Genome-wide haplotype association study identifies the FRMD4A gene as a risk locus for Alzheimer’s disease.


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Genome-wide haplotype association study identifies the FRMD4A gene as a risk locus for Alzheimer’s disease

Jean-Charles Lambert1,2,3,*, Benjamin Grenier-Boley1,2,3,*, Denise Harold4, Diana Zelenika5, Vincent Chouraki1,2,3, Yoichiro Kamatani6,6, Kristel Sleegers7,8, M. Arfan Ikram9, Mikko Hiltunen10, Christiane Reitz11, Ignacio Mateo12, Thomas Feulner13, Maria Bullido14, Daniela Galimberti15, Letizia Concar16, Victoria Alvarez17, Rebecca Sims4, Amy Gerrish4, Jade Chapman4, Candida Deniz-Naranjo18, Vincenzo Solfrizzi19, Sandro Sorbi20, Beatrice Arosio21, Gianfranco Spalletta22, Gabriele Siciliano23, Jacques Epelbaum24, Didier Hannequin25, Jean-François Dartigues26, Christophe Tzourio27,28, Claudine Bern29, Elisabeth MC Schrijvers30, Robert Rogers11, Guiseppe Tosto11, Florence Pasquier31,31, Karolien Bettens7,8, Caroline Van Cauwenberghe7,8, Laura Fratiglioni32,33, Caroline Graff33,34, Marc Delepine5, EADI consortium35, GERAD consortium35, Raffaele Ferri36, Chandra A. Reynolds37, Lars Lannfelt38, Martin Ingelsson38, Jonathan A. Prince39, Caterina Chilotti40, Alberto Pilotto41, Davide Seripa41, Anne Boland5, Michelangelo Mancuso23, Paola Bossù22, Giorgio Annoni21, Benedetta Nacmias20, Paolo Bosco36, Francesco Panza19, Florentino Sanchez-Garcia18, Maria Del Zompo42, Eli cer Coto17, Michael Owen4, Michael O’Donovan4, Fernando Valdivieso14, Paolo Caffara16, Elio Scarpini15, Onofre Combarros12, Luc Buée2,43, Dominique Campion25, Hilka Soininen10, Monique Breteler44,45, Mathias Riemenschneider13, Christine Van Broeckhoven7,8, Annick Alpérovitch27,28, Marc Lathrop5,6, David-Alexandre Trégouët46,47, Julie Williams4, Philippe Amouyel1,2,3,31

* both authors contributed equally to this work

Correspondance to:
Jean-Charles Lambert
Unité INSERM 744, Institut Pasteur de Lille,
BP 245, 1 rue du professeur Calmette, 59019 Lille cedex
jean-charles.lambert@pasteur-lille.fr

