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Different Apolipoprotein E Polymorphisms Are Not Associated with Different Carotid Intima-Media Thickness Values in a Sample of Spanish General Population

Pilar Calmarza [1,*], José María Trejo [2], Carlos Lapresta [1], María Fernández [3], Pilar López [2]

[1] Hospital Universitario Miguel Servet, Zaragoza, Spain
[2] Complejo Asistencial, Burgos, Spain
[3] Hospital Santiago Apóstol, Miranda de Ebro, Burgos, Spain

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ABSTRACT

Background: Apolipoprotein E (Apo E) is a key factor in the atherosclerosis process, but the influence of its genetic polimorphisms in southern Europe populations is not well known.
Objectives: The present transversal, population-based study aimed to determine if Apo E genetic polymorphism is associated with early atherosclerosis, as assessed by carotid Intima-Media Thickness (cIMT), or with presence of carotid atherosclerotic plaques.
Patients and Methods: The study sample included 171 individuals older than 40 years who were randomly selected from the population assigned to a district medical center of Burgos, Spain. The proportion of males and females in the sample and their ages matched the adult Spanish general population. cIMT and number of carotid atherosclerotic plaques were determined to assess if higher values were associated with a particular ApoE genetic polymorphism.
Results: Neither cIMT nor the number of atherosclerotic plaques differed among the subjects with Apo E2, E3, and E4 genotypes. However, both total cholesterol and Low Density Lipoprotein (LDL)-cholesterol were higher in the E4 than in the E2 group.
Conclusions: In this sample of adult Spanish population, no association was found between Apo E genotypes and cIMT.

►*Implication for health policy/practice/research/medical education:*
The novelty at this project lies in being a study on the role that Apolipoprotein E genotypes play in carotid intima media thickness, the most accepted marker of early atherogenesis. Apolipoprotein E represents a key factor in the atherosclerosis process. Apolipoprotein E is a component of some plasma lipoproteins and acts as LDL receptor ligand as well as ligand of chylomicron remnants.

1. Background

As atheroma plaques begin to develop in the arterial intima, increased carotid Intima-Media Thickness (cIMT) is an accepted marker of early atherosclerosis and has been reported to be associated with cardiovascular events in both transversal and longitudinal studies (1).

Although some recently published studies are slightly critical about the value of cIMT in cardiovascular risk prediction (2, 3), it has been found particularly useful in intermediate-risk individuals, leading to an improvement in their reclassification (4).

Apolipoprotein E (Apo E) can influence atherosclerosis because it is a component of some plasma lipoproteins and acts as Low Density Lipoprotein (LDL) receptor ligand (5) as well as ligand of chylomicron remnants.

Different Apo E polymorphisms can have variable effects on vessel wall homeostasis functions, such as platelet aggregation, smooth muscle cells proliferation, and lymphocytes migration. Apo E glycoprotein has three polymorphic forms, namely E2, E3, and E4, with Apo E3 being the most widespread (6). These three polymorphic forms are the result of three independent alleles acting at a single locus of the Apo E gene which is located on chromosome 19 and contains four exons which encode the 299 amino acids of Apo E. Expression of each of the three

*Corresponding author: Pilar Calmarza, Avda. Cesáreo Alierta, nº 38 - Esc 1ª 5º A, Zaragoza, Spain, Post code: 7193711351, Tel: +34-976765500,
E-mail: mpcalmarza@salud.aragon.es

alleles results in 3 homozygous (apo E2/E2, E3/E3, and E4/E4) and three heterozygous genotypes (apo E3/E2, E4/E3, and E4/E2). Given the low frequency of some Apo E genotypes, they are designated as E2 (E2/E2, E2/E3), E3 (E3/E3), and E4 (E4/E4, E3/E4). These three isoforms differ in the substitution of amino acids at positions 112 and 158. Apo E3, the predominant isoform, is characterized by containing cysteine at position112 and arginine at position 158. On the other hand, Apo E2 has cysteine at both positions and Apo E4 contains arginine at both sites.

Although Apo E polymorphism is considered as a genetic risk factor for coronary heart disease (7, 8), studies examining its association with different cIMT values have not been conclusive (9, 10). Apo E polymorphism has been reported to be associated with different lipid parameters, E2 allele with lower and E4 with higher total and LDL-cholesterol (11-16), but different studies have shown the effects of this polymorphism on Triglyceride (TG) and High Density Lipoprotein (HDL) cholesterol levels (17).

2. Objectives

The present study aims to determine if a particular Apo E genotype is associated with the early atherosclerosis marker, cIMT, or with presence of carotid atherosclerotic plaques in a randomized sample of Spanish general population.

