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The APOE E4 Allele Confers Increased Risk of Ischemic Stroke Among Greek Carriers

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Abstract

Background. Although several studies in various countries have indicated that the presence of the E4 allele of the apolipoprotein-E (APOE) gene is a risk factor for ischemic cerebrovascular disease, the strength of this association still remains a matter of debate.

Objectives. The aim of the study was to determine the frequency of the APOE E4 allele and various other gene polymorphisms in a well-characterized sample of Greek patients and to evaluate the potential associations with the risk of ischemic stroke (IS) and coronary heart disease (CHD).

Material and Methods. A total of nine gene variants/polymorphisms – F5 (Leiden – R506Q, rs6025), F2 (20210G > A, rs1799963), F13A1 (V34L, rs5985), MTHFR (677C > T – A222V, rs1801133), MTHFR (1298A > C – E429A, rs1801131), FGB (–455G > A –c.-463G > A; rs1800790), SERPINE1 (PAI14G/5G – rs1799889), ACE (ACE I/D, rs1799752), ITGB3 (GPIIIa L33P, rs5918) and the APOE E2/E3/E4 alleles (rs7412, rs429358) – were genotyped in 200 newly diagnosed ischemic stroke (IS) patients, 165 patients with ischemic coronary heart disease (CHD) and 159 controls with no cerebro- or cardiovascular disease (non-CVD). A statistical analysis was performed using univariate and multivariate logistic regression models.

Results. No significant association was found regarding most gene polymorphisms and the presence of IS or CHD in the patient cohort. However, the APOE E4 allele frequency was significantly higher ($p = 0.02$) among patients with ischemic stroke (IS) or IS + CHD (12.7%) when compared to the controls (5.1%). More accurately, E4 carriers had 2.66 and 2.71 times greater likelihood of IS or IS + CHD than non-carriers, respectively (OR = 2.66, 95% CI 1.39–5.07, OR = 2.71, 95% CI 0.98–7.48).

Conclusions. In contrast to some previous studies, these results support the role of the APOE E4 allele as an independent risk factor for ischemic stroke and ischemic coronary heart disease among Greek patients (*Adv Clin Exp Med* 2016, 25, 3, 471–478).

Key words: ischemic stroke, gene polymorphisms, apolipoprotein E gene, E4 allele.

Cerebro- and cardiovascular disease, in particular coronary heart disease (CHD) and stroke, are the leading causes of mortality and disability [1–2]. Data from the World Health Organization suggest that stroke affects more than 15 million people worldwide, and the incidence of the disease exhibits considerable geographic variability. Stroke is divided in two different subtypes: ischemic stroke (IS) and hemorrhagic stroke (HS);

both are complex multifactorial disease entities with a clear genetic predisposition, most likely expressed through interaction of multiple genes and diverse environmental risk factors [3–5]. Ischemic stroke constitutes the more frequent subtype and has a high recurrence risk and a high mortality rate, making early prognosis and diagnosis of extreme importance; however, genetic association studies have so far failed to identify clinically use-

ful genetic factors that may have an impact on disease risk assessment. Many case-control studies have been performed to assess the functional consequences of several gene polymorphisms and how they might influence the risk of stroke, and many candidate genes have been analyzed in this way. For example, recent reviews and a large scale meta-analysis point to a possible association between certain common gene polymorphisms and ischemic stroke [6–8]. In particular, polymorphisms of the apolipoprotein-E (APOE) gene have been associated with cardiovascular disease (CVD) risk, with the APOE E4 allele being implicated in lipid disorders, CHD and stroke [8–14]. Nonetheless, other studies have failed to demonstrate a statistically significant association between carriers of the E4 allele and IS, adding further to the confusion related to the precise role of this gene polymorphism [15–19].