Affiliations:
1 INSERM, U744, Lille, France
2 Institut Pasteur de Lille, Lille, France
3 Université Lille-Nord de France, Lille, France
4 Medical Research Council (MRC) Centre for Neuropsychiatric Genetics and Genomics, Neurosciences and Mental Health Research Institute, Department of Psychological Medicine and Neurology, School of Medicine, Cardiff University, Cardiff, UK.
5. Centre National de Genotypage, Institut Genomique, Commissariat à l'énergie Atomique, Evry, France
6. Fondation Jean Dausset- CEPH, Paris, France
7. Neurodegenerative Brain Diseases group, Department of Molecular Genetics, VIB, Antwerpen, Belgium
8. Laboratory of Neurogenetics, Institute Born-Bunge, University of Antwerp, Antwerpen, Belgium
9. Departement of Epidemiology and Radiology, Erasmus MC, Rotterdam
10. Department of neurology, University of Eastern Finland and Kuopio University Hospital, 70211 Kuopio, Finland
11. G.H. Sergievsky Center, Columbia University, New York, NY 10032
12. Service of Neurology, University Hospital Marques de Valdecilla, University of Cantabria, Ciberned, Santander Spain.
13. Department of Psychiatry and Psychotherapy, Universitätsklinikum des Saarlandes, Universität des Saarlandes Saarbruecken Germany.
14. Centro de Biología Molecular Severo Ochoa (UAM-CSIC) and CIBERNEt, Universidad Autonoma, Cantoblanco, S-28049, Madrid, Spain
15. Dept. of Neurological Sciences, Dino Ferrari Center, University of Milan, Fondazione Cà Granda, IRCCS Ospedale Maggiore Policlinico, Milan, Italy
16. Dept. of Neuroscience, University of Parma, Parma, Italy
17. Genética Molecular-Laboratorio de Medicina, Hospital Universitario Central Asturias, Oviedo, Spain
18. Affiliation: Servicio de Inmunología. Hospital Universitario de Gran Canaria Dr. Negrín. Bco. de la Ballena s/n, 35010 Las Palmas de Gran Canaria, Spain
19. Department of Geriatrics, Center for aging brain, Memory unit, University of Bari, Bari, Italy
20. Department of neurological and psychiatric Sciences, University of Florence, Florence, Italy
21. Department of Internal Medicine, Geriatric Unit, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Università degli Studi di Milano, Milan, Italy
22. Clinical and Behavioral Neurology, IRCCS Fondazione Santa Lucia, Via Ardeatina 354, 00179 Roma - ITALY
23. Neurological clinic, University of Pisa, Pisa, Italy
24. INSERM UMR 894, Paris Descartes University, 75014 Paris, France
25. Inserm U614, Faculté de Médecine-Pharmacie de Rouen, F-76183, Rouen, France
26. Inserm U897, Victor Segalen University, F-33076, Bordeaux, France
27. Inserm U708, Paris 75013, France
28. UPMC Univ Paris 06, F-75005, Paris, France
29. Inserm U1061, Hôpital La Colombière, F-34093 Montpellier, France
30. Department of Epidemiology and Neurology, Erasmus MC, Rotterdam
31. CHRU de Lille, F-59000 Lille, France
32. Aging Research Center, Department NVS, Karolinska Institutet and Stockholm University, Stockholm, Sweden
33. Department of Geriatric Medicine, Karolinska University Hospital, SE-141 86, Stockholm, Sweden
34. KI-Alzheimer’s Disease Research Center, Department NVS, Karolinska Institutet, KIADRC, Stockholm, Sweden
35. A full list of members is provided in the Supplementary Note
36. IRCCS Associazione Oasi Maria SS, Institute for Research on Mental Retardation and Brain Aging, 94018 Troina (EN), Italy
37. Department of Psychology, University of California at Riverside, 92521 Riverside, USA
38. Department of Public health and Caring Sciences, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden
39. Department of Medical Epidemiology and Biostatistics, Karolinska Institute, 171 77 Stockholm, Sweden
40. Unit of Clinical Pharmacology, Teaching Hospital of Cagliari, Cagliari, Italy
41. Geriatric Unit & Gerontology-Geriatrics Research Laboratory, Department of Medical Sciences, I.R.C.C.S. “Casa Sollievo della Sofferenza”, 71013 San Giovanni Rotondo (FG), Italy.
42. Section of Clinical Pharmacology, Department of Neurosciences “B.B. Brodie”, University of Cagliari, Cagliari, Italy
43. Inserm U837, Lille, France
44. Department of Epidemiology, Erasmus MC, Rotterdam
45. DZNE, Bonn, Germany
46. INSERM UMR_S 937, Paris, France
47. ICAN Institute of Cardiometabolism and Nutrition, Université Pierre & Marie Curie, Paris, France
ABSTRACT

Recently, several genome wide association studies (GWAS) have led to the discovery of 9 new loci of genetic susceptibility in Alzheimer’s disease (AD). However, the landscape of the AD genetic susceptibility is far away to be complete and in addition to single-SNP analyses as performed in conventional GWAS, complementary strategies need to be applied to overcome limitations inherent to this type of approaches.

