

Original

Correlation between serum content of the main COPs (cholesterol oxidation products) from autoxidation and cardiovascular risk factors

M.^a Menéndez-Carreño¹, N. Varo², C. Mugueta², P. Restituto², D. Ansorena¹ and I. Astiasarán¹¹Department of Nutrition, Food Sciences, Physiology and Toxicology. Faculty of Pharmacy. University of Navarra. Pamplona, Spain. ²Laboratory of Biochemistry. University Clinic of Navarra. Pamplona, Spain.

Abstract

Background/aims: Risk factors for cardiovascular disease (CVD) have been proven to be associated with an increased oxidative stress. Several studies have considered cholesterol oxidation products (COPs) as specific *in vivo* markers of oxidative stress. The aim of this study was to investigate the association between the levels of COPs derived from autoxidation processes and established cardiovascular risk factors, comparing the levels of serum COPs in subjects with or without showing values out of the reference ranges.

Methods: It was a cross-sectional study in which 88 subjects were recruited and individual and total COPs from autoxidation origin was analyzed in serum by GC-MS. The simultaneous correlation of COPs with different CVD risk factors have been analyzed.

Results and discussion: A great variability of total COPs concentrations were found. Subjects presented total COPs values from 0.091 to 2.052 µg/mL. Total COPs were significantly higher ($p < 0.05$) in patients with hypertriglycerolemia, hypertension, diabetes and overweight/obesity status compared to those subjects who did not present those CVD risk factors. Moreover, 7 α and 7 β -hydroxycholesterol and 7-ketcholesterol were significantly higher ($p < 0.05$) in patients with hypertension and diabetes. No significant differences in total COPs were found between patients with and without hypercholesterolemia.

Conclusions: The obtained results showed that the analyzed COPs correlate well with at least 4 out of 6 risk factors of development of CVD.

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Key words: Oxidation. Hypertriglyceridemia. Hypertension. Diabetes. Obesity. Atherosclerosis. GC-MS.

CORRELACIÓN ENTRE LOS PRODUCTOS DE OXIDACIÓN DE COLESTEROL (COPs) FORMADOS POR AUTOOXIDACIÓN Y FACTORES DE RIESGO CARDIOVASCULAR

Resumen

Introducción: Se ha demostrado que los factores de riesgo cardiovascular están estrechamente asociados con un elevado nivel de estrés oxidativo. Varios estudios consideran a los productos de oxidación del colesterol (COPs) como marcadores específicos *in vivo* de estrés oxidativo. El objetivo de este trabajo fue estudiar la asociación entre los niveles de COPs derivados de procesos de autooxidación de colesterol y factores de riesgo cardiovascular, comparando el contenido sérico de COPs en sujetos afectados o no por dichos factores.

Métodos: Se trata de un estudio transversal en el que se reclutaron 88 personas a las que se analizó el perfil de óxidos de colesterol en suero procedentes de autooxidación, por cromatografía de gases-espectrometría de masas. Se valoró la correlación de los niveles de COPs con diferentes factores de riesgo cardiovascular.

Resultados y discusión: Se encontró una gran variabilidad en el contenido en COPs totales, observándose valores entre 0,091 y 2,052 µg/mL. COPs totales fueron significativamente superiores ($p < 0,05$) en pacientes con hipertrigliceridemia, hipertensión, diabetes y sobrepeso/obesidad con respecto a aquellos sujetos que no presentaron estos factores de riesgo cardiovascular. Además, 7 α y 7 β -hidroxicolesterol y 7-ketocolesterol mostraron valores mayores ($p < 0,05$) en pacientes con hipertensión y diabetes. No se observaron diferencias en COPs totales entre pacientes con y sin hipercolesterolemia.

Conclusiones: Los resultados de este estudio mostraron que los COPs analizados presentan altos niveles de correlación con, al menos, 4 de 6 factores de riesgo cardiovascular considerados.

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Palabras clave: Oxidación. Hipertrigliceridemia. Hipertensión. Diabetes. Obesidad. Atherosclerosis. GC-MS.

