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Oxidised Low Density Lipoprotein (LDL) Modification with Statin Therapy is Associated with Reduction in Carotid Stenosis

Elias Skopelitis, Dimitrios Levisianou,
Helen Lydataki and Sofoklis Kougialis

Additional information is available at the end of the chapter

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1. Introduction

1.1. Carotid stenosis and atheromatous process

Carotid artery stenosis due to atherosclerosis is a major complication of hyperlipidemia, diabetes mellitus and hypertension. Moreover, the extent of carotid intima media thickness is a measure of atheromatosis and therefore of cardiovascular disease (CVD).

The effect of cholesterol in the process of atheromatosis is now well established. High levels of total cholesterol, as well as of low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), lipoprotein a (Lp- α), and triglycerides, coupled with decreased levels of high-density lipoprotein (HDL) are responsible for the creation of atheromatous plaques [1-3]. Of the above factors, LDL cholesterol, and especially the oxidized LDL is considered as the most important contributor of atheromatosis [4].

The atheromatous process is completed in the following three stages:

1. In the first stage, LDL cholesterol enters the vessel wall, binds to glucosaminoglycans, which are part of the extracellular matrix of the intima. This binding is facilitated by apolipoprotein B-100 (ApoB-100). The accumulation of LDL in the vessel wall contributes to the formation of fatty streaks. Following LDL adhesion in the vessel wall, it undergoes oxidation by free radicals produced locally, the molecule is altered and chemokines are produced by adjacent vessel wall cells, such as MCP-1, together with growth factors, which are responsible for the accumulation of monocytes and macrophages. The latter,

cause further oxidation of LDL, resulting in negative charge, recognition by scavenger receptors on macrophage membrane and increased uptake of LDL inside the macrophages, as these receptors are not inhibited by increased intracellular concentration of cholesterol. The final result is an enormous accumulation of LDL in the macrophages, which are transformed to foamy cells. These cells represent the first step in the atheromatous process [5] (figure 1).

- During the second stage, the atheromatous plaque is formed. Foamy cells produce growth factors and together with oxidized LDL result to the attraction of smooth muscle cells. The latter, are then differentiated to fibroblasts and start producing collagen. This collagen covers foamy cells, which either are destroyed or are forced to apoptosis. The final result is the formation of a pool of extracellular cholesterol trapped under a fibrous capsid (figure 2). The part which is close to the yet intact vessel wall is the active one of the plaque, where the foamy cells are produced. As the plaque extends to the inner layers of the vessel wall, the point of foamy cell formation becomes instable and may cause rupture of the plaque [5] (figure 3).

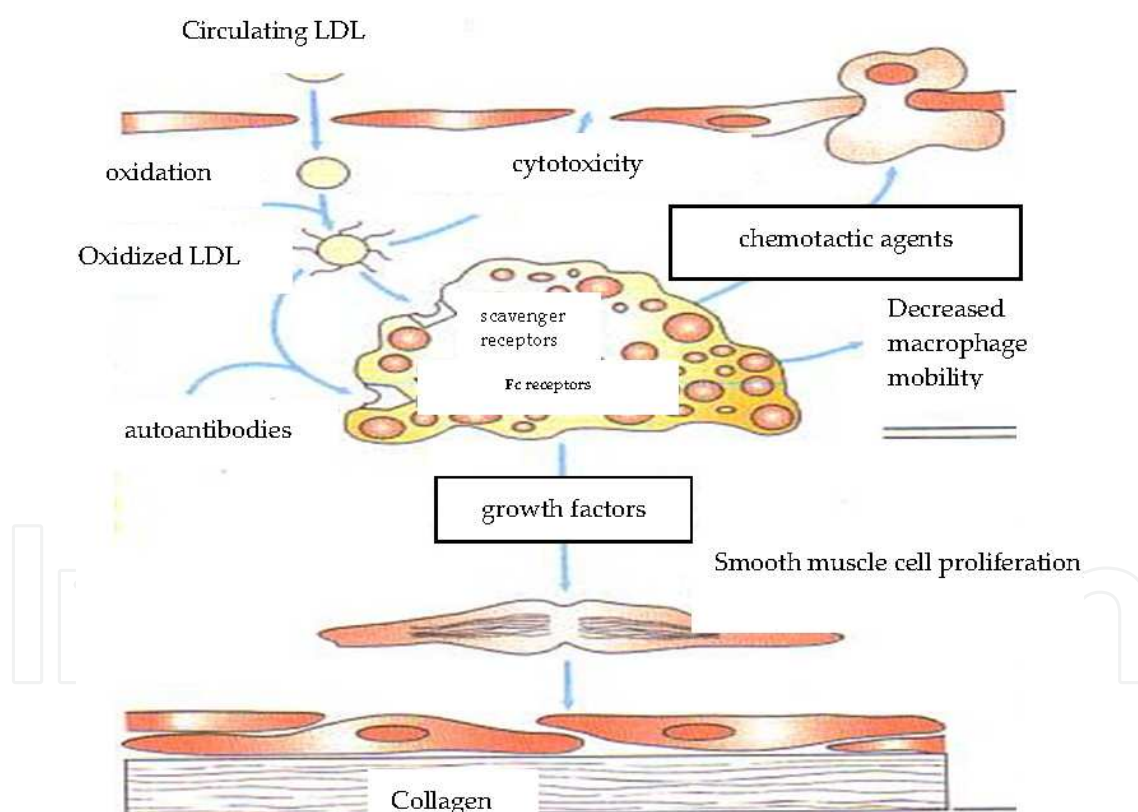


Figure 1. Atherogenesis. Fatty streaks are characterized by macrophages containing an excess of lipids (foamy cells). Foamy cells are derived by blood monocytes which are attracted to vessel intima and start phagocytosing lipoproteins, such as oxidized LDL. The conversion of fatty streak to atheroma depends on proliferation and differentiation of smooth muscle cells to fibroblasts. The latter produce collagen resulting in intima thickening. As the lesion extends further, foamy cells are destroyed releasing large amounts of cholesterol trapped in a fibrous capsid. The active site of atheroma is the point which is adjacent to normal endothelium, where foamy cells are formed (adopted from Durrington & Sinderman, 2002).

3. In the third stage, that of the complicated lesion, the rupture of the fibrous capsid of the atheromatous plaque leads to massive evacuation of the cholesterol reservoir. The artery may occlude due to the accumulation of platelets and clotting, leading to infarction (figure 4). If not so, then the plaque will be further enlarged [5].

