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Oxidized LDL and NO Synthesis as Biomarkers of Atherogenesis – Correlations with Metabolic Profile in Elderly

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1. Introduction

Atherosclerosis is a complex, multifactorial disease, developed in the arterial wall in response to various forms of injurious stimuli, resulting in excessive inflammatory and fibro-proliferative reactions. The endothelial cells are involved in all stages of atherogenesis and their dysfunction is a key initial event in the atherosclerotic plaque formation (Simionescu, 2007). The vascular endothelium, with its broad spectrum of paracrine and autocrine functions, can be regarded as a multifunctional organ and "chief governor" of body homeostasis. Occupying a strategic location between the blood and tissues, the endothelial cells exist in a "high-risk position" and react progressively to aggressive factors, at first by modulation of the constitutive functions - permeability and biosynthesis (Simionescu & Antohe, 2006; Sima et al., 2009). Atherogenesis is an intricate process involving hyperlipidemia, oxidative stress and vascular inflammation. Among the diversity of mechanisms implicated in the pathogenesis of atherosclerotic vascular diseases two of them have been discovered in parallel and studied extensively: the oxidation of low-density lipoprotein (LDL) and the synthesis of endothelium-derived nitric oxide (NO).

1.1 Relationship between oxidized LDL and NO as biomarkers of oxidative stress and endothelial dysfunction

Oxidized LDL and NO are recognized to exert contradictory actions within the vascular endothelium microenvironment and to influence the key events in the development of atherosclerosis such as leukocyte adhesion, platelet aggregation and vascular smooth-muscle cell proliferation and migration. While oxidized LDL (oxLDL) - a biomarker of lipoprotein-associated oxidative stress, is identified as a non-traditional pro-atherogenic emerging cardiovascular risk factor, NO is a free radical signal-transducing molecule that maintains the vasodilating tone, modulates *in vitro* lipid peroxidation reactions and alters proinflammatory gene expression (Figure 1).
Endothelial dysfunction - known to precede the development of atherosclerosis, is a systemic pathological state of the endothelium defined as an imbalance between

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vasodilating and vasoconstricting substances produced by (or acting on) the endothelium (Deanfield et al., 2007), leading to a reduced vasodilation, and even a proinflammatory and prothrombotic state (Cottone & Cerasola, 2008).
The most important of the vasodilating substances is nitric oxide, characterized as a non-eicosanoid component of endothelial-derived relaxation factor (EDRF), which is continuously synthesized by the endothelium under the action of different neurohumoral mediators such as acetylcholine, histamine, bradikinine, vasopressine, thrombine and serotonine (Rubbo et al., 1996).

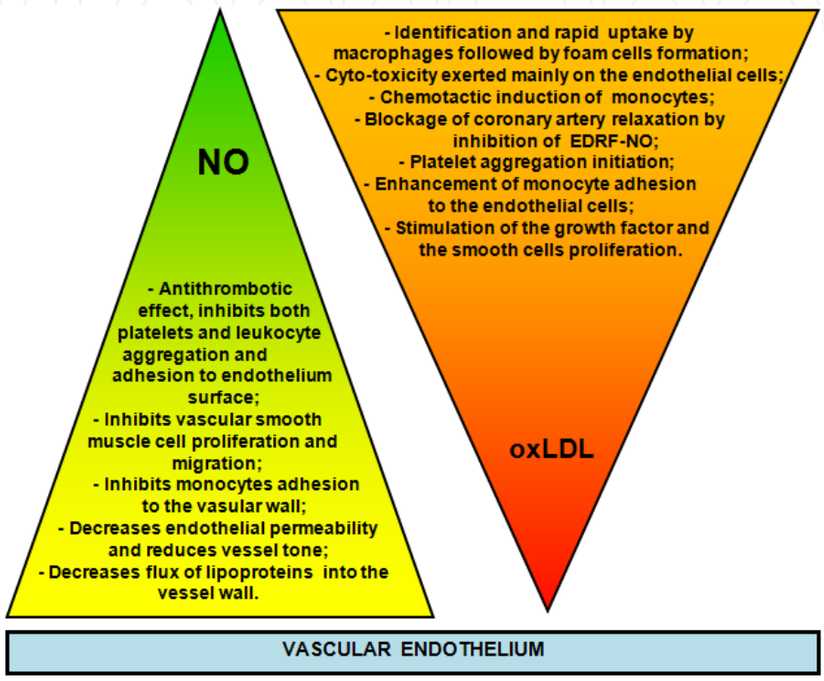


Fig. 1. Antiatherogenic effects and role of nitric oxide (NO) *versus* proatherogenic actions of oxidized LDL (oxLDL) exerted on vascular endothelium.

NO is produced by a variety of mammalian cells including: vascular endothelial cells, neurons, smooth muscle cells, macrophages, neutrophils, platelets, cardiomyocytes and pulmonary epithelium. The family of three enzymes responsible for the synthesis of NO, nitric oxide synthases (NOSs): endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) require calmodulin binding for their activities. The inducible nitric oxide synthases are transcriptionally regulated by cytokines and redox-sensitive transcriptional factors. Bacterial and parasitic antigens, which potently induce the expression of cytokines, also lead to induction of iNOS gene expression (Rubbo et al., 1996; Lundberg & Weitzberg, 2005).
In the endothelial microenvironment, concurrently, a variety of substances that adversely influence endothelial function have been recognized, including free fatty acids, cytokines such as TNF-α, and prooxidant molecules - including oxidized low-density lipoprotein (oxLDL). There are strong evidences for the role of oxidative stress in all stages of atherogenesis. Among different molecular targets affected by oxidative stress associated with hyperlipidemia and hyperglycemia, LDL is one of the most significant because is the major cholesterol carrier in the blood and contains also a relevant amount of polyunsaturated fatty acids (PUFAs) - the major substrate for lipid peroxidation.

The initial event in atherogenesis is the increased transcytosis of low-density lipoprotein, and its subsequent deposition, retention and oxidative modification in the subendothelium. It is followed by the infiltration of activated inflammatory cells from the coronary circulation into the arterial wall (Hulsmans & Holvoet, 2010).

The oxLDL is a byproduct of exposure to reactive oxygen species (ROS), and several potential mechanisms have been proposed for LDL oxidation: cell-mediated lipoxygenase and myeloperoxidase activities, non-enzymatic metal ion-mediated oxidation (iron, copper), superoxide generators (xanthine oxidase, NADPH-oxidase), thiol-dependent oxidation, peroxynitrite and other radical generation compounds (Parthasarathy et al., 2008).

Oxidatively modified lipoproteins lead to progression of atherosclerosis through macrophages engulfing oxLDL at the level of scavenger receptors, intracellular depositing of cholesterol esters and at last macrophages transformation into foam cells. Also, oxLDL can induce an immune response leading to anti-oxLDL autoantibodies production, which will determine formation of immune complexes (Steinberg et al., 1989; Tsimikas & Witztum, 2001; Parthasarathy et al., 1999, 2008).

