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ORIGINAL ARTICLE

A genome-wide association meta-analysis of plasma Aβ peptides concentrations in the elderly

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Amyloid beta (Aβ) peptides are the major components of senile plaques, one of the main pathological hallmarks of Alzheimer disease (AD). However, Aβ peptides' functions are not fully understood and seem to be highly pleiotropic. We hypothesized that plasma Aβ peptides concentrations could be a suitable endophenotype for a genome-wide association study (GWAS) designed to (i) identify novel genetic factors involved in amyloid precursor protein metabolism and (ii) highlight relevant Aβ-related physiological and pathophysiological processes. Hence, we performed a genome-wide association meta-analysis of four studies totaling 3 528 healthy individuals of European descent and for whom plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides levels had been quantified. Although we did not observe any genome-wide significant locus, we identified 18 suggestive loci ($P < 1 \times 10^{-5}$). Enrichment-pathway analyses revealed canonical pathways mainly involved in neuronal functions, for example, axonal guidance signaling. We also assessed the biological impact of the gene most strongly associated with plasma $A\beta_{1-42}$ levels (cortexin 3, CTXN3) on APP metabolism *in vitro* and found that the gene protein was able to modulate $A\beta_{1-42}$ secretion. In conclusion, our study results suggest that plasma Aβ peptides levels are valid endophenotypes in GWASs and can be used to characterize the metabolism and functions of APP and its metabolites.

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INTRODUCTION

The amyloid beta (Aβ) peptides are the major components of senile plaques—one of the main pathological hallmarks of Alzheimer disease (AD). Abnormal Aβ levels and aggregation of oligomeric Aβ peptides in the brain are thought to trigger a cascade of events that leads to synapse and neuron loss, progressive cognitive impairment and ultimately dementia.

The two major Aβ species ($A\beta_{x-40}$ and $A\beta_{x-42}$) are produced by sequential endoproteolysis of amyloid precursor protein (APP) by β-secretase and γ-secretase complex. Amyloid precursor protein can also undergo non-amyloidogenic proteolysis by α-secretase, which cleaves APP within the Aβ sequence and thereby precludes Aβ generation.[1] Although APP metabolism has been extensively studied, it is likely that some of the factors involved in this complex process have yet to be identified and characterized.

Beyond the role of Aβ peptides in the AD process, little is known about their physiological roles which seem to be highly pleiotropic. Several lines of evidence suggest that Aβ peptides have a broad spectrum of biological functions: (i) they are produced by many different cell types; (ii) they are present in peripheral tissues as well as in the brain; (iii) they may act as ligands for various receptors and other molecules;[2,3] (iv) they variously display neurotrophic, antioxidant, antimicrobial, vaso-constrictive and platelet aggregation-modulating properties at physiological concentrations.[4–8]

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[23]Data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.ucla.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be found at: http://adni.loni.ucla.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf.

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With a view to identifying novel factors involved in APP metabolism and characterizing Aβ-related physiological and pathophysiological processes, we hypothesized that Aβ peptides concentrations in biological fluids may constitute a suitable endophenotype for a genome-wide association study (GWAS). We decided to focus on plasma Aβ peptides concentrations for several reasons: (i) plasma samples are easy to collect and thus facilitate meta-analyses of individuals with both Aβ peptides concentrations and GWAS data; (ii) plasma Aβ peptides are associated with risk of incident dementia and hypertension;[9–12] (iii) plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ levels are heritable traits.[13]

Within this context, we developed a genome-wide association meta-analysis of four studies totaling 3 528 healthy individuals of European descent and for whom plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides levels had been assayed.

MATERIALS AND METHODS

Populations

The three-city (3C) study. The 3C study is a prospective cohort study of vascular risk factors for dementia. The methodology of the study has been described in detail elsewhere.[14] The 3C study's protocol was approved by the independent ethics committee at Kremlin-Bicêtre University Hospital (Paris). In 1999–2000, a sample of 9 294 community dwellers aged 65 and over was selected from the electoral rolls of three French cities: Bordeaux ($n = 2\,104$), Dijon ($n = 4\,931$) and Montpellier ($n = 2\,259$). Of these, 880 participants were excluded due to lack of a blood sample or lack of participation in any of the follow-up examinations. This left a sample of 8 414 participants. A case-cohort study (3C1) was performed after 4 years of follow-up, in order to investigate non-standard risk markers for dementia, stroke and coronary heart disease.[10] This sub-cohort was composed of 1 254 participants randomly selected in strata according to center, age (in 5-year age groups) and gender. Participants diagnosed with prevalent dementia at baseline or incident dementia during follow-up were excluded from the present analysis. Participants for whom at least one plasma Aβ concentration or covariate was missing and participants with an aberrant plasma Aβ concentration (more than four standard deviations above or below the mean) were also excluded. Finally, we excluded individuals of non-European descent or with missing genetic information. These selection steps allowed us to define a sample of 909 individuals. Another subset of 1 169 participants from the 3C Dijon Center (3C2) in whom plasma Aβ levels had been recently assayed was also available. A sample of 911 individuals was analyzed after application of the selection steps mentioned above.

The Rotterdam study. The Rotterdam study is an ongoing, prospective, population-based cohort study investigating risk factors and incidence of cardiovascular, neurodegenerative, locomotor and ophthalmological diseases in elderly people.[15,16] From 1990–1993, all 10 275 residents of Ommoord (a district of Rotterdam) aged 55 years or older were invited to participate in an extensive home interview and two visits to the research center; 7 983 (78%) agreed. At the baseline clinical examination, blood samples were drawn from 7 050 individuals, of whom 7 047 underwent screening for dementia. Prevalent dementia was diagnosed in 334 of the latter. Hence, the cohort at risk of dementia comprised 6 713 participants. A random sub-cohort of 1 756 people was drawn from this source population for plasma Aβ concentration assessment.[9] Individuals for whom at least one plasma Aβ concentration or co-variable measurement was missing were excluded, leading to final analysis set of 1 490 individuals.

