

Oxidized Phospholipids Predict the Presence and Progression of Carotid and Femoral Atherosclerosis and Symptomatic Cardiovascular Disease

Five-Year Prospective Results From the Bruneck Study

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OBJECTIVES	The purpose of this work was to determine the predictive value of oxidized phospholipids (OxPLs) present on apolipoprotein B-100 particles (apoB) in carotid and femoral atherosclerosis.
BACKGROUND	The OxPLs are pro-inflammatory and pro-atherogenic and may be detected using the antibody E06 (OxPL/apoB).
METHODS	The Bruneck study is a prospective population-based survey of 40- to 79-year-old men and women initiated in 1990. Plasma levels of OxPL/apoB and lipoprotein (a) [Lp(a)] were measured in 765 of 826 (92.6%) and 671 of 684 (98.1%) subjects alive in 1995 and 2000, respectively, and correlated with ultrasound measures of carotid and femoral atherosclerosis.
RESULTS	The distribution of the OxPL/apoB levels was skewed to lower levels and nearly identical to Lp(a) levels. The OxPL/apoB and Lp(a) levels were highly correlated ($r = 0.87$, $p < 0.001$), and displayed long-term stability and lacked correlations with most cardiovascular risk factors and lifestyle variables. The number of apolipoprotein (a) kringle IV-2 repeats was inversely related to Lp(a) mass ($r = -0.48$, $p < 0.001$) and OxPL/apoB levels ($r = -0.46$, $p < 0.001$). In multivariable analysis, OxPL/apoB levels were strongly and significantly associated with the presence, extent, and development (1995 to 2000) of carotid and femoral atherosclerosis and predicted the presence of symptomatic cardiovascular disease. Both OxPL/apoB and Lp(a) levels showed similar associations with atherosclerosis severity and progression, suggesting a common biological influence on atherogenesis.
CONCLUSIONS	This study suggests that pro-inflammatory oxidized phospholipids, present primarily on Lp(a), are significant predictors of the presence and extent of carotid and femoral atherosclerosis, development of new lesions, and increased risk of cardiovascular events. The OxPL biomarkers may provide valuable insights into diagnosing and monitoring cardiovascular disease. (J Am Coll Cardiol 2006;47:2219–28) © 2006 by the American College of Cardiology Foundation

The pathogenesis of atherosclerosis involves a complex interplay of lipid abnormalities, oxidative stress, inflammation, and thrombogenicity that leads to clinical sequelae (1,2). Traditional risk factors, such as diabetes mellitus,

hyperlipidemia, and hypertension are well accepted in contributing to atherosclerosis. The significance of emerging risk factors such as circulating oxidized low-density lipoprotein (OxLDL) is not as well established.

Plasma levels of oxidized phospholipids (OxPLs) present on individual apolipoprotein B-100 particles (apoB), measured by the murine monoclonal antibody E06 (3), have recently been shown to be correlated with angiographic coronary artery disease (CAD) (4), to be elevated after acute coronary syndromes (ACS) (5) and immediately after percutaneous coronary intervention (PCI) (6). In addition, OxPL/apoB levels have been shown to strongly correlate with lipoprotein (a) [Lp(a)] (3–7) and to be preferentially bound by Lp(a) compared with other apoB-containing lipoproteins (8).

In this prospective study using the Bruneck population (9–11), we examine OxPL/apoB levels and their relationship to traditional risk factors, Lp(a), and the presence, extent, and development of carotid and femoral atherosclerosis and symptomatic cardiovascular disease (CVD).

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Abbreviations and Acronyms

ACS	= acute coronary syndromes
apoB	= apolipoprotein B-100 particles
CAD	= coronary artery disease
CCA	= common carotid artery
CVD	= cardiovascular disease
ELISA	= enzyme-linked immunoadsorbent assay
ICA	= internal carotid artery
Lp(a)	= lipoprotein (a)
OxLDL	= oxidized low-density lipoprotein
OxPL	= oxidized phospholipid
PC	= phosphorylcholine
PCI	= percutaneous coronary intervention
POVPC	= 1-palmitoyl-2-(5-oxovaleroyl)-phosphocholine

METHODS

Study subjects. The Bruneck study is a prospective population-based survey of the epidemiology and pathogenesis of atherosclerosis (10–14). The study population was recruited as a gender- and age-stratified random sample of all inhabitants of Bruneck (125 women and 125 men in the fifth to eighth decades each, $n = 1,000$). At the 1990 baseline, 93.6% of recruited subjects participated, with data assessment completed in 919 subjects. During follow-up (1990 to the re-evaluations in 1995 and 2000), subgroups of 63 and 97 individuals, respectively, died or moved away. Among survivors, ultrasonographic follow-up of the carotid and femoral arteries was 96.5% (1995, $n = 826$) and 93.8% (2000, $n = 684$) complete. The current study focuses on the 1995 examination and the follow-up period between 1995 and 2000. Plasma samples for assessment of OxPL/apoB were available in 765 of the 826 subjects in 1995 (92.6%) and 671 of the 684 subjects in 2000 (98.1%). The study protocol was approved by the appropriate ethics committees, and all study subjects gave their written informed consent before entering the study.

Clinical examination and laboratory methods. All participants underwent a clinical examination and completed standardized questionnaires on current and past exposure to vascular risk factors as described previously (10–14). Blood samples were taken from the antecubital vein after subjects had fasted and abstained from smoking for ≥ 12 h. Low-density lipoprotein cholesterol was calculated with the Friedewald equation and corrected for the contribution of Lp(a) cholesterol, which was determined by multiplying Lp(a) mass by 0.45 (14). Lipoprotein (a) mass was measured by a double-antibody enzyme-linked immunoadsorbent assay (ELISA) using a polyclonal antiapolipoprotein (a) antibody for capture and monovalent antiapolipoprotein (a) Fab fragment coupled with peroxidase for detection (interassay coefficients for variation 1.5% to 6.3%) and apolipoprotein (a) phenotypes, measured as the number of kringle IV-2 repeats, as previously described (14).

Determination of OxPL/apoB levels with the OxLDL-E06 assay. The term OxLDL-E06 is used in a generic sense to describe the assay. Chemiluminescent ELISA was used to measure OxPL/apoB levels, as previously described (3), using the murine monoclonal antibody E06, which specifically binds to the phosphorylcholine (PC) head group of oxidized but not native phospholipids (15,16). Plasma from each sample was diluted 1:50 in phosphate-buffered saline and 50 μl added to microtiter wells coated with the monoclonal antibody MB47 (5 $\mu\text{g}/\text{ml}$), which specifically binds apoB. Under these conditions, a saturating amount of apoB is added to each well, and, consequently, an equal number of apoB are captured in each well for all assays. Thus, the assay measurement of OxPL/apoB by design is independent of apoB levels. The saturating concentration of apoB on the wells is approximately 1 to 2 $\mu\text{g}/\text{ml}$, which corresponds to plasma concentrations of 10 to 20 mg/dl , below all apoB concentrations of any patient in this cohort. Biotinylated E06 was then added to determine the amount of OxPL present on each captured apoB (OxPL/apoB) and the amount bound detected by a chemiluminescent technique, expressed as relative light units (RLUs) per 100 ms (3).

