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# Lack of association between carotid intima-media thickness and apolipoprotein (a) isoforms in a sample of Spanish general population

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ABSTRACT

*Objectives:* The purpose of this study was to examine the relationship between apolipoprotein (a) isoforms and early atherosclerosis, assessed by carotid artery intima-media thickness in a sample of adults of the general population of Burgos, a city in the north of Spain.
*Design and methods:* Lipids, lipoprotein (a), number of carotid atherosclerotic plaques, if any, and the intima-media thickness in the far wall of both common carotid arteries by B-mode ultrasound were determined in a group of 171 adults from the general population of Burgos, Spain. Apolipoprotein (a) isoforms were determined in a random subset of 119 subjects.
*Results:* Increasing age, male sex, and past personal cardiovascular history were significantly associated with increased left, right, or average intima-media thickness of both carotid arteries in multivariate analysis.

No statistically significant association was found between apolipoprotein (a) isoforms and mean carotid intima-media thickness by bivariate or multivariate regression analysis.
*Conclusions:* In this sample of the general Spanish population, no association was found between apolipoprotein (a) isoforms and carotid artery intima-media thickness.


## Introduction

Intima-media thickness (IMT) is an echographic measurement that estimates both inner arterial layers (intima and media) where atheroma begins, and therefore it is considered a good measure of atheroma in its initial stages. Both carotid atherosclerotic plaques and increased common carotid artery IMT are associated with a higher risk of ischemic stroke, presence and severity of coronary artery disease, and cardiovascular mortality [1–4]. A review of eight epidemiological studies showed that the IMT of the common carotid artery had independent predictive power with respect to cardiovascular events [5].

Lipoprotein (a) [Lp (a)], an emerging risk factor for cardiovascular disease, is a cholesterol rich lipoprotein composed of a low-density lipoprotein (LDL) particle and a highly polymorphic apolipoprotein (a) [Apo (a)], which is covalently linked to the apolipoprotein-B moiety of the LDL by a single disulphide bridge.

Apo (a) shares a striking structural similarity to plasminogen [6] and contains multiple repeated copies of a sequence that closely resembles plasminogen kringle 4 (KIV), followed by two sequences homologous to kringle V and serine protease domains of plasminogen, respectively.

The KIV-like sequences can be classified into 10 types, based on amino acid sequence. Each Apo (a) molecule contains a single copy of KIV types 1 and 3–10, but variable identical repeats (3 to more than 40) of kringle IV type 2, thus leading to different Apo (a) isoforms sizes [7,8], with molecular weights ranging from 280 to 800 kDa.

The concentration of Lp (a) is controlled by a single gene in the region 6q26-27 with multiple alleles, and each allele influences the concentration of Lp (a) differently [9], according to the number of expressed kringle IV repeats [10,11]. Other sequence variations of the Apo (a) locus may also influence Lp (a) plasma levels.

Lp (a) phenotypes have different functional properties as regards lysine binding affinity, the small Apo (a) isoforms showing the greatest affinity for fibrin [12], suggesting that the low molecular weight (LMW) Apo (a) isoform's size itself plays a role in atherogenesis [12].

Although a relationship has frequently been reported between Apo (a) isoforms with less than 22 KIV repeats and cardio- or cerebro-vascular diseases [13–15], few studies have investigated the relationship between phenotypes of Apo (a) and carotid artery IMT. In this study the association between Apo (a) isoforms and

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carotid IMT in a group of individuals from the general population of Burgos, northern Spain was studied.

## Materials and methods

### *Population studied*

This study was performed on 171 subjects randomly selected from the records of the 200,000 referral population of "Gamonal Antigua" Health Care Center of the city of Burgos, and their age and sex percentage distribution were those of the Spanish population in the 2004–2010 census. A sample size of 180 individuals was calculated in order to have an alpha error of 0.05, a power of 60% to detect a 0.2 mm difference from a normal IMT of 0.65 mm ± 0.15 mm and a 10% of expected losses of recruited participants. After data collection, losses were less than predicted, 9 participants, thus leaving 171 subjects valid for analysis.

Apo (a) isoforms were determined in a subset of 119 randomly selected subjects due to economic limitations. Lp (a) levels and established cardiovascular risk factors, such as blood pressure, total cholesterol, high-density lipoprotein (HDL)-cholesterol, LDL-cholesterol, and triglycerides were also determined.