Correspondence: Iciar Astiasarán.
Department of Nutrition, Food Science, Physiology and Toxicology.
Faculty of Pharmacy. University of Navarra.
Irunlarrea, s/n.
31008 Pamplona, Spain.
E-mail: iastiasa@unav.es

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Abbreviations

COPs: Cholesterol oxidation products.
CVD: Cardio vascular disease.

Introduction

Cholesterol is an unsaponifiable lipid prone to oxidation leading to the formation of cholesterol oxidation products (COPs). Certain oxysterols of biomedical interest (e.g., 26-OH-cholesterol, 24-OH-cholesterol, and 22-OH-cholesterol) are generally considered to not represent significant products of the autoxidation of cholesterol, while others (including 7-ketocholesterol, 7 β -OH-cholesterol, 7 α -OH-cholesterol, 25-OH-cholesterol, 5 β ,6 β -epoxycholesterol, 5 α ,6 α -epoxycholesterol, and 5 α ,6 β -diOH-cholesterol) are recognized products of cholesterol autoxidation.¹ These last ones could be both from endogenous origin or also they can be absorbed from the diet.^{2,3}

The presence of COPs in plasma or serum has been evident, and it has been demonstrated that they are potentially involved in the initiation and progression of major chronic diseases.⁴ They are present in high concentrations in atherosclerotic plaques, contributing to the development of atherosclerosis, the most common cause of death in Western world.⁵

Chronic and acute over-production of reactive oxygen species (ROS) under pathophysiologic conditions are associated with the development of CDV.⁶ There is growing evidence that oxidized LDL (oxLDL) plays a major role in the injury of endothelium, being its content in COPs the reactive mediator of structural and functional changes of the vascular endothelium affected by atherosclerotic process.^{7,8} oxLDL is not recognized by LDL receptors, but it is instead taken up by scavenger receptors on macrophages of arterial walls. The macrophages then develop into foam cells that form a fatty streak that ultimately turn into an atherosclerotic plaque.⁹

Zhou et al.¹⁰ found that plasma from catheterized patients showed much higher total free oxysterols than control ones, being the most abundant those from autoxidation origin. Larsson et al.¹¹ showed that increased levels of 7 β -hydroxycholesterol and 7-ketocholesterol may play an important role in the induction of oxidative stress in atheroma plaques by stimulating ROS production and decreasing cellular antioxidants.

Several diseases are associated with local sustained imbalance of the ratio between oxidative and reductive biochemical reactions towards oxidation, causing oxidative stress. Increased COPs levels (specially 7-ketocholesterol and 7 β -hydroxycholesterol) have been reported in disease states where oxidative stress was increased such as diabetes mellitus¹² or familial combined hyperlipidemia.¹³ For instance, functional impairment of the vascular endothelium is one of the first steps in the development of atherosclerosis, and

vascular adhesion molecules in plasma are indicators of endothelial damage in diabetes mellitus showing significant correlation with 7-ketocholesterol.¹⁴ Significantly higher concentrations of blood COPs were found in the blood of diabetic and hypercholesterolemic patients than in blood of control subjects.¹⁵ Other authors cannot exclude the role of COPs (7-oxysterols) in pathogenesis of arterial hypertension and non-insulin dependent diabetes mellitus in morbidly obese patients.¹⁶ Obesity is linked with enhanced inflammatory stress and increased atherosclerosis, which are associated with oxidative stress and greater formation of COPs. A recent study has established that serum oxysterol concentrations in adolescents increase with obesity, insulin, and ApoB indicating its relevance as potential indicators for assessing certain metabolic derangements.¹⁷

The ultimate goal of research on COPs should be to link findings regarding the biological roles of COPs to the prevalence of COPs in tissues or fluids, contributing with interesting data on the "focused" lipidomics research area.¹⁸ In this context, the aim of this study was to investigate the association between the levels of COPs derived from autoxidation processes and established cardiovascular risk factors,¹⁹ comparing the levels of serum COPs in subjects with or without showing values out of the reference ranges.