Endothelium relaxant factor is a central molecule in vascular homeostasis as a modulator of endothelial tone and reactivity, exerting pleiotropic positive effects on the cardiovascular system. Important for the cardiovascular biology is the consumption of NO by reactive oxygen species. Oxidative modification of NO not only leads to reduced bioavailability but also produces the toxic oxidant peroxynitrite ($ONOO^-$), which further aggravates the imbalance of protective and aggressive factors (Cai & Harrison, 2000; Schnabel & Blankenberg, 2007). Subsequently, LDL oxidative modifications are made possible through simultaneous NO and superoxide anion radical ($O_2^{\cdot -}$) actions.

A key determinant of the pro-oxidant *versus* oxidant-protective influences of NO is the underlying oxidative status of tissue. When NO is in excess of surrounding oxidants, lipid oxidation and monocyte margination into the vascular wall are attenuated, producing antiatherogenic effects. However, when endogenous tissue rates of oxidant production are accelerated or when tissue oxidant defenses become depleted, NO gives rise to secondary oxidizing species that can increase membrane and lipoprotein lipid oxidation as well as foam cell formation in the vasculature, thus promoting proatherogenic effects (Bloodsworth et al., 2000).

Therefore, targeting particularly upstream targets – substrates for oxidation and inflammation, will be important to better understand interactions of hyperlipidemia, inflammation and oxidation.

Current evidence suggests that endothelial function is an integrative marker of the net effects of damage from traditional and emerging risk factors on the arterial wall and its intrinsic capacity for repair. This endothelial-dependent vascular biology is critical, not only in the initiation and progression of atherosclerosis, but also in the transition from a stable to an unstable disease state with attendant risks. As a result, study of endothelial function has emerged as an important endpoint in clinical research (Deanfield et al., 2007).

1.2 Oxidized LDL and NO endothelial synthesis as factors affecting the vascular ageing

Diseases of the vascular system have long been considered to be age-related in terms of their onset and progression. Longevity is a vascular question. More than 50 years ago, a famous anatomist – Rudolf Altschul stated that we have the age of our blood vessels: "a man is as

old as his arteries". Senescent cells undergo distinct changes in gene expression that may cause an impairment of cellular function. In endothelial cells these changes result in a phenotype that is pro-inflammatory, pro-atherosclerotic, and prothrombotic. Endothelial cell senescence can be induced by a number of factors implicated in vascular pathologies, particularly by sustained cell replication and oxidative stress (Erusalimski, 2009).

Oxidative stress and inflammation are major determinants of arterial and biological ageing. Recent studies underscore the association between white blood cell (WBC) telomere length, as index of systemic aging, oxidized LDL, and human vascular aging, expressed by the distensibility of the carotid artery. Results showed that higher levels of oxidized LDL are associated with shorter WBC telomeres and increased stiffness of the carotid artery (Nawrot & Staessen, 2008; Nawrot et al., 2010).

Ageing is characterized not only by a reduced arterial compliance and alteration of the contractile properties of the vascular wall, but also by endothelial dysfunction (Alvarez de Sotomayor, et al., 2005; Brandes et al., 2006). At present, there are several reasons to believe that *in vivo* NO synthesis from L-arginine could indeed be impaired in atherosclerosis, hypertension, dyslipidemia, diabetes, obesity, insulin resistance, metabolic syndrome, as well as in ageing (Lind, 2002; Laroia et al., 2003; Hsueh & Quinones, 2003; Holvoet et al., 2003, 2008a; Vickers et al., 2009; Park et al., 2009; Njajou et al., 2009; Huang, 2009; Park et al., 2011; Tabit et al., 2010).

Recent studies support the fact that advancing age increases the LDL susceptibility to oxidation and decreases the nitric oxide availability and bioactivity (Heffernan et al., 2008). Not only LDL but also very low-density lipoprotein (VLDL), beta–VLDL and even HDL undergo oxidative modification that must be taken into consideration in the complex process of atherosclerosis (Parthasarathy et al., 2008). In elderly, higher oxLDL levels were associated with high coronary risk before any clinical manifestation of CHD (Holvoet et al., 2003), and with higher arterial stiffness, independent of cardiovascular disease risk factors (Brinkley et al., 2009). The oxLDL/Apo-B100 ratio and to a lesser extent the oxLDL/LDL-C ratio were significantly negative associated with the flow-mediated-dilation (FMD) of the brachial artery (van der Zwan et al., 2009).

1.3 Methods for measuring the circulating oxidized LDL and NO endothelial synthesis

Oxidative biomarkers are now showing strong associations with progression of coronary artery disease (CAD) and predict cardiovascular events, suggesting that they may serve as surrogates and may complement diagnostic investigations. Both *in vitro* and *in vivo*, low-density lipoprotein (LDL) particles are susceptible to oxidation and peroxidation by all of the causes of oxidative stress. Therefore, oxidized LDL are included among the "downstream markers" of oxidative stress. During the last decade, several monoclonal antibodies have been generated, each recognizing at least a substantial subset of the whole spectrum of oxLDL particles, leading to a myriad of new reports on the relation between circulating ox-LDL and cardiovascular pathological processes (Itabe and Ueda, 2007; Tsimikas, 2006).

Currently used assays for oxLDL detect minimally oxidized LDL particles. In addition, concentrations of oxLDL depend on the sensitivity of LDL to oxidation; small dense LDLs contain smaller amounts of antioxidants and are, therefore, more prone to oxidation. The widely applied sensitive immunoassay quantifying the circulating levels of oxLDL uses a monoclonal antibody – 4E6, directed against oxidized apolipoprotein B-100 moiety of LDL (Rietzschel et al., 2008; Holvoet et al., 2008b).

The NO activity is assessed representatively using a variety of clinical invasive and noninvasive methods among which, the use of acetylcholine that induces endothelium-dependent dilation and smooth muscle–mediated constriction. The coronary artery diameter is compared by quantitative angiography before and after infusion of acetylcholine. The functional status of the coronary microvasculature can also be assessed using intracoronary Doppler ultrasound to measure blood flow in resistance vessels in response to substances that produce either endothelial-dependent or endothelial-independent vasodilation. Another noninvasive method of detecting endothelial dysfunction uses high-resolution ultrasound to measure the brachial artery diameter in response to reactive hyperemia, which stimulates NO release and FMD (Davignon & Ganz, 2004).
NO present in the circulation is originating from endothelial, smooth muscle cells, thrombocytes, leukocytes and cardiomiocytes. NO activity is the net result of a balance between its production and its inactivation by oxygen free radicals. NO released "in vivo" by nitric oxide synthase (NOS) activity in endothelial cells and platelets, rapidly autooxidizes to yield nitrite (NO_2^-), which interacts with oxyhemoglobin yielding nitrate (NO_3^-). Because nitrite plus nitrate are relatively stable compounds in blood, their levels may be a biochemical index of systemic NO production. This is convenient because direct *in vivo* measurements of NO can be very difficult due to the extremely low levels and its short half life. When combined measurements of nitrate and nitrite are conducted, this is usually denoted by the term NOx (Lundberg & Weitzberg, 2005; Hirata et al., 2010).

