Clinical and Population Studies

Oxidized Phospholipids and Risk of Calcific Aortic Valve Disease
The Copenhagen General Population Study

Pia R. Kamstrup, Ming-Yow Hung, Joseph L. Witztum, Sotirios Tsimikas, Børge G. Nordestgaard

**Objective**—Lipoprotein(a) is causally associated with calcific aortic valve disease (CAVD). Lipoprotein(a) carries proinflammatory and procalcific oxidized phospholipids (OxPL). We tested whether the CAVD risk is mediated by the content of OxPL on lipoprotein(a).

**Approach and Results**—A case–control study was performed within the Copenhagen General Population Study (n=87980), including 725 CAVD cases (1977–2013) and 1413 controls free of cardiovascular disease. OxPL carried by apoB (apolipoprotein B-100; OxPL-apoB) or apolipoprotein(a) (OxPL-apo(a)) containing lipoproteins, lipoprotein(a) levels, LPA kringle IV type 2 repeat, and rs10455872 genetic variants were measured. OxPL-apoB and OxPL-apo(a) levels correlated with lipoprotein(a) levels among cases (r=0.75 and r=0.95; both P<0.001) and controls (r=0.65 and r=0.93; both P<0.001). OxPL-apoB levels associated with risk of CAVD with odds ratios of 1.2 (95% confidence interval [CI]:1.0–1.6) for 34th to 66th percentile levels, 1.6 (95% CI, 1.2–2.1) for 67th to 90th percentile levels, 2.0 (95% CI, 1.3–3.0) for 91st to 95th percentile levels, and 3.4 (95% CI, 2.1–5.5) for levels >95th percentile, versus levels <34th percentile (trend, P<0.001). Corresponding odds ratios for OxPL-apo(a) were 1.2 (95% CI, 1.0–1.5), 1.2(95% CI, 0.9–1.6), 2.1(95% CI, 1.4–3.1), and 2.9(95% CI, 1.9–4.5; trend, P<0.001) and were similar for lipoprotein(a). LPA genotypes associated with OxPL-apoB, OxPL-apo(a), and lipoprotein(a) levels and explained 34%, 46%, and 39%, respectively, of the total variation in levels. LPA genotypes associated with risk of CAVD; a doubling in genetically determined OxPL-apoB, OxPL-apo(a), and lipoprotein(a) levels associated with odds ratio of CAVD of 1.18 (95% CI, 1.10–1.27), 1.09 (95% CI, 1.05–1.13), and 1.09 (95% CI, 1.05–1.14), respectively, comparable to the corresponding observational estimates of 1.27 (95% CI, 1.16–1.39), 1.13 (95% CI, 1.08–1.18), and 1.11 (95% CI, 1.06–1.17).

**Conclusions**—OxPL-apoB and OxPL-apo(a) are novel genetic and potentially causal risk factors for CAVD and may explain the association of lipoprotein(a) with CAVD.

**Visual Overview**—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:1570-1578. DOI: 10.1161/ATVBHA.116.308761.)

**Key Words:** apolipoproteins B ■ case–control study ■ genotype ■ lipoprotein(a) ■ phospholipids

Recent data, encompassing genome-wide association and Mendelian randomization studies, have strongly implicated genetic variants in the LPA gene that are associated with elevated lipoprotein(a) plasma levels as risk factors for calcific aortic valve disease (CAVD).1–3 During the past decade, a large number of studies have shown that lipoprotein(a) is the preferential lipoprotein carrier of phosphocholine-containing oxidized phospholipids (OxPL) and that some of the clinical risk in mediating cardiovascular disease may be because of its content of OxPL.4–6 This naturally leads to the hypothesis that the risk of lipoprotein(a) in mediating CAVD may be because of its content of OxPL.
valve calcification. Many of these phosphocholine-containing OxPL are detected by the OxPL-apoB (apolipoprotein B) assay (ie, OxPL measured on apoB-100 lipoproteins using the murine monoclonal antibody E06). OxPL-apoB detects OxPL on all apoB-100–containing lipoproteins including lipoprotein(a), and we have previously shown most (85%–90%) of the OxPL in this assay format reflect OxPL on lipoprotein(a). Recently, a study using this assay and conducted in patients with pre-existing CAVD has demonstrated that patients with elevated OxPL-apoB, OxPL on apolipoprotein(a) (OxPL-apo(a)), and lipoprotein(a) levels have much faster progression of CAVD and more frequent need for aortic valve replacement.

In the present case–control study, we tested the hypothesis that observationally as well as genetically elevated OxPL-apoB and OxPL-apo(a) are associated with increased risk of CAVD.

We used 2 LPA genetic variants, the kringle IV type 2 repeat polymorphism (KIV-2) determining the number of apo(a) kringle structures and the rs10455872 intron single nucleotide polymorphism, partly tracking the KIV-2 genotype, both associated with CAVD, to conduct Mendelian randomization analyses testing whether genetically elevated OxPL-apoB or OxPL-apo(a) associate with increased risk of CAVD indicating causality.

Materials and Methods

A case–control study was designed from among 87’980 participants in the CGPS (Copenhagen General Population Study), a general population study initiated in 2003. For the present study, we included all CGPS participants diagnosed with CAVD from 1977 until 2013, and additionally for each CAVD case participant, 2 matched control participants free of cardiovascular disease, dependent on available blood samples (n=2138). The detailed Materials and Methods are available in the online-only Data Supplement.

