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Genetic association of complement receptor 1 polymorphism rs3818361 in Alzheimer's disease

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Abstract Complement receptor 1 gene polymorphism rs3818361 was recently shown to increase the risk of Alzheimer's disease (AD). We performed an independent replication study of this genetic variant in 2470 individuals from Spain. By applying an allelic model, we observed a trend toward an association between this marker and late-onset AD susceptibility in our case–control study (odds ratio = 1.114, 95% confidence interval: 0.958–1.296, $P = .16$). Meta-analysis of available studies (n = 31,771 individuals), including previous studies and public genome-wide association study resources (Alzheimer's Disease Neuroimaging Initiative, Translational Genomics Research Institute, and Multi-site Collaborative Study for Genotype-Phenotype Associations in Alzheimer's Disease), strongly supports the effect of rs3818361 (odds ratio = 1.180, 95% confidence interval: 1.113–1.252, $P < 2.99E\text{-}8$) and suggests the existence of between-study heterogeneity ($P < .05$). We concluded that the complement receptor 1 gene may contribute to AD risk, although its effect size could be smaller than previously estimated.

Keywords: Alzheimer's disease; Association; *CR1*; Genotype; Meta-analysis; Molecular genetics; Polymorphism

The genetic basis of Alzheimer's disease (AD) has been the focus of several genome-wide association studies (GWAS). Recently, a large case–control study performed in a French sample identified a genome-wide significant signal for a single nucleotide polymorphism (SNP) within the complement component receptor 1 locus (complement receptor 1 [*CR1*], complement component 3b/4b receptor, C3-binding protein, C3BR, C4BR, or CD35, Mendelian Inheritance in Man code 120620). Importantly, this finding was replicated in multiple AD case–control series from Belgium, Finland, Italy, and Spain [1]. Consequently, the authors proposed that *CR1* markers may act as a modifying genetic factor for AD risk. Furthermore, a consistent signal within *CR1* gene was observed in a concurrent independent GWA study published by other European researchers, although no GWAS significance was achieved in that particular study [2].

The *CR1* gene is located on chromosome 1q32, and it is strongly expressed in myeloid cell lines, whole blood, breast,

Some of the data used in this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (www.loni.ucla.edu/ADNI). As such, the investigators within ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in the analysis or writing of this report. A complete listing of ADNI investigators is available at www.loni.ucla.edu/ADNI/Collaboration/ADNI_Authorship_list.pdf.

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ovary, or spleen and weakly expressed in other tissues, including pancreatic islets, brain cortex, hypothalamus, or salivary glands [3]. This gene is a member of the receptors of complement activation (RCA) family. It is located within the "cluster RCA" region of chromosome 1 [3], and its function with regard to AD is still poorly understood, although this protein could be involved in erythrocyte amyloid beta 42 sequestration and clearance from whole blood [1].

Because the follow-up and replication of GWAS findings provide further validation of proposed signals, we decided to evaluate a *CR1* marker with the most consistent association to AD (rs3818361) as a genetic modifying factor for AD risk in a sample of the Spanish population.

To conduct this research, we studied 2470 Spanish individuals previously selected to evaluate other SNPs associated with AD [4]. Specifically, the sample included 1140 patients with sporadic AD diagnosed as possible or probable AD in accordance with National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association criteria [5], 1209 control subjects with unknown cognitive status from the general population, and 121 neuropsychologically healthy elderly control (NHEC) subjects screened for the absence of cognitive impairment by a structured interview including neurological mental status examination, category fluency test, and Folstein Mini-Mental State Examination. Mean (SD) age at recruitment was 78.8 (7.9), 49.9 (9.2), and 77.5 (9.4) years in patients, control subjects, and NHECs, respectively. The total number (%) of females in these groups was 797 (69.9%), 638 (52.8%), and 63 (52.1%), respectively. Mean (SD) age at AD diagnosis was 77.6 (7.7) years. DNA extraction procedures and apolipoprotein E (*APOE*) genotyping have been previously described [4]. As reported for other European populations, the presence of *APOE* ε4 allele was strongly associated with AD in our series (cases vs all controls; odds ratio [OR] = 3.18, P = 2.48E-37).