The Pittsburgh cardiovascular health study cognition study (CHS-CS). This study began in 1992–1994, at the time when the participants underwent an initial brain MRI scan.[17] In 2002–2003, the incidence of dementia and mild cognitive impairment (MCI) diagnosed in 1998–1999 in the CHS-CS population was determined. Of the 924 participants examined in 1992–-1994, a total of 532 normal and MCI participants were available for study in 1998–1999. These participants had undergone annual cognitive tests from 1989–1990 to 1998–1999 and complete neurologic and neuropsychological examinations in 1998–1999 and 2002–2003. In addition to the brain MRI data set obtained in 1992–1994, a second MRI session was performed in 1998–1999 and 157 participants also underwent MRI in 2002–2003. The brain MRI in 2002–2003 was performed when a participant's status changed from normal to MCI, from MCI to dementia or from normal to dementia. Participants were included in this analysis if they were alive at both time points, had available blood samples from both 1998–1999 and 2002–2003 and had been classified according to the CHS' cognitive criteria. Application of the exclusion criteria used in the 3C and Rotterdam study lead to an analysis set of 73 participants.

The Alzheimer's disease neuroimaging initiative study (ADNI). The ADNI study is a prospective, multicentre, longitudinal neuroimaging study that was launched in the USA in 2004 by the National Institute on Aging, the National Institute of Biomedical Imaging and Bioengineering, the Food and Drug Administration, private pharmaceutical companies and non-profit organizations.[18] It includes 819 adult participants aged 55–90 who fulfilled the entry criteria (a clinical diagnosis of amnestic MCI, probable AD or cognitively normal). The collected data encompasses clinical information, neuroimaging data and biological samples for molecular biomarker measurement (for more details, see http://www.adni-info.org). We further classified participants as 'stable' if they did not change their diagnostic status from cognitively normal to MCI/AD or from MCI to AD during a follow-up period of at least 36 months. Participants other than stable cognitively normals were excluded. After applying the same criteria as in the 3C study, 145 participants were included in the analyses.

Genotyping and imputation

All the above-mentioned studies used different Illumina platforms for genome-wide genotyping; these are described in detail elsewhere[19–21] and summarized in Supplementary Material Table 1. In order to have a common set of single-nucleotide polymorphisms (SNPs) available for meta-analysis and after selecting individuals and genotyped SNPs on the basis of call rates, minor allele frequency and Hardy–Weinberg equilibrium test thresholds, SNPs were imputed in each study using the Hapmap 2 release 22 CEU population as a reference panel (see Supplementary Material Table 1 for precise filters used). All data sets were recoded to match the '+' strand.

Amyloid beta peptide assays

The assays used in the different studies have been described in detail elsewhere.[9,10,17,22] Briefly, in the 3C study, non-fasting plasma samples were collected in tubes containing sodium EDTA as an anticoagulant. After centrifugation, plasma samples were divided into aliquots in polypropylene tubes, stored at −80 °C and only thawed immediately before Aβ quantification. The plasma Aβ peptide assay was performed using an INNO-BIA kit (Innogenetics NV, Ghent, Belgium) based on a multiplex xMAP (Luminex, Austin, TX, USA) technique.

In the Rotterdam study, non-fasting blood samples obtained at baseline were placed in Vacutainer tubes (Becton Dickenson, Franklin Lakes, NJ, USA) containing sodium citrate. These samples were put on ice immediately and centrifuged within 60 min. Aliquots of plasma were stored at −80 °C. Plasma Aβ concentrations were determined using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) method (Pfizer, New York, NY, USA).

In the CHS-CS, the samples used for the assays (collected in 1998–1999 and 2002–2003) were defrosted in 2006 and measured at the same time using the same method. Plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ levels were measured with a double-antibody sandwich ELISA based on a combination of the mouse monoclonal antibody 6E10 (which is specific for an epitope present within amino acid residues 3–16 of Aβ) and two different, specific anti-$A\beta_{1-40}$ and -$A\beta_{1-42}$ antibodies.

In the ADNI study, plasma samples were obtained from the ADNI biofluid repository at the University of Pennsylvania. The plasma samples were collected at the participating centers. After overnight fasting, plasma was collected in the morning by venipuncture and placed in Vacutainer tubes containing potassium K3 ethylene tetraacetate as an anticoagulant. After centrifugation, samples were placed in polypropylene transfer tubes (13 ml, Sarstedt Inc., Newton, NC, USA catalog number 60.541), frozen and shipped on dry ice to the UPenn Biomarker Core Laboratory, where they were stored temporarily at −80 °C. Within several weeks of receipt, the samples were thawed, aliquoted by 500 µl into polypropylene tubes (1.5 ml, Thermo Fisher Scientific, Waltham, MA, USA catalog number 05-408-129) and stored at −80 °C pending biochemical analyses. The plasma levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ were quantified with the same INNO-BIA kit (Innogenetics NV, Ghent, Belgium) as in the 3C study.

Of note, all the characteristics of the populations are described in the Supplementary Table 3.

Cell culture and western blot analyses

The HEK293-APP^{695wt} cell line was maintained in DMEM supplemented with 10% FBS, penicillin and streptomycin at 37 °C in a humidified atmosphere with 5% CO2. Transient transfection was performed using X-tremeGENE HP DNA Transfection Reagent (Roche Applied Science, Rotkreuz, Switzerland) according to the manufacturer's recommendations. The pCNV6entry-mycCTXN3 vector (NM_001048252) containing RC215242 (a Myc-DDK-tagged ORF clone of the *Homosapiens* cortexin 3 gene) was purchased from Origene (Rockville, MD, USA). After 48 h of transfection, supernatants were replaced. After 4 h, cells were finally lysed and supernatants were recovered. Cell extracts (10–30 µg) were analyzed by SDS–PAGE. The antibody against the c-myc epitope (Life Technologies, catalog number 46-0603, Grand Island, NY, USA) was diluted 1/5000; The primary antibody used to measuring holo-APP and APP-CTF is the APPCter-C17, a well-characterized rabbit antibody raised against the last 17 amino acids of the human APP sequence[23,24] and was diluted 1/10 000. A monoclonal mouse antibody (Sigma-Aldrich, St Louis, MO, USA) was used to detect β-actin (dilution: 1/10 000). Immunoreactive complexes were revealed using the ECL western blotting kit (Amersham, Piscatway, NJ, USA). Membranes were digitized using the ChemiDoc MP System (Bio-Rad, Marnes-la-Coquette, France).

