Serum Concentration and Polymorphisms of Apolipoproteins A-I and B, and Risk of Coronary Artery Disease

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Abstract: *Introduction:* Variations in the apoprotein apoA-I and apoB genes have been associated with their serum concentration, and thus with Coronary Artery Disease (CAD). This study determined the relationship between polymorphism and the serum concentration of apoA-I and apoB in a group of patients with angiographically documented CAD.

Materials and methods: Polymorphic sites, XbaI (in apoB), and MspI (in apo A-I) and serum concentrations in these lipoproteins were determined in a population of 231patients classified into two groups: patients with significant CAD (coronary obstruction ≥50%) and patients with mild CAD (coronary obstruction <50%); the apoB/apoA-I ratio was then calculated.

Results: It was found that the M2 allele in the apoA-I acts as a risk factor for coronary artery disease, odds ratio=1.78(confidence interval: 1.21 – 2.61), p =0.002. Nosignificant differences were found between the apoA-I concentration values and the corresponding MspI polymorphism genotypes in the two study groups. Meanwhile, the apoB concentration presented significant differences among the XbaI (p=0.027) polymorphism genotypes, with greater values for the heterozygous genotype in patients with CAD≥50%. Likewise, a significantly abnormal difference was evident in the apoB/apoA-I ratio among the two groups, with a greater value for patients with mild CAD.

Conclusion: the data showed that the M2allele may be a risk factor for CAD in this population. Additionally, it showed that there is a relationship between apoB serum concentration and XbaI polymorphism; relationship which manifests itself more strongly in patients with CAD. There was no relationship established between the MspI polymorphism of apoA-I and its serum concentration. Other studies are necessary in order to establish the anomalous behavior of the apoB/apoA-I relation in this population.

Keywords: Apolipoprotein, Polymorphism, Coronary artery disease (CAD), ApoB/apoA-I ratio.

INTRODUCTION

Cardiovascular Diseases (CVDs) are the main cause of morbidity and mortality worldwide. Reducing cholesterol levels, controlling blood pressure, and renouncing cigarett cves are control strategies used to prevent cardiovascular diseases. However, there is an important causative genetic factor [1, 2]. Candidate genes related to lipoprotein metabolism and their related apoproteins are found, among others, to be associated with CDVs [3].

Recent studies have suggested that apolipoprotein B (apoB) and apolipoprotein A-I (apoA-I) may be non-traditional markers of cardiovascular risk [4, 5]. ApoA-I activates the enzymes that takes part in transport the cholesterol from tissues to a high-density lipoprotein (HDL). It also allows the HDL to be recognized by the liver receptors, thus joining them by the end of the transport. The apoA-I concentration can be measured directly and it tends to reflect the HDL concentration. This fact has led some experts to believe that apoA-I could be a better indicator of atherogenic risk than HDL [5].

ApoA-I deficit appears to correlate with an increased risk of developing CAD and peripheral vascular disease [6]. Generally an increase of apoA-I does not represent a problem, but a decrease is associated with low levels of HDL and reduced elimination of cholesterol in the body [7]. Low levels of apoA-I along with high levels of apoB are associated with an increased risk of coronary artery disease [8].

Some genetic alterations lead to apoA-I deficiency, and consequently to low concentrations of HDL [9, 10]. Individuals with these conditions tend to have hyperlipidemia and higher concentrations of low density lipoprotein (LDL). They often suffer from advanced atherosclerosis (formation of fatty plaques and hardened tissue in the arteries) that can lead to coronary and cerebral events [6].

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E-ISSN: 2410-2822/15

Likewise, apoB tends to indicate LDL concentrations and can be measured directly. Many experts believe that the concentration of apoB-100 may be a better risk indicator of atherosclerotic heart disease than LDL [4, 5, 8]. Others disagree, but claim that apoB and other cardiac risk markers, such as apoA-I, Lp(a), and C-reactive protein (CRP), can provide valuable additional information.

