Sphingomyelin of Erythrocytes Membranes is Related to Total Cholesterol and LDL-Cholesterol in Patients with Significant Coronary Arterial Disease

Jorge Alberto Jiménez[1,2], Nelsy Loango[1,2], Alejandra María Giraldo[1,3], Patricia Landázuri[1*] and Hugo Castaño[1,4]

[1]*Grupo de Investigación en Enfermedades Cardiovasculares y Metabólicas. Facultad de Ciencias de la Salud. Universidad del Quindío. Armenia-Quindío, Colombia*

[2]*Programa de Biología. Facultad de Ciencias Básicas y Tecnologías. Universidad del Quindío. Armenia-Quindío, Colombia*

[3]*Programa de Licenciatura en Biología y Educación Ambiental. Facultad de Educación. Universidad del Quindío. Armenia-Quindío, Colombia*

[4]*Centro de Hemodinamia del Quindío. Armenia-Quindío, Colombia*

Abstract: We quantified the main phospholipids of erythrocyte membranes and lipid profile from patients with coronary arterial disease (CAD). 202 patients between 22 and 86 years were classified in two groups: a group with CAD greater to or equal to 50% (CAD ≥ 50%) and a second group with CAD between zero and less than 50% (CAD<50%). Total phospholipids was,161.1 ± 12.0 nmol Ptotal/mg protein for patients with CAD and 137.7 ± 17.6 nmol Ptotal/mg protein for the patients with CAD < 50% (p=0.038). In the group of patients with CAD ≥ 50%, we found a linear correlation between sphingomyelin and total cholesterol (r = 0.69) and sphingomyelin and low-density lipoproteins cholesterol (LDL-c) (r = 0.74). This study found that sphingomyelin from membrane is directly related to plasma cholesterol and LDL in patients with CAD ≥ 50%.

Keywords: Coronary arterial disease, sphingomyelin, erythrocyte membranes, lipid metabolism.

INTRODUCTION

Experimental and epidemiological evidence supports the association between anomalies in plasma lipids and coronary artery disease (CAD). Recent research has sought to establish an association among some phospholipids present in plasma and in lipoproteins and CAD [1]. Many researchers have described that cholesterol and some phospholipid components of the erythrocyte membrane (EM) are related to the atherosclerotic plaque, suggesting intervention of the erythrocyte membrane or its constituents in the plaque formation, maturity, and stability [2-4]. Sphingomyelin is one main component of cell membranes and plasma lipoproteins and some studies describe an association between high levels of sphingomyelin and CAD [5]. Several works have described that they participate in the formation of foam cells, atherosclerotic plaques and in the modification of LDL in the subendothelial space. The sphingomyelinase (enzyme that hydrolyses sphingomyelin), seems to have an important role in the process [6,7]. It has been demonstrated that ceramide, a product of sphingomyelin hydrolysis by sphingomyielinase, accumulates on the surface of lipoproteins and promotes its aggregation on the damaged tissue [7]. With these antecedents, the aim of this study was to establish a relationship among lipid profile, phosphatidylserine, phosphatedylcholine, and sphingomyelin from erythrocyte membrane of patients with CAD documented by angiography.

MATERIALS AND METHODS

Population and Sample

The study included patients from both sexes sequentially admitted to the Quíndio Hemodynamics Center in Armenia-Colombia between 2008 and 2009, with clinical necessity for a coronary angiography, and history of cardiac ischemia and 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 nephritic syndrome. We just have access to basic dates of the patients (lipid profile and grade of coronary obstruction). All they were prospectively included and prior to the angiography they signed an informed consent and filled out a questionnaire to obtain basic demographic data (age, sex, diet, per-

*Address correspondence to this author at the Facultad de Ciencias de la Salud. Universidad del Quindío. Armenia-Quindío, Colombia Carrera 15 # 12N.; Tel: 57 6 7359305; Fax: 57 6 7359305;
E-mail: plandazu@uniquindio.edu.co

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sonal and family medical antecedents and other cardiovascular risk factors). The study was approved by the Bioethics Committee at Universidad del Quindío.

Coronary Angiography

Conducted according to standard methods by an intervening physician. The lesions were evaluated *via* the coronary quantitative analysis (CQA) method by two independent observers who were unaware of the laboratory results. Interobserver variability was at 3.8%. Obstructive disease of the coronary arteries was defined as one or more stenosis ≥50% in a major coronary artery or in any of its main ramifications.

