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Circulating oxidized low density lipoprotein, autoantibodies against them and homocysteine serum levels in diagnosis and estimation of severity of coronary artery disease

ELSA FAVIOU[1], GEORGIA VOURLI[1], CHARALAMPOS NOUNOPOULOS[2], ARIADNI ZACHARI[2], & AMALIA DIONYSSIOU-ASTERIOU[1]

[1]*Department of Clinical Biochemistry, Medical School, University Hospital "Attikon", 1 Rimini Street, 12462 Chaidari, Athens, Greece, and* [2]*Department of Biological Chemistry, Medical School, University of Athens, 75 M. Asias Street, 11527 Goudi, Athens, Greece*

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Abstract

The oxidative hypothesis of atherosclerosis proposes that oxidative modification of low density lipoprotein (LDL) plays a critical role in atherogenesis. The evaluation of LDL oxidation *in vivo* is therefore very important. However, data concerning the evaluation of the above biochemical marker is very limited in clinical practice. This study was conducted to test the hypothesis that plasma levels of ox-LDL reflect atherosclerosis and determine the clinical significance in the measurement of circulating ox-LDL and autoantibodies against them as well as their correlation with homocysteine and lipid parameters in the diagnosis and severity of coronary heart disease. A total of 273 individuals were examined: 41 suffering from unstable angina pectoris (UAP), 62 from stable angina pectoris (SAP) and 170 healthy control subjects. We used a sensitive method for detecting ox-LDL that is based on a direct sandwich technique (ELISA) in which two monoclonal antibodies are directed against separate antigenic determinants on the oxidized apolipoprotein-B molecule along with another enzyme immunoassay designed to determine human antibodies to oxidized LDL (anti-oxLDL) directly in serum. Total homocysteine (HCY) was evaluated by means of a fully automated fluorescence polarization immunoassay. Patients with UAP exhibited marked elevations in oxLDL levels as compared to patients with SAP (161.2 ± 28.4 vs. 119.2 ± 26.6, $p < 0.001$) and the control subjects (67 ± 18.8, $p < 0.001$). The difference in oxLDL levels between patients with SAP and the control group was also statistically significant. Similarly, patients with UAP showed marked elevations in anti-oxLDL antibodies compared to both patients with SAP (602.2 ± 62.2 vs. 510.8 ± 50.3, $p < 0.001$) and control subjects (368 ± 79.6, $p < 0.001$). The difference in anti-oxLDL levels between patients with SAP and the controls was also statistically significant. OxLDL levels were not correlated with age in any of the groups studied. Triglycerides, LDL-cholesterol and total cholesterol were elevated in patients with UAP as opposed to patients with SAP and the control subjects, while HDL levels were elevated in the control subjects when compared to patients with SAP and UAP. Homocysteine levels were elevated in patients suffering from UAP and SAP when compared to healthy subjects. Patients with UAP or SAP did not differ on homocysteine levels. Our findings demonstrate the presence of oxLDL *in vivo*, its strong association with coronary artery disease as well as with the severity of the clinical presentation.

Keywords: *Atherosclerosis, angina pectoris, LDL oxidation, homocysteine*

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Introduction

Oxidative modification of low-density lipoprotein (LDL) by reactive oxygen species (ROS) is believed to play a significant role in the development of atherosclerosis. Atherosclerosis is a chronic disease characterized by the presence of fibrous fatty plaques throughout the arterial wall. Its clinical consequences are cardiovascular diseases and especially coronary heart disease. Oxidized LDL (oxLDL) have antigenic properties which lead to an antibody response and

Correspondence: E. Faviou, 11 Tirteou Street, 17564, P. Faliro, Athens, Greece. Tel: 30 2109420247; 30 6974762427. E-mail: efaviou@in.gr

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an inflammatory reaction that is thought to be the driving force in the formation of atherosclerotic lesions. In addition, they presume to be responsible for the inability to self-regulate cholesterol metabolism [1,2]. The association between LDL oxidation and atherogenesis was first introduced by experiments showing that oxidized LDL caused injury to endothelial cells [3]. Antibodies against oxLDL are detected in the circulation while antioxidants have been shown to decrease the formation of atherosclerotic lesions and LDL oxidation in animal models [4–7]. Recently, oxLDL are being included in the spectrum of the potential risk factors for cardiovascular diseases along with infectious agents such as *Chlamydia pneumoniae* and *H. pylori*, inflammatory mediators such as high sensitive C-reactive protein and Interleukin-6 and haemostatic markers [8–15].

The evaluation of LDL oxidation *in vivo* is therefore very important. The measurement of circulating oxLDL and autoantibodies against them could help to determine the severity of atherosclerosis, to predict the risk of the development of cardiovascular disease as well as to estimate disease progression [16,17]. However, data concerning the evaluation of the above biochemical markers is very limited in clinical practice.