We performed a genome wide haplotype association (GWHA) study in the EADI1 study (n=2,025 AD cases and 5,328 controls) by applying a sliding-windows approach. After exclusion of loci already known to be involved in AD (APOE, BIN1 and CR1), 91 regions with suggestive haplotype effects were identified. In a second step, we attempted to replicate the best suggestive haplotype associations in the GERAD1 consortium (2,820 AD cases and 6,356 controls) and observed that 9 of them showed nominal association. In a third step, we tested relevant haplotype associations in a combined analysis of five additional case-control studies (5,093 AD cases and 4,061 controls). We consistently replicated the association of a haplotype within FRMD4A on Chr.10p13 in all the data set analysed (OR=1.68, 95% CI 1.43-1.96; p=1.1x10^{-10}). We finally searched for association between SNPs within the FRMD4A locus and Aβ plasma concentrations in three independent non demented populations (n=2,579). We reported that polymorphisms were associated with plasma Aβ42/Aβ40 ratio (best signal, p=5.4x10^{-7}).

In conclusion, combining both GWHA study and a conservative three-stage replication approach, we characterised FRMD4A as a new genetic risk factor of AD.
INTRODUCTION

The identification of genes involved in monogenic forms of Alzheimer's disease (AD) has significantly contributed to our knowledge of the disease mechanisms. The causal links between mutations, the functions of the mutated genes (APP, PS1 and PS2) and disease development prompted a pathophysiological hypothesis which radically changed our understanding of AD: the amyloid cascade hypothesis.¹ The systematic association of pathogenic mutations with changes in APP metabolism and, more particularly, a relative overproduction of Aβ42 peptides indicates that this metabolism is at the heart of the disease process (at least in the monogenic forms of the disease). The overproduction of these neurotoxic peptides is supposed to lead to or accentuate neuron-to-neuron propagation of the τ pathology (leading to neuronal death) by an unknown mechanism.²

By analogy, it was expected that the characterization of genetic factors involved in the common forms of AD (i.e. lacking classical Mendelian inheritance), the most frequent form of the disease, should also help to better understand the AD physiopathological process. However, the characterization of these genetic factors has encountered significant difficulties. Until 2009, the apolipoprotein E (APOE) gene was the only globally valid genetic determinant of AD to have been unambiguously identified in 15 years of intensive research.³⁴ As with other multifactorial diseases, this systematic inability to detect new genetic determinants has prompted more comprehensive investigations using genome-wide association studies (GWASs). We and others performed five large GWASs in this field and reported that the CLU (clusterin), PICALM (phosphatidylinositol binding clathrin assembly protein), CR1 (complement component [3b/4b] receptor 1), BIN1 (bridging integrator 1), ABCA7 (ATP-binding cassette, sub-family A, member 7), MS4A (membrane spanning 4A) cluster, EPHA1 (ephrin type-A receptor 1), CD33 (differentiation antigen 33) and CD2AP (CD2-associated protein) genes were associated with the AD risk.⁵⁶ Most of these susceptibility genes have been already systematically replicated in Caucasians in large case-control studies and in families.⁷

However, our understanding of the AD genetics is far away to be complete and strong efforts have still to be done. At this level, classical GWAS approaches present an important limitation with systematic application of a conventional, highly conservative Bonferroni correction leading to select only the most statistically significant associations (commonly, p<1x10⁻⁶). This involves the risk of rejecting biologically valid hypotheses on purely statistical grounds, i.e. false negatives.

To partly handle some of this limitation, other complementary approach consists in extracting pertinent information from SNPs nominally associated with the risk of developing AD in GWAS by using complex statistical and bioinformatics multiple-SNP analyses such as
genome-wide haplotype association (GWHA) study.\textsuperscript{11} We adopted this GWHA strategy to the Alzheimer’s disease (AD) genetic susceptibility through a three-step approach.

