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APOE loss-of-function variants: Compatible with longevity and associated with resistance to Alzheimer’s Disease pathology
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NOTE: This preprint reports new research that has not been certified by peer review and should not be used to guide clinical practice.
Summary

The ε4 allele of apolipoprotein E (APOE) is the strongest genetic risk factor for sporadic Alzheimer’s Disease (AD). Knockdown of this allele may provide a therapeutic strategy for AD, but the effect of APOE loss-of-function (LoF) on AD pathogenesis is unknown. We searched for APOE LoF variants in a large cohort of older controls and patients with AD and identified six heterozygote carriers of APOE LoF variants. Five carriers were controls (ages 71-90) and one was an AD case with an unremarkable age-at-onset between 75-79. Two APOE ε3/ε4 controls (Subjects 1 and 2) carried a stop-gain affecting the ε4 allele. Subject 1 was cognitively normal at 90+ and had no neuritic plaques at autopsy. Subject 2 was cognitively healthy within the age range 75-79 and underwent lumbar puncture at between ages 75-79 with normal levels of amyloid. The results provide the strongest human genetics evidence yet available suggesting that ε4 drives AD risk through a gain of abnormal function and support knockdown of APOE ε4 or its protein product as a viable therapeutic option.

Keywords

Alzheimer’s Disease; Neurodegenerative disorders; Human genetics; Loss-of-function; Apolipoprotein E
Introduction

Advancements in genetic engineering have resulted in treatments for monogenic neurological disorders previously considered intractable. The most tangible progress has occurred in spinal muscular atrophy (SMA), a neurodegenerative disease caused by the loss-of-function of \textit{SMN1}. The antisense oligonucleotide (ASO) nusinersen increases the attainment of motor milestones in infants with SMA by altering splicing of the paralogous \textit{SMN2} gene to rescue its function. Risdiplam, a small molecule acting on \textit{SMN2}, and abeparvovec, an AAV-based therapy that restores \textit{SMN1}, have also received FDA approval\(^1\). Knockdown approaches using ASOs have been employed in familial amyotrophic lateral sclerosis and Huntington’s disease\(^2,3\). While these ASO trials did not result in clinical efficacy, they significantly reduced the abnormal protein in both disorders and informed new trials. These gene-targeting therapies offer hope that similar approaches could be successful in Alzheimer’s disease (AD).

Individuals carrying the ε4 allele of apolipoprotein E (\textit{APOE}) have a significantly elevated risk of AD, suggesting that genetic modulation of \textit{APOE}ε4 could be therapeutic. An \textit{APOE}ε4/ε4 individual of European ancestry has a sixteen-fold increase in AD risk and eighteen year earlier age at AD onset compared to a European ancestry individual carrying two copies of the more prevalent \textit{APOE}ε3 allele\(^4\). A critical question regarding pathogenesis is whether ε4 is inherently detrimental (in which case one would want to knock it down) or is less functional than ε3 (in which case one might want to increase levels of the protein in ε4 homozygotes). Evidence from animal models of AD can be marshaled to support either possibility\(^5,6\). The bulk of the animal literature supports knockdown of \textit{APOE} as likely to reduce AD pathogenesis. For example, studies have shown that reducing \textit{APOE} results in reduced amyloid\(^6–8\) and tau pathology\(^9\) in animal models. However, other animal studies support increasing \textit{APOE} as a potential therapeutic approach\(^10–12\).
Evidence from human studies is generally lacking because APOE loss-of-function (LoF) variants are rare. Only one individual carrying an APOE LoF variant has been cognitively assessed and reported in the literature. The patient was a man in the age range 40-44 homozygous for APOE c.291del (p.E97fs) and presenting with severe hyperlipidemia. He had negative spinal fluid biomarkers for AD but impaired memory. Despite the normal biomarkers, the cognitive impairment and relatively young age make this case ultimately uninformative on the question of whether reducing apoE might be beneficial or detrimental in terms of AD pathogenesis. We have found no publications describing AD-relevant phenotypes in older subjects heterozygous for APOE LoF variants. Here we ask whether APOE LoF variants impact AD pathogenesis.

