

Association of Rare *APOE* Missense Variants V236E and R251G With Risk of Alzheimer Disease

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 Editorial

 Supplemental content

IMPORTANCE The *APOE* $\epsilon 2$ and *APOE* $\epsilon 4$ alleles are the strongest protective and risk-increasing, respectively, genetic variants for late-onset Alzheimer disease (AD). However, the mechanisms linking *APOE* to AD—particularly the apoE protein's role in AD pathogenesis and how this is affected by *APOE* variants—remain poorly understood. Identifying missense variants in addition to *APOE* $\epsilon 2$ and *APOE* $\epsilon 4$ could provide critical new insights, but given the low frequency of additional missense variants, AD genetic cohorts have previously been too small to interrogate this question robustly.

OBJECTIVE To determine whether rare missense variants on *APOE* are associated with AD risk.

DESIGN, SETTING, AND PARTICIPANTS Association with case-control status was tested in a sequenced discovery sample (stage 1) and followed up in several microarray imputed cohorts as well as the UK Biobank whole-exome sequencing resource using a proxy-AD phenotype (stages 2 and 3). This study combined case-control, family-based, population-based, and longitudinal AD-related cohorts that recruited referred and volunteer participants. Stage 1 included 37 409 nonunique participants of European or admixed European ancestry, with 11 868 individuals with AD and 11 934 controls passing analysis inclusion criteria. In stages 2 and 3, 475 473 participants were considered across 8 cohorts, of which 84 513 individuals with AD and proxy-AD and 328 372 controls passed inclusion criteria. Selection criteria were cohort specific, and this study was performed a posteriori on individuals who were genotyped. Among the available genotypes, 76 195 were excluded. All data were retrieved between September 2015 and November 2021 and analyzed between April and November 2021.

MAIN OUTCOMES AND MEASURES In primary analyses, the AD risk associated with each missense variant was estimated, as appropriate, with either linear mixed-model regression or logistic regression. In secondary analyses, associations were estimated with age at onset using linear mixed-model regression and risk of conversion to AD using competing-risk regression.

RESULTS A total of 544 384 participants were analyzed in the primary case-control analysis; 312 476 (57.4%) were female, and the mean (SD; range) age was 64.9 (15.2; 40-110) years. Two missense variants were associated with a 2-fold to 3-fold decreased AD risk: *APOE* $\epsilon 4$ (R251G) (odds ratio, 0.44; 95% CI, 0.33-0.59; $P = 4.7 \times 10^{-8}$) and *APOE* $\epsilon 3$ (V236E) (odds ratio, 0.37; 95% CI, 0.25-0.56; $P = 1.9 \times 10^{-6}$). Additionally, the cumulative incidence of AD in carriers of these variants was found to grow more slowly with age compared with noncarriers.

CONCLUSIONS AND RELEVANCE In this genetic association study, a novel variant associated with AD was identified: R251G always coinherit with $\epsilon 4$ on the *APOE* gene, which mitigates the $\epsilon 4$ -associated AD risk. The protective effect of the V236E variant, which is always coinherit with $\epsilon 3$ on the *APOE* gene, was also confirmed. The location of these variants confirms that the carboxyl-terminal portion of apoE plays an important role in AD pathogenesis. The large risk reductions reported here suggest that protein chemistry and functional assays of these variants should be pursued, as they have the potential to guide drug development targeting *APOE*.

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Late-onset Alzheimer disease (AD) is a highly polygenic neurodegenerative disorder with, to date, 75 risk loci associated with AD risk.¹ Most of the common single-nucleotide variants (SNVs) at these loci only contribute a small amount to an individual's risk of AD,² with the exception of the *APOE* ϵ 2 and ϵ 4 missense variants that are associated with substantially decreased³ and increased AD risk,⁴ respectively. It is estimated that 25% of the genetic variance of AD can be attributed to *APOE* ϵ 2 and *APOE* ϵ 4.⁵ Despite the outsized role of these 2 common *APOE* alleles, more than 25 years after the initial studies linking them to AD, their role in pathogenesis remains ill-defined. Human studies have shown that ϵ 4 speeds and ϵ 2 slows the age-related misprocessing of β -amyloid, although how this occurs at the molecular level remains uncertain.^{6,7} Even the most basic question, does ϵ 4 act via a loss-of-function or gain-of-function mechanism, remains a point of contention.⁸ Loss-of-function variants on *APOE* are exceedingly rare, and the sole case report describing a compound heterozygote with 2 loss-of-function variants involved a patient who was too young to be informative.⁹ The study of additional missense variants on *APOE* may also help to answer this critical question and further elucidate the role of *APOE* in AD. In addition to ϵ 2 and ϵ 4, the only common missense variant (with a minor allele frequency [MAF] greater than 1%) is Arg145Cys (R145C), an African-ancestry variant always found coinherited with *APOE* ϵ 3, which we have shown increases risk for AD.¹⁰ The Arg136Ser (R136S) Christchurch variant has recently been posited to play a protective role in early-onset AD related to *PSEN1* variants, but this study had no statistical genetics support as it was based on data from a single patient.¹¹ Finally, strong functional evidence has been marshalled recently to support a protective role for the Val236Glu (V236E) variant, although this was based on data from an earlier case-control study with only approximately 9000 participants,^{12,13} likely underpowered to provide firm estimates of disease risk.

