

LDL Oxidation and Extent of Coronary Atherosclerosis

Lucy P.L. van de Vijver, Alwine F.M. Kardinaal, Wim van Duyvenvoorde, Dick A.C.M. Kruijssen, Diederick E. Grobbee, Geert van Poppel, Hans M.G. Princen

Abstract—Accumulated evidence indicates that oxidative modification of LDL plays an important role in the atherogenic process. Therefore, we investigated the relation between coronary atherosclerosis and susceptibility of LDL to oxidation in a case-control study in men between 45 and 80 years of age. Case subjects and hospital control subjects were selected from subjects undergoing a first coronary angiography. Subjects with severe coronary stenosis (≥85% stenosis in one and ≥50% stenosis in a second major coronary vessel) were classified as case subjects (n=91). Hospital control subjects with no or minor stenosis (≤50% stenosis in no more than two of the three major coronary vessels, n=94) and population control subjects free of plaques in the carotid artery (n=85) were pooled for the statistical analysis into one control category. Enrollment procedures allowed for similar distributions in age and smoking habits. Case subjects had higher levels of total and LDL cholesterol and triglycerides and lower levels of HDL cholesterol. Resistance time, maximum rate of oxidation, and maximum diene production were measured ex vivo using copper-induced LDL oxidation. A borderline significant inverse trend was observed for coronary atherosclerosis risk at increasing resistance time. Odds ratios (95% confidence interval) for the successive quartiles were 1.0 (reference), 0.77 (0.39 to 1.53), 0.67 (0.33 to 1.34), and 0.55 (0.27 to 1.15) (P<0.07). No relation with maximum rate of oxidation was found, and higher maximum diene levels were found in control subjects (P<0.01). The main determinant of oxidation was the fatty acid composition of LDL. No effect of smoking or use of medication was observed. We conclude that although LDL resistance to oxidation may be a factor in atherogenesis, the ex vivo measure is not a strong predictor of severity of coronary atherosclerosis.

(Key Words: LDL oxidation ■ atherosclerosis ■ resistance time ■ propagation rate ■ LDL composition)

Oxidative modification of LDL by free radicals has been implicated as an important determinant in the development of atherosclerosis. Oxidative modification accelerates the uptake of LDL by macrophages, which is the beginning of formation of a fatty streak. LDL is thought to be protected against attacks of free radicals by antioxidants in plasma and the particle itself. Circumstantial evidence indicates that oxidation occurs in vivo in humans. Epitopes of oxidized LDL have been found in plasma and atherosclerotic lesions of experimental animals and humans, and autoantibodies against these epitopes have been detected in human plasma. Furthermore, an increased susceptibility of LDL to oxidation has been described in patients with coronary heart disease. The susceptibility of LDL to oxidation is decreased by vitamin E supplementation and increased by adding unsaturated fatty acids to the diet. A reduction in risk of CVD at higher plasma antioxidant levels has been reported, and higher dietary antioxidant levels have been proposed to be associated with a reduced risk of CVD.

To determine the relation between oxidative stress and atherosclerosis, lipid peroxidation and its consequences are of particular interest. Ex vivo, the peroxidation process can be mimicked by incubating isolated LDL with the pro-oxidant Cu2+ and by following the production of conjugated dienes from polyunsaturated fatty acids. The time elapsing until the onset of diene production, the resistance time, depends on the strength of the antioxidant defense in the LDL particle and may therefore reflect the resistance to oxidation in vivo. By using the copper-induced oxidation method, several investigators have described individual variation in susceptibility to LDL oxidation. In previous studies we detected subtle changes in susceptibility of LDL to oxidation after supplementation of only 25 mg/d vitamin E and by adding 5 g of fish oil to the diet using this oxidation method.

To address the question of whether LDL oxidation is related to the severity of coronary heart disease, we compared the susceptibility of isolated LDL to copper-induced oxidation between a large group of patients with angiographically determined coronary atherosclerosis and a control group.

