

A Pilot Study of Gene/Gene and Gene/Environment Interactions in Alzheimer Disease

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Background: Although some genes associated with increased risk of Alzheimer Disease (AD) have been identified, few data exist related to gene/gene and gene/environment risk of AD. The purpose of this pilot study was to explore gene/gene and gene/environment associations in AD and to obtain data for sample size estimates for larger, more definitive studies of AD.

Methods: The effect of gene/gene and gene/environment interaction related to late onset Alzheimer Disease (LOAD) was investigated in 153 subjects with LOAD and 302 gender matched controls enrolled in the Personalized Medicine Research Project, a population-based bio-repository. Genetic risk factors examined included *APOE*, *ACE*, *OLR1*, and *CYP46* genes, and environmental factors included smoking, total cholesterol, LDL, HDL, triglycerides, C-reactive protein, blood pressure, statin use, and body mass index.

Results: The mean age of the cases was 78.2 years and the mean age of the controls was 87.2 years. *APOE4* was significantly associated with LOAD (OR=3.55, 95%CL=1.70, 7.45). Cases were significantly more likely to have ever smoked cigarettes during their life (49.3% versus 38.4%, $p=0.03$). The highest recorded blood pressure and pulse pressure measurements were significantly higher in the controls than the cases (all $P<0.005$). Although not statistically significant in this pilot study, the relationship of the following factors was associated in opposite directions with LOAD based on the presence of an *APOE4* allele: obesity at the age of 50, *ACE*, *OLR1*, and *CYP46*.

Conclusions: These pilot data suggest that gene/gene and gene/environment interactions may be important in LOAD, with *APOE*, a known risk factor for LOAD, affecting the relationship of *ACE* and *OLR1* to LOAD. Replication with a larger sample size and in other racial/ethnic groups is warranted and the allele and risk factor frequencies will assist in choosing an appropriate sample size for a definitive study.

Keywords: *ACE*; *APOE*; Biobank; Body mass index; *CYP46*; Alzheimer Disease; Late onset; *OLR1*; Smoking

Alzheimer Disease (AD [MIM 104300]) is a common, complex genetic disorder with a prevalence rate of 5% to 10% and an incidence that increases exponentially after the age of 65 years. A majority of AD cases manifest as sporadic late onset form (LOAD [MIM 606626]), typically with onset above the age of 65 years.

Clinical manifestations of the disease are associated with alterations in some or all of the following neurotransmitter

networks: the forebrain cholinergic system, noradrenergic system, and telencephalon somatostatinergic system.¹ In addition, the interaction of cholesterol metabolism, inflammatory processes, and *APOE* has been implicated in contributing to the pathogenesis and/or progression of the disease.

Because evidence of genomic variation in association with LOAD is increasingly emerging in both mutational and

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susceptibility loci, it is becoming increasingly clear that complex neurodegenerative processes underlie development and susceptibility to LOAD. More recently a large number of genes have been implicated as a risk to LOAD, but only a few of these associations have been replicated.²⁻⁴

Besides *APOE4*, which is currently the most established and validated genetic risk factor for LOAD in most populations,⁵ the *ACE* gene has emerged as another potential risk factor for LOAD. Specifically, the A262T polymorphism (rs4291) in the promoter region of the *ACE* gene showed strong association with LOAD and was present in up to 35% of subjects with AD in five independent populations representing approximately 3,100 individuals.⁶ Moreover, recent meta-analysis has determined a highly significant association of a 289bp Alu insertion/deletion-polymorphism (I/D) in intron 15 of the *ACE* gene for patients with AD.^{6,7}

Another interesting candidate gene is the oxidized low-density lipoprotein (LDL) receptor gene (*OLRI*).⁸ Three novel sequence variations in the *OLRI* gene (chromosome 12) exhibiting strong association with LOAD have been described in intron 4 (rs391640 [GG]), intron 5 (rs2742113 [TT]) and 3' untranslated region (rs1050283 [TT]). All three polymorphisms demonstrated significant association with AD after stratification for *APOE4* following screening of 800 AD cases and 700 controls (odds ratio [OR] 2.39, confidence interval [CI] 1.34-4.27), $P=0.0033$; OR 2.11, CI 1.18-3.78, $P=0.012$; OR 2.63, CI 1.44-7.1, $P=0.0012$, respectively).

