Valvular Heart Disease

Autotaxin Derived From Lipoprotein(a) and Valve Interstitial Cells Promotes Inflammation and Mineralization of the Aortic Valve

Rihab Bouchareb, PhD*; Ablajan Mahmut, MD, MSc*; Mohamed Jalloul Nsaibia, MSc*; Marie-Chloé Boulanger, PhD; Abdellaziz Dahou, MD, MSc; Jamie-Lee Lépine; Marie-Hélène Laflamme; Fayezy Hadji, MSc; Christian Couture, MD, MSc; Sylvain Trahan, MD; Sylvain Pagé, MD; Yohan Bossé, PhD; Philippe Pibarot, PhD; Corey A. Scipione, BSc; Rocco Romanguolo, PhD; Marlys L. Koschinsky, PhD; Benoît J. Arsenaault, PhD; André Marette, PhD; Patrick Mathieu, MD

Background—Mendelian randomization studies have highlighted that lipoprotein(a) [Lp(a)] was associated with calcific aortic valve disease (CAVD). Lp(a) transports oxidized phospholipids with a high content in lysophosphatidylcholine. Autotaxin (ATX) transforms lysophosphatidylcholine into lysophosphatic acid. We hypothesized that ATX-lysophosphatidic acid could promote inflammation/mineralization of the aortic valve.

Methods and Results—We have documented the expression of ATX in control and mineralized aortic valves. By using different approaches, we have also investigated the role of ATX-lysophosphatidic acid in the mineralization of isolated valve interstitial cells and in a mouse model of CAVD. Enzyme-specific ATX activity was elevated by 60% in mineralized aortic valves in comparison with control valves. Immunohistochemistry studies showed a high level of ATX in mineralized aortic valves, which colocalized with oxidized phospholipids and apolipoprotein(a). We detected a high level of ATX activity in the Lp(a) fraction in circulation. Interaction between ATX and Lp(a) was confirmed by in situ proximity ligation assay. Moreover, we documented that valve interstitial cells also expressed ATX in CAVD. We showed that ATX-lysophosphatidic acid promotes the mineralization of the aortic valve through a nuclear factor κB/interleukin 6/bone morphogenetic protein pathway. In LDLR−/−/ApoB100/100/IGFII mice, ATX is overexpressed and lysophosphatic acid promotes a strong deposition of hydroxyapatite of calcium in aortic valve leaflets and accelerates the development of CAVD.

Conclusions—ATX is transported in the aortic valve by Lp(a) and is also secreted by valve interstitial cells. ATX-lysophosphatic acid promotes inflammation and mineralization of the aortic valve and thus could represent a novel therapeutic target in CAVD. (Circulation. 2015;132:677-690. DOI: 10.1161/CIRCULATIONAHA.115.016757.)

Key Words: aortic stenosis, calcific • aortic valve stenosis • autotaxin • calcific aortic valve disease • ENPP2 • lipoprotein(a) • lipoproteins, • lysophosphatidic acid • lysophosphatidylcholine

Mineralization of the aortic valve is the major culprit involved in the development of calcific aortic valve disease (CAVD). There is, so far, no medical treatment for CAVD. The main process that triggers pathological mineralization of the aortic valve remains elusive. Recently, 3 successive studies with a Mendelian randomization design have reported a significant association between the LPA gene variant (rs10455872), which genetically determines the lipoprotein(a) [Lp(a)] plasma level, and CAVD. These studies thus suggested a causal relationship between Lp(a) and CAVD risk. Lp(a) is a low-density lipoprotein (LDL)–like particle in which an apolipoprotein(a) is linked by a disulfide bridge to apolipoprotein B. Lp(a) is a major carrier of oxidized phospholipids (OxPLs) and has been associated

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with atherosclerosis. However, the mechanism(s) by which Lp(a) could promote the mineralization of the aortic valve is still unresolved. Mahmut et al showed that lipoprotein-associated phospholipase A2 (Lp-PLA2) is highly expressed in mineralized aortic valve and is related to several indices of tissue remodeling. Lp-PLA2 uses OxPLs, which are incidentally transported by Lp(a), as substrate and produces lysophosphatidylcholine (LPC). Also, through nonenzymatic pathways, the oxidation of LDLs generates LPC, a highly reactive metabolite with pro-osteoinductive properties and present in mineralized aortic valves. Autotaxin (ATX), encoded by the ENPP2 gene, is a lysophospholipase D enzyme that is transported in the blood plasma and secreted by different cell populations. ATX uses LPC as a substrate and produces lysophosphatidic acid, which has potent proinflammatory properties. The expression and role of ATX in CAVD has never been explored. In this work, we hypothesized that ATX may play an important role in mediating inflammation and mineralization of the aortic valve.

Methods

Expanded materials and methods sections are in the online-only Data Supplement.

Procurement of Tissues for Analyses

We examined stenotic aortic valves (CAVd) that were explanted from patients at the time of aortic valve replacement. The protocol was approved by the local ethical committee and informed consent was obtained from the subjects.

Valve Interstitial Cell Isolation

Human valve interstitial cells (VICs) were isolated from control non-mineralized aortic valves obtained from patients undergoing heart transplantation.

ATX Activity

Control nonmineralized and CAVD valve tissues were homogenized. The reaction was initiated with the addition of FS-3 (Echelon Biosciences, Salt Lake City, UT) at a final concentration of 3 μmol/L. The reaction was allowed to proceed at 37°C for 1 hour, and the fluorescence was monitored at 520 nm.

Western Blotting

Tissue pieces were mixed with lysis buffer. Membranes were blocked with TBS-tween containing 5% nonfat dry milk, incubated with ATX (Fisher ThermoScience, QC, Canada) and β-actin (Sigma-Aldrich, ON, Canada) antibodies overnight at 4°C. Membranes were then washed and incubated with horseradish peroxidase–labeled secondary antibodies (Cell Signaling Technology, Boston, MA).

Quantification of Lysophosphatidylcholine and Lysophosphatidic Acid by Thin Layer Chromatography

Aortic valve was homogenized for 30 seconds in 3 mL CHCl₃:CH₃OH (2:1) containing 1% butylated hydroxytoluene. Samples were first migrated in CHCl₃:CH₃OH:H₂O (65:24:4) and then in hexane:diethyl ether:acetic acid (75:35:1). Visualization was performed by using 0.1% Amido Black 10b in 1 mol/L NaCl.

Immunostaining Analyses

Immunostaining analyses were performed with the following antibodies: ATX (Fisher ThermoScience, QC, Canada), oxidized LDL (ox-LDL; Accurate Chemical, ON, Canada), E06 (Avanti Polar

Lipids, Alabaster, AL), Lp(a) (Novus Biologicals, ON, Canada) and Lp-PLA2 (Abgent, San Diego, CA). Slides were incubated with primary antibodies overnight at 4°C in 1% bovine serum albumin in TBS1X and then washed with TBS1X. The EnVision Dual Link System-HRP and the AEC substrate (Dako, Carpinteria, CA) were used to detect signal.

Proximity Ligation Assay

Six-micrometer slices were cut from OCT-embedded human mineralized aortic valve leaflets. Following permeabilization, the proximity ligation assay (Olink Bioscience, Sweden) was performed according to the manufacturer’s instructions using monoclonal antibody 4H1 (Fisher ThermoScience, QC, Canada) that recognizes apolipoprotein (a) (fragment 4330–4521) and polyclonal antibody against ATX (Fisher ThermoScience, QC, Canada).

Real-Time Polymerase Chain Reaction

RNA was extracted from valves explanted from patients and mice and from cells during in vitro experiments. Quantitative real-time polymerase chain reaction was performed with Quantitect SYBR Green PCR kit from Qiagen on the Rotor-Gene 6000 system (Corbett Robotics Inc, San Francisco, CA).

In Vitro Analyses of Calcification

Cells were incubated for 7 days with a procalcifying medium containing: Dulbecco’s modified Eagle’s medium + 5% fetal bovine serum, 10−7 mol/L insulin, 50 μg/mL ascorbic acid, and Na₂HPO₄ at 2 mmol/L.

Quantification of IκB Kinase α and Phosphorylated IκB Kinase α

VICs were collected in cell lysis buffer. CAVD tissues were homogenized in lysis buffer. Lysates were used directly in enzyme-linked immunosorbent assay (ELISA) kit (PathScan IκKα/Phospho-IκB Kinase α [IKKα] [Ser176/180], Cell Signaling Technology, Inc, Danvers, MA). The quantification of IKKα and phospho-IκKα was determined in accordance with the manufacturer’s instructions and normalized with protein content.