Elisa

$A\beta_{1-40}$ and $A\beta_{1-42}$ peptides concentrations were measured using sandwich ELISAs (the Human Amyloid β (1–40) Assay Kit (IBL-Hamburg, Germany) and the INNOTEST β-Amyloid (1–42) (Innogenetics NV)), according to the manufacturer's recommendations. The results were read at 450 nm with a spectrophotometer (Labsystems Multiskom MS, Waltham, MA, USA). At least three independent triplicate experiments in were performed. All ELISAs were performed twice on a same sample.

Immunofluorescence

Cells were washed with PBS and fixed in PBS containing 4% paraformaldehyde for 30 min at room temperature. Cells were permeabilized with 0.25% Triton X-100 in PBS for 10 min. After blocking in 1% bovine serum albumin (BSA), cells were incubated for 2 h at room temperature with primary antibodies listed above (diluted 1/100 in 1% BSA in PBS). Cells were then washed three times with PBS. Appropriate secondary antibodies (Alexa Fluor, Invitrogen, Grand Island, NY, USA) diluted at 1/400 were used. After washing, slides were mounted with Fluoromount (Sigma-Aldrich).

Proximity ligation assay (PLA)

All reagents used in the PLA were purchased from Olink Bioscience (Uppsala, Sweden). The PLAs were performed according to the manufacturer's instructions by using anti-myc (diluted 1/100) and anti-holoAPP (diluted 1/100) as primary antibodies.

Genome-wide association analyses

Linear regression models were used to assess associations between genetic markers and plasma Aβ levels in each study. In each data set, plasma Aβ levels were transformed as z-scores by subtracting the mean and dividing by the standard deviation, in order to account for differences in assay methods. SNPs data were analyzed either as allele dosages or imputation probabilities, depending on the imputation software used (see Supplementary Material Table 1). All models were systematically adjusted for age at blood collection and gender. An additional adjustment for study center was done in the 3C1 sample. Population substructures were taken into account by adjustment for principal components when they were significantly associated with plasma Aβ levels. Of note, all these adjustments did not notably modify the association observed for the best signals (see Table 1 and Supplementary Table 5 for comparison).

Genome-wide meta-analysis

Single-nucleotide polymorphisms with a minor allele frequency below 1% or bad imputation quality (info score < 0.8 or r-squared < 0.3, depending on the imputation software used) were excluded (see Supplementary Table 2 for number of SNPs removed during filtering). A fixed-effect, inverse-variance weighted meta-analysis was then performed. Quantile-quantile (QQ) plots and the genomic inflation factor (lambda) were used to detect potential population stratification in each data set and in the meta-analyses (see Supplementary Material Figure 1). Additional correction on genomic inflation factor was performed and did not change the results. Genome-wide significance was defined for *P*-values below 5×10^{-8} and suggestive association was defined for *P*-values comprised between 1×10^{-5} and 5×10^{-8}. SNPs showing suggestive associations with plasma Aβ levels were examined manually and forest plots were used to detect extreme heterogeneity or aberrant values. Heterogeneity was also assessed by performing random-effect meta-analyses and the Cochran's *Q*-test.

Of note, the METAL script used for the GWAS analysis is described in the Supplementary method.

Pathway analyses

We selected a list of 1 762 genes containing at least one SNP associated with both plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ ($P < 0.05$) concentrations with the same direction of effect for pathway analyses using two softwares: Ingenuity Pathway Analysis and WebGestalt.

Ingenuity Pathway Analysis (IPA, November 2012) was used to detect enriched canonical pathways. Canonical pathways were determined by analyzing a ratio of the number of genes that map to the pathway divided by the total number of genes in the pathway using the Ingenuity knowledge base. This base contains expertly curated biological interactions and functional annotations from literature. We considered only direct and indirect experimentally observed relationships for this analysis. A Fisher exact test was used and to assess the significance of a pathway, we applied a Benjamini–Hochberg multiple testing correction.

WebGestalt (WEB-based Gene SeT AnaLysis Toolkit) incorporates different public resources and provides a gene-set enrichment analysis. We used the web version updated on 30th of January, 2013 and conducted a gene-set enrichment analysis on our gene list using Gene Ontology (version 1.2, 11/11/2012) as a reference set. We selected only gene ontology categories with a minimum of two genes. The enrichment analysis is based on an hypergeometric test and we corrected the *P*-value by a Benjamini–Hochberg multiple testing correction.

Software

Individual study analyses were performed with either R version 2.15.1 (2012-06-22),[25] Plink,[26] ProbABEL[27] or SNPTEST version 2.2 (https://mathgen.stats.ox.ac.uk/genetics_software/snptest/snptest.html). Genome-wide meta-analysis was performed with METAL.[28] Manhattan plots and quantiles-quantiles plots were generated for each study and for meta-analyses with *R* functions taken from the Getting Genetics Done blog (http://gettinggeneticsdone.blogspot.com/2011/04/annotated-manhattan-plots-and-qq-plots.html). For the top SNPs, random-effect meta-analyses were performed with the metafor *R* package.[29] Forest plots were generated using the rmeta *R* package (http://CRAN.R-project.org/package=rmeta). Regional association plots were generated using LocusZoom version 1.1 (http://genome.sph.umich.edu/wiki/LocusZoom_Standalone). Pathway analyses were performed using Ingenuity Pathway Analysis (http://www.ingenuity.com/products/ipa) and WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/).[30]

RESULTS

The total sample was composed of 3 528 non-demented participants from European descent from the three-city study from France ($n = 1\,820$), the Rotterdam study from the Netherlands ($n = 1\,490$), the Cardiovascular Health Study–Cognition Study from the US (CHS-CS; $n = 73$) and the Alzheimer's Disease Neuroimaging Initiative from the US (ADNI; $n = 145$). As expected, plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ concentrations were strongly correlated in each study and significantly differed across studies (one-way analysis of variance (ANOVA) *P*-values $< 10^{-16}$ for plasma $A\beta_{1-42}$ and $A\beta_{1-40}$ concentrations, see Supplementary Material Table 3). This latter observation led us to transform plasma Aβ levels into z-scores for each study. In each population, associations between $A\beta_{1-42}$ or $A\beta_{1-40}$ levels and SNPs were then evaluated in linear regression models adjusted for age, gender, center and principal components. After removing SNPs with low minor allele frequencies or bad imputation quality scores from each study (see Supplementary Material Table 2), we selected 2 316 178 SNPs present at least in the three-city and Rotterdam studies for meta-