Further, *CYP46* (chromosome 14q) is the gene encoding cholesterol 24-hydroxylase. The function of this molecule is hydroxylation of cholesterol, a prerequisite to its removal from brain. Recent studies demonstrated that *CYP46* (rs754203) is associated with increased β -amyloid load in brain and cerebral spinal fluid.⁹

As with most complex diseases, LOAD is likely to be influenced not only by genetic, but also by environmental components.^{10,11} Based on outcomes of studies in twins, Plassman et al¹² reported simple heritability estimates among various European populations at approximately 0.8, suggesting that environmental factors account for approximately 20% of variance in population susceptibility to AD.

One environmental factor whose role in AD pathogenesis remains controversial based on systematic review of case-control and cohort studies is smoking history.¹³⁻¹⁵ Several studies have demonstrated that smoking is inversely proportional to AD progression as a risk factor due to the demonstrated ability of nicotine to stimulate neurotransmitter systems that are compromised in AD. Others have defined potential risk, especially in association with heavy smoking.¹⁵⁻¹⁷

Another important environmental factor implicated in AD is cholesterol. Increased serum concentrations of total

cholesterol, low density lipoprotein (LDL) cholesterol and *APOE* have been reported in patients with AD.¹⁸⁻²⁰ Although cholesterol has been implicated in contributing to disease progression and etiology, and numerous hypotheses have been advanced to explain its role in disease pathogenesis, how *APOE* or other candidate genes investigated here mechanistically promote etiology, progression, or pathology remains to be defined.

The influence of environmental factors is further emphasized in a recent study of 4,615 subjects where authors reported significant association of increasing age, less education, and presence of *APOE4* allele with AD, and decreased risk in association with use of non-steroidal anti-inflammatory drugs, daily coffee consumption, wine consumption, and regular physical activity. The study was unable to demonstrate significant association with respect to family history of dementia, smoking, gender, depression history, estrogen use, hypertension, or cardiovascular conditions. However, while the authors adjusted for gender, education, and age, no adjustment was made for the genotype; thus it is unclear whether risk of other factors was influenced by presence of the *APOE4* allele or other candidate genes proposed for AD.²¹

Using a defined population cohort from the Personalized Medicine Research Project (PMRP) this study examined polymorphisms in the *APOE*, *ACE*, *OLRI*, and *CYP46* genes, as well as the effect of several environmental factors, including smoking, cholesterol, triglycerides, C-reactive protein (a marker for inflammation), and blood pressure in order to explore potential interactions of environmental factors and genes in index cases and controls.²²

Material and Methods

Personalized Medicine Research Project (PMRP)

The current study was conducted using a nested case-control design utilizing DNA and phenotypic data from a subset of participants in the PMRP cohort, details of which have been published previously.²² The PMRP is a population-based biobank located in central Wisconsin. Nearly 20,000 residents aged 18 years and older have participated, consenting to medical record access for purposes of defining phenotype and provision of a blood sample from which DNA, plasma, and serum were extracted and banked. The study was approved by the Marshfield Clinic Institutional Review Board, and all subjects, or their medical power of attorney, gave written informed consent prior to study participation.

Phenotyping of Cases and Controls

For the present study, the Marshfield Clinic medical records were searched electronically to identify potential cases of AD and controls. Chart abstraction was used to confirm case status and the required inclusion criterion of age ≥ 65 years at diagnosis. The LOAD diagnosis of cases was made using National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer Disease and Related Disorders

Table 1. Assay primers and expected product sizes.

