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Lipoprotein-Associated Phospholipase A2 activity, Genetics, and Calcific Aortic Valve Stenosis in Humans

Running title: Lp-PLA2 and calcific aortic valve stenosis

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Abstract

Background: Lipoprotein-associated phospholipase A2 (Lp-PLA2) activity has been shown to predict calcific aortic valve stenosis (CAVS) outcomes. Our objective was to test the association between plasma Lp-PLA2 activity and genetically-elevated Lp-PLA2 mass/activity with CAVS in humans.

Methods and results: Lp-PLA2 activity was measured in 890 patients undergoing cardiac surgery including 476 patients undergoing aortic valve replacement (AVR) for CAVS and 414 control patients undergoing coronary artery bypass grafting. After multivariable adjustment, Lp-PLA2 activity was positively associated with the presence of CAVS (OR=1.21 [95%CI, 1.04-1.41] per standard deviation increment). We selected four single nucleotide polymorphisms (SNPs) at the *PLA2G7* locus associated with either Lp-PLA2 mass or activity (rs7756935, rs1421368, rs1805017, and rs4498351). Genetic association studies were performed in 8 cohorts: Quebec-CAVS (1009 cases/1017 controls), UK Biobank (1350 cases/349,043 controls), EPIC-Norfolk (504 cases/20,307 controls), GERA (3469 cases/51,723 controls), Malmö Diet and Cancer Study (682 cases/5963 controls) and 3 French cohorts (3123 cases/6532 controls) totalising 10,137 CAVS cases and 434,585 controls. A fixed-effect meta-analysis using the inverse-variance weighted method revealed that none of the four SNPs were associated with CAVS (OR=0.99 [95%CI, 0.96-1.02, p=0.55] for rs7756935, 0.97 [95%CI, 0.93-1.01, p=0.11] for rs1421368, 1.00 [95%CI, 1.00-1.01, p=0.29] for rs1805017, and 1.00 [95%CI, 0.97-1.04, p=0.87] for rs4498351).

Conclusions: Higher Lp-PLA2 activity is significantly associated with the presence of CAVS and might represent a biomarker of CAVS in patients with heart disease. Results of our genetic association study suggest that Lp-PLA2 is however unlikely to represent a causal risk factor or therapeutic target for CAVS.

Keywords: Lp-PLA2 activity, PLA2G7, Calcific Aortic Valve Stenosis, Coronary Artery Disease

Key messages

What is already known about this subject? Lipoprotein-associated phospholipase A2 (Lp-PLA2) activity has been shown to predict calcific aortic valve stenosis (CAVS) outcomes.

What does this study add? The results of our observational study suggest that Lp-PLA2 activity could represent an independent biomarker for CAVS. However, we show for the first time that genetic variants influencing Lp-PLA2 mass or activity are not associated with CAVS in this large genetic association study.

How might this influence on clinical practice? Lp-PLA2 might represent a biomarker of CAVS presence. Results of our genetic association study suggest that inhibition of Lp-PLA2 activity might not be beneficial for CAVS prevention and/or treatment.

Introduction

Calcific aortic valve stenosis (CAVS) is the most common form of heart valve disease and currently affects almost 3% of the population older than 65.^{1,2} Replacement of the aortic valve is the only effective treatment for CAVS. Similar to atherosclerosis or coronary artery disease (CAD), the molecular mechanisms that initiate CAVS include infiltration of oxidized lipids, lipoproteins and inflammatory cells as well as extra-cellular matrix remodeling, degradation and calcification. CAD and CAVS also share similar clinical risk factors. In a large population-based study, we showed that risk factors associated with poor cardiovascular health (an unhealthy diet, physical inactivity, smoking, obesity, type 2 diabetes, hypertension and hypercholesterolemia), often referred to as Life's Simple 7, are associated with CAVS incidence.³ Despite the important overlap between pathobiological mechanisms and clinical risk factors between CAD and CAVS, traditional CAD treatments have so far been ineffective in treating CAVS. Low-density lipoprotein (LDL) cholesterol-lowering therapies such as statins and ezetimibe had no effect in the prevention and treatment of CAVS in previous studies.⁴⁻⁶ Whether other cardiovascular drugs under development could be effective for the treatment of CAVS is unknown.

