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Use of local genetic ancestry to assess *TOMM40*-523′ and risk for Alzheimer disease

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Abstract

Objective

Here, we re-examine *TOMM40*-523′ as a race/ethnicity-specific risk modifier for late-onset Alzheimer disease (LOAD) with adjustment for local genomic ancestry (LGA) in *Apolipoprotein E* (*APOE*) ε4 haplotypes.

Methods

The *TOMM40*-523′ size was determined by fragment analysis and whole genome sequencing in homozygous *APOE* ε3 and *APOE* ε4 haplotypes of African (AF) or European (EUR) ancestry. The risk for LOAD was assessed within groups by allele size.

Results

The *TOMM40*-523′ length did not modify risk for LOAD in *APOE ε4* haplotypes with EUR or AF LGA. Increasing length of *TOMM40*-523′ was associated with a significantly reduced risk for LOAD in EUR *APOE* ε3 haplotypes.

Conclusions

Adjustment for LGA confirms that *TOMM40*-523′ cannot explain the strong differential risk for LOAD between *APOE* ε4 with EUR and AF LGA. Our study does confirm previous reports that increasing allele length of the *TOMM40-523′* repeat is associated with decreased risk for LOAD in carriers of homozygous *APOE* ε3 alleles and demonstrates that this effect is occurring in those individuals with the EUR LGA *APOE* ε3 allele haplotype.

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Glossary

AF = African; **APOE** = Apolipoprotein E; **EUR** = European; **IRB** = institutional review board; **LOAD** = late-onset Alzheimer disease; **LGA** = local genomic ancestry; **SNP** = single nucleotide polymorphism; **WGS** = whole genome sequencing.

Apolipoprotein E (APOE) has long been the strongest and most consistently identified gene associated with the risk for developing late-onset Alzheimer disease (LOAD).[1,2] Of interest, the *APOE* ε4 allele in AFs and African Americans confers a much lower risk for disease than the identical allele in Europeans (EUR).[3–5] It was recently discovered that this racial difference can be attributed to the local genetic ancestry (LGA) within the *APOE* haplotype.[6] The responsible "protective" locus within the *APOE* LGA remains unknown, however. *TOMM40* is a gene lying within the *APOE* haplotype that codes for a channel-forming subunit required for protein import into mitochondria. It contains an intronic poly-T repeat known as "*TOMM40*-523′," which varies in length by individual and race/ethnicity. The *TOMM40*-523′ length has been proposed to influence the transcription of *APOE* and thus modify the risk for LOAD,[7–13] although the significance of its association with LOAD remains controversial.[14–19] The *TOMM40*-523′ LOAD relationship has been analyzed by race in the context of global genetic ancestry but not by using adjustment for LGA within the *TOMM40-APOE* haplotype, allowing for misclassification, given sometimes common differences between LGA and global ancestry.[6]

To confirm whether varying sizes of the *TOMM40*-523′ allele can truly explain the reduced risk for LOAD in African Americans expressing the *APOE* ε4 allele, we re-examine *TOMM40*-523′ with adjustment for LGA. We further investigate racial differences in effect of *TOMM40*-523′ on *APOE* ε3 carriers.

Methods

Standard protocol approvals, registrations, and patient consents

All ascertainment activities were approved by the institutional review boards (IRBs) at the respective universities and adhered to the tenets of the Declaration of Helsinki. Informed written consent was obtained for all participants. The current study is approved by the University of Miami IRB.

Samples and ascertainment

DNA samples used for this study were part of a larger study of Alzheimer disease (AD) genetics and were ascertained at Case Western University, Wake Forest University, and the University of Miami between 2012 and 2019. Participants were selected if they were homozygous for either the *APOE* ε3 or *APOE* ε4 allele, eliminating the need to phase *TOMM40*-523′ alleles by haplotype. All participants were enrolled under a standard ascertainment protocol. As part of this protocol, sociodemographic and clinical historic data were obtained for all participants. This included demographic variables, diagnosis, age at onset for cases, method of diagnosis, history of illness, and the presence of other relevant comorbidities. The Clinical Dementia Rating Scale,[20,21] National Institute on Aging (NIA)-Late Onset Alzheimer's Disease cognitive battery,[22,23] and Mini-Mental State Exam[24] or the Modified Mini-Mental State Exam[25] readings were collected for all participants.