Gene	SNP ID	Variant (UCSC genome browser)	Forward	Reverse	Extension primer	Amplicon length (bp)	Terminator mix
<i>APOE</i>	rs429358	C/T	TCGCCGCGGTACTGCACCA	CTGTCCAAGGAGCTGCAGG	CGGACATGGAGGACGTG	106	ACG
<i>APOE</i>	rs7412	C/T	CTCGCGGATGGCGCTGAGG	TCCGCGATGCCGATGACCTG	CCGATGACCTGCAGAAG	112	ACG
<i>ACE</i>	rs1800764	C/T	GAGTCTTGAATGTACCCAC	AACCCATGGGATGAGAGAAG	ATTTGCAAAGTATGTACAGCA	100	ACT
<i>ACE</i>	rs4291	A/T	TCGGGTGTCCGGCAAACCTG	GCAGAGGAAGCTGGAGAAAG	AAGGGCCTCCTCTCTTT	91	CGT
<i>ACE</i>	rs4646994	(289bp Alu)/-	GGCCATCACATTCTGTACGAT AGCGAGACTCCGTCTCAAAA	GACCACTCCCATCCTTTCTC	ggaTGCCTATACAGTCACTTTT	201 (del) 104 (ins)	iplex
<i>ACE</i>	rs4343	A/G	CATGCCATAACAGGTCTTC	CCTACCAGATCTGACGAATG	GACGAATGTGATGGCCAC	83	ACT
<i>OLR1</i>	rs3912640	A/G	CACAGCAGAGGAATTGAGAC	AACCTGAAACAGACCCAC	ACTATTAGTAGGAAATGTCATTAG	112	ACG
<i>OLR1</i>	rs2742113	A/C	ACACTCTAGAAGCCAGTAAG	GTGTGTTAGACTCCACAAATG	GAAGATAGAAACTTGACAGG	118	ACT
<i>OLR1</i>	rs1050283	C/T	CCTGGCACCTTTATGTCAAC	AAGCTTGGGACAAGCTAGGT	AAGCTAGGTGAAATAATACAG	90	iplex
<i>CYP46</i>	rs754203	A/G	CTACCAAAGAGTGTGTCC	ACATGACTCGGCAAGTGAGC	GGCAGAGCCTTGCCCCC	99	ACG
<i>AMG</i>		(6bp ins/del)	CCTCATCTGGGCACCCTGG	GGCTTGAGGCCAACCATCAG	GGACCACTTGAGAAAC	230(X) 236(Y)	ACG

Amplicon length includes the added 10-mers tail. The small letters in the extension primer for the ACE ins/del were added to improve mass discrimination in the multiplex reaction.

SNP, single nucleotide polymorphism.

Association (NINCDS-ADRDA) criteria.¹ Exclusion criteria included non-genetic causes for dementias and other conditions that make the diagnosis of AD less likely or uncertain, such as focal neurological findings. All cases were diagnosed by a single neurologist (SFM), who also reviewed all questionable cases in the study. Gender frequency-matched controls were required to be age 80 years and older to minimize misclassification bias in the controls, as is commonly done for strongly age-related conditions, and to have achieved a score ≥ 27 on a telephone-administered Mini Mental State Examination (MMSE, Psychological Assessment Resources, Inc).²³

Data Abstraction

The highest value for total cholesterol, LDL cholesterol, high density lipoprotein (HDL) cholesterol, diastolic and systolic blood pressures, log highest triglycerides, and highest quartile of C-reactive protein were abstracted from the electronic medical records for cases before diagnosis of AD was confirmed and for controls before the date of MMSE administration. The highest values were chosen, assuming that they had occurred before administration of any medication to alter these levels. This may lead to overestimations of the actual values, such as occurs with “white coat hypertension,” but would only lead to bias if it differentially occurred in the cases or controls. Lifetime smoking information (never smoker, past smoker, present smoker) was collected from the self-administered study questionnaire that PMRP participants complete at the time of their enrollment in PMRP. Statin use was abstracted from the medical records. No other medication use was recorded.

Genotyping

Primer design: Both the amplification and extension primers were designed using the Assay Designer 2.05-software from Sequenom. This software designs the primers used for amplification and the base extension reactions. It also

identifies the appropriate termination mix to use in the homogeneous mass extend (hME) or in the i-PLEX reactions. The amplification primers were designed with a 10-mer tag sequence ACGTTGGATG to increase their mass so that they would fall outside the range of detection of the matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry. The amplification and extension primers and expected product sizes are presented in table 1. The amylogenin gene (*AMG*) I/D gender-specific marker was typed in all the samples for quality control monitoring.