Before performing the OxPL/apoB measures, to confirm that a saturating amount of apoB was captured in each well, a validating assay was set up in parallel plates where MB47 (5 $\mu\text{g}/\text{ml}$) was plated to capture apoB similar as the methodology above. Then, biotinylated MB24, a murine monoclonal antibody specific for a different epitope of apoB (5 $\mu\text{g}/\text{ml}$), was added to quantitate the amount of apoB captured on the plates by MB47. In samples from the 1995 and 2000 time points combined ($n = 1,436$), the Spearman rank correlation coefficient between OxPL measured on single plates (expressed as RLU of E06 bound) and OxPL/apoB derived by using the parallel plates to measure apoB and calculating a *ratio* of E06 bound (E06 RLU) divided by the amount of apoB bound (MB24 RLU) was $r = 0.995$ ($p < 0.001$) (Fig. 1). Therefore, in this study, the OxPL/apoB value is presented as a ratio (rather than RLU) between E06 (OxPL) RLU divided by MB24 (apoB) RLU measured in parallel plates. All samples for a given assay were run in a single assay, and internal controls consisting of high and low standard plasma samples were included to detect potential variations between microtitration plates. Each sample was assayed in triplicate. The intra-assay coefficients for variation were 6% to 10%.

Assessment of atherosclerosis and CVD. The ultrasound protocol involves the scanning of the right and left common carotid arteries (CCA), internal carotid arteries (ICA), and femoral arteries with a 10-MHz imaging probe at the following locations: proximal CCA (15 to 30 mm proximal to the carotid bulb), distal CCA (<15 mm proximal to the carotid bulb), and proximal ICA (carotid bulb, identified by loss of the parallel wall present in the CCA and the initial 10 mm of the vessel above the flow divider between external and ICAs) and femoral artery (40 mm proximal and 10 mm distal to the bifurcation into the superficial and deep

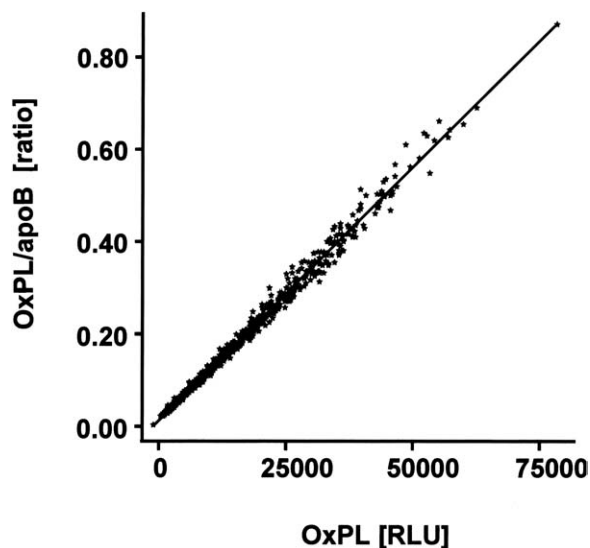


Figure 1. Plot of oxidized phospholipids (OxPL) values (in relative light units [RLU]) against OxPL/apolipoprotein B-100 particles (apoB) (ratio) values indicating a very high correlation of $r = 0.995$, $p < 0.001$ ($n = 1,436$).

branches) (10,11,13). Atherosclerotic lesions were defined according to two ultrasound criteria: 1) wall surface (protrusion or roughness of the arterial boundary); and 2) wall texture (echogenicity). The maximum axial diameter of plaques (in millimeters) was assessed on the near and far walls at each of two (femoral arteries) and eight vessel segments (carotid arteries). Femoral and carotid atherosclerosis scores were calculated by summing all diameters (carotid and femoral artery atherosclerosis score: intra-observer coefficients of variation, 13.5% and 14.0% [$n = 100$]) (17). The scores serve as measures of atherosclerosis severity (ranges, 0 to 29.7 mm [carotid atherosclerosis score] and 0 to 13.6 mm [femoral atherosclerosis score]).

All scans were performed in 1995 and 2000 by the same experienced sonographer, who was unaware of the subjects' characteristics. Measurements were performed on-line. On the basis of the follow-up evaluation, two different stages of atherosclerosis development/progression were differentiated: 1) incident atherosclerosis was defined by the occurrence of atherosclerotic lesions in subjects initially free of atherosclerosis in given vascular beds (early atherogenesis); and 2) incident vessel stenosis was assumed whenever the relative increase in the maximum plaque diameter exceeded the double measurement error of the method and a narrowing of the lumen $>40\%$ (diameter stenosis) occurred (advanced atherogenesis) (10–13). The two progression categories were highly reproducible (kappa coefficients >0.8 [$n = 100$]). Further details of imaging procedures and measurement errors have been published elsewhere (11,12).

Assessment of CVD was based on the patient's medical history, a detailed review of the Bruneck Hospital databases, and the results of clinical and various laboratory examinations (12,18). Myocardial infarction was deemed confirmed when World Health Organization criteria for definite dis-

ease status were met. Stroke and transient ischemic attack were classified according to the criteria of the National Survey of Stroke. The diagnosis of symptomatic peripheral artery disease required a positive response to the Rose questionnaire (typical claudication), with the vascular nature of complaints confirmed by standard diagnostic procedures (ankle-brachial pressure index or angiography), or an acute peripheral artery occlusion requiring revascularization.

Statistics. All calculations were performed using the SPSS 11.5 (SPSS Inc., Chicago, Illinois) and BMDP software packages (Statistical Solutions, Saugus, Massachusetts). Continuous variables were presented as means \pm SD or medians (interquartile range), and dichotomous variables as percentages. Spearman correlation coefficients were used to estimate the association between OxPL/apoB and Lp(a), and the associations between these variables and various population characteristics. In Table 1, a Bonferroni correction was performed to account for multiple comparisons. In all other analyses, a two-sided $p < 0.05$ was considered significant.

The associations of OxPL/apoB and Lp(a) with carotid and femoral artery atherosclerosis and with CVD were tested by means of logistic and linear regression analysis (Table 2). Base models were adjusted for age and gender. Multiple regression analyses were adjusted for fixed sets of covariates that were assessed in previous analyses of the vascular risk profiles of the Bruneck study population (13). Alternative models built by a forward stepwise selection procedure (allowing for all variables in Table 1) yielded identical results (data not shown); OxPL/apoB and Lp(a) were treated as continuous variables and \log_e -transformed to satisfy the assumption of normality and constant variance of the residuals. For comparability purposes odds ratios (95% confidence interval) and regression coefficients (95% confidence interval) were calculated for a 1-SD-U increase in \log_e -transformed OxPL/apoB and Lp(a) (Table 2). In separate equations tertile groups of OxPL/apoB were modelled as a set of indicator variables. To test for linear trend, we used the median level in each tertile group of OxPL/apoB as a continuous variable.

RESULTS

Demographics. Demographic, clinical, and laboratory characteristics of the 765 study subjects in 1995 are shown in Table 1. There were no differences between the 765 subjects with OxPL/apoB measurements and the 61 without (data not shown). All subjects are of Caucasian origin.