**METHODS**

**Population description**

The main characteristics of the populations used for the GWHA study are described in the supplementary notes and supplementary Table 1. All AD cases met the criteria for either probable AD (NINCDS-ADRDA, DSM-IV)\textsuperscript{12} or definite AD (CERAD)\textsuperscript{13}. All elderly controls were screened for dementia using the MMSE or the Alzheimer’s Disease Assessment Scale-cognitive subscale or were determined to be free from dementia at neuropathological examination or had a Braak score of 2.5 or below. All subjects or, in those with substantial cognitive impairment, a caregiver, legal guardian, or other proxy gave written informed consent for participation in this study. The study protocols for all populations were reviewed and approved by the appropriate Institutional review boards of each country. The main characteristics of the populations (non demented individuals) used for the Aβ plasma study (3C study, Rotterdam and CHS) are described in the supplementary notes and supplementary Table 2.\textsuperscript{14-16}

**Genotyping**

Participants in the French GWA study (including the 3C study) were genotyped using an Illumina 610-quad array. Quality control and analytical parameters have been described in detail elsewhere.\textsuperscript{5} The GERAD participants were genotyped using an Illumina 610-quad array, a HumanHap550 array or a HumanHap300 array. Again, QC and analytical parameters have been described in detail elsewhere.\textsuperscript{6} The Rotterdam and CHS studies were genotyped using the affymetrix 500K array. Again, QC and analytical parameters have been described in detail elsewhere.\textsuperscript{7}

In the European populations (stage 3), genotyping was performed using Sequenom assays at the exception of the German population genotyped with a 610K ILLUMINA chip. The primer and probe sequences used in the genotyping assays are available upon request. In order to avoid any genotyping bias, cases and controls were randomly mixed while genotyping and laboratory personnel were blinded to case/control status. The genotyping success rate was at least 95%. Departure from Hardy-Weinberg equilibrium (HWE) was observed for rs2446581 in the Swedish control and case samples (p=9.1x10\textsuperscript{-7} for the whole population). The Swedish sample was consequently excluded from further analysis, since haplotype analyses that do not comply with HWE are likely to bias observations.
Statistical Analyses

Missing age or gender data. Any individuals with missing age or gender data were excluded. This gave a maximum of 2,025 AD cases and 5,328 controls in step 1, 2,820 AD cases and 6,356 controls in step 2 and 5,093 AD cases and 4,061 controls in step 3.

Detection of haplotype effects using a sliding-window approach. This approach has been fully described elsewhere.\textsuperscript{11,17} Briefly, the search for haplotype effects was carried out by applying a sliding-window approach\textsuperscript{16,19} to the French GWA dataset for each chromosome. After excluding SNPs not in HWE or with a MAF<0.02, the first step of the strategy was to eliminate part of the redundancy between SNPs by using haplotype-tagging SNPs (htSNPs). For this, the same binning procedure as in\textsuperscript{11} was used: within each bin of 10 adjacent SNPs, we identified a minimal set of htSNPs that were able to characterize more than 95% of the inferred haplotypes with estimated frequency greater than 0.02. Once a bin had been characterized by a set of htSNPs, the same strategy was applied to the bin composed of the next 10 adjacent SNPs. The final set of htSNPs (n=287,956) was then fed into the sliding-windows approach.

Given a window of 10 htSNPs, the search for the most informative and parsimonious haplotype configuration in terms of disease prediction was performed for all possible 1- to 4-loci combinations of not necessarily adjacent SNPs. We used a strategy based on Akaike’s information criterion, which has been previously described for candidate gene haplotype analysis\textsuperscript{19,20}. It relies on the stochastic expectation-maximisation module\textsuperscript{21} in THESIAS software\textsuperscript{22}. If required, missing genotypes were inferred by applying multiple imputation.\textsuperscript{21} In all, 37,330,050 combinations were investigated in our genome scan and this investigation was conducted thanks to the use of the grid technology developed by the European Grid Infrastructure (http://www.egi.eu).\textsuperscript{20} This technology enables several thousand computations to be run in parallel on a large number of different CPUs. The sliding-window haplotype approach was developed into a GridHaplo grid package for the EGI grid (http://genecanvas.ecgene.net).\textsuperscript{11}