2. Study on correlations of oxLDL and NOx with the metabolic profile in elderly with hyperlipidemia

The LDL oxidation and nitric oxide are the key mediators involved in all stages of atherosclerosis: initiation, progression and complications. Their role is antagonistic: oxLDL have pro-atherogenic and NO antiatherogenic functions on vascular endothelium (Figure 1). A reduction in NO production or activity has been proposed as major mechanisms of endothelial dysfunction and a contributor to atherosclerosis. The endothelial dysfunction is considered an early marker for atherosclerosis and can be detected before structural changes in the vascular wall. An impairment of NO bioactivity or synthesis will reduce its braking effect on processes involved in atherogenesis.

2.1 Purpose

In the present study we evaluated the levels of circulating oxidized LDL (oxLDL) and the basal plasma levels of the NO metabolic pathway products, NOx (NO_2^- + NO_3^-), and examined their relationships with the global metabolic profile in a group of elderly patients with hyperlipidemia. We explored the determinants of oxLDL and NOx, as well as the relation between oxLDL and NOx in order to investigate whether the oxLDL/NOx and oxLDL/HDL-cholesterol ratios are more informative than the individual variables.

2.2 Materials and methods

2.2.1 Study design

The study population included 170 subjects (72 men and 98 women) aged 60 - 70 years, of the patients hospitalized at the Ana Aslan- National Institute of Gerontology and Geriatrics (NIGG), Bucharest, Romania, who were selected according to clinical and biochemical

criteria. The subjects did not have diabetes or any liver, kidney, hematological or oncological overt diseases. We selected in a first group 125 subjects with a high cardiovascular risk lipid profile characterized by hypercholesterolemia [serum total cholesterol (TC) > 200 mg/dL and LDL-cholesterol (LDL-C) > 130 mg/dL], associated or not with hypertriglyceridemia [serum triglycerides (TG) < or > 150 mg/dL]. Subjects were not previously diagnosed with cardiovascular disease and were not under treatment with any vasoactive or cardiovascular drugs. None of the patients used lipid-lowering therapy or antioxidants. The second group considered as the control group included 45 apparently healthy subjects with normal lipid profile (TC < 200 mg/dL, LDL-C < 130 mg/dL and TG < 150 mg/dL). Anthropometric and clinical characteristics were collected after a complete clinical examination. All the participants in this study gave their written informed consent, and the study protocol was approved by the Ana Aslan - NIGG ethics committee. All the procedures followed were in accordance with the institutional guidelines. Venous blood samples were drawn after an overnight fast and 24-hours refraining from smoking, caffeinated foods and beverages.

2.2.2 Biochemical methods

Total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), triglycerides (TG) and glycemia (G) were determinated by standard enzymatic methods. Results were expressed in mg/dL.

The circulating plasma oxLDL was evaluated by a competitive ELISA kit with the monoclonal antibody 4E6 (kit 10-1158-01, Mercodia, Sweden) directed against an epitope in the apolipoprotein B-100 moiety of oxLDL, formed from substitution of lysine residues of apoB-100 with aldehydes (Holvoet et al., 2008). Results were expressed in U/L plasma.

The total amount of plasma stable metabolic pathway products of NO, [NOx, the sum of nitrites and nitrates (NO_2^- + NO_3^-)] was determined using the Griess reagent, following the quantitative conversion of nitrates (NO_3^-) to nitrites (NO_2^-), with nitrate reductase (kit 23479, SIGMA). Results were expressed in µmols NOx/L plasma. All the biochemical and immunoenzymatic tests were performed on a ChemWell 2190 Analyser (Awareness Technology, USA).

The plasma atherogenic index (Ai) was calculated by the logarithmically transformed ratio of triglycerides on HDL-cholesterol (TG/HDL-C) (1) (Dobiasova, 2006; Dobiasova et al., 2011).

$$Ai = \log(TG/HDL\text{-}C) \quad (1)$$

2.2.3 Statistical analysis

Data are expressed as means±SD. The subjects clinical characteristics were compared using the Mann Whitney Wilcoxon non-parametric test. Differences in means of studied parameters between the groups (hyperlipidemic *vs.* control group) were assessed by Student's paired *t* test. The Pearson's correlation test was used to perform bivariate correlation analysis. Multiple regression analysis was performed to evaluate the independent relation between studied parameters using the Statistical Package for Social Sciences software (SPSS) version 15. Significance was defined at the 0.05 level of confidence.

2.3 Results

The study population included 170 subjects aged 60-70 years. In order to establish the link of the traditional markers for the evaluation of the cardiovascular risk (TC, TG and LDL-C) at

systemic level with the oxidative stress and endothelial function parameters, patients were divided into two groups: a group with normal lipid profile (TC<200 mg/dL, LDL<130 mg/dL, TG<150 mg/dL; n=45), and a group with high cardiovascular risk lipid profile (TC>200 mg/dL, LDL>130 mg/dL, TG < or > 150 mg/dL; n = 125).

We firstly collected anthropometric data (body weight, height), clinical parameters, including systolic blood pressure, body mass index (BMI), fasting plasma glucose and the lipid profile in the two groups of our interest.

Both cardiovascular disease risk and healthy control subjects showed not significantly different values of weight and body mass index, but systolic blood pressure, fasting plasma glucose and triglycerides were significantly higher in hyperlipidemic patients. The HDL-cholesterol concentrations of the two groups were comparable. The atherogenic index and the TC /HDL-cholesterol ratio were significantly higher in hyperlipidemic group *versus* control (Table 1).

Also, the increased lipid profile group had a significantly higher circulating levels of oxLDL associated with a significant decrease of the plasma nitric oxide metabolic pathway products (NOx) compared to the normolipidemic group (Table 1).

Variables	**Control Group (n = 45)**	**Hyperlipidemic Group (n = 125)**
Age (years)	65±3	66±4
Sex (males/females)	15/30	55/70
Systolic blood pressure (mmHg)	115.6±16.0	129.0±21.5**
Diastolic blood pressure (mmHg)	74.6±9.7	73.5±12.7
Body mass index (kg/m^2)	22.5±2.9	23.2±5.3
Glucose (mg/dL)	91±12	100±12**
Total cholesterol (mg/dL)	182±22	285±32**
Triglycerides (mg/dL)	77±22	103±48**
LDL-cholesterol (mg/dL)	105±24	214±35**
HDL-cholesterol (mg/dL)	56±11	54±9
Total cholesterol/HDL-C ratio	3.40±0.83	5.34±1.11**
Atherogenic index (Ai)	0.13±0.15	0.24±0.19**
Uric acid(mg/dL)	5.87±2.07	6.09±1.94
oxLDL (U/L)	71.51±13.11	85.50±20**
NOx (μmol/L)	32.52±10.63	23.52±8.66**
oxLDL/HDL cholesterol ratio	1.35±0.46	1.62±0.58*
oxLDL/NOx ratio	2.44±0.92	4.13±1.87**

Values are expressed as means±standard deviation LDL, low-density lipoprotein; HDL-C high-density lipoprotein cholesterol; oxLDL, oxidized low-density lipoprotein; NOx, nitric oxide metabolic pathway products * p values derived from Student *t* test: significantly different *vs.* control group;
* $p < 0.01$; ** $p < 0.001$

Table 1. Clinical characteristics and metabolic variables in control and hyperlipidemic subjects.