On this background, we aimed to investigate, at large scale, the association of rare missense variants on *APOE* with AD risk. We used the Alzheimer's Disease Sequencing Project (ADSP) whole-genome sequencing (WGS) and whole-exome sequencing (WES) data as our discovery sample (stage 1) and sought to replicate significant variants (stages 2 and 3) in multiple cohorts using microarray data imputed on the Trans-Omics for Precision Medicine (TOPMed) reference panel (National Institutes of Health),¹⁴ or by using directly sequenced and genotyped variants from a large Danish general prospective population cohort,¹⁵ as well as using the proxy-AD phenotype¹ in the UK Biobank WES data. After filtering, 3 variants, Leu28Pro (L28P), Val236Glu (V236E), and Arg251Gly (R251G), were tested for their association with AD risk after adjusting for ϵ 2 and ϵ 4 dosages. In complementary analyses, we assessed these associations in an *APOE*-stratified approach to account for the complete linkage disequilibrium of these variants with either the ϵ 2, ϵ 3, or ϵ 4 allele. In secondary analyses, combining stage 1 and 2 data sets, we tested their association with age at onset in individuals with AD and with risk of conversion to AD using competing-risk regression.

Key Points

Question Are *APOE* missense variants, other than the common *APOE* alleles ϵ 2 and ϵ 4, associated with Alzheimer disease (AD) risk?

Findings In this genetic association study including 544 384 participants, multiple studies including 67 896 individuals with AD, 28 484 with proxy-AD, and 340 306 healthy controls were meta-analyzed. Two rare missense variants (*APOE* ϵ 3 [V236E] and *APOE* ϵ 4 [R251G]) substantially reduced the risk of AD (by more than 60% and more than 50%, respectively).

Meaning Single amino acid alterations of the *APOE* ϵ 3 and *APOE* ϵ 4 isoforms can result in substantial risk reduction for AD.

Methods

Participants and Sources of Data

Participants or their caregivers provided written informed consent in the original studies. The current study protocol was granted an exemption by the Stanford University Institutional Review Board because the analyses were carried out on deidentified, off-the-shelf data; therefore, additional informed consent was not required. For stage 1 and stage 2, phenotypic information and genotypes were obtained from publicly released genome-wide association study data sets assembled by the Alzheimer's Disease Genetics Consortium (ADGC) and derived from WES and WGS data generated by the ADSP, with phenotype and genotype ascertainment described elsewhere.¹⁶⁻²⁰ The cohorts' queried accession numbers, as well as the sequencing technology or SNV genotyping platforms are described in eTables 1 and 2 in [Supplement 1](#). Information about stage 3, which included external replication cohorts and UK Biobank, is provided in the eMethods in [Supplement 1](#). Briefly, these included European Alzheimer's Disease DNA Biobank (EADB) core, European Alzheimer's Disease Initiative (EADI), Genetic and Environmental Risk in Alzheimer's Disease Consortium (GERAD), Norwegian Dementia Genetics Network (DemGene), and Genome Research at Fundació Alzheimer Center Barcelona (GR@ACE)/Dementia Genetics Spanish Consortium (DEGESCO) cohorts for which phenotype, genotype quality control, and imputation have already been described in Bellenguez et al¹; and the Copenhagen City Heart Study (CCHS) and the Copenhagen General Population Study (CGPS) *APOE* sequencing and genotyping were described in Rasmussen et al.¹⁵ The following sections describe quality control procedures and ancestry determination applied to the ADSP and ADGC samples, respectively, used as stage 1 and stage 2. This study followed the Strengthening the Reporting of Genetic Association Studies (STREGA) reporting guideline. UK Biobank WES data were analyzed under Application Number 45420.

Quality Control Procedures

Prior to ancestry, principal components, and relatedness determination, in each cohort platform, variants were excluded based on genotyping rate (less than 95%), MAF less than 1%,

and Hardy-Weinberg equilibrium in controls ($P < 10^{-6}$) using PLINK version 1.9.²¹ gnomAD²² database-derived information was used to filter out SNVs that met one of the following exclusion criteria^{23,24}: (1) located in a low-complexity region, (2) located within common structural variants (MAF greater than 1%), (3) multiallelic SNVs with MAF greater than 1% for at least 2 alternate alleles, (4) located within a common insertion or deletion, (5) having any flag different than PASS (passed all variant filters) in gnomAD version 3, and (6) having potential probe variants. The latter are defined as SNVs for which the probe may have variable affinity owing to the presence of other SNV(s) within 20 base pairs and with MAF greater than 1%. Individuals with more than 5% genotype missingness were excluded. Duplicate individuals were identified with KING (Kinship-based Inference for GWAS)²⁵ and their clinical, diagnostic, and pathological data (including age at onset of cognitive symptoms, age at examination for clinical diagnosis, age at last examination, and age at death), as well as sex, race, and *APOE* genotype were cross-referenced across cohorts. Duplicate entries with irreconcilable phenotype or discordant sex were flagged for exclusion. For individuals with duplicated genotype in sequencing and imputed data, the sequencing entry was used in the stage 1 discovery set and the imputed entry was not included in the stage 2 replication set. To apply the PC-AiR and PC-Relate methods, we simply considered the intersection of the variants passing quality control in both ADSP WES and ADSP WGS in the discovery set and similarly the intersection of the variants across cohorts genotyping platform in the replication set.