Distribution of OxPL/apoB and Lp(a) levels. The distribution of OxPL/apoB levels (1995 dataset) was skewed with most subjects having low levels (Fig. 2A) and strikingly similar to that of Lp(a) (Fig. 2B). The range of OxPL/apoB levels was 0.018 to 0.860, and mean and median levels were 0.109 and 0.051. A very strong correlation was noted between OxPL/apoB and Lp(a) levels (Spearman rank correlation coefficient $r = 0.87$, $p < 0.001$) (Fig. 2C). This

Table 1. Characteristics of the Study Subjects (1995) and Their Correlation With Oxidized Phospholipids/Apolipoprotein B-100 Particles and Lipoprotein (a) Levels (n = 765)

Variables	Mean ± SD, Median (IQR),* or %	Correlation With OxPL/apoB		Correlation With Lipoprotein (a)	
		r†	p Value	r†	p Value
Age (yrs)	57.7 ± 11.2	−0.003	0.929	0.009	0.807
Female gender (%)	49.5%	—	—	—	—
Vascular risk factors					
Hypertension (WHO) (%)	36.3%	—	—	—	—
Systolic BP (mm Hg)	148.0 ± 20.5	−0.073	0.044	−0.036	0.317
Diastolic BP (mm Hg)	86.9 ± 9.1	−0.036	0.326	0.011	0.768
Current smoking (%)	19.9%	—	—	—	—
Smoking (cigarettes/day)	2.6 ± 6.2	0.025	0.490	0.031	0.387
Diabetes (WHO) (%)	9.4%	—	—	—	—
Fasting glucose (mg/dl)	102.4 ± 25.0	−0.014	0.705	−0.007	0.857
Ferritin (μg/l)	135.0 ± 155.4	−0.061	0.090	−0.046	0.208
Microalbuminuria (g/l)*	9.0 (7.0–18.0)	−0.030	0.414	−0.043	0.230
Uric acid (mg/dl)	4.7 ± 1.3	−0.072	0.048	−0.012	0.738
Coagulation					
Fibrinogen (mg/dl)	288.2 ± 72.8	0.101	0.005	0.133	0.001
Antithrombin III (%)	99.3 ± 11.5	0.111	0.003	0.132	0.001
Activated protein C ratio	3.2 ± 0.6	−0.076	0.036	−0.097	0.007
Lipids and lipoproteins					
Total cholesterol (mg/dl)	229.7 ± 42.4	0.089	0.014	0.178	<0.001‡
Triglycerides (mg/dl)*	111 (81–159)	−0.114	0.002	−0.019	0.597
HDL cholesterol (mg/dl)	58.9 ± 16.4	−0.075	0.038	0.030	0.413
LDL cholesterol (mg/dl)	145.1 ± 37.9	0.125	0.001	0.239	<0.001‡
LDL cholesterol corr (mg/dl)§	135.9 ± 36.9	−0.010	0.788	0.066	0.070
Apolipoprotein A (mg/dl)	166.0 ± 27.6	0.025	0.489	0.002	0.956
Apolipoprotein B (mg/dl)	115.7 ± 31.3	−0.008	0.830	0.093	0.010
Lipoprotein (a) (mg/dl)*	12.1 (4.7–36.8)	0.874	<0.001‡	—	—
Infection and inflammation					
Alpha-antithrypsin (mg/dl)	198.4 ± 36.6	0.035	0.330	0.019	0.608
C-reactive protein (mg/l)	2.8 ± 7.1	−0.008	0.826	0.021	0.569
Hsp65 antibody (titer)	3.4 ± 1.2	−0.025	0.496	−0.066	0.069
Cytomegalovirus IgG (titer)	3.1 ± 1.6	−0.003	0.940	−0.014	0.689
<i>Chlamydia pneumoniae</i> IgA (titer)	3.1 ± 1.6	−0.001	0.969	0.002	0.948
<i>Helicobacter pylori</i> IgG (titer)	3.6 ± 1.4	0.011	0.770	−0.003	0.931
Vitamins					
Retinol (μmol/l)	2.4 ± 1.1	−0.140	0.007	−0.135	0.008
Alpha-tocopherol (μmol/l)	24.8 ± 10.6	−0.145	0.006	−0.157	0.002
Carotenoids (μmol/l)	1.4 ± 0.7	0.022	0.466	−0.071	0.162
Nutrition, activity, and body composition					
Sports index (Beacke score)	2.4 ± 0.9	−0.034	0.351	−0.049	0.177
Energy intake (kcal)	2,830 ± 864	−0.052	0.153	−0.043	0.140
Fat intake (g/day)	147.7 ± 48.3	−0.064	0.077	−0.050	0.171
Alcohol (g/day)	23.9 ± 31.1	−0.107	0.003	−0.103	0.004
Body mass index (kg/m ²)	25.6 ± 3.8	−0.065	0.038	−0.031	0.390
Waist-hip ratio (cm/cm)	0.9 ± 0.1	−0.035	0.329	−0.002	0.953

*Median and interquartile range (IQR) is presented for skewed variables; †values presented are Spearman's rank correlation coefficients; ‡correlations are significant after accounting for the multiple comparisons performed (Bonferroni adjustment); §LDL cholesterol corrected for the contribution of Lp(a) cholesterol; ||vitamin measurements were available in a sample of 390 subjects only. For the computation of correlation coefficients, the vitamins/LDL ratio was applied. To convert values for cholesterol to mmol/l, multiply by 0.02586. To convert values for triglycerides to mmol/l, multiply by 0.01129. **Bold** values are statistically significant.

BP = blood pressure; HDL = high-density lipoprotein; Hsp65 = heat shock protein 65; LDL = low-density lipoprotein; WHO = World Health Organization.

correlation applied to men and women and all other subgroups. The OxPL/apoB levels in paired samples (n = 671) from 1995 and 2000 were highly correlated (Spearman rank correlation coefficient r = 0.78, p < 0.001) (Fig. 3). These correlation coefficients were higher than for low-density lipoprotein (r = 0.62, p < 0.001), even excluding subjects on statin therapy (r = 0.71, p < 0.001), and

high-density lipoprotein (r = 0.75, p < 0.001) in years 1995 versus 2000. Similarly, a strong correlation was noted between Lp(a) levels assessed in 1990 and 1995 (r = 0.83, p < 0.001).

