Nutrición Hospitalaria

Original

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

M.ª Menéndez-Carreño[1], N. Varo[2], C. Mugueta[2], P. Restituto[2], D. Ansorena[1] and I. Astiasarán[1]

[1]Department of Nutrition, Food Sciences, Physiology and Toxicology. Faculty of Pharmacy. University of Navarra. Pamplona. Spain. [2]Laboratory 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, 7α and 7β-hydroxycholesterol and 7-ketocholesterol 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.

(*Nutr Hosp.* 2011;26:144-151)

DOI:10.3305/nh.2011.26.1.4690

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, 7α y 7β-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.

(*Nutr Hosp.* 2011;26:144-151)

DOI:10.3305/nh.2011.26.1.4690

Palabras clave: *Oxidación. Hipertrigliceridemia. Hipertensión. Diabetes. Obesidad. Aterosclerosis. 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

Abbreviations

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

Introduction

Cholesterol is an unsaponificable 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, 7β-OH-cholesterol, 7α-OH-cholesterol, 25-OH-cholesterol, 5β,6β-epoxycholesterol, 5α,6α-epoxycholesterol, and 5α,6β-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 entothelium affected by atherosclerotic process.[7,8] oxLDL is not recognized by LDL receptors, but it is instead taken up by scavenger receptors on macrophagues of arterial walls. The macrophagues 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 7β-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 substained imbalance of the ratio between oxidative and reductive biochemical reactions towards oxidation, causing oxidative stress. Increased COPs levels (specially 7-ketocholesterol and 7β-hydroxycholesterol) have been reported in disease states where oxidative stress was increased such as diabetes mellitus[12] 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 morbidity obese patients.[16] Obesity is linked with enhanced inflamatory 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

7α-Hydroxycholesterol, 7β-hydroxycholesterol, 5,6β-epoxycholesterol, 5,6α-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, Massachussets, U.S.A.).

Study population

This cross sectional study was performed in 88 subjects (55.7 % males; 63.5 ± 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/m^2, and it was calculated using the following formula: weight (kg)/height^2 (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 derivates 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 7α-hydroxy-cholesterol, 7β-hydroxycholesterol, 5,6α-epoxide, 5,6β-epoxide, triol, 25-hydroxycholesterol and 7-keto-cholesterol 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 ± 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 ± 49 mg/dL (HDL = 56 ± 15 mg/dL, LDL = 124 ± 47 mg/dL and VLDL = 21 ± 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 ± 20 mmHg and mean DBP was 77 ± 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 μg/mL. Various authors have reported total plasma/serum COPs values in normal volunteers ranging from 0.02 μg/mL to 9.6 μg/mL.[2,22,23] The main cholesterol oxidation products found in serum were 7α-hydroxycholesterol and 7β-hydroxycholesterol (0.304 and 0.247 μg/mL, respectively), followed by 7-ketocholesterol and 5,6β-epoxycholesterol (0.062 and 0.060 μg/mL, respectively). No 25-hydroxycholesterol was detected in serum samples.

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

COPs (μg/mL)	7α-hydroxy cholesterol	7β-hydroxy cholesterol	5,6β-epoxy-cholesterol	5,6α-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 [µg/mL] according to total lipids levels

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

Results are expressed as mean ± 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 ≥ 140 mm Hg systolic or ≥ 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 µg/mL) than in subjects with high HDL levels (0.70 µg/mL),but no statistical significance was noticed (table II).

Subjects with hypertriglyceridemia showed significantly higher total COPs levels (1.02 µg/mL) than those without hypertriglyceridemia (0.71 µg/mL) (table II). These differences were due to 7α-hydroxycholesterol, which showed significant differences between both groups, and also to the quantitatively great differences (although not statistically significant $p = 0.08$) found for 7β-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 µg/mL for hypertensive patients and 0.61 µg/mL for normal subjects), detecting significant increased levels of cholesterol oxides at C7 position. The rest of the COPs, 5,6β-epoxycholesterol, 5,6α-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). 7α-hydroxycholesterol, 7β-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 µg/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 µg/mL in the first case and 0.71 µg/mL in the latest. In obese population, 7α-hydroxycholesterol and 7β-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 µg/mL, compared to 0.60 µg/mL that was observed for the group with BMI < 24.9 kg/m². 7α-hydroxycholesterol was the only COP that presented significant differences between these two groups.

