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Association and linkage disequilibrium analyses of *APOE* polymorphisms in atherosclerosis

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Abstract. *Background*: Apolipoprotein E (apo E) plays a major role in lipid metabolism, and its genetic variations have been associated with cardiovascular risk. The objective of this study was to investigate the influence of the *APOE* promoter (−491 A/T, −427 T/C and −219 G/T) and coding region (*APOE* $\varepsilon 2/\varepsilon 3/\varepsilon 4$) polymorphisms in atherosclerosis disease by association and linkage disequilibrium analyses. *Materials and methods*: We analyzed these polymorphisms in a sample of 286 subjects with atherosclerosis disease: 153 subjects with atherothrombotic stroke (ATS) and 133 subjects with ischemic heart disease (IHD); and in two control groups, 103 newborns and 114 elderly subjects. *Results*: The $\varepsilon 4$ allele was associated with more severe carotid stenosis in the ATS group, being the percentages of $\varepsilon 4$ carriers 26.7% and 11.4% for the higher and lower carotid stenosis groups, respectively ($p = 0.066$). The −491 T/T IHD subjects presented higher vessel scores than subjects A/A and A/T genotypes at that position ($p = 0,041$), and the frequencies of $\varepsilon 2$ (5.1% versus 14.1%, $p = 0.060$) and −427C (10.3% versus 24.4%, $p = 0.019$) alleles were lower in IHD subjects with higher extent score versus lower extent score. The $\varepsilon 2$ allele was in linkage disequilibrium with the −427C allele in all studied groups, and the −219T allele was associated with the $\varepsilon 4$ allele in the IHD group. *Conclusion*: In summary, the $\varepsilon 2$ allele was in linkage disequilibrium with the −427C allele in all studied groups, and only slight associations between the analyzed APOE polymorphisms in the promoter and in the coding region and carotid and coronary vascular disease have been observed.

Keywords: Apolipoprotein E, atherosclerosis, atherothrombotic stroke, ischemic heart disease, polymorphisms

1. Introduction

Apolipoprotein E (apo E), a glycoprotein produced mainly by the liver but also by other peripheral cells such as macrophages [30,33], is a component of chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL) and high density lipoproteins (HDL) [30], and it plays a mayor role in lipoprotein metabolism and lipid transport. Several hepatic receptors recognize apo E, in whose organ it acts as a ligand for receptor-mediated clearance of lipoproteins. Apo E is synthesized endogenously by foam cells, enabling the cholesterol efflux from intima lesions via HDL [1].

The human *APOE* gene is located at chromosome 19, and three major codominant alleles exist: $\varepsilon 2$, $\varepsilon 3$ and $\varepsilon 4$; coding for three isoforms: E2, E3 and E4. The apo E4 isoform is associated with higher total cholesterol (TC) and LDL cholesterol (LDLC) levels compared to the

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apo E3 isoform [8,13]. Moreover, apo E4 may decrease HDL cholesterol (HDLC) and raise triglyceride (TG) levels [12]. It has been estimated that carriers of the $\varepsilon 4$ allele have a 1.4 fold higher risk of coronary heart disease than $\varepsilon 3$ carriers [13]. Consequently, apo E4 is associated with heightened risk of coronary disease. The risk attributable to apo E4 persists even after adjusting for other major risk factors [27]. Furthermore, the $\varepsilon 4$ allele has been associated with an increased risk of developing Alzheimer's disease [6,34].

Several single nucleotide polymorphisms (SNPs) have been described in the 5' regulatory region (-491 A/T, -427 T/C, -219 G/T and $+113$ G/C) of *APOE* gene [25]. These SNPs have been reported to affect the transcriptional activity of *APOE* promoter in vitro assayed in a human hepatoma cell line [24], and they have been associated with coronary artery disease risk [29], Alzheimer's disease [18] and Parkinson disease [28]. However, no studies have analyzed their association with ischemic stroke.