Blood pressure was assessed by the mean of two systolic and diastolic readings after 10 min of rest in the supine position. Hypertension was defined as a systolic/diastolic blood pressure > 140/95 mm Hg or current use of antihypertensive drugs.

Body mass index (BMI), waist-to-hip ratio, and demographic data were also recorded. A standardized questionnaire on current and past personal or familial cardiovascular history, alcohol or tobacco consumption, sociodemographic variables, diabetes mellitus, or other previous disease was completed by each participant.

Informed consent for the procedures to be used was obtained from each subject. The study was approved by the Clinical Studies Committee of the referral hospital.

### *Analytical methods*

Venous blood samples were obtained under standardized conditions and after 12 or more hours of fasting. Total cholesterol and triglycerides where determined enzymatically (Roche Diagnostics, Basel, Switzerland), LDL-cholesterol using the Friedewald formula, and very low-density lipoprotein (VLDL)-cholesterol by dividing the triglyceride concentration by 2.18. Both LDL- and VLDL-cholesterol were calculated in mmol/l.

Lp (a) in serum was quantified by a commercially available rate nephelometry method (Siemens, Marburg, Germany) with an analytical sensitivity of 0.002 g/l, no cross-reactivity with apolipoprotein B (<1%), a <5% cross-reactivity with plasminogen, and minimally affected by Apo (a) isoforms size heterogeneity [16].

Apo (a) phenotypes were determined by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and immunoblotting by a method modified from that described by Utermann et al. [17] according to Kraft et al. [18].

The samples were prepared by mixing 10 μl of serum with 85 μl of a buffered solution and 5 μl of β-mercaptoethanol. Aliquots of samples were then subjected to SDS PAGE in a 4–15% gradient polyacrylamide gel. The separated proteins were transferred to a nitrocellulose membrane and antigens were visualized on the nitrocellulose membrane by a double-antibody procedure. The first antibody was a polyclonal antihuman Lp (a), which is raised in sheep. The second antibody was an anti-sheep immunoglobulin G antibody conjugated to alkaline phosphatase. When the appropriate substrate was applied the bands became visible.

The different isoforms of Lp (a) were designated according to their electrophoretic mobility on SDS PAGE relative to a mix of five recombinant Apo (a) isoforms containing kringles 35, 27, 23, 19, and 14 KIV repeats that was used as standard reference.

The Apo (a) phenotypes were stratified into 2 subgroups according to the molecular weight of the smaller Apo (a) isoform, the LMW Apo (a) phenotype was determined by the occurrence of at least 1 Apo (a) isoform with 11–22 KIV repeats, and the high molecular weight (HMW) Apo (a) phenotype when all isoforms had >22 KIV repeats as they are conventionally designated [19].

### *Intima-media thickness determination*

IMT was determined by B-mode ultrasound in the far wall of the left and right common carotid arteries, 1 cm proximal to its bifurcation. The ultrasound equipment was an HP Image Point (Hewlett-Packard, Palo Alto, CA, USA) with a 10 MHz linear probe that was placed on the neck of the subjects who lay supine, parallel to its longitudinal axis, in an anterolateral plane (60° angle, with 0° being the horizontal). Each IMT measurement was performed twice in both the left and right carotid of each subject, and the average right and left carotid IMT calculated. Measurements were conducted blind to the rest of the data, by the same investigator. An atheroma plaque was defined echographically as a hyperechogenicity or protrusion in the vascular lumen of the intima of at least twice the thickness of the adjacent intima media.

### *Statistical analysis*

For each variable, the validity of assumption of normality was checked. This assumption held true for all variables, except for IMT, waist-to-hip ratio and Lp (a) concentration, in which results are expressed as medians and interquartile ranges (IR). In the rest, results are expressed as means ± standard deviation (SD).

To assess the relationship between IMT and Lp (a), a bivariate linear regression analysis was performed. The same analysis of IMT and secondary independent variables such as age, BMI, waist-to-hip ratio, systolic blood pressure, HDL-cholesterol, LDL-cholesterol, plasma triglycerides, and fasting glucose followed. To determine the association of Lp (a) and IMT after adjusting for secondary independent variables, a multivariate linear analysis was performed in which nominal variables such as gender and current or past personal or familial cardiovascular history were included as dummy variables.