Ancestry Determination

For each cohort, we first determined the ancestry of each individual with SNPWeights version 2 (Harvard)²⁶ using reference populations from the 1000 Genomes Consortium.²⁷ By applying an ancestry percentage cutoff greater than 75%, the samples were stratified into 5 super populations: South Asian, East Asian, United States, African, and European individuals and an admixed group composed of individuals not passing the 75% cutoff in any single ancestry (eTable 3 in Supplement 1).^{10,23} Since the *APOE* missense variants of interest L28P, V236E, and R251G are too rare to assess reliably in non-European ancestry populations (eTable 4 in Supplement 1), we restricted our analysis to European and admixed European individuals. Admixed European individuals were also included in the main analysis and were part of the admixed group defined above and had at least 15% European ancestry. We performed sensitivity analyses in increments of 30%, including admixed European individuals at 45% and 75% cutoffs. The latter corresponding to the super population threshold.

Imputation

Each cohort genotyping platform was imputed on the TOPMed imputation server per ancestry group to obtain an imputation quality (R^2) per ancestry group. We retained cohorts with R^2 greater than 0.70 at rs199768005 for the V236E analyses and at rs26760661 for the R251G analyses. As there was no significant association signal for rs769452 (L28P) in the stage 1

primary analysis, we did not check its imputation quality in stage 2 samples.

APOE Genotype Ascertainment

We directed specific attention to the genotyping of the SNVs determining the main *APOE* genotype (rs429358 and rs7412), rs769452-C (*APOE* [L28P]), rs199768005-A (*APOE* [V236E]), and rs267606661-G (*APOE* [R251G]) and followed the procedure described in Le Guen et al.¹⁰ Note that Leu28Pro (L28P), Val236Glu (V236E), and Arg251Gly (R251G) are also sometimes referred to as *L46P*, *V254E*, and *R269G*, respectively, when the first 18 codons of *APOE* encoding a signal peptide are included.

Samples Analyzed

Our discovery sample (stage 1) was composed of individuals of European and admixed European ancestry from the ADSP WES and WGS, corresponding to 11 868 individuals with AD and 11 934 cognitively normal controls (Table 1). eFigure 1 in Supplement 1 provides a flowchart of the filtering steps leading to the inclusion of these individuals and describes how these data sets were combined. To build a replication sample (stage 2) for V236E and R251G, we queried for individuals of European and admixed European ancestry in all the publicly available microarray genetic data sets that we had access to at the time of the study in July 2021 (Table 1). These data sets are largely part of the ADGC, and as such, this replication will be referred to hereafter as the ADGC replication in stage 2. After quality control and duplicate removal, 7768 individuals with AD and 8059 controls remained in the ADGC replication sample. eTable 5 in Supplement 1 presents the demographic characteristics of the remaining individuals with AD and cognitively unimpaired controls. In stage 3, we pursued additional replication in external data sets (not publicly available) and in the UK Biobank WES using the proxy-AD phenotype (Table 1; eMethods in Supplement 1). Overall, the external replications included 36 393 individuals with AD and 150 943 controls, and the UK Biobank replication included 28 484 individuals with proxy-AD and 157 436 controls. Across cohorts reported in Table 1, the *APOE* genotype were split as follows: $\epsilon 2/\epsilon 2$, 0.5%; $\epsilon 2/\epsilon 3$, 10.4%; $\epsilon 3/\epsilon 3$, 54.5%; $\epsilon 2/\epsilon 4$, 2.5%; $\epsilon 3/\epsilon 4$, 27.6%; $\epsilon 4/\epsilon 4$, 4.4%.

Study Design and Statistical Analysis

In our analysis, we only considered missense variants with a minor allele count greater than 10 in any *APOE* main genotype groups in our next-generation sequencing discovery (stage 1) to avoid outlier-confounded effect size estimates.²⁸ Three *APOE* missense variants were retained for further analyses: L28P, V236E, and R251G (eTable 4 in Supplement 1). The V236E variant is always coinherited with *APOE* $\epsilon 3$, and the L28P and R251G are always coinherited with *APOE* $\epsilon 4$ (eTable 6 in Supplement 1). Two variants are coinherited when they are on the same chromosome copy and close enough to each other that a meiotic crossover event never occurs between them. We thus developed 2 complementary approaches to take into account these linkage disequilibrium structures. In primary analyses, we estimated the AD risk associated with L28P, V236E, and