PCR reaction setup for hME: For the hME reaction, a total of 25 ng of genomic DNA was amplified in a 5 ml reaction containing 0.1 U of HotStar Taq enzyme (Qiagen, Valencia, CA), 1X of HotStar buffer, 2.5 mM (total) MgCl₂, 0.2 mM (each) deoxynucleotide triphosphate, and 150 nM of the forward and reverse primers for each assay. The PCR step was initiated with a 95°C soak for 15 min, followed by 45 cycles consisting of 95°C for 20 sec, 56°C for 30 sec, 72°C for 60 sec, and a final extension of 3 min at 72°C.

PCR reaction setup for i-PLEX: A total of 25 ng of genomic DNA was amplified in a 5 ml reaction containing 0.5 U of HotStar Taq enzyme, 1.25 X buffer, 1.6 mM MgCl₂, 0.5 mM each dNTP and 100 nM of each gene-specific forward and reverse primer. The reactions were prepared in a 384-microtiter-plate format. The amplifications were done in thermocyclers equipped with 384 well blocks. Cycling conditions were 15 min at 94°C followed by 45 cycles of 20 sec at 94°C, 30 sec at 56°C, 60 sec at 72°C, and a final extension time of 3 min at 72°C.

Shrimp alkaline phosphatase (SAP) reaction: After PCR, the remaining unincorporated dNTPs were dephosphorylated by adding 2 μ l of the SAP cocktail containing 1.53 μ l of water, 0.17 μ l of hME reaction buffer (Sequenom), and 0.3 μ l of SAP (Sequenom). The 384-well plate was then sealed and

Table 2. Baseline characteristics of the controls and Alzheimer's Disease cases.

Baseline Characteristics	Controls (N=302)	Cases (N=153)	P value
Age	87.2±3.0	78.2±6.4	<0.001
Female gender	198 (65.6%)	92 (60.1%)	0.25
Ever Smoked	116 (38.4%)	74 (49.3%)	0.03
BMI at age 50	25.9±4.0	27.7±5.1	0.19
Obese (BMI≥30) at age 50	100 (52.9%)	49 (55.7%)	0.67
Total cholesterol	259.0±43.3	259.3±47.1	0.82*
Statin use	146 (48.3%)	74 (51.6%)	0.51
LDL cholesterol	167.6±41.1	169.3±43.9	0.50*
HDL cholesterol	62.9±17.0	59.5±16.8	0.08*
Triglycerides	203.4±112.5	234.8±199.3	0.67*
C-reactive protein	2.6±4.5	2.3±3.2	0.66*
Diastolic blood pressure	84.3±10.1	79.3±9.6	<0.001*
Systolic blood pressure	165.2±21.0	152.9±22.9	<0.001*
Pulse pressure	81.0±18.7	73.7±19.3	0.004

* Mann-Whitney U-test. BMI, body mass index. LDL, low-density lipoprotein. HDL, high-density lipoprotein.

placed in a thermal cycler under the following conditions: 37°C for 20 min, 85°C for 5 min, and 4°C until hME reaction.

hME reaction: After the SAP treatment, a 2 µl cocktail consisting of 1.728 µl water, 0.2 µl hME EXTEND mix containing 10X buffer and appropriate d/ddNTPs (Sequenom), 0.54 µl of 10 µM each extension primer mix and 0.018 µl of 32 U/µl ThermoSequenase (Sequenom) was added. After the hME cocktail addition, the plate was again sealed and placed in a thermal cycler with the following program: 94°C for 2 min followed by 55 cycles of 94°C for 5 sec, 52°C for 5 sec, and 72°C for 5 sec.

i-PLEX reaction: The extension primers, iPLEX enzyme, iPLEX buffer, and the iPLEX termination mixture of mass-modified dideoxynucleotide triphosphates were added to initiate the iPLEX primer extension reaction. This reaction generates allele-specific primer extension products that are one base longer than the original MassEXTEND primer. The EXTEND mixture (2 µl) was added to the PCR/SAP mixture (7 µl) for a total volume of 9 µl containing 0.222X iPLEX buffer, 1X iPLEX termination mix, 0.625 µM to 1.25 µM of each extension primer, and 1X iPLEX enzyme. The extension reaction consisted of a two-step 200 short cycles program consisting of one loop of 5 cycles inside a loop of 40 cycles. The sample was denatured at 94°C for 30 sec. Strands were then annealed at 52°C for 5 sec and extended at 80°C for 5 sec. The annealing and extension cycle was repeated 4 more times for a total of five cycles and then looped back to a 94°C denaturing step for 5 sec and then entered the 5 cycle annealing and extension loop again. The five annealing and extension steps with the single denaturing step were repeated an additional 39 times for a total of 40 runs, which equated to a total number of 200 cycles.

