Original Research *Highlight article*

Evaluation of *APOE* polymorphisms and the risk for age-related macular degeneration in a Southeastern Brazilian population

Marina GM Viturino[1], Jamil M Neto[1], Flávia F Bajano[2], Sueli MS Costa[2], Alicia B Roque[1], Gessica FS Borges[1], Galina Ananina[2], Priscila HH Rim[1], Flávio M Medina[3], Fernando F Costa[4], José PC de Vasconcellos[1] and Mônica B de Melo[2]

[1]Department of Ophthalmology, Faculty of Medical Sciences, University of Campinas, Campinas, SP 13083-887, Brazil; [2]Laboratory of Human Genetics, Center for Molecular Biology and Genetic Engineering (CBMEG), University of Campinas, Campinas, SP 13083-875, Brazil; [3]Department of Ophthalmology, Faculty of Medical Sciences, University of State of Rio de Janeiro, Rio de Janeiro, RJ 20551-030, Brazil; [4]Hematology and Hemotherapy Center, University of Campinas, Campinas, SP 13083-878, Brazil
Corresponding author: Mônica B de Melo. Email: melomb@uol.com.br

Impact statement

This is the first Brazilian study to suggest genetic relationship between variants in the *APOE* gene and the risk for age-related macular degeneration etiology and severity. The Brazilian population has unique characteristics, with ethnic peculiarities and miscegenation, encouraging replication of these findings in other regions of the country. Such studies are also relevant because they evaluate not only the risk for general AMD, as most of published studies have done, but also the risk for different disease subphenotypes, allowing an association with AMD severity and possibly with disease progression.

Abstract

This study aimed to evaluate the role of *APOE* polymorphisms (rs429358 and rs7412) in the risk of age-related macular degeneration in a sample of the Southeastern Brazilian population. Seven hundred and five unrelated individuals were analyzed, 334 with age-related macular degeneration (case group), and 371 without the disease (control group). In the case group, patients were further stratified according to disease phenotypes, divided into dry and wet age-related macular degeneration, and non-advanced and advanced age-related macular degeneration. *APOE* polymorphisms (rs429358 and rs7412) were evaluated through polymerase chain reaction and direct sequencing. In the comparison of cases vs. controls, none of the associations reached statistical significance, considering the Bonferroni-adjusted *P*-value, although there was a suggestive protection for the E3/E4 genotype (OR = 0.626; *P*-value = 0.037) and E4 carriers (OR = 0.6515; *P*-value = 0.047). Statistically significant protection for both the E3/E4 genotype and E4 carriers was observed in the comparisons: advanced age-related macular degeneration vs. controls (OR = 0.3665, *P*-value = 0.491×10^{-3} and OR = 0.4031, *P*-value = 0.814×10^{-3}, respectively), advanced age-related macular degeneration vs. non-advanced age-related macular degeneration (OR = 0.2529, *P*-value = 0.659×10^{-4} and OR = 0.2692, *P*-value = 0.631×10^{-4}, respectively). In the comparison of wet age-related macular degeneration vs. control, protection was statistically significant only for E3/E4 (OR = 0.4052, *P*-value = 0.001). None of the comparisons demonstrated any significant association for E2 genotypes or E2 carriers in age-related macular degeneration risk in this study. Findings suggest a protective role of the E4 haplotype in the *APOE* gene in the risk for advanced and wet forms of age-related macular degeneration, in a sample of the Brazilian population. To our knowledge, this is the first Brazilian study to show the association between *APOE* polymorphisms and age-related macular degeneration.

Keywords: Apolipoprotein E, polymorphism, age-related macular degeneration, pathogenesis, lipid transport pathway, genetic risk

***Experimental Biology and Medicine* 2021; 246: 1148–1155. DOI: 10.1177/1535370220985466**

Introduction

Age-related macular degeneration (AMD) is a neurodegenerative retinal disorder that is one of the leading causes of irreversible visual loss worldwide.[1] AMD is considered a complex condition, with aging, genetic, and environmental factors participating in its development and progression.[2]

Some independent lines of evidence indicate that cholesterol homeostasis in the retinal pigmented epithelium (RPE) and Bruch's membrane is unregulated in AMD. These include accumulation of cholesterol in Bruch's membrane that increases with aging, lipid-rich lesions found in subretinal and sub-RPE deposits, being a hallmark of AMD

findings and the association of variants in cholesterol-related genes with AMD risk in different studies.[3]

Apolipoprotein E (APOE) is a lipid transport protein with an important role in the maintenance and repair of neural cell membranes.[4] APOE has the ability to bind to membrane receptors, mediating lipid transfer between circulating lipoproteins and tissues. Additionally, APOE non-specifically binds to lipophilic inflammatory components, contributing to their clearance and participating in the innate immune response.[5] In the retina, APOE is mainly related to RPE cells and mononuclear phagocytes. In the RPE, lipoproteins transported by APOE are absorbed from the circulation via the low-density lipoprotein receptor (LDL-R), and used for retinal growth and metabolism. In addition, RPE cells participate in reverse lipid transport, by secreting APOE, which associates with high-density lipoproteins (HDL). In mononuclear phagocytes, APOE plays an important role in the survival and activation of immune cells.[4]

Different findings have strongly related APOE to AMD: the composition of drusen, rich in lipids, and the expression of APOE in its deposits; the role of APOE in the maintenance and repair of neuronal membranes in the nervous system and its important production in the retina; the participation of the *APOE* gene in the risk for other neurodegenerative diseases, such as Alzheimer's disease.[6]