Table 1. Demographic Characteristics per APOE Genotype^a

Sample	Diagnosis	APOE ε2/ε2			APOE ε2/ε3			APOE ε3/ε3			APOE ε2/ε4			APOE ε3/ε4			APOE ε4/ε4		
		Individuals, Total, No.	Female, No. (%)	Age, mean (SD), y	Total, No.	Female, No. (%)	Age, mean (SD), y	Total, No.	Female, No. (%)	Age, mean (SD), y	Total, No.	Female, No. (%)	Age, mean (SD), y	Total, No.	Female, No. (%)	Age, mean (SD), y	Total, No.	Female, No. (%)	Age, mean (SD), y
ADSP	CN	11 934	73	82.6 (8.3)	1481	924 (62.4)	83.0 (8.0)	7429	4636 (62.4)	82.3 (8.1)	195	137 (70.3)	79.8 (8.9)	2561	1590 (62.1)	79.7 (8.2)	195	123 (63.1)	76.6 (7.5)
	AD	11 868	29	82.5 (8.3)	583	369 (63.3)	80.1 (9.7)	5313	3236 (60.9)	77.0 (10.1)	258	158 (61.2)	75.3 (8.5)	4919	2853 (58.0)	73.2 (8.5)	766	406 (53.0)	67.9 (8.1)
ADGC	CN	8059	56	79.1 (10.2)	978	629 (64.3)	76.2 (9.5)	4795	2968 (61.9)	74.5 (9.4)	209	132 (63.2)	73.8 (10.1)	1847	1143 (61.9)	71.4 (9.3)	174	106 (60.9)	68.7 (9.3)
	AD	7768	10	72.5 (8.2)	323	181 (56.0)	75.8 (10.4)	2494	1586 (63.6)	74.7 (10.5)	237	150 (63.3)	75.7 (8.8)	3258	2059 (63.2)	73.0 (8.6)	1446	830 (57.4)	69.7 (7.2)
EADB core	CN	21 160	121	68.6 (13.2)	2503	1457 (58.2)	66.8 (15.1)	13 365	7725 (57.8)	67.0 (14.5)	396	220 (55.6)	66.7 (13.3)	4390	2445 (55.7)	66.3 (13.6)	385	212 (55.1)	64.2 (12.6)
	AD	19 873	27	76.4 (11.7)	877	522 (59.5)	74.2 (11.2)	8285	5128 (61.9)	72.9 (11.0)	435	287 (66.0)	73.2 (10.7)	8003	5042 (63.0)	71.7 (9.7)	2246	1289 (57.4)	67.6 (8.8)
GR@ACE	CN	8539	33	53.1 (17.6)	858	448 (52.2)	57.5 (18.7)	6005	3009 (50.1)	56.7 (18.0)	99	49 (49.5)	56.7 (17.6)	1459	727 (49.8)	56.7 (17.6)	85	37 (43.5)	54.9 (14.8)
	AD	7355	16	84.6 (8.6)	389	274 (70.4)	81.4 (8.1)	3840	2703 (70.4)	80.9 (7.9)	115	84 (73.0)	78.7 (7.4)	2590	1808 (69.8)	78.7 (7.4)	405	262 (64.7)	74.8 (7.3)
EADI	CN	6331	38	82.6 (7.5)	772	457 (59.2)	81.0 (7.5)	4247	2582 (60.8)	80.1 (7.7)	109	66 (60.6)	78.8 (7.1)	1106	655 (59.2)	79.0 (7.6)	59	42 (71.2)	77.1 (6.7)
	AD	2397	7	79.3 (6.0)	128	88 (68.8)	78.0 (10.8)	1078	704 (65.3)	76.5 (10.6)	71	42 (59.2)	73.4 (8.8)	888	586 (66.0)	72.6 (9.2)	225	146 (64.9)	70.0 (7.0)
GERAD	CN	7007	47	49.3 (11.0)	853	427 (50.1)	51.5 (12.6)	4127	2142 (51.9)	50.9 (11.9)	180	93 (51.7)	49.8 (10.9)	1627	843 (51.8)	49.9 (10.9)	173	86 (49.7)	49.9 (11.0)
	AD	2989	10	81.2 (9.7)	140	88 (62.9)	79.3 (11.3)	1092	677 (62.0)	79.3 (9.6)	90	57 (63.3)	80.4 (7.6)	1306	838 (64.2)	77.7 (8.9)	351	219 (62.4)	74.2 (8.4)
DemGene	CN	5911	32	68.7 (11.2)	685	336 (49.1)	69.2 (12.4)	3236	1540 (47.6)	68.9 (11.0)	167	76 (45.5)	70.6 (10.6)	1595	769 (48.2)	67.3 (10.5)	196	87 (44.4)	64.7 (11.0)
	AD	1687	5	74.0 (1.4)	72	42 (58.3)	71.6 (10.6)	537	359 (66.9)	73.7 (9.6)	43	31 (72.1)	75.4 (7.0)	769	512 (66.6)	72.2 (8.4)	261	161 (61.7)	69.3 (8.1)
CCHS and CGPS	CN	101 995	705	57.0 (13.2)	12 818	7063 (55.1)	57.6 (13.6)	57 115	31 299 (54.8)	57.5 (13.4)	2936	1627 (55.4)	56.8 (13.0)	25 616	14 063 (54.9)	56.7 (12.8)	2778	1600 (57.6)	55.3 (12.7)
	AD	2092	12	72.6 (5.3)	129	69 (53.5)	73.3 (8.4)	844	496 (58.8)	73.3 (8.4)	70	43 (61.4)	71.2 (8.0)	821	512 (62.4)	70.9 (8.0)	216	123 (56.9)	68.8 (7.9)

Abbreviations: AD, Alzheimer disease; ADGC, Alzheimer's Disease Genetic Consortium; ADSP, Alzheimer's Disease Sequencing Project; CCHS, Copenhagen City Heart Study; CGPS, Copenhagen General Population Study; CN, cognitively normal; DemGene, Norwegian Dementia Genetics Network; EADB, European Alzheimer's Disease DNA Biobank; EADI, European Alzheimer's Disease Initiative; GERAD, Genetic and Environmental Risk in Alzheimer's Disease Consortium; GR@ACE, Genome Research at Fundació Alzheimer Center Barcelona. ^a UK Biobank demographic characteristics are not reported in this table since cases correspond to proxy-AD phenotype mostly relying on self-report of first-degree relatives' diagnosis without age at onset being specified.

R251G on case-control diagnoses using linear mixed-model regression (stages 1 and 2 and UK Biobank) and logistic regression model (stage 3), adjusted for $\epsilon 2$ and $\epsilon 4$ dosages, in addition to the covariates described below for all analyses. The adjustment by the common $\epsilon 3$ and $\epsilon 4$ APOE alleles is necessary because the rare variants tested here are always co-inherited with either the $\epsilon 3$ or $\epsilon 4$ APOE allele. In complementary analyses, we also estimated the AD risk associated with V236E and R251G stratified by their associated common APOE allele genotype. V236E was assessed in APOE $\epsilon 3/\epsilon 3$ and R251G was assessed in the APOE $\epsilon 3/\epsilon 4$ stratum. An association was considered significant in stage 1 if it reached a Bonferroni-corrected P value threshold of .017 (.05/3) in the model adjusted for $\epsilon 2$ and $\epsilon 4$ dosages; all P values were 2-tailed. L28P was not associated with AD risk in this model and was not studied further.