Lipoprotein-associated phospholipase A2 (Lp-PLA2) was suggested as a driver of aortic valve calcification.⁷ In a prospective observational study of patients with mild-to-moderate CAVS, Lp-PLA2 activity was shown to be a predictor of CAVS progression.⁸ An important proportion of Lp-PLA2 in the bloodstream can be found on LDL and Lipoprotein(a) (Lp[a]) particles and both elevated LDL cholesterol and Lp(a) levels are potentially causal risk factors for the development of CAVS.⁹⁻¹² However, the causality of Lp-PLA2 in the etiology of CAVS has not been established and whether Lp-PLA2 inhibitors such as darapladib could be used for the prevention and/or treatment of CAVS is unknown. On the other hand, the causality of Lp-PLA2 in cardiovascular disease (CVD) prevention was severely questioned following the publication of two large trials showing no effect of darapladib treatment on cardiovascular events and by the lack of association between genetic variant influencing Lp-PLA2 mass/activity on CVD.¹³

The objective of this study was to assess the relationship between Lp-PLA2 activity and the presence of CAVS in patients with documented heart disease undergoing cardiac surgery. We also sought to determine whether the relationship between Lp-PLA2 and CAVS risk was potentially causal by examining genetic variants at the *PLA2G7* locus associated with circulating Lp-PLA2 mass or activity in patients undergoing cardiac surgery as well as in the general population.

Methods

Laboratory analyses and study population (biomarker study)

Lp-PLA2 activity was measured by a commercial colorimetric activity method (Platelet-activating factor acetylhydrolase (PAF-AH) assay kit, Cayman Chemical, Ann Arbor, Michigan) in patients undergoing heart surgery at the *Institut universitaire de cardiologie et de pneumologie de Québec* (IUCPQ), Québec, Canada. Briefly, the assay uses 2-thio PAF, which serves as a substrate for all PAF-AHs. Upon hydrolysis of the acetyl thioester bond at the *sn*-2 position by PAF-AH, free thiols are detected using 5,5'-dithio-*bis*-(2-nitrobenzoic acid) which absorbs 412 nm wavelength light. The intra- and inter-assay coefficient of variation of the assay were both below 10%. Only patients with a tricuspid aortic valve were selected in order to study a homogeneous population. These patients were compared to controls scheduled for a coronary artery bypass grafting who had no valvular heart disease (including no aortic sclerosis) at preoperative echocardiography. Patients with moderate to severe aortic and/or mitral valve regurgitation (grade >2) were excluded. Patients with a history of rheumatic disease, endocarditis, and inflammatory diseases were also excluded. The study protocol was approved by the Ethics Committee of the IUCPQ and all patients signed a written informed consent. Patients were not involved in the design of the study.

Study populations and single nucleotide polymorphism selection (genetic association study)

Genetic association studies were performed in 8 cohorts: Quebec-CAVS (1009 CAVS cases and 1017 controls), UK Biobank (1350 CAVS cases and 349,043 controls), EPIC-Norfolk (504 CAVS cases and 20,307 controls), GERA (3469 CAVS cases and 51,723 controls), Malmö Diet and Cancer Study (682 CAVS cases and 5963 controls) and 3 French cohorts (3123 CAVS cases and 6532 controls) totalising 10,137 CAVS cases and 434,585 controls. The cohorts are described in details in the Supplement. SNPs were selected based on their association with Lp-PLA2 mass or activity levels in the study of Grallert *et al.*¹⁴ This meta-analysis included data from five community-based cohorts from the US and Europe as part of the Cohorts for Heart and Aging Research in Genetic Epidemiology (CHARGE) consortium, including 13,664 subjects. Lp-PLA2 activity was assessed in the Cardiovascular Health Study (CHD), Framingham Heart Study (FHS) and Rotterdam Study (RS) studies using a colorimetric method (diaDexus CAM kit, diaDexus, Inc, San Francisco, CA, USA) or a radioactive method (Perkin Elmer Life Sciences, Inc, Waltham, MA, USA). Lp-PLA2 mass concentration was measured in the Atherosclerosis Risk in Communities (ARIC), CHD, FHS and Cooperative Health Research in the Region of Augsburg Study (KORA) studies using a commercial sandwich enzyme immunoassay (PLAC® test, diaDexus, Inc, San Francisco, CA, USA). Genotyping was performed using high-throughput assays and genetic imputation was performed to obtain results on >2.5 million SNPs. The SNP-phenotype association (Lp-PLA2 mass/activity) was performed using linear regression on log-transformed values in an additive model adjusted for age, sex and, if applicable, recruitment site. There was no sample overlap between the cohorts used to derive the exposure and the outcomes. We selected genome-wide significant and independent SNPs ($r^2 < 0.40$) at the *PLA2G7* locus to increase the probability of true effects (Table 1). We investigated the association between the 4 selected SNPs (2 associated with Lp-PLA2 mass and 2 associated with Lp-PLA2 activity) at the *PLA2G7* locus and CAVS in 8 cohorts (described in the supplementary data file). All study protocols were approved by local Ethics Committees and all patients provided informed consent.