Avoiding the formation and the instability of the atheromatous plaque is top priority for patients at risk for cardiovascular events. Statins may contribute towards this direction [6,7]

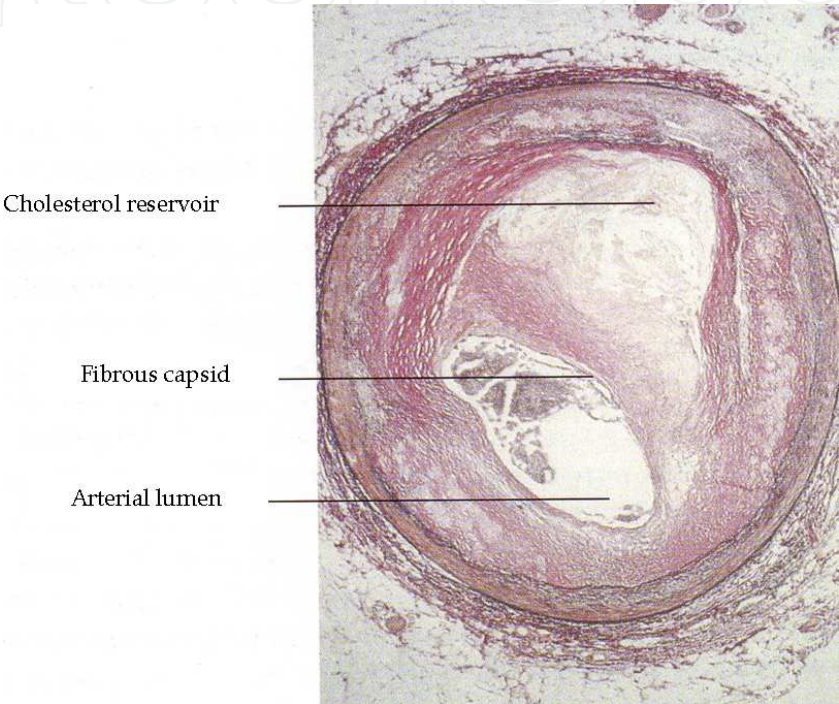


Figure 2. Advanced atheromatous plaque causing arterial lumen occlusion of 70% (adopted from Durrington & Sinderman, 2002).

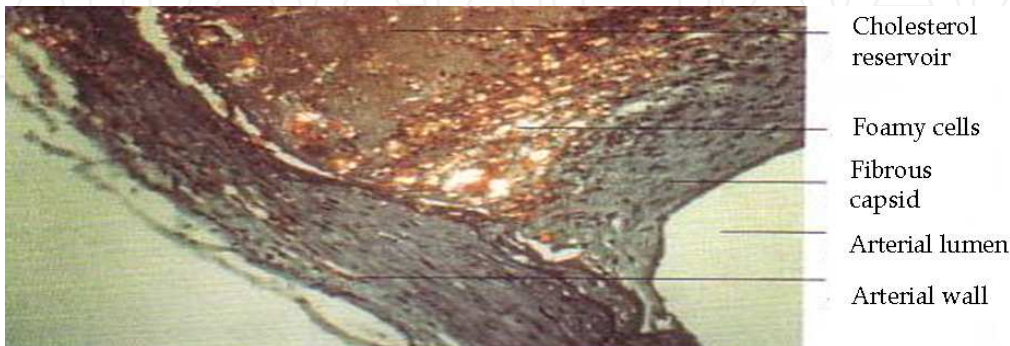


Figure 3. The point of the atheromatous plaque which active enlargement is occurring. Formation of new foamy cells, increased cholesterol uptake and increased instability of the plaque (adopted from Durrington & Sinderman, 2002).

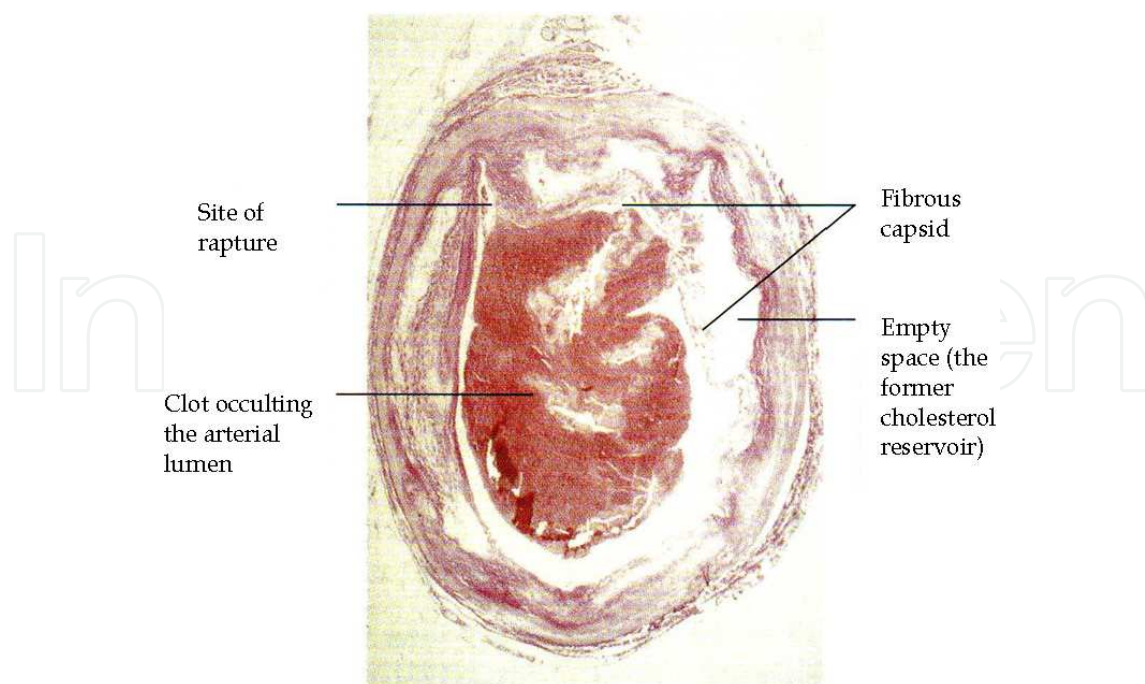


Figure 4. A ruptured atheromatous plaque, in which the cholesterol reservoir has evacuated itself under the fibrous capsid. A clot has in the endothelial surface at the site of rupture is completely occulting the lumen (adopted from Durrington & Sinderman, 2002).

1.2. Oxidised LDL

Oxidized low density lipoprotein LDL (oxLDL) cholesterol in humans is found mainly in two types:

- a. conjugated form, attached to the atheromatous plaque and
- b. circulating form found in serum.

Oxidized LDL is produced following oxidation of LDL by free radicals and other oxidative factors, a procedure called oxidative stress. The circulating oxidized LDL is the measurable fraction of oxidized LDL in plasma. Oxidised LDL is a key element of the pathway leading to the formation of the atheromatous plaque and has been extensively studied both as a marker of atheromatosis and as a possible target of therapeutic intervention. Circulating oxLDL is considered a risk marker for atherosclerosis [8] and coronary heart disease (CHD) [8-10]. Increased oxLDL levels in circulation and the vessel wall are associated with endothelial dysfunction [11] in such patients [9,10,12], contributing to atheromatous plaque instability [9].