Results

Baseline Characteristics of the Study Participants

Baseline characteristics of the 2138 participants selected from 87’980 CGPS participants and stratified by CAVD case–control status are shown in Table (and stratified by LPA genotypes shown in Table I in the online-only Data Supplement). Patients with CAVD had more diabetes mellitus, lower levels of total cholesterol (and were more often on lipid-lowering therapy), higher body mass index, higher levels of high-sensitivity C-reactive protein, higher levels of lipoprotein(a), and higher levels of oxidized phospholipids bound to apolipoprotein(a) and lipoprotein(a) are associated with increased risk of CAVD.

<table>
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<tr>
<th>Table. Baseline Characteristics of Participants From the Copenhagen General Population Study</th>
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<tr>
<td>No. of individuals</td>
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<td>Women, %</td>
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<td>Age, y</td>
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<tr>
<td>Total cholesterol, mmol/L</td>
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<td>HDL cholesterol, mmol/L</td>
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<td>Systolic BP, mm Hg</td>
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<td>BMI, kg/m²</td>
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<td>eGFR, mL/min</td>
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<td>hs-CRP, mg/L</td>
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<tr>
<td>Smoking, %</td>
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<tr>
<td>Diabetes mellitus, %</td>
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<td>Lipid-lowering therapy, %</td>
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<td>Lipoprotein(a), mg/dL</td>
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<td>OxPL-apoB, nmol/L</td>
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<td>OxPL-apo(a), nmol/L</td>
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<td>LPA rs10455872, % carriers</td>
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<tr>
<td>LPA KIV-2, no. of repeats in both alleles combined</td>
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</table>

Continuous covariates are reported as median (interquartile range). P values were obtained from Kruskall–Wallis tests for continuous variables and from chi² tests for categorical values. BMI indicates body mass index; BP, blood pressure; CAVD, calcific aortic valve disease; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; KIV-2, kringle IV type 2; NA, not applicable; OxPL-apo(a), oxidized phospholipids bound to apolipoprotein(a); and OxPL-apoB, oxidized phospholipids bound to apolipoprotein B.

*Number of participants for individual covariates may vary dependent on availability of covariates.
of lipoprotein(a), OxPL-apoB, and OxPL-apo(a), smaller numbers of LPA KIV-2 repeats, and higher prevalence of LPA rs10455872. Overall, 25% and 24% of cases had OxPL-apoB levels ≥5 nmol/L and lipoprotein(a) levels ≥50 mg/dL, with corresponding percentages in controls of 15% and 13%.

Association of OxPL-apoB and OxPL-apo(a) With Lipoprotein(a) Levels
The concentration distributions for OxPL-apoB (and OxPL-apo(a)) and lipoprotein(a) levels were similarly skewed in both controls and cases (Figure 1; Figure I in the online-only Data Supplement). For both controls and cases, OxPL-apoB and lipoprotein(a) levels were highly correlated with correlation coefficients of 0.65 (P<0.001) and 0.75 (P<0.001; Figure 2). OxPL-apo(a) and lipoprotein(a) levels were likewise highly correlated (Figure II in the online-only Data Supplement), with corresponding correlation coefficients of 0.93 (P<0.001) and 0.95 (P<0.001). Figures 1 and 2 and Figures I and II in the online-only Data Supplement thus demonstrate the close relationship between lipoprotein(a) measurements and measurements of OxPL-apoB and OxPL-apo(a).

Risk of CAVD as a Function of OxPL-apoB and Lipoprotein(a) Levels
A stepwise higher risk of CAVD for progressively higher OxPL-apoB and lipoprotein(a) levels (Figure 3) was observed. OxPL-apoB levels ranged from 0 to 23 nmol/L; a 1-nmol/L increase associated with a multivariable-adjusted odds ratio for CAVD of 1.10 (95% confidence interval [CI], 1.07–1.14). Lipoprotein(a) levels ranged from 0 to 327 mg/dL; a 10-mg/dL increase associated with a multivariable-adjusted odds ratio for CAVD of 1.10 (95% CI, 1.06–1.13). OxPL-apoB levels were associated with risk of CAVD with multivariable-adjusted odds ratios of 1.2 (95% CI, 1.0–1.6) for 34th to 66th percentile levels, 1.6 (95% CI, 1.2–2.1) for 67th to 90th percentile levels, 2.0 (95% CI, 1.3–3.0) for 91st to 95th percentile levels, and 3.4 (95% CI, 2.1–5.5) for levels >95th percentile, versus levels <34th percentile (trend, P<0.001). Corresponding odds ratios for OxPL-apo(a) were 1.2 (95% CI, 1.0–1.5), 1.2 (95% CI, 0.9–1.6), 2.1 (95% CI, 1.0–1.5), 1.2 (95% CI, 1.0–1.5), and 3.4 (95% CI, 2.1–5.5) for levels >95th percentile, versus levels <34th percentile (trend, P<0.001). These findings support the hypothesis that OxPL-apoB and lipoprotein(a) are individually associated with risk of CAVD.