Statistical analyses

The differences between biomarkers levels in patients with versus without CAVS were tested using one-way analyses of variance and unpaired t-tests. The association between measured Lp-PLA2 activity and the presence of CAVS was determined using logistic regression and odds ratios (OR) for an increment of one standard deviation unit before and after adjusting for cardiovascular risk factors (age, sex, body mass index, diabetes, smoking, lipid-lowering therapy use and creatinine levels). Lp-PLA2 activity was transformed into a z score ($z = (x - \text{mean}(x))/\text{sd}(x)$) to fit the logistic model. For each of the four selected SNPs, we used logistic regression models to verify their association with CAVS per-allele in each cohort. Models were adjusted for age, sex and the first 10 ancestry-based principal components when available. In the GERA cohort genetic variants were modelled using PLINK2 in logistic regression models adjusted for age, age squared, and sex. In the French cohorts, models were not adjusted for age. We performed a fixed-effect meta-analysis using the inverse-variance weighted method as implemented in the *rmeta* package (version 3.0) in R (version 3.3.1). Genetic study power was calculated using QUANTO v1.2.4 with a log-additive mode of inheritance using alleles frequencies provided in table 1, with the unmatched case-control ratio of our study cohorts and a two-tailed p-value threshold of 0.05. Statistical analyses were performed with R and SAS.

Results

Lp-PLA2 activity and calcific aortic valve stenosis

The clinical characteristics of the study participants included in the biomarker study are presented in Table 2. By design, the proportion of patients with CAD was higher in controls compared with cases. Mean Lp-PLA2 activity was higher in patients with CAVS compared to controls (Figure 1).

Table 3 presents the positive association between higher Lp-PLA2 activity and the presence of CAVS. The association between Lp-PLA2 activity and CAVS remained significant after adjusting for potential confounders (age, sex, body mass index, diabetes, smoking, lipid-lowering therapy use and creatinine levels).

Genetic variants at the PLA2G7 locus, calcific aortic valve stenosis and aortic valve calcium

The association between each SNP at the PLA2G7 locus and CAVS is presented in Figure 2. None of the Lp-PLA2 mass/activity raising SNPs were significantly associated with CAVS in the individual cohorts or in the meta-analysis. We found no evidence of heterogeneity across these meta-analyses (p-value for heterogeneity >0.05 for all). In addition, none of the selected SNPs showed a significant association with aortic valve calcification (AVC) assessed by CT and defined by an Agatston score >0 in 6942 participants from the CHARGE consortium: p-values for rs7756935, rs1421368, rs1805017 and rs4498351 were 0.15, 0.71, 0.99 and 0.052 respectively. To determine whether SNPs included in this analysis showed pleiotropic associations, we used the PhenoScanner^{15,16}, an online tool that provides the phenotypic spectrum of these SNPs. Results presented in Supplementary Table 1 suggest no association between these SNPs and traits (other than Lp-PLA2 mass/activity) at p-value <1,05E⁻⁵, with the

exception of rs1421368 associated with viral infections of unspecified site in the UK Biobank.

Discussion

Lp-PLA2 is an enzyme that hydrolyses oxidized phospholipids into lysophosphatidylcholine (lyso-PC), which is an important chemoattractant and pro-inflammatory mediator. Lp-PLA2 is secreted by inflammatory cells that is transported in the bloodstream by atherogenic lipoprotein particles such as LDL and Lp(a). Lp(a) is an important a likely causal risk factor for CAVS. On top of Lp-PLA2, Lp(a) transports oxidized phospholipids (Ox-PL) in the blood. Given the lack of a causal association between genetically-elevated Lp-PLA2 mass/activity and CAVS, we believe that Lp(a) might drive the initiation and progression of CAVS via mechanisms that are independent of Lp-PLA2, such as Ox-PL mediated inflammation and calcification.

