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ERYTHROCYTE MEMBRANE, PLASMA AND ATHEROSCLEROTIC PLAQUE LIPID PATTERN IN CORONARY HEART DISEASE

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Abstract The objective was to analyze the lipid composition of the atherosclerotic plaque (AP), plasma and erythrocyte membrane (EM) in patients with advanced coronary heart disease (CHD). AP were obtained through endarterectomy in 18 patients. Ten normolipemic healthy subjects were selected to obtain the normal lipid pattern profile. Total lipids of AP and EM were determined by HPTLC, and the fatty acid profile from AP, EM and plasma using TLC-FID. The relative amount of the lipid species analyzed in AP was in line with the data in the literature [phospholipids: 23.5 mol% ± 3.5; total cholesterol 68.9 mol% ± 7.9; triglyceride 7.6 mol% ± 3.4]. Plasma and EM from CHD patients compared to controls, showed a decrease in polyunsaturated fatty acids and an increase in saturated fatty acids leading to a decrease in the unsaturation index (plasma: 1.67 ± 0.06 vs. 1.28 ± 0.03, P<0.05; EM: 2.28 ± 0.04 vs. 1.25 ± 0.010, P<0.05) and an enhancement in the saturated/unsaturated ratio (plasma: 0.35±0.02 vs. 0.52 ± 0.02, P<0.05; EM: 0.45 ± 0.01 vs. 0.83 ± 0.04, P<0.05). These data are consistent with an essential fatty acid deficiency. Total cholesterol was increased in the CHD's EM (32.3 ± 0.8 vs. 40.6 ± 2.5, P<0.05) with a decrease in phospholipid percentage (67.7 ± 0.7 vs. 59.4 ± 2.6, P<0.05) indicating an alteration in membrane fluidity. These findings suggest changes in EM lipids in CHD patients in spite of different pathological conditions such as age, smoking status and diabetes. The analysis of the lipid composition of EM could provide a useful tool to monitor the evolution of the CHD.

Key words: coronary arteriosclerosis, dyslipidemia, endarterectomy, erythrocyte membranes, fatty acids

Resumen ***Perfil lipídico de membrana de eritrocito, plasma y placa ateromatosa en la enfermedad coronaria.*** El objetivo fue analizar la composición lipídica de las membranas de eritrocitos (ME), plasma y placas ateromatosas (PA) en pacientes con enfermedad coronaria avanzada (ECV). Las PA fueron obtenidas de endarterectomías coronarias de 18 pacientes. Fueron seleccionados 10 sujetos sanos, normolipémicos, como grupo control. Los lípidos totales de PA y ME se determinaron utilizando HPTLC, y el perfil de ácidos grasos de las PA, ME y plasma mediante TLC-FID. La cantidad relativa de las especies lipídicas obtenidas de las PA coinciden con la literatura [fosfolípidos 23.5 mol% ± 3.5; colesterol total 68.9 mol% ± 7.9; triglicéridos 7.6 mol% ± 3.4]. En el plasma y en las ME de los pacientes con ECV se observó, comparando con los pacientes controles, una disminución de los ácidos grasos poli-no saturados acompañado de un aumento de los ácidos grasos saturados que provocó el descenso del índice de instauración (plasma: 1.67 ± 0.06 vs. 1.28 ± 0.03, P<0.05; ME: 2.28 ± 0.04 vs. 1.25 ± 0.010, P<0.05) y el incremento del cociente AG saturados/insaturados (plasma: 0.35 ± 0.02 vs. 0.52 ± 0.02, P<0.05; ME: 0.45 ± 0.01 vs. 0.83 ± 0.04, P<0.05). Estos datos indicarían una deficiencia de ácidos grasos esenciales. Se observó una elevación en los valores de colesterol total (32.3 ± 0.8 vs. 40.6 ± 2.5, P<0.05) y una disminución de los valores de fosfolípidos (67.7 ± 0.7 vs. 59.4 ± 2.6, P<0.05) en las ME de los pacientes con ECV. Estos hallazgos sugieren cambios en los lípidos de las ME en los pacientes con ECV a pesar de presentar diferencias con respecto a edad, tabaquismo y diabetes. El conocimiento del perfil lipídico de las ME podría constituirse en una herramienta útil para monitorear la evolución de la enfermedad.