Table III

Serum COPs concentrations [µg/mL] regarding hypertension and diabetes

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

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

Table IV

Serum COPs concentrations [µg/mL] regarding obesity (IMC > 30.0 kg/m²) and overweight + obesity (IMC > 25.0 kg/m²)

COPs (µg/mL)	Total COPs	7α-hydroxy cholesterol	7β-hydroxy cholesterol	5,6β-epoxy-cholesterol	5,6α-epoxy-cholesterol	Cholestanetriol	7-keto-cholesterol
Obesity							
BMI < 29.9 kg/m² (n = 71)	0.71 ± 0.80	0.28 ± 0.28	0.23 ± 0.18	0.06 ± 0.06	0.04 ± 0.04	0.03 ± 0.03	0.06 ± 0.05
BMI > 30 kg/m² (n = 13)	1.05 ± 0.60	0.44 ± 0.32	0.37 ± 0.27	0.06 ± 0.04	0.05 ± 0.03	0.03 ± 0.03	0.07 ± 0.06
p	*	P = 0.07	P = 0.08	n.s.	n.s.	n.s.	n.s.
Overweight + Obesity							
BMI < 24.9 kg/m² (n = 26)	0.60 ± 0.40	0.22 ± 0.22	0.20 ± 0.15	0.05 ± 0.04	0.04 ± 0.02	0.03 ± 0.03	0.06 ± 0.05
BMI > 25 kg/m² (n = 58)	0.84 ± 0.57	0.35 ± 0.31	0.28 ± 0.22	0.06 ± 0.05	0.05 ± 0.04	0.04 ± 0.03	0.07 ± 0.05
p	*	*	P = 0.06	n.s.	n.s.	n.s.	n.s.

Results are expressed as mean ± 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-
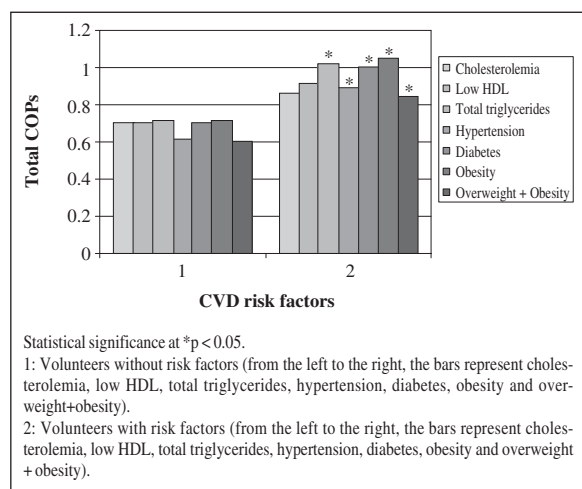

Statistical significance at * $p < 0.05$.
1: Volunteers without risk factors (from the left to the right, the bars represent cholesterolemia, low HDL, total triglycerides, hypertension, diabetes, obesity and overweight+obesity).
2: Volunteers with risk factors (from the left to the right, the bars represent cholesterolemia, low HDL, total triglycerides, hypertension, diabetes, obesity and overweight + obesity).

Fig. 1.—Concentration of Total Cholesterol Oxidation Products (µg/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, 7α-hydroxycholesterol, is partially formed *in vivo* by the liver specific cholesterol

M.ª Menéndez-Carreño et al.

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).

7α-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] 7β-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 periphericall 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, 5α-cholestane-3β,5,6β-triol and 5,6α-epoxy-5α-cholestan-3α-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 proinflamatory 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 7α-hydroxycholesterol, 7β-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/m^2 had significantly increased serum COPs levels, and even higher quantitative differences were detected for COPs values between those subjects suffering obesity (BMI > 30 kg/m^2) 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 patophysiologic interest are positively related to five CVD risk factors (hypertrigyceridemia, 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.

