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INFLUENCE OF THE POLYMORPHISM OF APOLIPOPROTEIN E IN CEREBRAL VASCULAR DISEASE

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ABSTRACT - The genetic heterogeneity of apolipoprotein E (apo E) has been associated with lipid profile and atherothrombotic stroke, however this association remains inconclusive. *Objective:* To evaluate the relationship between the isoforms of apo E and atherothrombotic stroke, by ascertaining the frequency of its alleles and genotypes associated with the lipid profile in patients with stroke. *Method:* A total of 207 individuals were divided into two groups, consisting of 107 patients with stroke and 100 individuals without clinical symptoms of the disease. Blood samples were taken from patients and controls for molecular investigation of the apo E (ε2, ε3 and ε4 alleles) for the analysis of the lipid profile. *Results:* The ε3 allele was the most common and its prevalence was significantly higher in patients (0.93) compared to the controls (0.86; p=0.024). The ε2 allele was rarely seen specifically in patients (0.02 versus 0.05 in controls, p=0.191). The ε4 allele was not associated with stroke showing a reduced frequency in patients (0.05) when compared to controls (0.09; p=0.011). Although higher average levels of lipid profile were found in patients when compared to controls, with statistical significance for the values of total cholesterol (TC) (203.6mg/dL±57.98 and 181.9mg/dL±68.47 respectively; p=0.003) and low-density lipoprotein cholesterol (LDLc) (131.4mg/dL±52.60 and 116mg/dL±56.38, respectively; p=0.014), these were independent of the presence of the ε4 allele. In control group the higher TC and LDLc values occurred in the absence of the ε4 allele, confirming the conflicting effect of the alleles of apo E on the plasmatic lipids and atherothrombotic stroke. *Conclusion:* The isoforms of apo E cannot be regarded as an isolated risk factor for stroke and do not show association with lipid profile in this study.

KEY WORDS: stroke, apolipoprotein E, atheromathosis, lipid profile.

Influência do polimorfismo da apolipoproteína E na doença cerebrovascular

RESUMO - A heterogeneidade genética da apolipoproteína E (apo E) tem sido associada com o perfil lipídico e acidente vascular cerebral aterotrombótico (AVC at); no entanto, esta associação permanece inconclusiva. *Objetivo:* Avaliar a relação entre as isoformas da apo E e o AVC at pela determinação da frequência de seus alelos e genótipos, associados ao perfil lipídico, em pacientes com a doença. *Método:* Foram estudados 207 indivíduos distribuídos em 2 grupos, consistindo de 107 pacientes com AVC e 100 indivíduos sem sintomas clínicos da doença. Amostras de sangue periférico foram obtidas de pacientes e controles para investigação molecular da apo E (alelos ε2, ε3 e ε4) e para análise do perfil lipídico. *Resultados:* O alelo ε3 foi o mais comum e sua prevalência foi significantemente mais elevada em pacientes (0,93) comparada com controles (0,86; p=0,024). O alelo ε2 foi raramente encontrado, especialmente em pacientes (0,02 versus 0,05 em controles; p=0,191). A presença do alelo ε4 não foi associada com AVC, apresentando frequência reduzida em pacientes (0,05) quando comparada com controles (0,09; p=0,011). Apesar de níveis médios elevados do perfil lipídico terem sido encontrados em pacientes quando comparados com controles, com significância estatística para valores de colesterol total (CT) (203,6md/dL±57,98, e 181,9mg/dL±68,47, respectivamente; p=0,003) e colesterol de baixa densidade (LDLc) (131,4mg/dL±52,60 e 116mg/dL±56,38, respectivamente; p=0,014), esses resultados se mostraram independentes da presença do alelo ε4. No grupo controle, os valores mais elevados de CT e LDLc ocorreram na ausência do alelo ε4, confirmando o efeito conflitante dos alelos da apo E sobre os lipídios plasmáticos e sobre o AVC at. *Conclusão:* As isoformas da apo E não podem ser consideradas fator de risco isolado para AVC at e não mostram associação com o perfil lipídico neste estudo.