Measurement of Interleukin 6 in Supernatants

Interleukin 6 (IL-6) was measured in supernatants of human VICs exposed to lysophosphatidylcholine, Ki6425, or lysophosphatidic acid. IL-6 was measured by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Measurement of ATX in Supernatants

ATX was measured in supernatants of human VICs using ELISA (Echelon Biosciences, Salt Lake City, UT) according to manufacturer’s instructions and normalized with protein content.

Animals

All animal protocols were conducted according to guidelines set out by the Laval University Animal Care and Handling Committee and conform with the National Institutes of Health guidelines for the care and use of laboratory animals. LDLR−/− /ApoB100/100 /IGF2 (on C57Bl/6J background) were generated from an established colony at the Quebec Heart and Lung Institute. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Male mice were fed ad libitum a high-fat, high-sucrose, cholesterole diet (55% calories from fat, 28% from sucrose, 0.2% cholesterol) for 6 months starting at 12 weeks of age.

Echocardiography in Mice

The investigator performing echocardiography analyses was blinded to the group allocation. Transthoracic echocardiography was
performed under 2.5% isoflurane anesthesia, with a L15-7io (5–12 MHz) and S12-4 (4–12 MHz) probes connected to a Philips HD11XE ultrasound system (Philips Healthcare Ultrasound, Netherlands).

**Statistical Analyses**

Results were expressed as mean±standard error of the mean. Continuous data were tested for normality of distribution with the Shapiro-Wilk test and compared with the Student t test. For continuous data with a nonnormal distribution or with a n≤10 the values were compared between groups with nonparametric Wilcoxon-Mann-Whitney or Kruskal-Wallis test when 2 or >2 groups were compared respectively. Post hoc Steel-Dwass multiple comparisons tests were performed when the P value of the Kruskal-Wallis test was <0.05. Categorical data were expressed as a percentage and compared with the Fisher exact test. A P value of <0.05 was considered statistically significant. Statistical analyses, with the exception of the Wilcoxon-Mann-Whitney and Kruskal-Wallis tests, were performed with commercially the available software package JMP 10.0. Wilcoxon-Mann-Whitney and Kruskal-Wallis tests were performed with Prism 6.0.

**Results**

**Expression of ATX in CAVD**

We measured the ATX enzyme-specific lysophospholipase activity in 47 aortic valves from both control (n=12) and CAVD (n=35; Table 1) by using the FS-3 substrate, a fluorogenic analogue of LPC. We documented that ATX activity was increased by 60% in mineralized aortic valves in comparison with control nonmineralized aortic valves (Figure 1A). The increase in ATX activity in mineralized aortic valves was similar in tricuspid (56%) and bicuspid valves (68%; Figure 1B). Considering the age difference between the groups, in subanalysis we next matched for age and sex the 12 controls with CAVD (n=12; Table 2). In valves that were age and sex matched, we found that ATX activity was increased by 49% in stenotic aortic valves in comparison with control nonmineralized valves (Figure 1C). To further confirm these results we documented the expression of ATX in 12 controls and 15 stenotic aortic valves by Western blotting. We found that protein level of ATX was increased by 2.7-fold in mineralized aortic valves (Figure 1D). Considering that ATX produces lysosphosphatic acid from LPC, we next measured by thin layer chromatography the levels of lysosphopholipids in control and mineralized stenotic aortic valves. This analysis indicated that lysosphosphatic acid was increased by 1.5-fold in stenotic aortic valves in comparison with control nonmineralized tissues (Figure 1E and 1F). In stenotic aortic valves, the level of lysosphosphatic acid surpassed LPC by 11.9-fold (Figure 1G). Hence, these data suggest that a high level of ATX activity in a mineralized aortic valve is associated with the production of lysosphosphatic acid.

**ATX Is Transported by Lp(a) in the Aortic Valve**

By using immunohistochemistry studies (control n=15 and CAVD n=15), we found that ATX staining was weak in control nonmineralized aortic valves (Figure 2A). However, ATX was highly expressed in mineralized aortic valves and appears as a diffuse and cellular staining, suggesting that it is secreted and also possibly transported in the aortic valve (Figure 2A and 2B). We next immunostained aortic valves for lipoproteins and determined on adjacent sections the colocalization with ATX. By using an antibody reacting with malondialdehyde-modified ox-LDL, we documented that the staining in stenotic aortic valves was intense and localized in the fibrosa layer, often in the vicinity of mineralized nodules where ATX was expressed (Figure 2B). We next used the EO6 antibody, which reacts against OxPLs and we documented that it codistributed with ox-LDL and ATX stainings (Figure 2B). Because a large fraction of OxPLs is transported by Lp(a) in circulation, we next stained aortic valve sections with an antibody against Lp(a). The immunostaining for Lp(a) codistributed with ox-LDL, OxPL, and ATX (Figure 2A). Finally, on adjacent sections, we also documented that OxPL, Lp(a), and ATX colocalized with Lp-PLA2 (Figure 2B). In confocal immunofluorescence studies, ATX colocalized with Lp(a) (Figure 2C). Taken together, these data raised the hypothesis that ATX is transported by lipoproteins in the aortic valve. To examine this possibility we measured the ATX enzyme activity in isolated Lp(a) fractions in healthy volunteers. In isolated Lp(a) fractions, ATX enzyme activity was increased by 4.6-fold in comparison with the blood plasma (Figure 2D). These data further supported the hypothesis that ATX is transported in the bloodstream by lipids and enriched in the Lp(a) fraction. To further substantiate this finding, we next used in situ proximity ligation assay, which allows the detection of protein-protein interactions in tissue sections (Figure 2E). We used the monoclonal antibody 4H1 that recognizes the apolipoprotein(a) (fragment 4330–4521) and a polyclonal antibody against ATX. By using proximity ligation assay in mineralized aortic valves, we found that ATX was forming a complex with apolipoprotein(a) (each red dot represents apolipoprotein(a) and ATX interaction; Figure 2F). Taken together, these findings suggest that ATX is possibly transported in the aortic valve by Lp(a).

**Table 1. Clinical Characteristics of Patients for ATX Activity**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control Valves (n=12)</th>
<th>CAVD (n=35)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>60±1</td>
<td>68±2</td>
<td>0.0001</td>
</tr>
<tr>
<td>Male, %</td>
<td>67</td>
<td>57</td>
<td>0.73</td>
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<tr>
<td>Smoking, %</td>
<td>0</td>
<td>20</td>
<td>0.17</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>33</td>
<td>69</td>
<td>0.044</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>42</td>
<td>31</td>
<td>0.72</td>
</tr>
<tr>
<td>Coronary heart disease, %</td>
<td>67</td>
<td>46</td>
<td>0.31</td>
</tr>
<tr>
<td>Bicuspid aortic valves, %</td>
<td>0</td>
<td>34</td>
<td>0.021</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.2±1.7</td>
<td>27.6±0.8</td>
<td>0.56</td>
</tr>
<tr>
<td>Statins, %</td>
<td>100</td>
<td>94</td>
<td>1</td>
</tr>
<tr>
<td>Aortic valve area, cm²</td>
<td>–</td>
<td>0.79±0.03</td>
<td>–</td>
</tr>
<tr>
<td>Aortic mean gradient, mm Hg</td>
<td>–</td>
<td>40.6±1.9</td>
<td>–</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.44±0.08</td>
<td>1.44±0.12</td>
<td>0.50</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>2.33±0.46</td>
<td>2.20±0.16</td>
<td>0.59</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.03±0.07</td>
<td>1.27±0.06</td>
<td>0.017</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>135.4±17.8</td>
<td>90.7±4.4</td>
<td>0.015</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>55.6±6.4</td>
<td>74.2±3.5</td>
<td>0.011</td>
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</tbody>
</table>

Values are mean±SEM or %; P values: Student t test or Fisher exact test. ATX indicates autotaxin; BMI, body mass index; CAVD, calcific aortic valve disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and SEM, standard error of the mean.
ATX Is Expressed by VICs: Relationships With Remodeling and Inflammation

We next hypothesized that, in addition to being transported by lipoproteins, ATX could also be produced and secreted by VICs. By using confocal microscopy, we documented in stenotic aortic valves that ATX-positive cells coexpressed vimentin, a marker expressed by VICs (Figure 3A). Human VICs were isolated from nonmineralized aortic valves obtained during heart transplantation and we determined the expression of ATX in isolated cells by using immunofluorescence. We found that isolated VICs also widely expressed ATX, which is located in the cytosol and enriched in the perinuclear...
region (Figure 3B). We next measured the mRNA levels of ATX in 67 stenotic aortic valves (Table 3) and compared its expression with the remodeling score. In valves with a higher level of mRNA encoding for ATX (≥13.2 copies/hypoxanthine guanine phosphoribosyltransferase, median), there was a higher remodeling score (Figure 3C). The expression of ATX was associated with the expression of IL-6 (Figure 3D) and indices of inflammation (Figure I in the online-only Data Supplement). To confirm that the nuclear factor κB (NF-κB) pathway is activated in CAVD and related to ATX, we next measured in 10 mineralized aortic valves the phosphorylated IKKα (Ser 176–180)/IKKα ratio, which is upstream in the NF-κB pathway. In valves with an elevated ATX mRNA level, the total amount of IKKα was not modified (Figure 3E), whereas the phosphorylated IKKα (Ser 176–180)/IKKα ratio was increased by 1.7-fold (Figure 3F). These findings confirmed that ATX is related to tissue remodeling and the activation of the NF-κB pathway in CAVD.

