Clin Genet 2006: 70: 49–56
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CLINICAL GENETICS
doi: 10.1111/j.1399-0004.2006.00638.x

Short Report

Genetic association between endothelial nitric oxide synthase and Alzheimer disease

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Clin Genet 2006: 70: 49–56. © Blackwell Munksgaard, 2006

Evidence suggests that vascular and inflammatory factors may be important in the etiology of Alzheimer disease (AD). The Glu/Glu genotype at the Glu298Asp variant of the endothelial nitric oxide synthase (NOS3) gene has been tested for association with AD in several Caucasian and Asian populations, with conflicting results. We tested the Glu298Asp variant for association in African American and Caucasian AD patients, unaffected siblings, and unrelated controls from the MIRAGE Study. To explore whether the inconsistent results in previous studies might be due to linkage disequilibrium with a polymorphism or haplotype not previously tested, we genotyped 10 additional NOS3 single nucleotide polymorphisms (SNPs) spanning 25.3 kb. Finally, we compiled results of previous studies of Glu298Asp using meta-analysis, to determine whether the aggregate studies support an association between Glu298Asp and AD. We found that the Glu298 allele was associated with higher risk of AD in the MIRAGE African American ($p = 0.002$) but not Caucasian ($p = 0.9$) groups. None of the additional SNPs were associated with AD in the Caucasians, whereas two showed evidence for association in the African Americans. The meta-analysis showed a small effect of the Glu298Asp GG genotype on AD risk across all studies (summary odds ratio = 1.15, 95% confidence interval: 0.97–1.35) and significant heterogeneity of this association among studies ($p = 0.02$).

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Key words: Alzheimer disease – endothelial nitric oxide synthase – genetic association – Glu298Asp – haplotype analysis – single nucleotide polymorphism

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Received 20 January 2006, revised and accepted for publication 3 May 2006

There is a growing impression that intermittent ischemia-reperfusion, endothelial dysfunction, alterations in the blood–brain barrier, and reductions in neuronal reserves due to prior stroke-related tissue losses may be directly or indirectly involved with the neurodegenerative and/or functional impairments that define the pathological and clinical identity of Alzheimer disease (AD) (1–3). Abnormalities of cerebral small vessels occur early in AD, and it has been demonstrated that the presence of β-amyloid in blood vessels and hippocampal pyramidal neurons, the principal site of AD pathology (4), is associated with an excess of superoxide radicals (5–7). These reactive oxygen species react with nitric oxide (NO) to produce peroxynitrate, which can cause lipid peroxidation that further accelerates degenerative changes including those leading to AD via β-amyloid/lipid interactions (8). Oxidant stress can also lead to hypertension, ischemic heart disease and other cardiovascular diseases that indirectly contribute to progression of AD by reduced blood flow to the brain (9).

NO is produced from L-arginine by the action of the nitric acid synthase enzymes (NOS). There are three NOS isoforms encoded by genes located on chromosomes 12 (NOS1), 17 (NOS2) and 7 (NOS3). However, NOS3, also known as eNOS

(endothelial form), is the predominant isoform present in most tissues (10, 11). Genetic association studies between NOS3 and AD have lead to mixed results. An over-representation of the Glu/Glu genotype for the polymorphic variant at amino acid position 298 of NOS3 (Glu298Asp) was observed among late-onset AD cases in UK and Italian populations (12, 13), while studies in two other Italian samples and one UK sample, as well as in Japanese, US Caucasian, Swedish, Hungarian, Polish and Hispanic samples, did not find significant association (14–23). It is unclear whether the discrepant results among these studies is due to limited sample size, suboptimal matching between cases and controls, or varying effects of the Glu298Asp polymorphism across diverse populations.

