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ORIGINAL PAPER

Oxidized LDL and its correlation with lipid profile and oxidative stress biomarkers in young healthy Spanish subjects

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Abstract The biological effects of oxidized LDL (oxLDL) may contribute to initiation and progression of the atherosclerotic process, and the association between cardiovascular disease and oxidation of LDL has been largely demonstrated. The objectives of this study were to establish the reference values of oxidative stress biomarkers in a young healthy Spanish population to determine the concentration of oxLDL and its relationship with lipid profile and with these biomarkers. oxLDL, F_2-isoprostanes, protein carbonyls (PC), and 8-hydroxy-2′-deoxyguanosine (8-OHdG) were determinate by ELISA in 72 healthy subjects. Antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR) were carried out on a Hitachi 912 analyzer; lipid profile were assayed using automated systems (Cobas 711, Roche Diagnostics). All statistics were analyzed by using SPSS for Windows 15.0. SPSS Inc, Chicago, IL, USA. (Normal mean reference values): oxLDL (63.23±16.23 U/L), (Male/Female 68.06±17.69/58.39±13.6 U/L), F_2-isoprostanes (2.26±0.9 μg/g creatinine), PC (0.34±0.15 nmol/mg), 8-OHdG (23.27±10.58 ng/ml), SOD (931.97±271.09 U/g Hb), GR (46.56±11.68 U/L), GPx (27.58±6.89 U/gHb (Male/Female 25.91±5.03/29.2±8.07 U/L)). OxLDL (63.23 U/L) was significantly ($p<0.05$) positively correlated with BMI (22.53 Kg/m^2), total cholesterol (175.79 mg/dl), triglycerides (87.58 mg/dl), LDL cholesterol (96.25 mg/dl), and uric acid (4.78 mg/dl), while negatively correlated with HDL-cholesterol (62.25 mg/dl). We have found different correlation between oxLDL and isoprostanes by gender with the rest of parameters. Normal reference values have been found significantly different for oxLDL and GPx by gender. Oxidized LDL is correlated with lipid profile but not with the oxidative stress biomarkers. Urinary isoprostanes are positively correlated with triglycerides and negatively with GR and GPx.

Keywords Oxidized LDL · Oxidative stress · Lipid profile · F_2-Isoprostanes

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Introduction

The most plausible and biologically relevant modification of the LDL particle appears to be due to oxidation [2, 22]. The biological effects of oxidized LDL may contribute to initiation and progression of the atherosclerotic process and the association between cardiovascular disease and oxidation of LDL has been

largely demonstrated [5, 11, 30]. In middle-aged people, obesity and dyslipidemia are the strongest predictors of levels of oxidized LDL (oxLDL) [12]. The concentration of oxLDL depends not only on the degree of oxidative stress but also on the amount of substrate for oxidation (i.e., the number of LDL particles). Indeed, oxLDL levels were consistently found to be strongly correlated to LDL-cholesterol (LDL-c) [4, 17, 18, 27, 32, 34].

Oxidative stress was defined by Sies [28] as "a disturbance in the prooxidant–antioxidant balance in favor of the former." Thus, oxidative stress is essentially an imbalance between the production of various reactive species and the ability of the natural protective mechanism of the organism to cope with these reactive compounds and prevent adverse effects. To minimize free radical damage, there is a complex antioxidant defense system so that antioxidants prevent the organism from the harmful effects of free radicals by scavenging or inhibiting their formation. Cells maintain their vital functions against oxidative damage with the help of a system of antioxidants categorized into three groups: enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), or glutathione reductase (GR); proteins, which include albumin, ferritin, or transferrine; and low molecular weight molecules, including uric acid. The oxidative stress biomarkers can be split into two categories: formation of modified molecules by radical oxygen species such as 8-hydroxy-2′-deoxyguanosine (8-OHdG), F_2-isoprostanes, protein carbonyls (PC); and consumption or induction of enzymes or antioxidants [31].

Oxidative stress has been suggested to be involved in the etiology of a host of chronic diseases including cancer, cardiovascular disease, cataracts, age-related maculopathologies, and aging in general [25]. Reactive oxygen and nitrogen species can attack various substrates in the body including lipids, nucleic acids, and proteins, and it might be appropriate to evaluate plasmatic concentration stress oxidative biomarkers in the routine laboratory assessment.

