $A\beta_{1-42}$. These findings are in line with the results previously reported where *SNAR-I* was found as a novel *locus* associated with tau levels and as genetic variants that influence risk for AD via tau-dependent mechanism [11].

The gene *GLIS3* is also highly expressed in the brain and has been implicated in diabetes mellitus

pathogenesis [23]. In our study, rs9349407 was associated with increased CSF t-tau levels, supporting previous data [11].

A new association was identified on chromosome 20q13.31 within *CASS4* (encoding Cas scaffolding protein family member 4). SNP rs7274581 that was found to be an AD protective variant in the largest meta-analysis to date, with 74,046 individuals [10], in our study was associated with decreased t-tau and p-tau levels and thus in line with the suggested protective effect. The function of the encoded protein is not fully known, but it seems to be involved in cytoskeletal function and axonal transport, and was also implicated in AβPP and tau metabolism [10, 29].

Another novel association was found for *INPP5D* in chromosome 2 (encoding inositol polyphosphate-5-phosphatase). *INPP5D* is expressed at low levels in the brain, but the encoded protein has been shown to interact with *CD2AP*, whose corresponding gene is one of the AD genes previously identified by GWAS, and to modulate, along with GRB2, metabolism of AβPP [10]. Additionally, it has been demonstrated that *INPP5D* could regulate the gene expression and post-translational modification of proteins [10], as well as microglial and myeloid cell function [3]. The SNP rs35349669 seems to decrease t-tau levels possibly having a protective role. The *INPP5D* locus was also recently reported to be associated with p-tau levels, in a recent large genome-wide association study [16].

Another novel association was detected on chromosome 6 at *CD2AP*, where SNP rs9349407 was associated with increased t-tau and presumably with a deleterious effect on AD. This gene encodes a scaffolding protein involved in cytoskeletal reorganization and intracellular trafficking [30]. SNPs within 6q12 locus have been found associated with increased AD risk in several GWAS [8–10]. Additionally, *CD2AP* (rs9349407) has also been found associated with neuritic plaque burden in AD brains [31], thus further supporting a pathogenic role for *CD2AP*.

SNAR-I, GLIS3, CASS4, INPP5D, and *CD2AP* genetic variants influence CSF tau biomarkers, but not the CSF amyloid biomarker Aβ$_{1-42}$. This suggests that they might specifically regulate tau aggregation, processing or clearance other than interfering with AβPP metabolism. As far as we know from their function, reviewed above, they appear to be involved in cytoskeletal reorganization and intracellular trafficking, where they would cooperate or interfere with the tau-dependent processes of microtubule assembly.

We noted a number of changes in the strength of correlation when we corrected for *APOE*. *TOMM40* was no longer associated with CSF Aβ$_{42}$. This confirms previous findings that *TOMM40* is in strong linkage disequilibrium with *APOE*. The effect of *LOC100129500* and *PVRL2* on Aβ$_{42}$ and *CD2AP*, *GLIS3,* and *PVRL2* on tau also became nonsignificant. This suggests that the effect was not independent of *APOE*. Regarding *INPP5, SNAR-I,* and *CASS4*, we found no or minor changes on correlation with CSF markers after *APOE* correction implying a largely independent effect from *APOE*.

In conclusion, our results emphasize the usefulness of exploring AD associated genetic variants and relevant endophenotypes, reinforcing the involvement of specific genes in AD pathogenesis through biologic mechanisms that directly alter CSF levels of Aβ$_{1-42}$, p-tau, and t-tau. In particular, the finding of novel genetic variants that specifically influence CSF tau biomarkers may point out new mechanisms and pathways, largely independent of amyloid processing, which specifically regulate tau aggregation, processing or clearance, eventually suggesting innovative targets for the treatment of AD.

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SUPPLEMENTARY MATERIAL

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