Our study had three primary aims. The first was to evaluate the association of the Glu298Asp polymorphism with AD in African American and Caucasian families in the MIRAGE Study. Our second goal was to test additional SNPs and haplotypes comprising these SNPs across the NOS3 gene region to explore the possibility that the inconsistent results found in prior studies could be due to linkage disequilibrium (LD) between the Glu298Asp polymorphism and an unexamined causal variant. Our final goal was to compile the results of the prior studies and the MIRAGE Study to determine whether, taken together, the studies supported the association between Glu298Asp and AD and to determine whether there was evidence for heterogeneity of effect across the studies.

Materials and methods

Subjects and data collection

The MIRAGE Study is a multi-center family-based study of genetic and environmental risk factors for AD. Details of data collection procedures, protocols for obtaining family histories, and reports of validity of study questionnaires have been published elsewhere (24–26). In brief, probands were ascertained at 17 sites in the USA (14), Canada (1), Germany (1) and Greece (1) through research registries or specialized memory clinics. All AD cases were living individuals with probable AD or recently deceased individuals with definite AD verified by brain autopsy. AD was diagnosed in accordance with the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) criteria for probable or definite AD (27). Medical history and risk factor information and blood samples were obtained from AD patients and their available siblings and spouses after obtaining informed consent from the non-demented subjects and a combination of consent or assent along with informed consent by proxy on living demented subjects. In some instances when non-demented siblings were unavailable, neighborhood controls of similar age and ethnic background and living near the AD proband were also enrolled in the study. Cognitive status of individuals identified as non-demented was confirmed by administration of the modified Telephone Interview of Cognitive Status (28). Subjects were classified as African American or Caucasian according to reported ethnicity of the parents. The study sample is described in Table 1. AD patients were much more likely to be carriers of an APOE ε4 allele than their unaffected sibs or spouses ($p \leq .0001$ for all comparisons).

SNP selection and genotyping

SNPs were selected from the National Center for Biotechnology Information SNP database based on (i) prior implication in disease, (ii) predicted function, (iii) minor allele frequency greater than 5%, (iv) LD structure in the locus, (v) quality of validation evidence, and (vi) compatibility with the genotyping platform. Genomic DNA was

Table 1. Sample description

Ethnicity	Relationship	Alzheimer disease status	*n*	Male (%)	Mean age[a] (SD)	APOE ε4 carriers (%)
Caucasian	Proband	Affected	259	34	68.3 (9.0)	66
	Sib	Affected	11	25	71.5 (8.3)	73
	Sib	Unaffected	326	40	70.3 (9.3)	45
	Spouse	Unaffected	127	59	71.7 (9.3)	25
African	Proband	Affected	235	24	71.4 (8.1)	71
American	Sib	Affected	6	0	72.0 (6.3)	100
	Sib	Unaffected	144	34	72.9 (10.6)	52
	Spouse	Unaffected	48	35	72.0 (6.7)	36
	Unrelated	Unaffected	34	24	72.8 (7.9)	15

[a]Age of onset of Alzheimer disease symptoms for affected individuals, exam age for unaffected individuals.

extracted from peripheral blood lymphocytes using the Qiagen (Valencia, CA) DNA isolation kit. Twenty nanograms of DNA was used in a GenomiPhi (GE Healthcare Systems, Piscataway, NJ, USA) reaction to amplify the amount of DNA. SNP genotyping was performed on 5 ng of DNA using an ABI 7900 (real-time) platform with the manufacturer's protocols and Taqman (Applied Biosystems, Foster City, CA) assay-by-design and assay-by-demand products. Duplicate wells were scattered on DNA template plates. The duplicate discordance rate did not exceed 5% and was persistently localized to two samples, which were subsequently excluded from all analyses. The overall genotype call rate was found to be >95% for all SNPs typed.