Methods

Study Population

The study was conducted in several hospitals and clinical centers in Rotterdam and Dordrecht, The Netherlands, during 1993 to 1995. The study was approved by an ethics committee on human research.
and all participants gave informed consent. We selected a group of coronary atherosclerosis subjects, a group of hospital control subjects, and a group of population control subjects. The groups consisted of men between 45 and 80 years of age. Enrollment procedures allowed for similar distributions of age (in 5-year categories) and smoking habits (smoking and nonsmoking).

Selection of the two hospital groups was based on angiographic reports. To reduce the impact of the disease on dietary and lifestyle patterns, we selected only those patients who underwent their first angiography and who had not experienced an MI in the year before the study. For the same reason, blood was collected within 2 months after angiography. Subjects using HMG-CoA reductase inhibitors were excluded because of a possible influence of this medicine on LDL oxidation.

In the study period 2830 patients (1966 male) underwent coronary angiography for suspected coronary atherosclerosis. Subjects were ineligible if they met one of the following exclusion criteria: under 45 or over 80 years of age (n=144); not the first coronary angiography (n=389); MI during the 12 months before the study (n=180); diabetes mellitus (n=84); liver, kidney, or thyroid disease (n=15); alcohol or drug abuse (n=4); use of HMG-CoA reductase inhibitors (n=82); vegetarian diet (n=3); psychiatric complaints (n=2); or death (n=12). For 88 subjects more than 2 months had elapsed between angiography and case selection, leaving a population of 963 eligible subjects. Of this group 124 refused to participate, and 50 could not be contacted or were otherwise indisposed. From the remaining 789 men, subjects were selected on the basis of the extent of coronary stenosis. Nine angiographic reports lacked essential information, 500 subjects fulfilled all eligibility criteria, and 92 men were not included because they preselected subjects by age and smoking habits. Selected were 92 case subjects with at least 50% stenosis in one and at least 50% stenosis in a second of the three major coronary vessels and 95 hospital control subjects with less than 50% stenosis in no more than two of the three major coronary vessels. The percentage of stenosis was scored by the cardiologist performing the angiography.

Population control subjects were selected from participants in The Rotterdam Study. The rationale and design of this population-based prospective cohort study have been described previously. 7 No angiographic data were available, but the subjects who were selected had no plaques in the carotid artery as assessed by ultrasonic echography. Also, these subjects had no history of cardiac disease or treatment; had no diabetes mellitus or liver, kidney, or thyroid disease; did not use HMG-CoA reductase inhibitors; and did not eat a vegetarian diet. Because the participants in the Rotterdam Study were all 55 years and over at baseline, we recruited additional men between 45 and 55 years of age through an advertisement in a local newspaper. Recruitment took place in the same area from which the other population control subjects originated. A questionnaire was used to obtain information on medical history; candidates who fulfilled the inclusion criteria were invited to the research center. Enrollment in the study took place after medical history; candidates who fulfilled the inclusion criteria were invited to the research center.

Data Collection
For the hospital groups medical histories were obtained from medical files and through a questionnaire administered within 2 months after angiography. Information on dietary, smoking, and drinking patterns; drug use; use of vitamin supplements; occupation; and family history of CVD was obtained. Weight, height, and blood pressure were measured. A fasting venous blood sample was collected in EDTA Vacutainer tubes, immediately placed on ice, and cooled to 4°C. Plasma was prepared within 1 hour after blood collection by centrifugation at 1750g for 15 minutes, frozen in methanol (−80°C) or liquid nitrogen, and stored at −80°C.