In the present study, we tried to evaluate the clinical significance in the measurement of circulating oxLDL and autoantibodies against them as well as their correlation with lipid parameters, in the diagnosis and severity of coronary heart disease. Thus, we measured the above parameters in two clinical stages of coronary atherosclerotic disease, stable angina pectoris (SAP) and unstable angina pectoris (UAP). Furthermore, we estimated serum levels of homocysteine, this novel independent cardio-cerebrovascular risk factor, as it is believed to be involved in the oxidation process of atherosclerosis [18].

Material–methods

A total of 273 individuals were examined: 103 patients with proven coronary heart disease (41 suffering from UAP and 62 suffering from SAP) and 170 healthy control subjects.

UAP was defined as an increase in the frequency and/or severity of chest pain or new onset angina within two months after a previous bout or angina occurring at rest and lasting for >20 min during the preceding 24 h. SAP was defined as chest pain typical of cardiac ischemia on exertion [19,20].

Serum and EDTA plasma samples were collected after 12 h of fasting early in the morning. The samples were taken upon hospital admission. The serum was centrifuged within an hour after collection and separated into aliquots. The aliquot for the assessment of human antibodies against oxLDL was frozen at -20°C until the assay was performed. The remaining aliquots were used for the analysis of each subject's lipid profile. For the measurements of oxLDL concentration and homocysteine levels, plasma was separated by centrifugation at 4°C immediately after its collection. The specimens remained stored at 0–4°C for a maximum duration of one week.

OxLDL was measured by a sensitive direct sandwich technique (ELISA), using mAb-4E6 monoclonal antibody as the capture antibody and another antihuman apolipoprotein-B monoclonal antibody, directed against separate antigenic determinants on the oxidized molecule, labeled by horseradish peroxidase (Mercodia AB, Uppsala, Sweden). The mAb-4E6 is directed against a conformational epitope in the apolipoprotein B-100 (apoB-100) molecule of LDL that is generated as a consequence of substitution of at least 60 lysine residues of apoB-100 with aldehydes. This number of substituted lysines corresponds to the minimal number of substituted lysines required for scavenger-mediated uptake of oxLDL [21].

The quantification of antibodies against oxLDL was performed using an enzyme-linked immunoabsorbent assay, designed to determine human autoantibodies to oxidized LDL directly in serum (oLAB Biomedica, Vienna, Austria). Antigens used for this assay were Cu^{++} oxLDL precoating a 96 microwell plate.

Serum total cholesterol (t-CHL), high density lipoprotein cholesterol (HDL-C) and triglyceride (TG) levels were determined by enzymatic methods (Biosis, Athens, Greece and Human Wiesbaden, Germany) on an automated analyzer (ROCHE Diagnostics) according to the manufacturer's recommendations. Low density lipoprotein cholesterol (LDL-C) was calculated according to the Friedewald formula.

Total homocysteine (HCY) was measured by means of a fully automated fluorescence polarization immunoassay (Abbott Laboratories).

Continuous data are presented as mean $\pm$ S.D. When two groups were compared, an unpaired Student's *t*-test or a Mann–Whitney U test was used depending on whether the variance was heterogeneous. When more than two groups were compared, one-way ANOVA followed by a Sheffe's test was performed.

Categorical data are summarized as percentages and comparisons were performed by a chi-square test. Pearson's correlation coefficients were used to evaluate the correlations between oxLDL and the other measured parameters.

In order to discriminate healthy subjects from patients (either SAP or UAP), a multivariate logistic regression model was fitted. The response of the model was the subjects' status (diseased/healthy). All laboratory characteristics were included as independent continuous variables.

A second multiple logistic regression model was fitted for discriminating the type of angina

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(unstable, stable) in the group of patients. Gender, smoking status, hypertension status and diabetes status were treated as categorical data, while HCY, HDL, LDL, triglycerides, oxLDL and anti-oxLDL were treated as continuous data.

For all tests performed, $p < 0.05$ was considered as significant.

Results

Characteristics of the study groups are presented in Table I. Forty one patients with UAP (31 males and 10 females, aged 60.8 ± 5.3), 62 patients with SAP (43 males and 19 females, aged 61.6 ± 6.0) and 170 healthy volunteers (89 males and 81 females, aged 63.4 ± 6.7) were examined. Twenty three of the patients with UAP and 37 patients with SAP were hypertensive (56.1 and 59.7%, respectively). Nine patients with UAP and 18 with SAP had diabetes (22 and 29%, respectively). None of the control subjects suffered from hypertension or diabetes.

Triglycerides, LDL-C and total cholesterol were elevated in patients with UAP as opposed to patients with SAP and the control subjects ($p < 0.001$). The observed difference regarding the triglycerides levels between patients with SAP and the control subjects was also significant ($p < 0.001$). As far as the LDL-C and total cholesterol levels are concerned, patients with SAP and the control subjects did not differ ($p = 0.625$ and 0.305, respectively). HDL levels were elevated in the control subjects when compared to patients with SAP and UAP ($p < 0.001$). The observed difference with reference to HDL levels between UAP and SAP was not significant ($p = 0.891$). HCY levels were elevated in patients suffering from UAP and SAP when compared to healthy subjects ($p < 0.001$). Patients with UAP or SAP did not differ on HCY levels ($p = 0.682$) (Table I).