Sample sizes and demographic characteristics for the stratified analyses are shown in eTable 5 in Supplement 1. In sensitivity analyses, we estimated AD risk associations for different European ancestry inclusion thresholds. In secondary analyses, combining stages 1 and 2 data sets, we estimated the influence of significant stage 1 variants on age at onset in AD cases using linear mixed-model regression and risk of conversion to AD using competing-risk regression. In secondary analyses, associations were considered significant when passing the nominal P value threshold of .05. The case-control and age-at-onset analyses used linear mixed-model regression available through the GENESIS package version 3.12.²⁹ Multivariate competing-risk regression and cumulative incidence estimation were implemented using the *cmprsk* package version 2.2.³⁰ In this time-to-event analysis, failure events were defined as age at onset for individuals who developed AD (conversion to AD) and age at death for controls. Controls without reported death were right-censored at age at last visit. Left censoring was set at age 50 years, and younger individuals were excluded from the analysis. All statistical analyses were adjusted for sex and 4 genetic principal components estimated with the PC-AiR method³¹ implemented in GENESIS. Linear mixed-model analyses were additionally covaried by a sparse genetic association matrix estimated with the PC-Relate method³² implemented in GENESIS. Case-control analyses were not adjusted for age given that correcting for age when individuals with AD are younger than controls leads to the model incorrectly inferring the age effect on AD risk, resulting in statistical power loss.²³

Case-control analyses in stage 3, external replication cohorts and proxy-AD phenotype in UK Biobank, were implemented to be consistent with the stage 1 primary analyses. Exact model/analysis details are described in the eMethods in Supplement 1. For the ADSP/ADGC cohorts, all statistical analyses were performed in R version 4.0.2 (The R Foundation). All meta-analyses were implemented with a fixed-effect inverse variance-weighted design implemented in the *metafor* R package version 3.0.2.³³

Results

A total of 544 384 participants were analyzed in the primary case-control analysis; 312 476 (57.4%) were female, and the

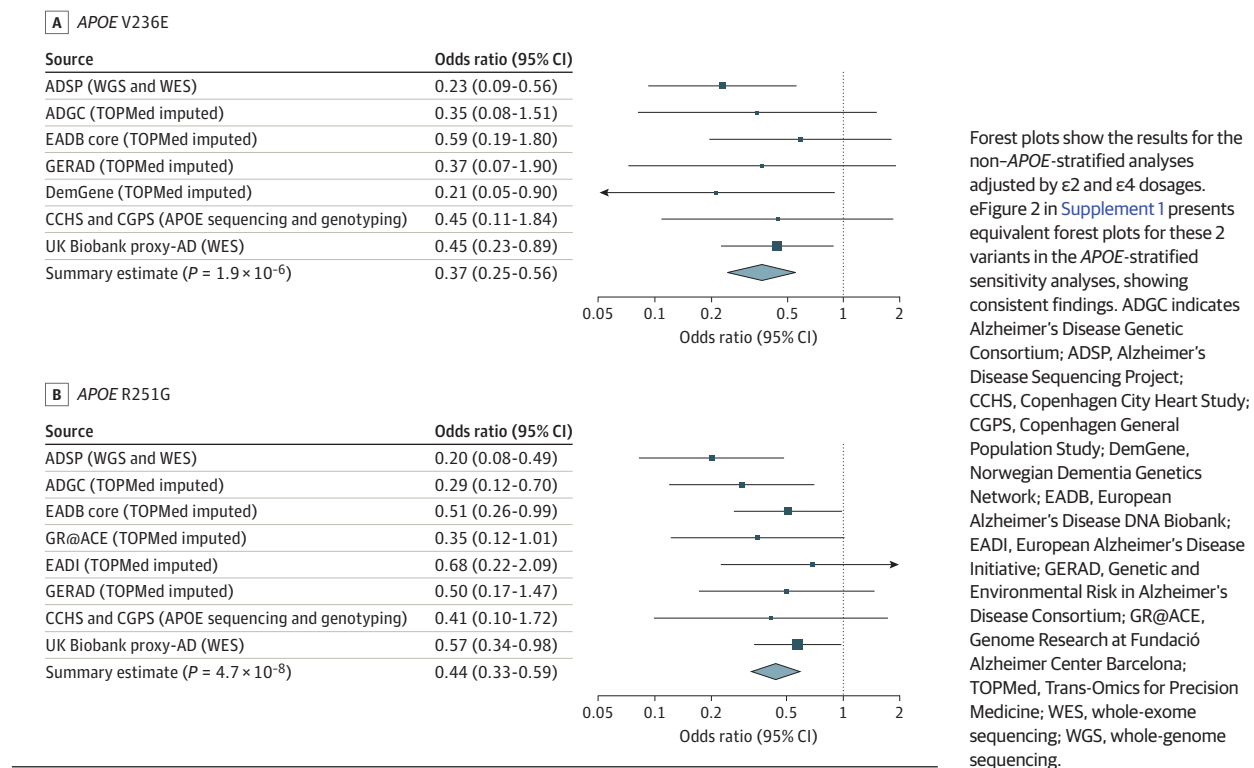
mean (SD; range) age was 64.9 (15.2; 40-110) years. In stage 1 primary analyses, V236E (rs199768005-A) and R251G (rs267606661-G) were associated with a 4-fold to 5-fold decreased AD risk in nonstratified analyses adjusted for $\epsilon 2$ and $\epsilon 4$ dosages (V236E: odds ratio [OR], 0.23; 95% CI, 0.09-0.56; $P = .001$; R251G: OR, 0.20; 95% CI, 0.08-0.49; $P = 3.7 \times 10^{-4}$) (Figure 1; Table 2). Similarly, in APOE-stratified analyses, V236E was associated with a 3-fold decreased AD risk in individuals with $\epsilon 3/\epsilon 3$ (OR, 0.31; 95% CI, 0.12-0.82; $P = .02$), and R251G was associated with a 5-fold decreased AD risk in individuals with $\epsilon 3/\epsilon 4$ (OR, 0.17; 95% CI, 0.06-0.48; $P = 7.8 \times 10^{-4}$) (Table 2). The L28P variant (rs769452-C) was not associated with AD risk in the nonstratified analyses (OR, 1.12; 95% CI, 0.77-1.62; $P = .56$). As such, it was not investigated further.