Replication of haplotype effects Regions with “window p-values” (i) below 10^{-5} and (ii) 100 times smaller than the smallest single-locus p-value (including all SNPs and not only htSNPs) were analysed in terms of replication in the GERAD1 dataset by using THESIAS software, with systematic adjustment for age and gender.

Association of FRMD4A SNPs with Aβ plasma concentrations. In each centre, Aβ plasma variables are normally distributed. We excluded prior analyses all samples with a +/- 2SD value in order to avoid potential associations driven by extreme observations. Finally, each quantitative variable was transformed into a z-score (equal to (observed value minus the sample mean), divided by the sample standard deviation). The association between the Aβi.
Aβ1-42 and Aβ1-42/Aβ1-40 z-scores on one hand and imputed FRMD4A imputed SNPs on the other (see below) were assessed using a general linear model (GLM) under an additive model adjusted for age, centre and gender.

We used inverse-variance weighting (also known as fixed-effects meta-analysis) to investigate the homogeneity of haplotype effects from one study to another and to provide meta-analysed, age- and gender-adjusted ORs for haplotype effect estimates in the seven studies. A similar strategy was used to provide meta-analysed, age- and gender-adjusted association levels between Aβ plasma Z-scores and FRMD4A SNPs.

Imputation analyses

We imputed SNPs by using MaCH (http://www.sph.umich.edu/csg/abecasis/mach/index.html) and minimac software (http://genome.sph.umich.edu/wiki/minimac). The reference haplotype data is provided by the MaCH website, which was built for the combined Caucasian populations as part of the 1000 Genomes project. In our dataset, all individuals were genotyped on the same platform (the Illumina Human660W-Quad Beadchip) and we used 492,941 observed SNP genotypes that passed quality filters as follows: genotyping call rate ≥ 98 %, Hardy-Weinberg Equilibrium Test p value ≥1x10^{-6} and MAF ≥1 %. We first inferred haplotype combinations of each individual using the “phase” option in the MaCH program and then imputed them with minimac. Since minimac is a newly developed software tool, we compared the correlation between the imputed genetic dosage from minimac and those from the standard MaCh program for SNPs in chromosome 22. The results were very similar (data not shown). Doses for 7,704,555 million SNPs with a MAF>0.01 were available from the French GWA dataset using the 1000 Genomes dataset. We selected 2,538 SNPs within the Chr. 10p13 locus of interest (chr10:13655705–14402866) and evaluated their associations with AD risk in an additive logistic regression model adjusted for age, gender and disease status. A graphic representation was then generated with Locuszoom software (http://csg.sph.umich.edu/locuszoom/).

In the 3C, Rotterdam and CHS cohorts, we used the genotype data to impute to the 2.5 million non-monomorphic, autosomal SNPs described in HapMap II (CEU population) as described elsewhere. We selected 1,486 SNPs (MAF>0.01) within the Chr. 10p13 locus of interest (chr10:13655705–14402866) and evaluated their associations with Aβ plasma concentrations as described above. Again, a graphic representation was then generated with Locuszoom software (http://csg.sph.umich.edu/locuszoom/).
RESULTS

We developed a three step approach. In the first step, the French GWA study (EADI1 for European Alzheimer’s initiative 1), including 2,025 AD cases and 5,328 controls was used to select regions with potential haplotype associations with AD. Following a sliding-windows approach (see material and methods), we applied two a priori criteria to select loci of interest as previously described\(^1\): level of association with a P-value (i) below \(10^{-5}\) and (ii) at least 100 times smaller than the smallest single-SNP P value observed in the corresponding locus. We were able to detect loci already known to be involved in AD (the APOE, BIN1 and CR1 locus\(^5-9\) from previous GWAS. The obtained signal was systematically highly stronger than the one observed for each SNP taken separately (Table 1). After exclusion of these loci, we retained 91 regions of interest.