In recent studies have expressed apoB and apoA-I in terms of apoB/apoA-I ratio and seems to be effective in cardiovascular disease risk characterization [4, 5].

The present study aims to determine the relationship between polymorphism and the serum concentrations of the apoA-I and apoB in a group of patients with mild versus significant coronary artery disease.

MATERIALS AND METHODS

Population and Sample

The study included 231 patients of both genders (92 females/139 males), who were sequentially admitted to the Quindío Hemodynamics Center in Armenia-Colombia between the years 2008-2009. These patients had a clinical necessity for a coronary angiography, a history of cardiac ischemia, and suffered at least one major cardiovascular event. The study did not include pregnant patients, or patients with dysbetalipoproteinemia, diabetes, hyperlipidemia, uncontrolled hypertension, kidney damage, or untreated idiopathic nephrotic syndrome, because these states or diseases alter plasma lipids. Only the patients' basic data (lipid profile and grade of coronary obstruction) was accessible. All patients were prospectively included and signed an informed consent prior to the angiography. They also filled out a questionnaire used to obtain basic demographic data (age, gender, diet, personal and family medical history, and other cardiovascular risk factors). The study was approved by the Bioethics Committee at Universidad del Quindío.

Coronary Angiography

The coronary angiography was conducted according to standard methods by an intervening physician. For the evaluation of the lesions, the coronary quantitative analysis (CQA) method was used by two independent observers unaware of the laboratory results. Results showed inter-observer variability was at 3.8%. Obstructive disease of the coronary arteries was defined as one or more stenos is ≥50% in at least one major coronary artery or in any of its main ramifications. This procedure permitted the classification of patients into two groups: the first group had a coronary obstruction greater to or equal to 50% (CAD ≥ 50%), and the second group had a coronary obstruction between zero and less than 50% (CAD < 50%).

Blood Samples and Biochemical Analyses

After 8-12 hours of fasting, the samples were obtained during the angiographic procedure and sent to the laboratory. The laboratory personnel did not know the patient information and could only identify the samples by number. For lipid profile analysis, blood collected in dry tubes was used. Serum was obtained through centrifugation at 2500 g for 15 minutes at 4 °C, separated in micro-tubes, and stored at -20 °C until use.

The quantification of apoA-I and apoB was performed in serum using the Biosystems commercial kit and following the manufacturer's instructions. Likewise, the apoB/apoA-I ratio was assessed as a risk factor for cardiovascular disease.

Molecular Analysis: XbaI and MspI Polymorphism

ApoB and ApoA-I genes have polymorphic sites, including those due to the presence/absence of the cutting site of the restriction enzymes (restriction fragment length polymorphism; RFLP), such as XbaI and MspI. RFLP produces two alleles and three genotypes in each gene, called M1 and M2 for MspI and X1 and X2 for XbaI. For this purpose DNA extraction was carried out using the Wizard Genomic DNA Purification Kit (Promega) in total blood and following the manufacturer's instructions.

ApoB Gene Amplification by PCR

The XbaI polymorphism of apoB was determined by allele specific amplification using the polymerase chain reaction (PCR). PCR was performed using the following procedure: 500 ng of genomic DNA was added to PCR 1X buffer, $MgCl_2$ (1.5 mM), primers (0.4 µM), dNTPs (200µM), dimethyl sulfoxide (DMSO) (10%), and Taq polymerase (3U) for a total volume of 50µl. The PCR was performed with 35 successive cycles with the following times and denaturation temperatures at 95°C for 1min, hybridization at 60°C

for 50sec, and an extension at 70°C for 2 min. Then, 25µl of the amplified product (144pb) underwent a digestion by applying 10UI of the XbaI restriction enzyme at 37°C during 3 hours. The resulting restriction fragments were separated by electrophoresis in a 1.5 % agarose and visualized on a UV transilluminator after staining the gel with ethidium bromide