In addition, parts of data used in the preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (www.loni.ucla.edu/ADNI). "The ADNI was launched in 2003 by the National Institute on Aging (NIA), the National Institute of Biomedical Imaging and Bioengineering (NIBIB), the Food and Drug Administration (FDA), private pharmaceutical companies and non-profit organizations, as a $60 million, 5-year public-private partnership. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), and other biological markers are related to the progression of mild cognitive impairment (MCI) and early AD. Determination of sensitive and specific markers of very early AD progression is intended to aid researchers and clinicians to develop new treatments and monitor their effectiveness, as well as lessen the time and cost of clinical trials. The Principal Investigator of this initiative is Michael W. Weiner, MD, VA Medical Center and University of California - San Francisco. ADNI is the result of efforts of many co-investigators from a broad range of academic institutions and private corporations, and subjects have been recruited from over 50 sites across the U.S. and Canada. The initial goal of ADNI was to recruit 800 adults, ages 55 to 90, to participate in the research—approximately 200 cognitively normal older individuals to be followed for 3 years, 400 people with MCI to be followed for 3 years and 200 people with early AD to be followed for 2 years." For up-to-date information, please refer to the Web site www.adni-info.org.

The *CR1* gene polymorphism rs3818361 was genotyped in a LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland) by using the LightCycler 480 Probes Master kit according to the manufacturer's instructions (Supplementary Methods and Supplementary Table 1). To conduct statistical analysis and phenotype–genotype correlations, we used tests adapted from the study conducted by Sasieni (available online at http://ihg2.helmholtz-muenchen.de/) [6]. Age-, sex-, and *APOE* genotype-adjusted binary logistic regression analyses were performed using SPSS 15.0 software (SPSS, Chicago, IL). Meta-analyses were conducted using Episheet (http://krothman.byethost2.com/Episheet.xls). Specifically, the pooled estimate and 95% confidence intervals (CIs) were estimated by assuming a random effects model. A forest plot with allelic ORs from published studies and the pooled OR was constructed using Episheet.

The referral centers' ethics committees and Neocodex have approved this research protocol, which is in compliance with Spanish national legislation and the Code for Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association Declaration of Helsinki.

Minor allele frequency, genotype distribution, and observed heterozygosity for rs3818361 marker in this sample of the Spanish population are in accordance with the National Center for Biotechnological Information database and previous investigations [1]. The average genotyping call rate in our study was >97.9%. Hardy–Weinberg equilibrium analyses in our population indicated no deviation for the SNP marker studied ($P > .31$; Supplementary Table 2). We explored the association of *CR1* rs3818361 marker with AD phenotype using different tests adapted from the study conducted by Sasieni and compared AD versus control subjects, AD versus NHECs, or AD versus all control subjects (Table 1). We found no evidence of significant association to AD in any comparison ($P > .16$). We also obtained adjusted estimates using binary logistic regression models adjusting for age, sex, and *APOE* (dominant model). Again, we did not detect any statistically significant effect on AD susceptibility in our series ($P > .60$; Table 1). To assess *CR1*–*APOE* (gene-gene) and *CR1*–sex interactions, we stratified our series according to *APOE* genotype and sex and applied Mantel–Haenszel stratified analysis. No evidence of interaction was observed (data not shown).

Overall, the *CR1* rs3818361 marker was not significantly associated to AD in this sample of the Spanish population. However, a trend toward association was observed when crude analysis of minor allele frequency differences between cases and controls (general population) is performed (OR =

Table 1
AD risk associated to *CR1* rs3818361 T allele in the Spanish population

Comparison	Model	Crude OR (T allele)	*P* value	Adjusted OR*	*P* value
Case vs controls	Allelic	1.114 (0.958–1.296)	.16	NA	NA
Case vs NHECs	Allelic	0.877 (0.631–1.218)	.43	NA	NA
Case vs all controls	Allelic	1.088 (0.939–1.260)	.26	NA	NA
Case vs controls	Dominant	1.100 (0.924–1.310)	.28	0.894 (0.583–1.372)	.60
Case vs NHECs	Dominant	0.856 (0.580–1.262)	.43	0.916 (0.599–1.400)	.68
Case vs all controls	Dominant	1.073 (0.905–1.272)	.41	0.990 (0.728–1.346)	.94

Abbreviations: OR, odds ratio; AD, Alzheimer's disease; *CR1*, complement receptor 1; NA, not applicable; NHECs, neuropsychologically healthy elderly control subjects.

* Estimates adjusted by sex, age, and *APOE* (ε4 dominant model) using a binary logistic regression model.

1.114, 95% CI: 0.958–1.296, $P = .16$). Importantly, the observed effect size and its direction were concordant with previous results [1]. The use of general population control subjects as opposed to age-matched NHECs deserves a comment here. It has been argued that this strategy could decrease our power to detect very small effects [7]. However, the results of recent longitudinal and case–control studies are very close to those observed in our population [7–9]. Conversely, it could seem that studies using unmatched general population control subjects may be more prone to bias than those using age-matched neurologically healthy control subjects. However, this is based on a wrong conception of the use of matching in case–control studies. Matching itself does not remove confounding in case–control studies (unlike in cohort studies). In fact, in contrast, it can introduce bias if not properly analyzed [10,11]. Furthermore, the use of "hypernormal" control subjects has been criticized because it could lead to biased results [12]. For all these reasons, we opted for including both types of control subjects in our study, and looked for consistency in the results across independent studies available. These new studies are suggesting that *CR1* rs3818361 effect on AD risk could have been initially overestimated. We believe that it is possible that we are facing a new example of what has become known as the winner's curse, in which the first study reporting a significant test (the winner) will also report an effect size larger than is likely to be seen in subsequent replication studies [13]. Alternatively, it has also been shown that the effect size of true causal genetic markers is higher in neuropathologically verified case–control studies compared with clinically based case–control series. Thus, between-study differences in diagnostic criteria and control selection could also explain observed effect size oscillations [14].