In order to address the issue regarding the possible association of several common gene polymorphisms with IS and CHD, the present authors genotyped nine known polymorphisms occurring in the F5, F2, F13A1, MTHFR, FGB, SERPINE1, ACE and ITGB3 genes, as well as the APOE E2/E3/E4 polymorphism, and carried out an extensive statistical analysis to determine any significant risk associations in a well-defined cohort of Greek IS and CHD patients.

Material and Methods

Study Design and Participants

The study protocol was in compliance with the Helsinki Declaration and was approved by the Institutional Ethics Committees of Eginition Hospital, Alexandra University Hospital and InterGenetics. Informed consent was provided by all patients and control individuals. A detailed medical history was recorded and a thorough physical examination was performed. Demographic features, Greek ethnicity, clinical features, body mass index (BMI), biochemical parameters and known risk factors for stroke and CHD were noted. Individuals who were taking antihypertensive drugs, or who had systolic blood pressure (BP) ≥ 140 mm Hg or diastolic BP ≥ 90 mm Hg, were classified as having arterial hypertension. Individuals who smoked daily, or who had quit smoking within the previous three months, were considered active smokers. Individuals were classified as hypercholesterolemic, when they had a history of or were currently on medical treatment for high serum cholesterol levels, or when a cholesterol concentration of > 6.5 mmol/L was found the day after

admission (in accordance with the National Cholesterol Education Program Adult Treatment Panel [NCEP-ATP] III) [20]. Further evaluation of the patients included triplex ultrasound imaging or CT angiography of extracranial and/or intracranial arteries, electrocardiogram (ECG), transthoracic or transesophageal echocardiography and clinical/laboratory assessment to rule out systemic thrombotic and autoimmune disorders. Each participant's IS type was assessed by CT angiography, typically within 24 h. As the authors have described in a previous publication, the patients were classified according to the TOAST criteria [21] and on the basis of etiopathogenetic mechanisms into the following groups: large artery atherosclerotic stroke, cardioembolic stroke, small artery occlusion or lacunar infarction, infarction of other determined origin and infarction of undetermined cause [22]. Cardioembolic strokes were excluded, since they could result from a different etiology. Strokes occurring in the course of systemic conditions, such as immunological disorders, coagulopathies or of undetermined etiology, were also excluded. The presence of coronary heart disease (angina pectoris, unstable angina, myocardial infarction or heart failure), heart valve disease or arrhythmias was assessed by means of a questionnaire and relevant medical confirmation.

The participants were adult patients newly diagnosed with a) ischemic stroke (IS); b) coronary heart disease (CHD); c) both IS and CHD; and d) randomly selected controls: adult participants entering the hospital for a confirmed reason unrelated to cerebro- or cardiovascular disease (CVD).

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes by standard procedures, utilizing a QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA). Genotyping of the F5 (Leiden – R5 06Q, rs6025), F2 (20210G > A, rs1799963), F13A1 (V34L, rs5985), MTHFR (677C > T, rs1801133), MTHFR (1298A > C, rs1801131), FGB (–455G > A, rs1800790), SERPINE1 (PAI14G/5G, rs1799889), ACE (ACE I/D, rs1799752), ITGB3 (GPIIIa L33P, rs5918) and the APOE rs7412 and rs429358 polymorphisms (defining the E2, E3 and E4 alleles) was performed by an in-house single fluorescent multiplex PCR reaction amplifying the relevant gene regions, followed by single multiplex mini-sequencing (SNaPshot multiplex kit, Applied Biosystems Inc., Carlsbad, USA). In a number of selected cases, samples were re-analyzed and the results confirmed through the reverse-hybridization ViennaLabStripAssay (ViennaLab Diagnostics, GmbH, Vienna, Austria).