PALAVRAS-CHAVE: acidente vascular cerebral, apolipoproteína E, ateromatose, perfil lipídico.

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Stroke is one of the most common causes of death in the industrialized countries and it affects the population by inflicting physical, emotional and economic injury to patients with serious consequences on their families and on the public health system. Studies have been searching for ways of controlling and preventing the stroke by investigating risk factors such as smoking, hypertension, dyslipidemia, diabetes and familial history[1,2]. Apolipoprotein E (apo E), associated with coronary arterial disease (CAD) and with the diversity of the serum lipid profile among individuals[3-6], has also been related as a risk factor for stroke[7,8] as it exerts an important role in the modulation of the atherogenic lipoproteins. The APOE gene, mapped on chromosome 19, exhibits at one unique locus three principal alleles ε2, ε3 and ε4 and their frequencies in Caucasian populations are estimated at 8%, 77% and 15% respectively. The most common phenotype E3/3 is represented in 60% of the population followed by E3/4 (22%), E2/3 (12%), E4/4 (3%), E2/4 (2%) and E2/2 (1%)[9]. Gender has no influence on the frequency of the alleles of apo E[10].

The association between the genetic heterogeneity of apo E and stroke is controversial. Some studies demonstrated a significantly higher frequency of the ε4 allele in patients with a familial history of stroke, compared to control individuals[7,11-13]. In this case, the etiological role of the ε4 allele is due to its association with arterosclerosis. However, other studies deny the existence of any relationship between apo E4 and the risk for stroke[14-17]. Therefore, the contribution of apo E in the outcome of the disease remains inconclusive, with the absence of any clear result in the Brazilian population.

The aim of this study was to verify the relationship between the isoforms of apo E and atherothrombotic stroke, by ascertaining the frequency of its alleles and genotypes associated with the lipid profile, in patients with clinical manifestations of cerebral atherosclerotic disease.

METHOD

A total of 207 white individuals, non-related were studied. Among them, 120 (58%) were male and 87 (42%) female, with ages greater than 55 years. The individuals were separated into Group 1, consisting of 107 patients with atherothrombotic stroke and an average age of 68.8 ±9.17 years; and Group 2, consisting of 100 individuals without clinical symptoms of the disease and a mean age of 69.4±8.29 years (p value = 0.6013). The groups were matched by age and gender. The diagnosis of stroke was based on clinical data and in the computed tomographic (CT) scan study. The neurological history and examination included loss of cerebral function, focal signs, with symptoms that lasted more than 24 hours. All the patients undergone a CT scan during the hospitalization, showing areas of low attenuation compatible with the clinical symptoms and signs. The patients were categorized into a possible subtype of stroke based on the Trial of Org 10172 in Acute Stroke Treatment - TOAST[19], including only large artery artherosclerosis as the cause of ischemic stroke. The subjects with a cardioembolic factor were not included in this study.

The lipid profile included the serum level of total cholesterol (TC), low-density lipoprotein cholesterol (LDLc), high-density lipoprotein cholesterol (HDLc), very low-density lipoprotein cholesterol (VLDLc), and triglycerides (TG) at the time of hospitalization or immediately after the first consultation in the outpatient clinic.

Molecular investigation of apo E included extraction of the DNA from leukocytes[20] and amplification of the polymorphic fragment by polymerase chain reaction (PCR). The resulting product was submitted to enzyme digestion using *Hha* I followed by electrophoresis in 6% polyacrylamide gel and ethidium bromide stain.

Statistical analysis included the Fisher test, the two-tail Mann-Whitney test and odds ratio with a confidence interval (CI) of 95%. The lipid profile and its association with the genotypes of apo E, related to the presence of ε4 allele, were analyzed in both groups. An error α 5% was admissible with a significance level for p value ≤ 0.05.

Informed consent was obtained from all subjects, and the study was approved by the Institution's Research Ethical Committee.