The human *APOE* gene is located on the 19q13.2 locus and consists of four exons and three introns. This gene is polymorphic in two single nucleotides (rs429358 and rs7412). The combination of these SNPs results in three different haplotypes (E2, E3, and E4; mostly referred to in literature using the term *allele*), leading to three isoforms of the APOE protein (e2, e3, and e4) and six (*APOE* diplotypes) diplotypes (E2/E2, E2/E3, E2/E4, E3/E3, E3/E4, and E4/E4; mostly referred in literature with the term *genotype*).[7] In this article, the terms *allele* and *genotype* will be used, since this is the most widely found terminology in current articles. These three APOE isoforms have different sequences of amino acids at positions 112 and 158. The e2 isoform has cysteine residues at positions 112 and 158 (cys112, cys158); the e3 isoform has a cysteine residue at position 112 and an arginine residue at position 158 (cys112, arg 158); and the e4 isoform is composed of arginine residues at positions 112 and 158 (arg112; arg 158). These isoforms have structural differences, which influence their modulation of lipid homeostasis, including their capacity for lipid association and receptor affinity.[7] Different properties among APOE isoforms are associated with the risk of several conditions related to aging, including Alzheimer's and cardiovascular diseases.

The relationship between *APOE* polymorphisms and AMD has been evaluated in other studies. Most of them agree that the E4 allele may be protective for this disease.[6,8–13] The association of E2 allele with AMD is controversial, with some studies showing that patients with E2 are at increased risk, while others did not support this relationship.[4] Thus, the association between *APOE* polymorphisms and AMD, and their roles in the different subtypes of the disease, need to be properly explored in different populations for a better understanding of AMD pathophysiology.

Materials and methods

A total of 705 patients were analyzed for *APOE* polymorphisms, including 334 with AMD (case group) and 371 without the disease (control group). All subjects were selected in the Ophthalmology Department, at the Clinical Hospital, University of Campinas (UNICAMP), Campinas, São Paulo, located in the Southeast of Brazil. This study adhered to the tenets of the Declaration of Helsinki, and was approved by the institution's Ethics Committee. Written informed consent was obtained from all participants.

Patients included were classified according to the *Clinical Age-related Maculopathy System* (CARMS), with the control group corresponding to CARMS 1 and the case group, to CARMS from 2 to 5.[14] Description of inclusion and exclusion criteria, stratification of the disease and ophthalmologic examination is presented below.

Case group formation

Patients included in the case group were aged over 50 years old and had an ophthalmologic examination compatible with CARMS 2 to 5. This group was stratified into dry (CARMS 2 to 4) and wet (CARMS 5) forms of AMD; and into non-advanced (CARMS 2 and 3) and advanced (CARMS 4 and 5) disease, considering the status of the worse eye.

Control group formation

The control group included individuals above the age of 50 years who did not show any evidence of AMD, such as numerous drusen or changes in macular RPE on ophthalmological examination (CARMS 1). These individuals consisted of non-family companions of patients examined in the retina clinic, as well as patients examined in the Ophthalmology Department with initial and/or intermediate cataracts.

Exclusion criteria

The exclusion criteria used for both case and control groups were: presence of any opacity of media that would prevent adequate fundus biomicroscopy and/or good quality retinography; patients with other diseases that could be related to the development of macular neovascularization, such as: polypoid choroidal vasculopathy, angioid streaks, high myopia (refractive error of at least −6.00 diopters), infectious, inflammatory or hereditary chorioretinal disease or trauma; presence of macular hemorrhage from any other cause; patients who did not agree to participate in the research study by signing the informed consent form.

Ophthalmologic examination

All studied patients underwent complete ophthalmological evaluation, comprising: best-corrected visual acuity; refraction; anterior segment biomicroscopy; tonometry, using

Goldmann tonometer; and fundus biomicroscopy, using a double aspheric lens of +78 diopters. Fundus color photography was performed in all subjects for the recording of fundoscopic aspect, with emphasis on the macular region. Additionally, optical coherence tomography and fluorescein angiography were obtained for AMD patients.

DNA extraction and APOE genotyping

Peripheral blood samples were collected from all subjects included in the study into sterile tubes with 10% ethylenediaminetetraacetic acid (EDTA). DNA was extracted from leukocytes using the phenol/chloroform method. Two single-nucleotide polymorphisms (SNPs) of the *APOE* gene (rs429358 and rs7412) were evaluated through polymerase chain reaction (PCR) and Sanger direct sequencing.

The primers used for PCR were sense 5′-TCCAAGGAGCTGCAGGCGGCGCA-3′ and anti-sense 5′-GCCCCGGCCTGGTACACTGCCA-3′ (IDT, Coralville, IA, USA). The reaction was standardized using: 0.25 μL of the primers (20 pmol/μL); 2.5 μL of 10× enzyme buffer (10× Buffer: 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.01% gelatin); 1.5 μL of 50 μM MgCl2; 0.5 μL of 10% DMSO; 0.5 μL of 10 mM nucleotide mixture (dATP, dCTP, dTTP, dGTP); and 0.1 μL of 10 U/μL Taq DNA polymerase (Invitrogen Life Technologies, Gaithersburg, MD, USA), added to 1.0 μL of genomic DNA (50 ng/μL). The final volume was completed with ultrapure water to 25.0 μL. The samples were amplified using Veriti 96-Well Thermal Cycler (Applied Biosystems-Applera Corporation, Foster City, CA, EUA), with an initial denaturation at 95°C for 5 min, followed by 35 cycles of DNA denaturation at 95°C for 1 min, annealing at 58°C for 1 min and 30 s, extension at 72°C for 1 min and 30 s, final extension at 72°C for 7 min. PCR products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide, and examined under ultraviolet light.