Statistical Methods

The statistical analysis was performed using SPSS v. 10 software (SPSS Inc., Chicago, USA) and STATA v. 8.2 software (Statacorp LP, College Station, USA). Continuous variables are presented as mean $\pm$ SD and categorical variables as frequencies. P-P and Q-Q plots were used to assess the normality of continuous variables. Age and total blood cholesterol levels were normally distributed, while systolic blood pressure, diastolic blood pressure and triglycerides were skewed. Comparisons of continuous variables between groups were performed using a one-way ANOVA F-test (for the normally distributed data) and the Kruskal-Wallis test (for the skewed). Associations between categorical variables were tested using Pearson's χ^2 test. Multinomial logistic regression analyses, adjusted for age and sex, and secondly for age, sex, smoking habits, the presence of obesity, diabetes and hypertension, total blood cholesterol levels, triglyceride levels and the use of anti-diabetic medication, were used to evaluate the main effect of the presence of the APOE E4 polymorphism in the study participants and the likelihood of the presence of IS, CHD, or both IS and CHD. Multinomial logistic regression analysis also evaluated the main effects of the presence of both the E4 allele and one other of the investigated polymorphisms on the likelihood of IS, CHD, or both IS and CHD. These last two models were adjusted for gender, the presence of obesity and smoking habits. The results are presented as odds ratios (OR) and the corresponding 95% confidence intervals

(CI). The Hosmer-Lemeshow statistic was used to test the models' goodness-of-fit. All the tested hypotheses were two-sided. A p-value of < 0.05 was considered statistically significant.

Results

In the study cohort, 31.7% of the participants were newly diagnosed with IS, 31.5% were newly diagnosed CHD patients, 6.5% were recently diagnosed with both IS and CHD, while 30.3% of the participants (the randomly selected controls) had entered the hospital for a non-CVD related reason. Of the 166 IS stroke patients, 101 (61%) represented the small-vessel lacunar subtype and 65 (39%) represented the large-vessel atherosclerotic subtype. Among the 165 CHD patients, 163 (99%) represented cases newly diagnosed with myocardial infarction (MI). Follow-up data regarding other possible neurological abnormalities that may have developed following the patients' initial assessment was not collected. Table 1 presents the various socio-demographic and clinical characteristics of the participants according to their CVD status. No statistically significant differences were observed between the groups in obesity and triglyceride levels ($p > 0.05$). However, patients with IS, CHD or both IS and CHD were older, male and current smokers ($p < 0.05$). Furthermore, participants with CHD or both CHD and IS had a higher proportion of diabetes and were more frequently under medication than the other groups ($p < 0.001$). Systolic and diastolic blood pressure as well as cholesterol levels

Table 1. Socio-demographic and clinical characteristics of the participants, by CVD status

	Non-CVD (controls)	CHD	Stroke	Stroke and CHD	Total sample	p-value ¹
N	159	165	166	34	524	
Age (years)	59 $\pm$ 13	66 11	60 $\pm$ 14	62 $\pm$ 12	62 13	< 0.001
Male sex (%)	47.5	82.9	68.3	88.2	67.9	< 0.001
Obesity (%)	28.0	20.5	23.5	25.0	24.1	0.48
Current smoking (%)	31.4	39.5	46.3	55.9	40.2	0.01
SBP (mm Hg)	141 $\pm$ 19	128 17	142 $\pm$ 24	148 $\pm$ 24	138 21	< 0.001
DBP (mm Hg)	87 $\pm$ 11	77 10	84 $\pm$ 13	82 $\pm$ 14	83 13	< 0.001
Total cholesterol (mg/dL)	206 $\pm$ 45	186 47	218 $\pm$ 43	202 $\pm$ 39	204 47	< 0.001
Triglycerides (mg/dL)	136 $\pm$ 78	145 89	142 $\pm$ 90	165 $\pm$ 66	142 85	0.06
Diabetes (%)	15.1	36.8	21.7	50.0	26.2	< 0.001
Anti-diabetic medication (%)	12.7	23.5	16.7	40.6	18.8	< 0.001

SBP – systolic blood pressure; DBP – diastolic blood pressure; ¹ p-values were derived through comparisons between non-CVD, CHD, stroke and stroke + CHD.

appeared to be lower in CHD participants (Bonferroni post-hoc multiple comparisons analysis, $p < 0.05$).

