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Oxidation of LDL and Extent of Peripheral Atherosclerosis

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Evidence has accumulated for oxidative modification of low-density lipoproteins (LDL) to play an important role in the atherogenic process. Therefore, we investigated the relation between susceptibility of LDL to oxidation and risk of peripheral atherosclerosis among 249 men between 45 and 80 years of age. The ankle-arm index was calculated for both legs as the ratio of systolic blood pressure in the leg divided by the arm systolic blood pressure. The lowest of both ankle-arm indices was used to categorize subjects. Thirty-nine men with an ankle-arm index < 1.00 (20% cut-off point of distribution) were classified as subjects with peripheral atherosclerosis.

Subjects with peripheral atherosclerosis reported more often the use of a special diet and the use of antihypertensive medication, aspirin and coumarin derivatives. No significant differences in total, LDL and HDL cholesterol and triglycerides were present between groups. Resistance time and maximum rate of oxidation were measured *ex vivo* using copper-induced LDL oxidation. Subjects with peripheral atherosclerosis had a significantly lower resistance time, whereas the maximum rate of oxidation tended to be increased in subjects with peripheral atherosclerosis. Odds ratios (ORs, and 95% confidence interval) for the successive tertiles of resistance time were 1.00 (reference), 0.37 (0.15–0.89) and 0.37 (0.16–0.86) ($p_{trend} < 0.01$). ORs for the successive tertiles of maximum rate of oxidation were 1.00 (reference), 1.34 (0.47–3.82) and 1.50 (0.55–4.15). This inverse association was borderline significant ($p_{trend} = 0.07$).

These results support an association between LDL oxidation and the development of peripheral atherosclerosis.

Keywords: LDL oxidation, peripheral atherosclerosis, ankle-arm index, resistance time, LDL composition

INTRODUCTION

Oxidative modification of low-density lipoprotein (LDL) by free radicals has been implicated as an important determinant in the development of atherosclerosis. By oxidative modification the

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uptake of LDL by macrophages is accelerated which is the beginning of formation of fatty streaks.[1,2] Antioxidants in plasma and in the LDL particle itself are thought to protect LDL against attacks of free radicals.[3]

Circumstantial evidence indicates that oxidation occurs *in vivo* in humans. Epitopes of oxidized LDL have been found in plasma[4,5] and atherosclerotic lesions of experimental animals and humans[6] and autoantibodies against these epitopes have been detected in human plasma.[7,8] The susceptibility of LDL to oxidation is decreased by vitamin E supplementation[9–11] and increased by adding unsaturated fatty acids to the diet.[12–14] A reduction in risk of cardiovascular disease (CVD) at higher plasma antioxidant levels[15] has been reported and higher antioxidant levels have been proposed to be associated with a reduced risk of CVD.[16–18]

Susceptibility of LDL to oxidative stress is measured *ex vivo* by determining the production of conjugated dienes from poly-unsaturated fatty acids after incubating isolated LDL with the pro-oxidant Cu^{2+}. The time elapsing until onset of diene production, the resistance time, depends on the strength of antioxidant defence in the LDL particle[19] and may, therefore, reflect the resistance to oxidation *in vivo*.[1]

A few studies have investigated the relationship between oxidation of LDL and risk of CHD.[20–23] Thus far, only one small study investigated the association between LDL oxidation and occurrance of peripheral atherosclerosis.[24,25] No association was detected, however, other studies reported higher levels of lipid peroxides[26,27] and autoantibodies against oxidized LDL[28] in patients with peripheral vascular disease. In the present study, initially designed to study the relationship between coronary atherosclerosis and susceptibility to oxidation,[23] we investigated the association between susceptibility to oxidation and peripheral atherosclerosis by measuring resistance time, maximum oxidation rate and determinants of oxidation in subjects with and without peripheral atherosclerosis.