Acknowledgements

References

1. Schroepfer GJ. Oxysterols: Modulators of cholesterol metabolism and other processes. *Physiol Rev* 2000; 80 (1): 361-554.
2. Guardiola F, Tres A, Codony R, Addis PB, Bergmann SD, Zavoral JH. Lack of effect of oral supplementation with antioxidants on cholesterol oxidation product concentration of human plasma, as revealed by an improved gas chromatography method. *Anal Bioanal Chem* 2007; 389: 277-289.
3. Menéndez-Carreño M, Ansorena D, Milagro FI, Campión J, Martínez JA, Astiasarán I. Inhibition of serum cholesterol oxidation by dietary vitamin C and selenium intake in high fat fed rats. *Lipids* 2008; 43: 383-390.
4. Sottero B, Gamba P, Gargiulo S, Leonarduzzi S, Poli G. Cholesterol oxidation products and disease: an emerging topic of interest in medicinal chemistry. *Curr Med Chem* 2009; 16 (6): 685-705.
5. Brown AJ, Jessup W. Oxysterols and atherosclerosis. *Atherosclerosis* 1999; 142: 1-28.
6. Singh U, Jialal I. Oxidative stress and atherosclerosis. *Pathophysiology* 2006; 13 (3): 129-142.
7. Guardiola F, Codony R, Addis PB, Rafecas M, Boatella J. Biological effects of oxysterols: Current status. *Food Chem Toxicol* 1996; 34 (2): 193-211.
8. Leonarduzzi G, Sottero B, Poli G. Oxidized products of cholesterol: dietary and metabolic origin, and proatherosclerotic effects (review). *J Nutr Biochem* 2002; 13: 700-710.
9. Stocker R, Keaney JF Jr. Role of Oxidative Modifications in Atherosclerosis. *Physiol Rev* 2004; 84: 1381-1478.
10. Zhou Q, Wasowicz E, Handler B, Fleischer L, Kummerow FA. An excess concentration of oxysterols in the plasma is cytotoxic to cultures endothelial cells. *Atherosclerosis* 2000; 149: 191-197.
11. Larsson DA, Baird S, Nyhalah JD, Yuan XM, Li W. Oxysterols mixtures, in atheroma-relevant prportions, display synergistic and proapoptotic effects. *Free Rad Biol Med* 2006; 41: 902-910.
12. Abo K, Mio T, Sumino K. Comparative analysis of plasma and erythrocyte 7-ketocholesterol as a marker for oxidative stresss in patients with diabetes mellitus. *Clin Biochem* 2000; 33 (7): 541-547.
13. Arca M, Natoli S, Micheletta F, Riggi S, Di Angelantonio E, Montli A, Antonini MT, Antonini R, Diczfalusy U, Iuliano L. Increased plasma levels of oxysterols, in vivo markers of oxidative stress, in patients with familial combined hyperlipidemia: Reduction during atorvastatin and fenofibrate therapy. *Free Rad Biol Med* 2007; 42: 698-705.
14. Murakami H, Tamasawa N, Matsui J, Yamato K, JingZhi G, Suda S. Plasma levels of soluble vascular adhesion molecule-1 and cholesterol oxidation products in type 2 diabetic patients with nephropathy. *J Atheroscl Thromb* 2002; 8 (1): 21-24.
15. Szuchman A, Aviram M, Musa R, Khatib S, Vaya J. Characterization of oxidative stress in blood from diabetic *vs.* hypercholesterolaemic patients, using a novel synthesized marker. *Biomarkers* 2008; 13 (1): 119-131.
16. Zwirska-Korczala K, Jagodzinska J, Wielkoszynski T, Jochem J, Bodzek D, Wylezol M, Pardela M. Assessment of plasma lipid profile oxysterols and vitamin E concentration in morbidity obese patients with coexisting arterial hypertension and non-insulin dependent diabetes mellitus. *Pol Arch Med Wewn* 2002; 107 (2): 141-147.
17. Alkazemi D, Egeland G, Vaya J, Meltzer S, Kubow S. Oxysterol as a marker of atherogenic dyslipemia in adolescence. *J Clin Endocrinol Metabol* 2008; 93 (11): 4282-4289.
18. Griffiths WJ, Wang Y. Sterol lipidomics in health and disease: Methodologies and applications. *Eur J Lipid Sci Technol* 2009; 111: 14-38.
19. National Cholesterol Education Program's Adult Treatment Panel III: Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) Final Report. *Circulation* 2002; 106: 3143-3441.
20. Menéndez-Carreño M, García-Herreros C, Astiasarán I, Ansorena D. Validation of a gas chromatography–mass spectrometry method for the analysis of sterol oxidation products in serum. *J Chromat B* 2008; 864: 61-68.
21. Rozner S, Garti N. The activity and absorption relationship of cholesterol and phytosterols. Colloids Surfaces A: *Physicochem Eng Aspects* 2006; 82, 283: 435-456.
22. Sevanian A, Hodis HN, Hwang J, Peterson H. In: Bellomo G, Finardi G, Maggi E, Rice Evans C (eds). Free radicals, lipoprotein oxidation and atherosclerosis. Richelieu, London, 1995, pp. 139-161.
23. Micheletta F, Natoli S, Misuraca M, Sbarigia E, Diczfalusy U, Iuliano L. Vitamin e supplementation in patients with carotid