Nanodispensing and mass spectrometry: Following the hME or the i-PLEX extension reactions, the reaction mix was

desalted by adding 20 µl of a cationic resin mix (6 mg), SpectroCLEAN (Sequenom). The plate was sealed and placed in a rotating shaker for 10 min for desalting. Completed genotyping reactions were spotted in nanoliter volumes onto a matrix arrayed silicon chip with 384 elements (Sequenom SpectroCHIP) using the MassARRAY Nanodispenser. SpectroCHIPS were analyzed using the Bruker Autoflex MALDI-ToF mass spectrometer and the spectra were processed using the SpectroTYPER software (Sequenom).

Data Analysis

All data were entered twice and verified. A de-identified data set was created after merging the genetic, phenotypic, and environmental data. SAS (Cary, NC) was used for the analyses and a *P* value <0.05 was considered statistically significant. Chi-square analysis and *t*-tests were used for univariate analyses, and multivariate logistic regression was employed to identify independent risk factors after adjusting for potential confounders. Haplotype analysis was performed using the HAP-Haplotype Resolution version 3.0²⁴ (<http://research.calit2.net/hap/WebServer.htm>).

Results

Our LOAD cohort was comprised of 153 cases (mean age 78.2 years) and 302 controls (mean age 87.2 years). Both the case and control groups were selected from PMRP and were carefully phenotyped as detailed previously in materials and methods (baseline characteristics in table 2). The percent of AD cases that had ever smoked cigarettes was significantly higher than controls (49.3% versus, 38.4%, *P* <0.03). Highest recorded systolic and diastolic blood pressures and pulse pressures were significantly higher in controls than cases (all *P* <0.005).

A total of ten single nucleotide polymorphisms (SNPs) from four genes were tested for associations with LOAD. Genes tested included *APOE* (rs429358, rs7412), *ACE* (rs1800764, rs4291, rs464994 [289bp Alu/-], and rs4343), *OLRI*

Table 3. Logistic regression analysis adjusting for age, gender, smoking and pulse pressure.

Genotype	Controls (N=302)	Cases (N=153)	Odds ratio (95%CI)
rs1800764 (<i>ACE</i>)			
TT	98 (32.5%)	61 (39.9%)	1.00
CT	141 (46.7%)	65 (42.5%)	0.83 (0.37, 1.88)
CC	63 (20.9%)	27 (17.7%)	1.13 (0.43, 2.92)
CC+CT	204 (67.6%)	92 (60.1%)	0.92 (0.44, 1.94)
rs4291 (<i>ACE</i>)			
AA	117 (38.7%)	73 (47.7%)	1.00
AT	136 (45.0%)	61 (39.9%)	0.81 (0.37, 1.75)
TT	49 (16.2%)	19 (12.4%)	0.82 (0.28, 2.38)
TT+AT	185 (61.3%)	80 (52.3%)	0.81 (0.40, 1.66)
rs4646994 (<i>ACE</i>)			
TT	76 (25.2%)	48 (31.4%)	1.00
AT	150 (49.7%)	71 (46.4%)	1.46 (0.61, 3.51)
AA	76 (25.2%)	34 (22.2%)	1.29 (0.48, 3.47)
AA+AT	226 (74.8%)	105 (68.6%)	1.39 (0.62, 3.16)
rs4343 (<i>ACE</i>)			
AA	73 (24.2%)	46 (30.1%)	1.00
AG	157 (52.0%)	72 (47.1%)	1.37 (0.56, 3.33)
GG	72 (23.8%)	35 (22.9%)	1.63 (0.59, 4.45)
GG+AG	229 (75.8%)	107 (69.9%)	1.45 (0.63, 3.36)
rs3912640 (<i>OLR1</i>)			
GG	239 (79.1%)	116 (75.8%)	1.00
AG	58 (19.2%)	35 (22.9%)	1.28 (0.53, 3.09)
AA	5 (1.7%)	2 (1.3%)	N/A
AA+AG	63 (20.9%)	37 (24.2%)	1.17 (0.49, 2.77)
rs2742113 (<i>OLR1</i>)			
CC	145 (48.0%)	75 (49.0%)	1.00
AC	125 (41.4%)	63 (41.2%)	1.20 (0.56, 2.57)
AA	32 (10.6%)	15 (9.8%)	0.96 (0.29, 3.16)
AA+AC	157 (52.0%)	78 (51.0%)	1.14 (0.55, 2.35)
rs1050283 (<i>OLR1</i>)			
CC	86 (28.5%)	40 (26.1%)	1.00
CT	144 (47.7%)	75 (49.0%)	1.24 (0.54, 2.83)
TT	72 (23.8%)	38 (24.8%)	0.72 (0.25, 2.02)
TT+CT	216 (71.5%)	113 (73.9%)	1.05 (0.48, 2.29)
rs754203 (<i>CYP46</i>)			
AA	130 (43.0%)	68 (44.4%)	1.00
AG	137 (45.4%)	67 (43.8%)	1.33 (0.63, 2.81)
GG	35 (11.6%)	18 (11.8%)	0.94 (0.26, 3.37)
GG+AG	172 (57.0%)	85 (55.6%)	1.25 (0.61, 2.57)
<i>APOE4</i> allele			
Absent	247 (81.8%)	61 (39.9%)	1.00
Present	55 (18.2%)	92 (60.1%)	3.55 (1.70, 7.45)