Materials and Methods

Materials and reagents

7 α -Hydroxycholesterol, 7 β -hydroxycholesterol, 5,6 β -epoxycholesterol, 5,6 α -epoxycholesterol, cholestanetriol, 25-hydroxycholesterol, 7-ketocholesterol and 19-hydroxycholesterol were purchased from Steraloids (Wilton, NH, U.S.A.). Tri-Sil reagent was obtained from Pierce (Rockford, IL, U.S.A.). Acetone, chloroform, diethyl ether, methanol, hexane, sodium sulphate anhydrous and potassium hydroxide were obtained from Panreac (Barcelona, Spain). Hexane for gas chromatography and butylhydroxytoluene (BHT) were from Merck & Co., Inc (Whitehouse Station, NJ, U.S.A.). Sep-pack Vac 6cc silica 1g cartridges were obtained from Waters (Milford, Massachusetts, U.S.A.).

Study population

This cross sectional study was performed in 88 subjects (55.7 % males; 63.5 \pm 14.7 yr) attending the Cardiovascular Risk Area of the University of Navarra for a general check-up.

All participants underwent a complete medical examination and anthropometric measurements were taken. Subjects were free from clinically apparent atherosclerotic disease on the basis of absence of history

of coronary disease, stroke, or peripheral artery disease and normal electrocardiogram. Exclusion criteria were: impaired renal or liver function, cancer, arteritis, inflammatory diseases and connective tissue diseases. The mean body mass index (BMI) was 28.8 kg/m², and it was calculated using the following formula: weight (kg)/height² (m). Blood pressure was measured on the right arm, with the subjects in a seated position and after a 5-min rest, with a mercury sphygmomanometer. The average of two measurements, at the beginning and end of the visit, was considered.

Risk factors for CVD were diagnosed according to the National Cholesterol Education Program's Adult Treatment Panel III guidelines with modification of waist criterion into body mass index (BMI).

The local committee on human research approved the study, performed in accordance with the Declaration of Helsinki, and all participants gave written informed consent.

Biochemical analysis

Following an overnight fast, serum and plasma were collected by venous puncture into Vacutainer tubes. Fasting serum glucose, cholesterol, triglycerides (TG), and high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol were measured by standard laboratory techniques.

Serum COPs analysis

A validated method for the analysis of COPs in serum was used, by means of GC-MS Menéndez-Carreño et al.²⁰ Gas chromatography-Mass spectrometry analysis was performed on a GC 6890N Hewlett Packard coupled to a 5975 Mass Selective Detector (Agilent Technologies, Inc., CA, USA). The TMS-ethers derivatives of sterol oxides were separated on a capillary column Varian VF-5ms CP8947 (50 m x 250 m x 0.25 m film thickness) (Varian, France). Identification of the peaks was made by the characteristic ion fragmentation of the standard substances and the quantification was made using selected ion monitoring (SIM) analysis. Integration was performed with Agilent G1701DA GC/MSD ChemStation (Agilent Technologies, Inc., CA, U.S.A.).

Seven different cholesterol oxidation products were analyzed as it has been demonstrated that 7 α -hydroxycholesterol, 7 β -hydroxycholesterol, 5,6 α -epoxide, 5,6 β -epoxide, triol, 25-hydroxycholesterol and 7-ketocholesterol are the most predominant COPs derived from autooxidation in vivo.²¹

Statistical analysis

The statistical analysis was performed with version 15.0; SPSS (Chicago, IL). The normal distribution of variables was tested with the Shapiro Wilks test. Differences between groups were evaluated with the Student t test and with the Mann-Whitney U test for non-normally distributed variables. Spearman correlation coefficients for continuous variables were used to assess univariate correlations. Results are presented as mean \pm EEM, $p < 0.05$ was considered significant.