Statistical methods

Each SNP was tested to determine if its genotype frequencies met Hardy–Weinberg equilibrium (HWE) expectations. Tests were conducted separately for groups of unrelated AD cases and controls in each ethnic group using an exact test of HWE (29). Because we expected population differences in LD and allele frequencies, we tested for association separately in the African American and Caucasian subgroups. To make use of all the genotype data available, we used a generalized estimating equation (GEE) approach (30) implemented in the GENMOD procedure of SAS to test the effects of genotype on the odds of AD with one genotype relative to another genotype group. Nuclear family members (including affected and unaffected siblings and spouses) were grouped, thus accounting for the correlation due to genetics and shared environment. Neighborhood controls also contributed to this analysis as independent observations. With age and gender as covariates, we performed GEE tests using a 2 degrees of freedom general genotype model that asks whether there is a difference in odds across the three genotype groups for each SNP. A second model including APOE ε4 allele carrier status as an additional covariate was tested to determine whether APOE genotype confounded the association.

We performed a meta-analysis to examine the evidence for association between the Glu/Glu genotype of Glu298Asp and AD across all published studies. This analysis included all early- and late-onset sporadic AD patients and unrelated controls from the African American and Caucasian subgroups in the current study and 12 previously published studies (12–23), omitting 88 AD patients specifically identified as having 'familial AD' in Tedde et al. (23). For the Dahiyat et al. (12) study, the two sets of late-onset AD and one of early-onset AD, all recruited from the same two institutions, were combined to form a single set of cases. The controls for this study were likewise combined, creating a single case–control cohort. A random-effects model was used to estimate the summary odds ratio (OR) of the effect of the Glu/Glu genotype on AD risk (31). This approach incorporates the heterogeneity of effects in the estimation of the overall effect of the polymorphism and assumes that the true effect size varies among populations. Heterogeneity in the OR among studies was tested using the method of Woolf (32).

To further investigate association between NOS3 and AD risk, we used haplotype-based methods for association implemented in the HAPLO.STATS package (33). This approach utilized information from the AD patients, unrelated spouses and neighborhood controls, and included age and gender as covariates. For reference, we also computed the age- and gender-adjusted single-SNP association tests under an additive model for this case–control subset of our data set to be able to compare the strength of the haplotype associations in this data set to the individual SNP associations. Sliding windows of two to five SNPs across the gene were tested for association with AD status, moving in steps of one SNP. Each window of SNPs was tested for an overall association using a global test. If the global test was significant at $p < 0.05$, haplotype-specific tests were performed for all haplotypes with an estimated frequency of greater than 0.005 in the sample.

Results

Evaluation of the genotype and allele distributions in the unrelated cases and controls (Table 2) revealed that one SNP (rs743507) was not in HWE in the African American cases ($p = 0.006$) and another SNP (rs1007311) was not in HWE in Caucasian controls ($p = 0.001$). Notably, these SNPs were not associated with AD in any of our analyses.

Figure 1 indicates that the Glu298Asp polymorphism (rs1799983) was significantly associated with AD in the African American (GEE general genotype test $p = 0.002$, case–control additive test $p = 0.003$) but not in the Caucasian group. Adjacent SNP rs1800780 (located 2768 bp 3′ to Glu298Asp) was associated in the African American case–control group ($p = 0.004$) but did not reach statistical significance in the family