Direct sequencing was performed with the following reagents: 1.0 μL of purified PCR product (20 ηg/μL); 0.5 μL of "Big Dye" 1× (ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit) (Applied Biosystem Applera Corporation, Foster City, CA, USA); 4.0 μL of 1× "Save Money" buffer (provided by the same manufacturer of the "Big Dye"), direct or reverse primer of the analyzed variant (1.0 μL of the 5 pmol/μL primer) and ultrapure H_2O to complete the reaction to a final volume of 20.0 μL. Conditions for the sequencing reaction were: initial denaturation of 96°C for 1 min, followed by 30 cycles at 96°C for 10 s, 57°C for 5 s, and 60°C for 4 min. Electrophoresis was performed in the ABI Prism 3530 DNA Analyzer (Applied Biosystems, Foster City, CA). Sequences were analyzed with the FinchTV program (Geospiza, Seattle, WA) and compared with the reference sequences using the BLAST algorithm (www.ncbi.nlm.nih.gov/Blast) and the MultAlin software (http://multalin.toulouse.inra.fr/multalin/).

Statistical analysis

Statistical analysis was performed using the R software (Foundation for Statistical Computing, Vienna, Austria). We used the Mann–Whitney nonparametric test and Fisher exact test to compare age and gender distribution, respectively, among affected and non-affected cohorts. Chi-square statistics were employed to test Hardy–Weinberg equilibrium in the studied groups. Associations and odds ratios (OR) estimates were modeled through logistic regression models, adjusted by age.

In the logistic regression models, we tested different types of categorical dependent variables: (1) AMD presence or absence (cases vs. controls comparison); (2) comparisons after AMD stratification in advanced and non-advanced disease, which included: non-advanced AMD form vs. controls, advanced AMD form vs. controls, and advanced AMD form vs. non-advanced AMD form; (3) comparisons after AMD stratification in dry and wet disease, including: dry AMD form vs. controls, wet AMD form vs. controls, and wet AMD form vs. dry AMD form. For each dependent variable, we tested three logistic regression models: (i) considering all APOE genotypes as independent co-variables in comparison to E3/E3, (ii) combining E2/E2 and E2/E3 into one independent variable (E2 carriers) in comparison to E3/E3 genotype, and (iii) combining E3/E4 and E4/E4 (E4 carriers) into one independent variable in comparison to E3/E3. The E2/E4 genotype was excluded from these last two analyses (E2 carriers and E4 carriers), due to the probable paradox effect among variants. In all our logistic regression models, age was included as a co-variable and the E3/E3 genotype was used as the reference genotype.

To reduce the possibility of type I error due to multiple testing, an adjusted *P*-value for Bonferroni correction was used in each comparison as a threshold to confirm statistical significance ($P = 0.05/21 = 0.002$; since we tested seven dependent variables using three logistic regression models).

Results

Overall, 705 patients participated in this study, including 334 in the case group and 371 in the control group. The percentage of females was 57.06% in the case group, and 52.29% in the control group. There was no difference in gender distribution between the two groups ($P = 0.2253$). The mean age of patients with AMD was 73.26 ± 9.24 years (median of 74.00 years; ranging from 50 to 103 years), and 65.3 ± 9.89 years (median of 66.50 years; ranging from 50 to 89 years) in the control group. The mean age presented a statistically significant difference between cases and controls ($P = 2.2 \times 10^{-6}$). Gender and age distributions among cases, controls and subgroups of AMD are described in Table 1.

To evaluate the role of *APOE* polymorphisms in different AMD forms, patients in the case group were stratified into non-advanced and advanced disease, and into dry and wet AMD. Ninety-nine (29.64%) patients presented the non-advanced form of AMD, and 235 (70.36%) had advanced disease; 134 (40.12%) had dry AMD, and 200 (59.88%) had wet AMD.

The distribution of *APOE* genotypes and alleles in all groups and subgroups analyzed is presented in Table 2. Frequencies of *APOE* genotypes were in Hardy–Weinberg

Table 1. Demographic characteristics of AMD patients and controls.

Study group (*n*)	Age (mean ± SD) (years)	Age range (years)	Female *n* (%)	Male *n* (%)
Control group (371)	65.3 ± 9.89	50–89	194 (52.30%)	177 (47.70%)
AMD case group (334)	73.26 ± 9.24*	50–103	190 (56.89%)$^{+}$	144 (43.11%)$^{+}$
Non-advanced AMD (99)	71.14 ± 9.20	50–103	35 (35.35%)	64 (64.65%)
Advanced AMD (235)	74.16 ± 9.13	51–93	126 (53.62%)	109 (46.38%)
Dry AMD (134)	72.75 ± 9.55	50–103	74 (55.22%)	60 (44.78%)
Wet AMD (200)	73.61 ± 9.03	51–93	116 (58.00%)	84 (42.00%)

n: number of subjects.
*Indicates a *P*-value $= 2.2 \times 10^{-6}$ when cases were compared to controls (Mann–Whitney test).
$^{+}$Indicates a *P*-value = 0.2253 when gender distribution was compared between cases and controls (Fischer's exact test).

Table 2. *APOE* genotypes distribution among all analyzed groups and subgroups.