Patients with UAP exhibited marked elevations in oxLDL levels as compared to patients with SAP (161.2 ± 28.4 vs. 119.2 ± 26.6, $p < 0.001$) and the control subjects (67 ± 18.8, $p < 0.001$). The difference in oxLDL levels between patients with SAP and the control group was also statistically significant ($p < 0.001$). Similarly, patients with UAP showed marked elevations in anti-oxLDL antibodies compared to both patients with SAP (602.2 ± 62.2 vs. 510.8 ± 50.3, $p < 0.001$) and control subjects (368 ± 79.6, $p < 0.001$). The difference in anti-oxLDL levels between patients with SAP and controls was also statistically significant ($p < 0.001$) (Figure 1).

With regard to the healthy individuals as well as the patients, oxLDL was found to be positively correlated with total cholesterol, triglycerides, LDL cholesterol and HCY levels, while it was found to be negatively correlated with HDL cholesterol. All correlations were significant, but their magnitudes differed (Figures 2 and 3). In patients with either SAP or UAP, oxLDL was correlated with anti-oxLDL ($r = 0.810$, $r = 0.578$ respectively, $p < 0.001$ in both cases) (Figure 2), while in the control group the correlation between oxLDL and anti-oxLDL was low and not significant ($r = 0.106$, $p = 0.423$) (Figure 3).

In order to examine whether the presence of hypertension and diabetes mellitus may affect oxLDL levels, we compared these levels in the presence and absence of the above pathological conditions. In patients with UAP, plasma levels of oxLDL were not significantly higher in patients with or without diabetes ($p = 0.531$). In addition, plasma levels of oxLDL did not differ among patients with or without hypertension ($p = 0.215$). Similarly, in the patients with SAP, plasma levels of oxLDL were not significantly higher in patients with or without diabetes ($p = 0.480$). Likewise, regardless of the absence or presence of hypertension, plasma levels of oxLDL were not significantly higher ($p = 0.156$) (Figure 4).

In the group of patients (SAP or UAP) there was no difference on oxLDL levels between men and women, or smokers and non-smokers. However, in the control group, a significant difference was found between men and women ($p < 0.001$) and between smokers and non-smokers ($p = 0.022$) (Figure 5).

OxLDL levels were not correlated with age in any of the groups studied.

Table I. Characteristics of CAD patients and control subjects, p_1 corresponds to the comparison between patients with SAP and patients with UAP, p_2 corresponds to the comparison between patients with UAP and controls and p_3 corresponds to the comparison between patients with SAP and controls.

	SAP ($N = 62$)	p_1	UAP ($N = 41$)	p_2	CONTROLS ($N = 170$)	p_3
Age	61.6 ± 6	0.820	60.8 ± 5.3	0.150	63.4 ± 6.7	0.058
Smoking	26 (41.9%)	0.843	18 (43.9%)	0.980	75 (44.1%)	0.767
Male gender	43 (69.4%)	0.490	31 (75.6%)	0.007	89 (52.4%)	0.021
Total cholesterol (mg/dl)	184.8 ± 34.3	<0.001	230.8 ± 39.7	<0.001	192.5 ± 31.7	0.305
Triglycerides (mg/dl)	153.5 ± 25.5	<0.001	178 ± 28.1	<0.001	134.8 ± 30.1	<0.001
HDL-cholesterol (mg/dl)	40.4 ± 4.8	0.891	39.7 ± 9.9	<0.001	46.9 ± 7.8	<0.001
LDL-cholesterol (mg/dl)	113.7 ± 33	<0.001	155.5 ± 40.9	<0.001	118.7 ± 33.2	0.625
Homocysteine (μmol/L)	12.7 ± 5.3	0.682	13.5 ± 4.7	<0.001	8.6 ± 3.6	<0.001

Results are expressed as mean ± SD.

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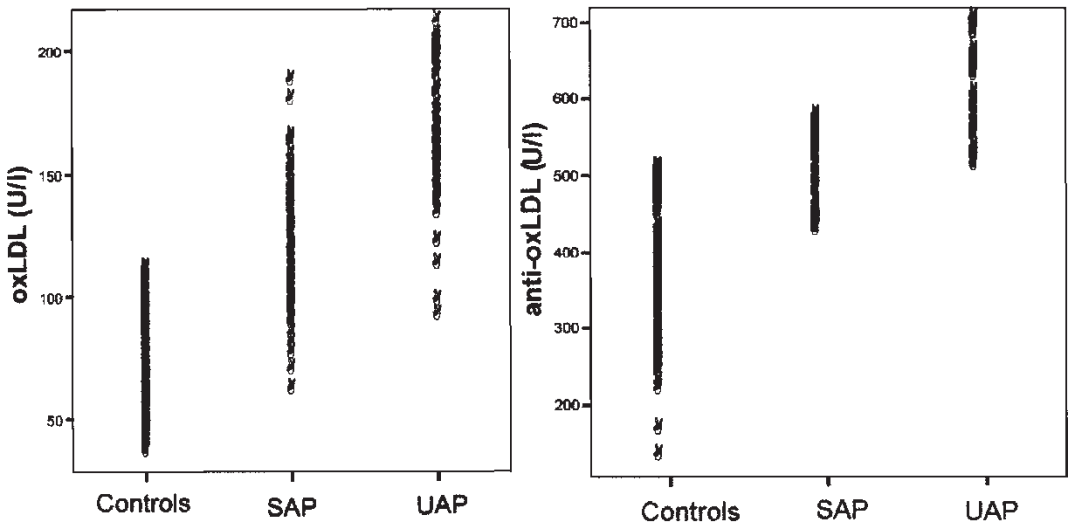