### Table 2. Clinical Characteristics of Age and Sex-Matched Patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control Valves (n=12)</th>
<th>CAVD (n=12)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>60±3</td>
<td>61±1</td>
<td>0.70</td>
</tr>
<tr>
<td>Male, %</td>
<td>67</td>
<td>67</td>
<td>1</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>0</td>
<td>25</td>
<td>0.21</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>33</td>
<td>83</td>
<td>0.036</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>42</td>
<td>42</td>
<td>1</td>
</tr>
<tr>
<td>Coronary heart disease, %</td>
<td>67</td>
<td>42</td>
<td>0.41</td>
</tr>
<tr>
<td>Bicuspid aortic valves, %</td>
<td>0</td>
<td>67</td>
<td>0.001</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.2±1.7</td>
<td>28.3±1.4</td>
<td>0.47</td>
</tr>
<tr>
<td>Creatinine clearance, mL/min</td>
<td>100</td>
<td>83</td>
<td>0.47</td>
</tr>
<tr>
<td>Aortic valve area, cm²</td>
<td>28.2±1.7</td>
<td>–</td>
<td>0.38</td>
</tr>
<tr>
<td>Aortic mean gradient, mm Hg</td>
<td>–</td>
<td>40.2±5.7</td>
<td>–</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.44±0.08</td>
<td>1.52±0.22</td>
<td>0.38</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>2.33±0.46</td>
<td>1.99±0.21</td>
<td>0.74</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.03±0.07</td>
<td>1.20±0.09</td>
<td>0.097</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>135.4±17.8</td>
<td>91.0±8.4</td>
<td>0.019</td>
</tr>
<tr>
<td>Creatinine clearance, mL/min</td>
<td>55.6±6.4</td>
<td>78.2±6.6</td>
<td>0.013</td>
</tr>
</tbody>
</table>

Values are mean±SEM or %; P values: Student t test or Fisher exact test. BMI indicates body mass index; CAVD, calcific aortic valve disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and SEM, standard error of the mean.

Lysophosphatidylcholine Stimulates the NF-κB Pathway in VICs: Role for Mineralization

Lp-PLA2, which is highly expressed in mineralized aortic valves and colocalizes with ATX, uses ox-LDL to produce the highly reactive LPC. Moreover, the oxidation of LDL leads to the production of LPC, which is incidentally present in human mineralized aortic valves. In turn, LPC is the natural substrate of ATX. We first assessed the role of LPC on the activation of the inflammatory pathway. Immunofluorescence studies in isolated VICs demonstrate a nuclear translocation of the p65 subunit of NF-κB in response to LPC, indicating that the inflammatory pathway is activated. In control nontreated VICs, we observed that p65 is mostly localized in the cytosol (Figure 4A). However, in VICs exposed to a low concentration of LPC (100 nmol/L; blood plasma levels ≈200 μmol/L)15 the p65 subunit is progressively translocated to the nucleus in 30 minutes, indicating the activation of the NF-κB pathway (Figure 4A). We previously showed that the activation of NF-κB pathway in VICs promotes the production of IL-6, which by a paracrine effect mediates the mineralization of cell cultures through the production of bone morphogenetic protein-2 (BMP2).16 We thus measured by ELISA the levels of IL-6 in supernatants of VICs. In isolated VICs, we found that a 24-hour exposure to LPC (100 nmol/L) increased the secretion of IL-6 by 3.5-fold (Figure 4B). VICs were next treated for 7 days with the mineralizing medium with or without LPC (100 nmol/L) and the levels of mineralization were measured. We documented that BAY11-7085 (an inhibitor of IκB phosphorylation, which is upstream of NF-κB) prevented the mineralization of VICs induced by LPC (Figure 4C and 4G). IL-6 silencing (Figure 4D) negated LPC-induced mineralization of VICs (Figure 4E and 4H). Also, LPC-induced mineralization of VICs was abrogated by a treatment of cell culture with noggin, an inhibitor of bone morphogenetic proteins (BMPs; Figure 4F and 4I). Thus, these data indicate that LPC mediates the mineralization of VICs through a NF-κB/IL-6/BMP pathway.

The Proinflammatory and Mineralizing Effects of LPC Are Mediated by ATX

We next hypothesized that LPC-mediated activation of NF-κB/IL-6/BMP pathway and mineralization was mediated by ATX, which is also secreted by VICs. To test this hypothesis, we performed a small interfering RNA (siRNA) for ATX, which reduced significantly the mRNA level and enzyme activity of ATX (Figure 5A and 5B). The siRNA also reduced the level of ATX secreted in the supernatant by 43% (Figure 5C). In isolated VICs, LPC (100 nmol/L) increased the level of mRNA encoding for IL-6 by severalfold, whereas a siRNA for ATX reduced this rise significantly (Figure 5D). These data suggested that LPC promotes the activation of the NF-κB pathway through an ATX-dependent process. To test this hypothesis, we measured the phosphorylation of IKKα. In this experiment we found that LPC (100 nmol/L) increased the phosphorylated IKKα (Ser 176–180)/IKKα ratio, whereas a silencing of ATX abrogated this activation (Figure 5E). The treatment of VICs with LPC (100 nmol/L) also increased the expression of BMP2 by 7.6-fold, whereas a siRNA for ATX significantly reduced this response (Figure 5F). A siRNA for ATX negated the LPC-induced rise of transcript encoding for runt-related transcription factor 2, a master transcription factor involved in osteogenic transition of cells (Figure 5G). Similarly, the silencing of ATX prevented the LPC-induced rise of osteogenic markers: osteonectin (SPARC; Figure 5H), alkaline phosphatase (ALP; Figure 5I), and collagen 1α (COL1A1; Figure 5J). The knockdown of ATX also negated the prominerization effect of LPC (Figure 5K). Hence, these data confirmed that ATX is promoting the inflammation and mineralization of VIC cultures. We next verified if, in turn, inflammation may promote the mineralization of VICs in an ATX-dependent manner. We first treated VICs with tumor necrosis factor α (TNF-α), a known promoter of mineralization
Figure 2. Autotaxin colocalized with Lp(a) in the aortic valve. A, Immunohistochemistry studies of ATX in control nonmineralized and CAVD tissues (representative of 15 controls and 15 CAVD). B, Immunostainings for ATX, oxidized-LDL (ox-LDL), oxidized phospholipids (OxPLs), Lp(a), Lp-PLA2, and control with secondary antibody alone in mineralized aortic valves. Scale bar, 150 μm. C, Confocal images showing the colocalization (appears in yellow) of Lp(a) and ATX. The DIC image shows the region where images were acquired on the tissue. Scale bar, 40 μm. D, Measurements of ATX activity in blood plasma and Lp(a) fraction from healthy donors (n=5). E, Cartoon of the proximity ligation assay. F, Confocal analysis of proximity ligation assay displaying interactions between ATX and apolipoprotein(a) (red dots; representative of n=3). Scale bar, 40 μm. Values are mean±SEM. P value: Wilcoxon-Mann-Whitney test. AB indicates antibody; ATX, autotaxin; CAVD, calcific aortic valve disease; CTL, control; Dapi, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast; Lp(a), lipoprotein(a); Lp-PLA2, lipoprotein-associated phospholipase A2; RFU, relative fluorescent unit; and SEM, standard error of the mean.
in the aortic valve. The addition of TNF-α (10 ng/mL) for 6 hours in VIC cultures increased the activity of ATX by 156% in cell supernatants, suggesting that it promoted the secretion of ATX by VICs (Figure 5L). In a mineralization assay over 7 days, a cotreatment with LPC (100 nmol/L) and TNF-α (10 ng/mL) significantly increased the calcium content of VIC cultures, whereas a silencing of ATX negated this effect (Figure 5M). Hence, the silencing of ATX prevented the TNF-α–induced mineralization of VICs.