Palabras clave: arteriosclerosis coronaria, dislipemia, endarterectomía, membrana de eritrocito, ácidos grasos

The increase in the number of patients with diffuse coronary disease and poor distal portion of vessels requires sometimes the use of a complex procedure with multiple graft and endarterectomy of one or more vessels. In our population 28% of patients were revascularized using coronary endarterectomy as a necessary procedure in order to obtain complete revascularization[1].

The development of tools for detection and assessment of vulnerable atherosclerotic plaque (AP) in different vascular beds is an important goal in current cardiovascular research. Several studies have shown that

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chemical composition and morphology, rather than anatomy (degree of stenosis), determine AP instability and predict either disease progression or regression[2,3]. Accumulation of lipids in AP causes progressive narrowing of the arterial lumen, often followed by thrombosis and ischemia. The lipid composition of the plaque is determined by chemical analysis of completely disrupted plaques[4-7]. However, clinical diagnostic methods that reliably document or predict plaque development or instability have not been described yet. It has been established that monitoring atherosclerotic inflammatory activity could provide important clues for the early diagnosis in individuals with potentially unstable disease[3]. It is well known that many alterations in plasma lipid patterns are predictors of coronary heart disease (CHD). However, recent papers described the importance of knowing the lipid pattern composition of erythrocyte membrane (EM) such as red blood eicosapentaenoic+docosahexaenoic acids (called Omega-3 index) that could be considered as a new risk factor for death from CHD[8-10]. The EM lipid composition adjusts to a change in dietary fatty acids (FA) almost completely in ten days[11]. Recently, it has been reported that EM FA composition may also be preferred over plasma because the n-3 FA family in EM was not affected by recent food consumption[8]. The erythrocyte lipid pattern was also analyzed in other pathologies such as female obesity[12], insulin sensitivity[13], chronic alcoholics[14] and diabetic retinopathy[15]. On the other hand, Thies et al.[16] described the importance the n-3 FA that are implicated in the prevention of coronary atherosclerosis. N-3 FA may additionally modulate atherogenesis by affecting processes of endothelial activation and cause a beneficial shift in the eicosanoid system decreasing platelet aggregation and blood pressure[17-19].

TABLE 1.– *Patients characteristics at study entry: with coronary heart disease (CHD) and controls*

	CHD	Control
Age (years, mean [SEM])	60.05 ± 11.2	35 ± 4.5
Body mass index (BMI) (kg/m^2, mean [SEM]	28.02 ± 0.9	27.0 ± 0.3
	CHD (N=18)	Control (N=10)
Gender:		
Men	17	6
Women	1	4
Smoking status:		
Current smokers	5	4
Ex-smokers	9	1
Clinical history:	CHD N=18	
Symptoms in the 6 months before study entry		
Myocardial infarction	12 (66.7%)	
Percutaneus coronary angioplasty	4 (22.2%)	
Stroke	1 (5.6%)	
Hypertension	17 (94.4%)	
Chronic renal insufficiency	1 (5.6%)	
Diabetes	Type I 1 (5.6%); type II 4 (22.2%)	
Stenosis (vessel % mean [SEM])	85.7 ± 2.2	
Drugs use:		
Aspirin	7 (38.9%)	
β Blockers	7 (38.9%)	
ACE inhibitors	4 (22.2%)	
Isosorbide mono nitrate	11 (61.1%)	
Calcium-channel blockers	10 (55.6%)	
Fibrates	1 (5.6%)	
Statins	8 (44.4%)	
Insulin	1 (5.6%)	
Oral antidiabetics	1 (5.6%)	
Aspect of the artery	Bony aspect 9 (50%); thrombosed	
	1 (5.6%); ulcerated plaque 2 (11.1%);	
	rechanneled 1 (5.6%); inflammatory 5 (27.8%)	