Genotype	Control group N (%)	Case group N (%)	Non-advanced AMD cases N (%)	Advanced AMD cases N (%)	Dry AMD cases N (%)	Wet AMD cases N (%)
E2/E2	1 (0.27)	3 (0.09)	1 (1.01)	2 (0.85)	1 (0.75)	2 (1.00)
E2/E3	48 (12.94)	43 (12.61)	18 (18.18)	25 (10.64)	24 (17.91)	19 (9.50)
E2/E4	6 (1.62)	4 (1.20)	0	4 (1.70)	2 (1.49)	2 (1.00)
E3/E3	231 (62.26)	235 (70.57)	54 (54.55)	181 (77.02)	80 (59.70)	155 (77.50)
E3/E4	78 (21.02)	44 (13.21)	24 (24.24)	20 (8.51)	25 (18.66)	19 (9.50)
E4/E4	7 (1.89)	5 (1.50)	2 (2.02)	3 (1.28)	2 (1.49)	3 (1.50)
E2 carriers[a]	49 (13.20)	46 (13.77)	19 (19.19)	27 (11.49)	25 (18.66)	21 (10.50)
E4 carriers[a]	85 (22.91)	49 (14.67)	26 (26.26)	23 (9.79)	27 (20.15)	22 (11.00)

[a]E2 carriers correspond to the groupment of patients with E2/E2 and E2/E3 genotypes; E4 carriers correspond to E3/E4 and E4/E4 genotypes. Subjects with E2/E4 genotype were excluded from both E2 carriers and E4 carriers groups.

equilibrium in both case and control groups (*P*-values were, respectively, 0.338 and 0.753).

In the comparison of cases versus controls, a suggestive protective role for AMD was conferred by the E3/E4 genotype (OR = 0.626; *P*-value = 0.037) and E4 carriers (OR = 0.6515; *P*-value = 0.047), when compared to E3/E3. However, these levels of significance were not maintained after Bonferroni correction ($P < 0.002$). All other genotypes and E2 carriers had no statistically significant association with AMD in this comparison (Table 3).

Results obtained after the stratification of the case group in non-advanced and advanced AMD are summarized in Table 4. When considering the comparison of non-advanced cases vs. controls, none of the evaluations demonstrated statistically significant difference between groups. In the evaluation of advanced cases vs. controls, statistical analysis revealed that the E3/E4 genotype (OR = 0.3665; *P*-value $= 0.491 \times 10^{-3}$) and E4 carriers (OR = 0.4031; *P*-value $= 0.814 \times 10^{-3}$) were significantly less frequent in the advanced cases, compared to the control group. In the comparison between advanced and non-advanced cases of AMD, statistically significant protection was observed for the E3/E4 genotype (OR = 0.2529; *P*-value $= 0.659 \times 10^{-4}$) and E4 carriers (OR = 0.2692; *P*-value $= 0.631 \times 10^{-4}$) versus E3/E3, in relation to the advanced form of the disease.

Regarding the comparisons after case group stratification in dry and wet AMD, data are described in Table 5. The evaluation of dry cases vs. controls did not reach statistical significance for any of the comparisons. When comparing wet cases vs. controls, statistical analysis demonstrated that the E3/E4 genotype was significantly more frequent in the control group than in wet AMD patients (OR = 0.4052; *P*-value = 0.001), conferring protection for this form of disease. Additionally, a suggestive protective effect for E4 carriers in relation to wet AMD was observed, but this result did not reach statistical significance after Bonferroni correction (OR = 0.4469; *P*-value = 0.003). In the evaluation of wet cases vs. dry cases, a suggestive protection for both E3/E4 genotype (OR = 0.3971; *P*-value = 0.005) and E4 carriers (OR = 0.4236; *P*-value = 0.007), when compared to E3/E3, was observed, although these findings did not survive Bonferroni adjustment for statistical significance.

Table 3. *APOE* genotype analysis in the comparison of case vs. control group, using age-adjusted logistic regression.

Genotype	OR	95% CI	*P*-value*
E2/E2	5.0761	0.5936–107.0217	0.173
E2/E3	0.8578	0.5225–1.4057	0.542
E2/E4	0.7173	0.1535–3.0344	0.656
E3/E3	Reference	–	–
E3/E4	0.6260	0.4004–0.9702	0.037
E4/E4	0.9656	0.2658–3.2649	0.955
E2 carriers	0.9286	0.5735–1.5020	0.763
E4 carriers	0.6515	0.4244–0.9931	0.047

*Bonferroni-corrected significance threshold ($P = 0.05/21 = 0.002$).
OR: odds ratio; CI: confidence interval.

Discussion

APOE variants were among the first reported genetic associations in AMD.[4] Reproduction of this relationship in independent cohorts[6,8,11,15–18] has provided consistency to the

Table 4. *APOE* genotype analysis in the comparisons after stratification of the case group in non-advanced and advanced AMD, using age-adjusted logistic regression.

Comparison	Non-advanced AMD vs. control		Advanced AMD vs. control		Advanced AMD vs. Non-advanced AMD	
	OR (95% CI)	*P*-value*	OR (95% CI)	*P*-value*	OR (95% CI)	*P*-value*
E2/E2	6.2593 (0.2377–165.1980)	0.205	3.6967 (0.3193–85.2566)	0.308	0.8497 (0.0776–18.8628)	0.896
E2/E3	1.6382 (0.8484–3.0776)	0.131	0.5944 (0.3290–1.0536)	0.078	0.3923 (0.1972–0.7886)	0.007
E2/E4	NA	0.981	0.9643 (0.1985–4.2496)	0.962	NA	0.984
E3/E3	Reference	–	Reference	–	Reference	–
E3/E4	1.4174 (0.7973–2.4742)	0.226	**0.3665 (0.2040–0.6339)**	**0.491×10^{-3}**	**0.2529 (0.1276–0.4954)**	**0.659×10^{-4}**
E4/E4	1.4484 (0.2043–6.5532)	0.660	0.9571 (0.1957–3.7119)	0.951	0.4636 (0.0740–3.6235)	0.409
E2 carriers	1.7087 (0.8991–3.1690)	0.094	0.6536 (0.3695–1.1370)	0.137	0.4114 (0.2103–0.8131)	0.009
E4 carriers	1.4310 (0.8169–2.4711)	0.203	**0.4031 (0.2327–0.6770)**	**0.814×10^{-3}**	**0.2692 (0.1406–0.5112)**	**0.631×10^{-4}**

Note: Results in bold represent statistically significant data after Bonferroni correction.
*Bonferroni-corrected significance threshold ($P = 0.05/21 = 0.002$).
OR: odds ratio; CI: confidence interval; NA: not available.