This procedure permitted classifying the patients into two groups; a first group with coronary obstruction greater to or equal to 50% (CAD ≥ 50%) and a second group with coronary obstruction between zero and less than 50% (CAD < 50%).

Blood Samples and Biochemical Analyses

The samples were obtained after 8-12 hours of fasting 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 tube was used. Serum was obtained *via* centrifugation at 2500 g *f*or 15 minutes, at 4 °C, separated in micro-tubes and stored at -20 °C until use. Lipid profile parameters were determined from serum, using as reference values those from the ATP III [8]. Total cholesterol (C_T), Trialcylglycerides (TAG), and high-density lipoprotein cholesterol (HDL-c) were measured through enzymatic methods (Sera-Pak plus kit from Bayer). For those with triglycerides below 400 mg/dl, the LDL Cholesterol (LDL-c) was calculated with the Friedewald equation [9] and the VLDL = TAG/5.

Lipid Extraction of Erythrocyte Membranes

5 ml of blood was collected in a tube with Ethylene diamine-tetraacetic acid (EDTA); it was centrifuged at 2280 rpm for 30 min., eliminating plasma and the leukocyte layer. The red blood cells were washed three times with five volumes of NaCl 0.89%; 1 ml was taken from the package of erythrocytes and 10 volumes of chloroform: methanol (2:1) were aggregated with 0.05% of Hydroxybutyrate (HB) as antioxidant; this was centrifuged for 15 min at 2500 rpm, the supernatant was eliminated and the lower phase was stored; the extraction procedure was repeated twice. The lower phases were mixed and dried under nitrogen atmosphere and resuspended in 1 ml chloroform: methanol (2:1) in HB [10].

Quantification of Total Proteins

This was carried out *via* bicinchoninic acid (BCA) method [11]: briefly: 1 ml BCA was added to the standard and to the samples, it was incubated for 10 min at 60 ºC and read at 562 nm in a spectrophotometer (Thermo electron corporation, Genesis 10uv model).

Quantification of Total Phospholipids (P_t): Phospholipids were Measured by their Content in Phosphorus

Briefly: 50 µl of $Mg(NO_3).6H_2O$ 10% in ethanol was added to 50 µl of the chloroform-methanol-BTH resuspension, incubating until completely evaporating the solvent, 300 µL of HCl 1N were added heating for 15 min., in boiling water, then 700 µl of (1:6) Ascorbic acid 10% and Ammonium molybdate 0.42% in H_2SO_4 1N were added and incubated for 30 min at 45 ºC. This was read at 820 nm in the spectrophotometer (Thermo Electron Corporation, Genesis 10uv model). The concentration was obtained from a calibration curve carried out with H_2KPO_4, and it was expressed as nmol P_i /mg of protein; where P_i = inorganic phosphorus.

Polar and Neutral Lipid Separation (Cholesterol)

Polar and neutral lipids were separated *via* thin layer chromatography (TLC) using 20 x 20 silica gel layers (silica gel 60, Aldrich). Briefly: 50 µl of the chloroform-methanol-HB re-suspension were placed on a plaque and circumvented with a mixture of Heptane: Isopropyl ether: acetic acid (60:40:4 v/v). The position of the lipids was revealed with iodine vapor. The bands corresponding to cholesterol were scraped and the total phospholipids and the scraping were resuspended in chloroform: Methanol (2:1). The cholesterol standard was used in each plaque.

Quantification of Cholesterol

The cholesterol obtained in the previous step was resuspended in Heptane: Isopropyl ether: acetic acid (60:40:4) and quantified *via* gas chromatography. An amount of 1 µl was injected to the gas chromatograph (Agilent 6890) with split 100:1 at 200 ºC. The column temperature is 275 º C with a Nitrogen flow at 25 ml/min, FID detector at 300 ºC.

Separation Phospholipids

100 µl of the total phospholipids obtained in the TLC, were fractioned by a second TLC (silica gel 60, 20x20, Aldrich) by using as running liquid a mixture of chloroform: methanol: 30% (17:7:1), the position of each phospholipid was revealed with iodine vapor, they were scraped and resuspended in chloroform: methanol (2:1). Phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin standards were placed on each plaque. The quantification of each phospholipid was conducted by its content in inorganic phosphorus according to the previously described procedure.