RESULTS

The allelic and genotypic frequencies for apo E and serum lipid levels were evaluated in 107 patients with stroke and 100 controls. Table 1 shows the distribution of the apo E polymorphism in relation to the presence of alleles and genotypes in both groups. The prevalence of the ε3 allele was significantly higher in the patients (0.93) compared with the controls (0.86; p=0.029). The ε4 allele had a significantly higher frequency in the controls (0.09), compared with the patients (0.046; p=0.016). The ε3/ε3 genotype was significantly more frequent in patients (87%) than in the control group (74%), (p=0.02), but the frequency of ε3/ε4 in controls (16%) and in patients (7%) was not statistically significant (p=0.08). Other genotypes were absent or rarely seen in both groups. Risk factors such as hypertension, smoking and diabetes mellitus were observed in patients at rates of 81%, 43% and 20% and in the control group at 39%, 49% and 13%, respectively.

The lipid profile remained within the desired limits in both groups (Table 2), although significantly higher levels of TC and LDLc were observed in patients

Table 1. Allelic and genotypic frequencies for apolipoprotein E in patients and controls.

	Patients		Controls		
Alleles	N	Frequency	N	Frequency	p*
ε2	5	0.02	10	0.05	0.1916 NS
ε3	199	0.93	172	0.86	0.0243 S
ε4	10	0.05	18	0.09	0.0116 S
Total	214	1.00	200	1.00	
Genotypes	N	%	N	%	p*
ε2/ε2	0	0	0	0	NS
ε3/ε2	5	5	8	8	0.3979 NS
ε3/ε4	0	0	2	2	0.2346 NS
ε3/ε3	93	87	74	74	0.0229 S
ε3/ε4	8	7	16	16	0.0814 NS
ε4/ε4	1	1	0	0	
Total	107	100	100	100	

*Fisher Test; NS, not significant; S, significant; N, number of individuals.

Table 2. Lipid profile of patients with cerebral vascular disease and controls.

Lipid profile (mg/dL)	Patients (N=107)		Controls (N=100)		p*	
	Mean	SD	Mean	SD		
TC	203.6	57.98	181.9	68.47	0.003	S
LDLc	131.4	52.60	116.0	56.38	0.0143	S
HDLc	47.8	15.90	42.0	16.92	0.3928	NS
VLDLc	30.0	32.95	25.8	12.89	0.3738	NS
TG	137.3	69.90	125.5	66.30	0.1401	NS

*Mann-Whitney Test; N, number of individuals; SD, standard deviation; NS, not significant; S, significant; TC, total cholesterol; LDLc, low density lipoprotein cholesterol fraction; HDLc, high density lipoprotein cholesterol fraction; VLDLc, very-low density lipoprotein cholesterol fraction; TG, triglycerides.

(203.6±57.98; 131.4±52.6 mg/dL, respectively) when compared with the control group (181.9± 68.47; 116.0±56.38mg/dL, respectively) (p=0.003; p=0.0143, respectively). However, the comparative study of the lipid profile in relation to the genotyping of apo E revealed similar lipid levels without any significant influence exerted by the ε4 allele, in patients and controls (Table 3). No significant effect was observed even when only borderline values were analysed with or without the presence of the ε4 allele (Table 4). In this case, slightly increased TC levels were measured in patients with at least one ε4 allele (323.5±62.93 mg/dL) compared to its absence (281.1±28.16 mg/dL), whilst in the control group the opposite was observed (260.2±11.53; 291.6± 67.08 mg/dL, respectively; p=0.1336).

Figure 1 demonstrates the effect of the presence and absence of the ε4 allele on the lipid profile in the study and control groups, assessed as a percentage variation of the average levels when taking into account only borderline values for TC, LDLc, HDLc, and VLDLc. The mean values of TC and LDLc for the patients increased in the presence of ε4 by 14.9% and 6.9%, respectively, while in the control group there was a reduction of 10.7% and 22.3%, respectively. The control group also showed a 3.7% reduction in HDLc levels.

Odds ratio confirmed that the ε4 allele did not significantly influence the lipid profile in either of the groups (Table 5).