To establish which variables other than LDL-C and NO were independent determinants of oxLDL and NO, we explored multiple linear regression models with oxLDL, NOx, oxLDL/HDL-C ratio and oxLDL/NOx ratio, as dependent variables, in control, hyperlipidemia, and whole study population. The results are presented in Table 2 and 3 and Figures 3-6.

In multiple regression analysis for estimating the association between the degree of endothelial dysfunction and metabolic parameters, we found different statistically significant correlations within the two study groups. Tables 2 and 3 show the correlations of oxLDL and NOx with serum metabolic variables, atherogenic markers and indices in normal and hyperlipidemic subjects.

In the control group (table 2) circulating oxLDL level positively correlated with glycemia and triglycerides, as well as the total cholesterol/HDL-cholesterol ratio.

In subjects with high cardio-vascular risk (table 3) significant positive correlations between oxLDL and LDL-cholesterol were pointed out. In both study groups oxLDL was significantly negative correlated with HDL-cholesterol, and significantly positive with the atherogenic index (Ai).

Variables	oxLDL	oxLDL/HDL-C ratio	NOx	oxLDL/NOx ratio
Control Group (n = 45)				
Glucose (mg/dL)	0.351*	0.273 (NS)	0.164 (NS)	- 0.004 (NS)
Total cholesterol (mg/dL)	0.257 (NS)	- 0.470**	- 0.088 (NS)	0.097 (NS)
Triglycerides (mg/dL)	0.358*	0.273 (NS)	- 0.259 (NS)	0.414**
LDL-cholesterol (mg/dL)	0.257 (NS)	0.396**	- 0.024 (NS)	0.031 (NS)
HDL-cholesterol (mg/dL)	- 0.471**	- 0.832**	0.081 (NS)	- 0.291 (NS)
Total cholesterol/HDL-C	0.528**	0.537**	- 0.075 (NS)	0.252 (NS)
Atherogenic index (Ai)	0.502**	0.605**	- 0.267 (NS)	0.234**
Uric acid(mg/dL)	0.200 (NS)	0.063 (NS)	0.184 (NS)	- 0.045 (NS)
oxLDL (U/L)		0.850**	0.074 (NS)	0.404**
oxLDL/HDL-C ratio	0.850**		0.045 (NS)	0.357*
NOx (μmol/L)	0.074 (NS)	0.045 (NS)		- 0.826**
oxLDL/NOx ratio	0.404**	0.357*	- 0.826**	

LDL, low-density lipoprotein; HDL-C, high-density lipoprotein cholesterol; oxLDL, oxidized low-density lipoprotein; NOx, nitric oxide metabolic pathway products

* $p < 0.05$; ** $p < 0.01$; NS, non-significant

Table 2. Interrelationships between studied markers of lipoxidative stress, endothelial function, and metabolic profile parameters, in control subjects, determined as Pearson's correlation coefficients (r).

As regards the nitric oxide metabolic pathway products, the statistical analysis of the data pointed out a significant negative correlation, between NOx and the total cholesterol/HDL-cholesterol ratio, but only in the hyperlipidemic group (Table 2 and 3).

Variables	oxLDL	oxLDL/HDL-C ratio	NOx	oxLDL/NOx ratio
Hyperlipidemic Group (n = 125)				
Glucose (mg/dL)	0.275**	0.329**	- 0.096 (NS)	0.245**
Total cholesterol (mg/dL)	0.390**	0.298**	- 0.340**	0.437**
Triglycerides (mg/dL)	0.320**	0.293**	0.037 (NS)	0.128 (NS)
LDL-cholesterol (mg/dL)	0.377**	0.391**	- 0.315**	0.411**
HDL-cholesterol (mg/dL)	- 0.445**	- 0.762**	0.011 (NS)	- 0.223*
Total cholesterol/HDL-C ratio	0.578**	0.796**	- 0.191*	0.416**
Atherogenic index (Ai)	0.549**	0.637**	0.054 (NS)	0.220*
Uric acid(mg/dL)	0.270**	0.201*	0.158 (NS)	- 0.006 (NS)
oxLDL(U/L)		0.895**	- 0.143 (NS)	0.641**
oxLDL/HDL-C ratio	0.895**		- 0.111 (NS)	0.546**
NOx (μmol/L)	- 0.143 (NS)	- 0.111 (NS)		- 0.729**
oxLDL/NOx ratio	0.641**	0.546**	- 0.729**	

LDL, low-density lipoprotein; HDL-C, high-density lipoprotein cholesterol; oxLDL, oxidized low-density lipoprotein; NOx, nitric oxide metabolic pathway products
* $p < 0.05$; ** $p < 0.01$; NS, non-significant

Table 3. Interrelationships between studied markers of lipoxidative stress, endothelial function, and metabolic profile parameters, in hyperlipidemic subjects, determined as Pearson's correlation coefficients (r)

To further estimate the extent of oxLDL involvement in endothelial dysfunction, the ratio of oxLDL to HDL-cholesterol and the newly introduced ratio of oxLDL to NOx, were calculated.

Significant differences as regards oxLDL/HDL-cholesterol ratio as well as oxLDL/NOx ratio were found out between the study groups; both ratios were higher in the hyperlipidemic subjects (Table 1).

Moreover, in the group of subjects with cardiovascular risk, oxLDL/NOx ratio correlated significantly with almost each of the traditional parameters of the metabolic profile, namely: glycemia, total cholesterol, LDL-cholesterol and HDL-cholesterol, as well as the Ai and cardiovascular risk markers (TC/HDL-C and oxLDL/HDL-C ratios) (Table 3).

We explored the metabolic determinants of oxLDL and NOx by performing the statistical multiple correlation test in the whole study population (n=170). Regarding the oxLDL we identified significant ($p < 0.01$) positive correlations with each studied parameters of the metabolic profile, such as glycemia ($r = 0.351$), total cholesterol ($r = 0.457$), LDL-cholesterol ($r = 0.456$), triglycerides ($r = 0.414$) and uric acid ($r = 0.253$). A strong significant negative association between oxLDL and HDL-C ($r= -0.425$, $p <0.01$) was pointed out.

In all 170 subjects we pointed out significantly negative correlations ($p < 0.01$) of NOx levels and lipid profile: total cholesterol ($r = - 0.470$), LDL-C ($r = - 0.451$), and the atherogenic risk marker TC/HDL ratio ($r= -0.365$) (Figure 2).