3. Patients and Methods

3.1. Study Population

The subjects were selected randomly from the registry of the 200,000 referral population of "Gamonal Antigua" Health Care Centre in Burgos, Spain. The individuals were enrolled into the study in case they were older than 40 years and their age and gender distribution matched the 2004 - 2010 Spanish population census. On the other hand, the subjects with major cardiovascular events (myocardial infarction, stroke, and limb amputation due to peripheral artery disease) were not included in the study.

A 180-subject sample size was calculated to detect a 0.2 mm difference from normal cIMT (0.65 +/- 0.15 mm) with alpha error of 0.05. However, 9 participants were excluded from the study, leaving 171 subjects valid for analysis. Yet, Apo E polymorphisms were determined in the first 158 individuals due to economic constraints.

The researchers recorded the main cardiovascular risk factors; i.e., total, HDL, and LDL-cholesterol, TG, blood pressure (blood pressure above 140/95 mmHg in the mean of two readings or current antihypertensive treatment were considered as hypertension), Body Mass Index (BMI), hip-waist ratio, and the demographic data. In addition, each participant completed a questionnaire on one's current and past personal and familial cardiovascular history, consumption or non-consumption of alcohol and tobacco, socio-demographic variables, diabetes mellitus, and other previous diseases.

Written informed consents for taking part in the study were obtained from all the participants and the study was approved by the Clinical Studies Committee of the referral hospital. Also, the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

3.2. Measurements Method

Total cholesterol and TG levels were determined enzymatically (Roche Diagnostics Basel, Switzerland), while LDL-cholesterol level was assessed using the Friedewald formula. All the lipids were expressed in mmol/L. Ultrasensitive C-Reactive Protein (UsCRP) was evaluated through particle enhanced inmunoturbidimetric assay (Tina Quant C-reactive protein latex ultrasensitive assay, Roche diagnostics) and homocysteine was assessed using Fluorescence Polarization Immunoassay (Architect, Abbott Laboratories).

3.3. Genotyping of Apo E

Apo E alleles were determined by reverse hybridization. In doing so, genomic DNA was extracted from EDTA anticoagulated whole blood (DNA extraction kit, Genomic DNA Purification System, Promega, USA) and was amplified by Polymerase Chain Reaction (PCR) in a thermocycler (Techne). Then, the amplified product was visualized by gel electrophoresis on 2% agarose to which ethidium bromide was added. Afterwards, the biotinylated and amplified DNA was hybridized with specific oligonucleotides immobilized on nitrocellulose strips. Then, streptavidin labelled with alkaline phosphatase, which binds to any biotinylated and hybridized product formed previously, was added. Incubation with chromogenic BCIP / NBT results in a brown-purple precipitate. This was carried out using Inno Lipa Apo E commercial kit (Innogenetics).

3.4. Carotid Intima-Media Thickness Determination

cIMT was determined by B-mode ultrasound (HP lmage Point equipment with a 10 MHz linear probe) in the far wall of both left and right common carotid arteries, 1 cm proximal to its bifurcation.

The subjects lay in supine position with the neck in a neutral position and the probe was applied to it, parallel to its longitudinal axis in an anterolateral plane (60º angle, with 0º being the horizontal axis). Each cIMT measurement was performed twice in both the left and right carotids of each subject, and the average of the right and left cIMTs was calculated. The measurements were performed blind to the rest of the data by the same investigator who had previous experience in making these measurements in all the segments of both common carotids, carotid bifurcations, and external and internal carotid arteries accessible to ultrasound. An atheroma plaque was defined echographically as a hyper-echogenicity or any protrusion of the intima-media in the vascular lumen of at least twice the thickness of the adjacent segment.

3.5. Statistical Analysis

Depending on their distributions, means and Standard Deviations (SD) were calculated for all the variables, except for age, TG, glucose, UsCRP, and cIMT where medians and

Table 1. The Association between Apo E Genotypes and cIMT

Mean Difference of Average IMT of Both Carotid Arteries	Univariate Analysis		Multivariate Analysis [a]		Multivariate Analysis [b]	
	B	95% CI	B	95% CI	B	95% CI
E3 vs. E2	0.042	-0.040 - 0.125	0.017	-0.044 - 0.078	0.009	-0.055 - 0.072
E2 vs. E4	0.024	-0.027 - 0.076	0.023	-0.020 - 0.066	0.004	-0.045 - 0.038
E3 vs. E4	0.006	-0.058 - 0.070	0.035	-0.013 - 0.083	0.036	-0.013 - 0.085

Abbreviations: Chol, cholesterol; LDL, low-density lipoprotein; Apo B, Apolipoprotein B; B, beta regression lineal coefficient; CI, confidence interval of the B coefficient.
[a] Adjusted for age and sex ;[b] Adjusted for age, sex, LDL-cholesterol, and Apo B levels