Because oxidative stress should be evaluated by looking at the whole spectrum of antioxidants and at biomarkers of oxidative damage, the objectives of this study are to establish the reference values of the oxidative stress biomarkers in a young healthy Spanish population to determine the serum concentration of oxLDL and to establish its relationship with the lipid profile and with these biomarkers.

Materials and methods

Seventy-two healthy volunteers, 36 women (26± 4.9 years) and 36 men (26±5.5 years), from the University of Murcia participated in this study. The inclusion criteria were (1) age 30±10 years old; (2) only subjects consuming four or less fruits and vegetables portions; (3) no vitamin supplements consumption for the 2 months before the study; (4) normolipidemic; (5) nonsmokers; (6) alcohol consumption <30 g/day; (7) subjects within 15% of ideal body weight with no major fluctuations in body weight; (8) no gastrointestinal, renal, or hepatic diseases, cancer, or allergies. All participants gave their informed consent to participate in the study, which was approved by the Ethical Committee of the University of Murcia.

Blood sampling In all patients, blood samples were obtained from the median cubital vein and placed in EDTA-containing vials. Serum samples were centrifuged at 3,000×*g* for 10 min at room temperature within 1 h of collection and stored at −80°C until the assays were performed.

Serum lipids and oxLDL The concentrations of cholesterol total, LDL, and HDL cholesterol (LDL-c and HDL-c, respectively), and triglycerides (TG) were assayed using automated systems (Cobas 711, Roche Diagnostics). A competitive enzyme-linked immunoabsorbent assay (ELISA; Mercodia, Uppsala, Sweden; with an intra-assay and interassay variation's coefficient of 4.8 and 4.5, respectively, for 82 U/L) was used to determine oxLDL concentrations in serum.

Antioxidant enzymes The determination of SOD and GPx was analyzed in total blood by a commercial kit (Randox Laboratories Ltd, UK). The concentrations of enzyme GR were analyzed in serum by a commercial kit (Randox Laboratories Ltd., UK). These assays were carried out on a Hitachi 912 analyzer (Roche Diagnostics Systems). Values for SOD and GPx were normalized by per gram of hemoglobin (Hb).

Oxidative stress biomarkers Urinary 15-isoprostane F_2 was analyzed by a commercially available ELISA kit (Oxford Biomedical Research, Inc. Oxford, MI, USA). Values for 15-isoprostane were normalized by per milligram of creatinine measured in urine.

Measurement of 8-OHdG was carried out with an ELISA kit (Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan). PC were determined by an ELISA kit (Biocell Corporation Ltd., New Zealand).

Statistical analysis All statistics were analyzed by using SPSS for Windows 15.0 (SPSS Inc., IL, USA). The characteristics of the subjects are described as numbers, percentage, means, and standard deviations. The Kolmogorov–Smirnov test was used for comparison of normally distributed variables across sex categories, and an independent-samples *t* test was applied to compare means between males and females. Pearson's correlation analysis was performed to examine the relation between oxLDL and the variables. Differences were considered statistically significant at $p \leq 0.05$.

Results

Table 1 shows the biochemical parameters, body mass index (BMI), lipid profile, antioxidant status: enzymes (SOD, GPx, and GR) and certain biomarkers of oxidative damage by separately by gender and altogether: oxLDL (endothelial damage), 8-OHdG (DNA damage), F_2-isoprostanes (lipid peroxidation), and PC (protein damage). The results are expressed as mean ± standard deviation.

According to the lipid profile results, the levels (mean values) of total cholesterol, LDL-c, TG, oxLDL, and uric acid in males were significantly higher ($p<0.05$) than those in females. However, the levels of HDL-c and GPx in males were significant lower ($p<0.05$) than those in females. There were no significant differences by sex in the other antioxidant parameters (Table 1).

In order to establish normal references values, we have analyzed the antioxidants enzymes and oxidative damage biomarkers by gender. We have obtained the results for men and women separately and altogether, and we have only found significant differences in GPx and OxLDL, so we consider only one reference value for both sexes in the rest of the biomarkers. The results obtained are SOD 931.97±271.09 U/g Hb; GR 46.56±11.68 U/L; GPx 27.58±6.89 U/g Hb (men 25.91±5.03 U/g Hb; women 29.2±8.07 U/g Hb); OxLDL 63.23±16.23 U/L (men 68.06±17.69 U/L; women 58.39±13.16 U/L); PC (0.34±0.15 nmol/mg); 8-OHdG (23.27±10.58 ng/ml), and F_2-isoprostanes 2.26±0.9 μg/g creatinine.