Table 5. *APOE* genotype analysis in the comparisons after stratification of the case group in dry and wet AMD, using age-adjusted logistic regression.

Comparison	Dry AMD vs. control		Wet AMD vs. control		Wet AMD vs. dry AMD	
	OR (95% CI)	*P*-value*	OR (95% CI)	*P*-value*	OR (95% CI)	*P*-value*
E2/E2	4.9486 (0.1857–132.2145)	0.272	4.1460 (0.3611–95.0873)	0.266	1.1545 (0.1075–25.2924)	0.907
E2/E3	1.4451 (0.7921–2.5949)	0.222	0.5493 (0.2899–1.0082)	0.058	0.4033 (0.2061–0.7789)	0.007
E2/E4	1.2872 (0.1636–6.7057)	0.781	0.5434 (0.0688–2.9619)	0.508	0.4821 (0.0565–4.1066)	0.471
E3/E3	Reference	–	Reference	–	Reference	–
E3/E4	1.0477 (0.5990–1.7954)	0.867	**0.4052 (0.2235–0.7061)**	**0.001**	0.3971 (0.2039–0.7622)	0.005
E4/E4	1.0640 (0.1475–4.9124)	0.942	1.0692 (0.2191–4.1353)	0.926	0.7867 (0.1277–6.0702)	0.795
E2 carriers	1.4988 (0.8302–1.4988)	0.173	0.6174 (0.3353–1.1059)	0.112	0.4322 (0.2260–0.8186)	0.010
E4 carriers	1.0530 (0.6116–1.7824)	0.849	0.4469 (0.2559–0.7555)	0.003	0.4236 (0.2249–0.7898)	0.007

Note: Results in bold represent statistically significant data after Bonferroni correction. Results underlined represent initially statistically significant data (P-value <0.05) that did not survive Bonferroni correction.
*Bonferroni-corrected significance threshold ($P = 0.05/7 = 0.002$).
OR: odds ratio; CI: confidence interval; NA: not available.

notion of the involvement of this gene in AMD susceptibility. Several studies[6,8–13] have shown a protective effect for the E4 allele. Although this relationship is widely supported, there is a lack of association in some cohorts from different populations,[19–26] including Caucasian,[19,22–24] Hispanic,[25] and Asian populations.[20,21,26] This protective effect of E4 could not be proven in some studies, even when analyzing non advanced and advanced AMD subgroups[26] or sporadic and familial cases of AMD.[22]

On the other hand, the E2 allele has a controversial association. Some studies have related it to an increased risk of AMD.[6,10–13,15,27] Beyond the role in AMD risk, Baird *et al.*[10] also associated the E2E3 genotype, in patients with late AMD, with a significantly earlier diagnosis of disease, compared to E3E3, both for women, men, and neovascular AMD. Other studies, on the other hand, have not supported this association.[17,23,24] Furthermore, some authors have suggested that E2 effect may be affected by environmental factors. Schmidt *et al.*[6] suggested that the E2 association with AMD may be modified by sex, with a protective effect for women and a risk effect for men; although these findings were not found to be statistically significant. Schmidt *et al.*[28] and Adams *et al.*[29] also related the E2 and AMD association with smoking status. A recent study[25] described, for the first time, a protective effect for the E2 allele in relation to wet AMD in a Spanish population (OR 0.42; 95% CI 0.19–0.95).

Particularities of the Brazilian population, including a great miscegenation and ethnic heterogeneity, increase the interest regarding the genetic profile of AMD and its relationship with data described in the literature in other populations. The only Brazilian study available in the literature[25] did not demonstrate a significant association between *APOE* polymorphisms and AMD risk. These results may be related to the sample size, which included 134 cases and 164 controls.

Our study suggested a protective role for E3/E4 genotype and E4 carriers in case vs. control comparison, but these results did not reach statistical significance when the Bonferroni correction was used. We believe that these findings do not invalidate the protective role of E4 in relation to the disease, considering that adjustments through Bonferroni's method reduce the chance of type I error, but increase the chance of type II error. In addition, the heterogeneity observed in the case group may explain why general results of the comparison between cases and controls did not achieve statistical significance, while comparisons after stratification in the most severe forms of AMD reached statistical significance for E3/E4 and/or E4 carriers.

We demonstrated a significant protection for the E4 allele after the stratification of the case group in the comparisons: advanced AMD vs. control and advanced AMD vs. non- advanced AMD, both for the E3/E4 genotype and E4 carriers, in relation to the E3/E3 genotype. In addition, a protective role for the E3/E4 genotype in relation to E3/E3 was found when comparing wet AMD vs. control, although data for E4 carriers did not reach a significant Bonferroni-corrected *P*-value. These results suggest a protective effect afforded by the E4 allele for the most severe forms of AMD. The comparisons between advanced vs. non-advanced AMD, and between wet vs. dry AMD have not been widely explored in the literature, with most studies using the control group as reference.[4] Findings indicating E4 protection in advanced disease may point to a protective role not only in AMD development, but also in disease progression. Longitudinal studies would be useful to clarify these associations.