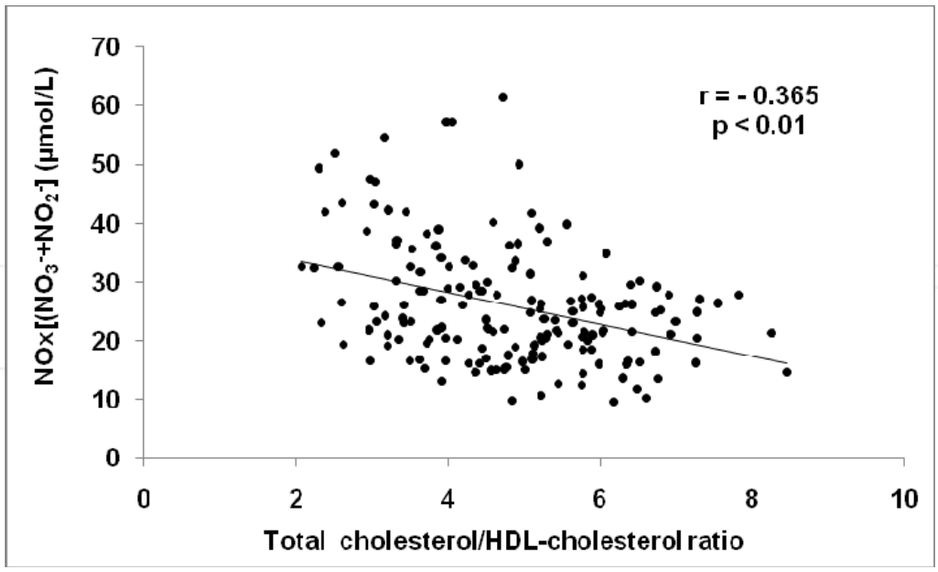


Fig. 2. Plasma nitric oxide metabolic pathway products (NOx) is inversely correlated with total cholesterol/HDL-cholesterol ratio in all the study subjects (n=170)

Finally, it is important to underscore the most interesting significant, negative, correlation, identified between oxLDL and NOx ($r = - 0.205$, $p < 0.01$; $n = 170$) in all study population (Figure 3). In the hyperlipidemic group this association was negative but not significant.

The newly introduced ratio oxLDL/NOx was significantly related to the ratio oxLDL/HDL ($r = 0.547$, $p < 0.01$, n=170), the atherogenic index (Ai) and also the total cholesterol/HDL-ratio ($r = 0.478$, $p < 0.01$ and $r = 0.537$, $p < 0.01$) (Figure 4 - A, B, C).

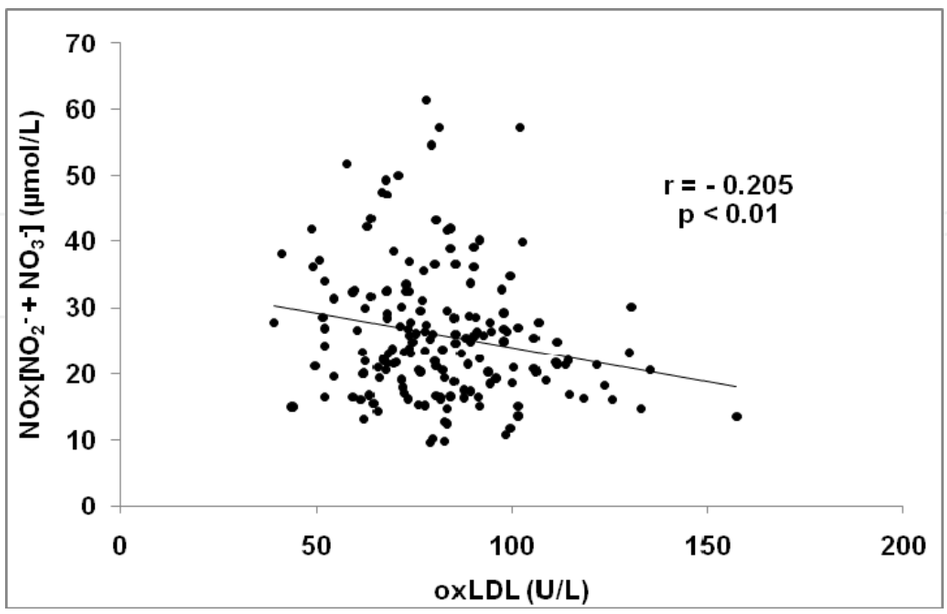


Fig. 3. Oxidized LDL (oxLDL) levels are inversely associated with plasma nitric oxide metabolic pathway products (NOx) in all the study subjects (n = 170).

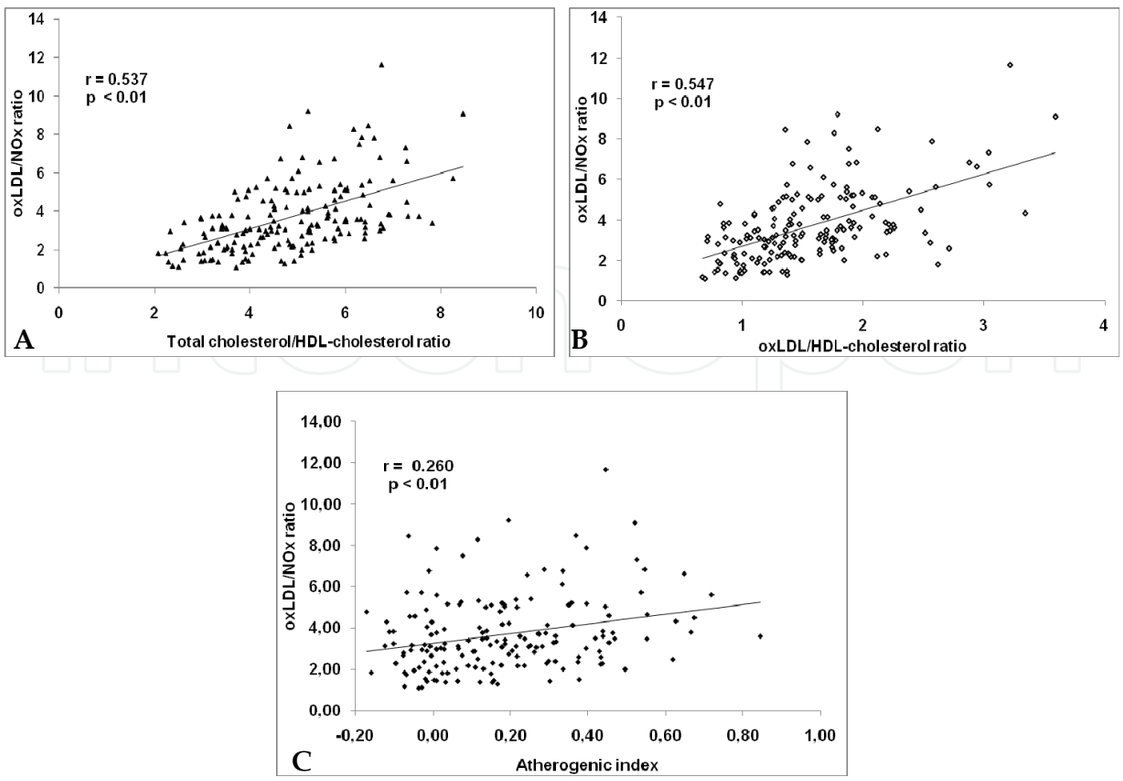


Fig. 4. oxLDL/NOx ratio is directly correlated with total cholesterol/HDL-cholesterol ratio (A), with oxLDL/HDL-cholesterol ratio (B), and with the Atherogenic index (C) in all the study subjects (n=170)

2.4 Discussion

The purpose of this work was to point to the interrelationships of oxLDL and NO as biomarkers of oxidative stress and endothelial function and the metabolic profile in elderly. We explored the literature on molecular mechanisms involved in the biochemical and metabolic links of NO and oxLDL. We investigated the metabolic determinants of oxLDL and NOx in 170 elderly subjects. Our research results focused mainly on the correlations between lipid and lipoprotein parameters as indices of atherogenic risk, and lipoxidative stress and endothelial dysfunction biomarkers, namely the nitric oxide metabolic pathway products (NOx) and the circulating oxidized LDL.

