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Oxidation-Specific Biomarkers, Prospective 15-Year Cardiovascular and Stroke Outcomes, and Net Reclassification of Cardiovascular Events

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Objectives This study sought to assess the long-term predictive value and net reclassification for risk of cardiovascular disease (CVD) of biomarkers reflecting oxidation-specific epitopes (OSEs).

Background OSEs are immunogenic, proinflammatory, and proatherogenic. The long-term predictive value and net reclassification of OSEs for risk of CVD events are not known.

Methods Oxidized phospholipids on apolipoprotein B-100 (OxPL/apoB) and immunoglobulin (Ig)-G (IgG) and IgM autoantibodies to malondialdehyde-modified, low-density lipoprotein (MDA-LDL) and copper-oxidized LDL (Cu-OxLDL) were measured in 765 subjects in 1995 and 656 subjects in 2000 in the Bruneck study, representing 45- to 84-year-old men and women from the general community.

Results Over 15 years of follow-up, 138 subjects reached the primary endpoint of incident CVD (ischemic stroke, myocardial infarction, new-onset unstable angina, acute coronary interventions, and vascular death). In a multivariable Cox model, the highest tertile of OxPL/apoB was associated with higher risk of CVD (hazard ratio [HR]: 2.4; 95% confidence interval [CI]: 1.5 to 3.7) and stroke (HR: 3.6; 95% CI: 1.8 to 7.4) compared with the lowest tertile. IgG Cu-OxLDLs were associated with higher risk of CVD, whereas IgM MDA-LDLs were associated with lower risk. Using OxPL/apoB, IgG Cu-OxLDL, and IgM MDA-LDL variables, the area under the curve (AUC) for CVD risk prediction increased from 0.664 (95% CI: 0.629 to 0.697) to 0.705 (95% CI: 0.672 to 0.737) ($p = 0.048$). The net reclassification index (NRI) was 0.163 ($p = 0.0044$) and 0.332 ($p < 0.0001$) in all subjects ($n = 765$) and in subjects with intermediate risk ($n = 305$), respectively. Of 627 subjects who remained free of CVD, 108 were correctly reclassified to a lower risk category, and 83 were reclassified to a higher category (categories: 15-year risk <15%, 15% to 30%, >30%).

Conclusions OSE biomarkers predict 15-year CVD and stroke outcomes and provide potential clinical utility by reclassifying a significant proportion of individuals into higher or lower risk categories after traditional risk assessment. (J Am Coll Cardiol 2012;60:2218–29) © 2012 by the American College of Cardiology Foundation

Atherosclerosis is initiated early in human life in an occult manner due to a variety of genetic, environmental, and behavioral risk factors, and is the leading cause of cardiovascular disease (CVD) events. It originates by the generation of subintimal macrophage foam cells that arise mainly due to the accumulation of oxidized lipids through unregulated uptake by macrophage scavenger receptors, an arm of the innate immune response in removing toxic substances to protect the host (1,2). After a long latent period of integrative and detrimental insults to the vessel wall, such as

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oxidation and chronic inflammation (3–5), it manifests clinically as either symptomatic obstructive disease, such as angina or claudication, or acute atherothrombosis, such as myocardial infarction and ischemic stroke.

Various degrees of subclinical atherosclerosis are almost universally present in people living in Western societies (6). However, the assessment of the specific future risk that subclinical atherosclerosis confers is difficult to assess at the individual level, even with invasive and expensive testing. This is due to the variable nature of its clinical expression, which is in part a consequence of the qualitative differences in plaque components in similar sized lesions. Furthermore, the current clinical paradigm of treating asymptomatic patients is appropriately focused on treating underlying risk factors and is not generally centered on treating only those patients with documented presence of atherosclerosis. In addition, aside from hypolipidemic therapies, specific treatments that target pathogenic mechanisms leading to clinical events, such as the oxidative, immune, and inflammatory components, do not yet exist.

In that regard, plasma biomarkers of CVD risk that provide value to easily measured clinical and laboratory variables are needed (7). In particular, biomarkers that function as a link between risk factors and clinical CVD and are also putatively involved in causal pathways of atherosclerosis would be an attractive addition to the clinical armamentarium. Oxidation-specific epitopes (OSEs), present in plasma on circulating lipoproteins and lipoprotein (a) [Lp(a)] and in the vessel wall on lipoproteins, apoptotic cells, and matrix proteins, are strong candidates as potentially causal biomarkers more proximal to the atherosclerotic disease process (8). OSEs represent "danger-associated molecular patterns (DAMPs)" (2) that are proinflammatory and are integrally involved in oxidative, innate, and adaptive immune responses. In this 15-year prospective study from the Bruneck population, we hypothesized that measurements reflecting risk from OSEs would predict the development of new CVD events and add clinical utility to established risk factors.

Methods

Study subjects. The Bruneck Study is a prospective population-based survey of the epidemiology and pathogenesis of atherosclerosis in a sex- and age-stratified random sample of all inhabitants of Bruneck, Italy (125 women and 125 men in their fifth to eighth decades [n = 1,000]) (9). The present study focuses on blood samples from the 1995 examination and the follow-up period for clinical events between 1995 and 2010 (100% follow-up). In 1995, the study population consisted of 826 subjects, and plasma samples for assessment of oxidized phospholipids on apolipoprotein B-100 (OxPL/apoB) were available in a random subsample of 765 subjects. The appropriate ethics committees approved the study protocol, and all study subjects gave written informed consent before entering the study. All risk factors were assessed by validated standard procedures. Study methodology and laboratory methods for factors listed in Table 1 were previously described in detail (9–11).

Determination of OSEs on lipoproteins. OxPL/apoB levels and immunoglobulin-G (IgG) and IgM autoantibodies to malondialdehyde-modified, low-density lipoprotein (MDA-LDL) and copper-oxidized (Cu-OxLDL) and apoB-immune complexes (apoB-IC) were measured in 765 subjects from the 1995 time point and in 656 subjects from the 2000 time point. OxPL/apoB levels were measured, as previously described, by chemiluminescent enzyme-linked immunosorbent assay using the murine monoclonal antibody E06, which binds to the phosphocholine (PC) head group of oxidized but not native phospholipids (10). When the OxPL/apoB levels in Bruneck were first published (10), they were reported in 2 ways: as relative light units (RLUs) and as a ratio of E06 (OxPL RLUs) binding to presence of apoB on the plate, measured by monoclonal antibody MB47 (apoB RLUs) (i.e., OxPL/apoB ratio), as previously described (10,12). It was demonstrated that these measurements provided nearly identical results and were essentially interchangeable, thus subsequent studies reported OxPL/apoB as RLUs due to the more simplistic methodology of their determination. In the present analysis, we show both levels, but the analyses are based on OxPL/apoB RLU data. IgG and IgM autoantibodies, apoB-IC, and Lp(a) were measured as previously described (13–15). The intra- and interassay coefficients of variation for OxPL/apoB were 6% to 10%. The University of California-San Diego Human Research Protection Program approved these studies. We previously showed that these biomarkers are stable with prolonged freezing or transport to processing sites on ice (10,16,17).