Although weak positive correlations of OxPL/apoB were noted with fibrinogen and antithrombin III and weak inverse correlations with levels of retinol and alpha-

Table 2. Association of OxPL/apoB and Lp(a) Level with Presence, Severity, and Progression of Carotid and Femoral Artery Atherosclerosis, and with Cardiovascular Disease

Models	OxPL/apoB		Lp(a)	
	Odds Ratio/Regression Coeff. (95% CI)*	p Value	Odds Ratio/Regression Coeff. (95% CI)*	p Value
Carotid arteries				
Presence of atherosclerosis 1995 (420 of 765)				
Age-/gender-adjusted model	1.21 (1.02–1.45)	0.031	1.22 (1.02–1.46)	0.028
Multivariate model†	1.30 (1.08–1.57)	0.006	1.28 (1.06–1.55)	0.012
Atherosclerosis score 1995‡				
Age-/gender-adjusted model	0.13 (0.03–0.23)	0.008	0.12 (0.02–0.22)	0.016
Multivariate model†	0.17 (0.07–0.26)	0.001	0.14 (0.04–0.24)	0.004
Incident atherosclerosis 1995–2000 (71 of 328)§				
Age-/gender-adjusted model	1.31 (0.98–1.76)	0.066	1.25 (0.94–1.67)	0.132
Multivariate model†	1.44 (1.06–1.96)	0.020	1.33 (0.97–1.81)	0.074
Incident stenosis >40% 1995–2000 (65 of 308)§				
Age-/gender-adjusted model	1.32 (1.03–1.70)	0.031	1.24 (0.93–1.65)	0.151
Multivariate model†	1.45 (1.09–1.92)	0.011	1.42 (1.02–1.96)	0.036
Femoral arteries				
Presence of atherosclerosis 1995 (332 of 765)				
Age-/gender-adjusted model	1.17 (1.00–1.38)	0.051	1.17 (0.99–1.38)	0.061
Multivariate model†	1.23 (1.04–1.46)	0.018	1.20 (1.01–1.43)	0.044
Atherosclerosis score 1995‡				
Age-/gender-adjusted model	0.14 (0.03–0.25)	0.013	0.15 (0.03–0.26)	0.011
Multivariate model†	0.16 (0.05–0.27)	0.003	0.15 (0.04–0.26)	0.006
Incident atherosclerosis 1995–2000 (106 of 390)§				
Age-/gender-adjusted model	1.29 (1.02–1.62)	0.032	1.15 (0.91–1.46)	0.252
Multivariate model†	1.34 (1.05–1.71)	0.018	1.25 (0.97–1.60)	0.085
Prevalent/incident stenosis >40% (39 of 246)§				
Age-/gender-adjusted model	1.38 (1.02–1.87)	0.039	1.70 (1.17–2.47)	0.006
Multivariate model†	1.51 (1.06–2.14)	0.022	1.88 (1.22–2.89)	0.004
Cardiovascular disease 				
Presence of vascular diseases 1995 (77 of 765)				
Age-/gender-adjusted model	1.47 (1.17–1.85)	0.001	1.39 (1.08–1.80)	0.012
Multivariate model†	1.46 (1.14–1.86)	0.002	1.35 (1.03–1.76)	0.029

*Odds ratios and regression coefficients (marked with ‡) were derived from logistic and linear regression analyses and calculated for a 1-SD-U increase of log_e-transformed oxPL/apoB and lipoprotein (a) level. The model included either OxPL/apoB level (left-hand columns) or lipoprotein (a) (right-hand columns); †multivariate analyses have been adjusted for age, gender, presence or absence of hypertension, presence or absence of hypothyroidism, smoking status, level of alcohol consumption, level of high-density lipoprotein and low-density lipoprotein (LDL) cholesterol, log_e-transformed level of urinary albumin, ferritin level, and white blood cell count. The model of incident carotid stenosis has been adjusted for age, gender, presence or absence of diabetes and impaired glucose tolerance, level of alcohol consumption, number of cigarettes smoked per day, level of LDL cholesterol, fibrinogen and antithrombin III, and factor V Leiden mutation; ‡values given are regression coefficients (95% confidence interval [CI]). Atherosclerosis scores were calculated by summing up the maximum diameter of atherosclerotic plaques at eight (carotid arteries) and two (femoral arteries) well-defined vessel segments. In the analyses log_e-transformed atherosclerosis scores were used; §incident (non-stenotic) atherosclerosis (early atherogenesis) was defined by the development of first atherosclerotic lesions in subjects initially free of atherosclerosis in given vascular beds. Incident stenosis >40% (advanced atherogenesis) was defined by the development of vessel stenosis >40% and focuses on subjects with pre-existing atherosclerosis. In the model targeting femoral artery stenosis, >40% prevalent (1995) and incident (1995–2000) stenosis were combined to ensure a sufficient number of outcome events; ||cardiovascular disease subsumes definite clinical end points (fatal and non-fatal myocardial infarction, fatal and non-fatal ischemic stroke, transient ischemic attacks, and symptomatic peripheral artery disease).

apoB = apolipoprotein B-100 particles; coeff. = coefficient; Lp(a) = lipoprotein (a); OxPL = oxidized phospholipids.

tocopherol, alcohol consumption, and triglyceride concentrations, these were no longer significant when adjusting for the multiple comparisons performed (Table 1). The significant association with low-density lipoprotein cholesterol disappeared when the Lp(a) contribution to the low-density lipoprotein cholesterol was adjusted for (Table 1). In a multivariate analysis Lp(a) and alcohol consumption were found to be independently significant determinants of log_e-transformed OxPL/apoB levels whereas all other variables were not. In line, Lp(a) did not show significant correlations with most of the demographic, lifestyle, and vascular risk factors.

Relationship of OxPL/apoB to apolipoprotein (a) phenotypes. The number of kringle IV-2 repeats was significantly and inversely related to Lp(a) mass ($r = -0.48$, $p <$

0.001) and, interestingly, similar to OxPL/apoB ($r = -0.46$, $p < 0.001$). Highly significant correlations between Lp(a) mass and OxPL/apoB existed for all apolipoprotein isoforms (Fig. 4) with the strength of this correlation being lowest for the largest phenotypes and highest with the lowest number of kringle IV-2 repeats (≥ 29 repeats $r = 0.66$, 23 to 29 repeats $r = 0.88$, and ≤ 22 repeats $r = 0.93$, $p < 0.001$ each). The smallest apolipoprotein (a) phenotypes were associated with the highest Lp(a) levels, as has been previously described (14,19). There was substantial variability of Lp(a) mass for each apolipoprotein (a) isoform. **OxPL/apoB levels by age and gender.** The OxPL/apoB levels were remarkably stable over the 40-year age range in the study population (Spearman rank correlation coefficient $r = -0.003$, $p = 0.93$) and nearly identical in men and

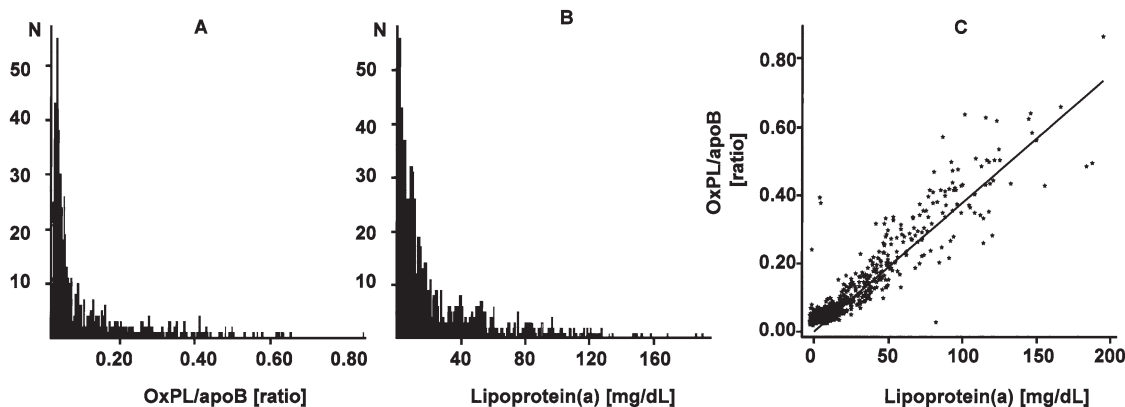


Figure 2. Comparative presentation of distributions of oxidized phospholipids (OxPL)/apolipoprotein B-100 particles (apoB) (A) and lipoprotein (a) (B) and plot of OxPL/apoB versus lipoprotein (a) (C) indicating a very high correlation (data from 1995; $r = 0.87$, $p < 0.001$, $n = 765$).

