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

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Key Points

Question

Are *APOE* missense variants, other than the common *APOE* alleles $\epsilon 2$ and $\epsilon 4$, 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* $\epsilon 3$ [V236E] and *APOE* $\epsilon 4$ [R251G]) substantially re-

duced 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.

This genetic association study evaluates whether rare missense variants on *APOE* are associated with Alzheimer disease risk.

Abstract

Importance

The *APOE* ϵ 2 and *APOE* ϵ 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* ϵ 2 and *APOE* ϵ 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* ϵ 4 (R251G) (odds ratio, 0.44; 95% CI, 0.33-0.59; $P = 4.7 \times 10^{-8}$) and *APOE* ϵ 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 coinherited with ϵ 4 on the *APOE* gene, which mitigates the ϵ 4-associated AD risk. The protective effect of the V236E variant, which is always coinherited with ϵ 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*.

Introduction

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 $\epsilon 2$ and $\epsilon 4$ 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 $\epsilon 2$, $\epsilon 3$, or $\epsilon 4$ 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.

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.^{16,17,18,19,20} 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 al.¹; 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 rs267606661 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 flow-chart 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* ϵ 3, and the L28P and R251G are always coinherited with *APOE* ϵ 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 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 ϵ 2 and ϵ 4 dosages, in addition to the covariates described below for all analyses. The adjustment by the common ϵ 3 and ϵ 4 *APOE* alleles is necessary because the rare variants tested here are always coinherited with either the ϵ 3 or ϵ 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* ϵ 3/ ϵ 3 and R251G was assessed in the *APOE* ϵ 3/ ϵ 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 ϵ 2 and ϵ 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 non-stratified 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 (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 *APOE*¹³ 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.^{36,37,38} 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 abundance of nonpolar residues, is thought to be a region that can foster oligomerization.^{39,40,41} 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 $5 \times \text{FAD}$ mice,

they observed that *APOE* ϵ 3 (V236E) reduced A β deposition, plaque-associated immune response, and neuritic dystrophy around amyloid plaques.¹² Chemically, they noted that *APOE* ϵ 3 (V236E) primarily remains as a monomer and is less likely to form oligomers compared with the canonical *APOE* ϵ 3 allele.¹² This propensity of V236E to reduce apoE aggregation was also observed when this variant was introduced on an *APOE* ϵ 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.^{42,43,44}

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 ϵ 4's ability to bind lipids rendering it more like ϵ 3 or ϵ 2 in this capacity.⁴⁵ Alternatively, the introduction of glycine could disrupt the α helix structure of the C-terminal impacting apoE ϵ 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* ϵ 3 (V236E) and *APOE* ϵ 4 (R251G). These findings validate the protective effect of the V236E variant and has uncovered a novel protective missense variant on *APOE* ϵ 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* - ϵ 4 allele protective supports the idea that *APOE* ϵ 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.

Notes

Supplement 1.

eAppendix. Additional acknowledgments.

eMethods.

eFigure 1. Flowchart describing the number of individuals remaining at each filtering steps.

eFigure 2. V236E and R251G are associated with decreased AD risk across dataset in *APOE*-stratified sensitivity analyses.

eFigure 3. *APOE* ϵ_3/ϵ_3 [V236E] individuals have a lower AD risk than *APOE* ϵ_2/ϵ_3 individuals and *APOE* ϵ_3/ϵ_4 [R251G] have a risk equivalent to ϵ_2/ϵ_3 carriers despite carrying 1 ϵ_4 allele, regardless of the EUR ancestry cutoff for admixed Europeans and Europeans.

eTable 1. Queried cohort overview to identify admixed and European ancestry individuals in the ADSP discovery and ADGC internal replication.

eTable 2. Overview of ADSP studies with whole-exome sequencing (WES) and/or whole-genome sequencing (WGS) available at NIAGADS DSS (NG00067).

eTable 3. Demographic characteristics of the cohorts queried for discovery and internal replication samples.

eTable 4. Missense variants on the *APOE* canonical transcript reported in gnomADv3.1.