Figure 1. Individual oxLDL and anti-oxLDL levels in the three groups of subjects.

According to our first logistic regression model, higher values of oxLDL and triglycerides as well as lower values of HDL cholesterol imply a higher risk for developing angina, stable or unstable (Table II).

According to the second fitted model, which had angina status (unstable/stable) as response and included gender, smoking, hypertension, age, triglycerides, HDL, LDL, HCY, oxLDL and anti-oxLDL levels as independent variables, UAP is associated with elevated oxLDL and anti-oxLDL levels, while SAP is associated with elevated HCY levels (Table III).

Discussion

Cardiovascular disease is the primary cause of death in westernized populations. Although the rate of death from ischemic heart disease is declining, the economic burden to society remains high. Since a number of cardiovascular fatalities may be preventable, the search for novel risk factors continues at a rapid pace. The oxidative modification of LDL is now considered to be a key event in the biological process that initiates and accelerates the development of the atherosclerotic lesion, the fatty streak [1,2]. Thus, a high oxLDL level is one of the most recent risk factors to be identified. The hypothesis that oxLDL is necessary in the development of atheroma was suggested many years ago when it was observed that the foam cell formation probably was not due to the uptake of native LDL by macrophages via classic LDL-receptors. In contrast, uptake of oxLDL via scavenger receptors resulted in the unregulated accumulation of lipid [17]. Macrophages express scavenger receptors for oxLDL which can bind and take up oxLDL. To date, more than ten receptors for oxLDL have been found and cloned [22–24].

OxLDL has been suggested as having a plethora of atherogenic effects [17,25]. The wide range of the atherogenic properties of oxLDLs include inducing the expression of adhesion molecules on endothelial cells, monocyte chemotaxis and adhesion, cytotoxicity, platelet aggregation and thrombus formation, and destabilizing plaques through several mechanisms [26–28]. OxLDL inhibits the vasodilatation that is normally induced by NO; OxLDL is immunogenic as well as mitogenic for macrophages [29]. The evaluation of LDL oxidation *in vivo* is therefore very important for it appears that the oxidative modification of LDL leads to an incredibly large number of consequences above and beyond the generation of foam cells, equally important in atherogenesis.

Oxidized LDL as well as antibodies against epitopes of oxLDL have been found in atherosclerotic lesions of both human and experimental animals in several studies [30–32]. LDL oxidation affects both protein and lipid content, resulting in the formation of many oxidative products [33]. These include esterified and unesterified peroxidized lipids, lysoPtdCho, cholesterol oxidation products, aldehydes derived from breakdown of both esterified and unesterified oxidized fatty acids, and perhaps proteolipids that may have peroxidized lipids bound to fragmented ApoB-100. It is therefore apparent that there is a broad spectrum of oxidatively modified LDL [34]. The extent of these modifications depends on the existence of prooxidant conditions and the length of time the particle is exposed to them. Moreover, it seems that these modified groups differ not only structurally, but also functionally [35].

Under oxidative stress, oxidative modification of LDL takes place in the subendothelial space of the arterial wall, and a small amount of oxLDL is released into the circulation [36,37]. Thus, measurement of plasma oxLDL is essential not only for investigating its relevance to arteriosclerotic diseases, but also for estimating the degree of the occurring oxidative stress.