Table 1. Results of a genome-wide meta-analysis of plasma Aβ levels

SNP	*Chr.*	*Position*	*Intragenic*	*Closest gene (50 kb)*	*Minor/major alleles*	*MAF (%)*	*$Aβ_{1–42}$*					
							Direction	*Effect size*	*s.e.*	*I^2 (%)*	*P for Cochran's* Q	*P in GWAS*
$Aβ_{1–42}$												
rs11241936	5	127006007	No	CTXN3	C/T	31.2	+++−+	0.08	0.02	38	0.17	2.32e−07
rs12761450	10	61555898	Yes	ANK3	T/G	41.5	−−−−−	−0.07	0.02	0	0.83	1.24e−06
rs12611088	19	48764642	Yes	XRCC1	A/G	37.1	+++++	0.07	0.02	0	0.54	1.30e−06
rs17655565	12	50978225	Yes	KRT86	C/T	10.9	+++++	0.12	0.02	0	0.61	1.76e−06
rs12656502	5	108009646	No	None	A/G	22.8	++++−	0.08	0.02	22	0.28	3.27e−06
rs2176862	3	46625997	No	LOC100132146	T/C	5.4	++++−	0.15	0.03	34	0.19	4.49e−06
rs7138951	12	79999764	Yes	ACSS3	G/A	9.4	++++−	0.12	0.03	0	0.56	5.03e−06
rs10819795	9	102408184	No	MURC	A/T	2.5	−++++	0.23	0.05	34	0.19	7.36e−06
$Aβ_{1–40}$												
rs3015469	14	50226910	No	SAV1	G/A	31.4	+++−−	0.04	0.02	34	0.19	2.39e−02
rs1335688	13	62292794	No	None	C/T	15.3	+++++	0.07	0.02	44	0.13	1.14e−03
rs1995809	4	148063874	Yes	TTC29	G/A	5.4	+++++	0.04	0.03	15	0.32	2.71e−01
rs7151302	14	32753868	Yes	NPAS3	C/T	13.6	−−−−+	−0.08	0.02	65	0.02	3.08e−04
rs12422267	12	131167549	Yes	EP400NL	G/A	9.5	+++++	0.04	0.02	23	0.27	1.04e−01
rs2403083	8	86295401	Yes	E2F5	C/A	26.1	−−−−−	−0.04	0.02	0	0.72	3.44e−02
rs1341320	1	57739116	Yes	DAB1	G/A	6.4	−−−−−	−0.09	0.03	0	0.88	2.33e−03
rs4263408	4	39461671	No	UBE2K	T/C	43.0	−+−++	−0.03	0.02	62	0.03	5.76e−02
rs8001893	13	33120261	Yes	STARD13	A/C	2.5	++++?	0.14	0.05	0	0.87	8.52e−03
rs108961	11	2714561	Yes	KCNQ1	T/C	47.4	+++++	0.03	0.02	72	0.007	2.85e−02

SNP	*Chr.*	*Position*	*Intragenic*	*Closest gene (50 kb)*	*Minor/Major alleles*	*MAF (%)*	*$Aβ_{1–40}$*					
							Direction	*Effect size*	*s.e.*	*I^2 (%)*	*P for Cochran's* Q	*P in GWAS*
$Aβ_{1–42}$												
rs11241936	5	127006007	No	CTXN3	C/T	31.2	+++−+	0.03	0.02	27	0.24	1.22e−01
rs12761450	10	61555898	Yes	ANK3	T/G	41.5	++−−+	−0.01	0.02	0	0.52	7.51e−01
rs12611088	19	48764642	Yes	XRCC1	A/G	37.1	++++−	0.07	0.02	22	0.28	7.58e−04
rs17655565	12	50978225	Yes	KRT86	C/T	10.9	+++++	0.07	0.03	0	0.79	3.74e−02
rs12656502	5	108009646	No	None	A/G	22.8	−−++−	0.04	0.02	53	0.07	1.45e−01
rs2176862	3	46625997	No	LOC100132146	T/C	5.4	−+++−	0.05	0.04	6	0.37	2.74e−01
rs7138951	12	79999764	Yes	ACSS3	G/A	9.4	++++−	0.10	0.04	15	0.32	1.06e−02
rs10819795	9	102408184	No	MURC	A/T	2.5	−++−+	0.09	0.07	37	0.17	2.18e−01
$Aβ_{1–40}$												
rs3015469	14	50226910	No	SAV1	G/A	31.4	+++++	0.11	0.02	54	0.07	8.35e−07
rs1335688	13	62292794	No	None	C/T	15.3	+++++	0.14	0.03	0	0.69	1.04e−06
rs1995809	4	148063874	Yes	TTC29	G/A	5.4	+++−+	0.22	0.05	51	0.08	1.60e−06
rs7151302	14	32753868	Yes	NPAS3	C/T	13.6	−−−−−	−0.14	0.03	0	0.57	1.71e−06
rs12422267	12	131167549	Yes	EP400NL	G/A	9.5	+++++	0.17	0.03	29	0.23	1.87e−06
rs2403083	8	86295401	Yes	E2F5	C/A	26.1	−−−−−	−0.11	0.02	0	0.96	3.27e−06
rs1341320	1	57739116	Yes	DAB1	G/A	6.4	−−−++	−0.18	0.04	0	0.56	5.40e−06
rs4263408	4	39461671	No	UBE2K	T/C	43.0	−−−++	−0.09	0.02	47	0.11	6.98e−06
rs8001893	13	33120261	Yes	STARD13	A/C	2.5	++++?	0.30	0.07	0	0.61	7.11e−06
rs108961	11	2714561	Yes	KCNQ1	T/C	47.4	+++++	0.09	0.02	34	0.20	9.34e−06

SNP: SNPs with the lowest *P*-value in a given locus for a particular plasma Aβ concentration. Chr.: chromosome. Position: positions of the SNPs (based on dbSNP build 130, Hg18 coordinates). MAF: minor allele frequency. Direction: directions of effect. The order is: 3C1, 3C2, Rotterdam, ADNI and CHS. s.e.: standard error.