Our purpose was to assess the association of the -491A/T, -427 T/C and -219 G/T polymorphisms in the promoter region of *APOE* gene, and the *APOE* $\varepsilon 2/\varepsilon 3/\varepsilon 4$ polymorphisms, with atherosclerosis disease, by studying a group of subjects with ischemic stroke of atherothrombotic origin and a group of subjects with ischemic heart disease. Moreover, we analyzed the possible existence of linkage disequilibrium between the three polymorphisms in the promoter region of *APOE* gene and the common *APOE* $\varepsilon 2/\varepsilon 3/\varepsilon 4$ alleles.

2. Materials and methods

2.1. Subjects

The atherothrombotic stroke (ATS) group consisted of 153 non-related Spanish subjects younger than 71 years of age (61.7 ± 6.8 (mean $\pm$ SE)) with an ischemic stroke defined as an abrupt onset of a focal neurological deficit attributable to a cerebral infarct by occlusion or stenosis of atheromatous etiology in intracranial or extracranial arteries. TOAST criteria were considered for inclusion [10]. Exclusion criteria were cardioembolic, lacunar or undetermined strokes, and intracerebral haemorrhage.

The ischemic heart disease group (IHD) consisted of 133 non-related Spanish male subjects younger than 65 years of age (55.2 ± 7.3) with stable angina pectoris. Inclusion criteria were the diagnosis of ischemic heart disease by coronary arteriography with at least one of the following conditions in the angiographic scores: vessel score $\geqslant 1$, stenosis score $\geqslant 4$, and extent score $\geqslant 12$. Exclusion criteria were acute myocardial infarction, coronary by-pass surgery or coronary angioplasty. Basal clinical characteristics of both study groups have been previously described [19].

Two control groups of Spanish subjects were selected. One of them consisted of 103 non-related and anonymous newborns (newborn group). Newborn cord blood samples were obtained from consecutive live births at Hospital Universitario Miguel Servet and served as unscreened, population-based controls. The other control group consisted of 114 non-related subjects older than 65 years of age (73.2 ± 5.0) randomly selected from the local area in the region of Aragon (elderly group). Exclusion criteria were previous documented cerebrovascular or coronary heart disease.

All procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983. The ethics committee of the Hospital Universitario Miguel Servet approved the study and all subjects or their representatives gave written informed consent.

2.2. Samples

Venous blood samples from ATS, IHD and elderly subjects were collected in tubes containing K_3EDTA (to obtain genomic DNA) and in tubes containing SST clot activating gel (to obtain serum) after 12 h fasting. For the newborn group, umbilical cord blood was collected only in K_3EDTA tubes. In the ATS group, samples were collected within 48 hours after the clinical onset of the stroke. In IHD subjects, samples were collected before a programmed coronary arteriography out of the acute event.

Serum samples were allowed to clot, and afterwards, serum was separated by centrifugation at 4 °C for 15 minutes at 3500 rpm, aliquoted and immediately stored at -80 °C. Subsequent analysis of TC and TG was carried out by enzymatic methods with a Beckman Syncron CX7 autoanalyzer (Boehringer Mannheim). HDLC was measured after precipitation of apolipoprotein B-containing lipoproteins with Mg-phosphotungstate (Boehringer Mannheim), and LDLC was calculated using the Friedewald formula [35].

Genomic DNA was isolated from peripheral blood cells using the Puregene Isolation System (Gentra) in accord with the manufacturer's protocol. DNA was quantified and diluted to a final concentration of 100 ng/mL to be used in polymerase chain reaction (PCR) analysis.

2.3. APOE genotypes analysis

APOE coding polymorphisms were analyzed using the method described by Hixson and Vernier [16]. The −491 A/T, −427 T/C and −219 G/T polymorphisms in the promoter region of *APOE* gene were determined by nested PCR and digestion by *Dra I*, *Alu I* and *Taq I*, respectively, as previously described [25].