Results
To characterize AD phenotypes of APOE LoF individuals, we searched the Alzheimer’s Disease Sequencing Project (ADSP) whole-exome and whole-genome sequencing datasets (Table 1) for predicted APOE LoF single nucleotide polymorphisms (SNPs) and structural variants (SVs). All SNPs on APOE were extracted, annotated with predicted variant type, and filtered for predicted high impact variants affecting the canonical transcript (Supplementary Table 1). The most common end-truncation variant, rs121918396 (p.W228*), was expressed in human hepatocytes and shown not to affect apoE protein level (Figure S1); thus predicted end-truncation variants were not explored further. We identified five participants carrying APOE LoF SNPs – three carriers of rs777551553 (p.W5*), one carrier of rs923895447 (p.L8*) and one carrier of 19:44907831:C:T (p.Q39*). These five carriers were normal at their last cognitive assessment (mean age at last assessment = 82, range 71-90). We also searched the ADSP SV dataset for insertions, deletions, duplications, inversions, and translocations overlapping APOE and further filtered these SVs for those predicted to be LoFs (Supplementary Table 1). We identified one individual with AD carrying a 1,798 base pair deletion (19:44905303-44907102) that eliminates the bulk of the APOE promoter region and exons 1 and 2 (including the start
codon and the signal peptide). APOE genotype, age, and diagnoses of the six APOE LoF variant carriers are shown in Figure 1a with variant positions shown in Figure 1b.

Subject 1 (carrier of rs777551553_A (p.W5*)) demonstrated striking resistance to amyloid pathology given his age at death (age 90+) and ε3/ε4 genotype. While he had evidence of hyperphosphorylated tau pathology (Braak stage IV of VI) there was no appreciable amyloid β (Aβ) pathology in the brain (CERAD neuritic plaque score 0 of 3, Thal Aβ stage 0 of 515, and no cerebral amyloid angiopathy). The absence of amyloid pathology and moderate extent of tau pathology at advanced age make this individual an outlier among ε3/ε4 heterozygotes (Figure 2). rs777551553_A was heterozygous and in phase with ε4 in this individual (Figure S2a, b). Sanger sequencing of reverse-transcribed mRNA detected both ε3 and ε4 transcripts (Figure S2c), as expected of a stop-gain variant. This variant would prematurely terminate translation to create a truncated 5 amino acid peptide, resulting in an effective ε3/- genotype.

Subject 2 (also an ε3/ε4 carrier of rs777551553_A (p.W5*)), was cognitively normal at 79. Whole-genome long-read sequencing established that rs777551553_A was in phase with ε4 (Figure S3). This subject underwent lumbar puncture at age 75-79 and had normal levels of Aβ and tau. In a meta-analysis of amyloid biomarker studies, by age 75 roughly 2/3rds of ε3/ε4 controls were amyloid-positive by spinal fluid measurement (Supplementary Figure 3B in Jansen et al.16). This underestimates the “protected” status of Subject 2 because it does not account for the fact that many ε3/ε4 individuals either have mild cognitive impairment or AD by age 75.

Three other carriers of early APOE stop-gain mutations were cognitively normal. Subject 3 is a 85-89 year-old ε3/ε3 female, Subject 4 is a 70-74-year-old ε3/ε4 female, and Subject 5 is an 80-84-year-old ε3/ε3 female. Subject 4 did not have mRNA or DNA available for phasing.
Subject 6 is an $\varepsilon3/\varepsilon4$ AD patient with a deletion including the APOE promoter, start codon, and two of four exons. PCR and Sanger sequencing of the patient’s post-mortem frontal cortex tissue confirmed the deletion (Figure S4a) and established that the deletion was heterozygous and in phase with $\varepsilon3$ (Figure S4b, c). Only the $\varepsilon4$ allele was detected on Sanger sequencing of reverse transcribed mRNA (Figure S4d), indicating that this deletion abolishes transcription of $\varepsilon3$.

Neuropathology in Subject 6 was consistent with AD (CERAD score = 2, Braak Stage VI). This individual had symptom onset at age 75-79, was diagnosed within age range 75-79 and died between ages 85-89. Mean age of onset is 69.73 for $\varepsilon4$ homozygotes ($N=1,689$) and 73.55 for $\varepsilon3/4$ heterozygotes ($N=6,223$) in the Alzheimer’s Disease Genetics Consortium (ADGC) and ADSP. Although this patient exclusively expressed the $\varepsilon4$ allele, their disease onset was later than that of a typical $\varepsilon4/\varepsilon4$ homozygote suggesting that an $\varepsilon4/-$ individual has a preferable disease course to an $\varepsilon4/\varepsilon4$ individual. Thus, partial knockdown of $\varepsilon4$ in $\varepsilon4/\varepsilon4$ patients may improve the trajectory of AD.