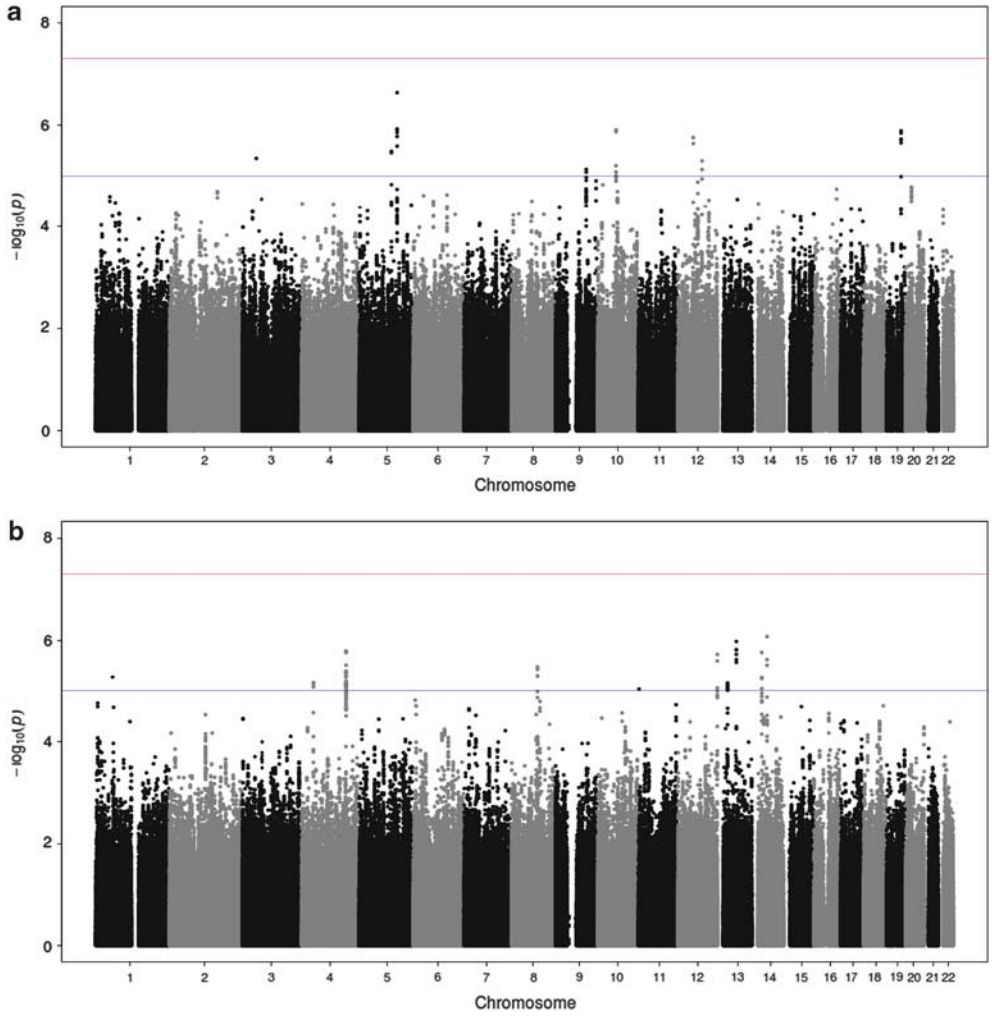

Figure 1. Results of a genome-wide meta-analysis of plasma Aβ levels. The plots represent the $-\log_{10}$(*P*-values) of the SNPs on *the y* axis and chromosome positions on the *x* axis for (**a**) $A\beta_{1-42}$ and (**b**) $A\beta_{1-40}$ plasma levels. The red line indicates the genome-wide significance threshold (5×10^{-8}) and the blue line indicates the suggestive significance threshold (1×10^{-5}). SNP, single-nucleotide polymorphism.

analysis. We filtered SNPs showing extreme heterogeneity (*P*-value in the Cochran's *Q*-test < 0.0001), excluding 1 725 SNPs for $A\beta_{1-42}$ and 1 744 SNPs for $A\beta_{1-40}$. QQ plots of the *P*-values were then generated (see Supplementary Material Figure 1). For both $A\beta_{1-42}$ and $A\beta_{1-40}$, no inflation of *P*-values was observed; the overall genomic inflation factors (lambda) were 1.02 for $A\beta_{1-42}$ and 0.99 for $A\beta_{1-40}$.

Manhattan plots of plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ *P*-values are presented in Figure 1. Although none of the meta-analyzed SNPs attained genome-wide significance (*P*-values $< 5\times10^{-8}$), several suggestive signals (with *P*-values between 5×10^{-8} and 1×10^{-5}) were identified. The SNPs with the lowest *P*-value in these loci are presented in Table 2 (see also detailed results in Supplementary Material Table 4 and forest plots and regional association plots in Supplementary Material Figures 2 to 19). We were unable to set up a replication stage due to the low number of available cohorts with both GWAS and plasma Aβ concentration data. We therefore decided to develop a number of complementary statistical, bioinformatics and biological approaches to assess the relevance of our results.

We first hypothesized that the genetic determinants of plasma Aβ concentrations were clustered within one or more specific biological pathways, rather than being randomly distributed. This type of analysis can indicate relevant pools of genes likely to genetically modulate APP metabolism and thus can provide clues about the function of Aβ peptides and APP. Hence, we generated a list of 1 762 genes containing at least one SNP associated with both plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ ($P < 0.05$) concentrations with the same direction of effect. Using Ingenuity Pathway Analysis (IPA), we observed 27 significantly enriched canonical pathways (out of 287) after multiple testing correction. These results are presented in Table 2. Interestingly, some of the pathways have been already described as being involved in APP metabolism (e.g., protein kinase A signaling and netrin signaling).[31–34] Other pathways appear to be related to potential physiological functions of APP in the brain (e.g., axonal guidance signaling; see Supplementary Material Table 6)[35,36] or directly associated with Aβ peptides' properties, for example, blood pressure modulation (renin-angiotensin signaling).[11] As pathway analyses depend on the tool, algorithm and pathway database used, we attempted to confirm our findings using the Webgestalt software. As a result, we observed 120 gene ontology categories significantly enriched ($P < 0.05$ after correction) (See Table 2 for the most significant pathways). In line with the IPA findings, most of the significantly enriched pathways were related to potential physiological functions of APP in the brain and particularly, axonal guidance (see Supplementary Material Table 7 for the list of genes associated with Aβ plasma level and involved in this pathway).