Preparation and Oxidation of LDL
The procedure for preparation and lipid peroxidation of LDL was adapted from Esterbauer et al9 with major modifications as described previously in detail. 15,17 Briefly, for each subject 2 mL of frozen plasma stored at −80°C was rapidly thawed and used for isolation of LDL by ultracentrifugation at 4°C in the presence of 10 μmol EDTA. To minimize the time between isolation and oxidation and to prevent loss of lipophilic antioxidants, 20 the LDL was not dialyzed. 15,17,20 Omitting dialysis allows a more stable LDL preparation that can be stored in the dark at 4°C under nitrogen for several days without affecting resistance time and maximum rate of oxidation to be obtained. 15,17,20 This improves the precision of the method because each LDL preparation can be oxidized consecutively in triplicate. In a representative experiment, resistance time was 90±2 minutes 1 hour after LDL isolation in an LDL preparation that had not been dialyzed; 24 hours after LDL isolation, resistance time was 91±3 minutes (n=3). Dialysis under nitrogen for 4 hours (two changes) at 4°C against 1000 vol of an oxygen-free buffer containing 150 mmol/L NaCl and 10 mmol/L sodium phosphate, pH 7.4, resulted in resistance times of 52±5 minutes immediately after dialysis and 23±4 minutes after storage of LDL under nitrogen for 24 hours (n=3). 20 In agreement with these observations, a loss of lipophilic antioxidants during dialysis was recently reported. 20

The kinetics of LDL oxidation was followed by continuously monitoring the change of absorbance at 234 nm. 15,17,25 Absorbance curves of LDL preparations obtained from an equal number (n=3) of subjects from each study group were determined in parallel. Each LDL preparation was oxidized in three consecutive oxidation runs on the same day. The means were calculated on the basis of these three observations. The intra-assay coefficients of variation for resistance time and maximum rate of oxidation calculated from measurements obtained at 1 day were 2.6% and 3.1%, respectively. The inter-assay coefficients of measurements performed on different days were 4.9% and 7.4%, respectively. 15,17 In every oxidation run one reference LDL, prepared from a reference plasma stored at −80°C, was used as a control. Oxidation runs with a deviation greater than 10% from the mean values of former reference measurements were omitted. 31,32 When this highly standardized method is used, resistance time and maximum rate of oxidation do not differ between LDL prepared from plasma frozen in liquid nitrogen and that from freshly collected plasma from the same subject. In addition, no differences in these parameters were found in plasma stored at −80°C for up to 18 months.

Analytical Measurements
Cholesterol and triglyceride concentrations were determined enzymatically using commercially available reagents (CHOD-PAP kit 236.691 and triglyceride kit 701.904, Boehringer-Mannheim, Mannheim, Germany). Phospholipid concentrations in LDL were determined using a commercially available color reagent (Wako Chemicals, Neuss, Germany). One hundred microliters of LDL (0.25 mg protein/mL) sample and 750 μL of color reagent were mixed for 10 minutes at 37°C, and the concentration was measured at a wavelength of 500 nm. The protein content of the LDL preparations was measured according to the method of Lowry et al. 30 HDL cholesterol was measured after precipitation of VLDL, IDL, and LDL using the precipitation method with sodium phosphotungstate-Mg2+. 31 LDL cholesterol concentrations were calculated by the formula of Friedewald et al. 31

Fatty acid composition of LDL was determined in duplicate by gas-liquid chromatography as previously described. 31 Heptadecanoic acid (C17:0) was added as an internal standard. We calculated the amount of polyunsaturated fatty acids (C18:2+C18:3+C20:2+...
Analyses were conducted using the BMDP statistical package.34 Regression to assess which variable was the most important predictor. Data distributions of oxidation parameters in the control group. The trend analysis was performed over the oxidation parameters as a continuous variable in the model. Results found (Table 4). The ORs calculated for the oxidation parameters as continuous variables in the model resulted in an OR of 0.97 (0.94 to 1.00) per minute increase of resistance time and maximum rate of oxidation, ie, maximum diene production, was found, with a higher maximum production being found in the control group. Parameters of LDL Oxidation

Table 3 lists parameters of LDL oxidation with age-adjusted differences. Resistance time and maximum rate of oxidation were not significantly different; however, a borderline significant lower resistance time was seen in the case subjects (P=0.07). Surprisingly, a significant difference in maximum oxidation, ie, maximum diene production, was found, with a higher maximum production being found in the control group.

ORs and 95% confidence intervals were calculated for the risk of coronary atherosclerosis per quartile of resistance time and maximum rate of oxidation. For resistance time a slightly (nonsignificant) decreased risk of coronary atherosclerosis was found. No associations for maximum rate of oxidation were found (Table 4). The ORs calculated for the oxidation parameters as continuous variables in the model resulted in an OR of 0.97 (0.94 to 1.00) per minute increase of resistance time and maximum rate of oxidation, ie, maximum diene production, was found, with a higher maximum production being found in the control group.