Impairment in NO, a common feature in patients with endothelial dysfunction, is considered to predict atherosclerosis and cardiovascular events. On the other hand, elevated levels of oxidized LDL, formed within the arterial wall, are commonly related to the atherogenic profile (Steinberg, 2009; Steinberg & Witzum, 2010). Therefore, in the present study, we evaluated the relationships of oxLDL and NOx as oxidative stress and endothelial dysfunction biomarkers with the metabolic profile and the cardiovascular high-risk profile markers, in 170 elderly patients.

Plasma NOx levels were significantly lower in patients with hyperlipidemia, further suggesting that physiologic levels of NO are necessary to maintain the normal, vasodilatatory and noninflammatory phenotype of the vascular wall. A major finding of this study is that NO release levels measured by its metabolic pathway products significantly

negatively correlated with circulating oxLDL concentrations. Overall, this work pointed out the link between the vascular endothelium vasodilating/vasoconstricting imbalance and the metabolic profile in hyperlipidemic elderly patients.

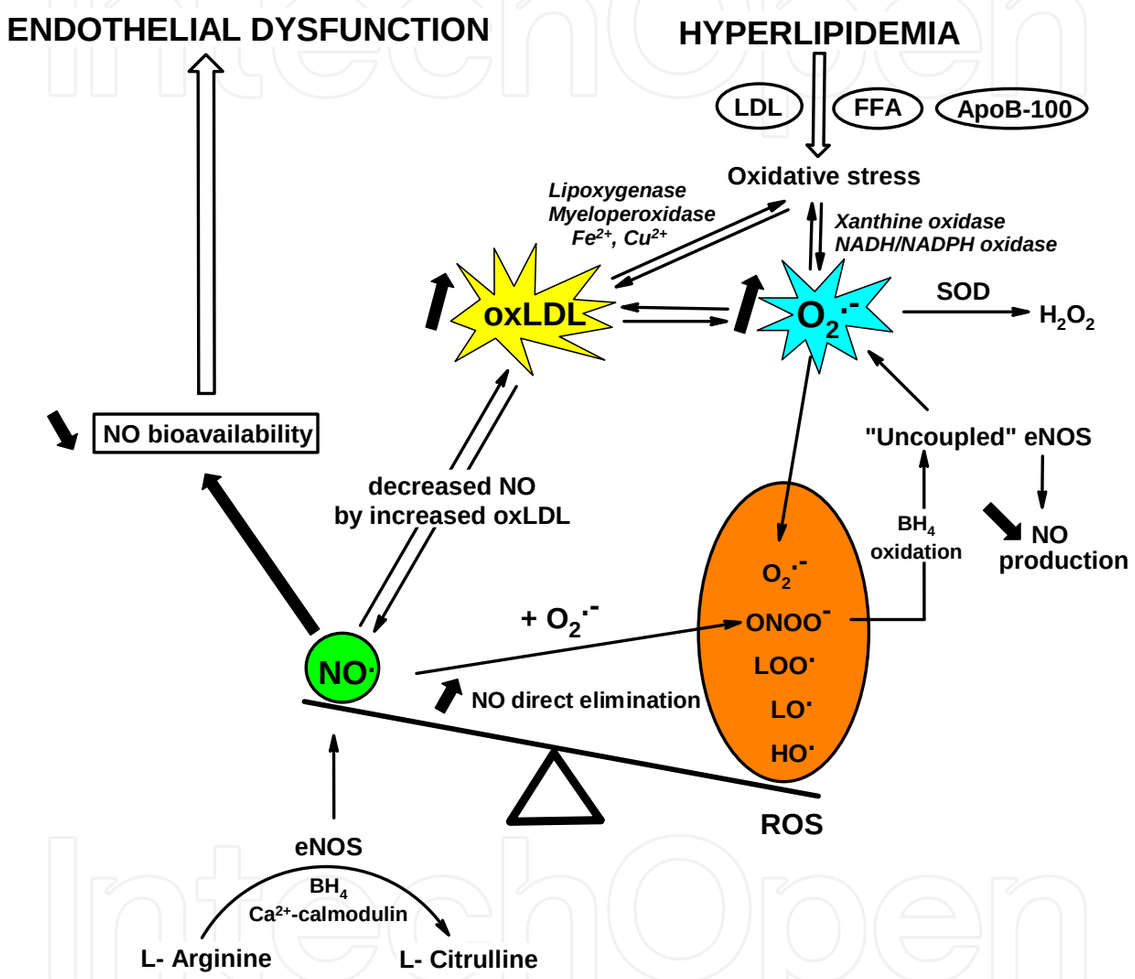
The evaluations of LDL-cholesterol, high-density lipoprotein-cholesterol, and triglycerides are the traditionally recommended lipid screening tests for coronary heart disease (CHD). Several studies do suggest that total cholesterol/HDL-cholesterol ratio, a major lipid index, is better than the individual total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides parameters (Lemieux et al., 2001). This ratio is easily obtained and one of the most powerful important risk factors for CHD. Both oxLDL and NOx significantly correlated with all markers and calculated atherogenic indices (TC/HDL-C, oxLDL/HDL-C and Ai). Hence, our data support the fact that measurements of the oxLDL and NOx levels at different times may help to monitor the state and severity of endothelial dysfunction.

Based on the multiple correlations analysis in both study groups and all subjects we found that TC, TC/HDL-C, and oxLDL/HDL-C ratios are major determinants of oxLDL and NO. These associations are stronger for the newly introduced oxLDL/NOx ratio. As well, the oxLDL/NOx ratio is strongly correlated with the atherogenic index and more importantly, with the oxLDL/HDL-C ratio, the best lipid biomarker used for discriminating between coronary artery disease patients and healthy control subjects, and also the best blood biomarker that reflects atherosclerotic disease activity in the arterial wall (Huang et al., 2008; Lankin et al., 2011)