In stages 2 and 3, across multiple replication cohorts, the effects of V236E and R251G in nonstratified analyses were concordant and both were significantly associated with AD risk (V236E: OR, 0.42; 95% CI, 0.27-0.66; $P = 2.0 \times 10^{-4}$; R251G: OR, 0.48; 95% CI, 0.35-0.66; $P = 5.8 \times 10^{-6}$). The overall meta-analysis (Figure 1; Table 2) provides robust effect size estimate for these 2 variants and confirmed their association with a 2-fold to 3-fold decreased AD risk (V236E: OR, 0.37; 95% CI, 0.25-0.56; $P = 1.9 \times 10^{-6}$; R251G: OR, 0.44; 95% CI, 0.33-0.59; $P = 4.7 \times 10^{-8}$). Similar results were obtained in APOE-stratified meta-analyses (Table 2; eFigure 2 in Supplement 1). We further estimated the odds per APOE genotype group, using individuals with $\epsilon 3/\epsilon 3$ who did not carry V236E as the reference (ie, OR of individuals with APOE $\epsilon 3/\epsilon 3$ equals 1), by meta-analyzing the ADSP discovery and ADGC replication cohorts. Compared with the reference $\epsilon 3/\epsilon 3$ group, the $\epsilon 3/\epsilon 3$ (V236E) and $\epsilon 3/\epsilon 4$ (R251G) groups had AD risk lower than or similar to the $\epsilon 2/\epsilon 3$ group (Figure 2).

Results of sensitivity analyses evaluating different European ancestry cutoffs are shown in eTable 8 and eFigure 3 in Supplement 1. Briefly, the results remained unchanged when selecting individuals with admixed ancestry with at least 45% European ancestry or when restricting the analysis to individuals with European ancestry (75% cutoff). We note that the ORs in the combined ADSP/ADGC data sets for V236E and R251G remain unchanged at different ancestry cutoffs. For example, using an ancestry cutoff at 75%, the nonstratified meta-analysis yielded an OR of 0.27 (95% CI, 0.12-0.58; $P = 8.6 \times 10^{-4}$) for V236E compared with an OR of 0.26 (95% CI, 0.12-0.56; $P = 5.4 \times 10^{-4}$) using a cutoff of 15%. Similar observations were made for the R251G variant. As additional supplementary analyses, we assessed the effect of the inclusion of all dementia (rather than AD specifically) in the CCHS and CGPS data set, and we estimated the significance without including UK Biobank. Overall, the significance of the results slightly improved when including a broader dementia category (R251G: OR, 0.44; 95% CI, 0.33-0.59; $P = 3.5 \times 10^{-8}$) (eTable 9 in Supplement 1). While removing UK Biobank proxy-AD phenotype samples reduced the significance of our results slightly, the ORs became slightly more protective (R251G: OR, 0.39; 95% CI, 0.27-0.56; $P = 1.2 \times 10^{-7}$) (eTable 10 in Supplement 1).

In secondary analyses, including data from stages 1 and 2, we considered the meta-analysis of ADSP/ADGC samples

Figure 1. Association of V236E and R251G With Alzheimer Disease (AD) Risk Across All Cohorts



(eTable 5 in Supplement 1). In non-APOE stratified analyses adjusted for $\epsilon 2$ and $\epsilon 4$ dosages (eTable 7 in Supplement 1), V236E carriers had a mean age at AD onset 10.5 years older than non-carriers ($\beta = 10.64$; 95% CI, 1.78-19.49; $P = .02$) and slower incidence with age (hazard ratio [HR], 0.30; 95% CI, 0.12-0.76; $P = .01$). While R251G's association with age at onset was not significant ($\beta = 0.97$; 95% CI, -2.96 to 4.91; $P = .63$), its association with reduced AD incidence with age was just nominally significant (HR, 0.67; 95% CI, 0.46-0.97; $P = .04$). In APOE-stratified analyses (eTable 7 in Supplement 1), a similar association of V236E with age at AD onset was observed in individuals with $\epsilon 3/\epsilon 3$ ($\beta = 10.93$; 95% CI, 1.06-20.81; $P = .03$). R251G carriers had a mean age at AD onset 6 years older than noncarriers of $\epsilon 3/\epsilon 4$, but this association was not significant ($\beta = 6.04$; 95% CI, -0.71 to 12.79; $P = .08$). The competing risk results emphasized that the cumulative incidence of AD in participants with $\epsilon 3/\epsilon 3$ grows slower with age in individuals carrying the V236E variant (HR, 0.40; 95% CI, 0.17-0.97; $P = .04$) and similarly in participants with $\epsilon 3/\epsilon 4$ carrying the R251G variant (HR, 0.26; 95% CI, 0.13-0.54; $P = 2.9 \times 10^{-4}$).