When we correlated BMI and lipid profile, we observed a significant ($p<0.05$) positive correlation between BMI and total cholesterol, LDL-c, TG,

Table 1 Body mass index, biochemical parameters, and oxidative stress biomarkers in young healthy Spanish subjects

Variable		Men	Women	*p* Value
	Mean±σ	Mean±σ	Mean±σ	
	n=72	*n*=36	*n*=36	
BMI (kg/m^2)	22.53±3.03	23.82±2.77	21.21±2.73	
Cholesterol (mg/dl)	175.79±28.02	184.86±32.44	166.72±19.26	0.005
HDL-c (mg/dl)	62.25±14.41	58.47±15.25	66.03±12.61	0.025
LDL-c (mg/dl)	96.25±27.09	105.86±29.67	86.64±20.44	0.002
TG (mg/dl)	87.58±54.12	103.03±70.12	72.14±23.10	0.014
Uric acid (mg/dl)	4.78±1.31	5.73±0.99	3.83±0.78	0.000
Homocysteine (μmol/L)	10.69±6.17	11.49±5.55	9.9±6.71	
SOD (U/gHb)	931.97±271.09	896.12±275.72	966.8±265.83	
GPx (U/gHb)	27.58±6.89	25.91±5.03	29.2±8.07	0.046
GR (U/L)	46.56±11.68	48.17±13.56	44.95±9.35	
OxLDL (U/L)	63.23±16.23	68.06±17.69	58.39±13.16	0.011
8-OHdG (ng/ml)	23.27±10.58	21.86±7.49	24.67±12.91	
PC (nmol/mg)	0.34±0.15	0.32±0.15	0.36±0.15	
F_2-isoprostanes (μg/g creatinine)	2.26±0.9	2.26±0.94	2.26±0.87	

BMI body mass index; *HDL-c* HDL-cholesterol; *LDL-c* LDL-cholesterol; *TG* triglyceride; *GR* glutathione reductase; *SOD* superoxide dismutase; *GPx* glutathione peroxidase; *oxLDL* oxidized LDL; *8OHdG* 8-hydroxy-2′-deoxyguanosine; *PC* carbonyl protein; *σ* standard deviation.

oxLDL, homocysteine, and uric acid and a negative correlation with HDL-c.

The results of Pearson's correlation analysis between oxLDL and F_2-isoprostanes (men and women together) and the other variables are shown in Table 2:

- OxLDL (63.23 U/L) was significantly ($p<0.05$) positively correlated with BMI (22.53 Kg/m^2), total cholesterol (175.79 mg/dl), TG (87.58 mg/dl), LDL-c (96.25 mg/dl), and uric acid (4.78 mg/dl), while negatively correlated with HDL-c (62.25 mg/dl). We have not found any correlation between oxLDL and antioxidants; no relationship between oxLDL and oxidative stress biomarkers has been found either.
- Urinary F_2-isoprostanes were significantly ($p<0.05$) positively correlated with TG and negatively correlated with GPx and GR. No relationship between F_2-isoprostanes and oxLDL has been found. No relationship between urinary F_2-isoprostanes and the rest of oxidative stress biomarkers has been found either.

We have made an analysis of Pearson's correlation between oxLDL and F_2-isoprostanes by gender. The results are shown in Table 3.

We observed a significant ($p<0.05$) positive correlation between oxLDL and total cholesterol and LDL-c in men. In women, we have also found a significant ($p<0.05$) positive correlation between oxLDL and LDL-c and uric acid. A negative correlation between oxLDL and HDL-c has been found in women but not in men.

When we correlated F_2-isoprostanes with the other biomarkers by gender, there is a significant ($p<0.05$) positive correlation with TG in men but not in women. A negative correlation between F_2-isoprostanes and GR in both genders was observed. We have also found a significant ($p<0.05$) negative correlation between F_2-isoprostanes and GPx ,but we did not find it in men.

Discussion

The oxidative modification of LDL is one of the key events in early atherogenesis, both directly contributing to cholesterol accumulation in arterial wall macrophages and in promoting pro-inflammatory events that accelerate lesion development [9]. We observed positive correlation of oxLDL concentration with cholesterol levels and body mass index, in agreement with the results of Ozata et al. [21] and with Holvoet et al. [12], who established the relationship between circulating oxLDL levels and cardiovascular risk factors for subjects without any clinical evidence of cardiovascular disease.