Statistics

This was a descriptive-type study. The Kolmogorov-Smirnov test was performed to determine the parametric and non-parametric behavior of the variables included in the study, and then a descriptive analysis was conducted on each of them. Student t and Mann Whitney tests were applied to compare each of the variables against coronary obstruction. Lastly, Pearson's and Kendall's tests were conducted to determine the la relationship of the erythrocyte membrane variables with the lipid profile parameters; all this by means of the SPSS 14 statistical package.

RESULTS

Population and Sample

the study included 202 patients, (69.8%) were classified with significant coronary obstruction (CAD ≥ 50%) and (31.6%) with insignificant coronary obstruction (CAD <

50%). Table **1**, shows demographics characteristics of the population.

Lipid Profile

El test de Mann-Whitney test for lipid profile among groups revealed that only VLDL cholesterol was highest with the group with CAD ≥ 50% compared to the group with CAD < 50%, (p= 0.05) (Table **2**).

Erythrocyte Membrane Cholesterol (EMC)

The median and the 25-75 percentiles of the EMC concentration of the total population was 10.7 (7.1 - 15.0) nmol/mg protein; for the group with CAD ≥ 50% it was 10.6 (6.9 – 15.8) nmol/mg protein, and for the group with CAD<50%, it was 10.9 (7.6 – 14.2) nmol/mg protein, without significant differences among the groups (P = 0.91).

Total Phospholipids

The mean phospholipids concentration for the total population was of 154.1 ± 10.0 nmol P_{total}/mg protein. The concentration of total membrane phospholipids was greater for patients with CAD ≥ 50% (161.1 ± 12.0 nmol P_{total}/mg protein) compared to patients with CAD < 50% (137.7 ± 17.6 nmol P_{total}/mg protein), con significant differences among groups (p = 0.038).

Individual Phospholipids

Three of the majority phospholipids were separated from the total phospholipids, sphingomyelin, phosphatidylethanolamine, and phosphatidylcholine. Their concentration (median and 25-75 percentiles) in EM of the general population was 13.3 (0.2 – 32.1), 12.5 (0.2 – 29.6), and 13.1 (0.2 - 29.6) Pi/mg protein, respectively, without significant differences among them. Analysis of the three phospholipids between the two study groups did not show significant differences (Table **3**).

Membrane Lipids and Lipid Profile Relationship

For the total population the correlation analysis between erythrocyte membrane lipids and lipid profile revealed a linear association between sphingomyelin and total cholesterol (r = 0.75) and between sphingomyelin and LDL cholesterol (r = 0.83), (Table **4**).

Table 1. Demographic Characteristics of the Study Population

Characteristics	Total	CAD	
		≥50%	<50%
n	202	141	61
Age (years)	61.58 ± 1.63	63.4±1.9	57.4±3.1
Gender (M/F)	137/65	105/36	32/29

M=male; F= female; CAD = Coronary Arterial Disease.

Table 2. Plasma Lipid Profile in Study Groups

MEMBRANE LIPIDS	Study Groups		
	Mann-Whitney Test		
	Median (P_{25}-P_{75})		P
	CAD ≥ 50%	CAD < 50%	
Triglycerides (mg/dl)	143.0 (119.0-194.0)	143.0 (98.3-174.5)	0.12
VLDL (mg/dl)	28.6 (28.6-38.8)	27.4 (19.5-33.9)	0.05
LDL (mg/dl)	102.2 (79.0-124.6)	110.6 (76.0-136.6)	0.41
	T-Student		
	Mean		CI 95% / P
	CAD ≥ 50%	CAD < 50%	
Total cholesterol (mg/dl)	173.2	180.7	6.9-21.7 / 0.31
HDL (mg/dl)	33.4	35.5	0.7-4.9 / 0.14

CAD= coronary arterial disease

The correlation analysis in the groups revealed a linear relation between sphingomyelin and total cholesterol and between sphingomyelin and LDL cholesterol in the group of patients with CAD ≥ 50% (r = 0.69, p = 0.04; r = 0.74, p = 0.01 respectively), but not in the group with CAD < 50% (Table **5**).