To test this possibility, we decided to conduct a large meta-analysis using available data from Alzgene forum (www.alzgene.com) and genotypes from public repositories [15–17]. We excluded data from Cohorts for Heart and Aging in Genomic Epidemiology longitudinal studies [7] and two cases–control studies [8,9] because crude genotypes for rs3818361 were not available. By merging data from other 12 independent case–control studies and using imputation and meta-analysis techniques, we were able to estimate the overall effect of rs3818361 on AD risk. Specifically, we analyzed the genotypes of 31,771

Table 2
Meta-analysis results for *CR1* rs3818361 SNP using genotypes from 31,771 individuals

Study/year (country)	OR	LL	UL	Cases (n)	Controls (n)	MAF (controls)	Relative weight
Lambert 2009 (France)	1.281	1.171	1.401	2018	5324	0.18	0.139
Lambert 2009 (Belgium)	1.086	0.900	1.312	972	436	0.23	0.065
Lambert 2009 (Finland)	1.497	1.244	1.802	690	634	0.19	0.067
Lambert 2009 (Italy)	0.994	0.869	1.138	1423	1232	0.20	0.098
Lambert 2009 (Spain)	1.220	1.019	1.462	732	792	0.18	0.069
Harold 2009 (Germany)	1.133	0.942	1.362	554	824	0.21	0.068
Harold 2009 (UK/Ireland)	1.181	1.079	1.293	2226	4836	0.17	0.138
Harold 2009 (USA)	1.142	1.009	1.292	1159	2188	0.20	0.107
ADNI (USA)	1.455	1.032	2.053	187	229	0.17	0.025
TGEN 2007 (USA/Neth)*	1.105	0.914	1.335	859	582	0.19	0.065
GenADA 2008 (Canada)*	1.232	1.033	1.469	799	778	0.18	0.072
Present study (Spain)	1.114	0.958	1.296	1127	1170	0.17	0.087
Pooled (random effects)	1.181	1.113	1.252	12,746	19,025	NA	NA

Abbreviations: SNP, single nucleotide polymorphism; LL, lower limit; UL, upper limit (95% confidence interval); MAF, minor allele frequency; UK, United Kingdom; USA, United States of America; TGEN, Translational Genomics Research Institute; GenADA, Multi-site Collaborative Study for Genotype-Phenotype Associations in Alzheimer's Disease; Neth, The Netherlands.

NOTE. Genotypes from ADNI [13] were obtained from ADNI repositories (http://www.adni-info.org/Home.aspx). Weight for each study was calculated using Episheet software assuming a random effects model.

* rs3818361 genotypes for GenADA [14] and TGEN [15] public databases were imputed using MACH 1.0 software (http://www.sph.umich.edu/csg/abecasis/MACH/download/).

individuals (12,746 cases and 19,025 controls) and observed that the *CR1* rs3818361 association to AD risk was highly significant ($P < 2.99E-8$, random effects model). However, its estimated effect size was very modest (OR = 1.180, 95% CI: 1.113–1.252; Table 2 and Supplementary Fig. 1).

Importantly, borderline evidence of between-study heterogeneity was also detected in our meta-analysis (*P* heterogeneity = .049). Remarkably, heterogeneity completely disappeared when original data reported by Lambert et al [1] were removed from meta-analysis (OR = 1.164, 95% CI: 1.102-1.230, $P = 6.35E-8$, random effects model, *P* heterogeneity = .81). This observation could be fully explained by the classic regression to the mean or winner's curse effect [13].

Overall, our meta-analysis results clearly indicate that *CR1* involvement in AD is uncontroversial. In fact, even after excluding the data from the original studies, the association remained highly significant for pooled data. The magnitude of the effect size for this variant could be smaller than previously suspected, in yet another example of the winner's curse. This pervasive phenomenon in genetic research when using hundreds of thousands of markers should be taken into account in the design of replication or follow-up of GWAS signals in population-based series.