ApoA-I Gene Amplification by PCR

The polymorphism of the apoA-I gene was determined by PCR. PCR was performed using the following procedure: 150 ng of genomic DNA containing PCR 1X buffer, Cl_2Mg (1.5mM), primers (20pmol), dNTPs (20mmol), and Taq polymerase (2.5U) for a total volume of 50µl. The PCR was performed with 35 successive cycles with the following times and denaturation temperatures at 90°C during 30sec, hybridization at 60°C for 30sec, and an extension at 72°C for 1 min. The amplified product was incubated for 3 hours with the MspI enzyme. The resulting restriction fragments were separated by agarose electrophoresis at 1.5 % and visualized on a UV transilluminator after dyeing the gel with ethidium bromide.

Statistical Analysis

The estimate of allelic differences was found by Allele-count method. The chi-square test was used to evaluate whether the allelic frequencies observed were consistent with those expected in accordance with the "Hardy-Weinberg law". These data were organized according to descriptive statistics such as mean and confidence intervals at 95% of the variables identified in the study, initially as a whole. The concentrations of apolipoproteins in each group, according to the genotypes of apoA-I and apoB were compared using the Kruskal-Wallis test. The differences according to gender or obstruction were determined by the Mann-Whitney test, considering there is statistically significance when $p < 0.05$.

RESULTS

General Characteristics of the Study Population

The 231 patients in the study were classified into two groups according to the degree of coronary arterial disease: 1) patients with CAD ≥ 50% (114 (49.35%)), considered patients with significant obstruction and, 2) patients with CAD <50% (117 (50.61%)), considered patients without significant obstruction (mild CAD). Of the total population, 92 patients were women and 139 were men. Out of the patients with CAD <50%, 58 were women and 59 were men; whereas in the group of patients with CAD ≥ 50%, 34 were women and 80 were men.

The general data of the population and the groups is presented in Table **1**.

Age was significantly different between the two study groups; the oldest were the patients in the group with (more severe) CAD. Differences were also evident with respect to gender, where for every woman with coronary artery disease there were 2.3 men with the disease (OR = 2.3; 95% CI 1.34 – 3.97). No significant differences were found in the apoA-I and apoB serum concentrations.

Apolipoprotein A1 and B Polymorphism

The genotype and allele frequencies are presented in Table **2**. The polymorphisms analyzed are not distributed according to the Hardy Weinberg law and

Table 1: Baseline Characteristics of the Two Groups

Variable	Total n = 231	CAD < 50% n = 117	CAD > 50% n = 114	P Value
Age (years)	61.6 ± 11.5	59.6 ± 12.5	63.6 ± 9.8	0.021*
Sex Distribution (Female/Male)	92/139	58/59	34/80	0.002*
Apolipoprotein A-1 (mg/dl)	74.1 ± 29.5	72.0 ± 31.6	76.2 ± 27.2	NS
Apolipoprotein B (mg/dl)	135 ± 62.7	139.5 ± 66.4	130.4 ± 58.7	NS
apoB/apoA-1	2.23 ± 1.5	2.38 ± 1.4	2.07 ± 1.6	0.013*

*p≤0.05; significant; NS Not significant.

Table 2: Frequencies of apoA-I and apoB Alleles and Genotypes in of the Two Groups

Polymorphisms	Total (%)	CAD< 50%	CAD> 50%	P Value	
XbaI genotype	n = 231	n = 117	n = 114		
X1/X1	-	-	-	NS	
X1/X2	160 (69.3)	78 (66.7)	82 (71.9)	NS	
X2/X2	71 (30.7)	39 (33.3)	32 (28.1)		
XbaI allele	80 (34.6)	39 (33.3)	41 (36)		
X1	151 (65.4)	78 (66.7)	73 (64)		
X2					
MspI genotype	n = 231	n = 117	n = 114		
M1/M1	127 (55)	73 (62.4)	54 (47.4)	NS	
M1/M2	44 (19)	20 (17.1)	24 (21.1)	*0.002	
M2/M2	60 (26)	24 (20.5)	36 (31.6)		
MspI allele	149(64.5)	83 (70.9)	66 (57.9)		
M1	82 (35.5)	34 (29.1)	48 (42.1)		
M2					

*p≤0.05; significant; NS Not significant.

showed X^2 values of 78.8 and 64.9 for apoA-I polymorphism and XbaI polymorphism, respectively.