Figure 1. Concentration distributions of oxidized phospholipids apolipoprotein B (OxPL–apoB) and lipoprotein(a) in controls and cases. One lipoprotein(a) measurement of 327 mg/dL among cases was not included in the depicted lipoprotein(a) data (bottom right).
CI, 1.4–3.1), and 2.9 (95% CI, 1.9–4.5; trend, \( P < 0.001 \); Figure III in the online-only Data Supplement), and for lipoprotein(a) levels 1.1 (95% CI, 0.9–1.4), 1.2 (95% CI, 0.9–1.6), 2.0 (95% CI, 1.3–3.0), and 3.5 (95% CI, 2.2–5.6; trend, \( P < 0.001 \)). Overall, results were similar in age and sex adjusted and in multivariable-adjusted analyses. Figure 3 and Figure III in the online-only Data Supplement thus demonstrate the similar and stepwise associations of increasing levels of lipoprotein(a) and OxPL-apoB and OxPL-apo(a) with increasing risk of CAVD. On additional adjustment for lipid-lowering therapy, risk estimates for lipoprotein(a), OxPL-apoB, and OxPL-apo(a) remained significant although they were somewhat attenuated (Figures IV and V in the online-only Data Supplement) likely because of reverse causation as case status is highly associated with prescription of lipid-lowering therapy in this cross-sectional study. On additional adjustment for lipoprotein(a) levels, risk estimates for OxPL-apoB measurements lost statistical significance (trend, \( P = 0.14 \)) and vice versa (trend, \( P = 0.34 \)). In the present study, we found no association of elevated low-density lipoprotein (LDL) cholesterol with increased risk of CAVD (Figure VI in the online-only Data Supplement).

### Association of LPA Genotypes With OxPL-apoB and Lipoprotein(a) Levels

Low number of LPA KIV-2 repeats and minor allele carrier status for LPA rs10455872 were associated with high levels of OxPL-apoB (Figure 4A; both \( P < 0.001 \)) and OxPL-apo(a) (Figure VII in the online-only Data Supplement; both \( P < 0.001 \)) and with elevated lipoprotein(a) levels (Figure 4B; both \( P < 0.001 \)), the latter finding in accordance with previous results.\(^2\) Figure 4 and Figure VII in the online-only Data Supplement thus demonstrate that not only lipoprotein(a) plasma levels but also measurements of OxPL-apoB and OxPL-apo(a) are at least partly determined by common variation in the LPA gene. On analysis...

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**Figure 2.** Association of oxidized phospholipids apolipoprotein B (OxPL-apoB) with lipoprotein(a) levels in controls and cases. The best fit linear regression line is shown in grey. One lipoprotein(a) measurement of 327 mg/dL among cases was not included in the depicted data (bottom); the OxPL-apoB value in this individual was 22.0 nmol/L.
of variance, the \textit{LPA} KIV-2 genotype explained 24% and 35% of the variation in OxPL-apoB and OxPL-apo(a) levels, and 29% of the variation in lipoprotein(a) levels. For the \textit{LPA} rs10453798 genotype, the corresponding percentages were 26%, 34%, and 30%, respectively. Combined, the 2 \textit{LPA} genotypes explained 34%, 46%, and 39% of the variation in OxPL-apoB, OxPL-apo(a), and lipoprotein(a) levels, respectively.

**LPA Genotypes and Risk of CAVD**

Consistent with previous results, a low number of KIV-2 repeats and minor allele carrier status for rs10455872 was associated with higher risk of CAVD (Figure VIII in the online-only Data Supplement). On instrumental variable analysis, a doubling in genetically determined OxPL-apoB, OxPL-apo(a), and lipoprotein(a) levels was associated with odds ratios of CAVD of 1.18 (95% CI, 1.10–1.27), 1.09 (95% CI, 1.05–1.13), and 1.09 (95% CI, 1.05–1.14), respectively, comparable to the corresponding observational estimates of 1.27 (95% CI, 1.16–1.39), 1.13 (95% CI, 1.08–1.18), and 1.11 (95% CI, 1.06–1.17) for a doubling in OxPL-apoB, OxPL-apo(a), and lipoprotein(a) plasma levels (Figure 5; Figure IX in the online-only Data Supplement). Figure 5 and Figure IX in the online-only Data Supplement thus demonstrate that the higher levels of lipoprotein(a) in cases and controls and provided similar risk prediction. Notably, both assays rely on the same monoclonal antibody to detect OxPL, and this antibody preferentially detects the phosphocholine headgroup of specific OxPL covalently bound to apo(a) influenced by the lysine-binding site of KIV-10.5 The close correlation of OxPL-apoB and OxPL-apo(a) with lipoprotein(a) measurements in both cases and controls reflects that lipoprotein(a) is a carrier of OxPL while the higher risk of CAVD found in cases likely reflects the higher levels of OxPL-containing lipoprotein(a) in cases versus controls.

**Discussion**

The present case–control study conducted within the CGPS demonstrates an observational and genetic associations of OxPL-apoB and OxPL-apo(a) with risk of developing CAVD. Notably, this effect was independent of all other measured risk factors for CAVD, except for lipoprotein(a). Levels of OxPL-apoB, OxPL-apo(a), and lipoprotein(a) were closely correlated and could explain the effect in a similar fashion. This suggests that the risk of OxPL in mediating CAVD is mechanistically linked through its presence on lipoprotein(a). Furthermore, \textit{LPA} genotypes explained similar proportions of the total variation in plasma OxPL-apoB, OxPL-apo(a), and lipoprotein(a) levels, a novel finding, and instrumental variable analyses demonstrated higher risk of CAVD as a function of genetically higher levels. Taken together, these observations identify OxPL on lipoprotein(a) as a likely causal and genetic risk factor that may explain the association of lipoprotein(a) with CAVD.