In the second step, we replicated these haplotype associations in the GWA database from another consortium involved in the study of AD genetic susceptibility, the GERAD1 consortium,\(^6\) including 2,820 AD cases and 6,356 controls. All 91 regions were available for investigation and 9 of them showed nominal association (P<0.05; Table 2). We decided to further investigate two regions showing the same best haplotypes associated with AD risk in both EADI1 and GERAD1, with similar magnitude and direction of association (Table 2). These two loci, not previously detected in single-SNP GWAS analyses, were located on chromosomes 6p21 and 10p13. The identified AD-associated haplotypes at the 6p21 region were tagged by rs2395760, rs991762 and rs4711652. The rs7081208, rs2446581 and rs17314229 tagged the 10p13 haplotypes. For the Chr. 6p21 locus, the best association was attributable to the GGT haplotype in both GWAS (OR (Odds Ratio): 1.53; 95%CI: [1.31-1.79], \(P=8.1x10^{-8}\) after adjustment for age and gender when both EADI1 and GERAD1 studies were combined). For the Chr. 10p13 locus, the highest level of association was attributable to the AAC haplotype (OR:1.76 ; 95%CI: [1.44-2.15], \(P=2.3x10^{-8}\) after adjustment for age and gender when both EADI1 and GERAD1 studies were combined. Additional adjustment for the four main principal components did not modify the results, data not shown) (Table 2).

In the third step, the six tagging SNPs were further genotyped in five additional AD case-control studies from Flanders-Belgium (842 cases and 489 controls), Finland (560 cases and 623 controls), Germany (728 cases and 961 controls), Italy (1,846 cases and 904 controls) and Spain (1,117 cases and 1,084 controls) (supplementary Tables 3 and 4). The same common haplotypes were inferred from the six SNPs in the two loci, with the exception of the AGT haplotype in Chr.10p13 in the Italian population (frequency<0.01 in controls) (Supplementary Table 5 and 6).

In a combined analysis of the five replication datasets, the overall difference in haplotype distribution for the 6p21 locus was not significant between AD cases and controls (\(P=0.13\)) and the GGT haplotype was not associated with AD risk (OR: 0.89; 95%CI: [0.74-1.08],
P=0.23 after adjustment for age and gender). We therefore considered that the haplotype association in this locus was not confirmed (see Supplementary Table 5).

Conversely, an overall significant difference in haplotype distribution between AD cases and controls was observed at the 10p13 locus in the replication sample (P=4.3x10^{-2} after adjustment for age, gender and country) and the AAC haplotype was associated with increased AD risk in the five datasets meta-analysis (OR: 1.55; 95%CI: [1.19-2.00], P=9.2x10^{-4} after adjustment for age and gender). When the seven datasets (EAD1, GERAD1 and the follow-up studies) were analysed together, the AAC haplotype had a meta-analysed OR of 1.68 (95% CI: [1.43-1.96]; p=1.1x10^{-10} adjusted for age and gender) when compared with the most frequent GGC haplotype, with no evidence of heterogeneity across the 7 countries (p=0.92) (Fig. 1). Among the whole sample, 9 cases were homozygous for the AAC haplotype versus only 5 controls (OR=2.85, 95% CI [0.88-9.76], p=0.09 with Yates correction), which is consistent with a dose-dependent effect of the AAC haplotype.

Importantly, none of the individual SNPs were associated with AD risk in the total sample with a P value lower than 10^{-5}. The best meta-analysed SNP was rs2446581 (OR: 1.15; 95%CI: [1.08-1.24]; p=2.1x10^{-5} after adjustment for age and gender; supplementary Table 7). The hypothesis that the rs2446581 could solely explain the association observed with the 10p13 haplotypes was rejected by use of the likelihood ratio test (P=9.1x10^{-7}).