The study showed that the M2 allele behaves as a risk factor for coronary artery disease, since an odds ratio of OR = 1.78 (CI: 1.21–2.61) was obtained with a value of p=0.002 when comparing the allele frequencies of this polymorphism among the patients with CAD ≥50 and the patients with mild CAD. There were no patients with the genotype X1/X1 of the XbaI polymorphism of the apoB. The most common alleles were the X2 allele for the XbaI polymorphism and the M1 allele for the apoA-I polymorphism

Polymorphism and Levels of ApoA-I and ApoB

In the total population, the results of the relationship between the MspI genotypes of apoA-I and its serum concentration presented higher levels in the heterozygous genotype M1/M2 with an average of 77.4mg/dl. The lowest results, 72.8mg/dl, were found in the genotype M2/M2. However, there were no statistically significant differences between the concentration and the genotypes of this polymorphism (Figure **1**).

In contrast, the serum concentrations of apoB showed statistically significant relationships with the

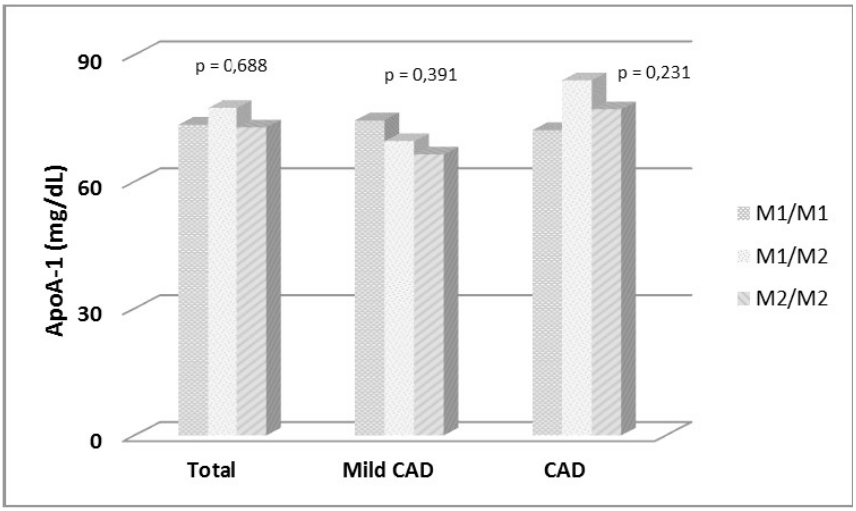

Figure 1: Polymorphism and Levels of ApoA-I.
In the all patients and patients with CAD the MspI genotypes of apoA-Iand its serum concentration presented higher levels in the heterozygous genotype M1/M2. However, there were no statistically significant differences. CAD = Coronary arterial disease. M1 and M2 alleles from MspI polymorphism in apo A-I.