Successful and full genotyping results for all nine gene polymorphisms and the three APOE E2/E3/E4 alleles were obtained in ~99% of participants. Individual allele frequencies for the nine gene polymorphisms are presented in Table 2, according to the patients' CVD status. As is evident, the data do not support a statistically significant dependence regarding the observed genotypes and the presence of IS or CHD.

However, the initial statistical analysis revealed that the APOE E4 allele appears to be more frequent in the group of patients with IS, CHD or both IS and CHD ($p = 0.02$, Table 3).

In order to more accurately assess the dependence of CVD status and the E4 allele polymorphism, an additional multi-adjusted statistical analyses were carried out. Particularly when age and gender were taken into account, a multinomial logistic regression analysis revealed that compared to non-carriers, E4 carriers had a 2.66 times greater likelihood of IS vs. non-CVD presence (OR = 2.66, 95% CI 1.39–5.07), and a 2.71 times greater likelihood of both IS and CHD vs. non-CVD presence (OR = 2.71, 95% CI 0.98–7.48), as well as a 2.15 times greater likelihood of CHD vs. non-CVD presence (OR = 2.15, 95% CI 1.07–4.30).

Table 4 also illustrates the results from the multi-adjusted analyses where socio-demographic and relevant clinical characteristics of the participants were also taken into account. As the table shows, models with the presence of IS or CHD as the main outcome maintained a significant correlation between the presence of the E4 allele polymorphism and the likelihood of the presence of these two types of CVD phenotype. Specifically, E4 carriers had a 2.51 and 2.05 times greater likelihood of IS or CHD vs. non-CVD presence, correspondingly, than non-carriers (OR = 2.51, 95% CI 1.25–6.17, OR = 2.05, 95% CI 1.04–5.92). However, no significant effect of the E4 polymorphism on the likelihood of both IS and CHD was observed in the multi-adjusted analysis ($p = 0.16$). Finally, the presence of the APOE E4 allele did not exhibit any statistically significant differences between its association with small-vessel IS (61%) and with large vessel IS (39%) in this patient cohort.

Lastly, the possible effect of the presence of both the E4 allele and any one of the other nine investigated polymorphisms was assessed in relation to the likelihood of IS, CHD, or both IS and CHD (always adjusting the models for gender, the presence of obesity and smoking habits). The results of the multinomial logistic regression analysis showed that compared with non-carriers, par-

Table 2. Allele frequencies of nine polymorphisms, by CVD status

Polymorphism (dbSNPs ID)	Non-CVD controls (%)	CHD (%)	Stroke (%)	Stroke and CHD (%)	Total (%)	p-value ¹
<i>F5</i> G > A (rs6025)	G = 95.9, A = 4.1	G = 97.0, A = 3.0	G = 98.8, A = 1.2	G = 98.4, A = 1.6	A = 2.8	0.13
<i>F220210G</i> > A (rs1799963)	G = 96.2, A = 3.8	G = 98.8, A = 1.2	G = 97.9, A = 2.1	G = 95.2, A = 4.8	A = 2.5	0.20
<i>MTHFR</i> C > T (rs1801133)	C = 59.4, T = 40.6	C = 60.4, T = 39.6	C = 58.7, T = 41.3	C = 52.9, T = 47.1	T = 40.6	0.53
<i>MTHFR</i> A > C (rs1801131)	A = 67.6, C = 32.4	A = 70.6, C = 29.4	A = 66.5, C = 33.5	A = 67.6, C = 32.4	C = 32.0	0.15
<i>F13A1</i> G > T (rs5985)	G = 74.7, T = 25.3	G = 81.2, T = 18.8	G = 76.8, T = 23.2	G = 74.1, T = 25.9	T = 22.5	0.58
<i>FGB</i> G > A (rs1800790)	G = 78.1, A = 21.9	G = 75.7, A = 24.3	G = 71.4, A = 28.6	G = 74.2, A = 25.8	A = 25.0	0.43
<i>SERPINE1</i> 4G/5G (rs1799889)	4G = 43.3, 5G = 56.7	4G = 51.2, 5G = 48.8	4G = 50.0, 5G = 50.0	4G = 46.8, 5G = 53.2	4G = 47.5	0.52
<i>ITGB3</i> T > C (rs5918)	T = 83.5, C = 16.5	T = 83.2, C = 16.8	T = 84.7, C = 15.3	T = 81.7, C = 18.3	C = 16.2	0.64
<i>ACE</i> I/D (rs1799752)	I = 39.8, D = 60.2	I = 39.1, D = 60.9	I = 38.4, D = 61.6	I = 43.5, D = 56.5	D = 60.7	0.82