METHODS

Study Population

The study was conducted in several hospitals and clinical centres in Rotterdam and Dordrecht, The Netherlands (1993–1995), initially to study the relationship between angiographically documented coronary atherosclerosis and LDL oxidation.[23] The study was approved by an ethical committee on human research and all participants gave their informed consent. For the initial study a group of subjects with a history of CVD was selected out of subjects undergoing their first coronary angiography and who had experienced a myocardial infarction (MI) in the year prior to the study. Further, a group of subjects without a history of CVD was selected from participants in the Rotterdam Study, a population-based prospective cohort study[29] and through advertisement in a local newspaper. Of this latter group, only those subjects without any plaques in the carotid artery as assessed by echography participated. All subjects were men between 45 and 80 years of age. Exclusion criteria were: use of HMG-CoA reductase inhibitors because of a possible influence of this medicine on LDL oxidation; diabetes mellitus; liver, kidney or thyroid disease; alcohol- or drug abuse; vegetarian diet and psychiatric complaints. In total 187 hospital derived subjects and 86 population derived subjects were selected for the initial study.[23] Due to logistic reasons ankle-arm index as a measure of peripheral artery disease was measured in only 252 out of 273 eligible subjects. Classified as subjects with peripheral atherosclerosis were subjects with an ankle-arm ratio lower than the 20% cut-off point of distribution. This cut-off point was situated at 1.00. As 33 subjects had an ankle-arm ratio of 1.00, it was decided to categorize only subjects with an ankle-arm ratio lower than 1.00 as subjects with peripheral atherosclerosis (16% of the subjects). No oxidation parameters were measured in one subject without peripheral atherosclerosis and two subjects without peripheral atherosclerosis

were excluded from the statistical analysis because of extreme low resistance time values probably due to errors in blood handling (26 and 45 min)! This resulted in a study population of 39 subjects with and 210 subjects without peripheral atherosclerosis.

Data Collection

For the participants with a CVD history information on medical history was obtained from the medical file and through a questionnaire within two months after angiography. For the others information on medical history was obtained from baseline data of the Rotterdam Study and via questionnaires. For all subjects information on dietary, smoking, and drinking habits, drug use, use of vitamin supplements, occupation and family history of CVD was obtained and weight and height were measured.

Systolic and diastolic blood pressure in the right upper arm were measured in duplicate with the subject in sitting position. To evaluate the presence of atherosclerosis in the lower arteries systolic blood pressure of the posterior tibial artery at both left and right ankle was determined using an 8 MHz continuous wave doppler probe (Imex Pocketdop-II) and a random-zero sphygmomanometer with the subject in supine position. Ankle-arm index was calculated as the ratio of the systolic blood pressure in the ankle to the systolic blood pressure in the arm. The lowest ankle-arm index in either leg, as a sign for peripheral atherosclerosis, was used in the analysis.

Finally, a fasting venous blood sample was collected in EDTA Vacutainer tubes and immediately placed on ice and cooled to 4°C. Plasma was prepared within 1 h after blood collection by centrifugation at 3000 rpm for 15 min, frozen in methanol of −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 al.*[19] with some major modifications as described previously.[9,11,30,31] 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 μM EDTA. To minimize the time between isolation and oxidation and to prevent loss of lipophilic antioxidants,[32] the LDL was not dialyzed.[30] By omitting dialysis a more stable LDL preparation is obtained, which can be stored in the dark at 4°C under nitrogen for several days without affecting resistance time and maximum rate of oxidation.[9,11,14] This improves the precision of the method, since each LDL preparation can be oxidized consecutively in triplicate. In a representative experiment, resistance time was 90 ± 2 min one hour after LDL isolation in a LDL preparation which had not been dialyzed; 24 h after LDL isolation resistance time was 91 ± 3 min ($n = 3$). Dialysis under nitrogen for 4 h (2 changes) at 4°C against 1000 volumes 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 min directly after dialysis and 23 ± 4 min after storage of this LDL under nitrogen for 24 h ($n = 3$). In agreement with these observations a loss of lipophilic antioxidants during dialysis was reported.[32] Oxidation under hypersaline conditions (1.18 mol/l NaCl) results in a higher resistance time than oxidation in physiological saline (0.15 mol/l NaCl; data not shown). Because of this and to overcome the 10 μmol/l EDTA background, 40 μmol/l $CuSO_4$ was added to initiate lipid peroxidation.[9,11]