DISCUSSION

In this study the presence of the ε4 allele was not associated with stroke showing a reduced frequency in patients compared to control individuals. Although higher average levels of the lipid profile were seen

Table 3. Mean values for the lipid profile in the presence or absence of the ε4 allele for apolipoprotein E in patients and controls.

PL	Patients			Controls		
(mg/dL)	With ε4	Without ε4	p*	With ε4	Without ε4	p*
TC	207.22 (N=9)	203.32 (N=98)	0.9463 NS	175.06 (N=18)	183.44 (N=82)	0.7399 NS
LDLc	138.56 (N=9)	130.77 (N=98)	0.9910 NS	109.17 (N=18)	117.49 (N=82)	0.8051 NS
HDLc	43.89 (N=9)	44.84 (N=98)	0.8487 NS	46.022 (N=18)	41.43 (N=82)	0.3940 NS
VLDLc	25.22 (N=9)	30.43 (N=98)	0.6575 NS	21.39 (N=18)	26.77 (N=82)	0.2757 NS
TG	124.11 (N=9)	138.53 (N=98)	0.5482 NS	99.44 (N=18)	131.18 (N=82)	0.1671 NS

*Mann-Whitney Test; N, number of individuals; NS, not significant; S, significant; TC, total cholesterol; LDLc, low density lipoprotein cholesterol fraction; HDLc, high density lipoprotein cholesterol fraction; VLDLc, very-low density lipoprotein cholesterol fraction; TG, triglycerides.

Table 4. Mean values for the lipid profile considering only the altered levels of total cholesterol (TC>240mg/dL), LDLc (>160mg/dL), HDLc (<40mg/dL), VLDLc (>35mg/dL) and TG (>200mg/dL).

PL	Patients			Controls		
(mg/dL)	With ε4	Without ε4	p*	With ε4	Without ε4	p*
TC	323	281	0.2414 NS	260,2	291,6	0.1336 NS
SD	62.93 (N=2)	28.16 (N=27)		11.53 (N=4)	67.08 (N=15)	
LDLc	213	199	0.8357 NS	184,3	225,5	0.1011 NS
SD	72.86 (N=3)	28.26 (N=27)		20.55 (N=3)	50.62 (N=12)	
VLDLc	31	31	0.4822 NS	26,0	27	0.8693 NS
SD	1.53 (N=3)	6.51 (N=45)		8.40 (N=7)	8.82 (N=36)	
HLDLc	44	46	0.6895 NS	45		
SD	11.31 (N=2)	10.59 (N=25)		9.8 (N=0)	(N=23)	
TG	263	263		251		
SD	(N=1)	49.90 (N=16)		(N=0)	50.63 (N=15)	

*Mann-Whitney Test; N, number of individuals; SD, Standard Deviation; NS, not significant; S, significant; TC, total cholesterol; LDLc, low density lipoprotein cholesterol fraction; HDLc, high density lipoprotein cholesterol fraction; VLDLc, very-low density lipoprotein cholesterol fraction; TG, triglycerides.

in patients when compared to controls, with statistic significance for the values of TC and LDLc, these were independent of the presence of the ε4 allele. The ε2 allele was rarely found specifically in patients, suggesting a possible protecting effect[14].

The role of the ε4 allele in the pathogenesis of atherothrombotic stroke is still controversial, with inconsistent results in the Brazilian population. In this study, the ε4 allele was observed with a significantly reduced frequency in patients (0.05) when