In our experience we observed different macroscopic characteristics in plaques. In this multifactorial disease, this observation could belong to different stages of the same pathological condition or to dissimilar pattern of coronary disease. The aim of the present work was to analyze the lipid composition of the AP, plasma and EM in patients with CHD, and to compare plasma and EM lipid pattern with the data obtained from healthy control subjects in order to determine the lipid profile of the group of patients with advanced coronary heart disease.

Materials and Methods

The present study is a prospective, double blind trial where we included all patients with CHD who were revascularized with endarterectomy technique. Plaques were obtained from 18 patients (aged 39 to 71 years, 17 men and one woman) who were all hospitalized at the *Servicio de Cirugía Cardiovascular, Hospital Francés,* Buenos Aires, Argentina. We enrolled all those patients who agreed to participate. Permission for all procedures entailing patients was obtained from *Hospital Francés* Research Ethics Committee, and all the patients gave written informed consent. Table 1 shows patients' characteristics at study entry. All of them presented very important vascular compromise.

Plaque samples were taken by endarterectomy procedure, then preserved in chloroform/methanol 2:1 v/v. The control group consisted of 10 age-matched healthy volunteers (4 men, 6 women) who had no history of hematological or heart diseases (see Table 1).

Isolation of EM: blood was collected in test tubes containing an anticoagulant EDTA solution (*Wiener Lab*., Rosario, Argentina). Whole blood was centrifuged, the plasma was immediately separated, and packed red blood cells were washed four times with a buffered solution containing NaCl (140 mM), KCl (5 mM), $NaHSO_4$ (1 mM), Tris buffer (10mM), pH 7.4 at 4 °C. After agitation they were kept at 4ºC for 10 min and centrifuged at 16 000 g for 15 min. This procedure was done twice, leaving a substantially hemoglobin-free pellet of EM, which was suspended in a small amount of supernatant and stored (-70 °C) until assayed.

Lipid extraction and analysis: after the endarterectomy procedure, AP was weighed and placed in a tube with chloroform-methanol (2:1 v/v in 3:1 w/v) for 24 h. Plasma, EM and AP lipids were extracted using chloroform-methanol mixture as indicated above. An aliquot from the organic phase was methylated and analyzed using a Hewlett-Packard Model 5840-A gas liquid chromatograph equipped with flame-ionation detector (Hewlett-Packard, California, USA). Another aliquot from the same phase was separated to determine phospholipid and neutral lipid content: free cholesterol; esterified cholesterol; tryglicerides. They were separated by thin layer chromatography (TLC) on Whatman high performance-TLC plates of 20 X 10 cm, using ether petroleum-ether/diethyl-ether/acetic acid (80:20:1 v/v/v). They were quantified by comparison with pure standards (1-5 µg). After staining with ferric chloride, plates were developed at 37 °C for 30 min. The plates were scanned and the densitometry quantification was performed using ID Image Analysis Software (Kodak Rochester, NY, USA)[20].

Statistical analysis

The statistical package from Excel 97 (Microsoft Corporation) was used to calculate correlation coefficients according to Spearman, and to perform paired *t* Test and one-way ANOVAs as appropriate. Data are expressed as mean ± standard error of the mean (SEM). Significance was set at $P<0.05$.

Results

In Figures 1 and 2: fatty acid profile of plasma and EM from control and CHD patients are presented.