2.4. Assessment of the atherosclerotic lesion extent

Extension of atherosclerotic lesion in cerebral stroke was measured by duplex sonography combining continuous-wave Doppler and B-mode imaging to evaluate the degree of stenosis and the plaque morphology in common and internal carotids [17,31]. The extent of atherosclerosis was expressed as the stenosis grade on a 0–100% scale.

The extension of coronary atherosclerosis was evaluated in a blinded manner from results of coronary arteriographies. Three different scores were determined: (1) vessel score, the number of major vessels with significant coronary stenosis according to the BARI protocol [2]; (2) stenosis score, the sum of stenosis in 8 different proximal segments (stenosis >50% = 1, 50% to 74% = 2, 75% to 99% = 3 and total occlusion = 4) [11, 23,26]; and (3) extent score, the addition of segment longitudinal extension of all coronary lesions within the 8 proximal segments [5,32].

2.5. Statistical analysis

The χ^2 test was performed to assess the Hardy-Weinberg equilibrium of the studied polymorphisms in the four groups. Distribution of quantitative variables was tested for normality. Variables without a normal distribution were log-transformed before analysis. Quantitative variables were compared with ANOVA one factor adjusted for age, sex and body mass index (BMI). Stenosis score and its log-transformed variable were not normally distributed and, therefore, comparisons of this variable were made with non-parametric analysis, using the Mann-Whitney and Kruskal-Wallis non-parametric tests. Categorical variables were compared by χ^2 or Fisher's exact test. All statistical analyses were carried out using SPSS 6.1.3 statistical software package (SPSS Inc.).

Pairwise linkage disequilibrium between the *APOE* coding region polymorphisms and the −491, −427 and −219 regulatory region polymorphisms, haplotype estimation and expected frequencies were performed by the maximum likelihood method, using the 3Locus 5.0 program by Long et al. [15]. Logistic regression analyses were performed in order to evaluate the impact of the polymorphisms on atherosclerosis disease risk. A value of $p < 0.05$ was considered statistically significant for all the above analyses.

3. Results

3.1. APOE polymorphisms

The −491 A/T, −427 T/C and −219 G/T genotype and allele frequencies determined in the four groups are reported in Table 1. The observed genotype frequencies agreed with those expected according to the Hardy-Weinberg equilibrium. No significant differences in the distributions of the −491 A/T, −427 T/C and −219 G/T genotypes and alleles were observed between the ATS or IHD groups and the control groups. No homozygous C/C subject for the −427 T/C polymorphism was detected in any of the study groups.

The genotype and allele frequencies distribution for the coding polymorphisms is shown in Table 1. *APOE* genotype distribution is presented as $\varepsilon 3/\varepsilon 3$, $\varepsilon 2$ carriers and $\varepsilon 4$ carriers. Only two subjects, one in the ATS group and the other in the newborn group, presented the $\varepsilon 2/\varepsilon 4$ genotype. In the ATS group, one subject was a carrier of εR136S, a rare mutation of *APOE*, his genotype being $\varepsilon 4/\varepsilon$R136S. This mutated form was also identified in two subjects of the elderly group, whose genotypes were $\varepsilon 3/\varepsilon$R136S. The allelic frequencies of εR136S in the ATS and elderly groups were 0.003 and 0.009, respectively. One subject in the elderly group was a carrier of $\varepsilon\Delta$L149, another rare *APOE* mutation. The allelic frequency was 0.003. There were no differences either in *APOE* genotype or allelic distributions between cases and controls, as is shown in Table 1. However, it is worth emphasizing that the $\varepsilon 4$ allele frequency was 0.083 in the newborn group, higher than in the elderly group (0.061). Additionally, only the ATS group showed higher $\varepsilon 4$ allele frequency (0.098) than control populations, but without statistical significance.