Diagnostic criteria

Diagnosis was made via case conferences of all clinical, historical, and psychometric test data (e.g., laboratory tests, neurologic examination, neuroimaging, and neuropsychological screening and testing) by a multidisciplinary clinical adjudication panel consisting of a boarded neurologist (J.M.V.), neuropsychologist (M.L.C.), and clinical staff (P.R.M.). All individuals with AD met the internationally recognized standard National Institute of Neurological Disorders and Stroke - Alzheimer's Disease and Related Disorders Association[26] or updated NIA-Alzheimer's Association criteria and were further classified as definite (neuropathologic confirmation), probable, or possible AD.[27]

Individuals classified as unaffected controls were older than 65 years of age, cognitively normal on the NIA-Late Onset Alzheimer's Disease battery, and had a CDR of zero. The controls were matched to cases for age, sex, and ethnicity.

APOE genotyping and determination of local genetic ancestry genome-wide single nucleotide polymorphism (SNP) genotyping was performed using 3 different platforms: Expanded Multi-Ethnic Genotyping Array, Illumina 1Mduo (v3), and the Global Screening Array (Illumina, San Diego, CA). *APOE* genotyping was conducted as in Saunders et al.[28] Quality control analyses were executed using the PLINK software, v.2.[29] The samples with call rates less than 90% and with excess or insufficient heterozygosity (±3 SDs) were excluded. Sex concordance was checked using X chromosome data. To eliminate duplicate and related samples, relatedness among the samples was estimated using identity by descent. SNPs were eliminated from further analysis if they were present in samples with call rates less than 97%, had minor allele frequencies less than 0.01, or were not in Hardy-Weinberg equilibrium ($p < 1.e^{-5}$).[30]

Genotyping data were initially phased using the SHAPEIT tool ver.2 to identify local ancestry at the *TOMM40-APOE* haplotype.[31] The discriminative RFMix modeling approach was used to estimate the genetic ancestral background (AF vs EUR) at the region of interest.[32] The Human Genome Diversity Project (HGDP) data were used as the reference panel in the analysis.[33]

Genotyping of *TOMM40*-523′ allele using fragment analysis

The *TOMM40*-523′ poly-T repeat was first PCR-amplified using fluorescein amidite-labeled forward primer 5′–CCTCCAAAGCATTGGGATTA-3′ and reverse primer 5′-GATTCCTCACAACCCCAAGA-3′. PCR was performed using *Taq* polymerase in the presence of 4% DMSO with a final volume of 25 uL. PCR conditions were as follows: initial 6-minute denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 40 seconds. Final extension was performed for 10 minutes at 72°C. PCR products underwent subsequent fragment analysis with LIZ600 size standard using 3130xl Genetic Analyzer, and resolved fragments were visualized with GeneMapper v4.0 (Applied Biosystems Inc., CA).

The allele size was determined by subtracting the fragment analysis-determined size by 439, the number of base pairs surrounding each end of the poly-T stretch in our PCR product. The mode of each peak was selected and established as the "true" poly-T length (figure e-1, links.lww.com/NXG/A230). In cases where the mode was shared by 2 peaks, the mean length of the 2 peaks was used. In all cases, peak size was rounded to the nearest integer. A replicate fragment analysis was performed on 20 randomly selected samples to ensure consistency in length calls. A fluorescein amidite-labeled PCR fragment with known length and void of repeated sequences was run alongside samples to ensure accurate sizing.

Genotyping of *TOMM40*-523′ using Whole Genome Sequencing

To test whether whole genome sequencing (WGS) could be used to genotype the *TOMM40*-523′ repeat, WGS was performed on a subset of 15 samples using a PCR-free library preparation and paired-end 150bp sequencing on the Illumina Novaseq. Raw reads were aligned to the human reference genome GRCh38 using the Burrows-Wheeler transform alignment algorithm.[34] Resulting Binary Alignment Map files were visualized in the Integrative Genomics Viewer,[35] and the number of thymine bases at rs10524523 were calculated and compared with fragment analysis of the same set of samples.