Prospective observational studies have shown that circulating Lp-PLA2 mass and/or activity is associated with CAD risk.^{17, 18} For instance, in a meta-analysis that included more than 79,000 participants in 32 prospective studies, a continuous association between Lp-PLA2 activity and the risk of coronary heart disease was observed. In that study a relative increase in risk of 1.10 (95% IC, 1.05 to 1.16) for each 1-SD increase in Lp-PLA2 activity was observed after adjustment for conventional risk factors.¹⁹ The possibility that Lp-PLA2 might be causally related to atherosclerosis was further supported by treatment with the Lp-PLA2 inhibitor darapladib in a porcine model.²⁰ In that study, in diabetic pigs fed a hyperlipidemic diet, treatment with darapladib (n= 20) reduced the development of coronary atherosclerosis and inhibited the subsequent progression to advanced lesions, resulting in a more stable plaque phenotype compared to control pigs treated with placebo (n=17). A marked reduction in necrotic core development and a change in arterial lesion composition was observed following Lp-PLA2 inhibition. Following these promising observations, two large-scale cardiovascular outcomes trials that documented the impact of the Lp-PLA2 inhibitor darapladib on health outcomes were launched. In both studies however, treatment of patients with stable coronary heart disease (STABILITY trial)²¹ or acute coronary syndrome (SOLID-TIMI 52 trial)²² did not lead to reductions in cardiovascular outcomes, which has left many to suggest that Lp-PLA2 might simply represent a biomarker of, rather than a causal risk factor of CAD. This observation was further supported by the results of Mendelian randomization studies. Mendelian randomization relies on the random assortment of alleles during meiosis to divide a population into groups with and without an elevation in a risk factor. The random distribution of inherited variants affecting the risk factor minimizes the possibility that reverse causation or confounding is responsible for the association between the risk factor and the outcome. Mendelian randomization had “predicted the failure” of Lp-PLA2 inhibition for ACVD risk reduction by providing evidence that variants at the *PLA2G7* locus associated with lifelong reductions in Lp-PLA2 mass or activity were not associated with cardioprotection.^{23, 24}

Lp-PLA2 is involved in aortic valve mineralization. A recent transcriptomic study by Mahmut et al.⁷ revealed that the expression of Lp-PLA2 was increased more than 4-fold in calcified aortic valves compared to normal aortic valves in humans. In the same report, it was documented that

lyso-PC induced mineralization and apoptosis of the human valvular interstitial cells in vitro. A small study showed that Lp-PLA2 levels were higher in patients with CAVS compared to participants without CAVS.²⁵ Our group also showed that higher Lp-PLA2 activity was associated with the progression of CAVS⁸ and the structural degeneration of bioprostheses.²⁶

The present study shows that elevated Lp-PLA2 activity is associated with CAVS, even when compared to patients with documented CAD, thereby suggesting that Lp-PLA2 activity might be a stronger marker of the presence of CAVS compared to CAD. However, we found no association between genetic variants at the *PLA2G7* locus that are associated with Lp-PLA2 mass or activity and CAVS in 8 cohorts. Consequently, Lp-PLA2 mass or activity is unlikely to represent a causal risk factor or therapeutic target for CAVS (Figure 3).

Previous Mendelian randomization studies on CAD have used rare loss-of-function mutations with large effect on Lp-PLA2 activity (up to 94%) and/or common variants with modest-impact on the risk factor. Limitations of our study include the low absolute effects on Lp-PLA2 activity/mass of our selected SNPs. However, the per-allele effects of the two SNPs, rs7756935 and rs1421368, associated with Lp-PLA2 activity used in the genetic study correspond respectively to 34% and 43% of the difference of Lp-PLA2 activity observed between cases and controls in our biomarker study. It is also worth mentioning that the effect of these variants on Lp-PLA2 mass or activity are lifelong effects. As CAVS is a less prevalent medical condition than ACVD, and mutations with large effects on Lp-PLA2 are extremely rare, we could not use genetic instruments with a large effect on the biomarker. To our knowledge, our present study has the largest number of CAVS cases collected in a genetic association study. Our sample size had 80% power to detect per-allele odds ratios for CAVS of 1.05, 1.07, 1.05 and 1.04 respectively for rs7756935, rs1421368, rs1805017 and rs4498351. It must also be mentioned, however, that individuals included in this study were all of European ancestry. Therefore, caution is warranted prior to generalizing these findings to populations from other ethnic backgrounds. This limitation also applies to our observational study. Additional limitations of our observational study include the presence of a control group of patients with CAD and the fact that Lp-PLA2 activity, but not mass was measured. Although, the inclusion of our control of patients with CAD enabled us the study the association of Lp-PLA2 activity with CAVS possibly independently of the presence/absence of CAD, we believe that additional studies including healthy controls and measuring both Lp-PLA2 mass and activity would be required to confirm and extend our findings.