Results

Regarding demographic and clinical characteristics of the study population, the most prevalent risk factor was dislipemia (79.4%) (data not shown). Mean cholesterol was 200 \pm 49 mg/dL (HDL = 56 \pm 15 mg/dL, LDL = 124 \pm 47 mg/dL and VLDL = 21 \pm 9 mg/dL) and 47% of the study participants were normocholesterolemic. There were 37.5% of hypertensive patients, most of them (30.7% of the volunteers) under treatment with antagonists of the angiotensin II receptor or ACE inhibitors. Mean SBP was 136 \pm 20 mmHg and mean DBP was 77 \pm 11 mmHg. The prevalence of diabetes was 18.6%. 47.7% of the participants were smokers.

The minimum and maximum concentrations of total and individual serum COPs observed are shown in table I. Total analyzed COPs results ranged from 0.091 to 2.053 μ g/mL. Various authors have reported total plasma/serum COPs values in normal volunteers ranging from 0.02 μ g/mL to 9.6 μ g/mL.^{2,22,23} The main cholesterol oxidation products found in serum were 7 α -hydroxycholesterol and 7 β -hydroxycholesterol (0.304 and 0.247 μ g/mL, respectively), followed by 7-ketocholesterol and 5,6 β -epoxycholesterol (0.062 and 0.060 μ g/mL, respectively). No 25-hydroxycholesterol was detected in serum samples.

Table I
Serum concentration of individual COPs [μ g/mL] (n = 88)

COPs (μ g/mL)	7 α -hydroxy cholesterol	7 β -hydroxy cholesterol	5,6 β -epoxy-cholesterol	5,6 α -epoxy-cholesterol	Cholestanetriol	25-hydroxy-cholesterol	7-keto-cholesterol
Minimum concentration	0.011	0.016	0.002	0.003	0.003	n.d.	0.006
Maximum concentration	1.092	0.762	0.241	0.264	0.162	n.d.	0.242
Mean	0.304	0.247	0.06	0.046	0.034	n.d.	0.062

n.d.: non detectable.

Table II
Serum COPs concentrations [$\mu\text{g/mL}$] according to total lipids levels

CVD risk factor	COPs ($\mu\text{g/mL}$)						
	Total COPs	7 α -hydroxy cholesterol	7 β -hydroxy cholesterol	5,6 β -epoxy-cholesterol	5,6 α -epoxy-cholesterol	Cholestanetriol	7-keto-cholesterol
<i>Total cholesterol</i>							
< 240 mg/dL (n = 56)	0.70 \pm 0.50	0.29 \pm 0.27	0.22 \pm 0.19	0.06 \pm 0.05	0.04 \pm 0.03	0.03 \pm 0.03	0.06 \pm 0.05
> 240 mg/dL (n = 29)	0.86 \pm 0.57	0.32 \pm 0.32	0.30 \pm 0.21	0.07 \pm 0.05	0.05 \pm 0.05	0.04 \pm 0.03	0.08 \pm 0.06
p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	p = 0.08
<i>Low HDL</i>							
HDL > 40 mg/dL (women); HDL > 50 mg/dL (men) (n = 63)	0.70 \pm 0.49	0.27 \pm 0.25	0.23 \pm 0.20	0.06 \pm 0.05	0.05 \pm 0.04	0.03 \pm 0.03	0.06 \pm 0.06
HDL < 40 mg/dL (women); HDL < 50 mg/dL (men) (n = 22)	0.91 \pm 0.61	0.41 \pm 0.36	0.29 \pm 0.21	0.06 \pm 0.04	0.04 \pm 0.03	0.04 \pm 0.03	0.06 \pm 0.04
p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Total triglycerides</i>							
< 150 mg/dL (n = 71)	0.71 \pm 0.50	0.28 \pm 0.28	0.23 \pm 0.19	0.06 \pm 0.05	0.04 \pm 0.04	0.03 \pm 0.03	0.06 \pm 0.05
> 150 mg/dL (n = 11)	1.02 \pm 0.71	0.44 \pm 0.37	0.37 \pm 0.26	0.06 \pm 0.05	0.04 \pm 0.03	0.03 \pm 0.03	0.07 \pm 0.05
p	*	p = 0.08	*	n.s.	n.s.	n.s.	n.s.