Discussion

The cognitive phenotypes of APOE LoF carriers support the hypothesis that APOE $\varepsilon4$ increases risk through a gain of function. The variants identified in this study result in the following APOE loss of function mechanisms: stop-gain in the 18 amino acid APOE signal peptide that would preclude further translation of apoE (p.W5*, p.L8*, Subjects 1, 2, 3, 4); stop-gain early in the APOE coding region that would result in a severely truncated peptide without apoE’s key binding regions (p.Q39*, Subject 5); and deletion involving the APOE promoter and exons 1 and 2 (including the start codon and signal peptide) that abolishes APOE transcription altogether (19:44905303-44907102, Subject 6). If APOE $\varepsilon4$ increases risk owing to diminished protein
function or availability, the APOE LoF variants detailed here should be associated with increased risk of AD. Instead, of the six subjects reported here, five were older controls ranging in age from 71-90. The sole AD case had loss of ε3 resulting in an effective ε4/- genotype. If APOE ε4 increased risk due to diminished function we would expect such an ε4/- genotype to result in comparable or even increased risk compared to ε4/ε4 homozygotes. Instead, this patient had a later age-at-onset similar to patients with the ε3/ε4 genotype. The phenotypes of these carriers are most consistent with a model in which loss of APOE ε4 enhances AD resistance, suggesting that APOE ε4 increases AD risk through a gain-of-function.

Most compellingly, we describe two older controls with LoF variants in-phase with ε4 resulting in an effective ε3/- genotype. Subject 1 was cognitively normal at 90 and had no appreciable amyloid pathology. When compared to neuropathological profiles of age- and genotype-matched peers this subject is a clear outlier (Figure 2). Subject 2 was cognitively normal within age range 75-79 and had a normal spinal fluid biomarker profile within this age range (seen in only 1/3 of APOE ε3/ε4 controls over 75).

Taken together, these findings support reducing apoE as a therapeutic option in AD. Regarding safety, the identification of 6 long-lived individuals with heterozygous APOE LoF variants suggests that partial knockdown of APOE should be well-tolerated. The resistant phenotypes of two APOE ε3/ε4 individuals with a LoF variant on their ε4 allele provides the first human genetics data suggesting that knocking down APOE ε4 could reduce AD pathogenesis.
Author contributions
Conceptualization, data curation, methodology, investigation, writing and revising manuscript: AC, YLG. Methodology, investigation, revising manuscript: SC, EL, LL, JG, GX, MB, NK, APT, KW, IS, DDC. Supervision, revising manuscript: TW, JL, SJ, CH, EP, CDK, LC, EU. Conceptualization, methodology, writing and revising manuscript, supervision: CY, MDG.

Supplementary material
Supplementary Figures 1-4
Supplementary Table 1: Carriers of APOE LoF variant candidates
Supplementary Table 2: Primer designs for characterization of Subjects 1 and 6

Declaration of interests
The authors declare no competing interests.

Data sharing statement
Data from the Alzheimer’s Disease Sequencing Project is available via NIAGADS DSS (https://dss.niagads.org/datasets/ng00067).
References


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Table 1: Demographics of ADSP cohorts

Abbreviations: Whole-exome sequencing (WES), Whole-genome sequencing (WGS), Diagnosis (Dx), Alzheimer's Disease (AD), Control (CN)
Figure 1. **APOE** loss-of-function carrier demographics and variant positions

(a) Carrier demographics. Six carriers of high confidence **APOE** loss-of-function variants were identified among 26,605 older controls and 20,856 AD cases sequenced as part of the Alzheimer’s Disease Sequencing Project. (b) Three distinct single nucleotide polymorphisms and one structural variant were identified. Genomic coordinates are based on hg38.
Figure 2. Loss of \textit{APOE} $\varepsilon 4$ is associated with absent amyloid pathology and reduced tau pathology in a 90+ year-old control.