Table 2. Results of the ingenuity pathway or Webgestalt analysis for genes which presented at least one SNP associated with both plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ ($P < 0.05$) concentrations and with the same direction of effect

Ingenuity canonical pathways	*Corrected* P-*value*[a]	*Webgestalt gene ontology categories*	*GO ID*	*Corrected* P-*value*[a]
Protein kinase A signaling	3.72E – 06	Neuron development	GO:0048666	9.48E – 10
Axonal guidance signaling	2.00E – 05	Neuron projection development	GO:0031175	9.48E – 10
Synaptic long term potentiation	3.47E – 04	Nervous system development	GO:0007399	1.28E – 09
Melatonin signaling	1.07E – 03	Axon guidance	GO:0007411	1.60E – 09
Role of NFAT in cardiac hypertrophy	2.34E – 03	Neurogenesis	GO:0022008	2.04E – 09
Corticotropin releasing hormone signaling	2.34E – 03	Generation of neurons	GO:0048699	2.04E – 09
Dopamine-DARPP32 feedback in cAMP signaling	2.88E – 03	Cell projection organization	GO:0030030	2.09E – 09
ErbB signaling	2.88E – 03	Axonogenesis	GO:0007409	2.29E – 09
Fcγ receptor-mediated phagocytosis in macrophages and monocytes	3.02E – 03	Cell projection	GO:0042995	2.55E – 09
Synaptic long term depression	3.09E – 03	Cell junction	GO:0030054	2.55E – 09
CREB signaling in neurons	3.16E – 03	Neuron projection morphogenesis	GO:0048812	2.61E – 09
GNRH signaling	3.16E – 03	Cell morphogenesis involved in neuron differentiation	GO:0048667	2.61E – 09
Cellular effects of sildenafil (Viagra)	3.80E – 03	Neuron differentiation	GO:0030182	2.61E – 09
Netrin signaling	7.24E – 03	Multicellular organismal signaling	GO:0035637	3.43E – 09
Neuregulin signaling	8.13E – 03	Transmission of nerve impulse	GO:0019226	1.40E – 08
Hepatic cholestasis	1.15E – 02	Cell development	GO:0048468	3.13E – 08
Renin-angiotensin signaling	1.38E – 02	Neuron projection	GO:0043005	5.17E – 08
α-Adrenergic signaling	1.51E – 02	Cell projection morphogenesis	GO:0048858	5.33E – 08
Endothelin-1 signaling	1.74E – 02	Cell morphogenesis involved in differentiation	GO:0000904	5.43E – 08
Calcium signaling	1.74E – 02	Plasma membrane	GO:0005886	5.51E – 08
G beta gamma signaling	1.74E – 02	Cell part morphogenesis	GO:0032990	6.74E – 08
Gap junction signaling	2.04E – 02	Cell periphery	GO:0071944	7.59E – 08
GABA receptor signaling	3.02E – 02	Cell projection part	GO:0044463	8.12E – 08
P2Y purigenic receptor signaling pathway	3.24E – 02	Synapse	GO:0045202	1.03E – 07
PTEN signaling	3.24E – 02	Cell adhesion	GO:0007155	1.11E – 07
Sonic hedgehog signaling	3.24E – 02	Biological adhesion	GO:0022610	1.23E – 07
PI3K signaling in B lymphocytes	3.24E – 02	Membrane	GO:0016020	2.25E – 07

[a]Benjamini–Hochberg correction.

To account for the eventuality of an artificial enrichment due to linkage disequilibrium between SNPs, we repeated IPA and Webgestalt analyses after filtering SNPs with r^2 value $\geqslant 0.5$ when comparing different SNPs. This resulted in the exclusion of three and four SNPs, respectively, and we were still able to identify axon guidance pathways displaying the overrepresentation of genes associated with Aβ plasma levels in our GWA data set (data not shown). We finally assessed the degree of consistency among data sets. We did not independently test CHS and ADNI because of their restricted sample size, and focused on results obtained from the Rotterdam study and in the combined 3C study. We observed consistent results among these data sets. For instance, using IPA, the Axonal guidance pathway was enriched in both Rotterdam and 3C studies (respectively, $P = 7.1 \times 10^{-3}$ and $P = 4.4 \times 10^{-3}$ after multiple testing correction). A similar observation was obtained using Webgestalt ($P = 1.2 \times 10^{-6}$ in 3C and 8.0×10^{-4} in Rotterdam after multiple testing correction).

We next took the gene most strongly associated with the plasma $A\beta_{1-42}$ level (cortexin 3, CTXN3) in the meta-analysis and studied its influence on APP metabolism at the biological level. The results are presented in Figure 2. We tested the effect of CTXN3 overexpression on Aβ peptide secretion in a HEK cell line that stably expressed the APP^{695wt} isoform. Following CTXN3 overexpression, $A\beta_{1-42}$ secretion was significantly lower than in control experiments (37% lower; $P = 0.02$). A trend in the same direction was observed for $A\beta_{1-40}$ secretion (27% lower, $P = 0.10$). Furthermore, we observed colocalization of APP and CTXN3 in HEK293 cells overexpressing both proteins. In order to control partially for potential biases due to proteins overexpression, we repeated the immunofluorescence experiments from endogenous APP and overexpressed CTXN3 in HEK293 cells (HEK293 cells do not express CTXN3). We still observed colocalization of APP and CTXN3 in this model. In conclusion, these data suggest that CTXN3 may lead to a decrease in Aβ peptide production by directly modulating APP metabolism.

Finally, we tried to replicate previously reported associations between SNPs and plasma $A\beta_{1-42}$ levels and explored whether SNPs associated with cerebrospinal fluid concentrations of $A\beta_{1-42}$ or AD risk would also be associated with plasma Aβ levels (see Table 3 and Supplementary Material Table 8). Only rs2075650, located in the APOE locus presented nominal level of association with plasma $A\beta_{1-42}$ concentrations (P-value $= 2.81 \times 10^{-2}$; see Table 3).

DISCUSSION

To our knowledge, the present study constitutes the first genome-wide association meta-analysis of plasma Aβ concentrations in non-demented participants. Although we did not observe any genome-wide significant locus, we identified 18 suggestive loci. Pathway analyses showed enrichment of canonical pathways involved in neuronal functions, for example, axonal guidance signaling. We also assessed the biological impact of the gene most strongly associated with plasma $A\beta_{1-42}$ levels (cortexin 3, CTXN3) on APP metabolism *in vitro* and found that the gene protein was able to modulate $A\beta_{1-42}$ secretion.