(rs3912640, rs2742113, rs1050283), and *CYP46* (rs754203). All SNPs tested were within Hardy-Weinberg equilibrium (data not shown).

As expected, the *APOE4* allele had the strongest association with increased risk to LOAD (OR=3.55, 95%CL=1.70, 7.45, [table 3]). Although not statistically significant in this pilot study, all of the SNPs genotyped in the *ACE*, *OLR1*, and *CYP46* genes exhibited different allele frequencies between cases and controls.

Stratification by *APOE4* Status

When stratifying by the presence or absence of the *APOE4* allele, obesity at age 50 was found to be positively associated with LOAD only in subjects who carry one or two *APOE4* alleles ($P=0.13$, table 4). The direction of a potential association between statin use and LOAD also varied by *APOE4* allele status, supporting gene/environment interactions.

For all but one of the *OLR1* SNPs, the association between allele frequency and LOAD case/control status was markedly,

Table 4. Baseline characteristics stratified by presence and absence of *APOE4* allele.

Baseline Characteristics	Presence of one or two copies of <i>APOE4</i> allele			No copies of <i>APOE4</i> allele		
	Controls (N=55)	Cases (N=92)	P value	Controls (N=247)	Cases (N=61)	P value
Age	86.3±2.8	77.6±6.4	<0.001	87.4±3.0	79.1±6.4	<0.001
Female gender	30 (54.6%)	48 (52.2%)	0.78	168 (68.0%)	44 (72.1%)	0.53
Ever Smoked	23 (41.8%)	45 (50.0%)	0.34	93 (37.7%)	29 (48.3%)	0.13
Obese (BMI≥30) at age 50	15 (45.5%)	33 (62.3%)	0.13	85 (54.5%)	16 (45.7%)	0.35
Statin use	30 (54.6%)	54 (58.7%)	0.62	116 (47.0%)	25 (40.1%)	0.40
Total cholesterol	261.9±44.1	261.1±46.4	0.92*	258.4±43.2	256.7±48.4	0.72*
LDL cholesterol	177.1±38.2	169.3±43.0	0.55*	165.5±41.5	169.2±45.9	0.66*
HDL cholesterol	60.5±14.5	57.7±17.3	0.28*	63.5±17.4	62.5±15.5	0.92*
Triglycerides	206.5±96.9	260.7±236.4	0.84*	202.8±115.9	190.1±97.0	0.57*
C-reactive protein	2.5±3.9	2.7±3.9	1.00*	2.6±4.7	1.7±2.0	0.73*
Diastolic blood pressure	83.1±10.1	78.2±10.5	0.02*	84.5±10.1	81.1±7.7	0.10*
Systolic blood pressure	161.5±20.0	151.5±25.0	0.04*	166.0±21.2	155.3±18.9	0.01*
Pulse pressure	78.4±16.8	73.3±20.0	0.17	81.5±19.1	74.3±18.3	0.06

* Mann-Whitney U-test. BMI, body mass index. LDL, low-density lipoprotein. HDL, high-density lipoprotein.

although not significantly, different between cases and controls, suggesting gene/gene interactions with *APOE* (table 5). The distribution of the *ACE* alleles also varied by *APOE4* status with a different direction of effect by *APOE4* allele status; however, these findings are not statistically significant because the confidence limits overlap. This is also true for *CYP46*. For four of the eight markers studied, it appears that the risk allele varies by *APOE4* allele status, suggesting gene/gene interactions.