Statistical analysis

TOMM40-523′ poly-T lengths were first binned into short (<20 T's), long (20–29T's), and very long (≥30 T's) alleles, as originally defined.[18] To assess the effect of the *TOMM40*-523′ poly-T length on LOAD risk, we stratified our data set according to local ancestries (AF and EUR) and *APOE* alleles (ε3 and ε4). Next, we categorized the counts of individuals in our data set by length of *TOMM40*-523′ poly-T (short, long, and very long) and affection status (affected vs control). We applied the Fisher exact test on each subgroup to test the significance of a difference between the proportions in the length of *TOMM40*-523′ poly-T and affection status (short vs long, short vs very long, and short vs sum of long and very long).

Data availability

Any data pertaining to this article and not published within this article is publicly available and may be requested through collaboration.

Results

Descriptive statistics

A total of 205 samples composed of 75 controls and 130 cases were analyzed, making up 410 individual haplotypes. Within AF LGA samples, 48 were *APOE* ε3/ε3 (15 cases, 33 controls) and 47 were *APOE* ε4/ε4 (38 cases, 9 controls). Within EUR LGA samples, 47 were *APOE* ε3/ε3 (34 cases, 13 controls) and 63 were *APOE* ε4/ε4 (43 cases, 20 controls) (table 1).

The average age at onset for affected individuals was 71.3 years old (SD = 8.1 years). The average age of examination for controls was 73.0 year old (SD = 6.9 years). Affected and control groups consisted of 70.8% and 65.3% women, respectively. The entirety of each haplotype was either EUR LGA or AF LGA, with zero samples expressing mixed LGA. Within affected individuals, 56.0% of haplotypes exhibited AF LGA. 40.8% of control haplotypes contained AF LGA.

Genotyping

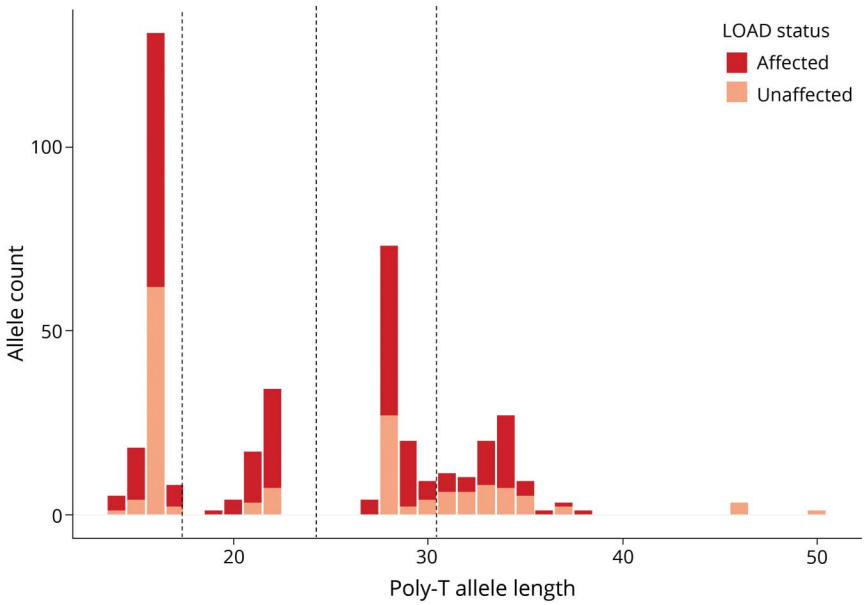
Polymerase stutter for the *TOMM40*-523′ allele, as described in previous *TOMM40* studies,[18] was observed with an average of 4 peaks per allele (figure e-1, links.lww.com/NXG/A230). There was 100% congruence of peaks between replicate samples. The length standard was consistently found to be 4bp shorter than its known value. To correct for this discrepancy, 4bp were added to poly-T allele lengths of each sample. Comparable with previous study,[36] our allele sizes appeared to distribute within 4 separate bins rather than the traditionally used 3 bin sizes (figure 1). For completeness, we also performed a similar 4-bin analysis in which the allele sizes were binned into very small (≤22 T's), small (23–28 T's), long (29–34 T's), and very long (≥35 T's). Allele comparisons made can be seen in table e-1, links.lww.com/NXG/A231.