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We investigated which variables were determinants of the oxidation parameters. The difference between the lowest 10% point of distribution and the 90% point produced an OR of 0.58 (0.32 to 1.03) for resistance time and 1.31 (0.67 to 2.57) for maximum rate of oxidation. When case and hospital control subjects and case and population control subjects were compared separately, ORs for the successive quartiles of resistance time and maximum rate of oxidation were 1.0, 0.77 (0.34 to 1.53), 0.67 (0.33–1.34), 0.55 (0.27–1.15) and 1.0, 0.72 (0.31 to 1.64), 0.55 (0.23 to 1.29), and 0.98 (0.43 to 2.21) for comparison between case and hospital control subjects and 1.0, 0.49 (0.21 to 1.13), 0.49 (0.21 to 1.13), and 0.60 (0.27 to 1.36) for comparison between case and hospital control subjects and 1.0, 0.49 (0.21 to 1.13), 0.49 (0.21 to 1.13), and 0.60 (0.27 to 1.36) for comparison between case and population control subjects.

Because more subjects in the case group had a history of MI and a history of MI could have had an impact on dietary patterns, we repeated the analyses with MI survivors excluded. No relevant differences in ORs were detected. For cases with versus cases without a history of MI, the resistance time (mean±SD) was 86±9 versus 88±8, maximum rate of oxidation was 10.5±1.1 versus 10.2±1.1, and maximum diene production was 408±16 versus 405±17.

Stratified analyses for smokers (n=95) and non- and ex-smokers (n=185) were performed. ORs were 0.97 (0.91 to 1.03) and 0.97 (0.93 to 1.01) per unit of resistance time and 1.11 (0.78 to 1.59) and 1.08 (0.81 to 1.45) per unit of maximum rate of oxidation in smokers and non- plus ex-smokers, respectively.

**Determinants of Oxidation Parameters**

We investigated which variables were determinants of the oxidation parameters in the control group. For this analysis age, body mass index, smoking status, plasma lipids, α-tocopherol and fatty acid content of the LDL particle, and LDL composition were considered. By univariate analysis determinants that were significantly correlated to the oxidation parameters were detected and included in a multiple linear regression analysis to identify those that substantially contributed to the outcome. The only significant association with resistance time was found in the percentage of saturated fatty acids in the LDL particle (r=.18). Maximum rate of oxidation was positively related to percentage of polyunsaturated fatty acids (r=.55) and inversely related to the percentage of monounsaturated (r=-.36) and saturated fatty acids (r=-.53). In addition, an association was found between maximum rate of oxidation and body mass index (r=-.18) and between the percentage of total cholesterol (r=.24) and triglycerides (r=-.23) in the LDL particle. Multiple linear regression ascribed the most relevant contributions to the maximum rate of oxidation to the percentages of saturated and monounsaturated fatty acids in the LDL particle (R²=.53).

**Discussion**

We investigated the relation between parameters of LDL oxidation and severity of coronary atherosclerosis in a case-control study. Resistance time, as a reflection of resistance to oxidation in vivo, was expected to be lowest in subjects with coronary atherosclerosis. We indeed found a slight, but not significant, decreased risk of coronary atherosclerosis with increasing resistance time. Propensity to oxidation may also be reflected in a higher maximum rate of oxidation. However, this could not be confirmed in our study. Unexpectedly, we found higher maximum diene levels in the control group.