In our study, we found that HDL-c was a negative determinant of oxLDL, likes Navab et al. [20], or Van

Table 2 Pearson's correlations of oxidized LDL and F_2-isoprostanes with the lipid profile and oxidative stress biomarkers in young healthy Spanish subjects

	Oxidized LDL			F_2-Isoprostanes		
	r	*p*	*n*	*r*	*p*	*n*
OxLDL (U/L)	1		72	−0.64	0.593	72
BMI (kg/m^2)	0.33	0.005	71	0.036	0.765	71
Cholesterol (mg/dl)	0.538	0.000	72	0.047	0.698	72
HDL-c (mg/dl)	−0.352	0.002	72	−0.2	0.87	72
LDL-c (mg/dl)	0.635	0.000	72	−0.4	0.741	72
Triglyceride (mg/dl)	0.285	0.015	72	0.264	0.025	72
Uric acid (mg/dl)	0.377	0.000	71	0.116	0.333	72
Homocysteine (μmol/L)	0.06	0.619	72	−0.16	0.892	72
GR (U/L)	0.009	0.941	72	−0.441	0.000	72
SOD (U/g Hb)	0.013	0.915	69	0.072	0.557	69
GPx (U/gHb)	0.043	0.724	69	−0.351	0.003	69
Protein carbonyls (nmol/mg)	−0.095	0.427	72	0.055	0.644	72
8-OHdG (ng/ml)	−0.117	0.328	72	−0.003	0.98	72
F_2-Isoprostanes (μg/g creatinine)	−0.064	0.593	72	1		72

Significant Pearson's correlation $p \leq 0.05$

OxLDL oxidized LDL; *BMI* body mass index; *GR* glutathione reductase; *SOD* superoxide dismutase; *GPx* glutathione peroxidase; *8OHdG* 8-hydroxy-2′-deoxyguanosine

Table 3 Pearson's correlations of oxidized LDL and F_2-isoprostanes with the lipid profile and oxidative stress biomarkers by gender

	Oxidized LDL				F_2-Isoprostanes			
	Male		Female		Male		Female	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
OxLDL (U/L)	1		1		−0.192	0.262	0.114	0.509
BMI (kg/m^2)	0.227	0.183	0.248	0.151	0.137	0.425	−0.095	0.586
Cholesterol (mg/dl)	0.576	0.000	0.299	0.077	0.098	0.571	−0.035	0.841
HDL-c (mg/dl)	−0.235	0.168	−0.399	0.016	−0.076	0.66	0.051	0.766
LDL-c (mg/dl)	0.642	0.000	0.496	0.002	−0.044	0.797	−0.039	0.82
Triglyceride (mg/dl)	0.23	0.178	0.226	0.185	0.404	0.015	−0.024	0.888
Uric acid (mg/dl)	0.123	0.474	0.445	0.007	0.091	0.596	0.281	0.097
Homocysteine (μmol/L)	0.043	0.802	−0.001	0.996	0.134	0.437	−0.15	0.381
GR (U/L)	−0.113	0.511	0.118	0.492	−0.524	0.001	−0.335	0.046
SOD (U/g Hb)	0.102	0.565	−0.01	0.954	0.04	0.824	0.122	0.486
GPx (U/gHb)	0.167	0.345	0.1	0.568	−0.303	0.081	−0.402	0.017
Protein carbonyls (nmol/mg)	−0.227	0.183	0.165	0.336	0.038	0.825	0.075	0.665
8-OHdG (ng/ml)	−0.076	0.660	−0.098	0.568	−0.129	0.453	0.075	0.662
F_2-Isoprostanes (μg/g creatinine)	−0.192	0.262	0.114	0.509	1		1	

Significant Pearson's correlation $p \leq 0.05$

OxLDL oxidized LDL; *BMI* body mass index; *GR* glutathione reductase; *SOD* superoxide dismutase; *GPx* glutathione peroxidase; *8OHdG* 8-hydroxy-2′-deoxyguanosine

der Zwan et al. [33]. There are several hypotheses about two possible mechanisms could contribute to the protective role of HDL in the formation of oxLDL [10, 13, 15, 23]; they are related to two HDL-associated enzymes that have been shown to inhibit LDL lipid-peroxidation in vitro: Paraoxonase 1 [16] and platelet-activating factor acetylhydrolase [35], respectively.