eTable 5. Demographic characteristics per cohort in ADSP discovery and ADGC internal replication after ancestry selection, quality control, and duplicates removal.

eTable 6. *APOE* missense variants rs769452-C (*APOE*[L28P]), rs199768005-A (*APOE*[V236E]), and rs267606661-G (*APOE*[R251G]) allelic breakdown by *APOE* main genotype.

eTable 7. V236E and R251G association in primary and secondary analyses, nonstratified and *APOE* stratified.

eTable 8. Nonstratified sensitivity analyses at various European ancestry cutoffs.

eTable 9. Sensitivity analysis, including all dementia in the CCHS and CGPS data set, slightly strengthens the V236E and R251G associations with decreased AD risk.

eTable 10. Sensitivity analysis, excluding the UK Biobank proxy-AD phenotype from the meta-analysis, results in slight worsening of the P values of the V236E and R251G associations with decreased AD risk.

eReferences.

Supplement 2.

Collaborators of the EADB, GR@ACE, DEGESCO, DemGene, GERAD, and EADI Groups.

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Figures and Tables

Table 1.

Demographic Characteristics per *APOE* Genotype^a

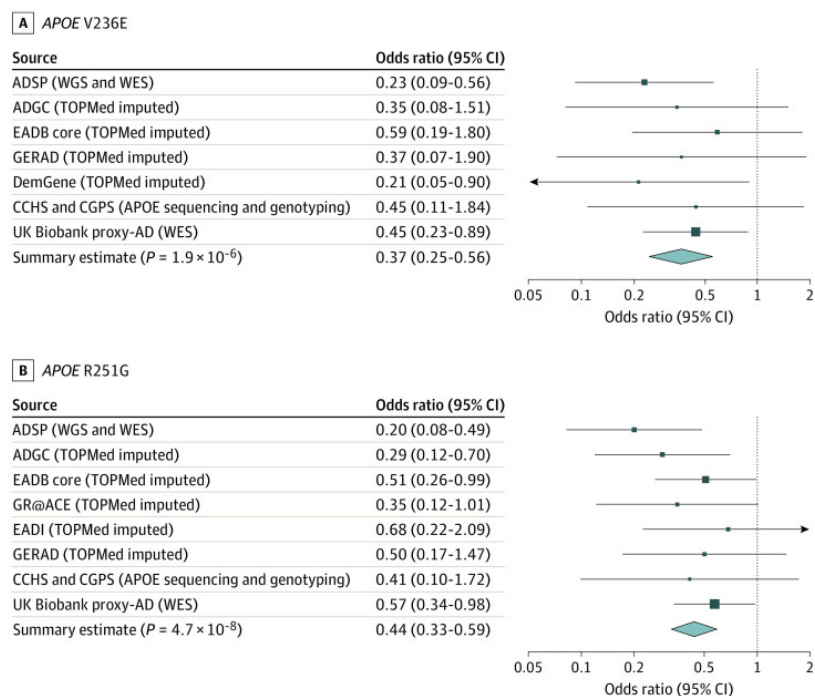
Sample	Diagnosis	Individuals, No.	APOE ε2/ε2			APOE ε2/ε3			APOE ε3/ε3		
			Total, No.	Female, No. (%)	Age, mean (SD), y	Total, No.	Female, No. (%)	Age, mean (SD), y	Total, No.	Female, No. (%)	Age, mean (SD)
ADSP	CN	11 934	73	40 (54.8)	82.6 (8.3)	1481	924 (62.4)	83.0 (8.0)	7429	4636 (62.4)	82.5 (8.1)
	AD	11 868	29	17 (58.6)	82.5 (6.9)	583	369 (63.3)	80.1 (9.7)	5313	3236 (60.9)	77.0 (10.0)
ADGC	CN	8059	56	26 (46.4)	79.1 (10.2)	978	629 (64.3)	76.2 (9.5)	4795	2968 (61.9)	74.5 (9.4)
	AD	7768	10	6 (60.0) (8.2)	72.5 (8.2)	323	181 (56.0)	75.8 (10.4)	2494	1586 (63.6)	74.7 (10.0)
EADB core	CN	21 160	121	72 (59.5)	68.6 (13.2)	2503	1457 (58.2)	66.8 (15.1)	13 365	7725 (57.8)	67.0 (14.0)
	AD	19 873	27	14 (51.9)	76.4 (11.7)	877	522 (59.5)	74.2 (11.2)	8285	5128 (61.9)	72.9 (11.0)
GR@ACE	CN	8539	33	19 (57.6)	53.1 (17.6)	858	448 (52.2)	57.5 (18.7)	6005	3009 (50.1)	56.7 (18.0)
	AD	7355	16	14 (84.6)	84.6 (3.5)	389	274 (70.4)	81.4 (8.1)	3840	2703 (70.4)	80.9 (7.9)
EADI	CN	6331	38	20 (52.6)	82.6 (7.5)	772	457 (59.2)	81.0 (7.5)	4247	2582 (60.8)	80.1 (7.7)
	AD	2397	7	6 (85.7) (6.0)	79.3 (6.0)	128	88 (68.8)	78.0 (10.8)	1078	704 (65.3)	76.5 (10.0)
GERAD	CN	7007	47	26 (55.3)	49.3 (11.0)	853	427 (50.1)	51.5 (12.6)	4127	2142 (51.9)	50.9 (11.0)
	AD	2989	10	6 (60.0) (9.7)	81.2 (9.7)	140	88 (62.9)	79.3 (11.3)	1092	677 (62.0)	79.5 (9.6)
DemGene	CN	5911	32	11 (34.4)	68.7 (11.2)	685	336 (49.1)	69.2 (12.4)	3236	1540 (47.6)	68.9 (11.0)
	AD	1687	5	2 (40.0) (1.4)	74.0 (1.4)	72	42 (58.3)	71.6 (10.6)	537	359 (66.9)	73.7 (9.6)