Table 2. Case and control genotype and allele distributions

Subgroup	Single nucleotide polymorphism	Major/ minor allele	Minor allele frequency		Case genotype distribution			Control genotype distribution		
			Case	Control	1/1	1/2	2/2	1/1	1/2	2/2
Caucasian	rs1800783	T/A	0.38	0.36	0.36	0.52	0.13	0.41	0.46	0.13
	rs1800779	A/G	0.38	0.36	0.35	0.52	0.12	0.41	0.47	0.13
	rs3918166	G/A	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00
	rs1007311	A/G	0.42	0.43	0.36	0.45	0.19	0.41	0.32	0.27
	rs1799983[a]	G/T	0.35	0.30	0.42	0.46	0.12	0.48	0.43	0.09
	rs1800780	G/A	0.45	0.50	0.32	0.46	0.22	0.30	0.41	0.30
	rs3918188	G/T	0.34	0.36	0.43	0.46	0.11	0.45	0.38	0.17
	rs743506	A/G	0.24	0.28	0.57	0.38	0.05	0.50	0.44	0.06
	rs743507	A/G	0.24	0.28	0.56	0.38	0.05	0.49	0.45	0.06
	rs891512	G/A	0.21	0.22	0.62	0.33	0.04	0.63	0.32	0.06
	rs1077872	G/C	0.31	0.37	0.48	0.41	0.11	0.40	0.46	0.13
African American	rs1800783	T/A	0.42	0.46	0.35	0.45	0.20	0.28	0.53	0.20
	rs1800779	A/G	0.14	0.18	0.74	0.24	0.02	0.65	0.35	0.00
	rs3918166	G/A	0.04	0.05	0.92	0.07	0.00	0.91	0.07	0.01
	rs1007311	A/G	0.43	0.51	0.20	0.46	0.33	0.27	0.47	0.26
	rs1799983[a]	G/T	0.09	0.16	0.82	0.18	0.00	0.68	0.30	0.01
	rs1800780	G/A	0.39	0.53	0.34	0.53	0.13	0.20	0.55	0.25
	rs3918188	G/T	0.38	0.33	0.41	0.43	0.17	0.46	0.43	0.11
	rs743506	A/G	0.42	0.41	0.34	0.48	0.18	0.31	0.57	0.12
	rs743507	A/G	0.22	0.22	0.64	0.28	0.08	0.61	0.36	0.04
	rs891512	G/A	0.03	0.07	0.94	0.06	0.00	0.86	0.14	0.00
	rs1077872	G/C	0.49	0.52	0.25	0.48	0.27	0.25	0.54	0.21

[a]Also known as Glu298Asp.

data set (GEE $p = 0.06$). SNP rs891512 (located 12 kb downstream from Glu298Asp) was also associated in the African American group (GEE $p = 0.03$). In the Caucasian group, no single SNP was significantly associated with AD. The patterns of association between NOS3 SNPs and AD were unchanged after adjustment for the presence or absence of at least one APOE ε4 allele (data not shown).

Haplotype analyses revealed some suggestion of association in between AD and SNPs at the 3′ end of NOS3 in the Caucasian subgroup (Fig. 1). Results for adjacent SNP sets with global p values less than 0.05 are presented. In the Caucasian group, all significant haplotype-specific associations include a G allele at SNPs 8, 9 and 10, and all haplotypes containing these three alleles have negative scores, indicating a protective effect. Because the associated haplotypes are uncommon, it is quite possible that these findings are spurious: the most significant haplotype was observed for the window including SNPs 7–10 (global p value = 0.003; adjusting for sliding window multiple testing, $p = 0.07$). In the African American group, no haplotype demonstrated considerably stronger association than the Glu298Asp polymorphism alone. The most significant global p value is 0.001 for haplotypes consisting of SNPs 5 (Glu298Asp) and 6 (adjusting for sliding window multiple comparisons, $p = 0.06$). For this SNP pair, the TA haplotype and GG haplotype have opposite effects of similar magnitude, which is consistent with the single-SNP analyses. Of note, the risk (GG) haplotype is very common (58% of all chromosomes) among African Americans.

Figure 2 depicts the OR estimates for AD associated with homozygosity of the Glu allele for the Glu298Asp polymorphism among the 12 published studies and the two MIRAGE ethnic groups. The studies are ordered by the frequency of the GG genotype in study cases. The ORs were significantly different among studies ($p = 0.02$). The summary OR is 1.15 [95% confidence interval (CI): 0.97–1.35], indicating an effect in the same direction as in the original study (12) that just misses statistical significance at the 0.05 level. Interestingly, the heterogeneity in effect appears to correlate with the GG frequency in cases rather than ethnicity: the seven studies with GG case frequency of greater than 0.50 all have OR estimates greater than or equal to 1, and three have significant associations at the 0.05 significance level. The six studies with GG frequency in cases of less than 0.50 all have non-significant OR estimates less than 1.