Table 1 Descriptive statistics

	AF LGA	EUR LGA
***APOE* ε3/ε3**	15/**33**	34/**13**
APOE ε4/ε4	38/**9**	43/**20**

Abbreviations: AF = African; *APOE* = *Apolipoprotein* E; EUR = European; LGA = local genetic ancestry.
Number of case (unbold) and control (bold) samples analyzed within each LGA (AF and EUR) and *APOE* genotype (ε3/ε3 and ε4/ε4).

Figure 1 Distribution of TOMM40-523′ allele sizes across all samples



Allele count (y-axis) assigned to respective poly-T allele lengths (x-axis) for both cases (dark red) and controls (light red). The dotted lines delineate the 4 bin sizes used in the four bin analysis. LOAD = late-onset Alzheimer disease.

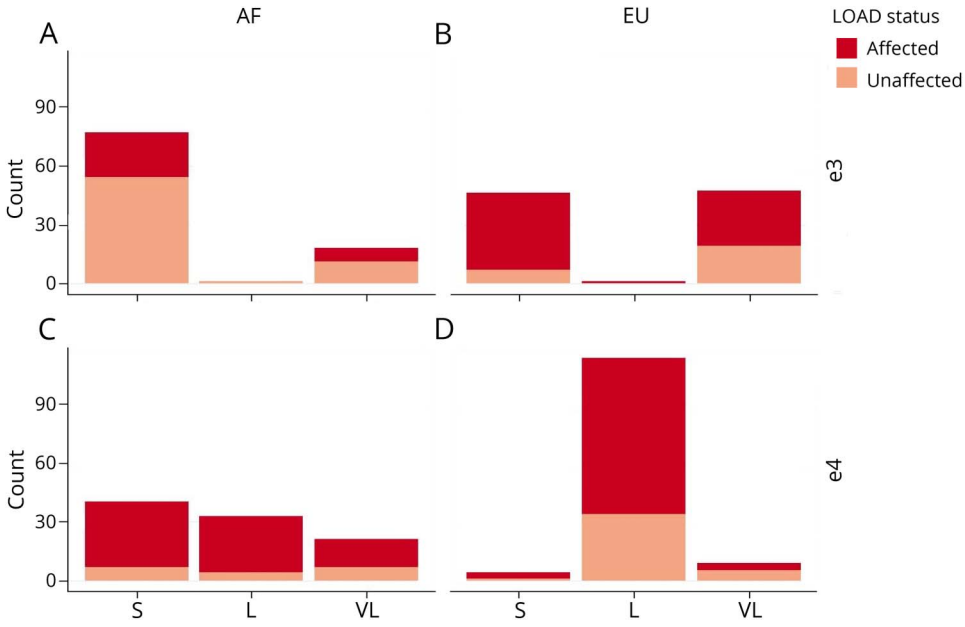
Allele frequencies differ by ancestry and *APOE* status

Allele frequencies varied between haplotypes harboring EUR vs AF ancestry and *APOE* ε3 vs *APOE* ε4 alleles (figure 2). AF LGA was more than 2 times more likely to display short (S) alleles compared with EUR LGA (148 vs 70). Long (L) alleles were commonly observed in *APOE* ε4 (123 alleles) but not *APOE* ε3 (24 alleles) haplotypes. AF *APOE* ε4 haplotypes exhibited the most variance in allele size, and EUR *APOE* ε4 contained more long (L) alleles than any other haplotype.

Effect of *TOMM40*-523′ length on risk for LOAD

There was no significant difference in risk for LOAD between "S" and "L" alleles, "S" and "very long (VL)" alleles, or "S" and "L + VL" alleles in the *APOE* ε4 haplotypes with AF (p = 0.2269, 0.2055, 0.7957, respectively) or EUR (p = 1, 0.9954, 1,

Figure 2 TOMM40-523′ allele frequencies



TOMM40-523′ allele frequencies in AF APOE ε3 (A), AF APOE ε4 (C), EUR APOE ε3 (B), and AF APOE ε3 (D) haplotypes for both cases (dark red) and controls (light red). AF = African; *APOE* = *Apolipoprotein E*; EUR = European; L = long; LGA = local genetic ancestry; LOAD = late-onset Alzheimer disease; S = short; VL = very long.