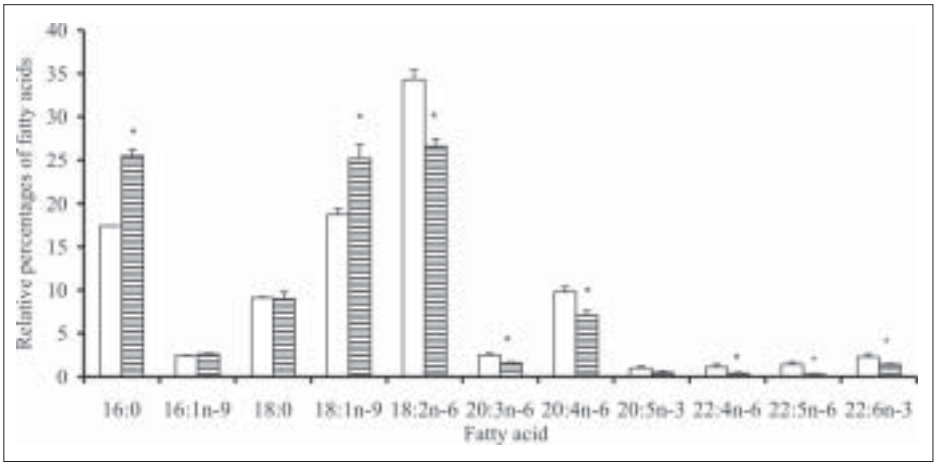

Fig. 1.– Fatty acid composition of plasma from 10 control ▤ and 18 CHD ▭ patients. Fatty acids are identified by: number of carbon atoms in the chain is given first, value following the double point represents number of double bond (zero means saturated fatty acids); number following "n" indicates the position of the last double bond counting the double bond from the terminal methyl group. Data are the mean ± SEM expressed as % of total fatty acids. (*) Significantly different from controls at $P<0.05$.

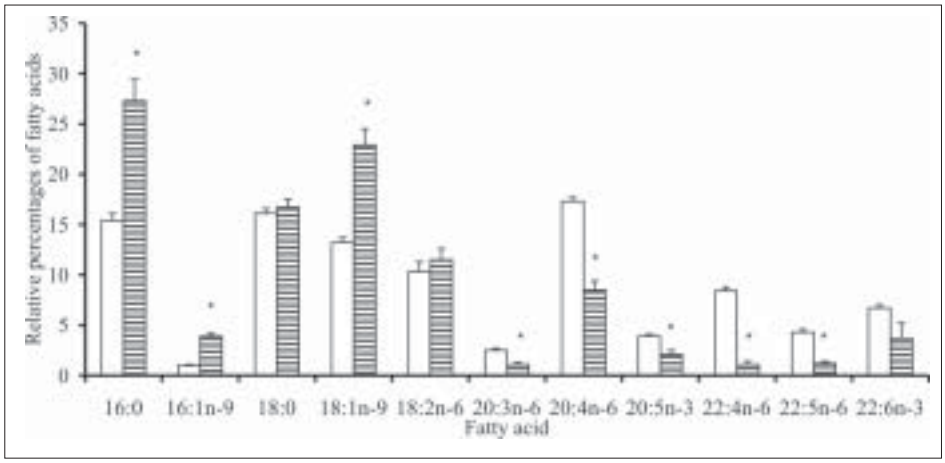

Fig. 2.– Fatty acid composition of erythrocyte membrane from 10 control ▤ and 18 CHD ▭ patients. Identification of fatty acids as in figure 1. Data are the mean ± SEM expressed as % of total fatty acids. (*) Significantly different from controls at P<0.05.