When evaluating wet vs. dry AMD, there was a suggestive protection conferred by E3/E4 and E4 carriers; however, as we used a corrected *P*-value, these results were not significant. As mentioned for the comparison of cases vs. controls, the use of Bonferroni correction may have limited such associations. E4 protection did not achieve statistical significance in the comparisons of the least severe forms of disease, in relation to the control group (non-advanced cases vs. control and dry cases vs. control). These results may be related to a smaller number of patients with non-advanced (99 cases) and dry (134 cases) forms in our sample; the percentages of E4 carriers were 26.26% and 20.15%, respectively.

In this study, none of the comparisons, even after case group stratification, demonstrated any significant association of E2 genotypes or E2 carriers with AMD risk. In contrast to our findings regarding the AMD subgroups, McKay *et al.*[13] found an increased risk for advanced AMD associated with the E2E2 genotype, although this relationship did not achieve statistical significance when analyzing advanced AMD sub-phenotypes (which included geographic atrophic (GA), neovascular (NV), and mixed GA and NV AMD) and also in cases of early AMD.

A recent meta-analysis[4] evaluated 12 independent studies, with a total of 12,842 cases and 38,647 controls, using the same genotype group analysis that was used in this study when comparing E2 carriers (E2/E2 and E2/E3) and E4 carriers (E3/E3 and E4/E4), in relation to the E3/E3 genotype. Patients were also subdivided according to ethnicity, allowing a better assessment of *APOE* polymorphisms in different populations. The association of E2 with AMD is still controversial, with divergences among subtypes of the disease and among different ethnicities, with significance observed in this meta-analysis only for Black individuals in the comparison of cases versus controls, and in Blacks and Asians in the comparison between early disease versus controls, with no statistical significance in any population when assessing the wet form of the disease. With regard to E4, its protective effect was confirmed in all populations, even after the stratification of the disease, in relation to controls.

APOE participates in cell membrane renewal in the central nervous system and retina. The high turnover of photoreceptor membranes, especially in the macular area, makes the cell membrane remodeling process of great importance for the maintenance of the physiology of the retina.[17] Failures in this process may result in retinal neurodegeneration and visual loss. In addition to its role in lipid metabolism, APOE seems to be related to the processes of modulation of cellular oxidative stress and aging.[30]

The isoforms of APOE have structural differences, leading to isoform-specific functional changes. Differences in load, total serum levels, brain levels, specific interaction properties with receptors, and lipid internalization and degradation between APOE e2, e3, and e4 have been demonstrated.[31] However, the mechanisms by which APOE isoforms are related to AMD remain unclear.

Some hypotheses have been proposed to explain the protective effect of the e4 isoform in AMD. Souied *et al*.[8] suggested that structural changes in the molecule of this isoform would allow greater permeability to Bruch's membrane, avoiding drusenoid deposits that serve as incubators for the peroxidation and deposit of complement fragments. The mechanisms for this alteration in the permeability of Bruch's membrane would be related to the positive charge of APOE e4, which would facilitate the clearance of cellular debris, and the absence of disulfide bridges, with less formation of dimers and easier transport of lipid particles.

A study by Levy *et al.*,[30] in animal models, associated the E4 allele with a lower inflammatory response in mice, reducing the levels of APOE and CCL2 (CC chemokine ligand 2), and preventing the accumulation of mononuclear phagocytes compared to APOE E3 transgenic mice, with a consequent reduction in retinal neurodegeneration.

Another possible explanation for E4 protection includes its association with lower concentrations of APOE in plasma, eye, and brain tissue, when compared to the E3 allele.[30,32] It was also demonstrated that the APOE e4 isoform may be involved in the transport of the macular pigments, lutein, and zeaxanthin, which may be related to the maintenance of macular function.[33]

On the other hand, the destructive effect of the APOE e2 isoform is still controversial in the literature. In our study, the relationship of E2 genotypes with increased risk for AMD did not prove to be statistically significant. Studies in animal models[30] have shown that transgenic APOE E2 mice express increased levels of APOE, IL-6 (interleukin-6), and CCL2, and develop accumulation of mononuclear phagocytes, photoreceptor degeneration, and exaggerated choroidal neovascularization, similar to AMD findings. This pro-inflammatory action could justify the relationship of this isoform with the increased risk for the disease reported in some studies. Further studies are needed to precisely understand the metabolism of different APOE isoforms in the retina, allowing a better evaluation of the mechanisms by which APOE and lipid levels modulate AMD pathogenesis.

This study has some limitations. One of them was not using a population-based sampling (population study), but, instead, using a sampling based on clinical convenience, since all the established inclusion and exclusion

criteria were met (case-control study). Despite this, both SNPs tested were in accordance with Hardy–Weinberg equilibrium in the case and control groups, and the allele frequencies in the control group were in accordance with data in the literature. The unmatched ages between case and control groups were another limitation that was overcome by the use of age-adjusted logistic regression for data analysis. It is important to reinforce that subjects in both the case and control groups were over 50 years old. In addition, environmental factors related to AMD and its interaction with *APOE* polymorphisms were not evaluated. Finally, the relatively low frequency of some genotypes may have limited the statistical power of some associations, especially in the comparisons after stratification of AMD in disease subgroups.

In conclusion, this study confirmed the protective role of the *APOE* gene E4 allele in the risk for advanced and wet forms of AMD in a sample of the Brazilian population, and also suggested a protection against general AMD risk. These findings are in agreement with data presented in the literature and reinforce the participation of the lipid transport pathway in AMD pathogenesis.[34] To our knowledge, this is the first study to show the association between *APOE* polymorphisms and AMD in the Brazilian population. The understanding of polymorphisms related to the risk of the disease in different populations and their functional consequences in the onset and progression of the disease may allow a better prediction of AMD risk, and provide new diagnostic and therapeutic targets.