Oxidative modification of LDL leads to rapid focal accumulation in macrophages [13], which is the first step in atheromatous process. The increased retention time of LDL in the intima offers enhanced probability to be oxidized by free radicals produced by endothelium, smooth muscle cells or macrophages [14]. Oxidized LDL then acts chemotactic for monocytes and smooth muscle cells through binding to scavenger receptors [15], leading to the formation of foam cells. Oxidized LDL is also capable to elicit endothelial dysfunction by altering the

secretory activity of endothelial cells [15], inhibiting the nitric oxide-mediated vasodilatation through reduction of the expression of endothelial nitric-oxide synthase (eNOS), inducing the expression of adhesion molecules on the endothelium thus mediating the adhesion of monocytes to intima [15], and inducing the expression of inflammatory cytokines [16]. Indeed, oxLDL is a potent inducer of inflammation [17], contributing to the chronic inflammatory process which results to atherosclerosis [18].

1.3. Statins

The 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, or statins, reduce total cholesterol (TC), LDL cholesterol, apolipoprotein B (apoB), and, to a lesser degree, triglycerides and lipoprotein a (Lp-a). Statins also have pleiotropic effects [19], such as the modulation of inflammatory molecules and monocyte maturation and differentiation [19], the suppression of smooth muscle-cells migration and proliferation [19], the reduction of the monocyte adhesion to the endothelium [20], the restoration of the impaired endothelium-dependent vessel wall relaxation [21], and the modification of cell-mediated LDL oxidation [22,23]. All of the above mechanisms contribute to the reversion of atheromatosis. Undeniably, statins reduce the incidence of coronary events and are a cornerstone in the primary and secondary prevention of CHD [24]. Previous studies have detected some efficacy in reducing the circulating oxLDL levels, but whether this effect is due to the reduction of LDL or is an independent, pleiotropic phenomenon remains a matter of controversy [25,26]. Furthermore, little is known about the definite clinical benefit of such oxidative marker reduction.

The aim of the present study was to evaluate the efficacy of atorvastatin in reducing stenosis, to investigate the effect on oxLDL and to search for possible associations of oxLDL modification with changes of stenosis in patients managed conservatively and in pre-treated with percutaneous catheter interventional procedures patients with carotid atheromatosis. We hypothesise that atorvastatin therapy will confer remission of oxLDL levels in vivo and this will be associated with significant reduction of carotid artery stenosis.

2. Patients and methods

Between January 2005 and February 2008 a total of 100 patients were randomly selected from the lipid clinic and the carotid angioplasty clinic of a large tertiary hospital in Athens for inclusion in the study. Informed consent was obtained from each patient at recruitment according to our institutional policies. Eligible were patients with carotid artery stenosis from various causes and with a range of predisposing factors. Exclusion criteria included: acute cardiovascular disease, severe or unstable angina pectoris, clinically evident cardiac failure, severe arrhythmias, recent surgical procedures, inflammatory diseases, active liver disease or liver impairment, excessive alcohol consumption (>4 units/day) or history of alcohol abuse, known allergic reaction to statins, poorly controlled diabetes mellitus as defined by a haemoglobin A1c (HbA1c) level of >7mg/dl, uncontrolled hypertension indicated by systolic blood pressure (SBP) >140mmHg and/or diastolic pressure >85mmHg, history of deep vein throm-

basis, bleeding tendency, serum triglycerides >350mg/dl, evidence of thyroid dysfunction, use of systemic steroids or other anabolics, pernicious anaemia, impaired vitamin B12 or folate acid levels, abnormal serum urate at baseline, serum creatinine phosphokinase elevation of >1.5fold at baseline, pregnancy or lactation, and end-stage renal disease or dialysis.

Patients were allocated into two groups according to the degree of carotid artery stenosis: those with arterial lumen occlusion of >70% in at least one common or internal carotid vessel consisted group A; those with stenosis <70% comprised group B. Patients in both groups were naive to statin therapy or if otherwise, a 6-month washout period was allowed before enrolment in the study. Group A underwent percutaneous transluminal carotid angioplasty with stenting by the same interventional cardiologist, prior to the initiation of statin therapy. Those patients were additionally administered clopidogrel and salicylate. Both groups had to follow an American Heart Association step II diet and were encouraged to exercise.

All patients were placed on atorvastatin once daily at bedtime in individualised doses, titrated to achieve and maintain serum LDL cholesterol levels of <100mg/dl (and ideally <70mg/dl, if hypertension, renal impairment, smoking, hyperlipidemia, symptomatic peripheral arterial obstructive disease, or diabetes mellitus were present). The most common doses used to achieve the above levels of LDL ranged between 10 to 40mg, while seldom it was required to administer higher doses such as 60mg (median atorvastatin dose for the total population = 20mg, range 10 – 60mg). The use of other drugs known to act synergistically with statins causing rhabdomyolysis was prohibited during the study. Adverse events were assessed in every visit in a non-specific manner: every newly reported symptom was documented as possible adverse reaction due to statin therapy and subsequently evaluated by an expert in clinical biochemistry. Adherence to the medication regimen was assessed indirectly by the low LDL levels compared with baseline.

Medical anamnesis, anthropometrics, smoking habits, blood pressure, and laboratory investigations comprising of complete blood count, fasting glucose, HbA1c, liver and kidney biochemistry, detailed lipid profile (TC, LDL cholesterol, high density lipoprotein [HDL] cholesterol, serum triglycerides [TG], apoB, and apolipoprotein A), urate, B12 and folate, thyroid function tests, homocysteine, Lp-a, and oxLDL were obtained at baseline and during follow-up visits, which were arranged at baseline, one, three, and six months; the final assessment was carried out in 12 months. Blood samples were collected after an at least 12-hour fast and a light, low-fat meal the night before sample collection was advised. Venous blood samples were collected in standard biochemistry vacutainer tubes. For the analysis of homocysteine and whole blood count, ethylenediaminetetraacetic acid (EDTA) vacutainer was used. Serum for biochemistry analysis was obtained by centrifugation (4000g) at 4°C for 7 min and was immediately tested.

Lipid profiles (TC, HDL, TG) were determined using commercially available enzymatic colourimetric methods (Dade Behring, Newark, USA) with a Dade Behring analyser. LDL was calculated with the use of Friedewald's formula as all had TG <350mg/dl [27]. For the measurement of circulating oxLDL, a commercially available kit (Mercodia, Uppsala, Sweden), based on a double antibody (4E6 and mouse monoclonal antiapoB) [28] capture ELISA test, was used. This method primarily detects malondialdehyde LDL (MDA-LDL). The normative

range (reference range) in our lab was 31-61mU/l. Apolipoprotein A, B and Lp-a were measured using immuno-nephelometry with rabbit antisera (Dade Behring, Newark, USA) in a Dade Behring analyser.