Discussion

As with other complex diseases, LOAD is not only a genetic disease, but also involves environmental components. In this study a number of genetic and environmental factors were examined to determine their relative contribution to LOAD in our PMRP cohort and to obtain pilot data to estimate needed sample size and staffing for a larger more definitive study. Several important strengths of this cohort are: (1) the homogeneity of the sample population (all subjects were European Caucasians from central Wisconsin with greater than 60% reporting their ethnicity to be German, (2) the LOAD diagnosis for patients was made by a single neurologist (SFM) using NINCDS-ADRDA criteria¹, (3) the controls from among the cohort were carefully selected after performing an MMSE to exclude any controls with possible signs of dementia who were older, and (4) a complete medical record including all laboratory testing that was electronically available for each patient in the cohort. The majority of the subjects receive their health care exclusively at Marshfield Clinic. A limitation of the pilot cardiovascular risk factors data is that only one measure was extracted. It is possible that lipids, or other risk factors, are associated with AD at different life points or that medications to lower risk of cardiovascular endpoints may impact LOAD risk.

The *ACE* gene has been a subject of controversy with respect to its association with AD. Our data confirm that the *ACE* gene is associated with increased risk to LOAD consistent with the three SNP haplotype TAA reported by Kehoe et al,⁶ not including the Alu I/D polymorphism. More interestingly, the association of the *ACE* SNPs with LOAD varied by *APOE4* allele status. These pilot data provide preliminary evidence that the *ACE* gene is an independent risk factor to AD and that gene/gene interaction is potentially occurring. More recently, Meng et al²⁵ reported a very strong association between the *ACE* gene and LOAD in an Israeli-Arab community where *APOE4* is in very low prevalence in both non-demented (2.4%) and demented subjects (3.6%). It is likely, therefore, that in the absence of *APOE4*, the *ACE* gene is a strong risk factor for LOAD. These data further support our finding that the *ACE* gene is an independent risk factor to AD. Recent cross sectional meta-analysis using thousands of samples established that the *ACE* I/D is associated with LOAD without stratification by *APOE4*.⁷

We also observed an association between the *OLRI* and LOAD which varied by *APOE4*. Again, this finding indicates that *OLRI* is an independent risk factor to LOAD and supports the initial finding by Luedeking-Zimmer et al.⁸ Interestingly, Bertram et al²⁶ showed a similar trend, with the *APOE4* negative group exhibiting a much smaller *P* value than other groups (with one or two copies of *APOE4*). However, since their study only achieved a trend toward significance for association of *OLRI* with LOAD in the *APOE4* negative group (*P*=0.074), this finding went unrecognized.

Preliminary evidence indicated that the *CYP46* intron 2 (rs754203) gene polymorphism is associated with increased

Table 5. Logistic regression analysis adjusting for age, gender, smoking and pulse pressure stratified by presence and absence of *APOE4* allele.