Assessment of future cardiovascular events. The primary composite cardiovascular endpoint included ischemic stroke, myocardial infarction, new-onset unstable angina, acute coronary interventions, and vascular death (due to

Abbreviations and Acronyms

apoB = apolipoprotein B
apoB-IC = apoB-immune complexes
AUC = area under the curve
CFH = complement factor H
CI = confidence interval
CRP = C-reactive protein
Cu-OxLDL = copper-oxidized low-density lipoproteins
CVD = cardiovascular disease
DAMPs = danger-associated molecular patterns
FRS = Framingham Risk Score
HDL = high-density lipoprotein
HR = hazard ratio
hsCRP = high-sensitivity C-reactive protein
IDI = integrated discrimination index
Ig = immunoglobulin
LDL = low-density lipoprotein
Lp(a) = lipoprotein (a)
MDA = malondialdehyde
NRI = net reclassification index
OSE = oxidation-specific epitopes
OxPL/ApoB = oxidized phospholipids on apolipoprotein B-100
PC = phosphocholine
RLUs = relative light units
TIA = transient ischemic attack

Table 1 Baseline Characteristics of Study Subjects (n = 765) According to Incident Cardiovascular Disease During Follow-Up (1995 to 2010)

Variable	Primary Composite CVD Endpoint		p Value
	No (n = 627)	Yes (n = 138)	
Demographic parameters			
Age, yrs	61.4 ± 10.9	68.8 ± 10.5	<0.0001
Female, %	51.5%	40.6%	0.020
Oxidation-specific biomarkers			
OxPL/apoB, ratio	0.098 ± 0.109	0.161 ± 0.172	<0.0001
OxPL/apoB, RLU	9,094 ± 9,966	14,773 ± 15,590	<0.0001
Lp-PLA_2 activity, μmol/min/L	768.8 ± 195.2	858.6 ± 201.1	<0.0001
MDA-LDL IgM, RLU	17,504 ± 9,739	16,271 ± 7,956	0.165
Cu-OxLDL IgM, RLU	4,848 ± 3,854	4,227 ± 2,493	0.018
ApoB-IC IgM, RLU	5,159 ± 2,826	4,544 ± 2,255	0.006
MDA-LDL IgG, RLU	17,642 ± 10,713	18,109 ± 11,886	0.650
Cu-OxLDL IgG, RLU	9,577 ± 6,284	10,769 ± 11,017	0.086
ApoB-IC IgG, RLU	7,033 ± 3,871	6,787 ± 3,419	0.491
Lipids and lipoproteins			
Triglycerides, mg/dl	109 (78–15)	119 (93–170)	0.010
HDL cholesterol, mg/dl	59.5 ± 16.2	56.4 ± 17.3	0.049
LDL cholesterol, mg/dl	143.3 ± 37.1	153.1 ± 40.5	0.006
Lipoprotein(a), mg/dl	10.8 (4.5–31.0)	21.5 (7.0–55.4)	0.001
ApoA, mg/dl	166.5 ± 27.1	163.8 ± 29.9	0.296
ApoB, mg/dl	113.6 ± 30.2	125.4 ± 34.5	<0.0001
Vascular risk factors			
Hypertension, %	66.0%	75.4%	0.034
Systolic BP, mm Hg	146.6 ± 19.8	154.3 ± 22.4	<0.0001
Diastolic BP, mm Hg	86.7 ± 8.9	88.0 ± 9.7	0.118
Current smoking, %	20.4%	17.4%	0.420
Smoking, cigarettes/d	2.6 ± 6.2	2.6 ± 6.5	0.992
Diabetes (ADA), %	6.9%	13.8%	0.007
Fasting glucose, mg/dl	100.8 ± 22.7	109.5 ± 32.8	0.004
Ferritin, μg/l	131.3 ± 153.7	151.8 ± 162.8	0.162
Uric acid, mg/dl	4.6 ± 1.3	5.2 ± 1.3	<0.0001
Urinary albumin, g/l	9.0 (7.0–16.0)	12.0 (8.8–54.8)	<0.0001
Inflammation			
Fibrinogen, mg/dl	284.8 ± 72.9	303.6 ± 70.5	0.006
C-reactive protein, mg/l	1.5 (0.8–3.0)	2.1 (1.1–4.5)	0.010
Physical activity and body composition			
Sports index (Baecke), score	2.4 ± 0.9	2.2 ± 0.8	0.002
Alcohol, g/day	24.2 ± 31.4	22.9 ± 30.1	0.640
Body mass index, kg/m^2	25.5 ± 3.8	26.1 ± 4.1	0.088
Waist/hip ratio, cm/cm	0.93 ± 0.07	0.95 ± 0.07	0.001
Pre-existent CVD, medication and antioxidant supplements			
Statins, %	3.3	4.3	0.609
Platelet inhibitors, %	9.6	23.9	<0.0001
Antioxidant supplements,* %	4.0	2.0	0.304
CVD, %	6.9	25.4	<0.0001

Values are mean ± SD, median (interquartile range)†, or %. To convert values for cholesterol to millimoles per liter, multiply by 0.02586. To convert values for triglycerides to millimoles per liter, multiply by 0.01129. *Antioxidant supplements include vitamins A, C, and E, and selenium. No subjects were taking beta-carotene. †Median and interquartile range are presented for markedly skewed variables.

ADA = American Diabetes Association: apoB = apolipoprotein B; BP = blood pressure; apoB-IC = apoB-immune complexes; CRP = C-reactive protein; Cu-OxLDL = copper-oxidized low-density lipoproteins; CVD = cardiovascular disease; HDL = high-density lipoprotein; Ig = immunoglobulin; LDL = low-density lipoprotein; Lp-PLA_2 = lipoprotein-associated phospholipase A_2 activity; MDA= malondialdehyde; OxPL/ApoB = oxidized phospholipids on apolipoprotein B-100; RLU = relative light unit.

ischemic stroke, myocardial infarction, sudden cardiac death, or aortic aneurysm rupture). The extended composite cardiovascular endpoint additionally included transient ischemic attacks (TIAs) and all revascularization procedures. Acute coronary artery disease included myocardial infarction (fatal and nonfatal), new-onset unstable angina defined as angina at rest, crescendo angina or new-onset severe angina, acute coronary interventions, and sudden cardiac

deaths. Myocardial infarction was confirmed when World Health Organization criteria for definite disease status were met. Stroke and TIA were classified according to the criteria of the National Survey of Stroke. All other revascularization procedures (angioplasty and surgery) were carefully recorded. Ascertainment of events or procedures did not rely on hospital discharge codes or the patient's self-report, but on a careful review of medical records provided by the general practitioners and files of the Bruneck Hospital and the extensive clinical and laboratory examinations performed as part of the study protocols. Cases were ascertained from 1995 to 2010, and 100% follow-up was achieved.