3.2. Apo E and arterial atherosclerotic lesion extent

In order to investigate the possible effect of *APOE* promoter polymorphisms on atherosclerosic lesion extent, we compared the carotid stenosis grade and the coronary arteriographic scores in the ATS and IHD groups, respectively, according to genotype at −491,

Table 1
Comparison of genotypic and allelic distributions of the −491 A/T, −427 T/C, −219 G/T and *APOE* ε2/ε3/ε4 polymorphisms between cases and controls in this study

	Genotypes n (%)			P value vs elderly	P value vs newborn	Alleles				P value vs elderly	P value vs newborn
−491 A/T	**AA**	**AT**	**TT**			**A**	**T**				
ATS	94 (61.4)	48 (31.3)	11 (7.1)	0.258	0.461	0.771	0.229			0.201	0.228
IHD	93 (71.0)	32 (24.4)	6 (4.6)	0.402	0.712	0.832	0.168			0.662	0.641
Elderly	74 (66.0)	35 (31.3)	3 (2.7)			0.817	0.183				
Newborn	69 (67.0)	30 (29.1)	4 (3.9)			0.816	0.184				
−427 T/C	**TT**	**TC**	**CC**			**T**	**C**				
ATS	124 (81.0)	29 (19.0)	–	0.134	0.667	0.905	0.095			0.153	0.683
IHD	107 (81.7)	24 (18.3)	–	0.183	0.768	0.908	0.092			0.203	0.779
Elderly	101 (87.8)	14 (12.2)	–			0.939	0.061				
Newborn	84 (83.2)	17 (16.8)	–			0.916	0.084				
−219 G/T	**GG**	**GT**	**TT**			**G**	**T**				
ATS	38 (24.8)	82 (53.6)	33 (21.6)	0.748	0.151	0.516	0.484			0.465	0.474
IHD	46 (35.1)	62 (47.3)	23 (17.6)	0.487	0.430	0.588	0.412			0.378	0.395
Elderly	32 (28.1)	61 (53.5)	21 (18.4)			0.548	0.452				
Newborn	35 (34.0)	43 (41.7)	25 (24.3)			0.549	0.451				
APOE	**ε3/ε3**	**ε2/εX**	**ε4/εY**			**ε2**	**ε3**	**ε4**	**Others**		
ATS	110 (71.9)	15 (9.9)	26 (17.0)	0.369	0.870	0.056	0.843	0.098	0.003	0.152	0.795
IHD	102 (73.9)	12 (8.7)	18 (13.0)	0.813	0.691	0.046	0.886	0.068	–	0.807	0.567
Elderly	90 (78.3)	8 (7.0)	14 (12.2)			0.035	0.891	0.061	0.013		
Newborn	74 (71.8)	12 (11.7)	16 (15.5)			0.063	0.854	0.083	–		

εX: allele ε2 or allele ε3; εY: allele ε4 or allele ε3; ATS: atherothrombotic stroke group; IHD: ischemic heart disease group. The genotype and allele frequencies distributions were compared by χ^2 or Fisher's exact test. A value of $p < 0.05$ was considered statistically significant.

−427 and −219 positions, as Table 2 shows. In the ATS group, subjects with −427 T/C genotype had a higher carotid stenosis grade than −427 T/T subjects. This difference was not statistically significant, although it was close to significant ($p = 0.068$). In the IHD group, homozygous subjects for T allele at −491 position presented higher vessel score than subjects with A/A and A/T genotypes at that position, presenting differences that reached statistical significance ($p = 0.041$).

We also compared the carotid stenosis and the angiographic scores in ε2 and ε4 carriers versus ε3/ε3 subjects, as shown in Table 2. The ε4 carriers in the IHD group presented similar angiographic scores to the ε3/ε3 group, while the ATS ε4 carriers had a slightly and not statistically significant higher carotid atherosclerotic lesion extent than ε3/ε3 subjects (67.91 ± 5.30 and 54.96 ± 2.67, respectively; $p = 0.485$). On the other hand, ε2 carriers did not show differences on these atherosclerotic lesion extent values compared to ε3/ε3 subjects.