The evaluation of stenosis was conducted by Triplex ultrasonography using an Apogee 800 plus scanner with a 7.5 MHz transducer (ATL Inc., Bothell WA, USA) at baseline and 12 months. The stenosis was calculated in three sections in each common and internal carotid artery, and the final measure was the mean value of the three. The value of stenosis in the most occluded vessel was used in the statistical analysis. Specifically, the internal carotid artery (ICA) and common carotid artery (CCA) bilaterally were evaluated for each patient using coloured and grey Doppler ultrasonography. An effort was made to completely visualize the vessels. Additionally, the pulse wave was estimated with Doppler phasmatometry as well as the blood flow velocity of the two vessels. Results were recorded in a validated form. Stenosis was defined as the presence of visual plaque in coloured or grey Doppler. The degree of stenosis was calculated by measuring the decrease of the lumen diameter and the maximum systolic blood flow velocity. In difficult cases, other parameters were taken into account, such as ICA/CCA max blood flow velocity ratio and the ICA end-diastolic velocity. A degree of stenosis >70% was considered as severe and angioplasty was advised. A degree of stenosis between 60 – 70% was defined as high, between 50 – 60% as moderate and <50% as mild. High, moderate and mild stenoses were treated conservatively. The intima media thickness (IMT) and plaque morphology were not studied due to specific lab requirements, not readily available in our institution.

2.1. Statistical analysis

Continuous variables were presented as mean values \pm standard deviation, while qualitative variables were presented as absolute and relative frequencies. Normality tests were applied using the Kolmogorov-Smirnov criterion as well as Shapiro-Wilk test. Univariate analysis was initially applied to test the associations of oxLDL with carotid stenosis for each patient group as well as to identify first order correlations with various clinical parameters. Correlations between skewed continuous or discrete variables were evaluated using Spearman's ρ -coefficient, whereas correlations of normally distributed variables were evaluated by calculating the Pearson's r -coefficient. Comparisons between normally distributed, continuous variables and categorical variables were made using the Student t -test. Analysis of categorical data was carried out with the χ^2 test or Fischer's exact test when appropriate.

The association of oxLDL with carotid stenosis was also tested through multiple Cox proportional hazard model. The results obtained were presented as Hazard Ratios (HR) and the 95% Confidence Intervals (CI). A backward elimination procedure was applied to all multivariate models (using $P < 5\%$ as the threshold for removing a variable from the models). All models were adjusted for age, gender, SBP and TC. Kaplan-Meier curves concerning stenosis over the study period were plotted and Log rank test was performed. All reported P -values were based on two-sided tests and compared to a significance level of 5%. STATA 8.0 software (Stata Corporation, 2003, Texas, USA) was used for the analysis.

3. Results

3.1. Patients' characteristics

A total of 612 patients were evaluated, of which 123 fulfilled the eligibility criteria; finally, 100 had complete data to enter the analysis, 76 males and 24 females, median age 68 years (range 45-81). Diabetes mellitus was recorded in 26 of the 100 patients and hypertension in 66. Twenty patients had metabolic syndrome according to the national cholesterol education programme-adult treatment panel III (NCEP-ATP III) criteria [29]. Active smoking (defined as current or discontinued as far back as 5 years) was reported by 58 patients. Mean atorvastatin dose at baseline was 24.31 ± 11.49 mg for group A and 20.62 ± 10.39 mg for group B ($p=0.1$). By the end of the study period, the respective mean values were significantly increased to 30.45 ± 16.27 mg for group A ($p=0.044$) and 28.75 ± 17.57 mg for group B ($p=0.007$).

Each of the study group (A and B) comprised 50 patients. The two groups were comparable with regard of their baseline characteristics (table 1).

3.2. Lipid profile and oxidised LDL

Mean serum TC, LDL-cholesterol, TG, Lp-a, homocysteine, HDL-cholesterol, and oxLDL were significantly reduced at 12 months compared to baseline (table 2). Specifically, mean oxLDL dropped from 62.26 ± 22.03 mU/l to 44.49 ± 21.75 ($p<0.001$). A marked decrease was noticed during the first 6 months and a plateau thereafter (Figure 5).

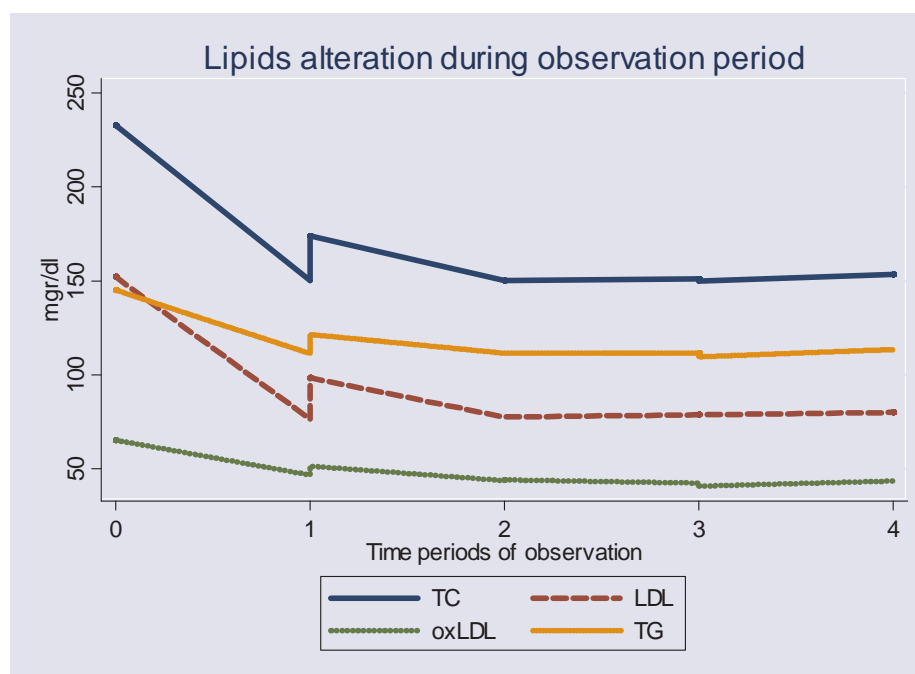
Characteristic	Total	Group A	Group B	p value
males / females	72 / 28	36 / 14	36 / 14	
mean age in years \pm SD	67.57 ± 7.15	68.46 ± 5.71	66.68 ± 8.31	0.21
number of pts with DM (percentage)	37 (37%)	18 (36%)	19 (38%)	0.83
number of pts with HTN (percentage)	67 (67%)	36 (72%)	31 (62%)	0.29
number of smokers (percentage)	54 (54%)	29 (54%)	25 (46%)	0.33
number of pts with CAD (percentage)	51 (51%)	24 (47%)	27 (53%)	0.55
mean \pm SD total cholesterol (mg/dl)	232.23 ± 47.8	235.24 ± 49.2	229.22 ± 46.7	0.53
mean \pm SD LDL cholesterol (mg/dl)	151.27 ± 41.7	154.16 ± 42.8	148.84 ± 40.9	0.52
mean \pm SD HDL cholesterol (mg/dl)	51.97 ± 12.7	52.12 ± 12.1	51.82 ± 13.4	0.9
mean \pm SD triglycerides (mg/dl)	145.59 ± 73.1	146.04 ± 73.2	145.14 ± 73.7	0.95
mean \pm SD oxidized LDL (mU/l)	64.66 ± 24.8	65.8 ± 25.3	63.53 ± 24.5	0.65
mean \pm SD homocysteine (mU/l)	13.99 ± 4.8	13.5 ± 4.6	14.47 ± 5.1	0.32

Pts: patients, DM: diabetes mellitus, HTN: arterial hypertension, MS: metabolic syndrome, SD: standard deviation, LDL: low density lipoprotein, HDL: high density lipoprotein, CAD: coronary artery disease

Table 1. Study population baseline characteristics

A significant correlation between LDL and oxLDL levels was detected (Pearson's correlation coefficient $r=0.7$, $p<0.01$) (Figure 6). Similar correlation was found between oxLDL and apoB levels ($r=0.65$, $p<0.001$), while no significant correlation was shown with Lp-a.