Lysophosphatidic Acid Generated by ATX Promotes the Mineralization of VICs

Considering that ATX is secreted by VICs and mediates the promineralizing effect of LPC through a NF-κB/IL-6/BMP pathway, we hypothesized that lysophosphatidic acid was the end product mediating these effects. We treated VICs with Ki16425, an antagonist of lysophosphatidic receptors 1 to 3, and measured the mRNA level of IL-6 in response to LPC (100 nmol/L). It is of interest that Ki16425 blocked the rise of the IL-6 transcript induced by LPC (Figure 6A). This finding was next corroborated by measuring IL-6 in supernatants of VIC cultures. In this assay, we found that LPC-induced secretion of IL-6 was prevented by Ki16425 (Figure 6B). Ki16425 also prevented the mineralization of VIC cultures induced by LPC (100 nmol/L; Figure 6C). These data suggested that LPC is transformed by ATX into lysophosphatidic acid, which has potent proinflammatory and promineralizing activities. To test this hypothesis we treated VICs with lysophosphatidic acid (10 μmol/L; the normal concentration of lysophosphatidic acid in the blood plasma and serum is in the low micromolar range and may surpass 10 μmol/L in atherosclerotic plaques),17 and we measured the production of IL-6 and mineralization levels. In VIC cultures treated with lysophosphatidic acid, we observed a significant increase of IL-6 in response to LPC (100 nmol/L). It is of interest that Ki16425 blocked the rise of the IL-6 transcript.
Lysophosphatidic Acid Promotes the Development of CAVD in Mice

Considering our in vitro findings, we next evaluated if lysophosphatidic acid could promote/accelerate the development of CAVD in a mouse model. LDLR/−/−ApoB100/100/IGFII transgenic mice (IGFII) develop CAVD under a high-fat/high-sucrose diet, whereas C57BL/6 mice under a high-fat/high-sucrose diet do not develop CAVD.25 After 6 months of high-fat/high-sucrose diet, whereas C57BL/6 mice under a high-fat/high-sucrose diet do not develop CAVD.25 After 6 months of high-fat/high-sucrose diet, a staining for OxPLs with E06 antibody showed an intense signal in leaflets of IGFII mice, whereas OxPLs were barely detectable in the aortic valves of C57BL/6 mice (Figure 7A). In immunofluorescence studies we found that the IGFI mice had a higher expression of ATX in aortic leaflets than control IGFII mice (Figure 7B). The level of mRNA encoding for ATX in the aortic root was significantly elevated in the IGFI mice in comparison with control IGFII mice (Figure 7F). Consistently, in comparison with baseline value, the aortic valve area measured at 6 months was significantly decreased in IGFII mice receiving lysophosphatidic acid (0.77±0.14 mm² versus 1.32±0.12 mm², P=0.03; Table II in the online-only Data Supplement). We next used a fluorescent imaging agent (OsteoSense 680EX) to detect hydroxyapatite of calcium in aortic valve leaflets. At 6 months, the signal for hydroxyapatite of calcium in aortic valve leaflets was increased by 1.7-fold in lysophosphatidic acid–treated mice in comparison with control IGFII mice (Figure 7G). Accordingly, alizarin red staining for minerals performed at 6 months showed a greater amount of staining in aortic valves of lysophosphatidic acid–treated mice (Figure 7H). In the aortic root, the level of mRNA encoding for BMP2 was increased in mice treated with lysophosphatidic acid (Figure 7I). Immunofluorescence studies showed a higher signal for BMP2 in aortic valve leaflets of IGFII mice receiving lysophosphatidic acid injections (Figure 7J). These data suggest that ATX is overexpressed in a mouse model of CAVD and that lysophosphatidic acid promotes and accelerates the development of calcific aortic valve stenosis.

Discussion

In this study, we identified for the first time that ATX and lysophosphatidic acid are involved in the pathobiology of CAVD (Figure 8). In this regard, we found that lysophosphatidic acid derived from ATX promoted a strong osteogenic response in VICs and accelerated the development of CAVD in a mouse model.

Lp(a) and ATX: Role in CAVD

ox-LDL has been associated with inflammation and the remodeling process of the aortic valve.19–21 One potent and bioactive mediator of ox-LDL is represented by the OxPLs. The proinflammatory property of Lp(a) is believed to be derived from its high content in OxPLs. A high level of OxPLs in small apolipoprotein(a) isoforms is associated with elevated levels of Lp(a).22 The recent discovery that LPA gene polymorphism (rs10455872) is causally related to CAVD has raised the question of how Lp(a) promotes the development of CAVD.23 OxPLs are substrate of Lp-PLA2, which is highly expressed in mineralized aortic valves.8,24 Lp-PLA2 produces LPC, a highly reactive lipid metabolite, which is incidentally the substrate for ATX. LPC is also an important component of circulating ox-LDL and present in the aortic valve. In this work, we established that the proinflammatory/mineralizing effects of LPC are mediated by ATX. In addition, we found that inflammatory stimuli such as TNF-α promoted the secretion of ATX and increased the mineralization of VICs. It is of interest that the promoter region of ATX contains a NF-κB consensus site, and, in isolated Hep3B cells, TNF-α induced the production of ATX.25 In the present work, a knockdown ATX prevented the promineralizing effect of TNF-α. Hence, ATX is induced by inflammatory mediators and is also a potent promoter of inflammation/mineralization. We also established that ATX is likely transported by Lp(a) in the aortic valve. To this effect, ATX activity was elevated by 4.6-fold in the Lp(a) fraction. In addition, by using in situ proximity ligation assay, which allows the detection of protein

Table 3. Clinical Characteristics of Patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>CAVD (n=67)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>70±1</td>
</tr>
<tr>
<td>Male, %</td>
<td>57</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>9</td>
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<tr>
<td>Hypertension, %</td>
<td>60</td>
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<tr>
<td>Diabetes mellitus, %</td>
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</tr>
<tr>
<td>Coronary heart disease, %</td>
<td>45</td>
</tr>
<tr>
<td>Bicuspid aortic valves, %</td>
<td>25</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.7±1</td>
</tr>
<tr>
<td>Statins, %</td>
<td>57</td>
</tr>
<tr>
<td>Aortic valve area, cm²</td>
<td>0.77±0.02</td>
</tr>
<tr>
<td>Aortic mean gradient, mmHg</td>
<td>40.6±1.7</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
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</tr>
<tr>
<td>LDL, mmol/L</td>
<td>2.3±0.11</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.35±0.04</td>
</tr>
<tr>
<td>Creatinine, µmol/L</td>
<td>88±5</td>
</tr>
<tr>
<td>Creatinine clearance, mL/min</td>
<td>73±2.3</td>
</tr>
</tbody>
</table>

Values are mean±SEM or %. BMI indicates body mass index; CAVD, calcific aortic valve disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and SEM, standard error of the mean.
complex interactions, we documented that ATX interacts with apolipoprotein(a) in mineralized aortic valves. These facts strongly suggest that ATX is transported in the bloodstream by Lp(a), which accumulates in the aortic valves. In addition, we found that VICs express a high level of ATX, which is secreted in the extracellular space where it transforms LPC into lysophosphatidic acid. In IGFII mice with CAVD, the expression of ATX was increased in the aortic valves and in the blood plasma. Although mice do not have Lp(a), we found a significant correlation between circulating ATX activity and the plasma cholesterol level, suggesting that, similarly to our human findings, ATX is possibly enriched in lipid fractions. Hence, during CAVD, there is a considerable build-up of lysophosphatidic acid, which mediates inflammation and mineralization of the aortic valve.

ATX: Role in Inflammation and Mineralization of the Aortic Valve

Human mineralized aortic valves with an elevated level of ATX had a higher remodeling score and a higher level of inflammation. In this regard, the level of transcript encoding IL-6 was higher in valves with an elevated amount of ATX. Isolated cell experiments confirmed that ATX plays a pivotal role in mediating the proinflammatory effect of LPC, an important lipid component derived from OxPL/ox-LDL. We found in isolated VICs that LPC-induced phosphorylation of IKKα, which is upstream in the NF-κB cascade, was inhibited by silencing ATX. Moreover, in mineralized aortic valves a higher level of ATX mRNA was associated with an elevated level of phosphorylated IKKα. We also found that LPC-induced secretion of IL-6 by VICs was negated by a siRNA targeting ATX.