To assess the relationship between the studied *APOE* polymorphisms and the arterial atherosclerotic lesion extent, we divided the ATS and IHD subjects into two subgroups on the basis of whether their carotid stenosis or their angiographic scores were above or below the median. We analyzed the distribution of −491T, −427C, −219T, ε2 and ε4 carriers in these groups. In relation to the ATS group, the percentages of ε4 carriers were 26.7% and 11.4% for the higher and lower carotid stenosis groups, respectively. The presence of the ε4 allele increased the severe carotid stenosis risk in the ATS studied population (odds ratio 2.84, 95% CI 0.91 to 8.88, $p = 0.066$). With regard to the IHD subjects, the percentages of ε2 carriers were 5.1% and 14.1%, and the percentages of −427C carriers were 10.3% and 24.4%, in the groups of higher and lower extent score, respectively. Therefore, the frequencies of ε2 and −427C carriers were lower in the group of subjects with higher extent score. In fact, the presence of ε2 and −427C allele was associated with a decreased coronary extension in the IHD population (odds ratios: 0.33, 95% CI 0.10 to 1.09, $p = 0.060$, and 0.35, 95% CI 0.14 to 0.87, $p = 0.019$, respectively). On the other hand, the prevalence of −219T carriers was increased in the group of subjects with higher stenosis score values (69.9% vs 58.9%), but these differences were not significantly different (odds ratio 1.62, 95% CI 0.84 to 3.14, $p = 0.152$). Finally, the studied *APOE* allele distributions among subjects with lower and higher vessel score values were not significantly different.

In summary, ε4 was associated with severe carotid atherosclerosis in the ATS group. The −491 T/T genotype was associated with a higher vessel score, and frequencies of ε2 and −427C alleles were lower in the group of subjects with higher extent score in IHD group.

Table 2
Arterial atherosclerotic lesion extent and *APOE* polymorphisms in ATS and IHD groups

Group	ATS	IHD		
Genotype	Carotid extent+	Vessel score*	Stenosis score*	Extent score+
−491 A/A	56 ± 3.0	1.0 ± 0.1	9.7 ± 0.4	43 ± 1.9
−491 A/T	55 ± 3.2	0.9 ± 0.1	8.8 ± 0.7	43 ± 3.2
−491 T/T	52 ± 6.9	1.8 ± 0.4	10.8 ± 2.0	49 ± 10.0
P	0.876	0.086	0.494	0.698
−427 T/T	53 ± 2.4	1.0 ± 0.1	9.3 ± 0.9	38 ± 4.2
−427 T/C	64 ± 5.1	0.9 ± 0.1	9.5 ± 1.8	44 ± 1.7
P	0.068	0.928	0.671	0.133
−219 G/G	55 ± 4.2	1.1 ± 0.1	9.2 ± 0.6	42 ± 3.1
−219 G/T	53 ± 2.8	0.9 ± 0.1	9.4 ± 0.5	42 ± 2.1
−219 T/T	61 ± 4.9	1.3 ± 0.2	10.4 ± 1.0	45 ± 4.1
P	0.305	0.370	0.573	0.820
ε3/ε3	55 ± 2.7	1.0 ± 0.1	9.4 ± 0.4	44 ± 1.9
ε2/εX	53 ± 6.6	1.3 ± 0.3	10.1 ± 1.2	38 ± 5.5
ε4/εY	68 ± 5.3	0.9 ± 0.2	7.8 ± 1.1	32 ± 4.2
P (ε2/εX vs ε3/ε3)	0.780	0.166	0.611	0.276
P (ε4/εY vs ε3/ε3)	0.485	0.740	0.856	0.250

Values are indicates as mean ± S.E. εX: allele ε2 or allele ε3; εY: allele ε4 or allele ε3; ATS: atherothrombotic stroke group; IHD: ischemic heart disease group.
+Quantitative variables were compared with ANOVA one factor (carotid extent and extent score).
*Stenosis and vessel scores were quantitative variables not normally distributed, therefore comparisons of these variables were made with non-parametric test.
A value of $p < 0.05$ was considered statistically significant.