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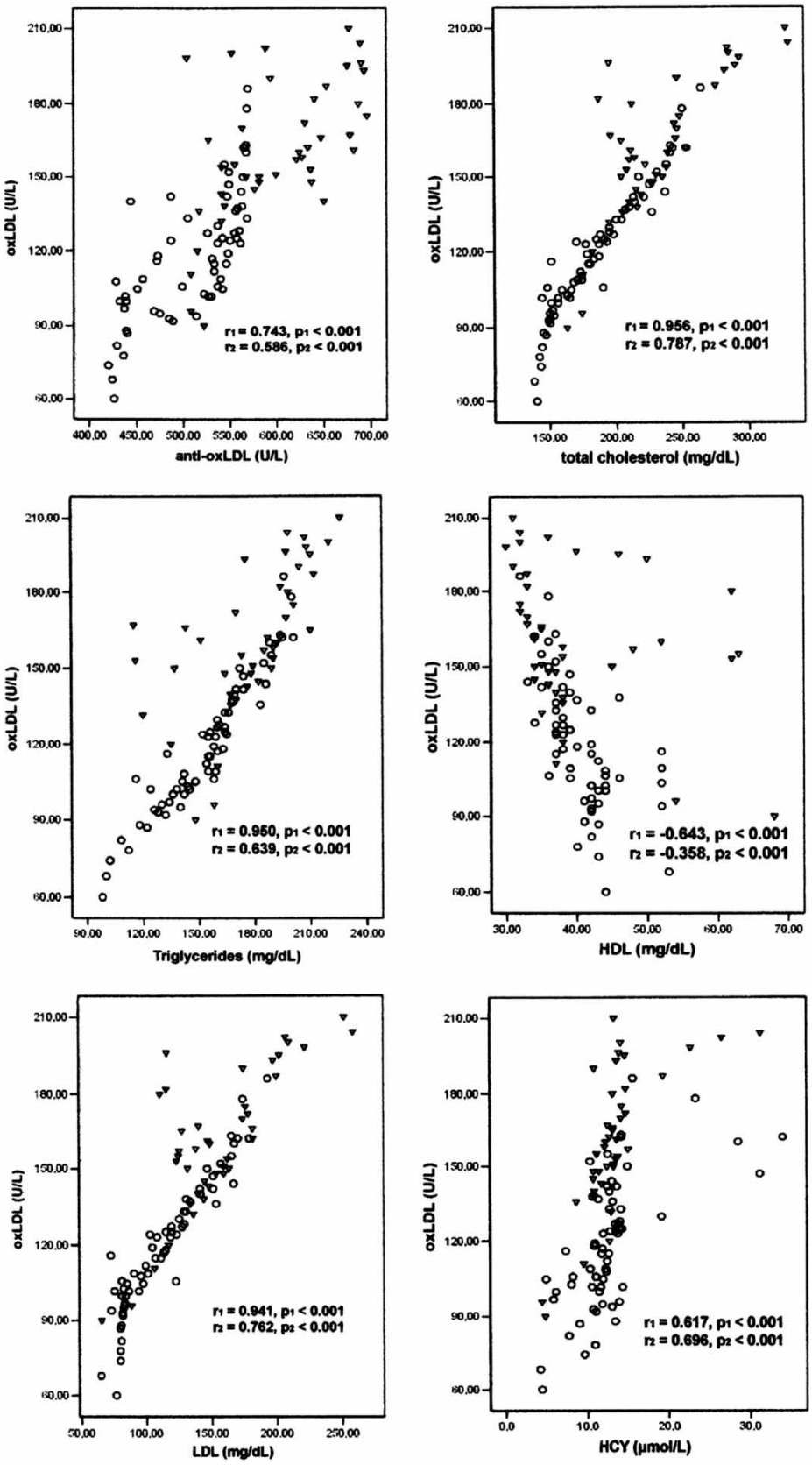

Figure 2. Plasma levels of oxLDL vs. anti-oxLDL, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, HCY in the group of patients. Solid circles and rectangles correspond to patients with SAP and patients with UAP respectively. Pearson's correlation coefficients and corresponding *p*-values are presented on each plot. Values with subscript 1 refer to patients with SAP while values with subscript 2 correspond to patients with UAP. All biochemical characteristics are significantly correlated with oxLDL, in both patients with SAP and UAP.