Discussion

We have shown that 2 missense variants V236E and R251G are each associated with a more than 2-fold reduction in AD risk (Figure 2). These variants have an allele frequency of less than 0.1% in gnomAD version 3.1, even when restricting this frequency estimate to individuals of European ancestry (eTable 4

in Supplement 1). Because of their rarity and linkage disequilibrium with the common APOE $\epsilon 3$ and $\epsilon 4$ alleles, they have not been identified in prior genome-wide association studies.¹ The protective effect of V236E has already been reported in a smaller prior study focused on APOE¹³ and was suggestive in a population-based study,¹⁵ but we validated this finding here in a large-scale genomic study and provide an improved estimate of its effect size. To our knowledge, the association of R251G with AD risk has not been previously reported. This variant, carried on the same haplotype as $\epsilon 4$, is the first APOE variant found to mitigate the AD risk attributable to the $\epsilon 4$ isoform of the apoE protein. Notably, having R251G in association with APOE $\epsilon 4$ results in a risk estimate similar to APOE $\epsilon 2$, as shown in Figure 2 where APOE $\epsilon 3/\epsilon 4$ (R251G) and APOE $\epsilon 2/\epsilon 3$ have an equivalent OR.

Regarding potential mechanisms driving these associations, it is notable that these 2 variants are on apoE's C-terminal domain. The common APOE $\epsilon 2$ and APOE $\epsilon 4$ alleles are located on the N-terminal domain of the protein near the receptor-binding region. Their outsized role in AD risk has, understandably, focused attention on the N-terminal domain and the differential capacity of these alleles to, for example, bind apoE's receptors.^{34,35} The current results add support to studies suggesting that the C-terminal domain is also of critical importance for AD pathogenesis.³⁶⁻³⁸ R251G is located within apoE's lipid-binding region (amino acid residues 244 to 272), while V236E is adjacent to this region.⁸ A 2021 publication¹² provided evidence for the protectiveness of V236E against AD pathology and explored the functional mechanism supporting its protective role. The lipid-binding region, with its abun-

Table 2. Association of V236E and R251G With Alzheimer Disease (AD) Risk^a

Sample	AD case-control regression (nonstratified)				AD case-control regression (APOE stratified)			
	Individuals, No.	MAC	OR (95% CI)	P value	Individuals, No.	MAC	OR (95% CI)	P value
V236E^b								
ADSP	23 427	20	0.23 (0.09-0.56)	.001	12 604	17	0.31 (0.12-0.82)	.02
ADGC imputed	11 652	10	0.35 (0.08-1.51)	.16	5741	10	0.40 (0.10-1.57)	.19
EADB core	41 033	27.17	0.59 (0.19-1.80)	.34	21 650	21.28	0.53 (0.15-1.92)	.30
GERAD	9996	17.72	0.37 (0.07-1.90)	.18	5219	9.43	0.77 (0.10-6.06)	.78
DemGene	7598	58.68	0.21 (0.05-0.90)	.009	3773	35.88	0.56 (0.13-2.46)	.40
CCHS and CGPS	104 084	240	0.45 (0.11-1.84)	.23	57 955	191	0.18 (0.01-2.97)	.27
UK Biobank proxy-AD	185 741	277	0.45 (0.23-0.89)	.02	109 120	219	0.47 (0.21-1.04)	.06
Meta-analysis	383 531	650.57	0.37 (0.25-0.56)	1.9×10^{-6}	216 062	503.59	0.43 (0.27-0.69)	4.4×10^{-4}
R251G^c								
ADSP	23 314	26	0.20 (0.08-0.49)	3.7×10^{-4}	7335	18	0.17 (0.06-0.48)	7.8×10^{-4}
ADGC imputed	14 134	29	0.29 (0.12-0.70)	.006	4630	16	0.19 (0.07-0.54)	.002
EADB core	41 033	59.16	0.51 (0.26-0.99)	.049	12 393	40.27	0.34 (0.15-0.76)	.008
GR@ACE	15 894	21.27	0.35 (0.12-1.01)	.049	4049	17.81	0.22 (0.06-0.77)	.01
EADI	8728	19.21	0.68 (0.22-2.09)	.49	1994	13.32	1.14 (0.32-4.04)	.84
GERAD	9996	23.17	0.50 (0.17-1.47)	.18	2933	16.82	0.57 (0.18-1.88)	.34
CCHS and CGPS	104 087	105	0.41 (0.10-2.72)	.23	26 437	75	0.33 (0.05-2.43)	.28
UK Biobank proxy-AD	185 735	335	0.57 (0.34-0.98)	.04	43 820	262	0.67 (0.36-1.22)	.19
Meta-analysis	402 921	617.81	0.44 (0.33-0.59)	4.7×10^{-8}	103 591	459.22	0.41 (0.29-0.57)	3.2×10^{-7}

Abbreviations: ADGC, Alzheimer's Disease Genetic Consortium; ADSP, Alzheimer's Disease Sequencing Project; CCHS, Copenhagen City Heart Study; CGPS, Copenhagen General Population Study; DemGene, Norwegian Dementia Genetics Network; EADB, European Alzheimer's Disease DNA Biobank; EADI, European Alzheimer's Disease Initiative; GERAD, Genetic and Environmental Risk in Alzheimer's Disease Consortium; GR@ACE, Genome Research at Fundació Alzheimer Center Barcelona; MAC, minor allele count; OR, odds ratio.