In the present study, and despite some differences in the assays, the OxPL-apoB and OxPL-apo(a) assays generally demonstrated similar correlations with lipoprotein(a) levels in both cases and controls and provided similar risk prediction. Notably, both assays rely on the same monoclonal antibody to detect OxPL, and this antibody preferentially detects the phosphocholine headgroup of specific OxPL covalently bound to apo(a) influenced by the lysine-binding site of KIV-10.5. The close correlation of OxPL-apoB and OxPL-apo(a) with lipoprotein(a) measurements in both cases and controls reflects that lipoprotein(a) is a carrier of OxPL while the higher risk of CAVD found in cases likely reflects the higher levels of OxPL-containing lipoprotein(a) in cases versus controls.
CAVD and atherosclerosis have many common mechanisms, although these are not entirely overlapping, because only ≈50% of patients with CAVD have concomitant obstructive coronary artery disease. Data from in vitro, animal, and large genetic association studies have provided ample support for proatherogenic and prostenotic effects of elevated lipoprotein(a) levels and possibly also for a prothrombotic effect at extreme levels. Furthermore, a large body of evidence suggests that a notable proportion of the atherogenicity of lipoprotein(a) may be mediated through its content of OxPL. A potential mechanism defining the ability of lipoprotein(a) and OxPL to induce CAVD includes the ability of lipoprotein(a) in binding to exposed or denuded valve surfaces through its potent lysine-binding site. In patients with elevated lipoprotein(a) levels enriched in OxPL, lipoprotein(a) may attach tightly to exposed valve surfaces, where it may then induce chronic inflammation and calcification of valvular cells through its associated OxPL leading to the progression of CAVD. Pathological studies of vulnerable human coronary and carotid plaques and explanted human valves have demonstrated an increased content of lipoprotein(a) and OxPL and oxidized LDL. However, elevated lipoprotein(a) levels seem not to be causally associated with increased low-grade inflammation as measured through C-reactive protein levels, and in the present study, adjustment for high-sensitivity C-reactive protein levels did not attenuate risk estimates. Nonetheless, recent data indicate that elevated lipoprotein(a) levels are indeed associated with increased arterial inflammation and enhanced peripheral blood mononuclear cells trafficking to the arterial wall, at least partly mediated through its OxPL content. Furthermore, lipoprotein(a)-associated autotaxin has recently been implicated in CAVD development through conversion of lysophosphatidylcholine to lysophosphatidic acid that promotes inflammation and mineralization of the aortic valve. In summary, these effects of OxPL and lipoprotein(a) may theoretically all contribute to CAVD development characterized by stages of lipid deposition, inflammation, fibrosis, and calcification eventually leading to symptomatic stenosis.
Although the association of lipoprotein(a) as a risk factor for CAVD was previously suggested,\textsuperscript{29,38} it was not until the recent meta-analysis of data from genome-wide association studies by Thanassoulis et al\textsuperscript{1} that a potential genetic and causal association was identified that appeared to be clinically relevant. The \textit{LPA} rs10455872 intron single nucleotide polymorphism was the only single nucleotide polymorphism that reached genome-wide significance for risk of aortic valve calcification and stenosis.\textsuperscript{1} We and others have since demonstrated a clear association of elevated lipoprotein(a) levels with increased risk of CAVD in prospective general population studies and provided further genetic data in support of a causal association of lifelong high lipoprotein(a) levels with increased risk of CAVD.\textsuperscript{2,3} The present study shows, for the first time in an epidemiological cohort without prior CAVD, the potential role of OxPL in the development of CAVD, and findings are pathophysiologically consistent with recent data showing that patients with pre-existing CAVD and elevated OxPL-apoB or OxPL-apo(a) and lipoprotein(a) levels have much faster progression of CAVD and more frequent need for aortic valve replacement.\textsuperscript{15}

Another important observation in this study was that there was no association of LDL cholesterol and CAVD after multivariable adjustment, including the use of lipid-lowering therapy. This is consistent with 4 randomized statin trials in patients with pre-existing CAVD where significant LDL cholesterol lowering did not affect echocardiographically determined progression of CAVD.\textsuperscript{39-42} suggesting LDL is unlikely to have a major causal association with either the development of CAVD, as shown in this study, or the progression of pre-existing CAVD as shown in these trials. Finally, in the ASTRONOMER trial (Aortic Stenosis Progression Observation: Measuring Effects of Rosuvastatin), rosuvastatin actually increased lipoprotein(a) and OxPL-apoB levels, whereas they did not change in patients on placebo. If indeed these are causal mediators, their increase may have negated any effect of LDL cholesterol lowering.

We applied a Mendelian randomization study design where association of genotypes, affecting a putative causal risk factor, with risk of disease may be taken as evidence of causality.\textsuperscript{17} The argument for causality is based on the fact that associations of genotypes with disease are generally unconfounded, because genotypes are distributed independent of environmental and lifestyle factors in homogenous populations, and may not result from reverse causality, as genotypes are invariant and not affected by disease status. Limitations of Mendelian randomization studies\textsuperscript{43} include genetic confounding and false-positive findings if the examined genotypes are in linkage disequilibrium with other genetic variation affecting disease. In the present study, the stepwise association of KIV-2 repeat genotype with risk of CAVD makes genetic confounding seem highly unlikely. Furthermore, pleitropic effects of genetic variants can make it difficult to be certain which intermediate parameter is causing the effect of the genotype on outcome, illustrated in the present study by both lipoprotein(a) per se and OxPL-apoB or OxPL-apo(a) being causally associated with CAVD. To the best of our knowledge, there are no known genetic variants that affect only OxPL, thus, clinical trials of, for example, lipoprotein(a) lowering