Figure 4. Lysophosphatidylcholine mediates mineralization by stimulating the NF-κB/IL-6/BMP pathway in VICs. A, Epifluorescence images showing translocation of p65 subunit of NF-κB from the cytosol to the nucleus in response to LPC (100 nmol/L) treatment. Scale bar, 40 μm. B, ELISA measurement of secreted IL-6 in isolated VIC cultures treated with LPC (100 nmol/L) for 24 hours (n=6). C, LPC-induced mineralization of VICs with or without IκB phosphorylation inhibitor, BAY11-7085 (n=6). D, Quantification of IL-6 silencing by real-time PCR (n=5). E and F, LPC-induced mineralization with or without siIL-6 treatment (E) or the BMP inhibitor noggin (F; n=6). Experiments in C, E, and F were independently corroborated by using alizarin red staining shown below the graphs showing biochemical determination of calcium (G through I; n=3). Values are means±SEM. LPC: 100 nmol/L; Noggin: 2.5 μmol/L. P values: Wilcoxon-Mann-Whitney or Kruskal-Wallis test; * P<0.05 for intergroup comparison with Steel-Dwass test; ** P<0.05 for intergroup comparison with Steel-Dwass test. BMP indicates bone morphogenetic protein; CTL, control; Dapi, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; HPRT, hypoxanthine guanine phosphoribosyltransferase; IL-6, interleukin 6; LPC, lysophosphatidylcholine; NF-κB, nuclear factor κB; PCR, polymerase chain reaction; SEM, standard error of the mean; siCTL, scramble small interfering RNA for negative control; siIL-6, small interfering RNA for IL-6; and VIC, valve interstitial cell.
Figure 5. Knockdown of autotaxin blocked LPC-induced inflammation and mineralization in VICs. A through C, Reduced ATX mRNA (A; n=6), ATX activity (B; n=5), and secreted ATX level (C; n=7), by siRNA silencing. D through J, Effect of silencing ATX on LPC-induced rise of IL-6 mRNA (D; n=5), phospho-IKKα/IKKα ratio (E; n=5), BMP2 mRNA (F; n=5), Runx2 mRNA (G; n=5), osteonectin mRNA (H; n=5), ALP mRNA (I; n=5), and COL1A1 mRNA levels (J; n=5). K, Effect of silencing ATX on LPC-induced mineralization of VICs (n=6). L, ATX activity in VIC supernatants in control and after TNF-α treatment for 24 hours (n=6). M, Mineralization of VICs after 7 days (n=6) following treatment with LPC with or without TNF-α and silencing of ATX. Values are means±SEM. LPC: 100 nmol/L; TNF-α: 10 ng/mL; P values: Wilcoxon-Mann-Whitney or Kruskal-Wallis test; *P<0.05 for intergroup comparison with Steel-Dwass test. ALP indicates alkaline phosphatase; ATX, autotaxin; BMP2, bone morphogenetic protein 2; IKKα, IκB kinase α; HPRT, hypoxanthine guanine phosphoribosyltransferase; IL-6, interleukin 6; LPC, lysophosphatidylcholine; Runx2, runt-related transcription factor 2; SEM, standard error of the mean; siATX, small interfering RNA for autotaxin; siCTL, scramble small interfering RNA for negative control; TNF-α, tumor necrosis factor α; and VIC, valve interstitial cell.
Husseini et al previously documented in VICs that secretion of IL-6, a downstream target of NF-κB, promoted the osteogenic transition of cells through the production of BMP2. We found that LPC induced the rise of osteogenic transcripts including BMP2, runt-related transcription factor 2, and osteonectin, whereas a silencing of ATX prevented this effect. Moreover, a knockdown of ATX prevented LPC-induced mineralization of VIC cultures. Hence, these findings indicate that ATX plays a central role in mediating the inflammation/mineralization of the aortic valve induced by LPC.

Lysophosphatidic Acid and CAVD
ATX is the major source of lysophosphatidic acid both in the circulation and at the tissue level. The present study showed that, in comparison with LPC, the level of lysophosphatidic acid was higher by severalfold in mineralized aortic valves. Hence, it strongly suggests that ATX, which is highly expressed and enzymatically active in mineralized aortic valves, promotes a sustained conversion of LPC into lysophosphatidic acid with osteogenic properties. In VIC cultures, lysophosphatidic acid induced the mineralization of cells that was negated by a silencing of IL-6, suggesting that lysophosphatidic acid promoted the osteogenic transition of VICs through an inflammatory pathway. Zhou et al showed that the administration of lysophosphatidic acid (at the same dose used in the present study) for 4 weeks accelerated inflammation and atherosclerosis development in apoE–/– mice. In the present study, we found that the administration of lysophosphatidic acid for 6 months in IGFII mice elevated the expression of BMP2 and greatly increased the deposition of hydroxyapatite of calcium in aortic valve leaflets and accelerated the development of aortic stenosis. Taken together, these findings indicate that lysophosphatidic acid promotes the mineralization of the aortic valve through a NF-κB/IL-6/BMP pathway.

Clinical Implications
This work has a potentially important translational impact. We demonstrated that a high level of ATX, which is transported by Lp(a) and also secreted by VICs, is an important driver of the mineralization of the aortic valve. In a mouse model, lysophosphatidic acid, a downstream product of ATX, increased the deposition of calcium in aortic valve leaflets and increased the rise of transaortic velocities by 91%. Mineralization of the aortic valve is regarded as a major culprit in the development/progression of CAVD. Hence, a pharmacological treatment that could stop or prevent ectopic mineralization of the aortic valve could lead to a novel form of therapy for CAVD. In this regard, inhibition of ATX or blocking lysophosphatidic receptors 1 to 3 could represent novel avenues that warrant further research.

Limitations
The expression of ATX was documented in human aortic valves with an advanced pathological process. Hence,
Figure 7. ATX and lysosphosphatic acid in a mouse model of CAVD. A, Immunohistochemistry analyses of oxidized phospholipids (OxPLs) in aortic valve leaflets of C57/BL7 mice (C57) and LDLR−/−/ApoB100100/IGFII transgenic mice (IGFII). Scale bar, 100 μm. B, Epifluorescence images of ATX and fluorescent signal quantification of aortic valve leaflets (n=16). Scale bars: 4×=500 μm, 10×=100 μm, and 40×=20 μm. C, Level of ATX mRNA in aortic root (n=14). D, ATX activity in blood plasma (n=10). E, Correlation between plasma ATX activity and blood cholesterol level (n=10). F, Delta (6 months – baseline) for transaortic velocity measurements in control (IGFII) and lysosphosphatic acid–treated IGFII mice (LPA; n=12). G, Spectral images showing signal intensities of calcified areas (hydroxyapatite) in mice aortic valve leaflets labeled with Osteosense 680EX. Scale bars: 10×=100 μm and 40×=20 μm. H, Alizarin red staining (for calcium) seen in polarized light and quantification of mineralized areas in control IGFII and LPA-treated IGFII mice (n=12). Scale bar, 100 μm. I, BMP2 mRNA levels in aortic root in control IGFII and LPA-treated IGFII mice (n=12). J, Epifluorescence images showing the expression of BMP2 in aortic valve leaflets of control IGFII and LPA-treated IGFII mice (n=11). Scale bar, 100 μm. Values are mean±SEM; P values: Wilcoxon-Mann-Whitney test. ATX indicates autotaxin; BMP2, bone morphogenetic protein 2; CAVD, calcific aortic valve disease; and SEM, standard error of the mean.
correlation between ATX and the remodeling score was limited to tissues with an advanced disease, thus limiting the range of variation.

Conclusion
In this work, we highlighted that ATX-lysophosphatidic acid promotes the mineralization of the aortic valve through a NF-κB/IL-6/BMP pathway. ATX and lysophosphatidic acid could represent novel potential targets in the treatment of CAVD.

Acknowledgments
Author contributions: Drs Bouchareb, Mahmut, Nsaibia, Boulanger, and Mathieu conceived and designed experiments. Drs Mahmut, Bouchareb, Nsaibia, and Boulanger performed immunofluorescence and confocal studies. M.-H. Laflamme, and Drs Mahmut and Nsaibia performed immunohistochemistry experiments. Drs Mahmut, Nsaibia, and Bouchareb performed q-PCR analyses. Drs Mahmut and Nsaibia performed enzyme activity assays. Dr Arsenaught performed lipid isolation fraction. Drs Mahmut, Nsaibia and, Hadji, J.-L., Lépine, and Dr Boulanger performed transfection experiments, ELISA, and Western blotting. Drs Couture, Trahan, and Pagé performed histological analyses for the remodeling score and measured the weight of aortic valves. Drs Koschinsky, Romagnuolo, and Scipione isolated Lp(a). Dr Pibarot helped supervise echo analyses. Dr Bouchareb performed the mouse studies. Drs Dahou and Bouchareb performed echocardiographic analyses in mice. Dr Marette helped supervise the mouse studies. Dr Mathieu wrote the manuscript. All the authors critically reviewed the manuscript.