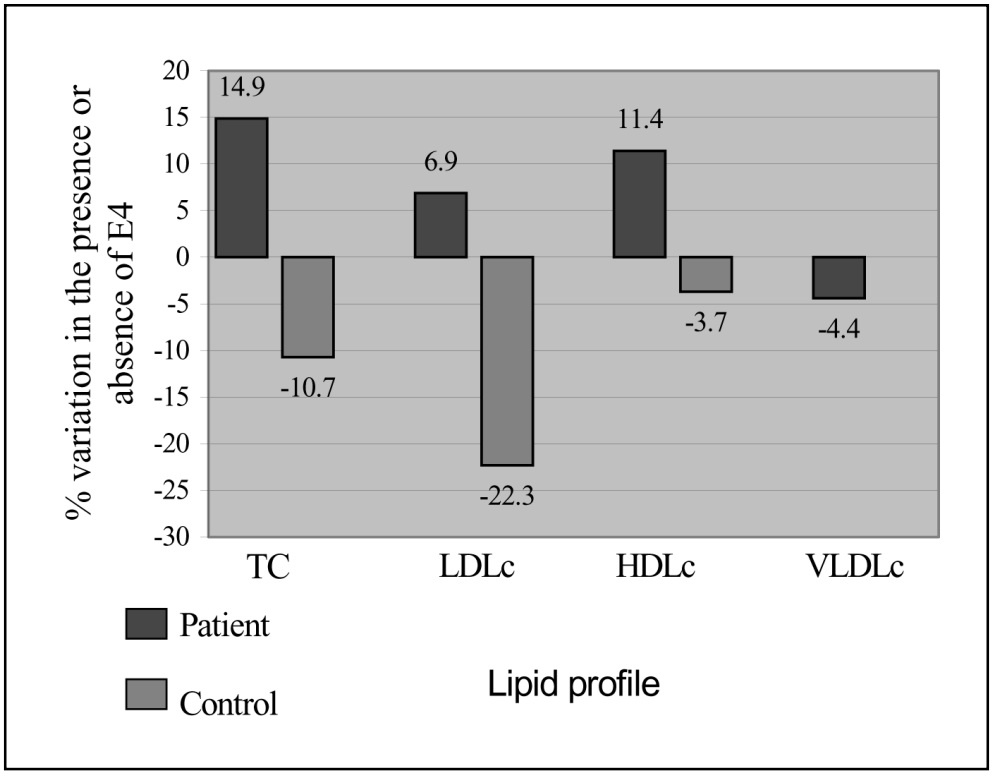

Fig 1. Effect of the ε4 allele for Apolipoprotein E on the levels of total cholesterol (TC) and lipoprotein cholesterol fractions of low (LDLc), high (HDLc) and very low (VDLc) density, considering the variation of the lipid profile in the presence or absence of the ε4 allele in patients and controls.

Table 5. Odds ratio for the lipid profile according to the frequency of the ε4 allele for apolipoprotein E in patients and controls.

Individual	Odds Ratio (CI)				
	TC	LDLc	HDLc	VLDLc	TG
Patients	0.75	1.25	1.69	5.83	0.64
CI	(0.1467-3.847)	(0.2921-5.35)	(0.4015-7.182)	(0.1625-4.285)	(0.0748-5.485)
Controls	1.27	1.17	1.23	0.01	0.12
CI	(0.3676-4.430)	(0.2926-4.65)	(0.4332-3.491)	(0.0039-1.183)	(0.0067-2.062)

CI, confidence interval; TC, total cholesterol; LDLc, low density lipoprotein cholesterol fraction; HDLc, high density lipoprotein cholesterol fraction; VLDLc, very low density lipoprotein cholesterol fraction; TG, triglycerides.

compared to controls (0.09). In others studies an association was seen between the ε4 allele and stroke[7,11,12]. McCarron et al.[7] observed in a meta-analytical study, a higher prevalence of the ε4 allele in patients with ischemic stroke compared to a control group (0.14 versus 0.09; odds ratio of 1.68 – CI 95%: range 1.36 - 2.09; $p < 0.001$). Margaglione et al.[12] detected a significantly higher frequency of the ε4 allele in patients with a familial history of stroke (0.18) compared to individuals without this familial history (0.07) and healthy individuals (0.09). However other authors have also discarded the relationship between apo E4 and risk of stroke[6,14,15]. On the other hand, it is possible that the ε4 allele carriers be more susceptible to complications, increasing the probability of an earlier mortality.