Subject 1 is a neuropathological outlier among age-matched $\varepsilon 3/\varepsilon 4$ individuals in their (a) CERAD staging of amyloid plaque density; (b) Thal staging of amyloid plaque regional distribution; (c) Cerebral amyloid angiopathy staging; and (d) Braak staging of neurofibrillary tangles (tau pathology).
Online Materials and Methods

Single nucleotide polymorphism identification

36,361 whole-genomes were downloaded from NIAGADS 10th release, 20,504 whole-exomes were downloaded from NIAGADS 4th release (https://dss.niagads.org/datasets/ng00067/#datareleases) and Plink 1.917 was used to extract all SNPs in the range chr19:44905796-44909393 corresponding to APOE. ENSEMBL Variant Effect Predictor (VEP)18 was used to annotate all SNPs with variant consequence and LoF flags19. We filtered for SNPs annotated as high impact (transcript ablation, splice acceptor variants, splice donor variants, stop gains, frameshift variants, stop loss, start loss, or transcript amplification) and causing LoF on the canonical transcript. Whole genomes and exomes were from patients with AD, healthy older controls, and a mix of subjects with other diagnoses including mild cognitive impairment, corticobasal degeneration, and other dementia not otherwise specified.

Structural variant identification

ADSP release 3 (R3) individual-level VCF structural variant (SV) calls from software packages Manta, Smoove, and joint genotyping VCF from Biograph were downloaded from NIAGADS 8th (Manta, Smoove) and 9th releases (Biograph) (https://dss.niagads.org/datasets/ng00067/#datareleases). Insertions (SVTYPE INS), deletions (SVTYPE DEL), inversions (SVTYPE INV or intra-chromosomal SVTYPE BND with INFO/EVENT field), duplications (SVTYPE DUP), and translocations (SVTYPE TRA or inter-chromosomal SVTYPE BND with INFO/EVENT field) up to 20 kilobases in length that overlap APOE (hg38; chr19: 44905796-44909393) were identified via the following approach. Variants matching the above criteria on chromosome 19 with start position within the range (44905796, 44909393), as well as variants on chromosome 19 with start position within the range (44905796 – 20000, 44909393) and end position within the range (44905796, 44909393 + 20000), were isolated. Breakend calls not matching a variant type described above (i.e. SVTYPE BND calls without INFO/EVENT field) were excluded.
Distribution of Tau Braak staging and neuritic plaques density in function of age-at-death in ε3/ε4

The uniform data set (UDS) obtained from the National Alzheimer’s Coordinating Center (NACC), December 2020 data freeze, was queried for individuals with APOE genotype, recorded age-at-death and neuropathological assessment available in NACC UDS (5,168 individuals). To assess where the W5*ε3/ε4 carrier stands compared to other ε3/ε4 subjects, we subset this dataset to ε3/ε4 individuals who died after 60 years old, leading to 1,758 individuals with CERAD (Consortium to Establish a Registry for Alzheimer’s Disease) score of neuritic plaques density and 1,750 individuals with tau Braak staging. The density plots (Figure 2) were made with the kdeplot function of the seaborn (v.0.12) package in Python (v.3.7.7).

DNA/RNA extraction from brain tissue

Genomic DNA and RNA were isolated from frozen post-mortem brain using the AllPrep DNA/RNA Mini Kit (Qiagen). Subject 6 nucleic acids were extracted from frontal lobe tissue and Subject 1 nucleic acids were extracted from cerebellum tissue. Nucleic acid concentrations were measured by NanoPhotometer (Implen), and DNA was stored at -20°C and RNA was stored at -80°C prior to use.

Reverse transcriptase (RT) reaction

Total RNA (100 ng) was used for each 20 μL RT reaction, and cDNA synthesis was performed with random primers using the PrimeScript RT Reagent Kit (Takara Bio USA).

DNA cloning

A primer pair AE-Ex1_F_pGL4-XhoI and AE-3’UTR_R_pGL4-Hind3 (Supplementary Table 2) was used to amplify full-length APOE cDNA that was reverse transcribed from Subject 1 RNA.
The PCR profile consisted of 15 min at 95°C, 35 cycles of 20 sec at 95°C, 20 sec at 55°C, and 3 min at 72°C. The amplified fragment was inserted into pGL4.10[luc2] vector (Promega) that was cut with XhoI and HindIII using the In-Fusion® HD Cloning Kit (Takara Clontech). The In-Fusion ligation mix was transformed into Stellar™ Competent Cells (Takara Clontech). The cells were plated on LB Agar Plate containing 100 µg/ml ampicillin and was incubated at 37°C for 16 hours.