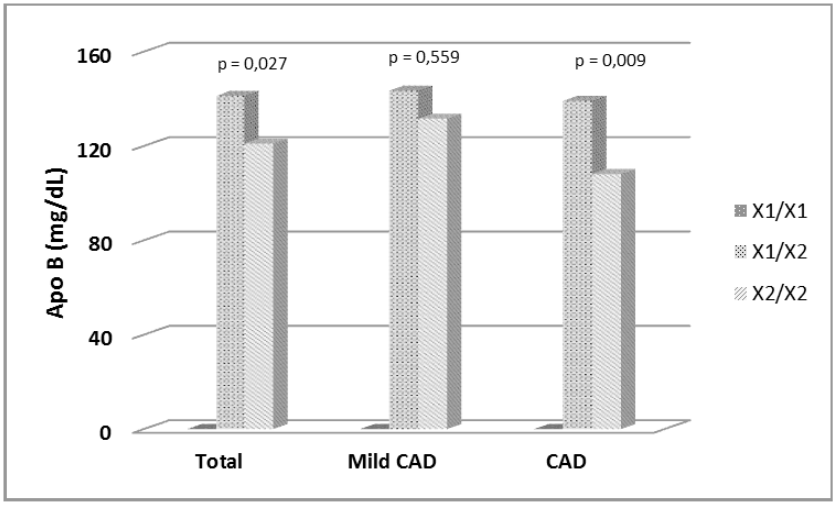

Figure 2: Polymorphism and Levels of ApoB.
In the all patients and patients with CAD and mild CAD, the XbaI genotypes of apoB and its serum concentration presented higher levels in the heterozygous genotype X1/X2. However in total population and patients with CAD there were statistically significant differences. CAD = Coronary arterial disease. X1 and X2 alleles from MspI polymorphism in apoB.

XbaI polymorphism genotypes, wherein the concentration of the heterozygous genotype X1/X2 was 141.2mg/dl, and 121.0 mg/dl p=0.027 for the genotype X2/X2 (Figure **2**).

In the group of patients with mild CAD, no significant differences were found between the values of apoA-I concentration and the corresponding MspI polymorphism genotypes. However, it should be noted that the genotype M1/M1 had the greatest concentrations with a value of 74.5mg/dl, followed by the genotype M1/M2 and the genotype M2/M2 with 69.6mg/dl and 66.5mg/dl, respectively (Figure **1**). Similarly, no statistical relationships were found between the XbaI polymorphism and apoB concentrations, being greater the values of concentration in the genotype X1/X2 than in the homozygous for X2 with 143.5mg/dl and 131.6mg/dl, respectively (Figure **2**).

By observing this group of patients according to gender, it was determined that there were no differences between the concentrations of both apolipoproteins and gender; in which the values of women in relation to apoA-I are greater than in men, whereas the opposite occurs with apoB. Reasonably, the apoB/apoA-I ratio presents the same tendency without statistical differences.

There were no significant differences between the values of concentration of apoA-I with its polymorphism in patients with CAD (Figure **1**). Contrary to what was discovered in the apoB, where its concentration varied with the polymorphism with significant differences (p=0.009). The heterozygous genotype had a greater concentration, 139.1mg/dl (CI 125.7 – 152.4), while that of the homozygous genotype for X2 was 108.1mg/dl (91.1 – 125.1) (Figure **2**). Finally, the concentration of apoA-I and apoB was not statistically different between men and women.

Analysis of Genotypes and Alleles by Gender

Of the 92 women studied, 34 had significant CAD and 58 had mild CAD. Neither of the two groups showed differences among the concentrations of both, apolipoproteins and their respective polymorphisms. By contrast, in the men studied a statistically significant relationship between XbaI genotypes and the concentration of apoB (p=0.014) was observed. Table (**3** and **4**).

DISCUSSION

In recent decades, studies focused on determining the factors that impact the development of cardiovascular disease have concentrated on linking biochemical and genetic aspects. There have analyzed the relationship between molecules that favor the appearance of the disease or that function as protector elements, with genetic variations in their genes and the importance that these relationships can have in the clinic, with respect to timely diagnosis and appropriate treatment [1, 2].