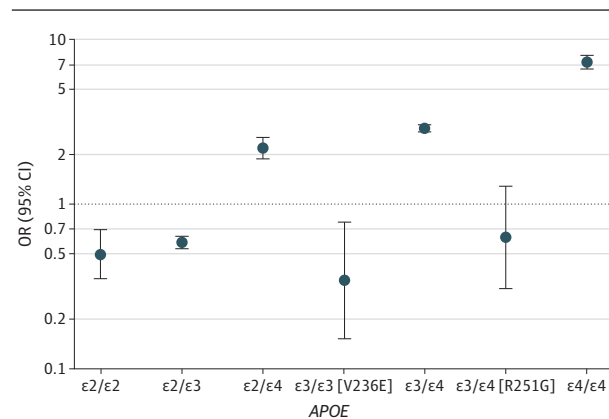
^a The significance of their association with AD risk was equivalent in

nonstratified analyses adjusted by APOE $\epsilon 2$ and $\epsilon 4$ dosages and in APOE-stratified analysis considering the main APOE genotype group with the most carriers for each variant, namely $\epsilon 3/\epsilon 3$ and $\epsilon 3/\epsilon 4$ for V236E and R251G, respectively.

^b For V236E, all APOE alleles are used in nonstratified analyses and the $\epsilon 3/\epsilon 3$ alleles only in APOE-stratified analyses.

^c For R251G, all APOE alleles are used in nonstratified analyses and the $\epsilon 3/\epsilon 4$ alleles only in APOE-stratified analyses.

Figure 2. Risk Equivalence of APOE $\epsilon 3/\epsilon 3$ (V236E) and APOE $\epsilon 3/\epsilon 4$ (R251G) to $\epsilon 2/\epsilon 3$ Carriers



Alzheimer disease risk per APOE genotype was compared with the APOE $\epsilon 3/\epsilon 3$ reference group (ie, odds ratio [OR] for APOE $\epsilon 3/\epsilon 3$ equals to 1), meta-analyzing results from the Alzheimer's Disease Genetic Consortium and Alzheimer's Disease Sequencing Project cohorts (stages 1 and 2). eFigure 3 in Supplement 1 presents equivalent results at different inclusion cutoffs for European ancestry.

dance of nonpolar residues, is thought to be a region that can foster oligomerization.³⁹⁻⁴¹ Switching a nonpolar valine for an

acidic glutamic acid might be predicted to reduce the hydrophobicity of this region and reduce its tendency to oligomerize. Notably, the authors showed reduced levels of insoluble β -amyloid and apoE aggregates in the brain of V236E carriers compared with noncarriers.¹² In 5 \times FAD mice, they observed that APOE $\epsilon 3$ (V236E) reduced A β deposition, plaque-associated immune response, and neuritic dystrophy around amyloid plaques.¹² Chemically, they noted that APOE $\epsilon 3$ (V236E) primarily remains as a monomer and is less likely to form oligomers compared with the canonical APOE $\epsilon 3$ allele.¹² This propensity of V236E to reduce apoE aggregation was also observed when this variant was introduced on an APOE $\epsilon 4$ allele. It is worth noting, however, that V236E also appears to increase dimerization (see Figure S10¹²), which may affect apoE's ability to bind to its receptors.⁴²⁻⁴⁴

Given that R251G is located squarely in the lipid-binding region of the protein, it is possible that R251G confers a protective effect by reducing apoE's ability to form insoluble oligomers. The switch from a charged arginine amino acid to a nonpolar glycine might, however, be expected to increase rather than decrease oligomerization. Changes in this region could also enhance apoE $\epsilon 4$'s ability to bind lipids rendering it more like $\epsilon 3$ or $\epsilon 2$ in this capacity.⁴⁵ Alternatively, the introduction of glycine could disrupt the α helix

structure of the C-terminal impacting apoE $\epsilon 4$'s hypothesized N-terminal-C-terminal domain interaction.^{34,35} In any case, pending protein chemistry experiments exploring potential structural and functional changes, the mechanism underlying the substantial protective effect of R251G remains to be elucidated.

Limitations

Our study has several limitations. The V236E association was not genome-wide significant. We included the UK Biobank data set that does not include a direct clinical diagnosis of AD. Because of the paucity of variant carriers of non-European ancestries, we did not assess these variants in other ancestries (although they can be found in African American individuals and admixed Latino individuals based on gnomAD estimates; eTable 4 in Supplement 1). These caveats point to the need for further confirmation of these variants as available AD data sets grow and become more ancestrally diverse.

Conclusions

Our work was performed on, to our knowledge, the largest available sample to date for APOE $\epsilon 3$ (V236E) and APOE $\epsilon 4$ (R251G). These findings validate the protective effect of the V236E variant and has uncovered a novel protective missense variant on APOE $\epsilon 4$. Each variant had a substantial association with reducing the risk of AD. While some compelling functional data suggest that V236E confers protection by reducing oligomerization of apoE, there are alternative mechanisms that merit consideration (increasing dimerization, for one). The protective mechanism of R251G remains unexplored, but finding a single amino acid substitution that renders the APOE - $\epsilon 4$ allele protective supports the idea that APOE $\epsilon 4$ -specific treatments are worth exploring.^{46,47} We anticipate that the findings reported here will spark additional mechanistic work on apoE's role in AD pathogenesis.

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