Table 2 Whole Genome Sequencing vs Fragment Analysis

Sample	FA Allele 1	WGS Allele 1	FA Allele 2	WGS Allele 2
201616933	15	14	32	32
201803389	16	15	16	15
201600975	16	15	16	15
201802895	15	14	28	28
201609823	21	21	34	30
201609813	16	15	33	14
201609811	16	15	32	31
201600974	16	15	16	13
201710863	16	15	34	15
201706978	15	14	38	13
201600962	16	15	16	15
201706996	16	15	21	21
201802891	16	15	28	15
201802829	16	15	28	28
201802899	16	15	28	28

Abbreviations: FA = fragment analysis; WGS = whole genome sequencing.
Comparison of *TOMM40*-523′ genotyping results between WGS and FA. Allele sizes are listed as number of T's.

respectively) LGA. However, there was a significant effect of *TOMM40*-523′ allele size within *APOE* ε3 haplotypes of EUR LGA. Both "VL" and "VL + L" allele groups had reduced risk compared with haplotypes with an "S" allele (VL: $p = 0.0104$) (VL + L: $p = 0.011$) (table 2). Our 4-bin analysis (VS, S, L, and VL) results were congruent with those of our 3-bin analysis (table e-1, links.lww.com/NXG/A231).

Genotyping *TOMM40*-523′ with WGS

Fifteen samples were genotyped using WGS. Their allele sizes, compared with the allele sizes determined via fragment analysis, can be seen in table 3. The correlations were calculated using fragment analysis as the reference length. The 2 methods correlated well for alleles <20 bp in length ($R^2 = 0.95$), though correlation decreased with inclusion of "L" ($R^2 = 0.85$) and "VL" ($R^2 = 0.57$) allele sizes.

Discussion

This *TOMM40*-523′ LOAD association study adjusted for local rather than global genetic ancestry, eliminating the potential for misclassification of ancestry housed within the *TOMM40*-523′ locus. This is an important distinction which cannot be overlooked. After re-examination of the *TOMM40*-523′ allele with adjustment for LGA, our study found no significant effect of the *TOMM40*-523′ poly-T size on the risk for LOAD within *APOE* ε4 haplotypes. This suggests that the *TOMM40*-523′ allele cannot explain the strong difference in risk conferred by the *APOE* ε4 allele between AF and EUR carriers of *APOE* ε4. A larger sample size is needed to test any possible weak to moderate significant differences, although given the current *p*-values, this seems unlikely. Therefore, we suggest that future efforts to explain the large risk difference between AFs and non-Hispanic whites should be directed toward the investigation of other candidate regions within the *APOE* haplotype to identify the protective variant for *APOE* ε4 found in the AF LGA.

Of interest, we did find a significant relationship between the poly-T length and risk for LOAD in *APOE* ε3 alleles harboring EUR LGA. The increasing number of T's was associated with decreased risk for LOAD. Our findings validate the findings of 2 previous studies reporting a significant under-representation of VL-*APOE* ε3 haplotypes in the LOAD cases vs controls[36] and significantly fewer VL alleles in EUR LOAD patients vs controls.[37] This suggests that the *TOMM40*-523′ variant may indeed play a role in modifying risk for disease in this subpopulation of *APOE* ε3 carriers. Given the tendency for human genomic repeats to have a negative phenotypic effect as they increase in length,[38,39] the finding that the largest expansion of the *TOMM40*-523′ repeat is protective is unusual at first glance. Our results align biologically in the case of the *TOMM40*-523′ repeat; however, as studies have shown that the expression of *TOMM40* increases with size of *TOMM40*-523′ poly-T repeat[11–13] and that augmented *TOMM40* expression confers subsequent mitochondrial protection.[11] *TOMM40*-523′ has additionally been identified as a transcriptional start site by the FANTOM project.[40] It is also possible, and perhaps likely, that the

Table 3 The effect of *TOMM40*-523′ allele size on the risk for LOAD

Haplotype	Comparison	OR	CI	*p* Value
AF ε4	S vs L	1.53	0.3–7.9	0.2269
	S vs VL	0.43	0.1–1.7	0.2055
	S vs (L + VL)	0.83	0.2–2.7	0.7957
EUR ε4	S vs L	0.78	0–10.1	1
	S vs VL	0.30	0–5.5	0.5594
	S vs (L + VL)	0.71	0–9.2	1
AF ε3	S vs L	—	—	—
	S vs VL	1.49	0.4–4.8	0.5742
	S vs (L + VL)	1.36	0.4–4.4	0.5873
EUR ε3	S vs L	—	—	—
	S vs VL	0.27	0.1–0.8	0.0104
	S vs (L + VL)	0.28	0.1–0.8	0.0110

Abbreviations: AF = African; *APOE* = *Apolipoprotein E*; EUR = European; L = long; LGA = local genetic ancestry; S = short; VL = very long.
Statistical test results between *TOMM40*-523′ allele sizes among *APOE ε3* and *APOE ε4* alleles in the presence of AF and EUR LGA.
"S vs L" could not be calculated because of insufficient "L" alleles.