**Sanger sequencing**

DNA was PCR amplified using the primers AE-Ex1_F_pGL4-XhoI and AE-3'UTR_R_pGL4-Hind3 (Supplementary Table 2). The PCR profile consisted of 15 min at 95°C, 35 cycles of 20 sec at 95°C, 20 sec at 55°C, and 3 min at 72°C. The PCR amplified DNAs were sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific). Primers Ch19_50103673_F and Ch19_50103049_R were used to obtain sequencing reads for SNP rs429358 and for the mutation located at rs777551553 on the APOE gene, respectively. The sequencing profile consisted of 1 min at 96°C, 35 cycles of 30 sec at 96°C, 10 sec at 55°C, and 4 min at 60°C.

**Long-read whole genome sequencing**

Whole blood collected in EDTA and stored at -80°C was used to extract genomic DNA using PureGene kit (Qiagen) for Subject 2. A sequencing library was prepared using Oxford Nanopore Rapid sequencing protocol (Oxford Nanopore Technologies, UK). The library was distributed over 3 R9 PromethION flow-cells and sequenced for a total of 24 hours on a PromethION48 sequencing device, achieving a total output of 116 gigabases of data. Fast5 files were base called using Guppy V6.4.2 Super Accurate(ONT) and aligned using MiniMap2.

**Determination of mean AAO by APOE genotype**
Genetic and phenotypic data from AD-related cohorts from the Alzheimer’s Disease Genetics Consortium (ADGC) and Alzheimer’s Disease Sequencing Project (ADSP) were processed as previously described. This includes state-of-the-art quality control of APOE ε4 status and resolving of phenotypes and age information across duplicated samples. Unique, non-duplicate subjects (identified using identity-by-descent; Plink v1.9) that were non-Hispanic and of European ancestry (SNPWeights v2.1) were retained to determine mean age-at-onset across APOE strata.

**Cell culture, transfection and monoclonal heterozygous p.W228* line generation**

Human hepatocyte line HepG2 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% Penicillin/Streptomycin (Gibco) at 37 °C, 5% CO2. For prime editing, 150,000 cells were seeded one day prior to transfection at a density of 150,000 cells/well in 24-well plate. On the day of transfection, using 1 μL jetOPTIMUS (Polyplus) per well, following the manufacturer’s instructions. Cells were transfected with 750 ng of the pCMV-PE2 (Addgene #132775), 83 ng of the pegRNA and 83 ng of the nicking sgRNA per well. 72 hr post transfection, single cells were sorted into 96-well plates using BD FACSAria II SORP and cultured until confluency.

To assess prime editing, loci were amplified from isolated single clone genomic DNA samples via two rounds of PCR then deep sequenced. Briefly, the first round PCR (PCR1) amplified the genomic sequence of interest using primers containing Illumina forward and reverse adapters:

NGS-ApoE-F: 5' CCATCTCATCCCTGCGTGTCTCCCAAGCTGCGTAAGCGGCTCCTC 3'  
NGS-ApoE-R: 5' CCTCTCTATGGGCAGTCGGTGATGCACCTGCTCCTTCACCTCGTC 3'

The second round PCR step (PCR2) added unique i7 and i5 index combinations to both ends of the PCR1 product:
CL_AmpNGS_BC_F: 5' AATGATACGGCGACCACCAGATCTACAC[8nt-barcode]ACACTCTTTCCCTACACGACGCTCTTCCGATCT[0-11nt stager]CCATCTCATCCCTGCGTGTCTCC 3'

CL_AmpNGS_BC_R: 5' CAAGCAGAAGACGGCATACGAGAT[8nt-barcode]GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT[0-11nt stager]CCTCTCTATGGGCAGTCGGTGATg 3'

The amplified products were quantified with Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific) and normalized by concentration, followed by sequencing using Illumina Miseq Reagent Kit v3 then analyzed with the CRISPResso2.

**ELISA Measurement of apoE protein level in prime-edited p.W228* cells**

Monoclonal gene-edited cells that harbor both the wild-type and the p.W228* alleles at the APOE locus were maintained in culture for at least 48 hours. Afterwards, the cells were harvested and lysed with RIPA buffer (Cell Signaling Technology) to extract the total proteins. Lysate containing the protein extracts were then subjected to ELISA detection using human apoE ELISA kit (Mabtech) following manufacturer’s recommended protocols. Data were analyzed using Prism9.
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