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Disclosures
None.

References
Calcific aortic valve disease (CAVD) is the most prevalent heart valve disorder. Mechanisms that promote CAVD are not fully understood. Lipid-derived products may drive aortic valve inflammation and mineralization. In this work, we identified that autotaxin was overexpressed in both human mineralized aortic valves and in a mouse model of CAVD. We found that autotaxin is possibly transported in the aortic valve by lipoprotein(a) and also is secreted by valve interstitial cells. We show in human valve interstitial cells and in a mouse model that autotaxin by its downstream product, lysophosphatidic acid, promotes inflammation, osteogenic transdifferentiation of valve interstitial cells, and the development of CAVD. Thus, autotaxin and lysophosphatidic acid could represent novel targets in CAVD.
Autotaxin Derived From Lipoprotein(a) and Valve Interstitial Cells Promotes Inflammation and Mineralization of the Aortic Valve
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Supplementary Materials for

Autotaxin derived from lipoprotein(a) and valve interstitial cells promotes inflammation and mineralization of the aortic valve

Rihab Bouchareb, PhD1*; Ablajan Mahmut, MD, MSc1*; Mohamed Jalloul Nsaibia, MSc1*; Marie-Chloé Boulanger, PhD1; Abdellaziz Dahou MD2; Jamie-Lee Lépine1; Marie-Hélène Laflamme1; Fayez Hadji, MSc1; Christian Couture, MD, MSc3; Sylvain Trahan, MD3; Sylvain Pagé, MD3; Yohan Bossé, PhD4; Philippe Pibarot, PhD2; Corey A Scipione, BSc5; Rocco Romagnuolo, PhD5; Marlys L. Koschinsky, PhD5; Benoît J. Arsenault, PhD2; André Marette, PhD2; Patrick Mathieu, MD1

1Laboratoire d’Études Moléculaires des Valvulopathies (LEMV), Groupe de Recherche en Valvulopathies (GRV), Quebec Heart and Lung Institute/Research Center, Department of Surgery, Laval University, Quebec, Canada
2Department of Medicine, Laval University, Québec, Canada
3Department of Pathology, Laval University, Québec, Canada
4Department of Molecular Medicine, Laval University, Québec, Canada
5Department of Chemistry and Biochemistry, University of Windsor, Ontario, Canada
*Authors contributed equally to this work

Corresponding author: P Mathieu; E-mail: patrick.mathieu@chg.ulaval.ca

This file includes:

Materials and Methods
Supplementary figure 1
Supplementary tables 1 and 2
References
Methods

Procurement of tissues for analyses

We examined stenotic aortic valves (CAVD) that were explanted from patients at the time of aortic valve replacement. Control non-calcified aortic valves with normal echocardiographic analyses were obtained during heart transplant procedures. Patients with a history of rheumatic disease, endocarditis, and inflammatory diseases were excluded. Valves with moderate to severe aortic valve regurgitation (grade>2) were excluded. The protocol was approved by the local ethical committee and informed consent was obtained from the subjects.

Remodelling score

The remodeling scores were determined by cardiovascular pathologists (C.C., S.T., S.P.) as previously described.¹

VICs isolation

Human VICs were isolated from control non-mineralized aortic valves obtained from patients undergoing heart transplantation. Aortic leaflets were incubated in 1mg/ml type I collagenase at 37°C for 30 minutes, then washed in HEPES1X, cut into pieces and incubated in 4.5mg/ml type I collagenase at 37°C for 30 minutes. Tissues were then washed in normal medium 3 times and seeded. Cells were used between passages 3 to 7.
ATX activity

Control non-mineralized and CAVD valve tissues were homogenized and harvested in Tris-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 50 mM Tris, pH 8.0). Samples were incubated for 15 minutes at 37°C. The reaction was initiated with the addition of FS-3 (Echelon Biosciences, UT, USA) at a final concentration of 3 μM. The reaction was allowed to proceed at 37°C for 1h and the fluorescence was monitored at 520 nm. ATX activity was also measured in human plasma and Lp(a) fraction according to the same protocol. Lp(a) was either purified from human plasma or purchased from (Biomedical Technologies, MA, USA). Results were normalized to protein contents.

Western blotting

Tissue pieces were mixed with lysis buffer (150mM NaCl, 20mM Tris pH7.5, 10% glycerol, 5mM EGTA, 5mM EDTA, 2mM sodium vanadate, 50mM sodium fluoride, 1% triton X-100, 0.1% SDS, 80mM β-glycerophosphate, 5mM sodium pyrophosphate, 1mM PMSF and protease inhibitor cocktail). Mechanical lysis was performed by using a polytron, following centrifugation, supernatants were harvested and protein loading buffer was added. Extracts were boiled 5 minutes, proteins were loaded onto polyacrylamide gels followed by electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked with TBS-tween containing 5% non-fat dry milk, incubated with ATX (Fisher ThermoScience, QC, Canada) and β-actin (Sigma-Aldrich, ON, Canada) antibodies overnight at 4°C. Membranes were then washed and incubated with HRP-labeled secondary antibodies (Cell Signaling Technology, MA, USA). Detection was done using clarity western ECL substrate (BioRad, ON, Canada). Images
were acquired and quantification analyses were performed using a ChemiDocMP system (BioRad, ON, Canada).

**Quantification of lysophosphatidylcholine and lysophosphatidic acid by thin layer chromatography**

Aortic cusps were homogenized for 30 seconds in 3mL CHCl₃:CH₃OH (2:1) containing 1% butylated hydroxytoluene. Solids were allowed to settle, then supernatants were decanted and pellets were stored at 4°C. The extraction procedure was repeated twice and samples were evaporated to dryness. Dry extracts were dissolved in 1mL CHCl₃:CH₃OH (10:1) and 20µl was applied to a thin-layer chromatography plate (20cm×20 cm). Samples were first migrated in CHCl₃:CH₃OH:H₂O (65:24:4) and then in hexane: diethyl ether:acetic acid (75:35:1). Visualization was performed using 0.1% Amido Black 10b in 1M NaCl.

**Immunostaining analyses**

Immunostaining analyses were performed with the following antibodies: ATX (Fisher ThermoScience, QC, Canada), ox-LDL (Accurate chemical, ON, Canada), E06 (Avanti Polar Lipids, AL, USA), Lp(a) (Novus Biologicals, ON, Canada) and Lp-PLA2 (Abgent, CA, USA). Slides were fixed in acetone: methanol (60:40) 10 minutes at -20°C, washed in TBS1X and incubated in dual enzyme block for 10 minutes (Dako, CA, USA). Slides were incubated with primary antibodies overnight at 4°C in 1% BSA in TBS1X and then washed with TBS1X. The EnVision Dual Link System-HRP and the AEC substrate (Dako, CA, USA) were used to detect signal. To assess the numbers of CD45 (Abcam, Toronto, ON, Canada) positive cells, representative regions, rich in cells, were detected in each section and the number of cells was counted by two observers blinded to clinical
results. Cells were counted at 400X in triplicates, and a mean value was attributed to each valve. Data were reported as the average number of cells per 400X field. To document the amount of ox-LDL in the aortic valve, a semi-quantitative score was used; score 1, 2 or 3 were given respectively when less than 25%, 25-50%, and more than 50% of the valve area was specifically immunostained.

**Immunofluorescence of human aortic leaflets**

Tissues were fixed in a solution of acetone-methanol (60:40), quenched in 50mM NH₄Cl for 30 minutes and blocked with 1% BSA in TBS1X for 30 minutes. Slides were then incubated with ATX (Fisher ThermoScience, QC, Canada) and Lp(a) (4H1, Fisher ThermoScience, QC, Canada) or vimentin antibodies (Sigma-Aldrich, ON, Canada) in 1% BSA in TBS1X overnight at 4°C. Slides were then incubated with alexa568-conjugated anti-rabbit and alexa488-conjugated anti-mouse secondary antibodies (Molecular Probes/Thermo Fisher Scientific, ON, Canada). Images were acquired using a confocal microscope system (FV1000, Olympus, ON, Canada, objective 60X oil, NA1.42) driven by Fluoview software (FV-10 ASW 3.1, Olympus). Merges were performed with ImageJ 1.47g (NIH, USA).