TOMM40-523′ repeat is a tagging variant for a distinct protective factor which is *APOE ε3* specific. Stratifying risk within patients carrying *APOE* ε3 on a EUR LGA, most non-Hispanic white patients, could be important in the clinical setting and perhaps in clinical trials. Further molecular experiments in inducible pluripotent stem cell lines with *APOE* ε3/ε3 possessing EUR LGA could deepen our understanding of this association and provide clues regarding LOAD pathophysiology.

Our study further used WGS to genotype the *TOMM40*-523′ allele. Genotyping long microsatellite regions such as *TOMM40*-523′ can be challenging. Current methods used to size poly-T and poly-A tracts such as *TOMM40*-523′ within the human genome include fragment analysis and Sanger sequencing.[14] Although our WGS data correlated extremely well with "S" *TOMM40*-523′ alleles, it grew less consistent with the inclusion of "L" and "VL" alleles. This is undoubtedly because of the shorter size fragments used in Illumina WGS. Long fragment WGS would likely increase the concordance to fragment analysis. Repetitive DNA regions are likely to gain importance in examining the risk for disease and use of WGS to assess these regions and may play a progressively large role.

As noted, there were very few "L" alleles within the *APOE* ε3 haplotypes. Because of this, we were unable to generate proper odds ratios for "S" vs "L" alleles in this subgroup. It was additionally difficult to obtain the AF LGA *APOE* ε4/ε4 control samples. Although we improved on previous study designs by adjusting for LGA, our reduced sample size, as compared to previous studies,[16,36] may have limited the resolution within groups.

In our study, the *TOMM40*-523′ poly-T variant could not explain the large difference in the risk for LOAD between AF and EUR LGA on the *APOE* ε4 haplotype. Increasing TOMM40-523′ length on the *APOE* ε3 haplotype may decrease the risk for LOAD and warrants further investigation. The differential effect of LGA in *APOE ε4*, and now shown in *APOE* ε3, is continuing the examples of the importance of considering LGA while interpreting the risk for disease. In the setting of personalized medicine, identical genetic variants do not always confer equal risk. Rather, genetic loci must be examined with an understanding of their contextual ancestry. As we increase the amount of diversity in our genetic studies, this is likely to become a common cofactor in assessing risk.

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Disclosure

The authors have no competing interests to declare. Go to Neurology.org/NG for full disclosures.

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Farid Rajabli, PhD	University of Miami	Interpreted the data and wrote the manuscript
Anthony Griswold, PhD	University of Miami	Interpreted the data and wrote the manuscript
Daniel A. Dorfsman, BA	University of Miami	Prepared the samples, maintained clinical database, and oversaw regulatory compliance
Patrice Whitehead, BS	University of Miami	Prepared the samples, maintained clinical database, and oversaw regulatory compliance
Larry D. Adams, BA	University of Miami	Designed and conducted ascertainment process
Pedro R. Mena, MD	University of Miami	Ascertained patients and adjudicated disease status
Michael Cuccaro, PhD	University of Miami	Ascertained patients and adjudicated disease status
Jonathan L. Haines, PhD	Case Western University	Designed and conducted ascertainment process
Goldie S. Byrd, PhD	Wake Forest University	Designed and conducted ascertainment process
Gary W. Beecham, PhD	University of Miami	Interpreted the data and edited the manuscript
Margaret A. Pericak-Vance, PhD	University of Miami	Coordinated collaboration between ascertainment centers, provided clinical samples, and secured funding
Juan I. Young, PhD	University of Miami	Conducted the experiment, analyzed and interpreted the data, and wrote the manuscript
Jeffery M. Vance, MD, PhD	University of Miami	Secured funding, designed the experiment, adjudicated disease status, wrote the manuscript

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