The kinetics of the LDL oxidation was followed by continuously monitoring the change of absorbance at 234 nm.[9,11,19] Absorbance curves of LDL preparations obtained from an equal number of subjects from each study group were determined in parallel. Each LDL preparation was oxidized in three consecutive oxidation runs on the same day. 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 of the

same LDL obtained in three consecutive runs at one 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.[9] 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 higher than 10% deviation from the mean values of former reference measurements were omitted.[9,11] By using this highly standardized method, 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 upon storage of plasma at −80°C up to 18 months.[31] In a representative experiment, the resistance time and the maximum rate of oxidation of a reference LDL prepared from freshly collected plasma were 91 ± 2 min and 8.7 ± 0.3 nmol/mg per minute ($n = 5$), respectively. After freeezing of the plasma in liquid nitrogen, storage for 3 h at −80°C, and rapid thawing at 37°C, these data were 90 ± 3 min and 8.8 ± 0.3 nmol/mg per min ($n = 5$) on oxidation in the same oxidation run. After storage of the same plasma for 18 months at −80°C the resistance time and maximum rate of oxidation were 92 ± 4 min and 8.9 ± 0.5 nmol/mg per min, respectively ($n = 4$ independent oxidations on different days).

Analytical Measurements

Cholesterol and triglyceride concentrations were determined enzymatically using commercially available reagents (CHOD-PAP) kit and Triglyceride kit, Boehringer-Mannheim, Mannheim Germany). Phospholipid concentrations were determined using a commercially available colour reagent (Wako Chemicals, Neuss, Germany). One hundred microlitres of LDL (0.25 mg/ml protein) sample and 750 µl colour reagent were mixed for 10 min 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 Lowry *et al.*[33]

High-density lipoprotein (HDL)-cholesterol was measured after precipitation of very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) and LDL using the precipitation method with sodium phosphotungstate-Mg^{2+} [34] LDL-cholesterol concentrations were calculated by the formula of Friedewald *et al.*[35]

Fatty acid composition of LDL was determined in duplicate by gas-liquid chromatography as previously described.[11] Heptadecanoic acid (C17 : 0) was added as internal standard. We calculated the amount of poly-unsaturated fatty acids (C18 : 2 + C18 : 3 + C20 : 2 + C20 : 3 + C20 : 4 + C20 : 5 + C22 : 6), mono-unsaturated fatty acids (C14 : 1 + C16 : 1 + C18 : 1 + C20 : 1 + C22 : 1), and saturated fatty acids (C12 : 0 + C14 : 0 + C15 : 0 + C16 : 0 + C18 : 0 + C20 : 0 + C22 : 0).

Concentrations of LDL antioxidants were determined by reversed-phase HPLC[36] and spectrophotometric detection. LDL-antioxidant concentrations were standardized by calculating antioxidant concentrations per mg LDL protein.

Statistical Analysis

Characteristics of the subjects with and without peripheral atherosclerosis were compared with Student's *t*-test for unpaired samples, after normality was established. Age-adjusted means were compared by covariance analysis. For comparison of LDL antioxidant concentrations log-transformed data were used to normalize the distribution. Odds ratios (ORs) were calculated to quantify the association between parameters of oxidation and peripheral atherosclerosis. Age was considered as a confounder in all the calculations of the ORs, and all calculations were therefore adjusted for age. Classification in tertiles for calculation of ORs was based on distributions of oxidation parameters in the group without peripheral atherosclerosis. The p for trend was assessed by calculating the ORs per unit of resistance time or maximum rate of oxidation

(continuous analysis). Data analysis was conducted using the statistical package BMDP.[37]

RESULTS

Table I lists characteristics of subjects with and without peripheral atherosclerosis. The mean ankle-arm ratios, based on the lowest ankle-arm ratio in either leg were 0.93 ± 0.11 and 1.20 ± 0.13 for subjects with and without peripheral atherosclerosis, respectively ($p < 0.01$). The left and right ankle-arm index were highly correlated ($r = 0.74$, $p < 0.001$), with a mean absolute difference between the left and right ankle-arm ratio of 0.08 (SEM 0.01). As shown in Table I, subjects with peripheral atherosclerosis were significantly older. No differences were seen in body mass index (BMI), plasma lipid levels, smoking status, systolic and diastolic blood pressure and family history of CVD.