Statistics. Calculations were performed with SPSS 18.0 (SPSS Inc., Chicago, Illinois) and Stata 12.0 MP (StataCorp., College Station, Texas) software packages. Continuous variables were presented as mean ± SD or medians (interquartile range) and dichotomous variables as percentages. Differences in baseline levels of vascular risk attributes between subjects with and without subsequent CVD (1995 to 2010) were analyzed with the Student t test and the chi-square test. Variables with a markedly skewed distribution (triglycerides, urinary albumin, C-reactive protein [CRP], and Lp[a]) were log-transformed to decrease the impact of extreme observations. To quantify the within-person variability of OSE biomarkers, regression dilution ratios (taking values between 0 and 1, with 1 indicating absence of variability [18]) were calculated based on 656 repeated measurements in samples taken 5 years apart (1995 and 2000). Long-term average levels of OSE markers were estimated by multivariate regression calibration models that regressed repeat measurements of the exposure on baseline exposure and confounder variables (19). Cox proportional hazard models were used to assess whether baseline OSE biomarker levels were independent predictors of CVD risk. For this purpose, OSE biomarkers were either entered as continuous variables or categorized in thirds (tertiles). We fitted 1 model adjusted for age, sex, and previous CVD and another model that additionally included systolic blood pressure, smoking, diabetes, ferritin level, LDL and high-density lipoprotein (HDL) cholesterol, alcohol consumption, social status, sports activity (Baecke score), and $\log_e$-transformed levels of CRP ("multivariable Cox model"). A third model included predicted long-term average levels instead of baseline levels of OSE biomarkers to account for the variability of OSE biomarker levels over time. ApoB and Lp(a) were not included because of the high correlation with LDL cholesterol and OxPL/apoB, respectively, and the potential problem of collinearity. Results remained virtually the same when apoB was used instead of LDL cholesterol or when Lp(a) was used instead of OxPL/apoB. To test for linear trend, we used the median level in each tertile group of OxPL/apoB as a continuous variable. All analyses were repeated using Lp(a) concentration instead of OxPL/apoB level. Proportional hazard assumptions were tested for OSE biomarkers and satisfied in all models. This was done by fitting interactions of the variables with survival time (Cox models with time-dependent covariates) and by scaled Schoenfeld residuals. Differential associations in subgroups were analyzed by inclusion of appropriate interaction terms. The incremental predictive value of OSE biomarkers over the Framingham Risk Score (FRS) was assessed by conventional C statistics, considering events but not time-to-events, and the Harrell's C-index for censored time-to-event data (20) (both measures for model discrimination with larger values indicate better discrimination). In addition, we calculated the net reclassification index (NRI) according to Pencina et al. (21), the continuous NRI, a category-free version of the NRI (22), and the integrated discrimination index (IDI), which integrates the NRI over all possible cutoffs and is equivalent to the difference in discrimination slopes (21) (all measures of reclassification). Comparison of the C statistics and Harrell's C for models, including and not including OSE markers, was performed according to the method of DeLong (23) and with the STATA procedures "somersd" and "lincom," respectively. NRIs were calculated across categories of predicted 15-year risk of <15%, 15% to 30%, and >30%. All reported p values are 2-sided.

Results

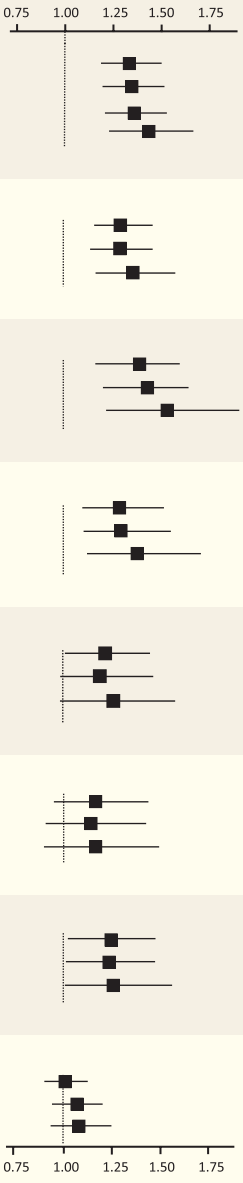
Baseline characteristics. Table 1 displays the baseline characteristics of study subjects (n = 765) according to incident CVD during follow-up (1995 to 2010). Subjects who developed the primary CVD endpoint (n = 138) were more likely to be older and male, have higher levels of LDL cholesterol, vascular, coagulation, inflammatory, and anthropomorphic and activity risk factors, and to have preexistent CVD at study entry in 1995.

OxPL/apoB levels and lipoprotein-associated phospholipase A_2 activity levels were higher in patients with incident CVD. In contrast, IgM antibodies to MDA-LDL, Cu-OxLDL, and apoB-IC were lower in subjects with CVD events, with the latter 2 associations achieving significance. IgG Cu-OxLDL antibodies tended to be higher in patients with subsequent CVD. Within-person variability of these markers was generally low. The regression dilution ratio of measurements taken 5 years apart was 0.83 (0.80 to 0.86) for OxPL/apoB levels; 0.91 (0.81 to 1.01) for IgG and 0.72 (0.64 to 0.79) for IgM Cu-OxLDL; 0.70 (0.64 to 0.76) for IgG and 0.87 (0.82 to 0.91) for IgM MDA-LDL; and 0.67 (0.61 to 0.74) for IgG and 0.69 (0.62 to 0.76) for IgM apoB-IC.

OxPL/apoB and incident CVD. The risk of the primary composite CVD endpoint increased across tertiles of OxPL/apoB and was particularly prominent in tertile 3 (Table 2). These associations were consistent across 3 models of multivariable adjustment: 1) following adjustment for age, sex, and previous CVD; 2) in a multivariable Cox model of 13 clinical variables, including high-sensitivity C-reactive protein (hsCRP); and 3) in the same Cox model with "usual" OxPL/apoB levels that considered the variability of

Table 2 Association of OxPL/apoB Tertile Groups With Cardiovascular Disease Risk in the Bruneck Study Cohort (1995 to 2010) (n = 765)