![Figure 5. Risk of calcific aortic valve disease per doubling in oxidized phospholipids apolipoprotein B (OxPL-apoB) or lipoprotein(a) levels in observational and genetic (instrumental variable) analyses. CI indicates confidence interval; OR, odds ratio; and RR, relative risk.](http://atvb.ahajournals.org/)

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**Table 1:**

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<th>OR/RR(95%CI)</th>
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<tr>
<td><strong>Observational</strong></td>
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<tr>
<td>OxPL-apoB (N = 2121)</td>
<td>1.27(1.16-1.39)</td>
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<tr>
<td>Lipoprotein(a) (N = 2121)</td>
<td>1.11(1.06-1.17)</td>
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<td><strong>Genetic</strong></td>
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<td><strong>OxPL-apoB</strong></td>
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<tr>
<td>LPA KIV-2 percentile groups (N = 2129)</td>
<td>1.15(1.05-1.25)</td>
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<tr>
<td>LPA rs10455872 (N = 2132)</td>
<td>1.21(1.11-1.32)</td>
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<tr>
<td>LPA genotypes combined (N = 2129)</td>
<td>1.10(1.10-1.27)</td>
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<td><strong>Lipoprotein(a)</strong></td>
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<td>LPA KIV-2 percentile groups (N = 2129)</td>
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<tr>
<td>LPA genotypes combined (N = 2129)</td>
<td>1.09(1.05-1.14)</td>
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versus inactivation of OxPL are needed to provide final proof for OxPL being the key biological variable in inducing CAVD.

Another limitation of this study is the inclusion of exclusively white individuals of Danish descent, which may limit the generalizability of our results. However, the inclusion of genetically homogenous individuals in the present study represents a strength in Mendelian randomization studies by minimizing risk of population stratification and false genetic associations.53

In conclusion, the current study identifies OxPL on lipoprotein(a) as a genetic and likely causal risk factor for CAVD. This finding suggests novel therapeutic approaches to halt the development or progression of CAVD, where no medical therapy exists today.36,37 Novel therapies may be specifically directed at lowering lipoprotein levels and their associated OxPL, as recently shown with an antisense oligonucleotide that lowers both lipoprotein(a) and OxPL-apoB and OxPL-apo(a) by >80%58 or by directly inactivating OxPL with specific antibodies.5,11

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Disclosures

P.R. Kamstrup has received lecture honoraries or consultancy fees from Fresenius Medical Care and Sanofi-Aventis/Regeneron. B.G. Nordestgaard has received consultancy fees or lecture honoraries from Sanofi-Aventis/Regeneron, Fresenius, Ionis Pharmaceuticals, Merck, AstraZeneca, Omthera, Aegerion, B. Braun, Dezzma, Kaneka, Kowa, and Amgen. S. Tsimikas and J.L. Witztum are coinventors and receive royalties from patents held by University of California San Diego (UCSD) on oxidation-specific antibodies and of biomarkers related to oxidized lipoproteins. S. Tsimikas currently has a dual appointment with UCSD and as an employee of Ionis Pharmaceuticals. J.L. Witztum is a consultant to Ionis, Cymbay, Intercept, and Prometheus Pharmaceuticals. The other author reports no conflicts.

References


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Oxidized Phospholipids and Aortic Valve Disease

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**Highlights**

- The present study shows, for the first time in an epidemiological cohort without prior calcific aortic valve disease (CVD), the potential role of oxidized phospholipids on apolipoprotein B or on apolipoprotein(a) in the development of CVD.
- This study demonstrates a genetic association of oxidized phospholipids on apolipoprotein B or on apolipoprotein(a) with future risk of developing CVD.
- This study identifies oxidized phospholipids on apolipoprotein B or on apolipoprotein(a) as a likely causal risk factor for CVD that may explain the association of lipoprotein(a) with CVD.
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DETAILED MATERIALS AND METHODS

Participants

A case-control study was designed from among 87980 participants in the Copenhagen General Population Study (CGPS), a general population study initiated in 2003 that is actively recruiting subjects. CGPS examinations included a self-administered questionnaire reviewed by an investigator on the day of attendance, a physical examination, and blood sampling including a sample for DNA analysis. Smokers were active smokers, and diabetes mellitus was diagnosed if self-reported, if insulin or oral hypoglycemic drugs were used, or in the presence of a non-fasting plasma glucose of >11 mmol/L. Body mass index was weight in kilograms divided by height in meters squared.

For the present study, we included all CGPS participants diagnosed with CAVD from 1977 until 2013, and additionally for each CAVD case participant, two matched control participants, dependent on available blood samples (N=2138). LPA KIV-2 genotype was available on 2129 and rs10455872 genotype on 2132 of these participants. CAVD was defined by International Classification of Diseases, 8th edition (ICD-8), codes 424.10, 424.12, 424.18, 424.19, and 10th edition (ICD-10) codes I35.0 and I35.2 and ascertained from the national Danish Patient Registry, and the national Danish Causes of Death Registry; public registers to which all hospitalizations and deaths in Denmark have been reported since 1977 (with outpatients and emergency treatments included from 1995). CAVD cases in the present study were diagnosed from 1982 to 2013, with the large majority diagnosed according to ICD-10 criteria using Doppler echocardiography and according to standard diagnostic criteria following international guidelines (peak transvalvular velocity >2.5 m/s, calculated aortic valve area <2.0 cm2). Controls were free of ischemic cardiovascular disease to exclude undiagnosed early CAVD mistaken for atherosclerotic disease, as symptoms of CAVD including chest pain, palpitations and shortness of breath are also all found in heart disease resulting from ischemic cardiovascular disease and atherosclerosis. Controls were matched to CAVD cases on sex, age (5-year strata), and recruitment-time (1 month strata), and selected randomly by computer from all CGPS participants free of cardiovascular disease at the end of follow-up in 2013.