IMT, age, Lp (a), and conventional analytical or anthropometric cardiovascular risk factors were compared between >22 k4 and ≤22 k4 repeats groups, using the Student's *t*-test when normally distributed or if not by the Mann–Whitney test as in the case of IMT, Lp (a), and waist-to-hip ratio.

We examined the association between IMT and Lp (a) carrying differently sized Apo (a) isoforms using multivariate linear regression analysis.

The Spearman's rank correlation coefficient was used to measure the statistical dependence between the molecular weight of the different isoforms of Apo (a) and the mean of the right and left IMT.

To determine the combined effect of increased Lp (a) concentrations and LMW, Apo (a) phenotypes were stratified into 4 groups according to Lp (a) concentrations (cut-off point 300 mg/l) and Apo (a) phenotypes (cut-off point 22 KIV repeats). All *p*-values less than 0.05 were considered to indicate statistical significance.

Statistics were calculated using the program SPSS 15 (SPSS Inc., Chicago, IL, USA).

## Results

Anthropometric and lipoprotein parameters are shown in Table 1. Lp (a) ranged from 10 to 1060 mg/l (IR = 307.5) with a

**Table 1**
Anthropometric and lipoprotein parameters.

| Variables | *N* | Descriptive statistics[a] |
|---|---|---|
| Age (years) | 171 | 63 (20) |
| Weight (kg) | 171 | 72.11 ± 11.80 |
| BMI (kg/m$^2$) | 170 | 28.13 ± 3.69 |
| Waist to hip ratio | 130[b] | 1.4 ± 0.2 |
| CVD personal history | 170[b] | 18 (10,53%) |
| SBP (mmHg) | 170[b] | 140.67 ± 17.6 |
| DBP (mmHg) | 169[b] | 80.43 ± 10.48 |
| Total cholesterol (mmol/l) | 171 | 5.53 ± 1.06 |
| HDL cholesterol (mmol/l) | 171 | 1.51 ± 0.4 |
| LDL cholesterol (mmol/l) | 171 | 3.43 ± 0.89 |
| Triglycerides (mmol/l) | 168[b] | 1.08 (0.62) |
| Lp(a) (mg/l) | 168[b] | 150 (307.5) |
| Fasting glucose (mmol/l) | 171 | 5.16 (0.89) |
| Right mean IMT (mm) | 169[b] | 0.73 (0.23) |
| Left mean IMT (mm) | 169[b] | 0.76 (0.22) |
| Overall mean IMT (mm) | 169[b] | 0.74 (0.22) |
| Subjects with at least one plaque | 171 | 41 (24%) |

[a] Nominal variables are expressed as percentages and quantitative variables are expressed as means ± standard deviation except for waist to hip ratio, triglycerides, Lp(a), glucose and IMT expressed as median (interquartile range). 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; Lp(a), Lipoprotein (a); IMT, intima-media thickness.
[b] The total number of patients does not sum up to 171 due to 31, 1, 2 or 3 missing values.

distribution skewed to lower values both in men and women. Age ranged from 44 to 93 years (mean 64.2 ± 12 years) and mean IMT of both carotid arteries from 0.53 mm to 1.27 mm (median 0.74 mm, IR = 0.22); 41 individuals (24%) had one or more atherosclerotic plaques.

Bivariate analysis showed that older age, male gender, higher BMI, higher waist-to-hip ratio, and elevated systolic blood pressure were associated with higher values of IMT, but in multivariate linear regression analysis, only older age, male sex, and past cardiovascular history were significantly associated with increased left, right, or average IMT of both carotid arteries (Table 2).

No relationship was found between IMT and Lp (a) levels in the univariate or multivariate analysis.

When we categorized Lp (a) levels into quartiles, we did not find thicker IMT in the higher quartiles of Lp (a) values and when we dichotomized Lp (a) into higher or lower than 300 mg/l groups, there were no differences in the number of plaques (or their thickness when present). Lp (a) in individuals with LDL-cholesterol levels in the highest quartile was not associated with a thicker IMT compared with people with LDL-cholesterol in the lowest quartile.

Lp (a) concentrations were inversely associated with the molecular weight of Apo (a) isoforms: Lp (a) concentrations were highest in individuals with Apo (a) phenotypes of LMW, in which Lp (a) concentrations were significantly higher compared with high molecular weight Apo (a) isoform individuals ($p < 0.001$).