Model	Hazard Ratio (95% CI) OxPL/apoB Tertile Groups I	II	III	p Value for Trend	Hazard Ratio (95% CI) per 1-SD Unit Increase in OxPL/apoB	p Value
Primary composite CVD endpoint* (n = 138)						
Adjusted for age, sex, and prior CVD	1.0	1.1 (0.7–1.7)	2.1 (1.4–3.1)	<0.0001		<0.0001
Multivariable Cox model†	1.0	1.1 (0.7–1.7)	2.2 (1.5–3.4)	<0.0001		<0.0001
Multivariable Cox model†‡	1.0	1.0 (0.7–1.7)	2.3 (1.5–3.5)	<0.0001		<0.0001
Multivariable Cox model†§	1.0	1.2 (0.7–1.9)	2.4 (1.5–3.7)	<0.0001		<0.0001
Extended composite CVD endpoint* (n = 154)						
Adjusted for age, sex, and prior CVD	1.0	1.0 (0.6–1.6)	2.1 (1.4–3.0)	<0.0001		<0.0001
Multivariable Cox model†	1.0	1.0 (0.6–1.5)	2.1 (1.4–3.1)	<0.0001		<0.0001
Multivariable Cox model†§	1.0	1.1 (0.7–1.8)	2.2 (1.4–3.4)	<0.0001		<0.0001
Stroke (n = 60)						
Adjusted for age, sex, and prior CVD	1.0	1.1 (0.5–2.4)	2.9 (1.5–5.4)	<0.001		<0.001
Multivariable Cox model†	1.0	1.0 (0.5–2.2)	3.2 (1.6–6.2)	<0.0001		<0.001
Multivariable Cox model†§	1.0	1.5 (0.7–3.4)	3.6 (1.8–7.4)	<0.0001		<0.001
Stroke/TIA (n = 76)						
Adjusted for age, sex, and prior CVD	1.0	1.1 (0.6–2.2)	2.8 (1.6–4.9)	<0.0001		0.001
Multivariable Cox model†	1.0	1.1 (0.5–2.1)	2.9 (1.6–5.1)	<0.0001		0.002
Multivariable Cox model†§	1.0	1.5 (0.8–3.1)	3.2 (1.7–6.1)	<0.0001		0.002
Acute coronary artery disease* (n = 70)						
Adjusted for age, sex, and prior CVD	1.0	1.2 (0.6–2.1)	1.5 (0.9–2.7)	0.148		0.032
Multivariable Cox model†	1.0	1.2 (0.7–2.3)	1.5 (0.8–2.7)	0.190		0.066
Multivariable Cox model†§	1.0	1.1 (0.6–2.2)	1.5 (0.8–2.9)	0.139		0.066
Myocardial infarction (n = 53)						
Adjusted for age, sex, and prior CVD	1.0	1.2 (0.6–2.3)	1.3 (0.7–2.5)	0.546		0.134
Multivariable Cox model†	1.0	1.2 (0.6–2.4)	1.2 (0.6–2.4)	0.662		0.244
Multivariable Cox model†§	1.0	1.0 (0.5–2.2)	1.2 (0.6–2.5)	0.525		0.244
Vascular death* (n = 64)						
Adjusted for age, sex, and prior CVD	1.0	0.9 (0.5–1.8)	1.3 (0.7–2.4)	0.244		0.031
Multivariable Cox model†	1.0	0.9 (0.5–1.7)	1.3 (0.7–2.4)	0.216		0.048
Multivariable Cox model†§	1.0	0.8 (0.4–1.7)	1.2 (0.6–2.3)	0.381		0.048
Death from all causes (n = 250)						
Adjusted for age, sex, and prior CVD	1.0	1.1 (0.8–1.5)	1.0 (0.8–1.4)	0.985		0.977
Multivariable Cox model†	1.0	0.9 (0.7–1.3)	1.2 (0.9–1.6)	0.157		0.264
Multivariable Cox model†§	1.0	1.3 (0.9–1.8)	1.4 (1.0–1.9)	0.176		0.264



Squares and lines are hazard ratios (HRs) and 95% confidence intervals. HRs were derived from Cox models and calculated for OxPL tertile groups (left-hand columns) and for a 1-SD unit increase in OxPL level (right-hand columns). In all analyses, only the first outcome event occurring in study participants was considered, whereas potential further or recurrent events were censored. *The primary composite cardiovascular endpoint subsumes ischemic stroke, acute coronary artery disease, and vascular death. The extended composite cardiovascular endpoint additionally considers TIAs and all revascularization procedures. Acute coronary artery disease subsumes myocardial infarction (fatal and nonfatal), new-onset unstable angina, acute coronary interventions, and sudden cardiac deaths. Vascular deaths subsume deaths due to ischemic stroke, myocardial infarction, sudden cardiac death, and aortic aneurysm rupture. †Multivariable adjustment: age, sex, previous cardiovascular disease, systolic blood pressure, smoking, diabetes, ferritin level, LDL and HDL cholesterol, alcohol consumption, social status, sports activity (Baecke score), and $\log_e$-transformed levels of C-reactive protein. ‡"Usual" OxPL levels were used instead of baseline levels. "Usual" OxPL level considers the variability of OxPL over time and was estimated by multivariate regression calibration. §Multivariable adjustment as previously described plus statin medication, platelet inhibitor medication, and antioxidant supplement use.

Abbreviations as in Table 1.

OxPL/apoB over time estimated by multivariate regression calibration based on measurements in 1995 and 2000. In model 3, the highest tertile of OxPL/apoB was associated with a hazard ratio (HR) of 2.4 and 95% confidence interval (CI) of 1.5 to 3.7 ($p < 0.001$) compared with the lowest tertile. Similar findings were present for the extended CVD endpoint that additionally included TIA and all revascularization procedures (Table 2). For individual endpoints, OxPL/apoB was a strong predictor of stroke (HR: 3.6; 95% CI: 1.8 to 7.4; $p < 0.001$) and combined stroke/TIA (HR:

Table 3 Association of Oxidation-specific Biomarkers With Cardiovascular Disease Risk in the Bruneck Study Cohort (1995 to 2010) (n = 765)

	Primary Composite CVD Endpoint (n = 138)		Stroke (n = 60)		Acute Coronary Artery Disease (n = 70)	
	HR (95% CI)	p Value	HR (95% CI)	p Value	HR (95% CI)	p Value
OxPL/apoB	1.34 (1.19–1.51)	<0.0001	1.42 (1.19–1.69)	0.0001	1.22 (1.01–1.46)	0.036
	1.37 (1.20–1.56)*	<0.0001*	1.47 (1.21–1.78)*	<0.0001*	1.20 (0.99–1.45)*	0.066*
	1.38 (1.21–1.57)†	<0.0001†	1.48 (1.22–1.80)†	<0.0001†	1.21 (1.00-1.46)†	0.052†
Cu-OxLDL IgG	1.11 (0.98–1.27)	0.104	1.25 (1.07–1.46)	0.005	0.91 (0.69–1.21)	0.516
	1.18 (1.03–1.37)*	0.022*	1.33 (1.12–1.58)*	0.001*	0.95 (0.71–1.28)*	0.738*
	1.18 (1.02–1.37)†	0.028†	1.32 (1.11–1.58)†	0.002†	0.94 (0.70–1.27)†	0.703†
MDA-LDL IgM	0.72 (0.53–0.98)	0.034	0.81 (0.63–1.05)	0.109	0.82 (0.69–0.97)	0.019
	0.69 (0.50–0.95)*	0.021*	0.79 (0.61–1.02)*	0.075*	0.79 (0.66–0.95)*	0.011*
	0.69 (0.50–0.94)†	0.018†	0.79 (0.61–1.03)†	0.084†	0.79 (0.66–0.95)†	0.010†

Hazard ratios (HRs) and 95% confidence intervals (CIs) were derived from Cox models and calculated for a 1-SD unit increase in OxPL level, Cu-OxLDL IgG, and MDA-LDL IgM antibodies. Cox models included age, sex, previous CVD, OxPL/apoB level, Cu-OxLDL IgG, and MDA-LDL IgM antibodies. *HRs (95% CIs) and p values are from multivariable Cox models with additional adjustment for systolic blood pressure, smoking, diabetes, ferritin level, LDL and HDL cholesterol, alcohol consumption, social status, sports activity (Baecke score), and $\log_e$-transformed level of C-reactive protein. †"Usual" OxPL/apoB level was used instead of baseline OxPL/apoB levels. "Usual" OxPL/apoB level considers the variability of OxPL/apoB over time and was estimated by multivariate regression calibration.