Additionally we determined the association of sphingomyelin levels with age and gender. We found a negative correlation, where the amount of sphingomyelin decreased when age increase (p = 0.05). The gender has no influence with concentration sphingomyelin in membranes.

Table 3. Analysis of Erythrocyte Membrane Lipids

MEMBRANE LIPIDS	Study groups		P
	CAD ≥ 50%	CAD < 50%	
sphingomyelin (nmol P_i/mg protein)	13.1 (0.2-32.2)	13.4 (0.2-32.5)	0.97
phosphatidylcholine (nmol P_i/mg protein)	12.4 (0.2-32.2)	12.6 (0.2-26.1)	0.54
phosphatidylethanolamine (nmol P_i/mg protein)	17.7 (0.2-30.4	12.3 (0.5-26.3)	0.79
Cholesterol	10.6 (6.9-15.8)	10.9 (7.6-14.2)	0.91

CAD= coronary arterial disease. Values are given in Median (P_{25}-P_{75})

Table 4. Plasma Lipid Profile Correlation and Erythrocyte Membrane Lipids in Total Population

MEMBRANE LIPIDS	LIPID PROFILE				
	Total Cholesterol r (p).	Triglycerides r (p).	HDL r (p).	VLDL r (p).	LDL r (p).
total lipids (nmol P_i/mg protein)	-0.03 (0.56)	0.03 (0.77)	-0.04 (0.66)	0.03 (0.46)	-0.04 (0.32)
sphingomyelin (nmol P_i/mg protein)	0.75 (0.03)	-0.04 (0.63)	0.04 (0,36)	-0.02 (0.64)	0.83 (0.02)
phosphatidylcholine (nmol P_i/mg protein)	0.00 (0.11)	-0.04 (0.33)	0.05 (0.25)	-0.02 (0.34)	-0.01 (0.10)
phosphatidylethanolamine (nmol P_i/mg protein)	0.04 (0.74)	-0.06 (0.66)	0.01 (0.67)	-0.05 (0.78)	0.05 (0.62)

Table 5. Plasma Lipid Profile Correlation and Erythrocyte Membrane Lipids in the Study Groups

MEMBRANE LIPIDS	LIPID PROFILE									
	Total Cholesterol r (p).		Triglycerides r (p).		HDL r (p).		VLDL r (p).		LDL r (p).	
	CAD ≥50%	CAD <50%	CAD ≥50%	CAD <50%	CAD ≥50%	CAD <50%	CAD ≥50%	CAD <50%	CAD ≥50%	CAD <50%
Total Lipids (nmol P_i/mg protein)	-0.02 (0.92)	-0.06 (0.26)	0.05 (0.54)	0.02 (0.67)	0.00 (0.12)	-0.12 (0.79)	0.04 (0.19)	-0.02 (0.36)	-0.04 (0.59)	-0.04 (0.09)
Sphingomyelin (nmol P_i/mg protein)	0.69 (0,04)	0.01 (0.54)	-0.05 (0.76)	-0.01 (0.39)	0.06 (0.15)	0.00 (0.78)	-0.05 (0.91)	0.04 (0.56)	0.74 (0.01)	0.12 (0.78)
Phosphatidylcholine (nmol P_i/mg protein)	0.01 (0.30)	-0.02 (0.26)	0.01 (0.65)	-0.17 (0.28)	0.03 (0.02)	0.14 (0.42)	0.01 (0.60)	-011 (0.28)	-0.03 (0.13)	0.01 (0.46)
Phosphatidylethanolamine (nmol P_i/mg protein)	0.05 (0.84)	0.01 (0.80)	0.11 (0.21)	-0.22 (0.09)	-0.02 (0.76)	0.14 (0.06)	0.01 (0.31)	-0.19 (0.06)	0.04 (0.49)	0.06 (0.90)
Cholesterol (nmol/mg protein)	0.06 (0.68)	-0.23 (0.00)	-0.02 (0.92)	-0.13 (0.22)	0.07 (0.24)	-0.01 (0.39)	-0.02 (0.91)	-010 (0.37)	0.03 (0.90)	0.20 (0.01)

CAD= Coronary Arterial Disease

DISCUSSION

This study describes the ratio of plasma lipids and some erythrocyte membrane lipids of patients with and without significant coronary obstruction. We first characterized the study population, then the lipid profile, and finally the total phospholipids, sphingomyelin, phosphatidylcholine, and phosphatidylserine and their ratios.