women (geometric means, 0.068 vs. 0.070; $p = 0.64$ for difference). There were no differences of OxPL/apoB levels in subjects with and without smoking history (0.067 vs. 0.071, $p = 0.36$) or subjects with and without diabetes (0.058 vs. 0.069, $p = 0.33$). Interestingly, subjects on statins had higher levels of OxPL/apoB in 1995 ($n = 25$, 0.102 vs. 0.068, $p = 0.021$) and in 2000 ($n = 60$, 0.096 vs. 0.061, $p < 0.001$), as previously noted in response to atorvastatin (3) and pravastatin (20).

Relationship between OxPL/apoB and Lp(a) with atherosclerosis and CVD. Log_e-transformed OxPL/apoB levels were significantly associated with the presence of carotid and femoral atherosclerosis and the carotid and femoral atherosclerosis scores. Moreover, log_e-transformed OxPL/apoB emerged as a significant risk predictor for the development of carotid and femoral atherosclerosis between 1995 and 2000 and for the manifestation of vessel stenosis >40% in the carotid arteries as well as the presence of femoral artery stenosis (Table 2). Finally, the significant relation extended to symptomatic CVD (Table 2). In 1995

there were 77 subjects who had experienced one or more of the following vascular diseases: ischemic stroke and transient ischemic attack, $n = 33$; myocardial infarction, $n = 17$; and symptomatic peripheral artery disease $n = 31$. The number of cases with incident CVD (1995 to 2000) was too low for a meaningful analysis. All associations for OxPL/apoB were independent of other cardiovascular risk factors, except Lp(a), and applied equally to men and women. The strength of association tended to decline with advancing age and to enhance with increasing low-density lipoprotein levels. Neither of these trends, however, achieved significance.

Existence of significant associations between Lp(a) and carotid atherosclerosis prevalence (1990) and progression (1990 to 1995) in the Bruneck study has been shown previously (14,21). Corresponding data for the 1995 examination and the 1995 to 2000 follow-up period are depicted in Table 2. Interestingly, the findings for Lp(a) are very similar to those obtained for OxPL/apoB.

Finally, we divided OxPL/apoB levels into tertile groups. The highest tertile of OxPL/apoB was associated with a higher odds ratio for the presence (in 1995) and development (1995 to 2000) of carotid and femoral atherosclerosis (Fig. 5), compared with the lowest tertile. In addition, the highest tertile of OxPL/apoB levels predicted the presence of symptomatic CVD in 1995.

DISCUSSION

This large population study representative of the general community demonstrates for the first time that OxPL/apoB levels are a significant predictor of the presence, extent, and development/progression of carotid and femoral atherosclerosis and are associated with an increased risk of cardiovascular events. The OxPL/apoB and Lp(a) levels are similarly distributed demographically, display long-term stability in levels, and lack correlations with demographic, major vascular risk, and lifestyle variables.

Recognition of PC on OxPL by murine monoclonal antibody E06. It was initially shown that E06 bound to OxLDL (22) and specifically to oxidized 1-palmitoyl-2-arachidonoyl-phosphatidylcholine and 1-palmitoyl-2-(5-

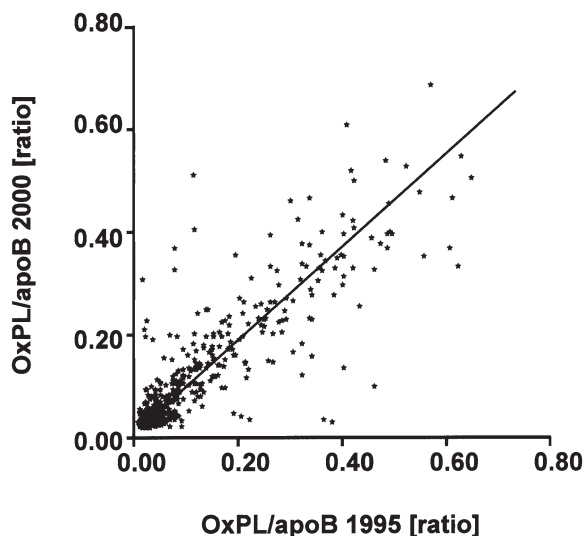


Figure 3. Plot of oxidized phospholipids (OxPL)/apolipoprotein B-100 particles (apoB) values in 1995 against OxPL/apoB values in 2000 indicating considerable consistency over time ($r = 0.78$, $p < 0.001$, $n = 671$).

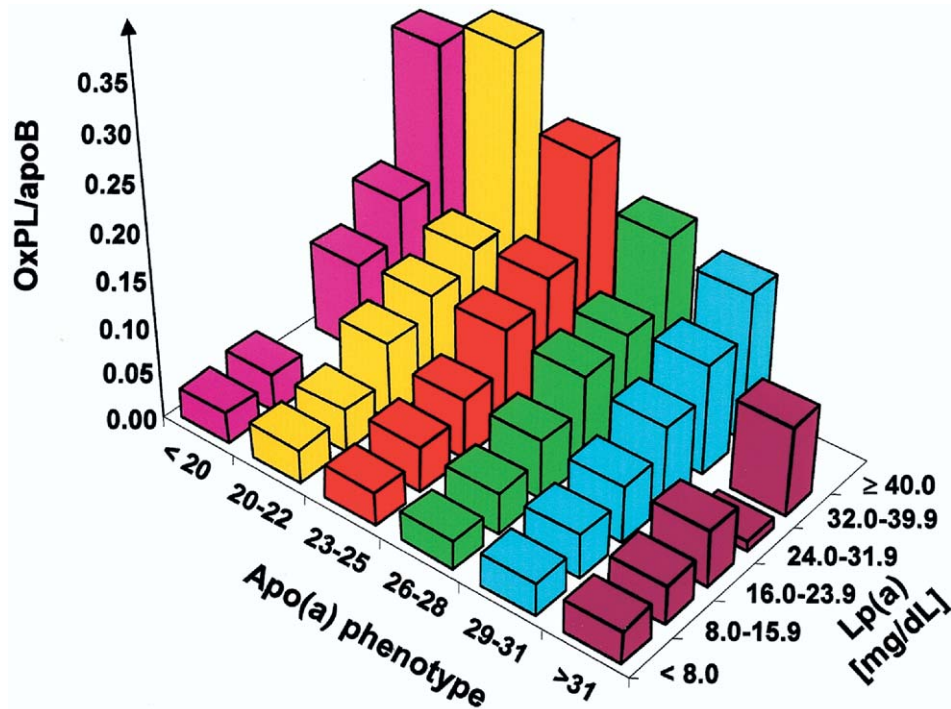


Figure 4. Three-dimensional plot of oxidized phospholipids (OxPL)/apolipoprotein B-100 particle (apoB) levels according to lipoprotein (a) [Lp(a)] mass and apolipoprotein (a) [Apo(a)] phenotypes expressed as the number of kringle IV-type 2 repeats. The OxPL/apoB levels presented are geometric means (taken as the antilog of the mean of \log_e -transformed OxPL/apoB values).