Abbreviations as in Table 1.

3.2; 95% CI: 1.7 to 6.1; $p < 0.001$). These findings were consistent when the data were evaluated as continuous variables per 1 SD in OxPL/apoB (Tables 2 and 3), showing HRs of approximately 1.25 to 1.5. In this analysis, OxPL/apoB also predicted vascular death (Table 2). All analyses were repeated using Lp(a) concentrations instead of OxPL/apoB levels, and similar results were obtained.

Cumulative hazard plots depicting the composite CVD endpoint (Fig. 1A) and stroke (Fig. 1B) indicated a progressive divergence in event frequency over 15-year follow-up in OxPL/apoB tertile 3 compared with tertiles 1 and 2.

Subgroup analysis revealed consistent findings across sex, age, CVD risk factors, Lp-PLA_2 activity, hsCRP, and FRS for both the tertile analysis and as continous variables for a 1-SD change for the primary composite CVD endpoint (Fig. 2) and for stroke as an individual endpont (Fig. 3).

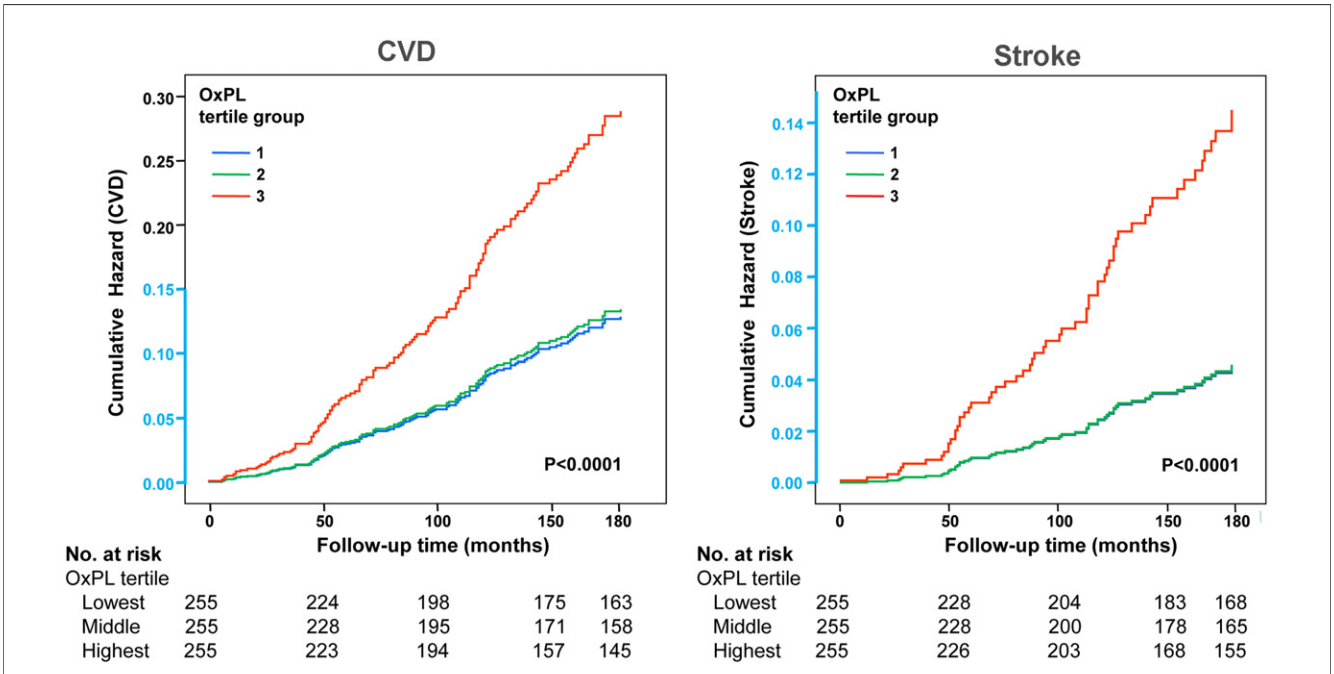


Figure 1 Cumulative Hazard Curves for CVD Incidence and Stroke Incidence by OxPL Tertile Groups, 1995 to 2010

The median oxidized phospholipids on apolipoprotein B-100 (OxPL/apoB) level (relative light units [RLUs]) for the lowest tertile was 2,908 (range 1,584 to 3,631); for the middle tertile, it was 4,862 (range 3,632 to 8,124); and for the highest tertile, it was 18,830 (range 8,125 to 79,541). There were 138 cases of incident cardiovascular disease (CVD) and 60 cases of incident stroke. Y-axis shown in **light blue** indicates range from 0 to 0.15.