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.

Figure 1.



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

Forest plots show the results for the non-*APOE*-stratified analyses adjusted by $\epsilon 2$ and $\epsilon 4$ dosages. eFigure 2 in [Supplement 1](#) presents equivalent forest plots for these 2 variants in the *APOE*-stratified sensitivity analyses, showing consistent findings. ADGC indicates 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; TOPMed, Trans-Omics for Precision Medicine; WES, whole-exome sequencing; WGS, whole-genome sequencing.

Table 2.

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

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

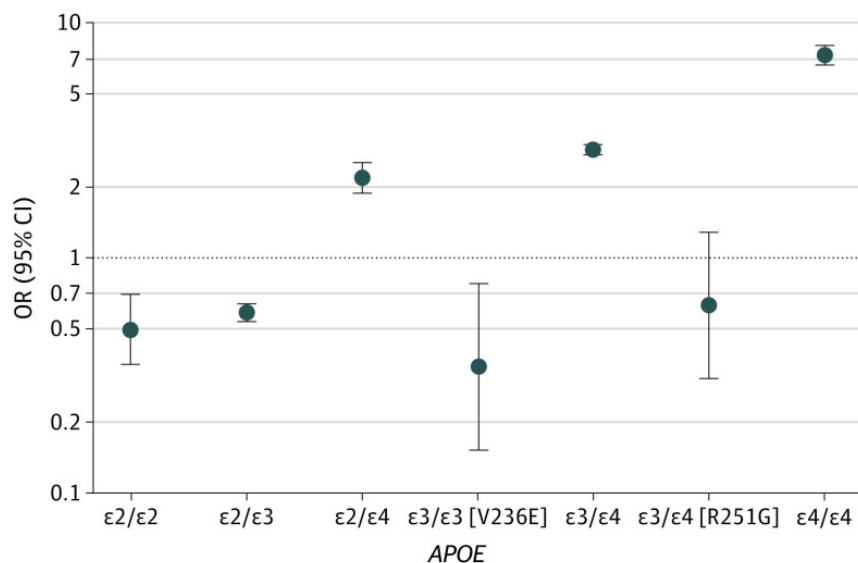
Center Barcelona; MAC, minor allele count; OR, odds ratio.

^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.