In 100 subjects (85.5%), only one Apo (a) isoform was detected, whereas two isoforms were found in 17 subjects (14.5%). Serum Lp (a) concentrations were significantly higher ($p < 0.001$) in "double band" subjects (487.65 mg/l) as compared to those with a single Apo (a) isoform (211.90 mg/l). There was a non-significant tendency of having one or more plaques in homozygous subjects (25.5%) compared to heterozygous subjects (5.9%) ($p = 0.061$).

Table 3 shows anthropometric and lipoprotein parameters when Apo (a) size was dichotomized into less than or greater than 22k. Apart from the difference in Lp (a) concentrations already mentioned, there were no differences between the groups in any other parameter studied.

IMT did not differ between subjects with LMW Apo (a) isoforms (IMT 0.71 mm) and HMW isoforms (IMT 0.72 mm), and between subjects with different Lp (a) levels or phenotypes. Number of plaques also did not differ among these groups.

In the subgroup with LDL-cholesterol levels in the highest quartile (LDL-C ≥ 39.57 mmol/l), LMW Apo (a) isoforms and IMT were also not associated.

Fig. 1 shows the average IMT according to the approximate molecular weights of the different isoforms of Apo (a).

The range of Lp (a) values was wider (20–1040 mg/l) within LMW (≤22k) Apo (a) isoforms than within the HMW (>22k) group in which Lp (a) ranged from 10 to 320 mg/l ($p < 0.001$).

Also, when the study participants were stratified into 4 groups according to low and high Lp (a) plasma concentrations (cut-off point 300 mg/l) combined with HMW and LMW Apo (a) phenotypes (cut-off 22 KIV repeats) there were no differences in the proportion of subjects with one or more atherosclerotic plaques.

The group with LMW Apo (a) phenotype and low Lp (a) plasma levels showed a borderline, non-significant trend to association with the presence of one or more atherosclerotic plaques: relative risk (RR) = 1.874; 95% CI 0.981–3.582.

## Discussion

As a relationship between carotid IMT and cardiovascular risk factors is accepted [20], our finding of one association of older age, male gender, BMI, waist-to-hip ratio, DBP, and personal cardiovascular history (Table 2) is not unexpected.

On the contrary, the relationship between Lp (a) and early atherosclerosis of carotid arteries is controversial. The exact mechanism by which Lp (a) increases cardiovascular risk is unknown and both proatherogenic and prothrombogenic effects have been hypothesized.

The few studies addressing this issue have failed to demonstrate a robust link between Lp (a) and surrogate markers of early atherosclerosis, such as IMT and brachial flow-mediated arterial dilation [20–25].

**Table 2**
Risk factors affecting carotid atherosclerosis. Multivariate linear regression analysis.

| Variables in the best explanation model | Right mean IMT *N* = 168 $R^2$ = 0.414 | | Left mean IMT *N* = 169 $R^2$ = 0.451 | | Overall mean IMT *N* = 167 $R^2$ = 0.461 | |
|---|---|---|---|---|---|---|
| | *B* | 95%CI | *B* | 95%CI | *B* | 95%CI |
| Age (years) | 0.009* | 0.007/0.011 | 0.008* | 0.007/0.010 | 0.008* | 0.006–0.010 |
| Sex (male vs. female) | 0.080* | 0.040/0.120 | 0.068* | 0.02870.109 | 0.078* | 0.043–0.114 |
| BMI (kg/m$^2$) | | | 0.004 | −0.001/0.009 | | |
| SBP (mmHg) | | | | | 0.001* | 0.000–0.002 |
| CVD personal history | 0.061 | −0.005/0.128 | 0.072* | 0.012/0.133 | 0.066* | 0.007–0.125 |
| LDL-Chol (mmol/l) | 0.020 | −0.003/0.043 | | | | |

*Abbreviations*: IMT, intima-media thickness; *N*, number; $R^2$, coefficient of determination; *B*, regression coefficient or carotid artery IMT change (mm); 95%CI, 95% confidence interval; BMI, body mass index; SBP, systolic blood pressure; CVD, cardiovascular disease; LDL, low density lipoprotein; Chol, cholesterol.
* *p*-Value < 0.05.