¹ p-values were derived through Pearson's χ^2 test of independence between CVD status and the presence of different allele frequencies.

Table 3. APOE genotypes and E4 allele frequency, by CVD status

APOE genotype	Non-CVD controls (%)	CHD (%)	Stroke (%)	Stroke and CHD (%)	Total (%)	p-value ¹
E2/E2	0.6	0	0	0	0.2	
E2/E3	10.1	8.0	4.9	6.4	7.4	
E2/E4	0	1.2	1.8	0	1.0	
E3/E3	79.1	72.8	72.6	71.0	75.0	
E3/E4	10.2	17.9	18.9	22.6	15.9	
E4/E4	0	0	1.8	0	0.6	
E4 carriers	10.2	19.1	22.6	22.6	17.5	0.02
E4 allele frequency	5.1	9.6	12.7	11.3	9.1	0.02

¹ p-values were derived through Pearson's chi-square test of independence between CVD status and the presence of different APOE allele frequencies.

Table 4. Results of the multinomial logistic regression analyses evaluating the main effect of the APOE E4 polymorphism on the likelihood of the presence of CHD and IS

	Odds Ratio (OR)	95% CI	p-value
Age- and sex-adjusted analysis			
IS vs. non-CVD	2.66	1.39–5.07	< 0.01
CHD vs. non-CVD	2.15	1.07–4.30	0.03
IS + CHD vs. non-CVD	2.71	0.98–7.48	0.05
Multi-adjusted analysis ¹			
IS vs. non-CVD	2.51	1.25–6.17	0.01
CHD vs. non-CVD	2.05	1.04–5.92	0.04
IS + CHD vs. non-CVD	1.40	0.70–8.55	0.16

¹ These models have been adjusted for age, sex, the presence of obesity, diabetes, hypertension, smoking habits, total blood cholesterol, triglyceride levels and the use of anti-diabetic medication.

ticipants harboring both the APOE E4 allele and the MTHFR rs1801133 T allele had 2.72, 3.67 and 4.72 times greater likelihood of the presence of CHD, IS or both IS and CHD vs. non-CVD presence, respectively (OR = 2.72, 95% CI 1.10–6.76, OR = 3.67, 95% CI 1.56–8.64 and OR = 4.72, 95% CI 1.36–16.33). In addition, the presence of both the E4 allele and the FGB rs1800790 A increased the likelihood of the presence of IS or both IS and CHD by 3.30 and 4.94 times, respectively, as compared with non-carriers (OR = 3.30, 95% CI 1.33–8.15 and OR = 4.94, 95% CI 1.47–16.62).

Discussion

In the study cohort of 365 clinically well-characterized Greek patients with first-time IS or CHD, the results reveal a statistically significant risk association between the presence of the E4 allele and

IS. Additionally, this strong association maintains its significance when adjusted for multiple conventional risk factors, such as age, sex, the presence of obesity, diabetes, hypertension, smoking habits, total blood cholesterol levels, triglyceride levels and diabetes. The results also demonstrate that the additional presence of the MTHFR rs1801133 T allele or the FGB rs1800790 A allele further increases this risk association.