Our results are in accordance with literature with regard to the damaging effects of hyperlipidemia, mediated or stimulated by oxidative stress. Numerous studies have supported the role of hyperlipidemia in atherosclerosis, endothelial dysfunction and progression of coronary heart diseases (Wallace et al., 2010; Deanfield et al., 2007; Highashino et al., 2010; Van den Oever et al., 2010). The hypothesised mechanisms for this effect are via hyperlipemia-induced oxidative stress, especially LDL oxidation and subsequent reduced NO bioavailibility. The strong significant association of oxidized LDL with plasma lipid profile (TC, LDL-C, TG), atherogenic risk markers (TC/HDL-c, oxLDL/HDL) and atherogenic index (Ai) found out in the hyperlipidemic group as well as the whole population studied, underscore the validity of the observation that hyperlipemia induces LDL oxidation and oxidative stress. The oxidative stress generates the superoxide radicals ($O_{2}^{\cdot -}$), which are scavenged by nitric oxide to form peroxynitrite ($ONOO^{-}$), a powerful oxidant. The overproduction of $O_{2}^{\cdot -}$ has direct and indirect effects on vascular NO bioavailability. Moreover, $O_{2}^{\cdot -}$ and $ONOO^{\cdot}$ can oxidize tetrahydrobiopterin (BH_4), the cofactor necessary for NO production by eNOS enzyme, leading to eNOS uncoupling, and thus to more $O_{2}^{\cdot -}$ generation and reduced NO production. Also, the significant negative correlation found out in this study between oxLDL and NOx shows that the excess of LDL oxidation itself may contribute to reduce NO level. Taken toghether, hyperlipidemia, oxidative stress and LDL oxidation result in reduced NO bioavailability via combinatory effects of direct elimination and decreased production of NO. This NO reduced bioavailability compromises all the antiatherogenic functions of the endothelium. This hypothesised mechanism shown above could be a target for interventions to protect against hyperlipidemia-induced atherogenesis and cardiovascular disease.

Based on the strong interrelationships pointed out in this clinical study and the numerous experimental and clinical research in the field of atherosclerosis we summarize in figure 5 the important relationships among hyperlipidemia, oxidative stress, LDL oxidation, nitric

oxide and endothelial dysfunction. Hyperlipidemia, oxidative stress and LDL oxidation are harmful at multiple steps in atherogenesis, including direct contributions to endothelial functions. As shown in figure 5, hyperlipidemia induces enhanced oxidative stress, superoxide ($O_2^{\cdot-}$) excessive generation and LDL oxidation. Increased $O_2^{\cdot-}$ generation as a result of excess mitochondrial lipid oxidation, LDL oxidation and other sources, is critically involved in reduced NO bioactivity and endothelial dysfunction, by direct elimination of NO.



LDL, low-density lipoprotein; oxLDL, oxidized low-density lipoprotein; FFA, free fatty acids; ApoB-100, apolipoprotein B-100; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase, ROS, reactive oxygen species; HO·, hydroxyl radical; LO·, alkoxyl radical; LOO·, peroxyl radical; $ONOO^-$, peroxynitrite; eNOS, endothelial nitric oxide synthase; BH_4, tetrahydrobiopterin.

Fig. 5. Simplified scheme of the interrelationships between oxidative stress, LDL oxidation and NO in the hyperlipidemic state leading to endothelial dysfunction.

There is abundant experimental evidence indicating the role of NO oxidative inactivation as a mediator of endothelial dysfunction and a pre-pathogenic vascular phenotype (Harrison, 1997; Bermudez et al., 2008). The NO is the kinetically preferred scavenger for $O_2^{\cdot-}$, because their reaction to generate $ONOO^-$ occurs three times faster than the $O_2^{\cdot-}$ elimination by

superoxide dismutase (SOD) (Beckman & Koppenol, 1996; Cai & Harrison, 2000). Both excess generation of reactive oxygen species (ROS) including $O_2^{\cdot-}$ and oxidized LDL, and decreased antioxidant defence mechanisms contribute to enhanced degradation of NO. Many studies support the role of the $O_2^{\cdot-}$ as an essential element in the decrease of NO bioavailability in oxidative stress conditions. Thus, studies on rabbbits with aortic atherosclerosis, demonstrated a remarkable decrease in endothelium-related relaxation, which was corrected by SOD treatment (Dulak et al., 1997).

Also, peroxynitrite is itself a powerful oxidant which contributes to enhance oxidative stress and turn the balance NO - ROS in the favour of ROS. Both radicals, $O_2^{\cdot-}$ and $ONOO^-$ can oxidize tetrahydrobiopterin (BH_4) leads to eNOS uncoupling, which in turn will produce $O_2^{\cdot-}$ instead of NO, and activate this vicious cycle (Fostermann, 2006). Uncoupling eNOS directly leading to decreased NO production. Not only BH_4 oxidation, but also decreases in BH_4 concentrations may reduce the NO production. Thus, many studies have shown a significant decrease in BH_4 activity in various pathological states, such as: hyperlipidemia, hypercholesterolemia, insulin resistance, probably through the oxLDL increase, as well as increased expression in some proinflammatory cytokine (TNF-alpha, interleukin-1 beta) (Bowers et al., 2011; Wever et al., 1997; Stroes et al., 1997). Furthermore, clinical and experimental studies have confirmed these mechanisms, showing that acute administration of BH_4 improve the endothelial dysfunctions related to hyperlipidemia, atherosclerosis and hypertension (Setoguchi et al., 2001). Also, a decrease in arginine and consequently a lack in eNOS substrate bioavailability leads to a failure in NO synthesis (Bermudez et al, 2005).

Recent *in vitro* studies (Bowers et al., 2011) demonstrated that tetrahydrobiopterin (BH4) could reduce oxLDL-induced $O_2^{\cdot-}$ production by NADPH oxidase, increasing NO synthesis in endothelial cells. The superoxide anion production was increased by pretreatment of cells with an inhibitor of BH_4 synthesis, and decreased following pretreatment with a BH_4 precursor. Thus, BH_4 concentrations can modulate the NADPH oxidase-induced imbalance of endothelial NO and $O_2^{\cdot-}$ production. BH_4 may be critical in combating oxidative stress, restoring proper redox state and reducing risk for cardiovascular disease including atherosclerosis.

Other mechanisms are also involved in the interrelations of LDL oxidation, nitric oxide and endothelial dysfunction. The oxidized LDL may reduce eNOS levels by inhibiting eNOS gene expression (Dulak et al., 1997) and also can displace eNOS from caveolae by binding to endothelial cell CD36 receptors and by depleting caveolae cholesterol content and therefore disrupt eNOS activity (Barbato et al., 2004). These adverse effects of oxLDL are prevented by HDL via binding to scavenger receptor BI (SR-BI), colocalized with eNOS in endothelial caveolae. This occurs through the maintenance of caveolae cholesterol content by cholesterol ester uptake from HDL. Moreover, HDL binding to SR-BI may stimulate eNOS activity in endothelial cells, and enhance endothelium- and NO-dependent relaxation. Thus, lipoproteins have potent effects on eNOS function in caveolae via actions on both membrane cholesterol homeostasis and the level of activation of the enzyme, processes that may be critically involved in the earliest phases of atherogenesis (Rigotti et al., 1997; Uittenbogaraard et al., 2000; Yuhanna et al., 2001; Schaul, 2003). The significant negative correlations between HDL and oxLDL, oxLDL/HDL ratio, atherogenic index and more important oxLDL/NO ratio pointed out in hyperlipidemic group and all subjects, underscore the beneficial effect of HDL on the endothelium.