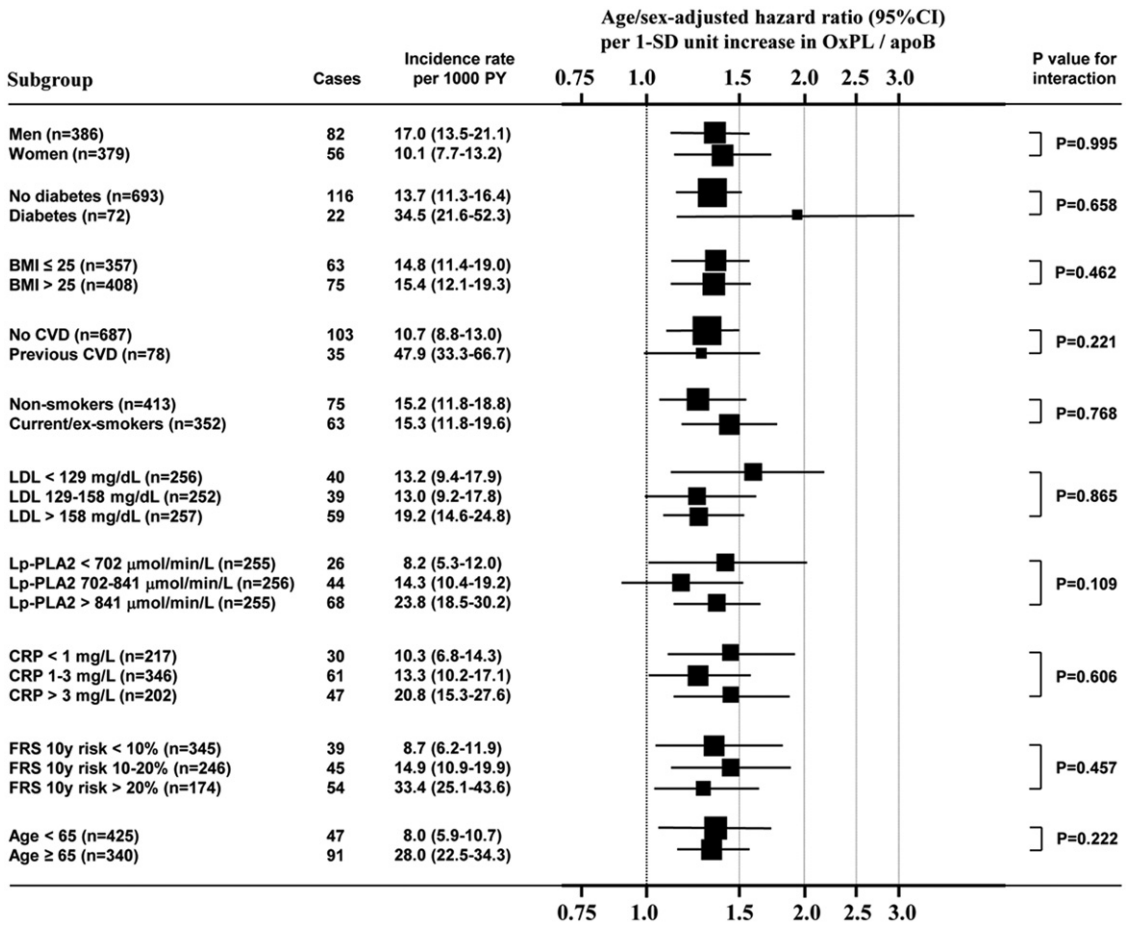


Figure 2 **Association Between OxPL/apoB and Incident CVD (Primary Composite Endpoint) in Various Subgroups (Bruneck Study, 1995 to 2010; n = 765)**

Squares and **lines** are hazard ratios and 95% confidence intervals (CIs) calculated for a 1-SD unit increase in OxPL/apoB ratio. The size of squares reflects the number of individuals in each subgroup. All models were adjusted for age, sex, previous CVD, systolic blood pressure, smoking, diabetes, ferritin level, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, alcohol consumption, social status, sports activity (Baecke score), and $\log_e$-transformed levels of CRP. Interactions were calculated by inclusion of appropriate interaction terms. BMI = body mass index; FRS = Framingham Risk Score; hsCRP = high-sensitivity C-reactive protein; Lp-PLA_2 = lipoprotein-associated phospholipase A_2 activity; PY = person years.

IgG and IgM autoantibodies and apoB-IC and incident CVD. In the next step, we fitted a model considering both OxPL/opaB and autoantibodies to OSEs. In Model 3, IgG Cu-OxLDLs were associated with higher risk of the composite endpoint (HR: 1.18; 95% CI: 1.02 to 1.37, p = 0.028 for 1-SD unit increase) and the individual endpoint of stroke (HR:1.32; 95% CI 1.11 to 1.58; p = 0.002) (Table 3). In contrast, in Model 3, IgM MDA-LDL (HR: 0.79; 95% CI: 0.66 to 0.95; p = 0.010) was associated with a lower risk of composite CVD, as well as stroke. If IgM MDA-LDL was replaced by IgM Cu-OxLDL, the respective HR was 0.78 (95% CI: 0.61 to 0.98; p = 0.037). IgG and IgM apoB-IC were not predictive of events in the multivariable models (data not shown).

Receiver-operating classification curves and reclassification. Receiver-operating classification curves for the primary endpoint were determined (Fig. 4). With inclusion of the oxidation markers (OxPL/apoB, Cu-OxLDL IgG, and MDA IgM), the area under the curve (AUC) increased from 0.664 (0.629 to 0.697) (FRS only) to 0.705 (0.672 to 0.737). This change, although modest (Δ AUC 0.042; 0.001 to 0.083, p = 0.048), was statistically significant. The NRI was 16.3% (2-sided p = 0.0044). Of 138 subjects with incident CVD, 35 were correctly reclassified to a higher risk category, 18 were reclassified to a lower category. In subjects who remained free of CVD [n = 627], 108 were correctly reclassified to a lower risk category, and 83 were reclassified to a higher category (categories of 15-year risk: <15%, 15% to 30%, >30%). Similar to other new biomarkers in vascular medicine, the NRI was higher in subjects at intermediate risk (33.2%; n = 305; p < 0.001).

In accordance with these findings, Harrell's C index increased from 0.675 to 0.705 (Δ C index 0.029; −0.005 to 0.064; p = 0.098), and the corresponding NRI, based on