Table 2. Anthropometric Parameters of Different Apo E Genotypes

	Apo E Genotypes			P value
	E2/2 and E2/3	E3/3	E4/3 and E4/4	
BMI (kg/m2) [a]	28.69 ± 5.68	28.18 ± 3.51	28.39 ± 3.19	0.866
Age (years) [b]	62 (17)	64.5 (21)	60 (20)	0.441
Waist to hip ratio [a]	1.33 ± 0.14	1.38 ± 0.17	1.41 ± 0.18	0.416
SBP (mmHg) [a]	136.67 ± 10.56	142.86 ± 17.62	138.07 ± 20.44	0.241
DBP (mmHg) [a]	78.07 ± 13.19	80.94 ± 10.36	79.72 ± 10.30	0.585
Smoking	40%	32%	40%	0.562
Alcohol abuse	20%	28%	40%	0.758

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.
[a] The results are expressed as mean ± standard deviation, [b] The results are expressed as median (interquartile range).

Table 3. Serum Levels of Lipid Parameters in Different Apo E Genotypes

	Apo E Genotypes			P value
	E2/2 and E2/3	E3/3	E4/3 and E4/4	
Total Cholesterol (mmol/L) [a]	5.17 ± 0.89 [c]	5.53 ± 1.01	5.78 ± 0.96	0.147
Triglycerides (mmol/L) [b]	1.34 (0.63)	1.28 (1.10)	1.40 (0.76)	0.828
HDL-cholesterol (mmol/L) [a]	1.46 ± 0.35	1.50 ± 0.38	1.46 ± 0.37	0.851
LDL-cholesterol (mmol/L) [a]	3.10 ± 0.82 [c]	3.45 ± 0.90	3.67 ± 0.84	0.121
Apolipoprotein A (mg/dL) [a]	161.6 ± 37.52	167.87 ± 31.09	164.9 ± 38.79	0.750
Apolipoprotein B (mg/dL) [a]	116.4 ± 34.28	113.22 ± 29.17	123.33 ± 28.54	0.253
Lipoprotein (a) (mg/dL) [a]	29.93 ± 33.43	23.10 ± 23.77	28.17 ± 25.05	0.437
Us CRP (mg/L) [b]	2.4 (2)	2.0 (3.7)	1.3 (3.1)	0.575
Homocysteine (mmol/L) [a]	13.27 ± 3.43	13.29 ± 3.53	13.00 ± 3.14	0.923

[a] The results are expressed as mean ± standard deviation, [b] The results are expressed as median (interquartile range), [c] Compared with E4 allele carriers, $P < 0.05$

interquartile ranges were calculated. The relationship between Apo E genotypes and cIMT was examined using univariate and multivariate linear regression analysis (Table 1). The rest of the variables were analyzed through Analysis of Variance (ANOVA) and Kruskal Wallis (if continuous) or chi-square test (if categorical) (Tables 2 and 3). Moreover, multivariate logistic regression analysis was used to assess the association between Apo E isoform groups and presence of atherosclerotic plaques. All the analysis were performed using the SPSS statistical software, version 15.0 and $P < 0.05$ was considered as statistically significant.

4. Results

The clinical characteristics and lipoprotein parameters of the study population have been presented in Table 4. The participants' age ranged from 44 to 93 years (M = 64.2, SD = 12 years) and 47.9% were female.

The mean cIMT of both carotid arteries (overall mean cIMT) ranged from 0.53 to 1.27 mm (median = 0.74, interquartile range = 0.22) and 41 individuals (24%) had one or more plaques. In this study, the patients were divided into three groups according to Apo E genotype: E2 (E2/E2, E2/E3), E3 (E3/E3), and E4 (E4/E4, E4/E3). The only patient with E2/4 genotype was excluded for the analysis. As expected, E3 was the most prevalent genotype (N = 112, 71.3%) followed by E4 (N = 30, 19.1%) and E2 (N = 15, 9.6%). The results revealed no significant differences among Apo E genotype groups regarding age, BMI, waist-to-hip ratio, alcohol consumption, smoking status, Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP) (Table 2), TG, Apolipoprotein A and B, Lipoprotein (a), Homocysteine, and UsCRP. However, total and LDL-cholesterol levels were higher in the ApoE4 (5.78 mmol/L and 3.67 mmol/L respectively) than in the Apo E2 group (5.17 mmol/L and 3.10 mmol/L, respectively) ($P < 0.05$) (Table 3).