We also tested whether this haplotype association might be explained by one or more untyped SNPs located nearby the genotyped SNPs. Genotypes for 7,704,555 million SNPs with a minor allele frequency (MAF) ≥0.01 were imputed from the French GWA data by using the 1000 genome dataset (http://www.1000genomes.org/). None of the single SNPs imputed at this locus (n=2,538) showed stronger evidence of association with the AD risk than the haplotypes initially identified (Supplementary Fig. 1). The 10p13 haplotype region appeared to be fully included within the FERM domain containing 4A (FRMD4A) gene, as indicated by the linkage disequilibrium map of this locus of interest (Fig. 2).

We finally explored how FRMD4A might be involved in the AD process. According to a recent report, the FRMD4A gene was described to interact with Arf6.24 Since this latter protein was reported to control APP processing,25 we postulated that the FRMD4A locus could be associated with endophenotypes susceptible to reflect modulation of the APP metabolism.

We accordingly analysed association of the FRMD4A locus with Aβ peptide plasma concentrations in three independent populations of non demented individuals (n=2,579) (Supplementary Table 2) for which FRMD4A imputed SNPs, Aβ_{1-40} and Aβ_{1-42} plasma concentrations were available. Strong associations were observed between several FRMD4A SNPs and Aβ_{1-42}/Aβ_{1-40} (9 SNPs reaching a significant level after Bonferroni correction, P=3.4x10^{-5} for 1,486 SNPs, Fig. 3). The best signal was obtained for rs7921545
(meta-analysed Z-score β coefficient : 0.12, CI 95% [0.07-0.17], P=5.4x10^{-7}) and was homogeneous between the three data sets (P for heterogeneity =3.7x10^{-1}, supplementary Fig. 2). Of note, rs2446581 showed nominal association with $A\beta_{1.42}/A\beta_{1.40}$ (P=3.1x10^{-2}, see supplementary Fig. 2). Furthermore, only nominal associations between FRMD4A SNPs, $A\beta_{1.40}$ or $A\beta_{1.42}$ were detected (see supplementary Fig. 3).

**DISCUSSION**
From a specific haplotype based GWAS approach, we were able to detect a new genetic susceptibility factor for AD that could not be identified through usual GWAS analyses. Due to the high number of association tests performed in this GWHA study (37,330,050, not all of which were independent), a robust replication strategy was necessary. Our three-step approach was thus particularly conservative and we cannot rule out the possibility that we failed to identify other haplotype-based loci associated with AD risk. Nonetheless, our GWHA immediately identified two potential AD susceptibility loci among which the 10p13 locus showed an AAC haplotype that was strongly and consistently associated with AD risk in seven independent European populations. Interestingly, this locus is included in the large AD linkage region regularly identified on chromosome 10.28

Additional work will be necessary to determine whether the AD risk-haplotype association indicates an interaction between SNPs or whether they tag non-genotyped, functional variants. However, our GWHA study could have handled several of the inherent limitations of GWAS. Since the AAC haplotype is rare (with a mean frequency of 2% in our Caucasian populations), our work suggests the possibility that rare variants may be responsible for the signal detected within the FRMD4A gene. This might explain why the locus was not detected (i) in our previous GWA studies based on single-SNPs analyses5,6 and (ii) through imputation, since SNPs with low frequency and/or SNPs within specific regions with low LD are poorly imputed even when using the 1000 genome data set (Fig. 2). Furthermore, we cannot rule out the possibility that the AAC haplotype tags insertion-deletion variants or copy-number variations (CNVs) – neither of which are captured by imputation. Interestingly, several CNVs within the FRMD4A locus have been described (http://projects.tcag.ca/variation/).