Genotype	Presence of one or two copies of <i>APOE4</i> allele (95% CI)	No copies of <i>APOE4</i> allele (95% CI)
rs1800764 (<i>ACE</i>)		
TT	1.00	1.00
CT	0.50 (0.13, 1.85)	1.28 (0.43, 3.85)
CC	1.75 (0.38, 8.07)	0.80 (0.17, 3.78)
CC+CT	0.77 (0.24, 2.47)	1.15 (0.40, 3.28)
rs4291 (<i>ACE</i>)		
AA	1.00	1.00
AT	0.59 (0.18, 1.99)	1.01 (0.37, 2.94)
TT	1.32 (0.24, 7.30)	0.53 (0.09, 3.05)
TT+AT	0.72 (0.23, 2.23)	0.91 (0.33, 2.46)
rs4646994 (<i>ACE</i>)		
TT	1.00	1.00
AT	0.93 (0.24, 3.61)	1.98 (0.60, 6.54)
AA	1.87 (0.39, 8.87)	0.84 (0.17, 4.03)
AA+AT	1.18 (0.33, 4.18)	1.56 (0.49, 4.91)
rs4343 (<i>ACE</i>)		
AA	1.00	1.00
AG	1.01 (0.24, 4.21)	1.60 (0.50, 5.18)
GG	3.29 (0.63, 17.20)	0.81 (0.17, 3.84)
GG+AG	1.47 (0.38, 5.66)	1.34 (0.43, 4.15)
rs3912640 (<i>OLR1</i>)		
GG	1.00	1.00
AG	1.11 (0.30, 4.15)	1.14 (0.32, 4.12)
AA	N/A	N/A
AA+AG	0.87 (0.25, 3.11)	1.16 (0.34, 3.99)
rs2742113 (<i>OLR1</i>)		
CC	1.00	1.00
AC	1.32 (0.40, 4.40)	0.76 (0.25, 2.28)
AA	0.62 (0.07, 5.25)	1.20 (0.29, 5.02)
AA+AC	1.18 (0.37, 3.78)	0.86 (0.32, 2.33)
rs1050283 (<i>OLR1</i>)		
CC	1.00	1.00
CT	2.78 (0.72, 10.7)	0.59 (0.18, 1.92)
TT	0.26 (0.03, 2.03)	1.13 (0.33, 3.84)
TT+CT	1.64 (0.48, 5.60)	0.77 (0.27, 2.19)
rs754203 (<i>CYP46</i>)		
AA	1.00	1.00
AG	1.88 (0.56, 6.38)	1.30 (0.45, 3.77)
GG	0.50 (0.06, 4.11)	1.51 (0.30, 7.72)
GG+AG	1.43 (0.46, 4.46)	1.34 (0.48, 3.71)

brain β -amyloid load and higher risk of AD.^{9,27-29} In our cohort, we found that a potential association between *CYP46* (rs754203) and LOAD varies by *APOE4* allele status. Recently a number of other studies in different populations excluded the association of *CYP46* (rs754203) with LOAD.³⁰⁻³⁵ It remains possible that such an association exists, and that *CYP46* acts synergistically with *APOE4* to increase the risk to LOAD as postulated by some investigators.^{9,28} However, a much larger sample size would be needed to confirm such an interaction.

We analyzed several environmental and personal risk factors before and after stratification by *APOE4* allele. After adjustment for the age difference between the cases and

controls in multivariate models, blood pressure differences persisted between cases and controls. Smoking showed a positive association with LOAD in subjects without an *APOE4* allele. Our findings pertaining to smoking are in line with previous findings,^{17,36} where the incidence of AD was shown to be more than double for smokers compared to non-smokers. Differences in the positive association of smoking and LOAD to studies showing a protective effect of smoking could be due to the stratification of results by *APOE4* observed in the current study.

We found obesity at the age of 50 years to be associated with LOAD in subjects who carry an *APOE4* allele. A review of the association between dementia and body mass index (BMI)

concluded that there is sufficient evidence to support an independent relationship of increased BMI as a risk factor for dementia.³⁷ Further research to determine if smoking cessation or weight loss results in a decreased risk of LOAD would strengthen these findings.

Identification of genetic risk factors has the potential to lead to both preventative and symptomatic treatment approaches targeting specific biochemical mechanisms supporting pathogenic developments. Currently, the mechanism in which *APOE*, *ACE*, and *OLRI* lead to increased risk for LOAD is unknown, but their definition may lead to development of pertinent models in which the mechanisms for increased risk may be explored. Understanding of mechanisms will lead to therapies targeted at the specific loci in the cascade of pathogenic events leading to LOAD.

In summary, the results from our pilot study suggest that both the *ACE* and *OLRI* genes are risk factors for development of LOAD independent of the *APOE4* risk allele, and that gene/gene and gene/environment interactions exist with *APOE4* allele status that need to be accounted for in study design and analysis in future studies of LOAD. The data from this pilot study can be used to estimate sample size and staffing requirements for larger, more definitive studies.

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