**Proximity ligation assay**

Six μm slices were cut from OCT embedded human mineralized aortic valve leaflets. Tissues were fixed with acetone:methanol (60:40) 10 minutes at -20°C, followed by quenching in 50mM NH₄Cl for 30 minutes at room temperature. Permeabilization was then performed in 0.2% triton (in PBS1X) for 10 minutes with constant agitation. Following permeabilization, the proximity ligation assay (Olink Bioscience, Sweden)
was performed according to the manufacturer’s instructions using monoclonal antibody 4H1 (Fisher ThermoScience, QC, Canada) that recognizes apolipoprotein (a) (fragment 4330-4521) and polyclonal antibody against ATX (Fisher ThermoScience, QC, Canada). Samples were analysed using a confocal microscope system (FV1000, Olympus, ON, Canada, objective 60X oil, NA1.42) driven by Fluoview software (FV-10 ASW 3.1, Olympus). Merges were performed with ImageJ 1.47g (NIH, USA).

**Immunofluorescence of cells**

Human VICs were seeded on poly-L-lysine coated glass coverslips. The following day, cells were washed once with PBS1X and fixed in 3.7% formaldehyde for 30 minutes at 37°C. Cells were treated 15 minutes with 50mM NH₄Cl in PBS1X. Cells were incubated in PBS1X containing 5% milk for one hour at room temperature with constant agitation. Incubation with anti-ATX (Fisher ThermoScience, QC, Canada) was performed in PBS1X containing 1% milk overnight at 4°C. Cells were incubated one hour with FITC-conjugated anti-rabbit secondary antibodies (Molecular Probes/Thermo Fisher Scientific, ON, Canada). Confocal images were acquired using a Zeiss microscope driven by the Zen software (Objective 40X oil, 1.4 NA, Zeiss, ON, Canada). Image processing and quantification were performed with ImageJ 1.47g (NIH, USA).

**P65 translocation**

Cells were treated with lysophosphatidylcholine for 30 minutes at 37°C, fixed in 3.7% formaldehyde for 30 minutes at 37°C and treated 15 minutes with a 50 mM NH₄Cl solution. Cells were then permeabilized 10 minutes with 0.2% triton in PBS1X, blocked for 1 hour in 5% milk PBS1X and incubated with mouse monoclonal NF-κB p65 antibody (Clone 2A12A7) (BioVision, CA, USA) at 4 °C overnight. Cells were incubated
with an alexa488 conjugated anti-mouse secondary antibody (Molecular Probes/Thermo Fisher Scientific, ON, Canada). Cells were mounted in DAPI-containing mounting medium and samples were analysed using an epifluorescence microscope system BX51 (Olympus, ON, Canada), mounted with an Evolution QEi camera (Media Cybernetics, MD, USA) driven by Image-Pro Plus 7.0 (Olympus, ON, Canada). Merges were performed with ImageJ 1.47g (NIH, USA).

**Real-time PCR**

RNA was extracted from valves explanted from patients, mice and from cells during *in vitro* experiments. Total RNA was isolated with RNeasy micro kit from Qiagen (ON, Canada). The RNA extraction protocol was performed according to manufacturer’s instructions. The quality of total RNA was monitored by capillary electrophoresis (Experion, Biorad, ON, Canada). One μg of RNA was reverse transcribed using the Quantitec Reverse Transcription Kit from Qiagen. Quantitative real-time PCR (qPCR) was performed with Quantitec SYBR Green PCR kit from Qiagen on the Rotor-Gene 6000 system (Corbett Robotics Inc, CA, USA). Primers for Lp-PLA2, IL6, TNF-α, ATX (human and mouse), BMP2 (human and mouse), Runx2, Osteonectin, ALP and COL1A1 were obtained from Qiagen (ON, Canada). The expression of the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene (human and mouse) (Life technologies/ Thermo Fisher Scientific,ON, Canada) was used as a reference gene to normalize the results.
In vitro analyses of calcification

Cells were incubated for 7 days with a pro-calcifying medium containing: DMEM + 5% FBS, $10^{-7}$ M insulin, 50 μg/ml ascorbic acid and NaH$_2$PO$_4$ at 2 mM. The medium was supplemented with lysophosphatidylcholine (100 nM) (Sigma-Aldrich, ON, Canada), lysophosphatidic acid (10 μM) (Sigma-Aldrich, ON, Canada), BAY11-7085 (20μM) (inhibitor of IκB phosphorylation; Calbiochem Gibbstown, NJ, USA), noggin (2.5μM) (inhibitor of BMP2 and 4, Calbiochem, Gibbstown, NJ, USA), Ki16425 (10 μM) (antagonist of lysophosphatidic receptors 1-3 (LPAR1) and (LPAR3), Tocris Bioscience, MI, USA).

Determination of calcium concentrations

Calcium content in cell cultures was determined by the Arsenazo III method (Synermed, Monterey Park, CA, USA), which relies on the specific reaction of Arsenazo III with calcium to produce a blue complex. Results are measured at 650 nm on the Modular P800 Elecsys of Roche Diagnostics apparatus (Roche Diagnostics, QC, Canada). This reaction is specific for calcium. Magnesium is prevented from forming a complex with the reactive. Results were normalized to protein contents.

Alizarin red staining of cultured cells

Cells were stained with 2% Alizarin red solution. Alizarin red solution was prepared by dissolving 2 g of Alizarin red (Sigma, Oakville, ON, Canada) in 100 ml distilled water, mixed well and pH was adjusted to 4.2 with 10% NH$_4$OH. The solution was filtered before use. Cells were washed one time with PBS and fixed with 3.7% formaldehyde (Sigma, Oakville, ON, Canada) for 30 minutes, and then washed once with distilled
water. Filtered 2% alizarin red solution was added to the cells for 2-3 minutes and washed with distilled water and acetone.

**siRNA transfection**

Cells were plated at a density of 6 x 10^4 cells / well (12-well plates) or 1 x 10^5 cells / well (6-well plates). The following day, cells were transfected by using HiPerfect reagent (Qiagen, ON, Canada) with 300-600 ng siRNA against IL6 or ATX (Qiagen, ON, Canada). Reduction of target genes was measured by qPCR, ELISA assay or enzymatic activity assay.

**Quantification of IKKα and pIKKα**

VICs were collected in cell lysis buffer. CAVD tissues were homogenized in lysis buffer. Lysates were used directly in ELISA kit (PathScan®IKKα/Phospho-IKKα (Ser176/180), Cell Signaling Technology, Inc., USA). The quantification of IKKα and phospho-IKKα was determined in accordance with the manufacturer's instructions and normalized with protein content.

**Measurement of IL-6 in supernatants**

IL-6 was measured in supernatants of human VICs exposed to lysophosphatidylcholine, Ki6425 or lysophosphatidic acid for 48 hours. IL-6 was measured by ELISA (R&D Systems, MN, USA) according to manufacturer's instructions.

**Measurement of ATX in supernatants**

ATX was measured in supernatants of human VICs using ELISA (Echelon Biosciences, UT, USA) according to manufacturer's instructions and normalized with protein content.
Animals

All animal protocols were conducted according to guidelines set out by the Laval University Animal Care and Handling Committee and are conform with the NIH guidelines for the care and use of laboratory animals. LDLR<sup>-/-</sup>/Apo<sub>B100/100</sub>/IGF2 (on C57Bl/6J background) were generated from an established colony at the Quebec Heart and Lung Institute of Laval University from original founders kindly provided by Dr. Seppo Ylä-Herttuala (University of Eastern Finland, Finland). C57BL/6 mice were purchased from Jackson Laboratories (MA, USA). Male mice were housed in a pathogen-free, temperature-controlled environment under a 12:12 hour light-dark cycle and fed ad libitum of a high fat, high sucrose, cholesterol diet (55% calories from fat, 28% from sucrose, 0.2% cholesterol) for 6 months starting at 12 weeks of age. At the end of protocol, mice were sacrificed by anesthesia under isoflurane (2–3%, inhalation) and cardiac puncture, which was performed by a qualified animal care technician.