**Table 3**
Lipids and lipoproteins levels in subjects with low and high molecular weight apolipoprotein (a) isoforms.

| | Total *N* | Apolipoprotein (a) phenotypes | | | |
|---|---|---|---|---|---|
| | | >22 kringles | | ≤22 kringles | |
| | | Descriptive statistics[a] | Total *N* | Descriptive statistics[a] | *p* |
| Age (years) | 47 | 60.53 ± 11.53 | 72 | 62.24 ± 10.54 | 0.408 |
| Sex (male) | 47 | 21 (44.68%) | 72 | 31 (43.06%) | 0.861 |
| BMI (kg/m$^2$) | 47 | 28.05 ± 3.73 | 72 | 28.32 ± 4.08 | 0.717 |
| Waist to hip ratio | 35 | 1.4 (0.1) | 59 | 1.32 (0.3) | 0.680 |
| CVD Personal history | 47 | 5 (10.64%) | 72 | 8 (11.11%) | 0.936 |
| SBP (mmHg) | 46 | 139.26 ± 17.88 | 72 | 140.21 ± 19.05 | 0.785 |
| DBP (mmHg) | 47 | 80.72 ± 10 | 72 | 80.97 ± 11.5 | 0.902 |
| Total cholesterol (mmol/l) | 47 | 5.68 ± 1.09 | 72 | 5.47 ± 0.9 | 0.262 |
| LDL cholesterol (mmol/l) | 47 | 3.57 ± 0.93 | 72 | 3.35 ± 0.83 | 0.168 |
| HDL cholesterol (mmol/l) | 47 | 1.47 ± 0.3 | 72 | 1.53 ± 0.42 | 0.382 |
| Triglycerides (mmol/l) | 47 | 1.03 (0.44) | 72 | 1.28 (0.81) | 0.608 |
| Lipoprotein (a) (mg/l) | 47 | 100 (20) | 70 | 315 (410) | <0.001[#] |
| Fasting glucose (mmol/l) | 47 | 5.27 (0.78) | 72 | 5.16 (1.03) | 0.442 |
| Subjects with at least 1 atheromatous plaque | 47 | 10 (21.28%) | 72 | 17 (23.61%) | 0.766 |
| Overall mean IMT (mm) | 47 | 0.72 (0.15) | 71 | 0.71(0.18) | 0.947 |

*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; Lp(a), Lipoprotein (a); IMT, intima-media thickness.

[a] Values are expressed as means ± standard deviation except for waist to hip ratio, triglycerides, Lp(a), glucose and IMT expressed as median (interquartile range).

[#] *p*-Value < 0.05.

Considering the importance of genetic background in Lp (a) levels, we attempted a population study in a Southern Europe ("Mediterranean") country, randomly selecting the subjects, stratified in order to be representative of the Spanish population.

No relationship was found between Lp (a) levels and IMT in bivariate or multivariate analysis, or when dichotomized into higher or lower than 300 mg/l Lp (a) groups. Although Kotani and Sakane [26] found an association between Lp (a) ≤300 mg/l in an asymptomatic group of individuals, they had a different genetic background and sex distribution (Japanese women) than our study.

There was also an absence of relationship when analyzing the atherosclerosis risk in the high LDL-cholesterol subgroup, suggesting that Lp (a) does not potentate early atherosclerosis by interacting with LDL-cholesterol.

On the other hand, an analysis of 36 prospective studies, including more than 126,000 subjects, showed a linear association between Lp (a) levels and coronary and cerebrovascular disease, independently from other conventional risk factors [27]. This association with clinical events suggests that Lp (a) has a more important role in fully developed, symptomatic atherosclerosis [28,29] than in its early phase [20,22–25].