AUTHORS' CONTRIBUTIONS

MGMV, JMN, JPCV, GA, and MBM participated in the design, interpretation of the studies and analysis of the data; MGMV, JMN, JPCV, FFC, and MBM reviewed the manuscript; MGMV, JMN, ABR, PHHR, and FMM performed ophthalmological examination in the patients included in the study; MGMV, JMN, ABR, and GFSB collected clinical data and blood samples; MGMV, FFB, and SMSC conducted the experiments; MGMV and MBM wrote the manuscript.

ACKNOWLEDGMENTS

Authors are grateful to Daniela Stancato and Yuri Oiamore for the help with sample storage, laboratory analysis, and data organization.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. .

ETHICAL APPROVAL

This research was approved by the Ethics Research Committee of the University of Campinas. Informed consent was obtained from all participants included. The procedures used were in accordance with the tenets of the Declaration of Helsinki for research involving human beings.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by the Fund for Support to Teaching, Research and Outreach Activities (FAEPEX) grants 1525/15 and 251/18 and by São Paulo Research Foundation (FAPESP) grant 2010/18353–9.

ORCID iD

Mônica B de Melo https://orcid.org/0000-0002-1801-5441

REFERENCES

1. Schwartz SG, Hampton BM, Kovach JL, Brantley MA, Jr. Genetics and age-related macular degeneration: a practical review for the clinician. *Clin Ophthalmol* 2016;**10**:1229–35
2. Pennington KL, DeAngelis MM. Epidemiology of age-related macular degeneration (AMD): associations with cardiovascular disease phenotypes and lipid factors. *Eye Vis* 2016;**3**:34
3. Pikuleva IA, Curcio CA. Cholesterol in the retina: the best is yet to come. *Prog Retin Eye Res* 2014;**41**:64–89
4. Xiying M, Wenbo W, Wangiy F, Qinghuai L. Association of apolipoprotein E polymorphisms with age-related macular degeneration subtypes: an updated systematic review and meta-analysis. *Arch Med Res* 2017;**48**:370–7
5. Huebbe P, Rimbach G. Evolution of human apolipoprotein E (APOE) isoforms: gene structure, protein function and interaction with dietary factors. *Age Res Rev* 2017;**37**:146–61
6. Schmidt S, Klaver CCW, Saunders AM, Postel E, La Paz M, Argawal A, Small K, Udar N, Ong J, Chalukya M, Nesburn A, Kenney C, Domurath R, Hogan M, Mah T, Conley Y, Ferrell R, Weeks D, de Jong Ptvm van Duijn C, Haines J, Pericak-Vance M, Gorin MA. Pooled case-control study of apolipoprotein E (APOE) gene in age-related maculopathy. *Ophthal Genet* 2002;**23**:209–23
7. Zhong L, Xie YZ, Cao TT, Wang Z, Wang T, Li X, Shen RC, Xu H, Bu G, Chen XF. A rapid and cost-effective method for genotyping apolipoprotein E gene polymorphism. *Mol Neurodegener* 2016;**11**:2
8. Souied EH, Benlian P, Amouyel P, Feingold J, Lagarde JP, Munnich A, Kaplan J, Coscas G, Soubrane G. The epsilon4 allele of the apolipoprotein E gene as a potential protective factor for exudative age-related macular degeneration. *Am J Ophthalmol* 1998;**125**:353–9
9. Shen L, Hoffmann TJ, Melles RB, Sakoda LC, Kvale MN, Banda Y, Schaefer C, Risch N, Jorgenson E. Differences in the genetic susceptibility to age-related macular degeneration clinical subtypes. *Invest Ophthalmol Vis Sci* 2015;**56**:4290–9
10. Baird PN, Guida E, Chu DT, Vu HT, Guymer RH. The epsilon2 and epsilon4 alleles of the apolipoprotein gene are associated with age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2004;**45**:1311–5
11. Simonelli F, Margaglione M, Testa F, Cappucci G, Manitto MP, Brancato R, Rinaldi E. Apolipoprotein E polymorphisms in age-related macular degeneration in an Italian population. *Ophthal Res* 2001;**33**:325–8
12. Thakkinstian A, Bowe S, McEvoy M, Smith W, Attia J. Association between apolipoprotein E polymorphisms and age-related macular degeneration: a HuGE review and meta-analysis. *Am J Epidemiol* 2006;**164**:813–22
13. McKay GJ, Patterson CC, Chakravarthy U, Dasari S, Klaver CC, Vingerling JR, Ho L, de Jong PTVM, Fletcher AE, Young IS, Seland JH, Rahu M, Soubrane G, Tomazzoli L, Topouzis F, Vioque J, Hingorani AD, Sofat R, Dean M, Sawitzke J, Seddon JM, Peter I, Webster AR, Moore AT, Yates JRW, Cipriani V, Fristche LG, Weber BHF, Keilhauer CN, Lotery AJ, Ennis S, Klein ML, Francis PJ, Stambolian D, Orlin A, Gorin MB, Weeks DE, Kuo CL, Swaroop A, Othman M, Kanda A, Chen W, Abecasis GR, Wright AF, Hayward C, Baird PN, Guymer RH, Attia J, Thakkinstian A, Silvestri G. Evidence of