The CGPS was approved by Herlev Hospital and by a Danish ethical committee, and was conducted according to the Declaration of Helsinki. Participants gave written informed consent.

Laboratory analyses

For the present study, measurements of OxPL-apoB and OxPL-apo(a), as well as lipoprotein(a) measurements were performed on blood samples stored at -80°C from the date of participant recruitment. Importantly, cases and controls were matched on recruitment date (1 month strata) to ensure comparable storage time of samples.

OxPL-apoB and OxPL-apo(a) levels were measured with a chemiluminescent immunoassay using the murine monoclonal antibody E06 that recognizes the phosphocholine (PC) group on oxidized but not on native phospholipids. E06 similarly recognizes the PC covalently bound to bovine serum albumin (BSA) in PC-BSA. A 1:50 dilution of plasma in 1% BSA in TBS was added to microtiter wells coated with the apoB-100 specific monoclonal antibody MB47, which binds a saturating amount of apoB-100 to each well, and biotinylated E06 was then added to determine the content of OxPL-apoB. These values are reported as nanomolar (nmol/L) PC-OxPL using a standard curve of nM PC equivalents, as recently described. Because each well contains equal numbers of apoB-100 particles, the OxPL-apoB value reflects the absolute content of OxPL per a constant amount of captured apoB lipoprotein. It thus represents an OxPL-apoB value that is independent of plasma levels of apoB-100 or of LDL cholesterol. Furthermore, the assay detects only the subset of OxPL detected by antibody E06 and not all species of OxPL. In prior studies, OxPL-apoB has been shown to be independently associated with the risk of CAVD, particularly for young individuals with CAVD. This finding supports the hypothesis that OxPL-apoB may be a biomarker of CAVD risk. The use of OxPL-apoB as a biomarker of CAVD risk has been supported by several studies, which have reported associations between OxPL-apoB levels and CAVD risk in various populations. Therefore, the measurement of OxPL-apoB in the present study provides valuable insights into the role of OxPL-apoB as a biomarker of CAVD risk.

Laboratory analyses

For the present study, measurements of OxPL-apoB and OxPL-apo(a), as well as lipoprotein(a) measurements were performed on blood samples stored at -80°C from the date of participant recruitment. Importantly, cases and controls were matched on recruitment date (1 month strata) to ensure comparable storage time of samples.

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this variable was expressed as OxPL/apoB, reflecting the fact that this measure quantitates the number of OxPL moles per unit mass of apolipoprotein B-100 present on microtiter well plates (and not the level in the circulation). The nomenclature is now changed to OxPL-apoB to minimize confusion that this measure represents a ratio of OxPL divided by plasma levels of apoB. Within-person 5-year reproducibility of frozen samples has been shown to be high ($r=0.78$) and pilot-tests showed that OxPL-apoB levels are stable over 24 hours on ice (intraclass correlation coefficient 0.96) as well as frozen samples stored under long term conditions. OxPL-apo(a) levels were measured in an analogous manner to OxPL-apoB, except that the capture antibody, LPA4, which detects apolipoprotein(a) was used to capture apo(a). The wells were coated with anti-apo(a) antibody, LPA4, plasma was added to saturate the plate with apo(a), and OxPL were then measured on the captured Lp(a) by the use of antibody E06, which only reacts with OxPL and not with the LPA4 antibody. The values are also reported in nmol/L. Please note that the absolute amounts of apoB-100 and apo(a) captured on the wells in the respective assays are different and therefore one can not directly compare the absolute OxPL-apoB and OxPL-apo(a) values.

Lipoprotein(a) total mass was measured on the same frozen samples using the commercial, immunoturbidimetric, isoform-insensitive Denka Seiken assay (Denka Seiken, Tokyo, Japan).

At the time of initial CGPS recruitment and blood sampling, enzymatic assays were used on fresh samples to measure plasma levels of total cholesterol and high-density lipoprotein cholesterol. Likewise, high-sensitivity C-reactive protein (hs-CRP) was measured on fresh samples using turbidimetry (Dako) or nephelometry (Dade Behring), and creatinine was measured using the Jaffe method with subsequent estimation of the glomerular filtration rate (eGFR) ad modum CKD-EPI.

The LPA KIV-2 repeat polymorphism was genotyped by real-time PCR analysis on the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) or on the CFX384 Real-time System (Bio-Rad) platform yielding comparable estimates of the sum of repeats on both alleles. Genotyping resulted in an estimate of the total number (sum of repeats on both alleles) of KIV-2 repeats. The single-copy gene albumin was used to normalize for different concentrations of DNA in different samples. Reactions were performed in 10 µL final volume, using 1xTaqMan Universal PCR Master Mix (Applied Biosystems), 900 nmol/L primers, and 200 nmol/L probe. Primer sequences were as follows: KIV-2 forward 5'-ATCCAGATGCTGTGGCAGCT-3', KIV-2 reverse 5'-GGCACGGCAGTCCCTTCTTCTTCT-3', albumin forward 5'-AACACGCCTTTGGCAACAATG-3', albumin reverse 5'-CCTTGGAAATAGGGCAAGCTA-3'. The sequence for the FAM labeled KIV-2 probe was 5'-ACAACGTGACGGCAATGC-3', while the sequence for the VIC labeled albumin probe was 5'-TGGTTAACCTTTATTTCAATGG-3'. All samples were run in duplicate for both the KIV-2 assay and the albumin assay. A discrepancy of more than 0.25 in Ct value (threshold cycle of the PCR) for duplicate samples for either the KIV-2 or the albumin assay resulted in a rerun of that sample. The LPA rs10455872 SNP was TaqMan genotyped and in Hardy-Weinberg equilibrium. SNP carriers were minor allele homozygotes and heterozygotes combined; 0.4% and 16%, respectively.