The present study further consolidates the strong association between elevated Lp-PLA2 activity and CAVS by reporting this association in a population with a high prevalence of ACVD. However, the lack of association between variants influencing Lp-PLA2 mass or activity and CAVS suggest that, as shown for CAD, elevated Lp-PLA2 is more likely to represent a biomarker for CAVS rather than a causal risk factor.

Contributorship statement

All authors have contributed to the manuscript either as substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data. All authors have contributed to the first draft the work or revising it critically for important intellectual content. They all gave final approval of the version published. The Corresponding Author has the right to grant on behalf of all authors and does grant on behalf of all authors, an exclusive licence on a worldwide basis to the BMJ Publishing Group Ltd and its Licensees to permit this article to be published in HEART editions and any other BMJPG products to exploit all subsidiary rights.

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Competing interests

BJA has received research funding from Pfizer, Merck and Ionis Pharmaceuticals and is a consultant for Novartis. PM is a consultant for Casebia Therapeutics.

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Table 1. Single nucleotide polymorphisms included in the genetic association study.

SNP	Position	Modelled allele	MAF	Beta (SE)
rs7756935	6:46675025	C	0.244	0.027(0.004)*
rs1421368	6:46696593	G	0.107	0.034(0.006)*
rs1805017	6:46684222	T	0.255	0.043(0.004) [#]
rs4498351	6:46676783	T	0.574	0.027(0.004) [#]

SNP indicates single nucleotide polymorphisms, MAF indicates modelled allele frequency from 1000 Genome European populations and SE indicates standard error. *Per allele difference in log Lp-PLA2 activity (nmol/min/mL). [#]Per allele difference in log Lp-PLA2 mass (ng/mL).

Table 2. Clinical characteristics of study participants.

	CAVS	CAD	P-Value
Number of patients	476	414	
Men	271 (57)	235 (57)	0.93
Age, years	72.0 (8.7)	71.1 (8.7)	0.10
Body mass index, kg/m ²	28.1 (5.2)	27.5 (4.3)	0.03
Active smokers	48 (10)	48 (12)	0.43
Coronary artery disease	244 (51)	414 (100)	<0.0001
Diabetes	140 (29)	126 (30)	0.75
Hypertension	339 (71)	291 (70)	0.54
Lipid-lowering therapy	306 (64)	341 (82)	<0.0001
Total cholesterol, mmol/L	4.35 (1.07)	4.06 (0.99)	<0.0001
LDL cholesterol, mmol/L	2.41 (0.91)	2.21 (0.80)	0.0004
HDL cholesterol, mmol/L	1.26 (0.36)	1.15 (0.34)	<0.0001
Apolipoprotein B, g/L	0.71 (0.21)	0.66 (0.20)	<0.0001
Triglycerides, mmol/L	1.51 (0.81)	1.56 (0.77)	0.30
Creatinine, µmol/L	96.6 (40.2)	91.9 (20.1)	0.02
Lp-PLA2, µmol/min/mL	0.017 (0.005)	0.016 (0.005)	<0.0001

Data are presented as means (SD) or N (%). CAVS indicates calcific aortic valve stenosis, CAD indicates coronary artery disease and Lp-PLA2 indicates lipoprotein-associated phospholipase A2.

Table 3. Association between Lp-PLA2 activity and CAVS risk.

	Per 1-SD Unit
Unadjusted	1.33 (1.16-1.52)*
Model 1	1.35 (1.17-1.55)*
Model 2	1.22 (1.05-1.42)*
Model 3	1.21 (1.04-1.41)*

Data are presented as odds ratios (95% confidence interval). SD indicates standard deviation. Model 1 is adjusted for age and sex. Model 2 is adjusted for age, sex, body mass index, diabetes, smoking and lipid-lowering therapy use. Model 3 is adjusted for age, sex, body mass index, diabetes, smoking, lipid-lowering therapy use and creatinine levels. *p<0.0001. (n= 890)

Figure Legends

Figure 1. Lp-PLA2 activity in patients with vs. without calcific aortic valve stenosis. * $p=4.3 \times 10^{-5}$

Figure 2. Meta-analysis of the per-allele association between four single nucleotide polymorphisms associated with Lp-PLA2 mass and/or activity and CAVS risk.

Figure 3. Higher Lp-PLA2 activity is significantly associated with the presence of CAVS in patients with heart disease, but variants influencing Lp-PLA2 mass or activity are not associated with CAVS in this large genetic association study.