Between smokers mean oxidized LDL was reduced from 60.68 ± 24.09 mU/l at baseline to 45.84 ± 24.89 mU/l at the end of study period (difference 14.84 mU/l, $p = 0.0036$). Similarly, between non-smokers it was reduced from 69.33 ± 25.11 to 40.36 ± 5.6 (difference 28.97, $p<0.001$). Non-smokers had approximately double decline of oxidized LDL levels compared to smokers. Carotid artery stenosis was reduced between smokers from $29.68\pm25.59\%$ at baseline to $23.06\pm21.71\%$ at 12 months ($p = 0.002$). Non-smokers also presented significant reduction of stenosis during the study period $24.67\pm26.22\%$ vs $20\pm21.45\%$, $p = 0.004$). Non-smokers and smokers had similar decline of carotid stenosis in 12 months (6.61% vs 4.67%, Table 3).



TC: total cholesterol, LDL: low density lipoprotein cholesterol, oxLDL: oxidised LDL, TG: triglycerides, 0=baseline, 1=one month, 2=three months, 3=six months, 4=twelve months.

Figure 5. Time curve of change of total cholesterol, LDL cholesterol, triglycerides and oxidised LDL levels during the observation period.

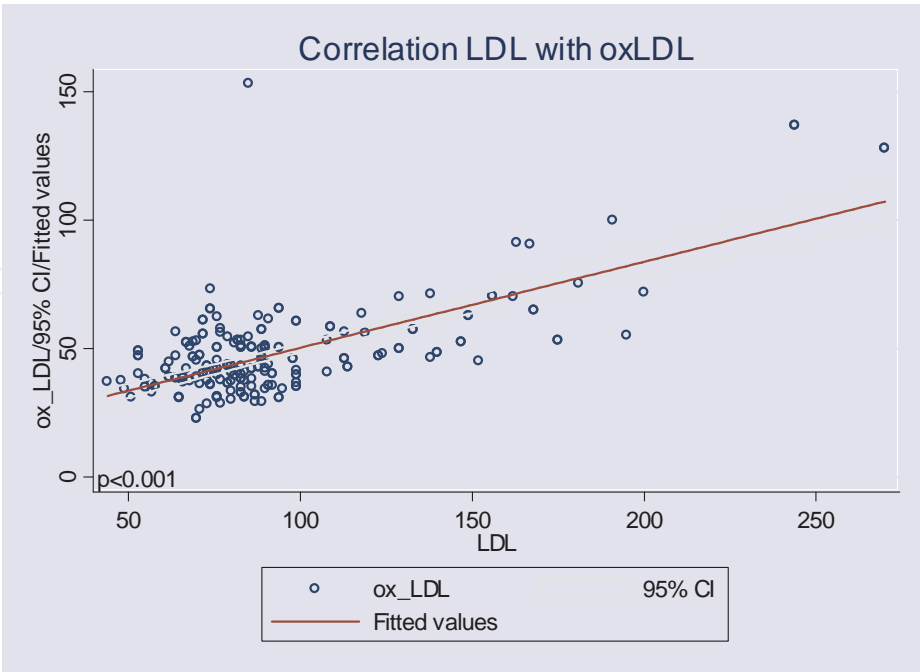
In further analysis, the group of smokers was subdivided to mild (≤ 5 cigarettes/day), moderate (5 – 15 cigarettes/day) and heavy (≥ 15 cigarettes/day) smokers. The statistical significant reduction of oxidized LDL levels and degree of carotid stenosis was apparent in the subgroup of mild smokers (oxidized LDL at baseline 48.24 ± 8.74 mU/l vs 41.54 ± 9 mU/l at 12 months, $p = 0.027$ and stenosis at baseline $27.63\pm25.68\%$ vs $23.42\pm21.74\%$ at 12 months, $p = 0.009$), while it was not apparent in the subgroups of moderate and heavy smokers (oxidized LDL at baseline 86.82 ± 37.7 mU/l vs 42.92 ± 10.77 mU/l at 12 months, $p = 0.077$ and stenosis at baseline $34\pm31.9\%$ vs $22\pm24.9\%$ at 12 months, $p = 0.186$, for moderate smokers; respective values for oxidized LDL were 66.29 ± 15.88 mU/l vs 34.81 ± 5.48 mU/l, $p = 0.06$ and for stenosis $32.14\pm24.13\%$ vs

22.86±22.8%, $p = 0.174$, for heavy smokers). The above described effect of smoking was taken into consideration during Cox-regression analysis.

Investigations	Total	Group A	Group B
total cholesterol (mg/dl) baseline	232.23 ± 47.8	235.24±49.1	229.22±46.7
total cholesterol (mg/dl) 12months	153.36±17.2	154.24±16.9	152.48±17.7
p value	<0.0001	<0.0001	<0.0001
LDL cholesterol (mg/dl) baseline	151.5±41.7	154.16±42.8	148.84±40.9
LDL cholesterol (mg/dl) 12months	79.75±12.7	79.54±13.2	79.96±12.3
p value	<0.0001	<0.0001	<0.0001
triglycerides (mg/dl) baseline	145.59 ± 73.1	146.04±73.2	145.14±73.7
triglycerides (mg/dl) 12months	111±53.1	112.1±54.7	109.9±51.96
p value	0.0002	0.01	0.0069
oxidized LDL (mU/l) baseline	64.67±24.8	65.8±25.3	63.53±24.6
oxidized LDL (mU/l) 12months	43.38±18.9	42.16±17.6	44.65±26.1
p value	<0.0001	<0.0001	0.0007
HDL cholesterol (mg/dl) baseline	51.97±12.7	52.12±12.1	51.82±13.4
HDL cholesterol (mg/dl) 12months	51.32±15.5	52.22±16.3	50.42±14.8
p value	0.74	0.97	0.62
homocysteine (mg/dl) baseline	13.99±4.8	13.5±4.6	14.48±5.1
homocysteine (mg/dl) 12months	11.89±3.5	11.88±3.8	11.9±3.4
p value	0.0006	0.057	0.0036
apolipoprotein A (mg/dl) baseline	156.57±26.7	156.46±27.3	156.68±26.4
apolipoprotein A (mg/dl) 12months	160.35±25.3	162.02±23.7	158.68±27.1
p value	0.3	0.28	0.7
apolipoprotein B (mg/dl) baseline	129.95±31.3	131.84±31.4	128.05±31.4
apolipoprotein B (mg/dl) 12months	77.1±11.8	77.58±13.1	76.62±10.47
p value	<0.0001	<0.0001	<0.0001
lipoprotein a [Lp(a)] (mg/dl) baseline	25.08±23.8	25.67±24.1	24.47±23.8
lipoprotein a [Lp(a)] (mg/dl) 12months	27.72±29.1	29.42±29.8	26.01±28.7
p value	0.48	0.49	0.77

LDL: low density lipoprotein, HDL: high density lipoprotein

Table 2. Comparison of mean ± standard deviation and respective p values of measured laboratory investigations at baseline and 12 months, in the total population, and the two groups.