Statistical analyses

We used Stata SE 13.1. A two-sided $p<0.05$ was considered significant. Kruskal-Wallis and Chi-square tests were used to compare continuous and categorical variables. Spearman’s rank correlation coefficient was used to estimate the linear associations between OxPL-apoB and OxPL-apo(a) and lipoprotein(a) levels. One-way analysis of variance (ANOVA) was used to estimate the contribution of the LPA genotypes to the variation in OxPL-apoB, OxPL-apo(a), and lipoprotein(a) levels (all log transformed due to skewness of the distributions). Cuzick non-parametric test for trend was used to test for differences in OxPL-apoB, OxPL-apo(a), and lipoprotein(a) levels across LPAGenotypes.
For further analyses, participants were divided into groups based on tertiles of OxPL-apoB, OxPL-apo(a), or lipoprotein(a) levels, and with further top tertile stratification to better examine the risk associated with extreme levels (i.e. 90th-95th and >95th percentiles). Due to the lack of standardization of Lp(a) assays, results are reported according to percentile cutpoints (and mg/dL) to ensure comparability with other studies using different Lp(a) assays and consistent with common practice. For genetic analyses, participants were divided into groups based on KIV-2 repeat percentile groups corresponding to plasma levels percentile groups, or based on rs10455872 carrier status.

We used conditional logistic regression analyses to estimate odds ratios with 95% confidence intervals. Cases and (if possible) two controls were matched perfectly for sex, age (5-year strata), and recruitment time (1 month strata), the latter to ensure similar storage time for samples from cases and controls. Analyses included only appropriately matched cases and controls. In multivariable adjusted analyses, we additionally adjusted for cardiovascular risk factors, i.e. total cholesterol, high-density lipoprotein cholesterol, systolic blood pressure, body mass index, estimated glomerular filtration rate (eGFR), high-sensitivity C-reactive protein (hs-CRP), smoking, and diabetes mellitus. Total cholesterol values were adjusted for the lipoprotein(a) contribution, as done previously. Further, to avoid possible non-linearity in the logit, continuous covariates were separated into eight categories. Information on covariates adjusted for were ~98% complete. For the relatively few participants who lacked information, continuous covariate values were imputed using multiple imputation based on age and sex, and for categorical variables a category for missing was defined. If only individuals with complete data were included, results were similar to those presented. We found no evidence of interaction with sex for Lp(a), OxPL, or LPA measurements on risk of AVS when comparing models with and without 2-factor interactions using maximum likelihood ratio tests; thus, results are presented combined for men and women to maximize statistical power.

Instrumental variable analysis (i.e. a Mendelian randomization analysis integrating the association of LPA genotypes with OxPL-apoB, OxPL-apo(a), or lipoprotein(a), and the association of genotypes with risk of CAVD) based on each of the 2 LPA genotypes separately, or based on the genotypes combined, was used to estimate causal relative risk estimates of CAVD for a doubling in OxPL-apoB, OxPL-apo(a), or lipoprotein(a) levels. We used an individual participant data approach, thus including only participants with complete information on genotypes and OxPL-apoB, OxPL-apo(a), or lipoprotein(a) levels. First, we estimated the strength of the genotypes as an instrumental variable (i.e. the association of genotype with OxPL-apoB, OxPL-apo(a), or lipoprotein(a) levels) by conducting a least-squares regression of levels on genotypes and examining the F-statistics, where F>10 indicates sufficient statistical strength (F-values ranged from 155 to 1086). Second, a causal relative risk was estimated using the multiplicative generalized method of moments estimator implemented in the user-written Stata command "ivpois", which for a binary outcome (i.e. CAVD) estimates the causal relative risk for a unit change in phenotype (i.e. a doubling in OxPL-apoB, OxPL-apo(a), or lipoprotein(a) levels). For comparison with genetic estimates, we also estimated the observational multivariable adjusted odds ratio of CAVD for a doubling in OxPL-apoB, OxPL-apo(a), or lipoprotein(a) levels.
REFERENCES


(13) Kamstrup PR, Tybjaerg-Hansen A, Steffensen R, Nordestgaard BG. Genetically elevated lipoprotein(a) and increased risk of myocardial infarction. *JAMA* 2009;301:2331-2339.