We also studied the relationship between circulating oxLDL and other potential cardiac risk factors, such as homocysteine, but we did not find any significant correlation. We have not found a relationship between oxLDL and other oxidative stress biomarker either. Galle et al. [8] found that formation of oxLDL is promoted by oxidative stress, but oxLDL itself has also been identified as a potent stimulus for vascular free radical formation. This, and the fact that our subjects are young and healthy, could explain our data.

When we studied the relationship between oxLDL and the others parameters by gender, we did not find the same correlations in men as in women. We have obtained a significant ($p<0.05$) positive correlation between oxLDL and total cholesterol in men, but we did not find it in women. However, we found a significant ($p<0.05$) positive correlation between oxLDL and uric acid in women but not in men. We believe that these differences could be explained by the small sample size by gender. Perhaps, with a higher population of each sex, we could obtain the same results that were obtained with the total number of subjects.

Isoprostanes are emerging as a new class of biologically active products of arachidonic acid metabolism of potential relevance to human vascular disease. Their formation in vivo seems to reflect primarily, if not exclusively, a nonenzymatic process of lipid peroxidation. F_2-isoprostanes formation is substantially increased in animal models of free radical-mediated injury [19] and is now used frequently as an accurate measure of oxidative stress in human studies. Measurements of specific F_2-isoprostanes in urine may provide a sensitive biochemical end point for dose-finding studies of natural and synthetic inhibitors of lipid peroxidation. We have measured this lipid peroxidation biomarker in a young healthy Spanish population, and although it has been a relatively small

group, we tried to establish normal reference values; we have not found any difference between gender (2.26±0.9 pg/g creatinine) conversely to Kaufman et al. [14] who found a strong, nonlinear relationship (indicated by the significance of the age), but it was in healthy children. The mean values of this study are lower than those we obtained before (5.17±4.74 Alcaraz et al. and 4.46±2.37 Cerdá et al.) [1, 3]. We think that these differences can be explained due to the difference in ages of the patients.

In the present trial, Urinary F_2-isoprostanes were significantly ($p<0.05$) positively correlated with TG according to several authors [7, 26] but conversely to them, no correlation with oxLDL has been found. We have also found a negative correlation between F_2-isoprostanes and antioxidants (GPx and GR).

When we studied the relationship between F_2-isoprostanes and the other parameters by gender, we did not find the same correlation in men as in women: F_2-isoprostanes were significantly ($p<0.05$) positively correlated with TG in men but not in women; the lower TG levels in women could perhaps explain it. We have found the same significant ($p<0.05$) negative correlation between F_2-isoprostanes and GR for men and women; for Gpx, only a significant ($p<0.05$) negative correlation was found in women; the higher results we found for this enzyme in women could explain it.

From this study, we have obtained our own reference values on oxidative stress biomarkers in a young healthy Spanish population, which differs from those found in the literature. Our SOD values are higher than Pincemail's values, obtained in 123 healthy people in Liège [24]. On the contrary, Pincemail et al. obtained GPx values higher than ours. In previous reports from this laboratory [6], we had obtained similar reference values for oxLDL in middle-aged subjects (58±11 years old) who were older than these in this study. All our previous laboratory reports showed oxLDL values lower than Sigurdardottir et al. [29], but they had established their control group with several degrees of obesity and insulin resistance.

In conclusion, the present study provides normal reference values in young healthy Spanish subjects for oxLDL and oxidative stress biomarkers. We have found normal reference values significantly different for oxLDL and GPx by gender.

We have also established the relationship between oxLDL, lipid profile and oxidative stress biomarkers in a young healthy Spanish population: oxLDL is correlated with lipid profile but not with the oxidative stress biomarkers. OxLDL was significantly and positively correlated with BMI, total cholesterol, TG, LDL-c, and uric acid while negatively with HDL-c, but it was not correlated to F_2-isoprostanes which are considered in the literature as one of the best lipid oxidative biomarkers. Urinary isoprostanes are positively correlated with triglycerides and negatively with GR and GPx.

We hope this work could contribute to detecting populations at risk of developing cardiovascular diseases evaluated through the damage of biological oxidative markers.

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