TABLE 2.– *Unsaturated index and fatty acid ratios in plasma and EM*

	Plasma		EM	
	Controls N=10	CHD. N=18	Controls N=10	CHD. N=18
UI	1.67 ± 0.06	1.28 ± 0.03*	2.28 ± 0.04	1.25 ± 0.10*
Sat/Unsat	0.35 ± 0.02	0.52 ± 0.02*	0.45 ± 0.01	0.83 ± 0.04*
16:1/16:0	0.14 ± 0.007	0.10 ± 0.03	0.07 ± 0.005	0.16 ± 0.03*
18:1/18:0	2.05 ± 0.07	2.87 ± 0.29*	0.82 ± 0.02	1.36 ± 0.13*
20:4/18:2	0.29 ± 0.02	0.27 ± 0.02	1.53 ± 0.05	0.88 ± 0.12*
20:4/20:3	4.19 ± 0.30	4.87 ± 0.50	7.37 ± 0.60	11.34 ± 3.83

Fatty acids were identified as in figure 1. UI: Unsaturated index (calculated as $\sum n_1x_1/FA$ *where* n_1 *is the number of double bonds in each fatty acid;* x_1*, moles of each fatty acid; FA, total moles of fatty acids). Sat/Unsat: saturated/unsaturated ratio. EM: erythrocyte membrane.*
N= number of samples. Data are the mean ± SEM. (*) Significantly different from controls at P< 0.05

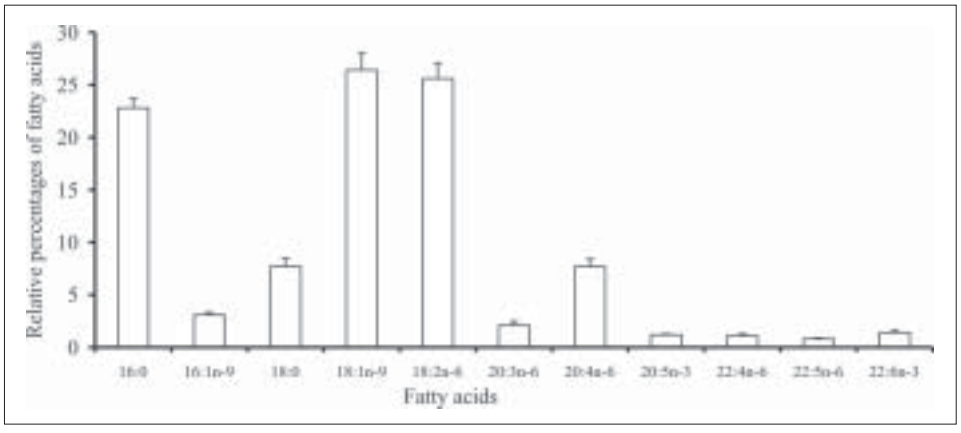

Fig. 3.– Fatty acid composition of atherosclerotic plaque from CHD patients. Identification of fatty acids as in figure 1. Data are the mean ± SEM expressed as % of total fatty acids.

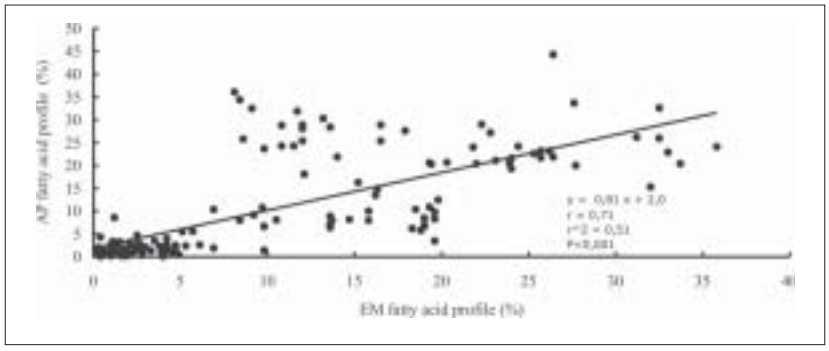

Fig. 4.– Significant correlation of erythrocyte membrane (EM) and atherosclerotic plaque (AP) of total lipids fatty acid composition according to the correlation coefficient by Spearman.