Subjects with peripheral atherosclerosis reported more frequently ($p < 0.05$) use of antihypertensive medication (76.9% vs. 55.2%, respectively in subjects with and without peripheral atherosclerosis), use of aspirins and coumarin derivatives (61.5% vs. 40.0%) and use of a special diet (20.5% vs. 8.2%). Of those subjects who used a diet, a fat restricted diet was used by 57.1% of the subjects with and by 35.3% of the subjects without peripheral atherosclerosis, use of a cholesterol lowering diet was reported by respectively 37.5% and 35.3% of the subjects with and without peripheral atherosclerosis. No significant differences in family history of CVD (23.1% vs. 23.8%) and history of MI (33.3% vs. 18.0%) were reported.

Age may potentially confound the association between the oxidation parameters and the risk of peripheral atherosclerosis. In the control group we calculated the correlation coefficients between age and resistance time ($r = 0.04$, $p = 0.56$) and age and maximum rate of oxidation ($r = 0.06$, $p = 0.42$). As the correlations in this study population were only weak, no large influence of age can be expected. To exclude the influence of age in the statistical comparison between the groups, p-values were calculated for the difference in age-adjusted means.

Table II lists potential determinants of LDL oxidation, i.e. LDL antioxidant concentrations, fatty acid content and LDL composition. Subjects with peripheral atherosclerosis had significantly lower lutein/zeaxanthin levels, whereas the other antioxidants did not differ. No significant differences were seen in total fatty acid content of LDL and LDL lipid composition. However, differences in proportions of poly-unsaturated and mono-unsaturated fatty acids were borderline

TABLE I Characteristics of the study population (mean ± SD)

	Subjects with peripheral atherosclerosis ($n = 39$)	Subjects without peripheral atherosclerosis ($n = 210$)
Ankle-arm index	0.93 ± 0.11	1.20 ± 0.13[b]
Age (years)	64.1 ± 8.1	59.9 ± 8.7[b]
Smokers (%)[a]	33.3	28.6
Ex-smokers (%)[a]	53.8	52.9
Body mass index (kg/m^2)	25.6 ± 3.1	26.1 ± 3.0
Total cholesterol (mmol/l)	5.7 ± 0.9	5.7 ± 1.0
Triglycerides (mmol/l)	1.8 ± 0.6	1.8 ± 0.9
HDL (mmol/l)	0.9 ± 0.2	1.0 ± 0.3
LDL (mmol/l)	4.0 ± 0.9	3.9 ± 1.0
Systolic blood pressure (mmHg)	137.7 ± 20.7	133.6 ± 17.0
Diastolic blood pressure (mmHg)	82.6 ± 9.4	83.0 ± 8.8
Under treatment by a cardiologist (%)	79.5	63.3
Years under treatment (years)	2.6 ± 4.1	3.1 ± 5.1

[a]Ex-smokers quit smoking more than one year ago, otherwise current smoker; [b]significant difference $p < 0.05$.

significant ($p = 0.053$ and $p = 0.07$, respectively). When separate long chain poly-unsaturated fatty acids were studied, a significantly higher percentage C20 : 4 was found in subjects with peripheral atherosclerosis, whereas the percentage C20 : 5 and C22 : 6 was not different between groups.

Parameters of LDL oxidation are listed in Table III. Resistance time was lower in the group with peripheral atherosclerosis, whereas a borderline significant difference was seen for the maximum rate of oxidation.

ORs were calculated per tertile of resistance time and maximum rate of oxidation (Table IV). The risk of peripheral atherosclerosis was significantly decreased in the two highest tertiles compared to the lowest tertile of resistance time. In the higher tertiles of maximum rate of oxidation risk of peripheral atherosclerosis was increased, though not significantly. The age-adjusted ORs (95% confidence interval) calculated for the oxidation parameters as a continuous variable in the model resulted in an OR of 0.94 (0.90–0.98) per minute increase in resistance time and 1.34 (0.97–1.85) per unit increase in maximum rate of oxidation. The difference between the lowest 10% point of distribution and the 90% point produced an OR of 0.34 (0.15–0.75) for resistance time and 2.21 (0.93–5.23) for maximum rate of oxidation.