Considering the main objective of this study, the results showed no significant difference among E3 (0.771 ± 0.154

Table 4. Anthropometric and Lipoprotein Parameters

Variables	N	Descriptive Statistics [a]
Age (years)	171	63 (20)
Weight (kg)	171	72.1 ± 11.8
BMI (kg/m2)	170	28.1 ± 3.7
Waist to hip ratio	130	1.4 ± 0.2
CVD personal history	170	18 (10.5%)
Smokers	170	57 (33.5%)
SBP (mmHg)	170	140.7 ± 17.6
DBP (mmHg)	169	80.4 ± 10.5
Total cholesterol (mmol/L)	171	5.53 ± 1.06
HDL cholesterol (mmol/L)	171	1.51 ± 0.40
LDL cholesterol (mmol/L)	171	3.43 ± 0.89
Triglycerides (mmol/L)	168	1.08 (0.62)
Fasting glucose (mmol/L)	171	5.2 (0.9)
UsCRP (mg/L)	141	1.8 (2.8)
Homocystein (mmol/L)	144	13.34 ± 3.50
Right mean cIMT (mm)	169	0.73 (0.23)
Left mean cIMT (mm)	169	0.76 (0.22)
Overall mean cIMT (mm)	169	0.74 (0.22)
Subjects with at least one plaque	171	41 (24%)

Abbreviations: N, number of subjects; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CVD, cardiovascular disease; HDL, high density lipoprotein; LDL, low density lipoprotein; cIMT, carotid intima-media thickness.
[a] Nominal variables are expressed as percentages and quantitative variables are expressed as mean ± standard deviation, except for age, triglycerides, glucose, UsCRP, and cIMT which are expressed as median (interquartile range).

Table 5. The Association between Apo E Genotypes and Presence of Atherosclerotic Plaques in Carotid Arteries

Presence of One or More Atherosclerotic Plaques	OR (95% CI)	OR (95% CI) Adjusted for Age and Sex	OR (95% CI) Adjusted for Age, Sex, LDL-Cholesterol, and Apo B Level
E3 vs. E2	0.214 (0.027 - 1.704)	0.251 (0.028 - 2.204)	0.234 (0.026 - 2.098)
E2 vs. E4	0.196 (0.026 - 2.114)	0.229 (0.025 - 2.113)	0.308 (0.031 - 3.065)
E3 vs. E4	1.095 (0.424 - 2.826)	0.839 (0.300 - 2.345)	0.088 (0.312 - 2.508)

Abbreviations: Chol, cholesterol; LDL, low-density lipoprotein; Apo B, Apolipoprotein B; OR, odds ratio; CI, confidence interval

mm), E4 (0.777 ± 0.171 mm), and E2 (0.729 ± 0.138 mm) groups regarding cIMT. Adjustment for age, gender, LDL-cholesterol, and Apolipoprotein B also did not change these results (Table 1). Moreover, the presence of one or more plaques was similar in ApoE3, ApoE2, and ApoE4 groups before and after adjusting for the above-mentioned factors (Table 5).

5. Discussion

Apo E4 allele has been considered as a risk factor for advanced atherosclerosis such as found in coronary artery disease (16, 18, 19). Besides, some studies have suggested that E2 allele protects against myocardial infarction (16, 19, 20). Nonetheless, the results concerning Apo E genotype and early carotid atherosclerosis remain controversial. Indeed, higher cIMT values have been found in Apo E2 (21, 22), Apo E3/E3 homozygous (23), and Apo E4/E4 homozygous (24) genotype individuals. The results are also varied depending on the country, gender, and age subgroups. cIMT values increased in the North American and Australian Apo E4/E4 men in ARIC and CUDAS studies (25, 26). However, no such differences were found among the genotype subgroups in Rotterdam study conducted on Central European elderly participants (27). Up to now, only few studies have been performed in this area in Southern-European countries, such as Spain. Yet, the current study results were consistent with those found in a Scandinavian study on young men and women, indicating that Apo E4 genotype was associated with higher levels of atherogenic LDL-fraction but not with early atherosclerosis as assessed by cIMT (28). Similarly, the studies carried out in Central-Southern France (29) and Japan (30) showed that Apo E4 genotype had no effects on cIMT. This also holds true for the conditions associated with accelerated atherosclerosis, such as diabetes and chronic renal failure (30, 31).

Moreover, it is still unsettled whether Apo E4 genotype is associated (32) or, in agreement with our findings, is not associated with carotid atherosclerotic plaques (28). The striking discrepancy of the deleterious role of Apo E4 in cardiovascular events associated with advanced atherosclerosis with its neutral influence on early atherosclerosis, as assessed through cIMT, has not been resolved. Apo E genotype can influence the arterial wall directly or through atherogenic compounds, such as LDL-cholesterol. The findings of the current study demonstrated higher levels of LDL-cholesterol in the subjects with E4 allele compared to those with Apo E2 allele, which is consistent with the results of another larger longitudinal study on asymptomatic subjects (33). In contrast to that study, however, ApoE groups were

not different regarding HDL-cholesterol in our sample. Apo E4 genotype could also exert its influence in the late stages of atherosclerosis or in the coagulation cascade, leading to cardiovascular events.

In spite of the small sample size, our study expanded the general finding of ineffectiveness of Apo E genotype in cIMT to a representative sample of the general population of Spain, as a southern European country.

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Authors' Contribution

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