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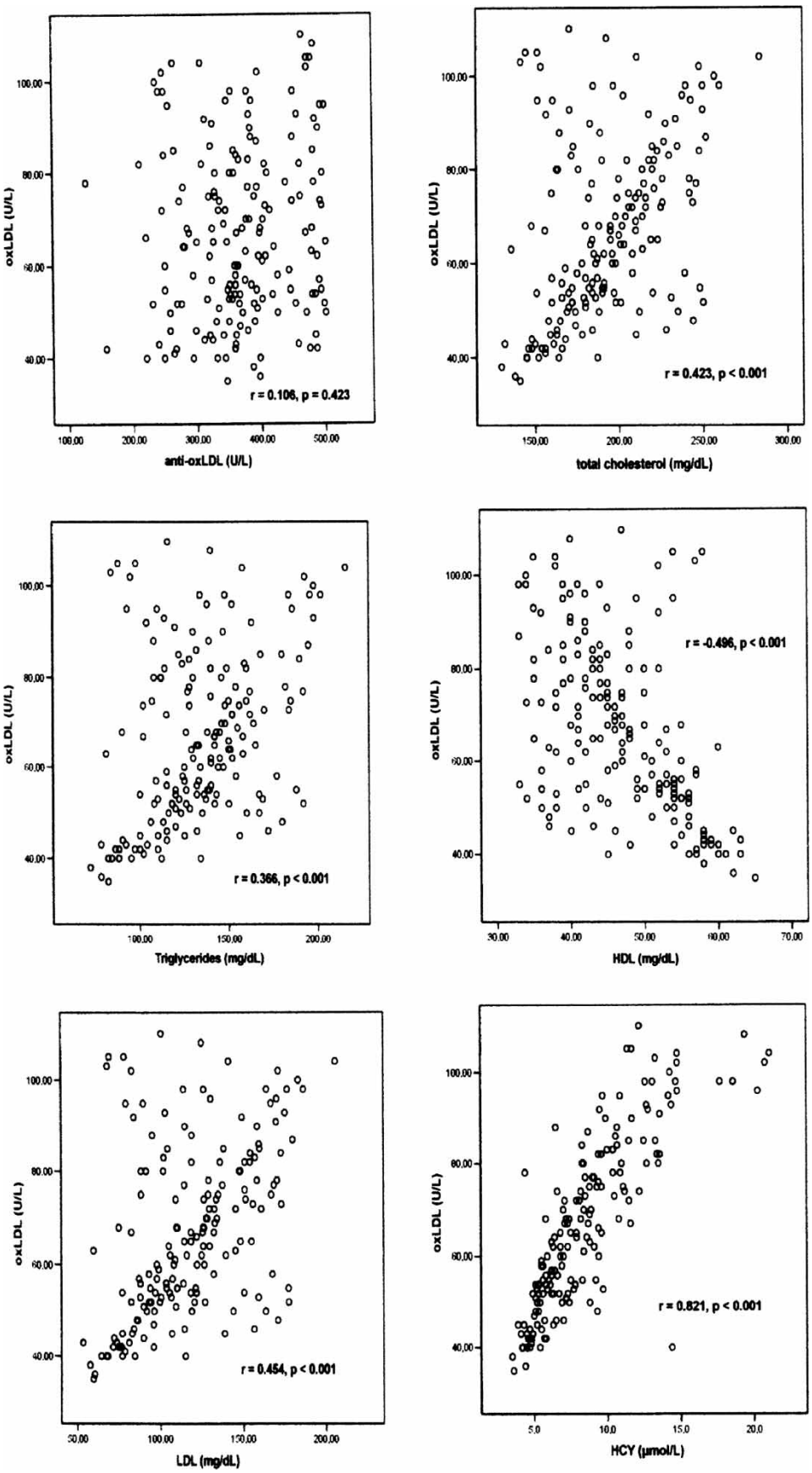

Figure 3. Plasma levels of oxLDL vs. anti-oxLDL, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, HCY in control subjects. Pearson's correlation coefficients and corresponding *p*-values are presented on each plot. In the group of healthy subjects, correlation between oxLDL and antibodies against them is not significant.

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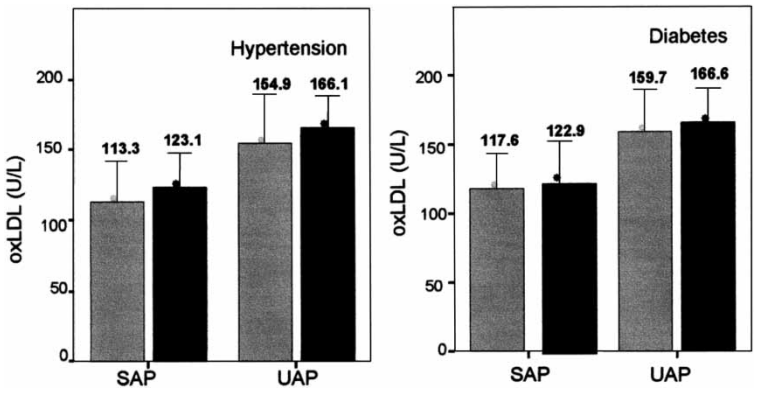

Figure 4. Relationship between oxLDL levels and hypertension (left plot) and between oxLDL levels and diabetes mellitus (right plot). Grey bars correspond to absence of the characteristic while black bars correspond to presence of the characteristic. Mean oxLDL levels are shown above each bar. Error bars indicate standard deviation.

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Until recently, it has been difficult to measure epitopes of circulating oxLDL in human plasma, mainly due to two reasons: the heterogeneity of the degree of oxidation of LDL and the lack of methods capable of specifically detecting them. Nowadays, there are plenty of methods available for assessing the oxidative modification of LDL, but they seem to reflect different processes or stages [4,38]. As far as immunoassays are concerned, the efforts of several researchers have been devoted to the measurement of circulating oxLDL using different anti-oxLDL antibodies [21,36,39–43]. Unfortunately, there is a clinical limitation regarding the number of immunoassays which require the isolation of LDL by means of density ultracentrifugation, therefore making their performance impractical outside of the investigational arena. In the present study, we measured the aldehydic modification of the apoB-100 molecule that takes place on the extensively oxidized LDL found in atherosclerotic lesions, by means of a commercially available ELISA kit utilizing two monoclonal antibodies. The capture antibody was the same monoclonal antibody (mAb-4E6) as in the assay described by Holvoet et al. [21].