More subjects in the case group reported the use of a prescribed diet or had a history of MI. This latter also could have had impact on dietary

TABLE II Concentrations of LDL antioxidants, LDL fatty acid content and LDL composition in subjects with and without peripheral atherosclerosis (mean ± SE)

	Subjects with peripheral atherosclerosis ($n = 39$)	Subjects without peripheral atherosclerosis ($n = 210$)	p^a
γ-Tocopherol (ng/mg protein)[b]	572.2 ± 97.4	640.1 ± 105.6	0.65
α-Tocopherol (ng/mg protein)[b]	5149.6 ± 191.8	4994.1 ± 80.0	0.43
Lutein/Zeaxanthin (ng/mg protein)[b]	50.0 ± 3.7	60.2 ± 2.1	0.03
β-Cryptoxanthin (ng/mg protein)[b]	58.8 ± 8.0	54.4 ± 3.0	0.67
Lycopene (ng/mg protein)[b]	86.2 ± 10.3	117.6 ± 7.1	0.46
α-Carotene (ng/mg protein)[b]	17.4 ± 2.8	21.4 ± 1.3	0.12
β-Carotene (ng/mg protein)[b]	124.6 ± 9.9	136.0 ± 5.9	0.66
Total fatty acids in LDL (mg per mg protein)	1364.8 ± 24.0	1406.7 ± 13.8	0.37
Poly-unsaturated fatty acids (%)	60.1 ± 0.8	58.2 ± 0.3	0.05
Mono-unsaturated fatty acids (%)	17.6 ± 0.8	19.4 ± 0.4	0.07
Saturated fatty acids (%)	22.1 ± 0.2	22.1 ± 0.1	0.76
Total cholesterol (%)[c]	41.3 ± 03	41.1 ± 0.1	0.42
Free cholesterol (%)[d]	8.4 ± 0.4	8.0 ± 0.2	0.49
Cholesterol ester (%)[d]	32.9 ± 0.5	33.1 ± 0.2	0.93
Triglycerides (%)[c]	6.3 ± 0.3	6.0 ± 0.1	0.73
Phospholipids (%)[c]	25.1 ± 0.3	25.6 ± 0.1	0.24
Protein(%)[c]	27.4 ± 0.3	27.3 ± 0.1	0.96

[a]p-value for age-adjusted difference; [b]test of significance on log transformed data; [c]number of cases ($n = 38$) and controls ($n = 205$); [d]number of cases ($n = 31$) and controls ($n = 174$).

TABLE III Oxidation characteristics of subjects with and without peripheral atherosclerosis (mean ± SE)

	Subjects with peripheral atherosclerosis ($n = 39$)	Subjects without peripheral atherosclerosis ($n = 210$)	p^a
Resistance time (min)	85 ± 2	89 ± 1	< 0.01
Maximum rate of oxidation (nmol diene/min per mg protein)	10.5 ± 0.2	10.1 ± 0.1	0.07

[a]p-value for age-adjusted difference.

TABLE IV Odds ratios per tertile of resistance time and maximum rate of oxidation

Tertiles	I	II	III	p_{trend}[a]
Resistance time (min)	< 86	86–92	> 92	
Number of cases	22	8	9	
OR[b]	1.0	0.37 (0.15–0.89)	0.37 (0.16–0.86)	0.008
Max. rate of oxidation	< 9.7	9.7–10.6	> 10.6	
Number of cases	8	12	19	
OR[b]	1.00	1.34 (0.47–3.82)	1.50 (0.55–4.15)	0.07

[a] p_{trend} calculated per unit increase; [b] age adjusted.

patterns. ORs calculated with diet users or MI survivors excluded, yielded no relevant differences in ORs (results not shown).

We performed analysis with exclusion of subjects (10 subjects without peripheral atherosclerosis) who had an ankle-arm index larger than 1.5, as these unusually high values may reflect a high degree of arterial calcification.[38] Exclusion, however, resulted in similar ORs (results not shown).