atherosclerosis - Reversal of altered oxidative stress status in plasma but not in plaque. *Arterioscler Thromb Vasc Biol* 2004; 24 (1): 136-140.

24. JNC VI: The Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Arch Intern Med* 1997; 157: 2413-2446.

25. Gavin III JR, Alberti KGMM, Davidson MB, DeFronzo RA, Drash A, Gabbe SG, Genuth S, Harris MI, Kahn R, Keen H, Knowler WC, Lebovizt H, Maclaren JK, Palmer JP, Raksin P, Rizza RA, Stern MP. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 1998; 21 (Suppl.):S5-S19.

26. National Institutes of Health: Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults-the evidence report. *Obes Res* 1998; (6 Suppl.): 51S-209S.

27. Crosignani A, Del Puppo M, De Fabiani E, Caruso, Gallisai D, Mela MG, Melzi M, Galli Kienle M, Colombo C. Plasma oxysterols in normal and cholestatic children as indicators of the two pathways of bile acid synthesis. *Clin Chim Acta* 2008; 395: 84-88.

28. Smith L. Cholesterol autoxidation 1981-1987. *Chem Phys Lipids* 1997; 44: 87-125.

29. Diczfalusy U. Oxysterols in biological samples. In Cholesterol and phytosterol oxidation products: analysis, occurrence, and biological effects. Ed. Guardiola F, Dutta P, Codony R, Savage GP. AOCS Press. 2002, pp. 204-240.

30. Diczfalusy U, Olofsson KE, Carlsson AM, Gong M, Golenbock DT, Rooyackers O, Flaring U, Bjorkbacka H. Marked upregulation of cholesterol 25-hydroxylase expression by lipopolysaccharide. *J Lipids Res* 2009; 50: 2258-2264.

31. Wilson PWF, D'Agostino RB, Levy D, Belanger AM, Silberhatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. *Circulation* 1998; 88: 1837-47.

32. Stamler J, Wentworth D, Neaton JD, for the MRFIT Research Group. Is relationship between serum cholesterol and risk of premature death from coronary heart disease using continuous and graded. Findings in 356 222 primary screenees of the Multiple Risk Factor Intervention Trial (MRFIT). *J Am Med Assoc* 1986; 256: 2823-2838.

33. Lipid Research Clinics Program. The Lipid Research Clinics Coronary Primary Prevention Trial results. I: Reduction in the incidence of coronary heart disease. *JAMA* 25: 351-364.

34. Iuliano L, Micheletta F, Natoli S, Ginanni-Corradini S, Iappelli M, Elisei W, Giovannelli L, Violi F, Diczfalusy U. Measurement of oxysterols and α-tocopherol in plasma and tissues samples as indices of oxidant stress status. *Anal Biochem* 2003; 312: 217-223.

35. Rimner A, Al Makdessi S, Sweidan H, Wischhusen J, Rabenstein B, Shatat K, Spyridopoulos I. Relevance and mechanism of oxysterol stereospecifity in coronay artery disease. *Free Rad Bio Med* 200; 535: 535-544.