Recent studies demonstrated that oxLDL causes impairment of endothelium-dependent, nitric oxide-mediated vasodilation involving L-arginine deficiency. The oxLDL may reduce L-arginine availability to eNOS for NO production, by up-regulating arginase. The experimental studies indicated that oxLDL increased arginase expression in the vascular wall without altering eNOS expression (Wang et al., 2011).

Experimental studies underscore the dual role of oxLDL on endothelial cells causing either proliferation or apoptosis, depending on its concentration and exposure time (Galle et al., 2001). Thus, oxLDL induced proliferation at low (5 to 10 microg/mL) and apoptosis at higher concentrations (50 to 300 microg/mL). Both effects are mediated by $O_2^{\cdot-}$ formation via NADPH oxidase as it major source. Thus, oxLDL contributes importantly to vascular cellular turnover through the induction of oxidative stress. More recently, was demonstrated that oxLDL at low concentrations (5 microg/mL) promotes *in vitro* angiogenesis and activate nitric oxide synthase through Pl3K/Akt/eNOS pathway in human coronary artery endothelial cells (Yu et al., 2011).

On the whole, the decline in nitric oxide bioavailability is caused by the cummulative effects of many factors and processes discussed above: the decreased expression of the endothelial NO synthase, a reduction of substrate or cofactors for eNOS, alterations of cellular signaling, eNOS inhibition by asymmetric demethyl arginine, reduced NO production and accelerated NO degradation by hyperlipidemia, oxidative stress and LDL oxidation.

Taking into account overall the atherogenic properties of oxidized LDL, involved in all stages of atherosclerosis (Steinberg et al., 1989; Steinberg, 2009), and the vasoprotective and antioxidant functions of NO (Bermudez et al., 2008; Yasa & Turkseven, 2005), we introduced for the first time the ratio oxLDL to NOx for quantifying their possible cumulative effect on vascular endothelium. The strong positive associations of this ratio with the atherogenic index and the atherogenic risk markers: TC/HDL and oxLDL/HDL ratios, supported us to propose this newly introduced ratio (oxLDL/NOx) as a potential marker of endothelial dysfunction. The future in depth studies, will take into consideration the association with clinical parameters of vascular endothelial functions using acethylcholine to induce endothelium dependent dilation, quantitative angiography, and high resolution ultrasound to measure brachial artery diameter, to further support this new candidate marker.

Wu et al., (2006) suggested in a prospective cohort study that circulating oxLDL as an individual parameter, measured with antibody 4E6, was not an independent overall predictor of coronary heart disease (CHD), after adjustment of lipid markers and less predictive in development of CHD than apoB and total cholesterol/HDL-cholesterol ratio (Wu et al., 2006). Therefore, based on the results obtained in our study it is important to examine in future research whether the ratio oxLDL to NOx correlates with endothelial function and predicts CHD independently of the lipid markers.

Data of this study support the relevance of oxLDL and NOx as biomarkers reflecting, at systemic level, the progressive damage at cellular level under the action of prooxidant pathogenic factors. These biomarkers could be valuable in the complex evaluation of oxidative stress in the endothelium.

Despite numerous evidences of oxidative processes involved in atherosclerosis and the multiple experimental research on their inhibition by traditional antioxidants, and the success in several animal trials, the human clinical trials using antioxidants have failed (Parthasarathy et al., 2008; da Luz et al., 2006). There were not taken into consideration all the factors, aspects, processes, steps and stages involved in early or advanced atherosclerotic

lesions, their interrelations, and the most important the pro-oxidant properties and actions of antioxidants in different oxidative process steps and disease stages.
These interrelatioships pointed out in our study could be very important in the management of new effective therapeutic strategies for atherosclerosis and cardiovascular disease. Because oxidative stress, LDL oxidation and endothelial dysfunction centrally contributes to cardiovascular disease, further sustained efforts must be undertaken to translate this knowledge into the characterization and identification of biomarkers that enable preventive or early detection of injuries and allow improved risk stratification by integration into cardiovascular risk stratification models.

2.5 Limitations of our study

The study population included only elderly and therefore the results may be different in other age-groups subjects, in order to have identified the early onset of hyperlipidemia-induced vascular impairment. Another important limitation was that we did not evaluate the endothelial function using the flow-mediated dilation (FMD) and ultrasound examination of the right brachial artery.

3. Conclusion

The results of this correlations study pointed out that in hyperlipidemic elderly patients the endothelial NO synthesis could indeed be impaired and associated with a higher oxidative stress exerted on circulating LDL particles. Oxidized LDL has a large range of biological effects that contribute to atherogenesis, but NO also has many biological effects that prevent atherogenesis. In this context, the interrelations pointed out between hyperlipidemia, oxidative stress, LDL oxidation and nitric oxide leading to endothelial dysfunctions, emphasized their implications in molecular mechanisms of endothelial dysfunction.
It is important to distinguish between the effect of oxidized LDL and the effect of a deficiency in the release of NO and to draw a link between these two biomarkers. According to the results obtained in this study, we propose the use of a new marker of endothelial dysfunction, the ratio of oxLDL to NOx, which could be a more accurate estimation of the *in vivo* cumulative implications of oxLDL and NO in atherogenesis. Future studies taking into account the association of this newly introduced marker with other markers of endothelial function will be undertaken to support the marker validity.
The strong interrelations pointed out in our study underscore the molecular mechanisms implicated in endothelial dysfunctions and atherosclerosis presented in this chapter. Future research is needed to translate this knowledge into the identification, characterization and validation of new and known biomarkers of lipoxidative stress-induced endothelial dysfunctions and atherosclerosis, and their integration into cardiovascular risk stratifications models.
These findings suggest the importance of understanding the senescent specific changes occurring in endothelium associated with age-related disease. Such an understanding may not only provide answers regarding mechanisms of disease development, but may also provide biomarkers of endothelium specific ageing.
As perspectives, the nutritional and therapeutic strategies should attempt to correct the lipid profile and lipoxidative stress in order to prevent the amplification of redox and inflammatory phenomena that lead to increased cardiovascular risk. As well, therapeutic

approaches in the prevention and treatment of atherosclerosis based on improving NO bioactivity and reducing LDL oxidation may become a challenge for future studies.

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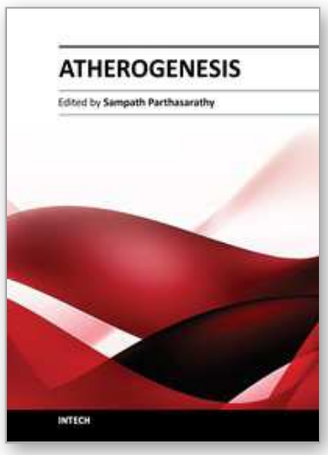

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This monograph will bring out the state-of-the-art advances in the dynamics of cholesterol transport and will address several important issues that pertain to oxidative stress and inflammation. The book is divided into three major sections. The book will offer insights into the roles of specific cytokines, inflammation, and oxidative stress in atherosclerosis and is intended for new researchers who are curious about atherosclerosis as well as for established senior researchers and clinicians who would be interested in novel findings that may link various aspects of the disease.

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