association of APOE with age-related macular degeneration: a pooled analysis of 15 studies. *Hum Mutat* 2011;**32**:1407–16
14. Seddon JM, Sharma S, Adelman RA. Evaluation of the clinical age-related maculopathy staging system. *Ophthalmology* 2006;**113**:260–6
15. Bojanowski CM, Shen D, Chew EY, Ning B, Csaky KG, Green WR, Chan CC, Tuo J. An apolipoprotein E variant may protect against age-related macular degeneration through cytokine regulation. *Environ Mol Mutagen* 2006;**47**:594–602
16. Baird PN, Richardson AJ, Robman LD, Dimitrov PN, Tikellis G, McCarty CA, Guymer RH. Apolipoprotein (APOE) gene is associated with progression of age-related macular degeneration (AMD). *Hum Mutat* 2006;**27**:337–42
17. Klaver CC, Kliffen M, Van Duijn CM, Hofman A, Cruts M, Grobbee DE, Van Broeckhoven C, De Jong PT. Genetic association of apolipoprotein E with age-related macular degeneration. *Am J Hum Genet* 1998;**63**:200–6
18. Zareparsi S, Reddick AC, Branham KE, Moore KB, Jessup L, Thoms S, Smith-Wheelock M, Yashar BM, Swaroop A. Association of apolipoprotein E alleles with susceptibility to age-related macular degeneration in a large cohort from a single center. *Invest Ophthalmol Vis Sci* 2004;**45**:1306–10
19. DeAngelis MM, Ji F, Kim IK, Adams S, Capone A, Jr, Ott J, Miller JW, Dryja TP. Cigarette smoking, CFH, APOE, ELOVL4, and risk of neovascular age-related macular degeneration. *Arch Ophthalmol* 2007;**125**:49–54
20. Gotoh N, Kuroiwa S, Kikuchi T, Arai J, Arai S, Yoshida N, Yoshimura N. Apolipoprotein E polymorphisms in Japanese patients with polypoidal choroidal vasculopathy and exudative age-related macular degeneration. *Am J Ophthalmol* 2004;**138**:567–73
21. Pang CP, Baum L, Chan WM, Lau TC, Poon PM, Lam DS. The apolipoprotein E epsilon4 allele is unlikely to be a major risk factor of age-related macular degeneration in Chinese. *Ophthalmologica* 2000;**214**:289–91
22. Schultz DW, Klein ML, Humpert A, Majewski J, Schain M, Weleber RG, Ott J, Acott TS. Lack of an association of apolipoprotein E gene polymorphisms with familial age-related macular degeneration. *Arch Ophthalmol* 2003;**121**:679–83
23. Bergeron-Sawitzke J, Gold B, Olsh A, Schlotterbeck S, Lemon K, Visvanathan K, Allikmets R, Dean M. Multilocus analysis of age-related macular degeneration. *Eur J Hum Genet* 2009;**17**:1190–9
24. Liutkeviciene R, Vilkeviciute A, Smalinskiene A, Tamosiunas A, Petkeviciene J, Zaliuniene D, Lesauskaite V. The role of apolipoprotein E (rs7412 and rs429358) in age-related macular degeneration. *Ophthalmic Genet* 2018;**39**:457–62
25. Fernández-Vega B, García M, Olivares L, Álvarez L, González-Fernández A, Artime E, Fernández-Vega Cueto A, Cobo T, Coca-Prados M, Vega JA, González-Iglesias H. The association study of lipid metabolism gene polymorphisms with AMD identifies a protective role for APOE-E2 allele in the wet form in a Northern Spanish population. *Acta Ophthalmol* 2020;**98**:282–91
26. Sun E, Lim A, Liu X, Snellingen T, Wang N, Liu N. Apolipoprotein E gene and age-related macular degeneration in a Chinese population. *Mol Vis* 2011;**17**:997–1002
27. Fritsche LG, Freitag-Wolf S, Bettecken T, Meitinger T, Keilhauer CN, Krawczak M, Weber BH. Age-related macular degeneration and functional promoter and coding variants of the apolipoprotein E gene. *Hum Mutat* 2009;**30**:1048–53
28. Schmidt S, Haines JL, Postel EA, Agarwal A, Kwan SY, Gilbert JR, Pericak-Vance MA, Scott WK. Joint effects of smoking history and APOE genotypes in age-related macular degeneration. *Mol Vis* 2005;**11**:941–9
29. Adams MK, Simpson JA, Richardson AJ, English DR, Aung KZ, Makeyeva GA, Guymer RH, Giles GG, Hopper J, Robman LD, Baird PN. Apolipoprotein E gene associations in age-related macular degeneration: the Melbourne collaborative cohort study. *Am J Epidemiol* 2012;**175**:511–8
30. Levy O, Calippe B, Lavalette S, Hu SJ, Raoul W, Dominguez E, Housset M, Paques M, Sahel JA, Bemelmans AP, Combadiare C, Guilonneau X, Sennlaub F. Apolipoprotein E promotes subretinal mononuclear phagocyte survival and chronic inflammation in age-related macular degeneration. *EMBO Mol Med* 2015;**7**:211–26
31. Guillaume D, Bertrand P, Dea D, Davignon J, Poirier J. Apolipoprotein E and low density lipoprotein binding and internalization in primary cultures of rat astrocytes: isoformspecific alterations. *J Neurochem* 1996;**66**:2410–8
32. Riddell DR, Zhou H, Atchison K, Warwick HK, Atkinson PJ, Jefferson J, Xu L, Aschimies S, Kirksey Y, Hu Y, Wagner E, Parratt A, Xu J, Li Z, Zaleska MM, Jacobsen JS, Pangalos MN, Reinhart PH. Impact of apolipoprotein E (ApoE) polymorphism on brain ApoE levels. *J Neurosci* 2008;**28**:11445–53
33. Loane E, McKay GJ, Nolan JM, Beatty S. Apolipoprotein E genotype is associated with macular pigment optical density. *Invest Ophthalmol Vis Sci* 2010;**51**:2636–43
34. Fritsche LG, Fariss RN, Stambolian D, Abecasis GR, Curcio CA, Swaroop A. Age-Related macular degeneration: genetics and biology coming together. *Annu Rev Genomics Hum Genet* 2014;**15**:151–71

(Received September 21, 2020, Accepted December 13, 2020)