LDL: low density lipoprotein cholesterol, oxLDL: oxidised LDL, CI: confidence intervals.

Figure 6. Correlation of low density lipoprotein (LDL) with oxidised LDL (oxLDL) levels at baseline (Pearson’s correlation coefficient $r=0.7$, $p<0.001$)

	Smokers	P value	Non Smokers	P value
ox LDL (mg/dl)				
baseline	60.68±24.09		69.33±25.11	
12 months	45.48±24.89	0.0036	40.36±5.6	0.001
difference	14.84		28.97	
stenosis (%)				
baseline	29.68±25.59		24.67±26.22	
12 months	23.06±21.71	0.002	20±21.45	0.004
difference	6.61		4.67	
Correlation of oxLDL change with stenosis change in 12 months	Pearson’s $r = 0.412$	0.021	Pearson’s $r = 0.198$	0.03

Table 3. Comparison of mean oxidized LDL values and degree of carotid stenosis change during the 1 year follow-up period, between smokers and non-smokers.

Within group B, the subgroup of patients with high degree of stenosis (>60%) had oxLDL 63.47±19.18 mU/l at baseline, while those with moderate and mild degree of stenosis (<60%) had 40.32±20.72 mU/l ($p<0.001$). Corresponding values at 12-months were 33.18±17.78 and

38.81±29.02, representing a marked decline for patients with >60% initial stenosis and a far less decline for patients with <60% initial stenosis; yet the differences were not significant (table 4).

	Stenosis >60<70%	Stenosis <60%	P value
Baseline			
Mean oxidized LDL	63.47±19.18 mU/l	40.32±20.72 mU/l	< 0.001
12 months Mean oxidized LDL	33.18±17.78 mU/l	38.81±29.02 mU/l	NS

Table 4. Comparison of mean oxidized LDL levels at baseline and 12 months within patients of group B (n = 50), according to degree of stenosis at enrollment.

3.3. Anthropometrics

Body mass index (BMI), weight, waist circumference and waist:hip ratio did not change significantly during the study period.

3.4. Carotid stenosis

Patients in group A had null stenosis at recruitment due to prior angioplasty with stenting. At the end of the 12-month statin therapy, no case of clinically important restenosis (>70%) was reported in this group (as restenosis was defined any increase of the carotid lumen diameter >5%). Patients in group B had mean percentage of stenosis at baseline 47.6±13.2%, which was significantly reduced following 12-month statin therapy (37.7±15.7%, p<0.001) (Table 5).

	baseline	12 months	p value
Mean % carotid stenosis ± standard deviation	47,6 ± 13,2	37,7 ± 15,7	0,001

Table 5. Change of the percentage of carotid artery stenosis between baseline and 12months for patients in group B.

3.5. Association of stenosis with oxidised LDL

Group B patients in the highest quartile of oxLDL values had a 12-month risk ratio for restenosis of 1.025, 95%CI=1.006-1.044, p=0.0083 (figure 7). After adjusting for gender, age, smoking, SBP, TC, and LDL levels, these patients demonstrated a HR for restenosis of 4.319 compared with those in the lowest quartile (p<0.001, figure 7). This means that an increase of oxidized LDL by one unit increases the degree of carotid stenosis by 2.5%, for patients in group B. A weak but significant correlation was detected between oxLDL levels and the degree of carotid artery stenosis (r=0.17, p=0.018). Similar correlation was found between LDL cholesterol levels and carotid stenosis (r=0.18, p=0.0085). The strength of Pearson’s correlation of mean oxidised LDL change with degree of carotid stenosis change during the 12-month period was greater for smokers compared to non-smokers (table 3).

3.6. The effect of LDL levels

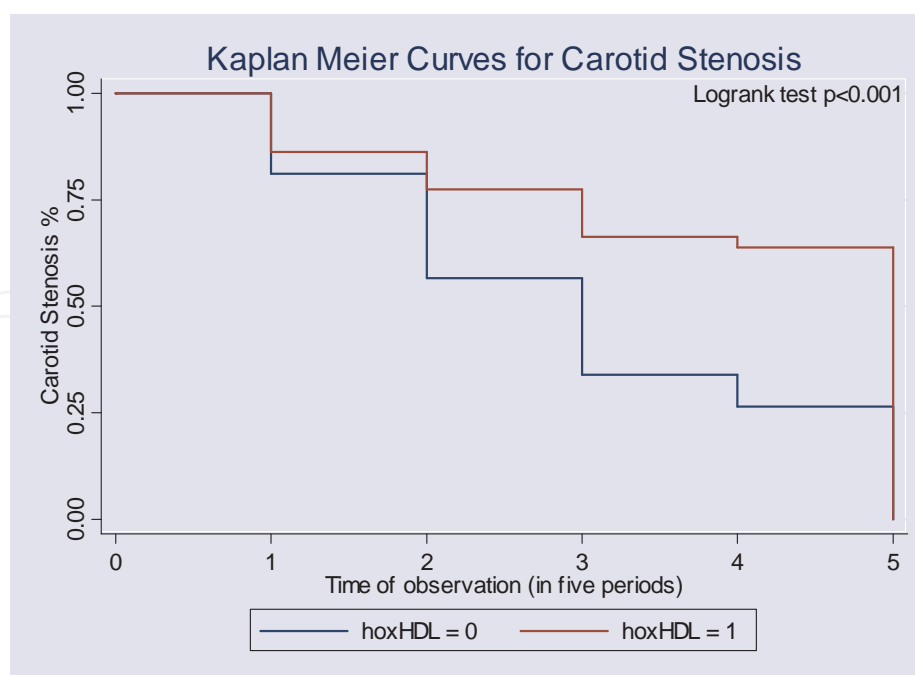
Patients in group B who achieved LDL levels $<70\text{mg/dl}$ during the observation period had a greater ($28.08 \pm 28\%$ vs $22.31 \pm 22.7\%$, difference 5.77% , $p = 0.06$) reduction of carotid stenosis compared to those with LDL levels between 70 and 100 mg/dl ($26.98 \pm 25.3\%$ vs $21.35 \pm 21.3\%$, difference 5.63% , $p < 0.001$), but this difference was not statistically significant. Thus, in conservatively treated group B, further reduction of LDL than the limit of 100mg/dl was not associated with additional improvement of stenosis.