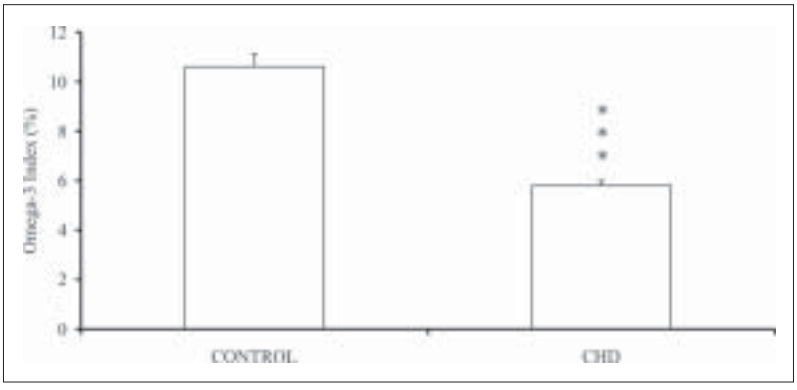

Fig. 5.– Omega-3 index (%)= eicosapentaenoic + docosahexaenoic acids obtained from the fatty acid profile of erythrocyte membranes from 10 controls and 18 CHD patients. Data are the mean ± SEM. (***) Significantly different from controls at P<0.001.

The FA composition of plasma and EM showed an increase in the relative percentage of palmitic and oleic acids in both tissues and an increment of palmitoleic acid in the EM in CHD patients. A significant decrease in n-6 and n-3 families long chain polyunsaturated fatty acids (PUFAs) was noted in both plasma and EM in CHD patients when compared with controls. This result was evidenced by the decrease of the unsaturated index and the increment in the saturated/unsaturated ratio observed in CHD patients (Table 2). This table also shows indirect evidence of desaturase activities in EM. Thus, an increment in Δ9 desaturase activity was inferred through the enhancement 16:1/16:0 and 18:1/18:0 ratios. The decrease in 20:4/18:2 ratio included the Δ6 and Δ5 desaturase activities. Considering that 20:4/20:3 ratio, in which Δ5 desaturase was involved, was not statistically different in CHD patients compared to controls, it was possible to infer that in these patients there was a decrease in Δ6 desaturase activity. Figure 3 shows the FA profile of AP. The major FA were represented by palmitic, oleic and linoleic acids. Stearic and arachidonic acids corresponded to 7% and the remaining FA expressed values below 5%. When EM and AP fatty acid profile was correlated, a statistically significant positive relationship was found, Fig. 4. The Omega-3 index shows a significant decrease in CHD patients, where the control group presented normal values (up to 10%, Fig. 5).

TABLA 3.– *Total lipid composition in erythrocyte membranes (EM) (mol %)*

Lipid	Control N=10	CHD N=18
PL	67.7 ± 0.7	59.4 ± 2.6*
TC	32.3 ± 0.8	40.6 ± 2.5*
TC/PL	0.47 ± 0.13	0.68 ± 0.13

PL: phospholipids, TC: total cholesterol. TC/PL: cholesterol to phospholids ratio. N= number of samples. Data are the mean ± SEM. (*) Significantly different from controls at P< 0.05.

The relative amount of different lipid species in EM and AP is shown in Tables 3 and 4 respectively. A significant decrease in phospholipid as well as an increase in total cholesterol levels and in total cholesterol/

TABLA 4.– *Total lipid composition in the atherosclerotic plaque (AP) (mol %)*

PL	TC	FC	EC	FC/EC	TG
23.5 ± 3.5	68.9 ± 7.9	39.7 ± 4.2	29.2 ± 3.7	1.1 ± 0.3	7.6 ± 3.4

PL: phospholipids, TC: total cholesterol, FC: free cholesterol, EC: esterified cholesterol. TG: triglycerides. Data are the mean of 18 samples ± SEM.

phospholipid ratio were found in EM of CHD patients. Table 4 shows that total cholesterol was the most important component of the AP where it represented over 60%.