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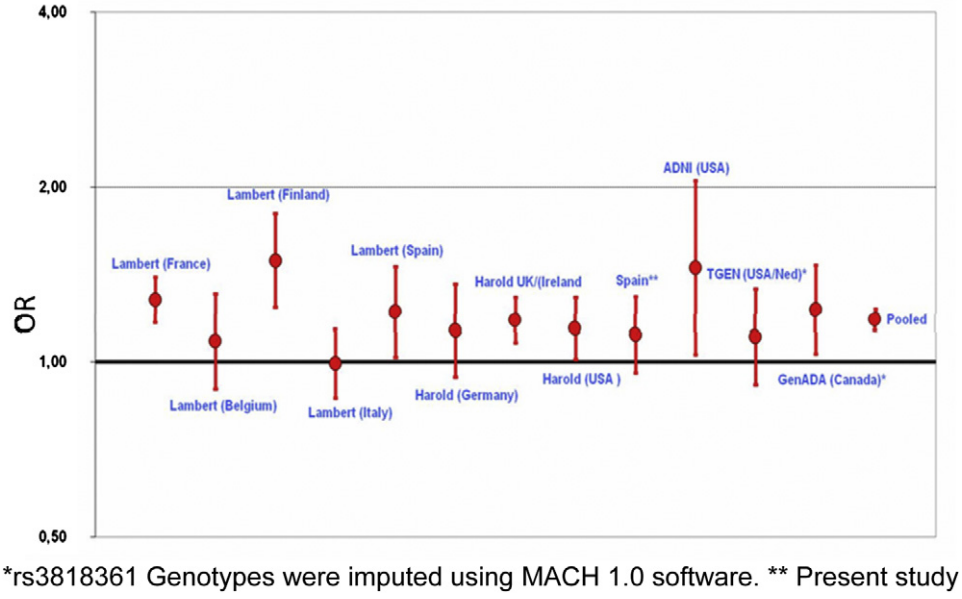
Supplementary Methods

CR1 rs3818361 genotyping:

Amplification primers and sequence-specific probes are included in Supplementary Table 1. Real-time PCRs were performed in a reaction volume of 20 μL with 0.5 μM of each amplification primer, 20 ng of genomic DNA, and 0.2 μM of each FRET (Fluorescence Resonance Energy Transfer) probe. Cycling conditions were as follows: 95°C for 5 minutes, and 50 cycles at 95°C for 30 seconds, 56°C during 20 seconds, and 72°C for 30 seconds. Fluorescence was monitored at the end of each annealing phase. After amplification, specific conditions to obtain melting curves were 95°C for 2 minutes s, 40°C for 30 seconds, and 65°C for 0 seconds (ramping rate: 1°C/s). In the last step, continuous fluorimetric registers were performed by the system at one acquisition register per second. Melting peaks and genotype calls were obtained by using the LightCycler 480 software 1.5.0 SP3 (Roche Diagnostics, Basel, Switzerland).



Supplementary Fig. 1. Forest plot with allelic odds ratios from published studies and pooled odds ratio. The pooled estimate and 95% confidence intervals were calculated using Episheet software assuming a random effects model. Individual and pooled estimates were plotted using Episheet. *rs3818361 genotypes were imputed using MACH 1.0 software. **Present study.

Supplementary Table 1
Primers and probes employed for real-time detection of *CR1* rs3818361 marker

PCR primers	Forward	GGAAAGGACAGTTCCAGAGC
	Reverse	AGCTGCACTCTGCAATGACG
FRET probes	Sensor	CAATTTCCTTTGCTATATCTT [Flc]
	Anchor	[Cy5]TGCTTACCAGAGGGCTTAAAAAT [Phos]

Abbreviations: FRET, fluorescence resonance energy transfer; [Flc], fluorescein; [Cy5], fluorochrome Cy5; [Phos], phosphorothioate.

All sequences are given in 5' to 3' direction.

Supplementary Table 2
Genotypes observed, MAF, and Hardy–Weinberg results for *CR1* rs3818361 SNP in 2470* Spanish individuals

rs3818361	AD cases	Controls	NHEC	All controls	All individuals
CC	748 (66.4%)	801 (68.5%)	76 (62.8%)	877 (67.9%)	1625 (67.2%)
CT	339 (30.1%)	340 (29.1%)	40 (33.1%)	380 (29.4%)	719 (29.7%)
TT	40 (3.5%)	29 (2.5%)	5 (4.1%)	34 (2.6%)	74 (3.1%)
MAF	0.19	0.17	0.21	0.17	0.18
HWE (*P* value, 1 df)	0.83	0.31	0.92	0.34	0.60

Abbreviations: MAF, minor allele frequency; SNP, single nucleotide polymorphism; AD, Alzheimer's disease; NHEC, neurologically healthy elderly control subjects.

* Fifty-two null genotypes were observed (call rate: 97.9%).