Set-up and performance of the study was subject to several methodological and technological limitations. First, environmental factors modulating Aβ plasma concentrations are poorly known. Thus, it is difficult to assess whether differences in alimentary habits or in medications across samples could influence our results. Second, plasma Aβ concentrations were assayed using

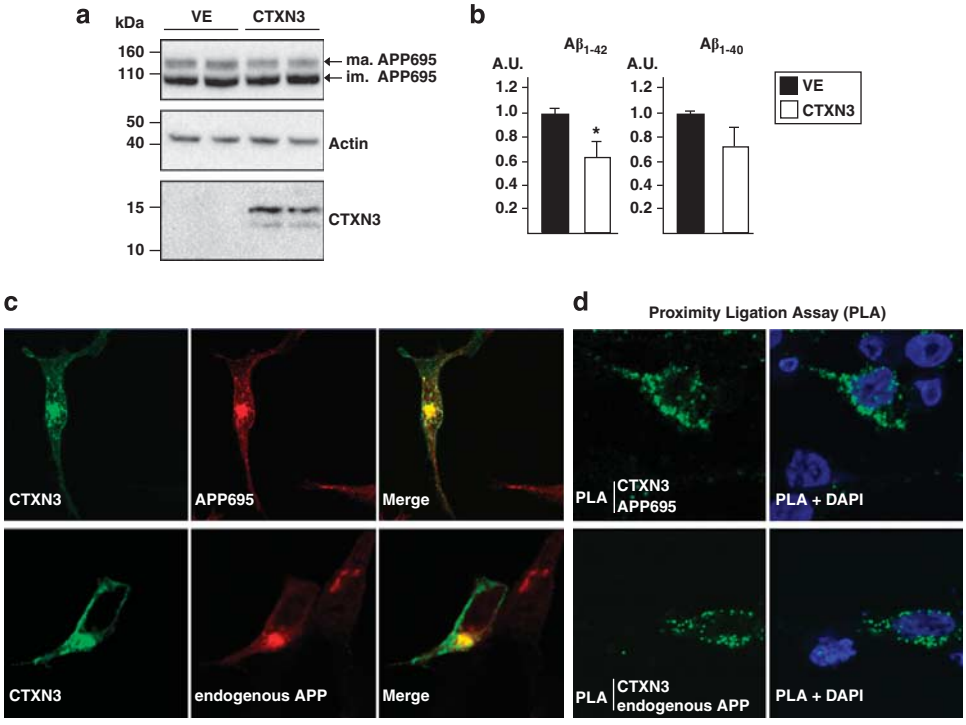

Figure 2. Overexpression of CTXN3-myc in a HEK293 cell line. (**a**) Representative western blots of extracts from HEK293-APP^{695wt}—stably transfected with APP^{695wt}—and transiently transfected with *CTXN3-myc* cDNA or a control empty plasmid (vector). Anti-APP (APPCter-C17), anti-myc (Invitrogen 46-0603) and anti-actin antibodies have been used for western blots. This experiment was repeated three times. (**b**) Measurement of secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ by ELISA following transfection of the CTXN3-myc expression vector in APP-HEK293 cells. Variations in $A\beta_{1-42}$ (left) and $A\beta_{1-40}$ (right) secretion from three independent experiments (performed in duplicate) are shown. (**c**) Representative confocal images from immunofluorence staining of HEK293-APP^{695wt} cells (APP695) or HEK293 (endogenous APP) transfected with CTXN3-myc using anti-myc (Invitrogen 46-0603, green) and anti-APP (APPCter-C17, red) antibodies. (**d**) An *in situ* proximity ligation assay measuring the interaction between overexpressed CTXN3 and holoAPP in HEK293 cells transfected as previously described above (in panel **c**). *$P < 0.05$ in a Mann–Whitney non-parametric test); AU: arbitrary units.

Table 3. Results of a meta-analysis of plasma Aβ levels for SNPs known to be associated with the Alzheimer's disease (AD) risk

SNP	*Chr.*	*Position*	*Gene*	*Minor/ major alleles*	*MAF (%)*	*$A\beta_{1-42}$*					*$A\beta_{1-40}$*				
						Direction	*Effect size*	*s.e.*	*P in GWAS*	*I^2 (%)*	*Direction*	*Effect size*	*s.e.*	*P in GWAS*	*I^2 (%)*
rs3818361	1	205851591	CR1	A/G	19.4	−−−−+	−0.0341	0.0184	6.41e−02	0	+−+−−	−0.0189	0.0255	4.61e−01	60
rs744373	2	127611085	BIN1	G/A	28.9	+−−++	−0.0046	0.0163	7.78e−01	0	+−−+−	−0.009	0.0225	6.91e−01	41
rs9296559	6	47560229	CD2AP	C/T	30.8	++−−+	0.005	0.0166	7.64e−01	0	++−++	0.0136	0.0228	5.49e−01	18
rs11767557	7	142819261	EPHA1	C/T	19.9	−−−+−	−0.0205	0.0181	2.56e−01	0	−−−++	−0.0193	0.0249	4.39e−01	54
rs11136000	8	27520436	CLU	T/C	39.2	−+−−+	−0.0032	0.0152	8.35e−01	11	−−−−+	−0.0151	0.0209	4.68e−01	0
rs610932	11	59695883	MS4A6A	T/G	43.8	++−−−	0.0035	0.0148	8.16e−01	26	+−+−−	0.0185	0.0203	3.62e−01	36
rs3851179	11	85546288	PICALM	T/C	36.7	−−−++	−0.0076	0.0151	6.16e−01	63	−−−++	−0.0366	0.0208	7.90e−02	58
rs3764650	19	997520	ABCA7	G/T	8.7	+−−++	−0.0136	0.0267	6.11e−01	0	+−−−+	−0.0305	0.0355	3.91e−01	0
rs2075650	19	50087459	APOE	G/A	14.0	−−−−+	−0.0466	0.0212	2.81e−02	77	−−+−+	0.0167	0.0307	5.87e−01	23
rs3865444	19	56419774	CD33	A/C	32.4	−−+−−	−0.0016	0.0159	9.21e−01	0	−−+−−	−0.0156	0.022	4.78e−01	0