4. Discussion

This study demonstrates that atorvastatin administered in individualised doses, titrated to maintain serum LDL cholesterol levels $<100\text{mg/dl}$, significantly decreased lipid profile and oxLDL, reduced carotid artery stenosis in patients managed conservatively and prevented restenosis in patients with prior angioplasty. Oxidised LDL in this study correlated positively with the degree of carotid artery stenosis; it was also shown by multivariate analysis that oxLDL represented an independent risk factor for restenosis. To our knowledge this is the first prospective study with a long observation period of 12 months to report such a clear, significant reduction of oxLDL levels following atorvastatin therapy for carotid atheromatosis of various causes and to report an association of the degree of oxLDL reduction with remission of carotid stenosis. It is also of major importance that this robust, long-standing decline of oxLDL was achieved with doses of atorvastatin used in everyday clinical practice. Interestingly, this beneficial effect was completed in the first six months, while practically no further reduction was noticed past this time point.

The mechanism by which statins modulate oxLDL levels has been controversial in the literature. Moreover, the association of oxLDL level modification with improvement of carotid atheromatosis and clinical outcome is not unequivocally established by large, double-blinded, randomised trials. Under this perspective, the present observational study provides reasonable evidence that reducing oxLDL may independently improve carotid stenosis.

Carotid intima media thickness (IMT) is a validated measure of carotid atherosclerosis. It is well established that carotid atherosclerosis, serves as an independent surrogate marker for CHD [30] and CVD [31]. Nevertheless, in the present study it was preferred to estimate the degree of carotid stenosis with a more direct approach, because this is more readily available in most hospital settings and because there is an obvious relation with clinical symptoms and signs. Besides, it represents a reliable method with sufficient reproducibility and it is practically the method of choice when evaluated patients candidate for endarterectomy or angioplasty. Evaluating carotid stenosis in turn, is an established method for estimating coronary risk [30] and cardiovascular risk [31]. Other parameters of vessel wall function, such as IMT and plaque morphology, even if clearly associated with cardiovascular risk in the literature, require well equipped laboratory and are not readily available in our hospital. Future research on the field should, ideally, comprise such measurements.



hoxLDL=0: low quartile of oxidised LDL levels, hoxLDL=1: high quartile of oxidised LDL levels, 0=baseline, 1=one month, 2=three months, 3=six months, 4=twelve months.

Figure 7. Kaplan Meier survival analysis for the estimation of the risk ratio for restenosis according to the levels of oxidised LDL (oxLDL). With red line those with oxLDL levels in the highest quartile of the values. With blue line those with oxLDL levels in the lowest quartile of the measurements (risk ratio 1.025, logrank test $p<0.001$).

Oxidised LDL has long been recognized as a risk factor for carotid atherosclerosis in asymptomatic men [32] and has also been linked with CVD [33]. Oxidized LDL levels [34], autoantibodies against epitopes of oxLDL [34] and oxLDL:LDL ratio [30] are independently associated with increased risk for coronary atheromatosis and ischemic heart disease. Increased levels of oxLDL [9] and MDA-LDL [10] in such cases are related to plaque instability. On the other hand, it has been reported that oxLDL is weakly associated with carotid IMT, but not with carotid plaque occurrence [35]. Oxidised LDL impairs endothelium relaxation [36] by inhibition of the expression of eNOS and of the transport pathways of nitric oxide (NO) from the endothelial cell, reduces the responsiveness of smooth muscle cell to NO [37], inhibits the NO-mediated vasodilation [16,36,38], induces the expression of adhesion molecules [39], acts directly chemotactic to circulating monocytes [16], stimulates endothelial cells to produce monocyte chemoattractant protein-1 (MCP-1) [40], facilitates monocyte adhesion to intima [41], exhibits cytotoxic properties against endothelial cells [16], and induces the expression of inflammatory molecules [16]. All of the above contribute directly to dysfunction of the endothelium [13] and foam cell formation, which is the first step in the development of fatty streaks [18], the first visible step of atherosclerosis. These effects are mediated by preferential binding of oxLDL with type A scavenger receptors (SRA, SRA-II and CD36) on subendothelial resident macrophages and smooth muscle cells [42] and lectin-like oxLDL receptor-1 (LOX-1) on endothelial cells [43] rather than the typical LDL receptor, resulting in an unrestricted uptake of cholesterol.

Statins reduce the incidence of cardiovascular events, an effect attributable to their hypocholesterolemic properties [44]. However, the extent of clinical benefit and accumulating laboratory evidence suggest additional mechanisms of action, the so-called pleiotropic effects [19]. The most important among such effects are the suppression of smooth muscle cell migration and proliferation [45], the reduction of monocyte adhesion to the vascular endothelium [20], the improvement of endothelial function [21], the inhibition of cell-mediated LDL oxidation [22,23], the immuno-modulation of monocyte maturation and differentiation, and the modification of production of inflammatory cytokines [46].

Atorvastatin suppresses cellular uptake of oxLDL from differentiating monocytes by reducing the expression of LOX-1 and scavenger receptors [47] and accelerates the LDL-receptor-mediated removal of the non oxidized LDL particles [48]. Hydroxymetabolites of atorvastatin protect the LDL against oxidation [31]. The antioxidant potency of atorvastatin metabolites has been confirmed by the reduction of IgG antibodies against LDL, a marker well-associated with CHD [23]. It has even been reported that these active atorvastatin metabolites may have greater anti-atherosclerotic effects than other statin molecules [49].

In acute coronary syndromes, atorvastatin therapy was linked to modulation of short- and long-term immune response towards LDL due to inhibition of lipoprotein-associated phospholipase A2 (Lp-LPA2) enzyme [34]. The apparent benefit from statin therapy after acute coronary events may also be attributed to the stabilization of the plaque and removal of oxLDL from the vessel wall [50]. Increased mobilization of oxidized phospholipids from the vessel wall, transient binding with apoB-100 particles and clearance from the circulation may be the possible underlying mechanism. Under this perspective the increase in oxLDL:apoB ratio detected with atorvastatin therapy might represent a marker of oxLDL efflux from the vessel wall. Removal of oxLDL contributes to improved endothelial function as oxLDL is highly immunogenic and vasoconstrictive. In our study there was no significant change in oxLDL:apoB ratio. Atorvastatin also inhibits the oxLDL-mediated LOX-1 expression by endothelial cells, the uptake of oxLDL in endothelium and the oxLDL-mediated reduction of protein kinase B (PKB) phosphorylation [24]. The activation of PKB is critical for the expression of eNOS, which promotes vessel relaxation. However, a meta-analysis provided no clear evidence that statin therapy have a favourable effect on oxLDL [51].

In STAT trial [52] the antibodies against oxLDL were equally decreased with both aggressive and conventional lipid-lowering therapy. This indicates that the statin-related reduction of oxLDL is not a dose-dependent phenomenon, a finding which is in agreement with our results. It might therefore represent a pleiotropic effect, independent -at least partially- from the hypocholesterolemic action. A study by Orem et al detected a significant decrease of autoantibodies against oxLDL with low doses of atorvastatin (10mg) [53], similar to doses used in our study. In statin exposed patients, intensification of the regimen offers no additional benefit and only those with LDL>125mg/dl benefited from a more aggressive statin therapy [52]. Statins have a dose-related response with regard to clinical outcome, but this dose-related response has not been confirmed with regard to oxidative stress [54]. This might alternatively be explained by the hypothesis that statins achieve their uttermost benefit on oxLDL after a certain time point [52], after which further continuation of treatment serves only the purpose of maintenance.