Discussion

The results of our cross-sectional family-based study indicate that homozygosity for the Glu

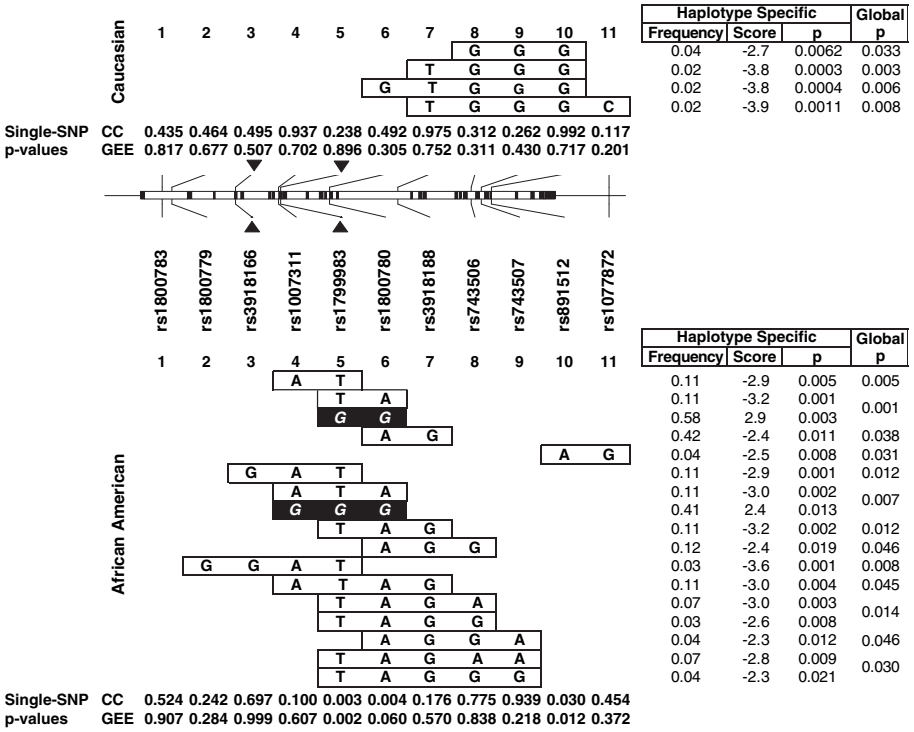

Fig. 1. Association of endothelial nitric oxide synthase with Alzheimer disease. The sliding window single nucleotide polymorphism (SNP) sets yielding haplotype global asymptotic p values <0.05, and at least one haplotype with nominal haplotype-specific asymptotic p value of <0.05, are shown. The 'Haplotype Specific' columns indicate the frequency of the displayed haplotype, its score, and the nominal p value for the specific haplotype listed. Positive scores indicate haplotypes associated with risk; negative scores indicate protective haplotypes. CC, individual SNP case-control association tests (additive model); GEE, individual SNP full data set association tests (general model).

allele of the Glu298Asp polymorphism (corresponding to the GG genotype of SNP 1799983) in NOS3 is associated with increased risk for AD in African Americans. This association is observed in the full sample of affected AD cases, their unaffected siblings, and unaffected controls, but also in the case-unrelated control subset (Fig. 2). In additional association analyses (data not shown), we found that the association is also significant when spouse and neighborhood controls are omitted from the GEE model ($p = 0.006$).