Individually, none of the other nine gene polymorphisms investigated exhibit any association with a risk of IS or CHD when compared to the non-CVD control group, and this is in accordance with the majority of similar recent studies [23]. It is important to note that the genotype frequencies of all nine polymorphisms observed in the control group are similar to the frequencies reported in the general Greek population [24–26]. Also, the observed frequency of the E4 allele in the non-CVD control group – 5.1% – is very similar to that of the gener-

al Greek population (6.5%) [27]. It is noteworthy that the E4 allele frequency in the Greek population is significantly lower than average European values, and follows the decreasing north-to-south European frequency for this allele – which interestingly enough parallels a similar decrease in the frequency of cerebro- and cardiovascular disease [11].

There are relevant issues that the authors wish to address. Firstly, as ischemic stroke may be caused by occlusion (~80% of the cases) or hemorrhage (~20% of the cases) of cerebral blood vessels, several gene polymorphisms with a potential functional effect on blood coagulation proteins have been investigated in the last decade as potential risk factors for IS or CHD, with inconclusive results [28–30]. Similarly, a large number of published genome-wide association studies (GWAS) investigating thousands of single nucleotide polymorphisms (SNPs) across the human genome have failed to identify DNA sequence variants which, even when combined, are in a position to provide clinically useful and robust genotype-phenotype associations [26–29]. On the one hand, the results of these studies confirm the complex and multifactorial basis of cerebro- and cardiovascular disease in general, and more specifically of IS and CHD, while at the same time they exhibit considerable phenotypic variations in patient selection and study design.

Secondly, it is well established that cerebrovascular disease is related to vascular dementia and also to the expression of Alzheimer's disease, and as the APOE E4 allele has been shown to be a considerable risk factor for both [31], the association between the E4 allele and IS and CHD has been widely investigated. Although there are conflicting results, it is perhaps not surprising that the consensus from the majority of well-designed studies, involving a more uniform patient cohort, appears to support a causal role for the E4 allele in stroke [14, 18, 31]. Since many other genetic and environmental factors obviously contribute to the expression of IS and CHD, it is logical to expect that a single variant may only explain a small proportion of cases. On the other hand, as environmen-

tal factors are tightly linked to ethnicity/race, and gene polymorphisms vary considerably among different racial groups, one would also expect that the effect of potential risk-associated polymorphisms will not be the same across heterogeneous groups of patients. In light of the above, the findings of the current study, involving a clinically well-characterized and racially homogeneous patient cohort are perhaps more meaningful.

Future studies should address more carefully the precise clinical phenotype of the study participants; strive to apply more rigorous multi-adjusted statistical analyses between gene polymorphisms and conventional risk factors; and, finally, involve a racially homogeneous group. Furthermore, the panel of polymorphisms examined in this study is by no means exhaustive and there is no doubt that multi-factorial disorders, such as the vast majority of strokes or CHD, are most probably related to an interplay between a multitude of other genetic, epigenetic and environmental factors [32]. It would therefore appear that the introduction of similar studies as a useful tool in everyday clinical practice may still be premature [33], and perhaps the main value of these approaches in the immediate future is not to provide predictive genetic testing, which may currently yield limited information, but instead to afford a better understanding of the underlying disease mechanisms.

As with most similar studies, the present study has certain limitations. Although the number of IS cases analyzed is not small, a larger number might have led to more convincing statistical association data, particularly in terms of differentiating the risk associated with the two major IS subtypes (small vessel and large vessel) and the presence of the E4 allele.

Nevertheless, the current study has revealed a significant risk association between carriers of the APOE E4 allele and the occurrence of first-time ischemic stroke in Greek patients. A further replication study is under way to confirm these findings in a separate and clinically well-defined cohort of Greek IS patients.

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