SUPPLEMENTAL FIGURES AND TABLES

Supplemental Figure I
Supplemental Figure II
Supplemental Figure III
Supplemental Figure IV
Supplemental Figure V
Supplemental Figure VI
Supplemental Figure VII
Supplemental Figure VIII
Supplemental Figure IX
Supplemental Table I
Supplemental Figure I. Concentration distributions of OxPL-apo(a) and lipoprotein(a) in controls and cases. One lipoprotein(a) measurement of 327 mg/dL among cases was not included in the depicted data (lower right panel).
Supplemental Figure II. Association of OxPL-apo(a) with lipoprotein(a) levels in controls and cases.
The best fit linear regression line is shown in grey. One lipoprotein(a) measurement of 327 mg/dl among cases was not included in the lower graph; the OxPL/apo(a) value in this individual was 82.8 nM.
Supplemental Figure III. Risk of calcific aortic valve disease as a function of OxPL-apo(a) or lipoprotein(a) levels. Analyses were adjusted for age and sex or multivariable adjusted. Analyses excluded cases without matched controls, and vice versa (N=17).
Supplemental Figure IV. Risk of calcific aortic valve disease as a function of OxPL-apoB or lipoprotein(a) levels. Analyses were adjusted for age and sex or multivariable adjusted incl. adjustment for lipid lowering therapy. Analyses excluded cases without matched controls, and vice versa (N=17).
Supplemental Figure V. Risk of calcific aortic valve disease as a function of OxPL-apo(a) or lipoprotein(a) levels. Analyses were adjusted for age and sex or multivariable adjusted incl. adjustment for lipid lowering therapy. Analyses excluded cases without matched controls, and vice versa (N=17).
Supplemental Figure VI. Risk of calcific aortic valve disease as a function of LDL-cholesterol.
Analyses were adjusted for age and sex or multivariable adjusted including adjustment for HDL cholesterol, systolic blood pressure, diabetes, smoking and the use of lipid lowering therapy. LDL cholesterol was available in 2125 study participants, however, analyses excluded cases without matched controls, and vice versa (N=21).
Supplemental Figure VII. OxPL-apo(a) levels as a function of LPA genotypes. Boxes show median and interquartile range (also given in numbers) and error bars depict the 10th and 90th percentiles. P values were obtained from Cuzick nonparametric test for trend or from Wilcoxon test.
Supplemental Figure VIII. Risk of calcific aortic valve disease as a function of LPA genotypes. Analyses were adjusted for age and sex or multivariable adjusted for total cholesterol, high-density lipoprotein cholesterol, systolic blood pressure, smoking, and diabetes mellitus. Analyses excluded cases without matched controls, and vice versa (N=22 for KIV-2 and N=21 for rs10455872). Abbreviations: OR, odds ratio; CI, confidence interval.
Supplemental Figure IX. Risk of calcific aortic valve disease per doubling in OxPL-apo(a) or lipoprotein(a) levels in observational and genetic (instrumental variable) analyses. Abbreviations: OR, odds ratio; CI, confidence interval; RR, relative risk.

<table>
<thead>
<tr>
<th></th>
<th>Observational</th>
<th>Genetic</th>
<th>OR/RR(95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-OxPL-apo(a)</td>
<td>(N = 2121)</td>
<td>P-OxPL-apo(a)</td>
<td>1.13(1.08-1.18)</td>
</tr>
<tr>
<td>P-lipoprotein(a)</td>
<td>(N = 2121)</td>
<td></td>
<td>1.11(1.06-1.17)</td>
</tr>
</tbody>
</table>

**Genetic**

**OxPL-apo(a)**

<table>
<thead>
<tr>
<th></th>
<th>(N = 2129)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LPA KIV-2 percentile groups</td>
<td></td>
<td></td>
<td>1.07(1.02-1.12)</td>
</tr>
<tr>
<td>LPA rs10455872</td>
<td>(N = 2132)</td>
<td></td>
<td>1.10(1.06-1.15)</td>
</tr>
<tr>
<td>LPA genotypes combined</td>
<td>(N = 2129)</td>
<td></td>
<td>1.09(1.05-1.13)</td>
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</tbody>
</table>

**Lipoprotein(a)**

<table>
<thead>
<tr>
<th></th>
<th>(N = 2129)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LPA KIV-2 percentile groups</td>
<td></td>
<td></td>
<td>1.08(1.02-1.13)</td>
</tr>
<tr>
<td>LPA rs10455872</td>
<td>(N = 2132)</td>
<td></td>
<td>1.11(1.06-1.15)</td>
</tr>
<tr>
<td>LPA genotypes combined</td>
<td>(N = 2129)</td>
<td></td>
<td>1.09(1.05-1.14)</td>
</tr>
</tbody>
</table>

Odds ratio/relative risk (95% CI) per doubling in OxPL-apo(a) or lipoprotein(a) levels
**Supplemental Table I. Baseline characteristics of participants stratified by LPA genotype.**

<table>
<thead>
<tr>
<th></th>
<th>LPA KIV-2 percentile</th>
<th>LPA rs10455872</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-5</td>
<td>6-10</td>
</tr>
<tr>
<td>No. individuals</td>
<td>108</td>
<td>105</td>
</tr>
<tr>
<td>Women, %</td>
<td>40</td>
<td>42</td>
</tr>
<tr>
<td>Age, years</td>
<td>74(67-79)</td>
<td>73(67-78)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.3(4.8-6.3)</td>
<td>5.7(5.1-6.4)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.5(1.2-2.0)</td>
<td>1.6(1.3-1.9)</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>146(134-162)</td>
<td>141(130-157)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26(24-29)</td>
<td>26(24-28)</td>
</tr>
<tr>
<td>eGFR, mL/min</td>
<td>67(57-78)</td>
<td>68(58-77)</td>
</tr>
<tr>
<td>hs-CRP, mg/L</td>
<td>1.7(1.2-3.2)</td>
<td>1.7(1.2-3.0)</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Lipid lowering therapy, %</td>
<td>42</td>
<td>25</td>
</tr>
</tbody>
</table>

Continuous covariates are reported as median (interquartile range). Abbreviations: HDL, high density lipoprotein; BP, blood pressure; KIV-2, kringle IV type 2.