Results are expressed as mean \pm standard deviations. * $p < 0.05$; n.s.: non significant.

Subjects were classified into two groups depending on the presence or absence of different CVD risk factors, following the criteria described by the ATP III guidelines.¹⁹ Hypercholesterolemia was defined as cholesterol levels higher than 240 mg/dL. Low HDL was characterized as HDL levels lower than 40 mg/dL for women and lower than 50 mg/dL for men. Hypertriglyceridemia was considered when triglycerides levels were higher than 150 mg/dL. In relation to hypertension, the Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure defined categorical hypertension as a blood pressure \geq 140 mm Hg systolic or \geq 90 mm Hg diastolic.²⁴ Diabetes was described as fasting blood glucose levels higher than 126 mg/dL.²⁵ Finally, overweight and obesity were classified according to body mass index (BMI) criteria described by the National Institutes of Health (BMI > 25 kg/m² for overweight and > 30 kg/m² for obesity).²⁶

Regarding hypercholesterolemia, there were no significant differences between the two groups of subjects, with and without hypercholesterolemia, for total COPs levels, 7-ketocholesterol being the only oxide that showed some trend to signification ($p = 0.08$) (table II). In relation to HDL levels, total COPs concentrations were higher in patients with low HDL levels (0.91 $\mu\text{g/mL}$) than in subjects with high HDL levels (0.70 $\mu\text{g/mL}$), but no statistical significance was noticed (table II).

Subjects with hypertriglyceridemia showed significantly higher total COPs levels (1.02 $\mu\text{g/mL}$) than those without hypertriglyceridemia (0.71 $\mu\text{g/mL}$) (table II). These differences were due to 7 α -hydroxycholesterol, which showed significant differences between both groups, and also to the quantitatively

great differences (although not statistically significant $p = 0.08$) found for 7 β -hydroxycholesterol.

Results regarding COPs concentrations depending on the blood pressure of subjects are shown in table III. There were significant differences between the two groups for total COPs levels (0.89 $\mu\text{g/mL}$ for hypertensive patients and 0.61 $\mu\text{g/mL}$ for normal subjects), detecting significant increased levels of cholesterol oxides at C7 position. The rest of the COPs, 5,6 β -epoxycholesterol, 5,6 α -epoxycholesterol and cholestanetriol did not show significant differences between both groups. Similar results were found when COPs were analyzed in subjects with and without diabetes (table III). 7 α -hydroxycholesterol, 7 β -hydroxycholesterol and 7-ketocholesterol showed significant increases in subjects with diabetes in relation to subjects which did not suffer from this disease. Total COPs were also significantly higher in those patients (1.00 and 0.70 $\mu\text{g/mL}$, respectively).

In relation to subjects which were classified according to their BMI, significant differences were found between subjects with BMI higher or lower than 30 kg/m², showing 1.05 $\mu\text{g/mL}$ in the first case and 0.71 $\mu\text{g/mL}$ in the latest. In obese population, 7 α -hydroxycholesterol and 7 β -hydroxycholesterol also showed a trend to signification, with p values of 0.07 and 0.08, respectively (table IV). An evaluation of the COPs content in overweight + obesity subjects was also done. In this case, significantly higher values were found for total COPs in the group with a BMI > 25 kg/m², with 0.84 $\mu\text{g/mL}$, compared to 0.60 $\mu\text{g/mL}$ that was observed for the group with BMI < 24.9 kg/m². 7 α -hydroxycholesterol was the only COP that presented significant differences between these two groups.