Chr.: chromosome. Position: positions of the SNPs (based on dbSNP build 130, Hg18 coordinates). MAF: minor allele frequency. Direction: directions of effect. The order is: 3C1, 3C2, Rotterdam, ADNI and CHS. s.e.: standard error.

different approaches and thus were not fully comparable. Therefore, we transformed the data into z-scores prior to the statistical analyses to minimize between-center variability. Third, few previous studies had both plasma Aβ concentrations and GWAS data available at the time of this study. This limited the power of our meta-analysis and we failed to generate results that were statistically significant on the genome-wide scale. Given the high number of association tests usually performed in GWASs, the

likelihood of false-positive results is high and is mitigated by applying highly conservative alpha-risk corrections (e.g., the Bonferroni correction in many cases). Furthermore, our results may be influenced by the simultaneous involvement of many biological processes modulating Aβ peptide concentrations in plasma (e.g., peptide production, secretion, degradation and/or clearance); this would have weakened our ability to detect significant genome-wide signals. Finally, the main limitation of our study relates to the difficulty in obtaining relevant replication samples; this made it impossible for us to discriminate between false and true positives. One option would have been to split our data sets between discovery and replication stages. However, we considered that an excessively underpowered discovery stage would have led to a large number of false negatives.

Given these caveats, we used complementary approaches. The first was based on pathway enrichment analyses using the IPA software. The most enriched canonical pathway was protein kinase A signaling, which had already been described as modulator of APP metabolism.[31,32] Remarkably, we also observed that a large proportion of the enriched canonical pathways were involved in neuronal functions and development. This is in line with the reported involvement of APP and its metabolites in neurogenesis and synapse formation/function.[35–37] Furthermore, we observed that canonical pathway renin-angiotensin signaling was enriched in genes associated with plasma Aβ concentrations. This is of particular interest, as blood pressure modulation is one of the suspected physiological functions of Aβ peptides.[5,11] With importance, we confirmed the enrichment of pathway involved in the development of neurons and in neuronal functions using the Webgestalt software.

The existence of equilibrium between plasma and brain Aβ peptides is debated even if some evidences seems to indicate that plasma Aβ peptides do not reflect a dynamic equilibrium between brain, CSF and plasma compartments.[38–43] One can argue that actors of the APP metabolism driving Aβ peptide production and/or degradation are not the same between organs/compartments. However, we detected enrichment of pathways in axon guidance using two different tools (IPA and Webgestalt) and a list of 1 762 genes containing at least one SNP associated with both plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ ($P < 0.05$) concentrations with the same direction of effect. We indeed postulate that correlation between plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ are representative of physiological processes leading to Aβ peptide productions (i.e., amyloid precursor protein metabolism). Of note, we observed, as expected, strong correlation between plasma $A\beta_{1-40}$ and $A\beta_{1-42}$ (Supplementary Table 3). Taken together, these observations indicate that we were able to detect processes involved in neuronal development although working on plasma phenotype. This suggests that mechanisms controlling Aβ peptide production and/or degradation could be in part constant across organs/compartments. As a consequence, analyzing such a plasma phenotype appears to be relevant to search for cerebral actors of the APP metabolism.

Our second approach was based on biological relevance and determination of whether genes corresponding to our best signals might be directly involved in APP metabolism. We noticed that the disabled homolog 1 (DAB1) gene was associated with plasma Aβ concentrations in our GWAS. The corresponding protein is a partner of APP and has been described as a modulator of metabolism.[44,45] In addition to characterizing genes known to be involved in Aβ peptides production, we also focused on the best signal obtained for plasma $A\beta_{1-42}$ concentration. This locus contained the CTXN3 gene, the product of which had not previously been described as a modulator of APP metabolism. We showed that CTXN3 overexpression was associated with significantly lower $A\beta_{1-42}$ secretion and that CTXN3 may co-localize with APP. Nevertheless, little is known about the CTXN3 protein (described for the first time in 2007): it has a single membrane-spanning domain and appears to be expressed only in the kidney and in the brain.[46] Another interesting gene of this locus, MEGF10, encodes a phagocytic receptor that could have a role in $A\beta_{1-42}$ uptake in the brain[47] and a suggestive association with cognitive decline in AD patients has been recently reported within this gene.[48]

Previous genetic studies of plasma Aβ levels have reported a linkage peak on chromosome 10q[49] and subsequently, several associations with variants in candidate genes of this region, including CTNNA3,[50,51] PLAU[52,53] and IDE.[54–57] Another group reported an haplotypic association spanning the MMP3 gene.[58] Using the same SNPs, we did not replicate theses findings in our study (Supplementary Table 8). Of note, our second best signal for plasma $A\beta_{1-42}$ levels was located within the 10q linkage peak, in an intron of the ANK3 gene. Further studies will be needed to assess whether this gene is responsible for our signal and the 10q linkage peak previously reported.[49] Variants in this gene are associated with risk of bipolar disorder,[59] schizophrenia[60] and austism spectrum disorders.[61] Links between these conditions and APP metabolism have been suggested,[62–64] which warrant further studies of ANK3.

Several GWAS have also analyzed genetic associations with $A\beta_{1-42}$ concentrations in CSF.[65–67] Only one SNP associated with CSF concentrations of $A\beta_{1-42}$ and tau was also associated with plasma concentrations of $A\beta_{1-42}$ at nominal significance level in our study (Supplementary Table 8). This SNP, rs2075650, is located in the APOE locus, which is suspected to influence AD pathophysiology through impaired Aβ peptides clearance from the brain.[68]

In addition, apart from this APOE SNP, we did not also find any significant association between known genetic risk factors for AD[18,69,70] and plasma Aβ concentrations (Table 3). As our analyses were performed in non-demented samples, the genes within our suggestive loci may not be involved in the AD process. Of note, we did not observe any significant associations between CTXN3 and AD risk in the European Alzheimer's Disease Initiative GWAS of 2 032 AD cases and 5 328 controls.[19]

In conclusion, our study results suggest that the use of plasma Aβ peptides are valid endophenotypes in GWASs and can be used to characterize the metabolism and functions of APP and its metabolites. Our identification of CTXN3's involvement in APP metabolism *in vitro* indicates that our genome-wide association meta-analysis was able to pick up new factors in this metabolic pathway. Consequently, systematic screening of the other suggestive loci might be relevant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)