Discussion

Patients with CHD exhibited a decrease in PUFAs and an increase in saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) in plasma and EM, indicating an essential FA deficiency. Some investigators have expressed the "double bond" or "unsaturated index" as a crude index of membrane fluidity where the number of double bonds relative to the number of FA was compared. When microviscosity of membranes increased, the unsaturated index decreased[21]; in this work we observed a significant decrease in the unsaturated index and in the saturated/unsaturated ratio in red blood cells as well as in plasma, suggesting an alteration in the fluidity in the lipid bilayer of red blood cells. Other factors are believed to affect membrane fluidity, including the cholesterol to phospholipid ratio that was increased in the EM of CHD patients.

Our results showed that the EM and plasma were strongly affected in the CHD group in contrast to healthy subjects. A remarkable fact is that we found a similar lipid pattern in plasma and red blood membranes of CHD patients. We also observed a positive correlation between the AP fatty acid composition and the red blood membranes, in spite of patients differences such as age, smoking status, serum lipids, body mass index, pharmacological treatments and sample size. This correlation was not evident in plasma values. The biochemical description of total lipids obtained from AP reported in this work, is in line with that published in the literature, confirming once again the presence of high content of free cholesterol and esterified cholesterol, phospholipid and minor amounts of triglyceride in the examined plaques[22, 23]. We observed that free cholesterol was more abundant than esterified cholesterol. This could be due to the fact that most analyzed patients were in the end-stage artery disease. The free cholesterol/esterified cholesterol ratio is in line with the literature too, demonstrating an indirect measure of the plaque texture[24]. As most of the "soft plaques" were esterified cholesterol rich, the ratio of free cholesterol to esterified cholesterol could be one of the key factors for determining the stability of the fibrous cap[2]. Guyton et al.[22] demonstrated, by chemical analysis, that the lesions that contained crystal cholesterol also showed relatively high levels of free cholesterol. Data in the li-terature have shown the mechanism by which lysosomal hydrolysis of esterified cholesterol could contribute to the accumulation of FC in cultured cells, that was exacerbated by inefficient transfer of cholesterol to extralyso-somal locations[22-25].

We processed nine calcified AP, four of them belonging to patients with diabetes type II. Burke et al.[26] showed that calcified lesions were more prevalent in patients with diabetes type II in comparison to non-diabetic subjects. Moreover, the inflammatory response was greater in diabetic than in non-diabetic plaques. However, in our study, patients with diabetes type II did not present differences in their AP lipid pattern profile when compared with "soft" plaques obtained from patients with diabetes type I and non-diabetic patients.

The high values of oleic and linoleic FA found in AP could show the fact that the FA esterified to cholesterol in the core contained a large amount of linoleic acid, similar to plasma lipoproteins and dissimilar from the oleic acid predominance of cholesteryl ester fatty acids resulting from lipid processing in lesion foam cell. The discrepancy in cholesteryl ester fatty acyl pattern has been confirmed in comparisons of foam cells-rich areas versus core areas within the same microdissected lesions[22]. The increment of arachidonic acid in AP could be ascribed to the enhancement of the lipoprotein-associated phospholipase A2 activity within the plaque considered as risk factor[27].

At early asymptomatic phases of the atherosclerotic process, important chemical changes not only in serum lipids but also in EM, this would contribute to predict the disease progression. Although this is a preliminary study that does not included a large number of patients we conclude that a simple laboratory technique which determines the EM lipid pattern (FA and total lipid) composition could be a complementary tool for monitoring the progression of the CHD in a safe and valid manner to select and evaluate the effect of various intervention therapies.

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El argentino, a diferencia de los americanos del Norte y de casi todos los europeos, no se identifica con el Estado. Ello puede atribuirse a la circunstancia de que, en este país, los gobiernos suelen ser pésimos o al hecho general de que el Estado es una inconcebible abstracción; lo cierto es que el argentino es un individuo, no un ciudadano.

Jorge Luis Borges (1899-1986)

Nuestro pobre individualismo. Otras inquisiciones (1952). Obras Completas. Buenos Aires: Emecé, 1974, p 658-66