Little is known about the function of the protein encoded by FRMD4A in animal cells. It belongs to the FERM super family, which includes ubiquitous components of the cytocortex involved in cell structure, transport and signalling functions. 27 According to a recent report, the FRMD4A gene product may regulate epithelial polarity by interacting with Arf6 and the PAR complex. 24 Interestingly, several other lines of evidence suggest that Arf6 modulates cell polarity in various systems – including neurons. Arf6 reportedly regulates dendritic branching in hippocampal neurons and neurite outgrowth in PC12 cells. 28,29 Finally, arf6 was recently
reported to control APP processing,\textsuperscript{27} suggesting that FRMD4A could also be implied in this metabolism. This hypothesis is sustained by the observation of an association of the \textit{FRMD4A} locus with plasma Aβ\textsubscript{1-42}/Aβ\textsubscript{1-40}, at once reinforcing the plausibility of the association of this gene with AD risk and its potential implication in a subtle control of the APP metabolism. Unfortunately, since the SNPs defining the AAC haplotype were only available by imputation in the Rotterdam and CHS study, search for association of the AAC haplotype with plasma Aβ\textsubscript{1-42}/Aβ\textsubscript{1-40} was not possible. Furthermore, it is important to keep in mind that this result is difficult to interpret in terms of AD pathophysiological process. First, it is not known whether plasma Aβ peptides reflect a dynamic equilibrium between brain, CSF and plasma compartments.\textsuperscript{30-34} Secondly, the source of plasma Aβ species is not known and the Aβ peptides’ physiological functions are still not fully understood.\textsuperscript{35,36} However, taken as a whole, these data suggest that \textit{FRMD4A} could be a relevant candidate gene for AD risk and the basis for another possible pathophysiological pathway for AD.
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Spanish sample collection : the Madrid site (MB) was supported by grants of the Ministerio de Educación y Ciencia and the Ministerio de Sanidad y Consumo (Instituto de Salud Carlos III), and an institutional grant of the Fundación Ramón Areces to the CBMSO. We thank I. Sastre and Dr. A Martínez-García for the preparation and control of the DNA collection, and Drs. P. Gil and P. Coria for their cooperation in the cases-controls recruitment. We are grateful to the Asociación de Familiares de Alzheimer de Madrid (AFAL) for continuous encouragement and help. The Gran Canaria site was supported by grants of the Ministerio
de Sanidad y Consumo (Instituto de Salud Carlos III), and the Fundacion Canaria de Investigacion en Salud (FUNCIS).

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**Conflicts of interest**

None
References:


Table 1: Best haplotype combination observed in CR1, BIN1 and APOE and comparison with the association obtained for single-SNP analysis within these loci.

Table 2: List of the haplotype combinations identified in the French GWA study and replicated at $p<0.05$ in GERAD. Exactly replicated combinations (i.e. the same best haplotypes associated with AD risk in the datasets from the French GWA study and GERAD, with similar magnitudes of association and the same direction of association) are indicated in grey.

Figure 1: Haplotypic Odd ratios (Ors) for AD risk with the AAC haplotype derived from rs7081208, rs2446581, rs17314229 at Chr. 10p13 in seven independent European populations.

Figure 2: Linkage disequilibrium map in the FRMD4A locus and localization of the region defined by the rs7081208, rs2446581 and rs17314229 at this locus (in bold).

Figure 3: Association of SNP in the FRMD4A locus with plasma Aβ$_{1-42}$/Aβ$_{1-40}$ level following meta-analyses of Z-score β coefficients under an additive model adjusted for age and gender using three independent healthy populations. SNPs in red are nominally associated with Aβ peptide levels. SNPs in are the three markers defining the AAC haplotype associated with AD risk.
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<tr>
<th>Locus</th>
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<th>Best associated SNP within the locus</th>
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Table 2

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Figure 1

Haplotype frequency

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Figure 2
Figure 3

- \log_{10}(p\text{-value})

recombination rate (cM/Mb)

position on chr10 (Mb)

- PRPF18
- FRMD4A