We performed stratified analysis for smoking habits. This, however, did not result in significantly different ORs for smokers ($n = 73$) and non- and ex-smokers ($n = 176$). ORs were 0.90 (0.82–0.98) and 0.95 (0.90–1.00) per minute increase in resistance time and 1.42 (0.85–2.36) and 1.27 (0.84–1.91) per unit increase of maximum rate of oxidation, respectively in both groups (smokers and non- and ex-smokers).

DISCUSSION

We investigated the relationship between parameters of LDL oxidation (*ex vivo*) and peripheral atherosclerosis. Resistance time was used as a measure of resistance to oxidation *in vivo*, which was expected to be lower in subjects with peripheral atherosclerosis. Further, a decline in resistance to oxidation may be reflected in an increased maximum rate of oxidation. In this study resistance time was reduced in subjects with peripheral atherosclerosis, whereas the maximum rate of oxidation was borderline significantly increased.

In this study a four minutes difference in mean resistance time between the two groups was observed. This difference was highly statistically significant. Whether this difference is also of biological relevance is not sure. With our method, even under high oxidative stress conditions, differences in resistance time between the groups were seen. This means that also *in vivo*, probably under milder oxidative conditions, a difference in oxidizability of LDL between the groups will exist. This may lead to increased atherogenesis and can thus explain our results regarding peripheral atherosclerosis.

This study was initially performed to study the association between oxidation parameters and risk of coronary atherosclerosis.[23] For the initial study three groups were selected: subjects with angiographically assessed severe coronary atherosclerosis, subjects with angiographically assessed no or minor coronary atherosclerosis and healthy population controls with no history of CVD. The group with severe coronary atherosclerosis had more that 80% stenosis in one and more that 50% stenosis in a second of the three major coronary vessels. Of these subjects, 55% had a narrowing of at least 50% in all three coronary vessels. The mean percentage of stenosis was 75% over all three coronary vessels. The group with minor coronary stenosis had a mean of 4% of stenosis and 76% of them had no substantial narrowing in the three major coronary vessels. The healthy population controls had no history of CVD and were free of stenosis in the carotid artery. In the present study, we recategorized subjects

according to their lowest ankle-arm index. In both groups subjects with known CVD were included. Adjustment for severity of coronary atherosclerosis was performed by including the initial study group as dummy variables in a model with the category of peripheral atherosclerosis as outcome variable and the oxidation parameters as continuous explanatory variables. This resulted in an OR of 0.95 (0.91–0.99) per minute increase in resistance time and 1.34 (0.96–1.86) per unit increase in maximum oxidation rate. These ORs are essentially similar to the ORs found without adjustment. This indicates that in our study the associations between oxidation parameters and peripheral atherosclerosis are independent of the extent of coronary atherosclerosis.

Because two-thirds of our study population underwent angiography for suspected CVD, changes in dietary and life-style habits as a result of their disease status may have occurred. To minimize dietary changes as a result of the angiography blood samples were taken within two months after catheterisation. Subjects who had undergone angiography did not differ in fatty acid composition of the LDL from those subjects without a CVD history, which indicates that dietary intake of fatty acids did not differ between these groups.[23] Another reason for changing dietary patterns could be the experience of MI, which was more commonly reported in the group with peripheral atherosclerosis. Exclusion of MI survivors, however, did not change the results.

This study did not intend to produce a representative sample of men aged 45–80. One-third of all participants ($n = 85$) had no history of CVD and was free of plaques in the carotid artery. As peripheral atherosclerosis is related to presence of carotid artery disease,[39] the likelihood of finding peripheral atherosclerosis subjects in the latter group was reduced. From looking at the distribution it showed that the arbitrary cut-off point 0.90 for ankle-arm index used commonly in studies on peripheral atherosclerosis[40,41] was not appropriate in this study (insufficient statistical power). Only 19 subjects had an index lower than 0.90. However, when the 0.90 cut-off point was chosen, the direction of the association was comparable to those previously reported.

Categorization of subjects was based on a blood pressure measurement at one point in time. As blood pressure can fluctuate over time, misclassification could have occurred. The ankle-arm index, however, is a ratio between two systolic blood pressures at one specific time point, which is more stable than blood pressure itself.