Echocardiography in mice

The investigator performing echocardiography analyses was blinded to the group allocation. Transthoracic echocardiography was performed under 2.5%-isoflurane anaesthesia, with a L15-7io (5-12 Megahertz) and S12-4 (4-12 Megahertz) probes connected to a Philips HD11XE ultrasound system (Philips Healthcare Ultrasound, Netherlands). Left ventricular (LV) M-mode imaging was obtained in parasternal short-axis view at the level of the papillary muscles. LV dimensions were measured at end-diastole (LVDd) and end-systole (LVDs), LV fractional shortening (LVFS) was calculated as (LVDd – LVDs)/LVDd× 100%. LV volumes and ejection fractions (EF) were calculated using the Teicholz formula. The diameter of the LV outflow-tract
(LVOTD) was measured in a zoomed parasternal long-axis view. LVOT cross sectional area (LVOTCSA) was calculated as \( \pi (D/2)^2 \). LVOT flow velocity was obtained by pulsed-wave Doppler (PW) in the apical 5-chamber view. The LV stroke volume (SV) was calculated as \( \text{LVOTVTI} \times \text{LVOTCSA} \), where LVOTVTI is the velocity-time integral measured in the LVOT. Aortic valve area (AVA) was calculated using the formula: 

\[
\frac{\text{LVOT area} \times \text{LVOT peak velocity}}{\text{peak jet velocity across the aortic valve}}.
\]

Cardiac output (CO) was measured as: \( \text{HR} \times \text{SV} \) and indexed for weight (cardiac index [CI]). Pulsed-wave Doppler was used to record transmural flow in the apical 4-chamber view and from this signal we measured peak velocity of E- and A- waves. Mitral annulus motion velocity was recorded by tissue Doppler imaging, velocity during early filling E’ was measured, and E/E’ ratio was calculated. Continuous-wave Doppler was also used to record aortic jet flow velocity in the apical 5-chamber view and peak aortic jet velocity and velocity-time integral were measured. The average of 3 consecutive cardiac cycles was used for each measurement. Special care was taken to get similar imaging planes at follow-up studies.

**Alizarin red staining of mouse tissues**

Hearts were perfused with HEPES, and embedded in OCT. Hearts were cut exposing leaflets and 6 µm slices were made. Sections were fixed with acetone-methanol (60:40), rinsed 2 minutes in water and stained with an alizarin red (Sigma-Aldrich, ON, Canada) solution (2% alizarin red in water, pH 4.2 adjusted by using 10% ammonium hydroxide in water) for 3 minutes. Sections were incubated in acetone for 30 seconds and then rinsed with water 30 seconds. Finally, sections were mounted in cytoseal (Dako, ON, Canada). Pictures were acquired using a Zeiss Axio Observer microscope using the Zen.
software (Zeiss, ON, Canada), with a LD A-Plan 10x/0.25 Ph1objective (Zeiss) in polarised light. Images were processed and quantifications were performed using Image J1.47g (NIH, USA).

**Osteosense 690EX calcium quantification**

Tissues were fixed in a solution of acetone-methanol (60:40), washed 3 times with PBS1X, tissue were then stained for 1 hour with OsteoSense 680EX (Perkin Elmer, MS, USA) diluted to 1:10 in PBS 1X. Slides were then washed 3 times with PBS1X and mounted. Images were acquired with Nikon Eclipse TE 2000 epifluorescence microscope (NY, USA) equipped with a MicroMax 512BFT CCD camera (Princeton Instruments, NJ, USA). Quantifications were performed using Image J1.47g (NIH, USA).

**Immunofluorescence of mouse aortic leaflets**

Tissues were fixed in a solution of acetone-methanol (60:40), washed 3 times with TBS1X, quenched in 50mM NH₄Cl for 30 minutes and blocked with 1% BSA in TBS1X for 30 minutes. Slides were then incubated with ATX (Fisher ThermoScience, QC, Canada) or BMP-2 (Novus Biologicals, ON, Canada) antibodies in 1%BSA in TBS1X overnight at 4°C. Slides were washed with TBS1X and incubated with an alexa568-conjugated anti-rabbit secondary antibody (Molecular Probes/Thermo Fisher Scientific, On, Canada). Images were acquired using an Olympus BX51 microscope (Olympus, ON, Canada), mounted with an Evolution QEi camera (Media Cybernetics, MD, USA) driven by Image-Pro Plus 7.0 (Olympus, ON, Canada). Quantifications were performed using Image J1.47g (NIH, USA).
Cholesterol and triglycerides measurements in mice

Plasma cholesterol and triglycerides assay was done according to the protocol Company (Randox, Crumlin, United Kingdom).

Statistical analyses

Results were expressed as means ±SEM. Continuous data were tested for normality of distribution with the Shapiro-Wilk test and compared with Student t-test. For continuous data with a non-normal distribution or with a n ≤ 10 the values were compared between groups with nonparametric Wilcoxon-Mann-Whitney or Kruskal-Wallis test when two or more than two groups were compared respectively. Post-hoc Steel-Dwass multiple comparisons test were performed when the p value of the Kruskal-Wallis test was <0.05. Categorical data were expressed as a percentage and compared with Fisher exact test. A p value <0.05 was considered as statistically significant. Statistical analyses except for the Wilcoxon-Mann-Whitney and Kruskal-Wallis tests were performed with commercially available software package JMP 10.0. Wilcoxon-Mann-Whitney and Kruskal-Wallis tests were performed with Prism 6.0.
Supplementary figure 1: Relationships between ATX level (dichotomized at the median value) and (a) ox-LDL score (b) CD45 positive cells (c) Lp-PLA2 mRNA levels (d) TNF-α mRNA levels and (e) weight of aortic valve. p values: Wilcoxon-Mann-Whitney.
<table>
<thead>
<tr>
<th></th>
<th>IGFII</th>
<th>IGFII LPA</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>16.2±2.0</td>
<td>16.0±2.8</td>
<td>0.89</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.2±0.3</td>
<td>1.4±0.4</td>
<td>0.25</td>
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<tr>
<td>Glucose (mmol/l)</td>
<td>8.5±0.8</td>
<td>8.8±1.2</td>
<td>0.60</td>
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Supplementary table 1: Assessment of biological parameters in IGFII mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>6 months</th>
<th>p</th>
<th>Baseline</th>
<th>6 months</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td>Mice weight (g)</td>
<td>24±3</td>
<td>39±2</td>
<td>0.001</td>
<td>24±5</td>
<td>35±5</td>
<td>0.001</td>
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<tr>
<td>LV systolic function</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>FS (%)</td>
<td>39±4</td>
<td>34±4</td>
<td>0.005</td>
<td>39±3</td>
<td>33±5</td>
<td>0.011</td>
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<tr>
<td>EF (%)</td>
<td>77±4</td>
<td>69±7</td>
<td>0.021</td>
<td>77±4</td>
<td>69±7</td>
<td>0.031</td>
</tr>
<tr>
<td>SV (ml)</td>
<td>0.06±0.01</td>
<td>0.06±0.01</td>
<td>0.080</td>
<td>0.06±0.01</td>
<td>0.06±0.01</td>
<td>0.70</td>
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<tr>
<td>Heart rate (bpm)</td>
<td>337±32</td>
<td>412±91</td>
<td>0.062</td>
<td>349±79</td>
<td>397±93</td>
<td>0.30</td>
</tr>
<tr>
<td>CO (ml)</td>
<td>19±3</td>
<td>28±8</td>
<td>0.012</td>
<td>19±6</td>
<td>21±6</td>
<td>0.70</td>
</tr>
<tr>
<td>LV diastolic function</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E/E’</td>
<td>26.6±3.2</td>
<td>24.7±3.4</td>
<td>0.30</td>
<td>23.2±1.8</td>
<td>38.5±2.1</td>
<td>0.003</td>
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<tr>
<td>Aortic Valve Hemodynamics</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Peak aortic jet velocity (cm/s)</td>
<td>96.1±18.0</td>
<td>138.2±8.6</td>
<td>0.003</td>
<td>89.8±10.8</td>
<td>171.0±22.3</td>
<td>&lt;0.0001</td>
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<tr>
<td>Peak LVOT velocity (cm/s)</td>
<td>90.7±14.5</td>
<td>100.0±17.6</td>
<td>0.12</td>
<td>82.4±6.7</td>
<td>88.2±13.5</td>
<td>0.18</td>
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<tr>
<td>LVOT area (mm²)</td>
<td>1.42±0.11</td>
<td>1.58±0.09</td>
<td>0.54</td>
<td>1.43±0.16</td>
<td>1.46±0.11</td>
<td>0.34</td>
</tr>
<tr>
<td>AVA (mm²)</td>
<td>1.31±0.14</td>
<td>1.18±0.20</td>
<td>0.43</td>
<td>1.32±0.12</td>
<td>0.77±0.14</td>
<td>0.030</td>
</tr>
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</table>
Reference