Table III
Serum COPs concentrations [$\mu\text{g/mL}$] regarding hypertension and diabetes

COPs ($\mu\text{g/mL}$)	Total COPs	7 α -hydroxy cholesterol	7 β -hydroxy cholesterol	5,6 β -epoxy-cholesterol	5,6 α -epoxy-cholesterol	Cholestanetriol	7-keto-cholesterol
Hypertension							
No (n = 42)	0.61 \pm 0.41	0.23 \pm 0.21	0.20 \pm 0.15	0.06 \pm 0.05	0.04 \pm 0.04	0.03 \pm 0.03	0.05 \pm 0.04
Yes (n = 45)	0.89 \pm 0.59	0.37 \pm 0.33	0.29 \pm 0.24	0.06 \pm 0.05	0.05 \pm 0.04	0.04 \pm 0.03	0.08 \pm 0.06
p	*	*	*	n.s.	n.s.	n.s.	*
Diabetes							
No (n = 70)	0.70 \pm 0.50	0.27 \pm 0.27	0.23 \pm 0.20	0.06 \pm 0.05	0.04 \pm 0.04	0.03 \pm 0.03	0.06 \pm 0.05
Yes (n = 16)	1.00 \pm 0.8	0.44 \pm 0.32	0.33 \pm 0.21	0.06 \pm 0.03	0.05 \pm 0.03	0.03 \pm 0.02	0.09 \pm 0.07
p	*	*	*	n.s.	n.s.	n.s.	*

Results are expressed as mean \pm standard deviations. * p < 0.05; n.s.: non significant.

Table IV
Serum COPs concentrations [$\mu\text{g/mL}$] regarding obesity (IMC > 30.0 kg/m²) and overweight + obesity (IMC > 25.0 kg/m²)

COPs ($\mu\text{g/mL}$)	Total COPs	7 α -hydroxy cholesterol	7 β -hydroxy cholesterol	5,6 β -epoxy-cholesterol	5,6 α -epoxy-cholesterol	Cholestanetriol	7-keto-cholesterol
Obesity							
BMI < 29.9 kg/m ² (n = 71)	0.71 \pm 0.80	0.28 \pm 0.28	0.23 \pm 0.18	0.06 \pm 0.06	0.04 \pm 0.04	0.03 \pm 0.03	0.06 \pm 0.05
BMI > 30 kg/m ² (n = 13)	1.05 \pm 0.60	0.44 \pm 0.32	0.37 \pm 0.27	0.06 \pm 0.04	0.05 \pm 0.03	0.03 \pm 0.03	0.07 \pm 0.06
p	*	P = 0.07	P = 0.08	n.s.	n.s.	n.s.	n.s.
Overweight + Obesity							
BMI < 24.9 kg/m ² (n = 26)	0.60 \pm 0.40	0.22 \pm 0.22	0.20 \pm 0.15	0.05 \pm 0.04	0.04 \pm 0.02	0.03 \pm 0.03	0.06 \pm 0.05
BMI > 25 kg/m ² (n = 58)	0.84 \pm 0.57	0.35 \pm 0.31	0.28 \pm 0.22	0.06 \pm 0.05	0.05 \pm 0.04	0.04 \pm 0.03	0.07 \pm 0.05
p	*	*	P = 0.06	n.s.	n.s.	n.s.	n.s.

Results are expressed as mean \pm standard deviations. *p < 0.05; n.s.: non significant.

Figure 1 shows COPs concentrations in relation to the different CVD risk factors evaluated in this work. Analysis of COPs concentrations revealed a significant increase in total COPs levels in patients presenting hypertriglycerolemia, hypertension, diabetes and over-

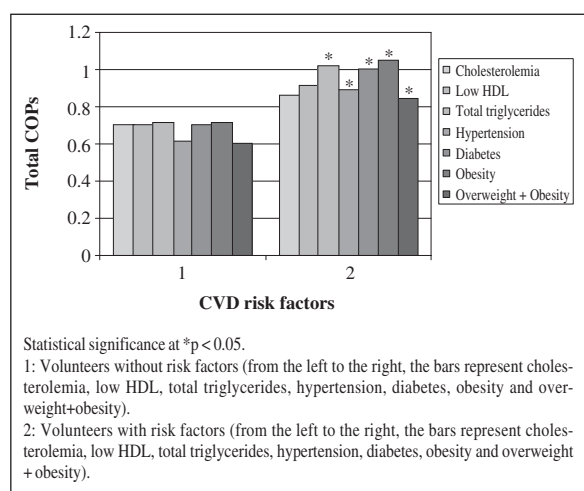


Fig. 1.—Concentration of Total Cholesterol Oxidation Products ($\mu\text{g/mL}$ serum) in relation to the absence (1) or presence (2) of different CVD risk factors in the studied volunteers (n = 88).