In this study, selection of both case and hospital control groups was based on results of angiography. The mean percentage of stenosis in the case group was 75%, and 55% of the case subjects had narrowing of at least 50% in all three coronary vessels, whereas the hospital control subjects had a mean of 4% stenosis, and 76% of these control subjects had no substantial narrowing in the three major coronary vessels. The

### Table 3. Parameters of LDL Oxidation (Mean±SE) and Age-Adjusted Differences

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Case Subjects (n=91)</th>
<th>Control Subjects (n=179)</th>
<th>Adjusted Difference*±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance time, min</td>
<td>87±1</td>
<td>89±1</td>
<td>-2±1</td>
</tr>
<tr>
<td>Maximum rate of oxidation, nmol/min - mg protein⁻¹</td>
<td>10.3±0.1</td>
<td>10.1±0.1</td>
<td>0.1±0.15</td>
</tr>
<tr>
<td>Maximum diene production, nmol/mg</td>
<td>406±2</td>
<td>420±3</td>
<td>-14±4†</td>
</tr>
</tbody>
</table>

*Case minus control.
†P<.01.

### Table 4. ORs for the Risk of Coronary Atherosclerosis Per Quartile of Resistance Time and Maximum Rate of Oxidation

<table>
<thead>
<tr>
<th>Quartile</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>Trend*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance time</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases, n</td>
<td>30</td>
<td>23</td>
<td>21</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>1.0</td>
<td>0.77</td>
<td>0.67</td>
<td>0.55</td>
<td>P=.07</td>
</tr>
<tr>
<td>Maximum rate of oxidation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases, n</td>
<td>20</td>
<td>26</td>
<td>19</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>1.0</td>
<td>1.27</td>
<td>0.91</td>
<td>1.30</td>
<td>P=.43</td>
</tr>
</tbody>
</table>

CI indicates confidence interval.
*The trend was calculated over the oxidation parameters as a continuous variable.
contrast between case and control subjects was thus sufficient with virtual exclusion of misclassification of disease. Moreover, we included a group of population control subjects, for whom we had a measure of CVD (echography of the carotid arteries).

In this study a positive correlation between the percentage of polyunsaturated fatty acids and maximum rate of oxidation was found. The percentages of monounsaturated and saturated fatty acids were inversely related to the maximum rate of oxidation, whereas resistance time was positively correlated with percentage of saturated fatty acids. Assessment of determinants important in predicting susceptibility to oxidation demonstrated that fatty acid composition of LDL may be most important. These results coincide with other studies reporting increased susceptibility to oxidation with the degree of unsaturation of fatty acids, which leads to a decreased resistance time and an increased maximum rate of oxidation and maximum diene production.19–20 In our study the mean level of fatty acids, however, did not differ between the groups. Despite equal amounts of polyunsaturated fatty acids in the LDL particle, we found significantly higher maximum diene production in control subjects, most pronounced in the population control group. We do not yet know how to interpret this result, but diene production seems to be an unsuitable parameter to study LDL oxidation as risk factor for coronary atherosclerosis.

In our study we found small but significant differences in LDL lipid composition between the case group and the control group, with higher total cholesterol and phospholipid levels in the LDL particle and lower LDL triglyceride levels in the control group. These differences, however, were not reflected by significant differences in resistance time and maximum rate of oxidation between the groups.26 The reason for this may be the small diversity of LDL particles despite significant differences between case and control subjects.

No difference in fatty acid composition of the LDL particle between case and control subjects was observed, indicating that dietary intake of fatty acids was similar in the groups. The use of a prescribed diet was not different between groups, and to further exclude dietary changes as a result of angiography, blood samples were taken within 2 months after catheterization. ORs for coronary atherosclerosis risk for the two separate control groups did not differ. Therefore, it seems unlikely that the hospital groups were more prone to dietary changes. Another reason for changed dietary patterns could have been the experience of MI, which was more common in the active stage of atherogenesis. Sclerosis may differ between individuals, and those in the most active stage of atherogenesis may be most susceptible to oxidation.

Results of several studies have indicated that medication may influence the oxidizability of LDL.35–37 Therefore, we investigated the effect of use of antihypertensive medication, use of coumarin derivatives or salicylic acid, and use of lipid-lowering medication. We compared the oxidation parameters within subgroups of the control group (both hospital and population control groups). No differences were found in oxidation parameters between users and nonusers of antihypertensive medications, ACE inhibitors, and calcium antagonists, nor between users and nonusers of coumarin derivatives and salicylic acid. Because of the small numbers of men using lipid-lowering drugs, we investigated the influence of lipid-lowering medication by excluding the users from the analysis. This had only a minor impact on the ORs. So, in contrast to others, we did not find an effect of medication in our study population.