3.3. Linkage disequilibrium analysis

In order to characterize the association analysis of *APOE* polymorphisms, we analyzed the existence of linkage disequilibrium between the studied polymorphisms in the four studied groups. No linkage disequilibrium was found between the three promoter polymorphisms in the ATS ($p = 0.718$; 2 d.f.), IHD ($p = 0.803$; 2 d.f.), or elderly ($p = 0.392$; 2 d.f.) groups (data not shown). However, −491 A/T polymorphism was found to be in linkage disequilibrium with −219 G/T in the newborn group ($p = 0.023$; 2 d.f.). The allele −491T was associated with the −219T allele. On the other hand, −427 T/C polymorphism was in strong linkage disequilibrium with *APOE* ε2/ε3/ε4 polymorphisms in all studied groups: ATS, IHD, elderly and newborn, as is shown in Table 3. The ε2 allele was found preferably associated with the −427C allele in all groups ($p < 0.022$; 0.009; 0.049; 0.017 for ATS, IHD, elderly and newborn, respectively). In the IHD group, the −219 G/T polymorphism was also found in linkage disequilibrium with *APOE* coding polymorphisms. The −219T allele was associated with the ε4 allele (expected and estimated frequencies, 0.0275 and 0.0461, respectively; χ^2 (2 d.f.) = 8.50; $p = 0.014$). However, this linkage disequilibrium was not observed in the other studied groups.

To sum up, the ε2 allele was associated with the −427C allele in all studied groups, and the −219T allele was preferably associated to the ε4 allele in the IHD group.

3.4. Predictors of atherosclerosis disease

Because of the linkage disequilibrium between ε2 and −427C, we considered whether the haplotype ε2/−427C might have a protective role in atherosclerosis disease. However, we did not observe significant differences between cases and controls. The same analysis was carried out with the haplotype ε4/−219T between IHD and controls groups to determine whether it might be a predictor of atherosclerosis disease, but no significant differences were observed.

Furthermore, to determine if ε4 and −427C alleles are independent predictors of atherosclerosis disease in the ATS group, we performed a multivariate logistic regression analysis. However, neither ε4 nor −427C alleles were independently predictors of atherosclerosis disease (odds ratios: 1.54, 95% CI 0.76 to 3.11, $p = 0.231$, and 1.74, 95% CI 0.87 to 3.48, $p = 0.118$, respectively). The same analysis was performed in the IHD group in order to analyze whether ε2, −427C and −219T alleles were predictors of atherosclerosis disease. Neither ε2 nor −427C nor −219T alleles were independent predictors of atherosclerosis disease (odds ratios: 0.99, 95% CI 0.36 to 2.69, $p = 0.979$, 1.60, 95% CI 0.76 to 3.37, $p = 0.220$, and 0.75, 95% CI 0.43 to 1.30, $p = 0.300$, respectively).

Table 3
APOE $\varepsilon 2/\varepsilon 3/\varepsilon 4$ / -427 T/C expected and estimated haplotype frequencies in cases and controls in this study

Haplotype	ATS		IHD		Elderly		Newborn	
	Expected frequencies	Estimated frequencies	Expected frequencies	Estimated frequencies	Expected frequencies	Estimated frequencies	Expected frequencies	Estimated frequencies
ε 2/-427 T	0.051	0.029	0.042	0.019	0.034	0.024	0.059	0.037
ε 3/-427 T	0.768	0.792	0.805	0.828	0.845	0.851	0.780	0.795
ε 4/-427 T	0.086	0.084	0.068	0.068	0.059	0.062	0.077	0.083
ε 2/-427 C	0.005	0.027	0.004	0.027	0.002	0.012	0.005	0.028
ε 3/-427 C	0.081	0.057	0.075	0.052	0.056	0.051	0.072	0.057
ε 4/-427 C	0.009	0.011	0.006	0.006	0.004	0.000	0.007	0.000
	χ^2 (2 df) = 12.26, $p = 0.022$		χ^2 (2 df) = 15.16, $p = 0.009$		χ^2 (2 df) = 6.01, $p = 0.049$		χ^2 (2 df) = 8.08, $p = 0.017$	