The difference in susceptibility of LDL to oxidation between subjects with and without peripheral atherosclerosis may partly be explained by differences in known determinants of LDL oxidation. An increased susceptibility to oxidation with increasing degree of unsaturation of fatty acids has been described in several studies.[12–14] In our study a borderline significant difference in unsaturated fatty acids was found, and the percentage C20:4 of LDL was significantly higher in subjects with peripheral atherosclerosis compared to subjects without peripheral atherosclerosis. Other studies reported increased susceptibility to oxidation with decreasing LDL vitamin E concentration[9–11] and with decreasing LDL particle size,[42,43] which could not be confirmed in our study. In the group without peripheral atherosclerosis no correlations were found between fatty acid composition and resistance time, but the fatty acid composition was correlated ($p < 0.05$) with maximum rate of oxidation ($r = -0.53$ for saturated, $r = -0.26$ for mono-unsaturated and $r = 0.47$ for poly-unsaturated fatty acids). For LDL antioxidant concentrations, no correlations were found between antioxidants and resistance time and only γ-tocopherol ($r = -0.22$) was inversely correlated with maximum rate of oxidation. In this study LDL particle size was not measured but the LDL composition can reflect this; small dense LDL particles are lower in cholesteryl esters and phospholipids and relatively richer in triglycerides and protein than large particles and have been reported as being more susceptible to oxidation.[42,43] However, we did not find differences in LDL lipid composition between the groups.

Several studies have indicated that medication may influence the oxidizability of LDL.[44–46] Therefore, the effect of use of antihypertensive medication, use of coumarin derivatives or salicylic acid and use of lipid lowering medication was investigated. Oxidation parameters within subgroups of the control group were compared. No differences were found between users and non-users of antihypertensive medication, ACE-inhibitors nor calcium antagonists, nor between users and non-users of coumarin derivatives and salicylic acid. Because of the small numbers of men using lipid lowering drugs, the influence of lipid lowering medication was investigated by excluding the users from the analysis. This had only minor impact on the ORs. So, in contrast to others we did not find an effect of medication in our study population.

Thus far one study has investigated the association between LDL oxidation and risk of peripheral atherosclerosis.[25] As in our study, a decrease in lag time was reported in patients with intermittent claudication compared to healthy controls, however, this association was not statistically significant which may be ascribed to the small number of subjects. Few previous studies have investigated the relation between oxidation parameters and CHD. Coronary bypass subjects with progression after seven years showed a higher susceptibility to oxidation than those without progression,[22] an inverse association between resistance time and severity of coronary stenosis was described in young MI survivors,[20] and resistance time was found to be lower in coronary artery patients than in hyperlipidaemic patients or valvular heart disease patients.[21] Further, higher TBARS-MDA concentrations were described in coronary artery patients[47] and in peripheral vascular disease patients[26,27] than in controls. Also elevated levels of auto-antibodies against oxidized LDL have been reported in young patients with peripheral vascular disease.[28] Although our results agree with these findings, it should be noted that in the initial study on the association between LDL oxidation and extent of coronary atherosclerosis only a borderline significant association between the oxidation parameters and risk of coronary atherosclerosis was found.[23] It seems, therefore, that LDL oxidation is more strongly related to the pathogenesis of peripheral atherosclerosis than of coronary atherosclerosis. The reason for this is not clear. Other studies have indicated that peripheral atherosclerosis is a strong independent predictor of mortality even in patients with known CHD, and that different vascular areas seem to be differently susceptible to different atherogenic risk factors.[48] This suggests that there may be differences in the pathogenesis of peripheral atherosclerosis and coronary atherosclerosis, and that oxidative modification of LDL may be a more important risk factor in peripheral atherosclerosis.

Another explanation for this finding may be that because of the presumably larger lesion size in the peripheral arteries compared to the coronary arteries, more mildly-oxidized LDL can return to the circulation.[49] Mildly oxidized LDL can oxidize faster that native LDL, which may explain differences in susceptibility to Cu-oxidation between peripheral and coronary atherosclerosis.

In this study oxidation of LDL has been investigated as a potential risk factor of peripheral atherosclerosis. We conclude that the susceptibility of LDL to oxidation measured as resistance time is associated with peripheral atherosclerosis, with a decreased resistance time seen in subjects with peripheral atherosclerosis.

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