In immunoassays using different anti-oxLDL antibodies, Ehara et al. [44] and Nishi et al. [37] found that plasma oxLDL levels increase in acute myocardial infarction and in carotid artery atherosclerosis respectively. Holvoet et al. have initially observed significant increases in oxLDL concentrations in plasma from patients who had undergone heart transplantation and hemodialysis [45,46]. They suggested that plasma levels of circulating oxLDL might be associated with coronary disease [41,45]. Increased levels of circulating oxLDL have also been measured in clinically healthy individuals with subclinical atherosclerosis [47,48]. These results suggest that circulating oxLDL could provide useful data concerning various manifestations of atherosclerosis and could be a potential biochemical marker of coronary artery disease that is correlated with the majority of risk factors [21].

In our study, patients with UAP showed marked elevations in oxLDL levels when compared to patients with SAP and the control subjects. The difference in oxLDL levels between patients with SAP and the control group was also statistically significant. Our findings show a significant positive correlation with

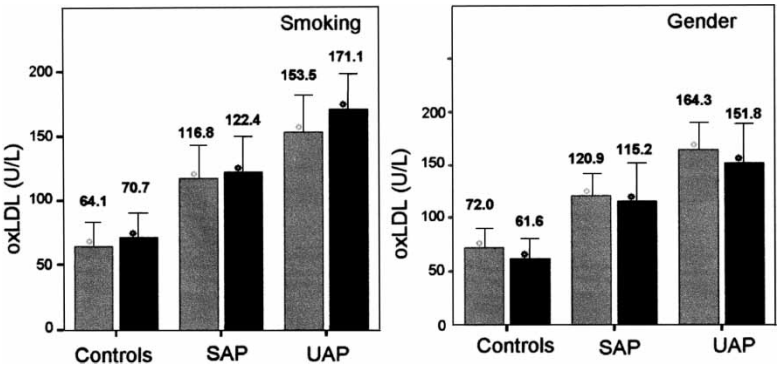

Figure 5. Relationship between oxLDL levels and smoking status (left plot) and between oxLDL levels and gender (right plot). Grey bars correspond to absence of the characteristic or males while black bars correspond to presence of the characteristic or females. Mean oxLDL levels are shown above each bar. Error bars indicate standard deviation.

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Table II. The multivariate logistic regression analysis revealed high levels of oxLDL and triglycerides and low levels of HDL-cholesterol as important factors for developing angina.

Covariate	OR	P
OxLDL	1.196	0.005
Abs vs. oxLDL	1.041	0.135
Triglycerides	1.103	0.008
HDL-cholesterol	0.568	0.006
LDL-cholesterol	0.800	0.190
Homocysteine	0.673	0.072

This model correctly classified 98.2% of the subjects using a cut-off of 0.5, while R2 was 0.709.

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regard to the severity of the clinical presentation and are in accordance with other studies conducted by Ehara et al. [44,49] who demonstrated that patients with acute myocardial infarction had the highest serum oxLDL concentrations followed by those with unstable angina. They also studied immunohistochemically the presence of oxLDL in coronary specimens and discovered that the more severe atherosclerotic lesions contain a significantly higher percentage of oxLDL-positive macrophages. All the above-mentioned findings suggest that oxLDL present within unstable plaques may be released into the blood stream in patients suffering from UAP with severe endothelial injuries, such as plaque erosion or rupture. In more stable patients, it is likely that the levels of oxLDL reflect the turnover of oxLDL in newly formed or progressing lesions not just in coronary arteries but also in systemic arteries. Similarly, Tsimikas and Witztum [39] reported that elevated serum levels of oxLDL could be explained by ruptured or permeable atherosclerotic plaques and ischemic injury. In addition, Nishi et al. [37] suggest that LDL levels reflect oxidative conditions, and that high plasma and plaque oxLDL levels are associated with the vulnerability of carotid atherosclerotic lesions to rupture. In contrary, data from a study of Hulthe et al. [47] in clinically healthy individuals (with mainly normal C-reactive protein levels) are refuting the above suggested mechanisms as the sole explanations. Moreover, Holvoet et al. [36] found no significant differences in plasma levels of oxLDL between patients with acute coronary syndromes and those with stable coronary artery disease.

Table III. Multiple logistic regression analysis for discriminating patients with UAP and SAP.

Covariate	OR	P
Gender	0.401	0.399
Smoking	0.490	0.408
Hypertension	4.213	0.082
Age	0.955	0.470
OxLDL	1.095	0.034
Abs vs. oxLDL	1.022	0.023
Triglycerides	0.964	0.244
HDL-cholesterol	1.112	0.208
LDL-cholesterol	1.032	0.222
Homocysteine	0.652	0.002
Diabetes	0.233	0.174

For this model, R2 was 0.552 and 87.4% of cases were correctly classified using a cut-off of 0.5.