Atorvastatin has been shown to reduce small dense LDL subfractions, remnant-like particles cholesterol and oxLDL, and improve endothelial function, after just few weeks of therapy [55,56]. Such time-related effect has not been fully elucidated, but may possibly account for our finding that in the first six months there was an accelerated decline of oxLDL levels followed by a milder reduction rate thereafter.

Additional pleiotropic effects of statins have been reported in the literature and might account for the observed beneficial effects in the current study. Lysophosphatidylcholine is elevated during LDL oxidation and is responsible for some of the biological effects of oxLDL. Atorvastatin alters the ability of oxLDL to impair the endothelium relaxation, by modulating the hydrolysis of phosphatidylcholine to lysophosphatidylcholine when LDL is being oxidized [57]. Statins remove predominately "aged LDL" from plasma, which is more prone to oxidation [53], through stimulation of hepatic LDL receptor activity and inhibition of very-low density lipoprotein (VLDL) and LDL production by the liver cells [53]. Statins also reduce oxygen species generation [54]. Atorvastatin promotes adipocyte uptake of oxLDL in rabbits by increasing the expression of CD36 and peroxisome proliferators-activated receptor γ (PPAR γ) in adipocytes [58]. The increased expression of such receptors by adipocytes results to internalization of oxLDL and clearance from plasma, converting adipocytes to an oxLDL-buffering pool [58]. Reduction of oxLDL in patients with CHD with atorvastatin 10mg parallel with an increase of adiponectin, which has anti-atherogenic [55], anti-inflammatory and anti-diabetic [55] properties through reduction of insulin resistance [55]. The CARDS study reported a significant degree of preventive activity of atorvastatin against myocardial infarction in eucholesterolemic diabetic patients, conceivably attributed to such improvement of insulin sensitivity [55]. Statins also diminish the expression of CD40 and CD40 ligand in vascular cells, smooth muscle cells and macrophages, which are promoted by oxLDL and are considered proatherogenic [59]. Other anti-inflammatory pathways include reduction of C-reactive protein [60], chemokines, major histocompatibility complex II molecules, matrix-degrading enzymes, and procoagulant tissue factor [59]. Atorvastatin reverses the oxLDL-mediated inhibition of vascular endothelial growth factor-induced endothelial progenitor cell differentiation via the phosphatidylinositol 3 kinase/Akt pathway [61], which restores the oxLDL-related inhibition of mature endothelial cells migration [61]. This could improve neovascularization and collateral vessel formation in response to tissue ischemia. Atorvastatin also suppresses platelet activity [62] by reducing the expression of CD36 and LOX-1, which are present in platelets [43,62], thus inhibiting the oxLDL-mediated platelet hyperactivity [62]. Statins reduce the oxLDL-derived expression of adhesion molecules (E- and P-selectins, vascular cell adhesion molecule 1 [VCAM-1] and intercellular adhesion molecule 1 [ICAM-1]) in human coronary artery endothelial cells [15], through up-regulation of eNOS expression [15], which regulates the expression of adhesion molecules in endothelial cells [15]. Statins also diminish the oxLDL-mediated activation of nuclear factor- κ B (NF- κ B) [15], which regulates the transcription of adhesion molecule genes [33]. In diabetic patients with dyslipidemia atorvastatin reduced CVD and markers of inflammation, adhesion and oxidation, such as CRP, soluble ICAM-1, soluble VCAM-1, E-selectin, matrix metalloproteinase 9, secretory phospholipase A2 (sPLA2), and oxLDL, the latter by 38,4% [60]. Moreover, the change of oxLDL levels correlated with the change of sICAM-1 and E-selectin levels, suggesting that statins could

possibly counteract the oxLDL-associated increase of NF- κ B, and therefore, the production of such cell adhesion molecules [60]. Statins also enhance scavenger receptor expression in macrophages [60], and increase plaque stability via reduction of metalloproteinases [60].

The reduction of oxidised LDL and of carotid stenosis in our study was relevant for both, smokers and non-smokers. However, during subgroup analysis showed that the beneficial effect of statin use concerns mostly the subgroup of mild smokers, while no such effect was noticed for moderate and heavy smokers. How smoking may diminish the beneficial effect of statins on oxidized LDL and carotid stenosis is not yet clarified in the literature. A reasonable assumption might be that, since smoking increases the oxidative stress, it contributes to enhanced LDL oxidation [63]. Moreover, studies in animal models, have demonstrated that smoking alters the immunologic response to oxidized LDL by reducing the production of antibodies against these molecules, i.e. causing a kind of immune suppression regarding the response to oxidized LDL. Thus, it has been shown to increase carotid IMT [64].

The Mercodia oxLDL detects the MDA-modified apoB [28]. It has been proposed that oxLDL loses its predictive value for CVD when adjustment for apoB level is performed [54]. In several studies though, a significant reduction of Mercodia oxLDL with atorvastatin 10mg was still detected even after adjustment for apoB, [10,31,54], while in other studies no adjustment for LDL or apoB levels was made [54,65]. In our study the oxLDL:apoB ratio remained unchanged, but in the multivariate analysis the reduction of oxLDL was still significant after adjustment for apoB and LDL levels.

In patients with familial hypercholesterolemia a lack of association between oxLDL and IMT was reported at baseline, however two years therapy with atorvastatin 80mg was associated with regression of carotid IMT [66]. The LDL subfraction profile and autoantibodies against oxLDL remained unchanged. Nevertheless, the rate of oxidation and the amount of dienes formed decreased and this was linked to lessening of atherosclerosis. In our study the reduction of carotid stenosis was associated with decreased oxLDL levels. Besides, the unchanged oxLDL autoantibodies levels do not preclude the reduction of oxLDL, as was indicated in another study involving dialysis patients, where atorvastatin therapy reduced plasma oxLDL, whereas oxLDL autoantibodies did not change significantly [67].

Disadvantages of the study were the relatively small size, the lack of a control group comprising of patients with carotid stenosis not on statin therapy, which would be unethical, the fact that researchers were not blinded to the patients' status, the lack of randomization of the dose-schedules and the use of only one method to detect oxLDL.

5. Conclusion

This prospective, cross-sectional study with such a long observation period provided enough evidence to postulate a favourable effect of low-dose atorvastatin therapy on oxLDL, which was additionally associated with improvement of stenosis in patients with carotid atheromatosis. We thus, assume that oxidised LDL may represent a far more sensitive risk factor for

carotid stenosis, than LDL itself or apoB. Further studying is required to confirm such findings and to establish a clear clinical and pathophysiologic link between oxLDL and carotid stenosis.

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Author details

Elias Skopelitis, Dimitrios Levisianou, Helen Lydataki and Sofoklis Kougialis

General Hospital of Nikaia and Piraeus, Athens, Greece

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