The goal of our haplotype analysis was to explore the possibility that SNPs in addition to or in lieu of Glu298Asp might be responsible for the conflicting association results in previous studies. By testing each of 11 SNPs including the Glu298Asp polymorphism, and sliding windows of SNPs across the gene, we hoped to identify evidence for association within the gene that was more consistent across the two ethnic groups in our sample. We hypothesized that the basis for the inconsistent evidence of association in the previous studies, which considered only the Glu298Asp SNP, is differing LD between Glu298Asp and the true causal variant. Our study was not successful in identifying another variant or haplotype that may be responsible for the reported associations. While we cannot definitively exclude the possibility that a mutation elsewhere in NOS3 is responsible for the heterogeneity in the AD/NOS3 association, we found no evidence in support of this explanation in our Caucasian and African American samples.

We performed a meta-analysis to determine whether there is heterogeneity in the association among the many studies examining the association between Glu298Asp and AD or when considered jointly, the studies indicate a clear conclusion. For one half of the previously published data sets, the Glu/Glu genotype increases AD risk, but for only two of these prior studies was the effect significant (Fig. 2). None of the studies showing the opposite genotype effect reached significance. The two MIRAGE samples mimic this pattern and give the first report of a significant association in African Americans. There are several possible explanations for the heterogeneous pattern of association observed. Since the allele frequencies of the polymorphism differ widely between

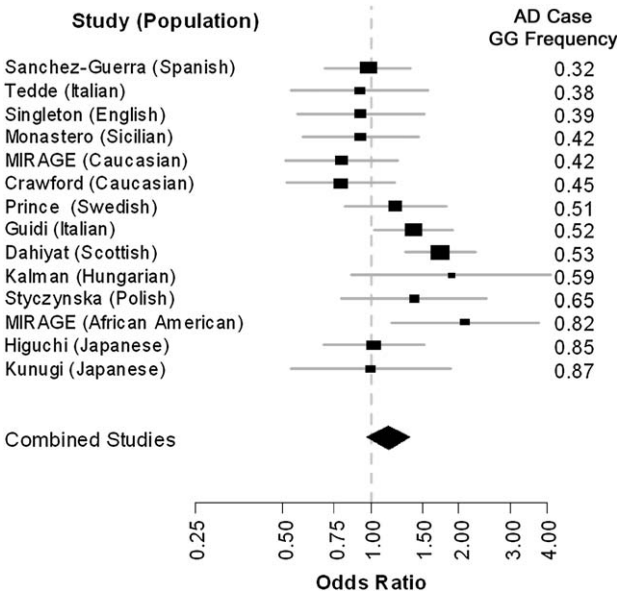

Fig. 2. Meta-analysis of the endothelial nitric oxide synthase Glu298Asp Glu/Glu genotype association with Alzheimer disease. Odds ratios (OR) and 95% confidence intervals (CI) are presented for 12 published studies and the two MIRAGE samples, ordered by case Glu/Glu genotype frequency. Boxes indicate study size—larger studies have larger boxes. Summary OR and CI were computed using a random-effects model using the DerSimonian–Laird method (31).

populations, it is possible that the observed association in the African American sample is due to confounding by admixture. Alternatively, the association could depend upon the general genetic and cultural background of the population. These explanations are unlikely since positive associations with the same genotype were identified in our African American cohort and samples from Scotland, Sweden, Italy, Hungary and Poland, but not in samples from Japan and various North American and European countries. Admixture would not account for our findings in African Americans because nearly all the controls in this group are siblings or spouses of the cases. A more likely epidemiological explanation is given that the GG genotype is prevalent in the general population (0.33–0.87), and the impact of the GG genotype compared to other Glu298Asp genotypes on AD risk is modest (summary estimate 1.15, 95% CI: 0.97–1.35) and the ability to discern a positive association is dependent on the frequency of this variant in the population and the sample size. Indeed, given the GG genotype frequency of 0.42 observed in our Caucasian cases, with a two-sided test and $\alpha = .05$, we had only 64% power to detect in this group an effect of the same size (OR $\approx$ 1.7) as that observed in the original study (12). Alternatively, multiple disease-related variants in the coding region of NOS3 or its regulatory elements may exist that act independently or synergistically with Glu298Asp. Our haplotype analysis of 11 NOS3 SNPs suggests that the Glu298Asp polymorphism, the adjacent SNP rs1800780, or an untyped polymorphism in LD with these polymorphisms is responsible for the association in African Americans, whereas a weakly acting variant in the distal portion of NOS3 may modulate AD risk in some Caucasian populations. A recent study of a polymorphism in the NOS3 promoter region (T-786C), which had been previously associated with vascular pathologies, showed an additive effect of the T allele on NOS3 mRNA levels; however, this pattern was unrelated to risk of AD in sample showing association with Glu298Asp (13, 34). Evaluation of multiple SNPs spanning the NOS3 region in independent samples will be necessary to elucidate all the AD susceptibility variants in NOS3.