Characterization of the population revealed that the frequency of coronary obstruction is greater in men than in women and that increased with age in both genders. Similar results has been reported by others in patients with coronary obstruction where gender and age are parameters that increase the probability of this condition [12,13].

Although high levels of total plasma cholesterol and cholesterol in the LDL have been identified as risk factors in coronary disease, in our study the population had normal values of both components but low concentrations of HDL cholesterol. No significant relation was found of these factors with coronary disease, suggesting that others could be the causal factors of the disease in our population.

In the search for some these factors, we investigated the lipids of erythrocyte membranes, among them the cholesterol. It has been demonstrated that cholesterol content in erythrocyte membranes is greater than that of the other cells of the organism [4]. Also, evidence is available for the presence of erythrocyte membranes in the necrotic center of the atherosclerotic plaques of individuals with sudden death by coronary causes [3], suggesting the participation of these membranes or their lipids in the development of atherosclerosis; however, this work found no differences in cholesterol content of membranes between both study groups. This result is coherent with the physiology of the membranes, if we understand that the plasma levels of cholesterol of the two study groups were similar and they were in the normality range, as occurred with the LDL cholesterol; hence, a substantial modification was not expected in the flow of these lipids between membranes and plasma, as shown by some studies where the membrane cholesterol content is modified by the lipid concentration in the plasma [14,15].

Other important components of cell membranes are the phospholipids, among which are sphingomyelin and phosphatidylcholine, as main lipids in the external monolayer, while phosphatidylserine and phosphatidylethanolamine are in the internal monolayer, one and the other joined to minority components [16]. This lipid asymmetry is maintained through diverse cell mechanisms; it loss leads to alterations in the composition of the membrane and, hence, of its physiological and structural functions, as has been shown by studies of diverse diseases – among them atherosclerosis [14,15]. Our work found greater content of total phospholipids in membranes of patients with CAD $\geq$ 50% compared to patients with CAD < 50%; the differences were significant, which suggests a variation in the content of one or several of the individual membrane phospholipids; however, the concentrations of phosphatidylserine, phosphatidylethanolamine and sphingomyelin were similar in both groups; this meaning that possibly other phospholipids (which are not measured here, for example phosphatidylserine) could be making the difference. Several studies have shown that externalization of phosphatidylserine is related to pro-inflammatory events or cardiovascular risk [17,18].

In this work, the exploration of the relationship between phospholipids and plasma lipids yielded a direct relation among membrane sphingomyelin, total cholesterol, and LDL cholesterol in the general population and in the group with CAD $\geq$ 50%; thus, this last result relates sphingomyelin with coronary disease in this population. These findings are coherent with other studies where an important role has been shown for sphingomyelin and sphingomyielinase in the aggregation of lipoproteins and the formation of foam cells [19], and some authors have even shown that sphingomyelin stimulates atherosclerosis when its circulating concentration is increased [20], attributing it atherogenic properties [1]. Several mechanisms are proposed for the atherogenic action of sphingomyelin, among them its hydrolysis in LDL to ceramide by the endothelial sphingomyielinase [7]. The ceramide increase in lipoproteins promotes their aggregation while also promoting their retention in the atherosclerotic lesions leading to the formation of foam cells [21,22]; the sphingomyielinase activity is promoted by atherogenic inflammatory cytokines [23].

The direct relationship among sphingomyelin, total cholesterol, and LDL-c found in the present study in the group of patients with significant coronary obstruction, possibly indicates that this would be one of the atherogenic mechanisms in this population, given that LDL-c levels were normal, it would be interesting to investigate if these particles are modified in this population group and if by some additional mechanism the low levels of HDL-c contribute to the genesis of the formation of atherosclerotic plaques in the coronary arteries in this population.

In conclusion, this study found that membrane sphingomyelin is directly related to total cholesterol and LDL-c in patients with CAD $\geq$ 50%. Further studies should be aimed at explaining the mechanisms that establish the relationship among sphingomyelin, plasma lipids, and coronary obstruction in patients from our population.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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