In this study the low frequencies of the ε2 and ε4 alleles in both patients and controls with ages of approximately 70 years, determined the prevalence of the ε3 allele, which was significantly more common in patients (93%) compared to controls (86%). These frequencies were more elevated compared to Caucasian population and more consistent to Asian samples (85%)[4]. In populations, the apo E polymorphism explains the variation in LDLc plasmatic levels[10,22].

The association between the apo E polymorphism and atherosclerosis was first described by Utermann et al.[23] in studies of patients with type III hyperlipoproteinemia, E2/2 genotypes and early onset of stroke. In the Finnish population the high frequency of E4, low frequency of E2 and the exceptionally high rate of stroke in the country, support a suggested association between this disease and the apo E phenotypes. In Finland, 34% of the individuals have the E4/4 or E3/4 phenotypes. Thus, the phenotypes which express the apo E4, associated with a traditional Finnish diet rich in saturated fats and cholesterol, can induce hyperlipoproteinemia especially in rural areas where the saturated fat proportion in the diet is considerably higher[24].

In this study, an increased effect of the ε4 allele was only observed when considering patients with borderline values. These patients presented on average increases of about 15% (42mg/dL) and 7% (14mg/dL) of TC and LDLc levels, respectively. But these values were not considered significant in relation to patients without the ε4 allele. However, epidemiological studies have alerted of the benefits of a reduction in plasmatic cholesterol levels, as an effective intervention to decrease the morbidity and mortality rates caused by cardiovascular disease. Gould et al.[9] in a meta-analytical study revealed that for every 10% decrease in plasmatic cholesterol levels there was a 15% reduction in the total risk of mortality by cardiovascular disease and an 11% reduction in the total risk of death. This suggests that, although there was a low representation in this study group, the ε4 allele could have an influence in the morbi-mortality rate, principally of patients with atherothrombotic stroke and an increased lipid profile.

The effect on the TC and LDLc values in the patients with at least one ε4 allele, could result from its interaction with other atherogenic risk factors, such as hypertension, smoking and diabetes observed in 81%, 43% and 20% of the patients, respectively. In this way, the results can indicate that these risk factors, associated with the genesis of stroke, can compromise the evaluation of the apo E polymorphism as a risk factor for this disease.

On the other hand, in the control group the higher TC and LDLc values occur in the absence of the ε4 allele, confirming its non-significant effect in these individuals with a mean age of nearly 70 years old and without clinical symptoms of stroke. In reality, the effect of the alleles of apo E on the plasmatic lipids is still conflicting. There is a reference of an important correlation between the ε2 allele and the plasmatic lipid levels in an ageing multi-ethnic population, while the ε4 allele remains without expression[25] and it is not associated with an altered lipid profile in a rural North American population[26].

Due to the similarities of the risk factors for atherothrombotic cerebral stroke and coronary disease, in which the lipid profile is an independent risk factor, a positive association between apo E4 with a well-known function on the lipid metabolism and cerebral infarction would be expected. This was not observed in this study. It is possible that the role of apo E in cerebral infarction is not etiological but that its genotype influences the degree of neuronal degradation after a cerebral event as was demonstrated by Tomimoto, et al.[27] Furthermore, studies in younger populations could clarify the effect of the apo E polymorphism with greater reliability. Ferruci et al.[14] detected a lower risk of stroke in under 80 years old individuals who were carriers of the ε2 allele which was not seen in an older group.

It is evident that a complex disease such as stroke is determined by multiple factors, both genetic and environmental. This makes it improbable that a single gene locus can identify a subset of individuals susceptible to the disease. The recent increase in life expectancy encourages the investigation of other risk factors of cerebral-vascular disease with the view of precocious intervention to control morbi-mortality associated with age.

CONCLUSION

In conclusion, this study shows that the ε4 allele, with a reduced frequency particularly in patients suffering from stroke, can not be regarded as an isolated risk factor for this disease. Variations in the lipid profile apparently demonstrate independence from apo E, although, increased levels of TC, LDLc and also HDLc appear to be poorly associated with ε4 allele in the patients, which needs further investigation in this subgroup.

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