36. Prunet C, Petit JM, Ecarnot-Laubriet A, Athias A, Miguet-Afonsi C, Rohmer JF, Steinmetz E, Néel D, Lizard G. High circulating levels of 7β- and 7α-hydroxycholesterol and presence of apoptotic and oxidative markers in arterial lesions of normocholesterolemic atherosclerotic patients undergoing endarterectomy. *Pathol Biol* 2006; 54: 22-32.

37. Toikka JO, Ahotupa M, Viikari JSA, Niinikoski H, Taskinen MR, Irjala K, Hartiala JJ, Raitakari OT. Constantly low HDL-cholesterol concentration relates to endothelial dysfunction and increased in vivo LDL-oxidation in healthy young men. *Atherosclerosis* 1999; 147: 133-138.

38. Tall AR. Cholesterol efflux pathways and other potential mechanism involved in the athero-protective effect of high density lipoproteins. *J Intern Med* 2008; 263: 256-273.

39. Austin MA, Hokanson JE, Edwards KL. Hypertriglyceridemia as a cardiovascular risk factor. *Am J Cardiol* 1998; 81: 7B-12B.

40. Hulley SB, Rosenman RH, Bawol RD, Brand RJ. Epidemiology a a guide of clinical decisions: the association between triglyceride and coronary heart disease. *N Engl J Med* 1998; 302: 1383-1389.

41. MacMahon S, Peto R, Cutler J, Collins R, Sorlie P, Neaton J, Abbot R, Godwin J, Dyer A, Stamler J. Blood pressure, stroke, and coronary heart disease: part 1: prolonged differences dilution bias. *Lancet* 1990; 335: 765-774.

42. Deckert V, Brunet A, Lantoine F, Lizard G, Millanvoye-van Brussel E, Monier S, Larost L, David-Dufilho M, Gambert P, Devynk MA. Inhibition by cholesterol oxides of NO release from human vascular endothelial cells. *Arterioscl Thromb Vasc Biol* 1998; 18: 1054-1060.

43. Moriel P, Sevanian A, Ajzen S, Zanella MT, Plavnik FL, Rubbo H, Abdalla DSP: Nitric oxide, cholesterol oxides and endothelium-dependent vasodilation in plasma of patients with essential hypertension. *Braz J Med Biol Res* 2002; 35: 1301-1309.

44. Hodis HN, Hashimoto S, Mack WJ, Sevanian A. Probucol reduces oxysterol formation in hypertensive rabbits. *Free Rad Biol Med* 2000; 25: 621-628.

45. Bierman EL. George Lyman Duff Memorial Lecture. *Arteriosclerosis Thrombosis* 1992; 12: 647-656.

46. Ferderbar S, Pereira EC, Apolinário E, Bertolami MC, Faludi A, Monte O, Calliari LE, Sales JE, Gagliardi AR, Xavier HT, Abdalla DSP. Cholesterol oxides as biomarkers of oxidative stress in type 1 and type 2 diabetes mellitus. *Diabetes/Metabolism Research and Reviews* 2007; 23: 35-42.

47. Endo K, Oyama T, Saiki A, Ohira M, Koide N, Murano T, Watanabe H, Nishii M, Miura M, Sekine K, Miyashita Y, Shirai K. Determination of serum 7-ketocholesterol concentrations and their relationships with coronary multiple risks in diabetes mellitus. *Diab Res Clin Pract* 2008; 80: 63-68.

48. Yoshioka N, Adachi J, Ueno Y, Yoshida K. Oxysterol increased in diabetic rats. *Free Rad Res* 2005; 39 (3): 299-304.

49. Calle EE, Thun MJ, Petrelli JM, Rodríguez C, Heath CW. Body-mass index and mortality in a prospective cohort of U.S. adults. *N Engl J Med* 1999; 341: 1097-1105.

50. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 2004; 114 (12): 1752-1761.

51. Njajou OT, Kanaya AM, Holvoet P, Connelly S, Strotmeyer ES, Harris TB, Cummings SR, Hsueh WC. Association between oxidized LDL, obesity and type 2 diabetes in a population-based cohort, the Health, Aging and Body Composition Study. *Diab Metab Res Rev* 2009; 25 (8): 733-739.