It is well documented that plasma lipoprotein concentrations, especially the increase in total

Table 3: Apolipoproteins Concentration by Gender in of the Two Groups

Variables	Women				Men			
	Total	CAD	Mild CAD	P	Total	CAD	Mild CAD	P
n	92	34	58		139	80	59	
Apo A-1 (mg/dL)	74.9 ± 31.4	77.7 ± 28.6	73.2 ± 32.1	0.62	73.6 ± 28.3	75.6 ± 26.7	70.81 ± 30.2	0.542
Apo B (mg/dL)	136.8 ± 70.1	139.2 ± 65.4	135.4 ± 73.2	0.62	133.8 ± 57.6	126.6 ± 55.7	143.57 ± 59.2	0.139
Ratio ApoB/ApoA-1	2.3 ± 1.6	2.28 ± 2.1	2.3 ± 1.4	0.43	2.2 ± 1.4	1.98 ± 1.4	2.47 ± 1.4	0.010

CAD = Coronary arterial disease.

Table 4: Apolipoproteins Concentration by Gender and Polymorphism in of the Two Groups

	Apolipoprotein B (mg/dL)				Apolipoprotein A -1 (mg/dL)			
	Polymorphism Xbal				Polymorphism MspI			
GENDER	X1/X1	X1/X2	X2/X2	p	M1/M1	M2/M2	M2/M2	p
Women (CI 95%)	0	139.0 ± 70.3	131.1 ± 70.6	0.579	76.0 ± 32,7	81.3 ± 30,5	69.3 ± 30	0.334
Men (CI 95%)	0	142.7 ± 60.2	115.1 ± 47	0.014	72.0 ± 28	75.2 ± 28.5	75.9 ± 29.3	0.827

CAD = Coronary arterial disease.

cholesterol (TC), low density lipoprotein (LDL), very low density lipoproteins (VLDL), and the decrease of high-density lipoprotein (HDL), behave as risk factors for the development of coronary artery diseases [11]. Moreover, it has been suggested that there is a protective effect of HDL and the majority of its apolipoprotein (apoA-I) against the onset of the disease. This effect may be mediated primarily by the activation or stimulation of cholesterol efflux from peripheral cells; even more so, if it is taking into account the anti-inflammatory and antioxidant properties of the apo contributing to its anti-atherogenic effects [12].

It is important to remember that genetic variations in the apoB gene, such as XbaI polymorphism, may affect its plasma concentration as well as the concentration or functions of lipoproteins that contain it (LDL and VLDL) [13]. This is due to the alteration in the rate of secretion, structural stability, receptor affinity, or interaction with other lipoproteins or enzymes (for example, lipoprotein lipase), disrupting the metabolism of these lipoproteins [14-17].

In this study the data showed that the concentration of apoB in plasma is greater in patients with mild CAD than in patients with CAD. The greatest values for both groups were found in the heterozygous genotypes X1/X2, finding a significant difference in the group of patients with CAD. This result is consistent with a report based on a Chinese population [14], in which the greatest apoB concentration values were found for X1 alleles, in both subjects and controls. Conversely, Hubacek *et al*. in 1998 [15], Rantala *et al*. in 2000 [16], and Scartezini *et al*. in 2003[17], reported that the concentration of apoB were significantly increased in homozygous genotype X2/X2. The results present a relationship between polymorphism and the concentration of apoB in plasma, which generated an important question; if polymorphism is a synonymous mutation of character where the amino acid sequence is not affected, what is the reason for, or how does the mutation affect the expression or the metabolism of the protein? A possible answer to this question could be that the change of this base (C→T) decreases the affinity of potential ribonucleases that inhibit the transcription of this gene or other circumstances [18].

On the other hand, the distribution of XbaI polymorphism for the mutated allele X2 was lower (64%) in patients with CAD than in patients with mild CAD (66.7%) and no significant differences were

found. Meanwhile, studies of a Brazilian populations reported a greater frequency of both, the allele X2 and its homozygote genotype X2/X2, in patients with coronary artery disease compared to that of the control patients [16, 17]. In our study population there was no genotype X1/X1 present, similarly to that discovered by Ju-Pin Pan *et al*. in 1995 [14], who studied a population of 301 Chinese individuals of both genders and found that there were more individuals exhibiting the allele X2 in the group of patients with mild CAD than in the group of patients with CAD. However, the results of genotypic and allelic distribution for the XbaI polymorphism contrast with those reported in Caucasian populations, in which the proportions of such alleles are very similar in both, controls and patients [19], similar to our study.