oxoaleroyl)-phosphatidylcholine (POVPC) (15) but not to other phospholipids, such as oxidized phosphatidylserine or phosphatidylethanolamine. Interestingly, E06 bound both to the isolated oxidized protein and lipid moieties of OxLDL after solvent extraction of the lipid phase (15,23). In competition experiments, Friedman et al. (16) showed that E06 binding to OxLDL was inhibited by diverse and unrelated OxPL peptides, including PC-keyhole limpet hemocyanin, PC-bovine serum albumin, and PC-lysine as well as by non-peptide antigens, such as aldol condensation products of OxPL not attached to any peptides and PC alone. Saponification of isolated POVPC or OxLDL (to remove the PC headgroup) showed that the E06 immunoreactivity was completely abolished, confirming the essential role of PC in antigen recognition. Furthermore, the E06 antibody gene is identical in the variable sequence to T15 natural antibodies that provide optimal protection against pneumococcal infection in mice. T15 antibodies bind PC present on gram-positive bacterial cell membranes, bound to teichoic or lipoteichoic acid and not present as OxPL (24). Thus, there is molecular mimicry between the PC of OxLDL and the PC of pathogen. These findings have allowed us to postulate that during oxidation of phospholipids, a conformational change occurs that “presents” the PC headgroup in an immunogenic manner, thus differentiating normal phospholipids from OxPL.

OxPL/apoB and atherosclerosis. The fact that OxPL/apoB levels predicted the presence, extent, and development/progression of carotid and femoral atherosclerosis and symptomatic CVD suggests that OxLDL markers may be

useful in risk stratification of CVD. The strong association between OxPL/apoB and Lp(a) and their similar relationship to carotid and femoral disease suggests a common biological influence on atherogenesis and supports the hypothesis that the atherogenicity of Lp(a) may be mediated by associated OxPL.

The OxPL are pro-inflammatory and are present on OxLDL, apoptotic cells, and various innate immune mechanisms have evolved to bind and potentially neutralize them, such as natural monoclonal IgM autoantibodies, C-reactive protein (25), and scavenger receptors CD36 (26) and SR-B1 (23). Monoclonal antibody E06 can inhibit the uptake of OxLDL by macrophages, suggesting it might retard foam cell formation. Indeed, immunization of low-density lipoprotein receptor^{-/-} mice with *Streptococcus pneumoniae* resulted in increased titers of OxLDL-specific immunoglobulin-M E06/T15 autoantibodies and decreased atherosclerosis progression (27). Several studies have also noted an inverse association between immunoglobulin-M titers to OxLDL and various manifestations of CVD (5,28–30).

Relationship of OxPL/apoB and Lp(a). This study extends our recent observations that a strong correlation exists between OxPL/apoB and Lp(a) in patients with ACS (5), PCI (6), stable CAD (4), and after statin therapy in ACS patients (3). This strong correlation appears to be due to enhanced binding and affinity of OxPL for Lp(a), a unique property in humans (6,8), as opposed to non-human primates that also have Lp(a) (31). For example, in the PCI study (6), we showed that immediately post-PCI there was

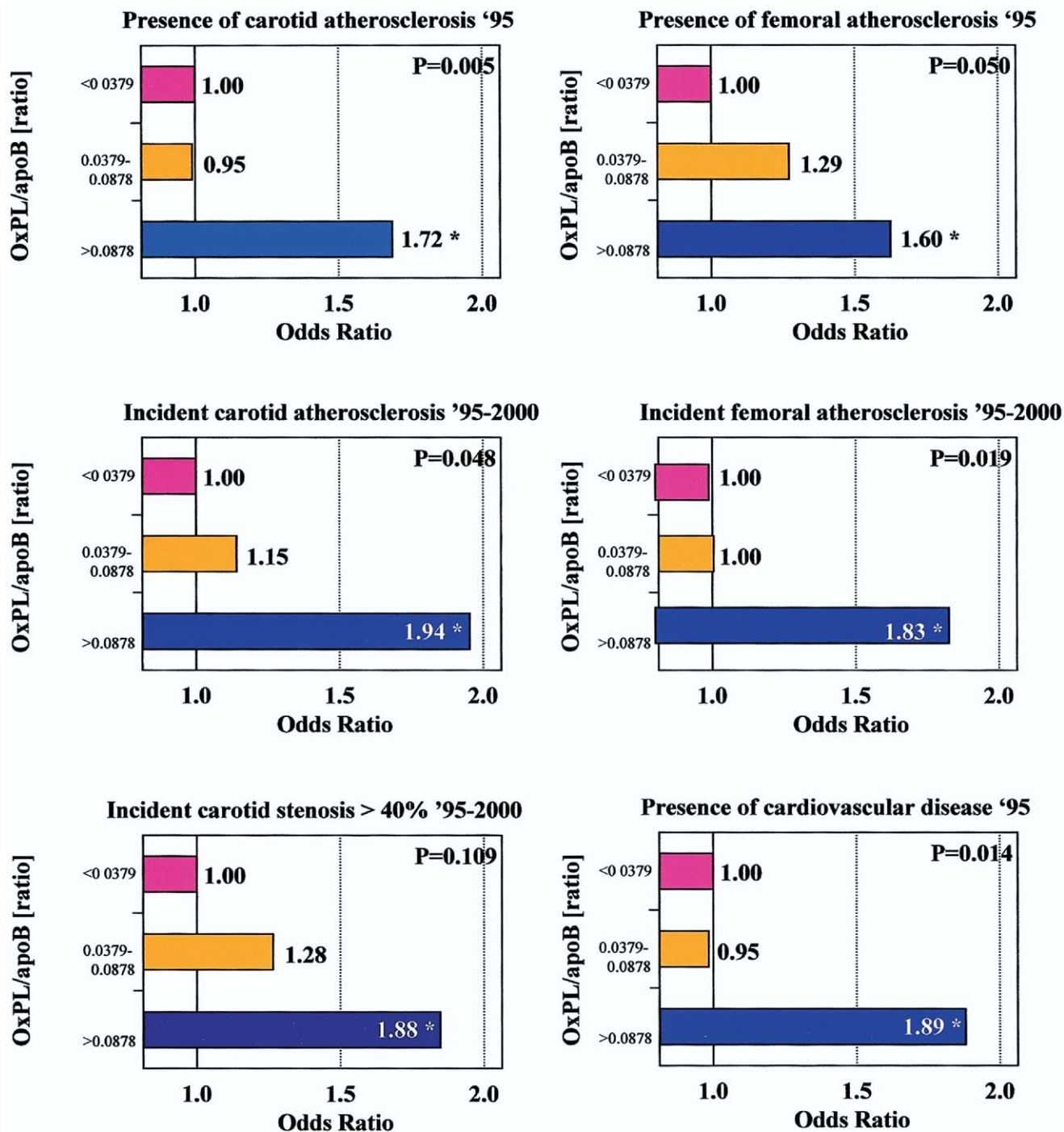


Figure 5. Multivariate analysis showing the association of oxidized phospholipids (OxPL)/apolipoprotein B-100 particle (apoB) tertile groups with presence and progression of carotid and femoral artery atherosclerosis and with cardiovascular disease. For adjustment see Table 2. * $p < 0.05$ for the comparison between the first tertile group (reference category) and the third tertile group. The p values presented in the figures are the overall p values for the three tertiles (test for trend).