Our study has several notable strengths. We evaluated large samples of Caucasian and African Americans using standardized research diagnostic criteria. Controls were immediate family members, thus lessening the possibility of spurious results due to population admixture and providing some degree of informal matching on social and environmental variables. In contrast to previous investigations of the AD/NOS3 association that studied exclusively the Glu298Asp polymorphism, we examined 11 SNPs spanning NOS3 and evaluated these data using haplotype methods that are more powerful at discerning association than analysis of individual SNPs.

The relatively modest increased risk of AD associated with NOS3, which is evident in many but not all populations, and the fact that abnormalities of NOS3 functioning could impact a variety of tissues in addition to the brain suggest that the AD risk variants in NOS3 likely interact with other genetic and environmental factors. For example, the presence of APOE ε4 results in elevated lipid levels (35), which, when modified by peroxynitrite, would be predicted to elevate levels of toxic lipids that can lead to AD. Lack of evidence in this study for specificity of the NOS3/AD association among APOE ε4 carriers does not negate this possibility since APOE levels are governed in part by polymorphic sites in the transcriptional regulatory portion of the APOE gene (36). Furthermore, genetic variation in enzymes, such as paraoxonases, that degrade toxic lipid isoforms could also be important. In fact, we observed significant

association between the paraoxonase genes and AD in this same population (37). In summary, our data support the hypothesis that oxidative stress plays an important role in the etiology of AD. Thus, additional experiments examining the NOS3 gene as well as other genes related to oxidant stress are warranted to identify the variants contributing to AD.

Acknowledgements

The MIRAGE Study Group members are Drs LA Farrer, RC Green, LA Cupples, CT Baldwin, KL Lunetta, and S Auerbach (Boston University); Drs A Akomolafe, P Griffith, and E Ofili (Morehouse School of Medicine); Drs J Browndyke, D Schmechel, and K Welsh-Bohmer (Duke University); Dr H Chui (University of Southern California); Dr C DeCarli (University of California—Davis); Dr R Duara (Mt. Sinai Medical Center, Miami); Drs T Foroud and M Farlow (Indiana University School of Medicine); Dr R Friedland (Case Western Reserve University); Dr R Go (University of Alabama—Birmingham); Dr A Kurz (Technical University, Munich, Germany); Dr T Obisesan (Howard University); Drs H Petrovitch and L White (Pacific Health Research Institute); Dr N Relkin (Cornell University); Dr M Sabbagh (Sun Health Research Institute); Dr D Sadovnick (University of British Columbia); and Dr M Tsolaki (University of Aristotle, Thessaloniki, Greece). We are grateful to the contributions of the MIRAGE Study coordinators (P Morey, B Adamiak, Dr A Ade-Okanlami, L Cowden, B Cramer, M Dandiilidou, E Dwosh, E Grady, S Humphrey, C Imamura, H Kolody, M Luis, K Martelli, M McCart, and J Wainwright), I Simkin for laboratory work, and J Farrell and J Blesso for database programming and electronic data capturing support. This work was supported in part by National Institute on Aging grants R01-AG09029 and P30-AG13846.

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