OxLDL particles cause *in vivo* formation of autoantibody which can be isolated from atherosclerotic lesions and measured in serum. The relationship between serum concentrations of such antibodies and atherosclerotic disease is not fully clarified [50]. Several studies indicated that higher levels of anti-oxLDL titer were associated with the presence of atherosclerotic disease [51,52]. Inoue et al. [53] have demonstrated that the anti-oxLDL titer was elevated in severe coronary disease (unstable angina or acute myocardial infarction) but not in mild coronary artery disease and Tsai et al. [54] concluded that anti-oxLDL is higher in patients with acute myocardial infarction and is correlated with myocardial damage to a greater degree than with the severity of coronary atherosclerosis. In a recent study, Wang et al. [55] suggest that the expression level of anti-oxLDL antibody play a role in the pathogenesis of disease. In other studies, however, no such relationships between atherosclerotic disease and the antibody titer have been found [56,57]. Rossi et al. [58] found no significant difference in anti-oxLDL titer between patients with and without coronary artery disease of different degrees of severity. In our study, patients with UAP showed marked elevations in anti-oxLDL antibodies when compared to patients with SAP and the control subjects. The difference in anti-oxLDL levels between patients with SAP and the control subjects was also statistically significant. In both patients with SAP and UAP, oxLDL was correlated to anti-oxLDL, while oxLDL was not correlated to anti-oxLDL in healthy subjects. The high titer of anti-oxLDL probably reflects the greatest atherosclerotic injury of arteries in patients with UAP, followed by SAP and the control subjects.

Thus, the higher levels of anti-oxLDL in patients with UAP could confirm that the humoral autoimmune response against oxLDL antigens in the atherosclerotic plaque plays a crucial role in the development of atherosclerosis. Recently, several authors investigated the role of circulating oxLDL as an independent predictor for cardiovascular manifestations either in healthy individuals or in patients with coronary artery disease. In a study performed by Shimada et al. [59] levels of circulating oxLDL were significantly higher in patients with a history of cardiac event (cardiac death, myocardial infarction or refractory angina requiring revascularization) than in patients without a cardiac event. In addition, Laaksonen et al. [18] attributed an important role to oxLDL and plasma homocysteine on early

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impairment of coronary reactivity in young adults. Our first logistic regression model (Table II) revealed the association of elevated levels of ox-LDL with the presence of angina, stable and unstable, indicating the involvement of oxLDL with regard to the extent of atherosclerosis and the severity of coronary artery disease.

In our study, we evaluated known risk factors such as lipid profile, homocysteine, diabetes and hypertension and their role in atherosclerosis. In the studied patients as well as the healthy individuals, oxLDL was found to be positively correlated with total cholesterol, triglycerides, LDL cholesterol and HCY levels, while it was found to be negatively correlated with HDL cholesterol. Negative correlation with HDL cholesterol may be attributed to antioxidant properties of HDL [60,61]. According to our second logistic regression model (Table III), SAP is associated with elevated homocysteine levels while unstable angina is associated with elevated oxLDL and anti-oxLDL levels. To the best of our knowledge this is the first study that attempts to correlate oxLDL with homocysteine in patients suffering from cardiovascular disease. Homocysteine is involved in vascular endothelial dysfunction [62], thus resulting in the acceleration of LDL oxidation. Holvoet et al. [21] investigated the correlation between circulating oxLDL and major cardiovascular risk factors, although omitting homocysteine, in subjects without clinical evidence of coronary artery disease.

In the patients with UAP, plasma levels of oxLDL were not significantly higher in patients in the absence or presence of diabetes. In addition, plasma levels of oxLDL did not differ among patients with or without hypertension. Similarly, in the patients with SAP, plasma levels of oxLDL were not significantly higher in patients with or without diabetes, or with or without hypertension. It is worthy to pay attention to our findings regarding oxLDL levels in healthy smokers. Hulea et al. [63] in a case control study suggested that oxidative LDL damage caused by oxidants present in cigarette smoke may be involved in the pathogenesis of coronary disease. Until now, according to the published reports, there still exists controversy over this subject [64–66]. In the group of the studied patients (SAP or UAP) no difference was found on oxLDL levels between men and women, or smokers and non smokers. However, in healthy individuals a significant difference was found between men and women ($p < 0.001$) and between smokers and non-smokers ($p = 0.022$). Zaratin et al. [67] evaluated the contribution of cigarette smoking on LDL oxidizability and plasma levels of antioxidized LDL in a healthy population of normolipidemic smokers and found similar results.

In conclusion, our findings demonstrate the presence of oxLDL *in vivo*, its strong association with coronary artery disease as well as with the severity of the clinical presentation. Undoubtedly, in order to obtain a potential application of oxLDL in the screening and follow-up of high risk individuals there is an imperative need for many clinical studies, pointing out the fact that an objective laboratory estimation of coronary artery disease can be achieved only by means of a biochemical test profile exclusively including multiple biochemical markers capable of significantly improving the diagnostic sensitivity and specificity. This would not only allow for early detection of subclinical stages of cardiovascular disease but it would also single out its various stages.

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