It has been established that the presence of high concentrations of HDL in plasma is a protective factor against CAD. However, it is important to emphasize that it is the quality and function of HDL that has a great beneficial impact on the health of patients. This lipoprotein presents apoA-I as the main protein component, thus playing an important role in its metabolism, since apoA-I is the primary inducer of the lecithin-cholesterol acyltransferase (LCAT) enzyme activity and it is the main component in the reverse transport of cholesterol [20]. In this sense its genetic variation influences its concentration and atheroprotective function.

In relation to this, in the present study, the MspI polymorphism of the apoA-I gene had a higher allele frequency for the mutant allele M2 in patients with CAD (42.1%) compared to the frequency found in patients without significant disease (29.1%). It was also determined that this allele acts as a risk factor for coronary obstruction in our population, with a value of OR = 1.78. These results are contrary to that reported by Dixit *et al.* in 2007[21], who observed that both allele frequencies and genotype have similar distribution in patients and controls, and also that the homozygous genotype M2/M2 was uncommon.

The concentration of apoA-I is similar to the three polymorphism genotypes of apoA-I, in patients with CAD as well as in patients with mild CAD. Similar results were reported by Pulkkinen *et al.* in a population of western Finland in 2000 [22], where the values of concentration of apoA-I showed no significant difference among the genotypes or among patients and controls. Nonetheless, the results of the study being presented showed a high frequency of the M2 allele (35.5%) as well as of the genotype M2/M2 (26%) in comparison to other studies [22,23], which report that this allele has a low frequency (4.1%). It has been suggested that this mutation may generate hypomethylation of the gene, which would cause an increase of its expression; whereas if there is no variation some issues/drawbacks may arise at the time of initiation of the transduction of mRNA [24, 25].

The apparent contradictions that can arise from different studies in relation to allele frequencies, genotypic of genotype concentration ratio for the XbaI and apoA-I polymorphism, may be associated to the variation and genetic recombination that occurs during the crossings of different races. In fact, the variations manifested within the same group can be possible caused by pressures of expression related to the influence of environmental factors.

Another situation of analysis within our results is the ratio of the concentration of apolipoproteins studied in relation to the disease. It has been documented that apoA-I in HDL is a protective factor, therefore a greater concentration was expected in patients with mild CAD; similarly, a lower concentration of apoB was expected, as described in other studies [19, 22]. Nonetheless, it should be noted that the concentration-disease ratio implies mechanisms more complicated than a simple in vitro measurement.

This study also found abnormal results for the apoB/apoA-I ratio of the concentrations of the two apoproteins, and therefore it cannot be used as a predictor of risk of CAD as described by some authors [26, 27]. For the population of the present study, the findings in the concentration of lipoproteins and apoB/apoA-I ratio suggest that the concentration of LDL and HDL as such do not have a great impact on the development of coronary artery disease in these patients, and that they are the factors involved in the stated pathology. This has been demonstrated through various studies of the group, in which it is demonstrated that patients with and with mild coronary artery disease have normal LDL and HDL at the lower limit of the reference point [28].

In conclusion, this study found that there is a relationship between the concentration of apoB and the XbaI polymorphism genotypes of this apolipoprotein. This relationship manifests itself most strongly in patients with CAD, whereas no relationship was

established between the MspI polymorphism of apoA-I and its serum concentration. It was also found that the M2 allele may be a risk factor for coronary artery disease in our population

ACKNOWLEDGEMENTS

This study was supported by a grant of Quindío University.

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Received on 23-03-2015 Accepted on 02-04-2015 Published on 14-04-2015

http://dx.doi.org/10.15379/2410-2822.2015.02.01.06