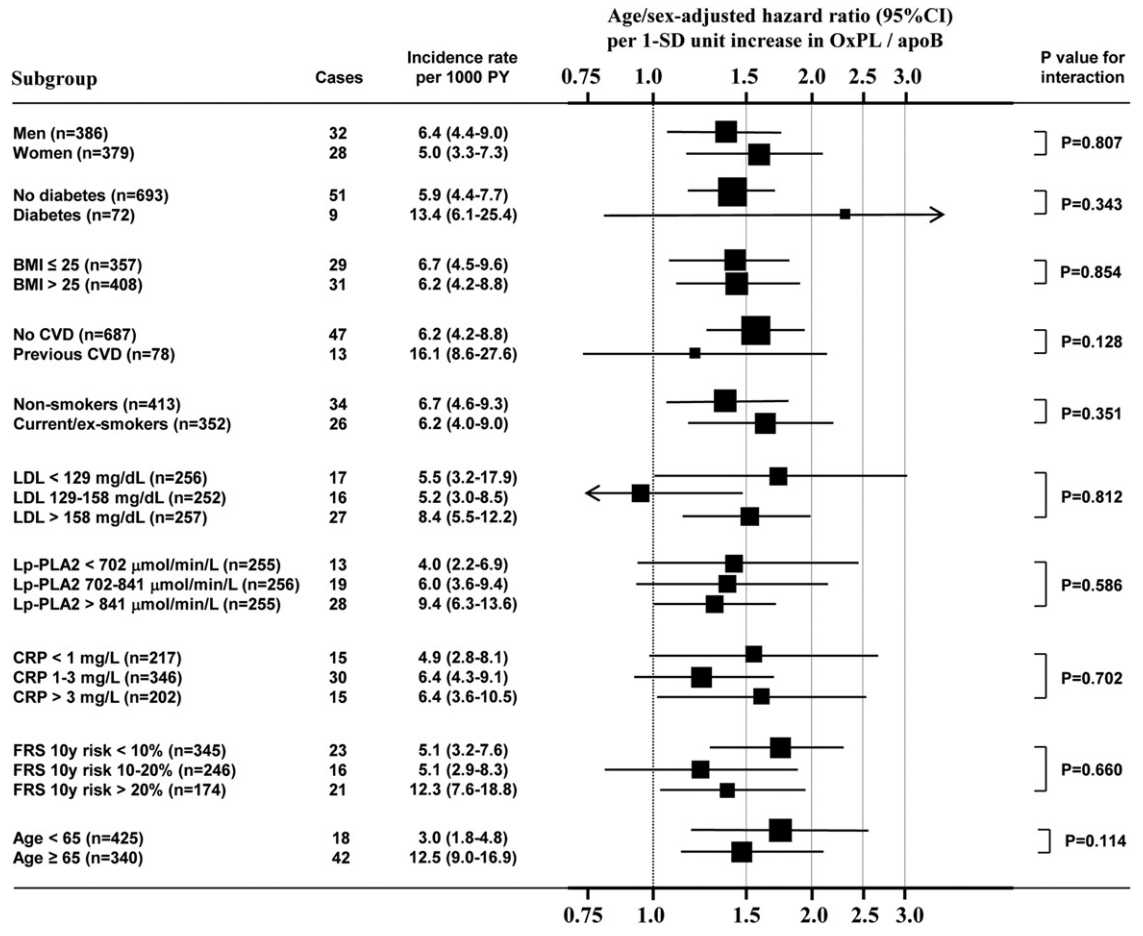


Figure 3 **Association Between OxPL/apoB and Incident Stroke in Various Subgroups (Bruneck Study, 1995 to 2010; n = 765)**

Squares and **lines** are hazard ratios and 95% CIs calculated for a 1-SD unit increase in OxPL/apoB. The size of squares reflects the number of individuals in each subgroup. All models were adjusted for age, sex, previous CVD, systolic blood pressure, smoking, diabetes, ferritin level, fibrinogen level, LDL and high-density cholesterol, waist-to-hip ratio, alcohol consumption, social status, Lp-PLA_2 activity, sports activity (Baecke score), uric acid level, fasting glucose, and $\log_e$-transformed levels of CRP and urinary albumin. Interactions were calculated by inclusion of appropriate interaction terms. Abbreviations as in Figures 1 and 2.

the previous 15-year risk categories (<15%, 15% to 30%, >30%), was 14.0% (2.6 to 25.3) ($p = 0.016$) (Table 4), whereas the category-free continuous NRI and the IDI, which integrates the NRI over all possible cutoffs, were 37.6% (17.6 to 57.6; $p < 0.001$) and 0.052 (0.027 to 0.076; $p < 0.001$), respectively.

The largest discrimination improvement was afforded by OxPL/apoB (AUC 0.664 to 0.698). Addition of the 2 other markers further increased the AUC from 0.698 to 0.705. In this population, both risk discrimination and risk reclassification improved significantly upon addition of all 3 parameters.

Discussion

This study demonstrates the clinical value of oxidation-specific biomarkers in predicting CVD events and stroke over a prospective 15-year follow-up in an unselected population from the general community. Individually, OxPL/apoB and IgG Cu-OxLDL autoantibodies predicted a higher event rate, whereas IgM MDA-LDL (or IgM Cu-OxLDL) autoantibodies predicted a lower event rate. Collectively, they significantly improved the predictive accuracy of CVD events in a model adjusting for 16 clinical variables, including hsCRP. Importantly, they allowed reclassification of subjects at initial low-, intermediate-, and high-risk categories, moving a significant number of subjects into both higher and lower categories. Because the Bruneck population reflects individuals seen in a primary care setting, broad application of oxidation-specific biomarkers, in addition to currently available clinical and laboratory risk factors, would allow optimal risk prediction and tailored therapies to appropriately match the risk with the intensity of treatment and follow-up.

These data support the unifying hypothesis that OSEs, such as OxPL and MDA epitopes, represent DAMPs that are detrimental to the host, and that the innate immune

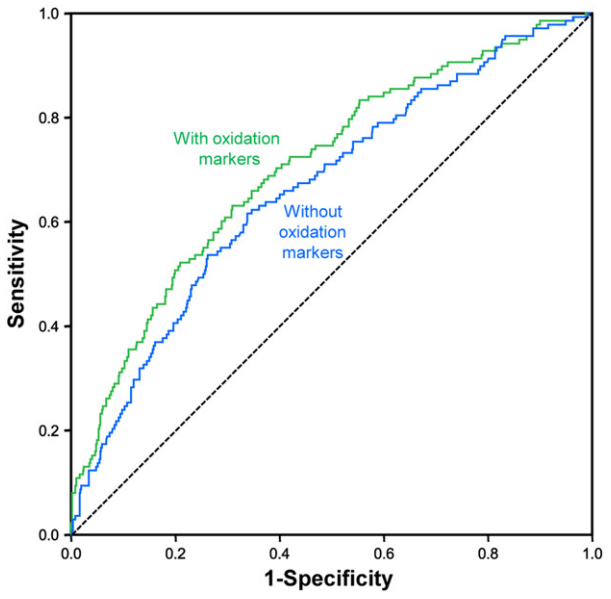


Figure 4 **Receiver-Operating Classification Curves for the CVD Endpoint With Inclusion of the Oxidation Markers (OxPL/apoB, Cu-OxLDL IgG, and MDA IgM) in the AUC**

The reference comparison was FRS without oxidation biomarkers included. Cu-OxLDL IgG = copper-oxidized low-density lipoprotein immunoglobulin-G; MDA IgM = malondialdehyde immunoglobulin-M; other abbreviations as in Figures 1 and 2.

system provides protective responses to them (2,24). Such DAMPs are strongly present on apoptotic cells (25,26), infectious pathogens such as pneumococcus (27), and oxidized lipids (28,29). In response to such DAMPs, evolutionary processes have preserved and amplified innate immune effector proteins to bind and neutralize their proinflammatory effects. The initial evolutionary pressure may have been derived from the need to clear trillions of apoptotic cells on a daily basis, which may have been subsequently amplified by repeated exposure to common infectious pathogens that share similar epitopes or molecular mimics (2), as well as oxidized lipids derived from the diet and those generated in vivo following oxidative stress in the setting of dyslipidemia and other risk factors. These innate effector proteins are represented by a variety of macrophage scavenger receptors, IgM natural antibodies such as E06, and IgM autoantibodies measured in this study (29), as well as innate plasma proteins, such as CRP, which binds not only the PC headgroup of OxPL, but the same PC (not as part of a phospholipid) on the cell wall of *Streptococcus pneumoniae* (30).

Recently, we demonstrated that complement factor H (CFH), which is a primary regulator of the alternative pathway of complement activation, strongly binds to MDA epitopes (24). The loss of function single nucleotide polymorphism Y402 of CFH exhibited limited ability to bind MDA. The Y402 allele is a major risk factor for development of age-related macular degeneration, and importantly, loss of function by CFH in binding and preventing proinflammatory responses of MDA was directly linked to enhanced oxidative stress in the retina in a murine model. This suggests an important link between limiting inflammatory responses to MDA and age-related macular degeneration, a disease with many common risk factors and features to atherosclerosis. Overall, this study supports the notion that OSEs are important factors in immune-inflammatory diseases such as CVD, and that their measurement may serve as useful biomarkers of CVD risk assessment and event prediction.