weight/obesity status. Regarding hypercholesterolemia and HDL levels, COPs did not show significant differences with the presence of the risk factor although their levels were increased in both cases.

Significant positive Spearman correlations between total COPs and total triglycerides (p = 0.012), between total COPs and systolic and diastolic arterial pressure (p = 0.038 and p = 0.06, respectively) and between total COPs and BMI (p = 0.015) were obtained (table V). On the contrary, as it could have been expected, a negative and significant Spearman correlation between total COPs and HDL was found (p = 0.029). No significant correlations between total COPs and glucose levels were found. Regarding correlations between serum cholesterol levels and different CVD, only negative Spearman significant correlations between cholesterol and HDL (p < 0.001) were found (table V).

Discussion

The high variability found for the amount of COPs in the analyzed subjects serum shows that their level is probably affected by several factors. The major oxysterol detected in this study, 7 α -hydroxycholesterol, is partially formed *in vivo* by the liver specific cholesterol

Table V
Correlation between serum COPs and cholesterol levels and CDV risk factors

CVD risk factor	HDL cholesterol	Triglycerides	Sistolic blood pressure	Diastolic blood pressure	BMI	Glucose
Correlation with serum COPS						
R Spearman	-0.236	0.273	0.31	0.291	0.264	0.092
p	0.029	0.012	0.038	0.006	0.015	n.s.
Correlation with serum cholesterol						
R Spearman	-0.226	0.197	-0.03	0.176	-0.096	-0.288
p	0.037	n.s.	n.s.	n.s.	n.s.	0.011

n.s.: non significant ($p > 0.05$).

7 α -hydroxylase (CYP7A), a P-450 enzyme of liver microsomes limiting step in the synthesis of primary bile acids,²⁷ but it is also a common non-enzymatic cholesterol oxidation product that may be formed by secondary lipid peroxidations.²⁸ 7 β -hydroxycholesterol is generally regarded as COPs formed *in vivo* by a non-enzymatic mechanism because no specific enzymes responsible for their formation have yet discovered in humans.²⁹ 25-hydroxycholesterol, the only non-detected COPs among those analyzed, it is a minor cholesterol oxidation product in human plasma and in atherosclerotic lesions,⁵ Diczfalusy 2009 et al.³⁰ have described values around 5 ng/ml for this oxysterol.

Increased plasma cholesterol levels, particularly LDL, are considered one of the most important risk factor for CVD by several institutions as the Framingham Heart Study,³¹ the Multiple Risk Factor Intervention Trial (MRFIT)³² or the Lipid Research Clinics trials.³³ Nevertheless, the mechanisms by which cholesterol contributes to the initiation and progression of atherosclerotic lesions are not still clear owing to its lack of reactivity *per se*.⁸ Although the concentration of LDL in circulation is important in determining its uptake into the endothelium during the development of atherosclerosis, LDL particles are taken especially after being oxidized and may be then deposited in the arterial intima, thus leading to the formation of atheroma. In a previous paper it was also found that no correlation exists between serum cholesterol and levels of serum COPs when analyzing the effects of feeding on rats with different diets.³ In this sense, the fact that levels of serum COPs did not show correlations with hypercholesterolemia, which is in agreement with other researchers,^{34,35} has induced to some authors to hypothesize that COPs could be used as more appropriate markers for the development of atherosclerosis than cholesterol or LDL in normocholesterolemic patients with atherosclerosis of the lower limbs.³⁶