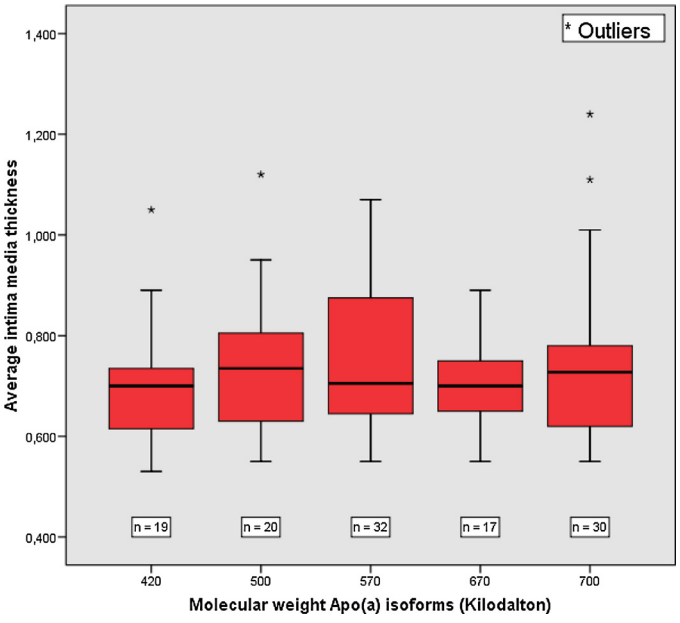


**Fig. 1.** Average intima media thickness of both carotid arteries according to the approximate molecular weights of the different apolipoprotein (a) [Apo (a)] isoforms.

Because the association between Lp (a) and CHD (coronary heart disease) is moderate in magnitude (RR of approximately 1.3 in a comparison between people in the top one-third and those in the bottom one-third of the population distribution [27]), certain subtypes of Lp (a) could be more strongly associated with disease risk than others. It has been proposed that increased Lp (a) concentrations and LMW Apo (a) phenotypes may synergistically contribute to Lp (a) pathogenicity [30], but there are conflicting reports on their relative roles in cardiovascular disease.

While the Physician's Health Study demonstrated that small Apo (a) size was an independent predictor of angina pectoris, regardless of Lp (a) concentration, and without additive or synergistic effect [31], Dieplinger et al. [32] found a synergistic effect between increased Lp (a) concentrations and the LMW Apo (a) phenotypes in subjects with symptomatic peripheral artery disease. Our results cannot confirm this: the group with high Lp (a) levels and LMW isoforms had no more atherosclerotic plaques than the rest.

Similarly, Erqou et al. [33] in a systematic review of 40 studies found that people with smaller molecular weight Apo (a) isoforms had an approximately 2-fold higher risk of CHD and ischemic stroke than those with higher molecular Apo (a) isoforms.

It remained unclear whether the deleterious vascular effect of LMW Apo (a) isoforms is primary or is due to higher Lp (a) concentrations that are associated with these isoforms [34,35].

There are few studies, as well as conflicting results on subjects with subclinical arteriosclerosis: a sub-study of the ARIC population found no differences in the Apo (a) phenotypes distribution between individuals with preclinical extracranial carotid atherosclerosis and those who were free of carotid atherosclerosis [36].

Similarly, the Bruneck study, a population-wide prospective study of all 49–79 inhabitants of this Italian city in which Apo (a) phenotypes also did not show a significant association with early carotid atherogenesis, but Apo (a) emerged as one of the strongest risk predictors of advanced stenotic atherosclerosis [34]. Also, in a recent study by Helgadottir et al. [37] no association was found between Apo (a) phenotypes and carotid IMT.

In agreement with this, a significant association between early atherosclerosis measured by IMT and Apo (a) phenotypes was not found in this study.

Taken together, these studies, suggest that the effects of the Lp (a) variants, and thus Lp (a) levels are manifested at later stages in the atherosclerotic process, and thereby are not reflected by variation in carotid IMT. In the same sense Abdullah et al. [38], studying patients with coronary artery disease (CAD) compared to controls, found a number of gene candidates as potential biomarkers of CAD in patients with different ethnicity and Atkov et al. [39] have developed an artificial neural network including traditional and genetic factors of coronary heart disease.

The high variability of Lp (a) concentrations in subjects with LMW Apo (a) isoforms compared to subjects with HMW Apo (a) isoforms found in our study, may be due to the fact that the number of k4 repeats is the main determinant of Lp (a) concentration when this number is more than 22, whereas other polymorphisms may be involved in the alleles with fewer than 22 k4.

The limitations of this study are a small sample size, that conditions a relatively small statistical power, and that Apo (a) isoforms were determined in two thirds of the sample, but bias is not likely as the subjects were randomly excluded. Although dedicated software may be more exact in the measurement of IMT, repeated manual measurements were made by the same investigator to decrease variability.

## Conclusions

The main conclusion of this study is a lack of association of Apo (a) isoforms with different IMT of the carotid artery in a sample of the general adult Spanish population.

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