The clinical role of autoantibodies to OxLDL has progressively evolved since the first demonstration of their presence in human and animal experimental lesions >20 years ago (28,31–33). The totality of data suggests that IgG autoantibodies are positively associated with anatomical CVD, whereas IgM autoantibodies are inversely associated. This area has been controversial due to mixed results arising for a variety of factors related to lack of prospective studies, small cohorts, lack of adequate power and extended follow-up. In addition, there has been a lack of standardization of antigens to precisely measure their levels in comparative studies (34,35). The present study represents the longest prospective follow-up of any study evaluating such biomarkers and demonstrates that increased baseline levels of IgG autoantibodies to Cu-OxLDL predict higher risk of cardiovascular events. Whether this increased risk association is a reflection of primary antibody mediated risk, for example, through proinflammatory effects mediated

Table 4 **Risk Reclassification Based on Oxidation Markers (OxPL/ApoB, Cu-OxLDL IgG, and MDA IgM) in Patients Who Experienced a Cardiovascular Disease Endpoint (n = 138) and in Those Who Remained Free of Cardiovascular Disease during Follow-Up (n = 627; 1995 to 2010)**

Model Based on the FRS only	Model Additionally Considering Oxidation Markers			
Frequency (n) Risk Categories (15-Year Cardiovascular Disease Risk)	<15% Risk	15–30% Risk	>30% Risk	Total
People who remained free of cardiovascular disease (n = 465)				
<15% risk	162	31	6	199
15–30% risk	95	119	22	236
>30% risk	0	12	18	30
Total	257	162	46	465
People who experienced a cardiovascular disease endpoint (n = 138)				
<15% risk	16	10	3	29
15–30% risk	20	34	14	68
>30%	0	2	39	41
Total	36	46	56	138

This reclassification table compares a model based on the Framingham Risk Score (FRS) only with a model considering the FRS and levels of oxidation markers (OxPL/apoB, Cu-OxLDL IgG, and MDA IgM). The shaded values reflect subjects who were reclassified.
Abbreviations as in Table 1.

by fragment-dependent events, or is simply a reflection that such IgGs are a surrogate of the presence of the enhanced generation of OSEs, cannot be determined from this clinical investigation.

In contrast, IgM autoantibodies to OSEs were associated with lower incident CVD event rates and stroke. These data are in keeping with evolving experimental developments suggesting that IgM autoantibodies may be atheroprotective. 1) IgM OxLDL autoantibodies are highest in younger patients and decline as patients age, when CVD risk is highest (reviewed in [35]). 2) OSEs represent a dominant, previously unrecognized target of IgM natural antibodies in both mice and humans, and ~30% of all natural antibodies bind to model OSEs, atherosclerotic lesions, and apoptotic cells (36). The high prevalence of such OSE-specific natural antibodies suggests strong evolutionary pressure in protecting hosts from the proinflammatory effects of OSEs. 3) The natural antibody IgM E06/T15 has the capacity to block macrophage uptake of OxLDL, and generation of high titers of E06 in response to pneumococcal vaccination in cholesterol-fed $LDLR^{-/-}$ mice results in reduced atherosclerosis (27). 4) Direct experimental evidence exists for a protective role of IgM autoantibodies in several different murine models of atherosclerosis; for example, the demonstration that the complete absence of B-1 cell-derived IgM led to accelerated atherosclerosis in $LDLR^{-/-}$ mice (37), and transfer of B-1 cells capable of secreting IgM into splenectomized $apoE^{-/-}$ mice was atheroprotective, but transfer of B-1 cells incapable of secreting IgM was not (38). This suggests that natural IgM antibodies are evolutionarily selected in nature and play an important role in mediating homeostatic functions. In the case of CVD, the presence of circulating IgM may lead to prevention of foam cell formation, as has been shown with passive immunization of human antibodies (39), and neutralization and/or clearance of apoptotic cells and other OSEs when generated by oxidative stress (36). In this way, they provide a beneficial innate immune function when DAMPs are generated.

Unlike OxLDL autoantibodies that represent indirect measures of OSEs, OxPL/apoB represents a direct measure of OSEs in plasma. The measurement of OxPL/apoB has been now validated in a large clinical database (reviewed in Taleb et al. [8]). OxPL/apoB levels reflect the presence of OxPL on apoB particles and are increased in a variety of atherogenic phenotypes (40,41). OxPL/apoB levels have been previously shown to correlate with Lp(a). Interestingly, they correlate best with Lp(a) in patients with high Lp(a) levels associated with small apo(a) isoforms, but do not correlate well in subjects with lower Lp(a) levels with large isoforms (42). OxPL/apoB, therefore, seems to reflect the most atherogenic Lp(a) particles. Because patients have 2 different apo(a) isoforms that are not measured clinically (43,44), the OxPL/apoB measure is a clinically useful surrogate for these highly atherogenic Lp(a) particles and may represent a unifying measure of their cardiovascular risk.

In this study, the predictive value of oxidation-specific biomarkers was extended in several ways. 1) The availability of 2 separate measures over 5 years and their stability over this time (more reproducible than LDL-C levels [10,40]) allowed us to analyze and report the usual levels of OxPL/apoB. This adds an enhanced level of robustness to the predictive value. 2) OxPL/apoB was predictive of stroke/TIA as an individual endpoint with a robust HR of 3.6 over a 15-year follow-up. Because stroke is an increasing cause of mortality and morbidity globally, it may serve as an important risk stratifier, particularly with the dearth of available stroke biomarkers (45). Such biomarkers will also be needed with the increasing aging of populations across the globe. 3) OxPL/apoB remained independently predictive of CVD over a prospective 15-year follow-up. 4) The fact that subjects in the third tertile of OxPL/apoB were as much higher risk than subjects in tertiles 1 and 2 may suggest a threshold effect of increasing CVD risk in patients with very high levels of OxPL/apoB. This needs to be validated in accumulating outcomes studies with this biomarker by plotting OxPL/apoB levels with risk of new CVD events and assessing for a threshold in risk prediction. The addition of OxPL/apoB, IgG, and IgM autoantibodies to the predictive models allowed enhanced risk prediction, and importantly, net reclassification of subjects into lower or higher risk categories. If these reclassification findings are confirmed in larger studies, it may allow clinically meaningful reclassification of primary care subjects into different categories, which would have significant public health implications from many perspectives, including prevention of events and cost of treatments. The OxPL/apoB assay has been recently licensed commercially and will be made available in the near future as a sendout laboratory measurement and as a kit for both clinical and research applications.

Study limitations. Limitations of this study include that the subjects were all Caucasian, and these findings need to be verified in other racial populations.

Conclusions

A combination of direct and indirect oxidation-specific biomarkers provide robust, independent predictive value for CVD and stroke events and enhances the ability to reclassify subjects into lower or higher risk categories. These biomarkers may have clinical utility in a broad range of patients at risk for CVD.

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Key Words: atherosclerosis ■ autoantibodies ■ lipoproteins ■ oxidation ■ oxidation-specific epitopes ■ oxidized phospholipids.