In addition, HDL has been proposed to protect against the development of CDV by facilitating the transport of cholesterol in peripheral cells back to the liver for removal from the body (reverse cholesterol transport). It has been demonstrated that there is an inverse association between plasma HDL level and incidence of CVD. Constantly low HDL concentration

in young healthy men free from other coronary risk factors, is associated with increased *in vivo* LDL oxidation and with arterial endothelial vasodilatory dysfunction.³⁷ HDL was found to protect macrophages from apoptosis induced by oxidized LDL or by loading with free cholesterol.³⁸ 7-ketocholesterol and related oxysterols modified at the C7 position are selectively exported to HDL by macrophages ATP-binding cassette transporter ABCG1 having a protective role in advanced atherosclerotic plaques. The negative correlation found between total COPs and HDL cholesterol pointed out the potential importance of HDL levels in preventing oxidative mechanisms which occur during the development of atherosclerosis. However, these results were not confirmed by the differences observed for COPs between both groups, high and low HDL cholesterol, which did not reach statistical significance.

Many studies have shown an association between high level of plasma triglycerides (TG) and CVD.³⁹ In contrast, there is a controversial debate whether hypertriglycerolemia represents a risk factor independent of other factors that are often related to it.⁴⁰ One remarkable finding of the present work is that total COPs analysed in this study were significantly higher in hypertriglycerolemic patients, showing also a positive and significant correlation between both variables.

This study also showed that hypertensive patients showed significantly higher COPs concentrations in serum than non-hypertensive ones. High blood pressure is a reversible risk factor and even small reductions in blood pressure can imply large beneficial effects on the risk of CVD.⁴¹ In hypertension, there is an enhancement of lipid peroxidation and antioxidant consumption in plasma. Furthermore, COPs inhibit nitric oxide radical production, considered as the major endothelium-derived relaxing factor.⁴² Moriel et al.⁴³ found higher concentrations of 7-ketocholesterol, 5 α -cholestane-3 β ,5,6 β -triol and 5,6 α -epoxy-5 α -cholestan-3 α -ol in LDL particles of hypertensive patients than in those of normotensive subjects, despite the normal concentrations of cholesterol and triglycerides found in hypertensive patients. Studies in rabbits showed that induction of hypertension by coarctation of the aorta gave rise to an enhancement of COPs in plasma and

aortic tissue.⁴⁴ There were significant differences between the two groups for total COPs levels, detecting significant increased levels of cholesterol oxides at C7 position.

Diabetes is a major independent CVD risk factor.⁴⁵ The finding that COPs levels were significantly increased in diabetic patients is relevant because COPs, in addition to be biomarkers of oxidative stress, also have cytotoxic and proinflammatory effects, which can be related to the implications of diabetes.⁸ Other authors^{46,47} also found high concentrations of plasma oxysterols in patients with diabetes mellitus. Yoshioka et al.⁴⁸ studied whether diabetes enhanced lipid peroxidation in diabetic Wistar rats detecting higher levels of 7 α -hydroxycholesterol, 7 β -hydroxycholesterol and 7-ketocholesterol in diabetic rats than in control ones.

Overweight/obesity is a major, modifiable risk factor for CDV although the link between increased fat mass and atherosclerosis is still unknown.⁴⁹ This study revealed that subjects with BMI > 25 kg/m² had significantly increased serum COPs levels, and even higher quantitative differences were detected for COPs values between those subjects suffering obesity (BMI > 30 kg/m²) compared to non obese subjects. Alkazemi et al.¹⁷ also found increased serum COPs concentration with obesity, detecting increased levels of 7-oxysterols. The increases of serum COPs from autoxidation origin with obesity could be related with data suggesting that oxidative stress is a risk factor for obesity.^{50,51}

In summary, serum levels of COPs with pathophysiologic interest are positively related to five CVD risk factors (hypertriglyceridemia, hypertension, diabetes, obesity and overweight). It could be interesting to determine in which extent these oxysterols are originated in the organism or proceed from the diet. Also the study of the viability of the use of these compounds as potential biomarkers of development of CVD would be of great interest.

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