Only a few studies have reported on the relation between oxidation parameters and coronary heart disease. de Rijke et al35 found a higher susceptibility of LDL to oxidation in coronary bypass patients who had shown progression in stenosis compared to those without progression after 7 years of follow-up. Regnström et al11 described an inverse association between resistance phase and severity of coronary stenosis in young MI survivors; however, in a subgroup analysis no association was detected.38 Cominacini et al12 observed a lower lag phase in coronary artery patients than in hyperlipidemic patients or valvular heart disease patients. The study of Croft et al13 did not reveal a difference in oxidation parameters between coronary atherosclerotic patients and healthy control subjects, whereas in our study a lower (though not significantly lower) risk of coronary atherosclerosis with increasing resistance time was seen. As in the study of de Rijke et al,13 no significant difference in maximum rate of oxidation was found in our study.

Differences in study design, methods used to assess LDL oxidation, and choice of subjects may account for differences in study results. In four studies only a one-point measure of CVD was used to relate to oxidation parameters. Only de Rijke et al13 studied the association between oxidation parameters and progression of stenosis. The progression of atherosclerosis may differ between individuals, and those in the most active stage of atherogenesis may be most susceptible to oxidation.

Susceptibility of LDL to oxidation can be measured by means of a fluorescence method12 or, as in our study, by measuring conjugated diene production.11,13,35 In our study and the study of de Rijke et al,13 LDL was not dialyzed before oxidation, in contrast with other studies using extensive dialysis11,35 (see “Methods”).

Another important difference in the studies may be the choice of study population. Our study population is the largest population studied thus far, and it consisted of relatively old, normolipidemic men. Regnström et al11 reported on young male MI survivors, of which the majority were hypertriglyceridemic. Cominacini et al12 studied young men and women, of which the case group had less severe coronary stenosis than our cases. de Rijke et al13 studied men and women with or without progression of stenosis after 7 years follow-up. The study population in the study of Croft et al13 is comparable to
ours, with the exception that our cases had more severe coronary atherosclerosis. The focus of our study was to investigate the effect of oxidation on atherosclerosis. To study only atherogenic and no thrombogenic effects, we excluded individuals who had experienced an MI less than 1 year before the study. Our results, therefore, may be interpreted as the association between LDL oxidation and atherogenesis solely, whereas in the other studies a thrombogenic effect cannot be excluded.

Intervention studies have shown a clear relationship between vitamin E14–17 and unsaturated fatty acid supplementation17–20 and LDL susceptibility to oxidation. From this it has been concluded that the ex vivo oxidation can mimic the oxidative process in vivo. In this study we found borderline significant associations between risk of coronary atherosclerosis and reduced resistance time, indicating that coronary heart disease does not lead to a significant contrast in oxidation parameters, as was found after intake of vitamin E or specific fatty acids.18–20 In addition, it is likely that this method of assessing oxidizability will not reflect the oxidative process active in development of atherosclerosis in vivo. The oxidative process is not only influenced by antioxidants and fatty acids in the LDL particles but is part of a larger mechanism in which plasma antioxidants and cell constituents also play a role. Direct methods for measuring oxidation in vivo may therefore be more successful as predictive parameters. Measurement of autoantibodies against oxidized LDL7–10 or epitopes of oxidized LDL16 appears to be promising, but so far results are contradictory. It is possible that autoantibodies are not an indicator of severity of atherosclerosis, ie, the extent of thickening of the vessel wall, but can be used as an indicator of the active atherogenic process.16 The same may hold true for LDL oxidation and thickening of the vessel wall.

In conclusion, our data do not support the presence of an inverse association between extent of coronary atherosclerosis and LDL oxidation in patients with severe coronary heart disease. This may be due to the phase of the atherosclerotic process or failure of the method used to measure LDL oxidation to sufficiently reflect in vivo oxidation.

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References
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