a sharp increase in plasma OxPL/apoB levels, which were distributed ~50% on Lp(a) and ~50% on non-Lp(a) apoB. However, by 6 h post-PCI, the majority (~85%) of OxPL were on Lp(a), suggesting transfer and selective binding of OxPL to Lp(a). We have also shown that kringle V of apolipoprotein (a) has the ability to covalently bind up to 2 mol of OxPL, which are recognized by E06 (8). In addition, preliminary data from our laboratory have shown that 40%

to 70% of OxPL are actually present in the lipid phase of Lp(a) whereas the remaining OxPL are on apolipoprotein (a) (31). Furthermore, in vitro transfer studies demonstrate that OxPL derived from OxLDL are preferentially transferred to Lp(a) compared with low-density lipoprotein (31).

The current data are also remarkable for the fact that OxPL/apoB levels were relatively constant between years 1995 and 2000, more so than high-density lipoprotein

cholesterol or low-density lipoprotein cholesterol. These data are consistent with other studies showing that Lp(a) levels remain relatively unchanged over time (19), except during acute phase responses or plaque disruption (3,5,6). In this study, OxPL/apoB levels were higher in patients taking statins, which is consistent with our previous data showing increases in OxPL/apoB levels in patients with ACS treated with atorvastatin (3) and in children with familial hypercholesterolemia treated with pravastatin (20). This is also consistent with data showing increases in Lp(a) after treatment with several different statins (3). We have hypothesized that this may represent efflux of OxPL from the vessel wall and transient binding in the circulation by Lp(a), potentially as a mechanism of early plaque stabilization. Such an increase in the OxPL/apoB ratio in response to an intervention may be a surrogate of effective therapy. Indeed, we recently observed a similar increase in OxPL/apoB ratio in healthy women in response to low fat diets, a situation where apoB levels did not change (7). Additional potential mechanisms of the increase in OxPL/apoB include, among others, preferential clearance of native apoB compared to minimally-modified PC-containing apoB, or differential clearance of Lp(a) particles that contain most OxPL epitopes measured by E06. The reasons for the increase in Lp(a) in response to statins and low fat diets are not yet determined. Proof of these hypotheses awaits further experimental studies.

The role of Lp(a) and OxPL/apoB in atherogenesis. Lipoprotein (a) is composed of apolipoprotein (a) covalently bound to apoB of low-density lipoprotein by one disulfide bond and has been associated with angiographically determined and clinically manifested CAD (32). This association appears to be particularly true in high-risk, as opposed to low-risk, populations. Interestingly, this study shows for the first time that the association of OxPL/apoB and Lp(a) was strongest in those subjects with the highest Lp(a) concentration but smallest apo (a) isoforms, suggesting that OxPL/apoB levels may be related to the number of K-IV2 repeats. Previous data from the Bruneck study showed that subjects with low-molecular weight apo (a) phenotypes and high Lp(a) levels had more rapid progression of established carotid atherosclerosis compared with those with high-molecular-weight phenotypes (14). Similarly, hemodialysis patients who are known for their enhanced oxidative stress show the most pronounced association with CAD if they are carriers of small apo(a) isoforms (33). Our findings suggest that low-molecular phenotypes may be more atherogenic, either due to larger number of particles, or perhaps through enhanced content of OxPL per unit of Lp(a) mass.

The strong association and binding of OxPL by Lp(a) may be part of an innate immune response to detoxify pro-inflammatory OxPL, analogous to the actions of C-reactive protein and natural immunoglobulin-M OxLDL autoantibodies (34). In support of this concept is the idea that Lp(a) is highly enriched in platelet-activating factor

acetyl hydrolase (seven-fold higher than low-density lipoprotein) (35,36), an enzyme that degrades OxPL, and that Lp(a) appears to increase in plasma in a manner similar to acute phase proteins. This concept implies that some minimal levels of Lp(a) may be beneficial. However, chronically elevated levels may be detrimental due to the associated pro-inflammatory OxPL, among other pro-inflammatory and -thrombotic properties, particularly due to the enhanced binding of Lp(a) to the vessel wall matrix.

OxPL as potential biomarkers. Given the very strong correlation between OxPL/apoB levels and Lp(a) concentration, both variables are exchangeable in the risk models (Table 2) and provide similar information. However, if the hypothesis holds true that scavenging of OxPL is an important physiological role of Lp(a) and OxPL primarily accounts for atherogenicity of Lp(a), direct assessment of OxPL should allow for a more precise estimate of risk than assessment of entire Lp(a) mass. Remarkably, in this study the associations between OxPL/apoB and all clinical and ultrasound end points were stronger than those assessed for Lp(a). Analogous findings have been obtained in a recent study, where we showed that in patients <60 years old OxPL/apoB levels were indeed independent predictors of angiographically determined CAD, with all the typical risk factors in the model (4). Measurement of pro-inflammatory OxPL on apoB-100 particles in plasma may be a surrogate that links atherogenesis and inflammation. Therefore, further research is required in distinct clinical populations to ascertain whether OxPL/apoB will provide clinically relevant information on cardiovascular risk. Whether these will lead to clinical utility awaits determination. Our data suggest the novel hypothesis that the enhanced content of OxPL helps to explain the increased risk of CVD associated with Lp(a). Further animal and human studies will be required to delineate these pathways and assess their pathophysiological and clinical relevance. Prospective studies evaluating the change in the OxPL/apoB ratio in intervention studies with clinical outcomes are also needed to determine how these changes in OxPL/apoB and their relationship to Lp(a) can be applied for clinical decision making.