ATS: atherothrombotic stroke group; IHD: ischemic heart disease group; df: degree freedom.
Haplotype estimation and expected frequencies were performed by the maximum likelihood method using the 3Locus 5.0 program.

4. Discussion

In this study, we have evaluated the influence of the *APOE* promoter and coding region SNPs in atherosclerosis disease. Our study is the first to analyze these polymorphisms in atherothrombotic stroke. However, in the domain of coronary heart disease and *APOE* promoter polymorphisms, two previous studies have been reported [14,29]. We carried out a precise selection of the patients included in the study to ensure that all of them had atherosclerosis disease. Especially in the stroke group, where the etiology may be very heterogeneous, we selected only those subjects with specific criteria of atherothrombotic cerebrovascular disease, excluding other possible etiologies. Moreover, the atherosclerotic lesion extent was quantified in all selected subjects as case groups. The importance of our study is that we have related carotid stenosis in the ATS group and the angiographic scores of the coronary arteries in IHD patients with genetic variations.

The allelic distributions of the *APOE* regulatory region polymorphisms in our sample were similar to those previously reported in a Spanish healthy group by Artiga et al. [25]. *APOE* coding region polymorphisms also had similar frequencies to those reported in previous studies in several regions of Spain [3,4,21]. In order to have a control group representative of the general population, we selected non-related consecutive newborns from Hospital Universitario Miguel Servet. The $\varepsilon 4$ allele frequency was 0.083 in the newborn group, higher than in the elderly group (0.061), probably due to the morbi-mortality associated with the $\varepsilon 4$ allele. Along this line, previous studies have demonstrated associations of $\varepsilon 4$ allele with coronary heart disease [7,29], stroke [20,36], and calcific valvular heart disease [9]. However, in the present study, only the atherothrombotic stroke group showed higher $\varepsilon 4$ allele frequency than control Spanish populations (0.098), but without statistical significance, and the $\varepsilon 4$ allele was associated with more severe carotid stenosis in ATS group. We identified four subjects with rare mutations of *APOE* in the elderly and ATS groups. These variants seem to be frequent in our region, as previous studies have reported [22].

An excess of -427C allele was observed in the ATS and IHD groups with respect to the elderly group, but this trend did not reach statistical significance. This allele was in strong linkage disequilibrium with the $\varepsilon 2$ allele in all the studied groups, in accordance with results reported by Corbo et al. [29]. The IHD subjects showed an inverse relationship between -427C allele and the lesion severity when this was evaluated by the extent score. In contrast, Corbo et al. found that this allele could be considered a risk factor for developing atherosclerosis [29].

The -491 A/T and -291 G/T polymorphisms presented a similar allele and genotype distribution in the ATS, IHD and control groups, suggesting an unimportant role in atherosclerosis. These results are in accordance with previously published case-control studies in Italy [29], but in contrast with the French study [14], in which the -219T allele was associated with a significantly increased risk of myocardial infarction.

In summary, the $\varepsilon 4$ allele was associated with severe carotid atherosclerosis in the ATS group. The -491 T/T genotype was associated with a higher vessel score and frequencies of $\varepsilon 2$ and -427C alleles were lower in the group of subjects with higher extent score in the IHD group. The $\varepsilon 2$ allele was in linkage disequilibrium with the -427C allele in all studied groups, and the -219T allele was associated with the $\varepsilon 4$ allele in the IHD group.

Our findings, however, must be interpreted with caution because any results did not reach the statistical significance, and therefore, further studies with larger numbers of subjects are needed to confirm our findings.

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