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REFERENCES

1. Tsimikas S, Glass C, Steinberg D, Witztum JL. Lipoproteins, lipoprotein oxidation and atherogenesis. Molecular basis of cardiovascular disease. In: Chien KR, editor. *A Companion to Braunwald's Heart Disease*. Philadelphia, PA: W.B. Saunders Co., 2004;385–413.
2. Libby P. Inflammation in atherosclerosis. *Nature* 2002;420:868–74.
3. Tsimikas S, Witztum JL, Miller ER, et al. High-dose atorvastatin reduces total plasma levels of oxidized phospholipids and immune complexes present on apolipoprotein B-100 in patients with acute

- coronary syndromes in the MIRACL trial. *Circulation* 2004;110:1406–12.
4. Tsimikas S, Brilakis ES, Miller ER, et al. Oxidized phospholipids, Lp(a) lipoprotein, and coronary artery disease. *N Engl J Med* 2005;353:46–57.
 5. Tsimikas S, Bergmark C, Beyer RW, et al. Temporal increases in plasma markers of oxidized low-density lipoprotein strongly reflect the presence of acute coronary syndromes. *J Am Coll Cardiol* 2003;41:360–70.
 6. Tsimikas S, Lau HK, Han KR, et al. Percutaneous coronary intervention results in acute increases in oxidized phospholipids and lipoprotein (a): short-term and long-term immunologic responses to oxidized low-density lipoprotein. *Circulation* 2004;109:3164–70.
 7. Silaste ML, Rantala M, Alfthan G, et al. Changes in dietary fat intake alter plasma levels of oxidized low-density lipoprotein and lipoprotein (a). *Arterioscler Thromb Vasc Biol* 2004;24:498–503.
 8. Edelstein C, Pfaffinger D, Hinman J, et al. Lysine-phosphatidylcholine adducts in Kringle V impart unique immunological and potential pro-inflammatory properties to human apolipoprotein (a). *J Biol Chem* 2003;278:52841–7.
 9. Kiechl S, Willeit J, Rungger G, Egger G, Oberhollenzer F. Quantitative assessment of carotid atherosclerosis in a healthy population. *Neuroepidemiology* 1994;13:314–7.
 10. Kiechl S, Willeit J. The natural course of atherosclerosis. Part I: incidence and progression. *Arterioscler Thromb Vasc Biol* 1999;19:1484–90.
 11. Kiechl S, Willeit J. The natural course of atherosclerosis. Part II: vascular remodeling. Bruneck Study Group. *Arterioscler Thromb Vasc Biol* 1999;19:1491–8.
 12. Kiechl S, Lorenz E, Reindl M, et al. Toll-like receptor 4 polymorphisms and atherogenesis. *N Engl J Med* 2002;347:185–92.
 13. Willeit J, Kiechl S, Oberhollenzer F, et al. Distinct risk profiles of early and advanced atherosclerosis: prospective results from the Bruneck study. *Arterioscler Thromb Vasc Biol* 2000;20:529–37.
 14. Kronenberg F, Kronenberg MF, Kiechl S, et al. Role of lipoprotein (a) and apolipoprotein(a) phenotype in atherogenesis: prospective results from the Bruneck study. *Circulation* 1999;100:1154–60.
 15. Hörkkö S, Bird DA, Miller E, et al. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *J Clin Invest* 1999;103:117–28.
 16. Friedman P, Hörkkö S, Steinberg D, Witztum JL, Dennis EA. Correlation of antiphospholipid antibody recognition with the structure of synthetic oxidized phospholipids: importance of Schiff base formation and Aldol condensation. *J Biol Chem* 2001;277:7010–20.
 17. Willeit J, Kiechl S. Prevalence and risk factors of asymptomatic extracranial carotid artery atherosclerosis. A population-based study. *Arterioscler Thromb* 1993;13:661–8.
 18. Kiechl S, Schett G, Wenning G, et al. Osteoprotegerin is a risk factor for progressive atherosclerosis and cardiovascular disease. *Circulation* 2004;109:2175–80.
 19. Utermann G. Genetic architecture and evolution of the lipoprotein (a) trait. *Curr Opin Lipidol* 1999;10:133–41.
 20. Rodenburg J, Vissers MN, Wiegman A, et al. Oxidized low-density lipoprotein in children with familial hypercholesterolemia and unaffected siblings: the effect of pravastatin. *J Am Coll Cardiol* 2006;47:1803–10.
 21. Willeit J, Kiechl S, Santer P, et al. Lipoprotein (a) and asymptomatic carotid artery disease. Evidence of a prominent role in the evolution of advanced carotid plaques: the Bruneck study. *Stroke* 1995;26:1582–7.
 22. Palinski W, Hörkkö S, Miller E, et al. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. *J Clin Invest* 1996;98:800–14.
 23. Gillotte-Taylor K, Boullier A, Witztum JL, Steinberg D, Quehenberger O. Scavenger receptor class B type I as a receptor for oxidized low density lipoprotein. *J Lipid Res* 2001;42:1474–82.
 24. Shaw PX, Hörkkö S, Chang MK, et al. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest* 2000;105:1731–40.
 25. Chang MK, Binder CJ, Torzewski M, Witztum JL. C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: phosphorylcholine of oxidized phospholipids. *Proc Natl Acad Sci U S A* 2002;99:13043–8.
 26. Boullier A, Gillotte KL, Hörkkö S, et al. The binding of oxidized low density lipoprotein to mouse CD36 is mediated in part by oxidized phospholipids that are associated with both the lipid and protein moieties of the lipoprotein. *J Biol Chem* 2000;275:9163–9.
 27. Binder CJ, Horkko S, Dewan A, et al. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between *Streptococcus pneumoniae* and oxidized LDL. *Nat Med* 2003;9:736–43.
 28. Wu R, de Faire U, Lemne C, Witztum JL, Frostegard J. Autoantibodies to OxLDL are decreased in individuals with borderline hypertension. *Hypertension* 1999;33:53–9.
 29. Hulthe J, Bokemark L, Fagerberg B. Antibodies to oxidized LDL in relation to intima-media thickness in carotid and femoral arteries in 58-year-old subjectively clinically healthy men. *Arterioscler Thromb Vasc Biol* 2001;21:101–7.
 30. Karvonen J, Paivansalo M, Kesaniemi YA, Hörkkö S. Immunoglobulin M type of autoantibodies to oxidized low-density lipoprotein has an inverse relation to carotid artery atherosclerosis. *Circulation* 2003;108:2107–12.
 31. Bergmark C, Tsimikas S, Witztum JL. A possible novel function for lipoprotein (a) as a carrier of oxidized phospholipids. *Arterioscler Thromb Vasc Biol* 2004;25:e71.
 32. Danesh J, Collins R, Peto R. Lipoprotein (a) and coronary artery disease. Meta-analysis of prospective studies. *Circulation* 2000;102:1082–5.
 33. Kronenberg F, Neyer U, Lhotta K, et al. The low molecular weight apo (a) phenotype is an independent predictor for coronary artery disease in hemodialysis patients: a prospective follow-up. *J Am Soc Nephrol* 1999;10:1027–36.
 34. Binder CJ, Shaw PX, Chang MK, et al. The role of natural antibodies in atherogenesis. *J Lipid Res* 2006;46:1353–63.
 35. Blencowe C, Hermetter A, Kostner GM, Deigner HP. Enhanced association of platelet-activating factor acetylhydrolase with lipoprotein (a) in comparison with low density lipoprotein. *J Biol Chem* 1995;270:31151–7.
 36. Karabina SA, Liapikos TA, Grekas G, Goudevenos J, Tselepis AD. Distribution of PAF-acetylhydrolase activity in human plasma low-density lipoprotein subfractions. *Biochim Biophys Acta* 1994;1213:34–8.