Valvular Heart Disease

Upregulation of the 5-Lipoxygenase Pathway in Human Aortic Valves Correlates With Severity of Stenosis and Leads to Leukotriene-Induced Effects on Valvular Myofibroblasts

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Background—The development of aortic valve stenosis is not only associated with calcification and extracellular matrix remodeling, but also with inflammation. The aim of this study was to determine the role of proinflammatory signaling through the leukotriene (LT) pathway in aortic stenosis.

Methods and Results—After macroscopic dissection of surgically removed human aortic valves, RNA was extracted from 311 preparations derived from 68 patients to differentiate normal, thickened, and calcified areas from each cusp. Subsequently, quantitative polymerase chain reaction analysis was used to correlate gene expression patterns with preoperative echocardiographic parameters. The messenger RNA levels of the LT-forming enzyme 5-lipoxygenase increased 1.6- and 2.2-fold in thickened and calcified tissue, respectively, compared with normal areas of the same valves. In thickened tissues, messenger RNA levels for 5-lipoxygenase (r = −0.35; P = 0.03), its activating protein (5-lipoxygenase activating protein; r = −0.39; P = 0.02), and LTA4 hydrolase (r = −0.48; P = 0.01) correlated inversely with the velocity–time integral ratio. In addition, leukotriene A4 hydrolase transcripts correlated inversely with aortic valve area, indexed for body surface area (r = −0.52; P = 0.007). Immunohistochemical stainings revealed LT receptor expression on valvular myofibroblasts. In primary cultures of human myofibroblasts derived from stenotic aortic valves, Leukotriene C4 (LTC4) increased intracellular calcium, enhanced reactive oxygen species production, reduced the mitochondrial membrane potential, and led to morphological cell cytoplasm changes and calcification.

Conclusions—The upregulation of the LT pathway in human aortic valve stenosis and its correlation with clinical stenosis severity, taken together with the potentially detrimental LT-induced effects on valvular myofibroblasts, suggests one possible role of inflammation in the development of aortic stenosis. (Circulation. 2011;123:1316-1325.)

Key Words: aortic valve stenosis ■ echocardiography ■ inflammation ■ leukotrienes ■ myofibroblasts

Although the primary risk factor for developing aortic stenosis is increasing age,1 stenotic aortic valves share morphological characteristics with atherosclerotic lesions. For example, in the active disease process, lipid accumulation,2 inflammatory infiltration,3–5 neovascularization,6,7 extracellular matrix degradation,8–10 and extensive calcification11,11 take place, which eventually cause the aortic valve to narrow. However, attempts to inhibit the hemodynamic progression of aortic stenosis using lipid-lowering drugs have not been successful.12–14 The poor prognosis and increased mortality from aortic stenosis after the onset of symptoms in the absence of surgical valve replacement15 provide a rationale for the pursuit of a medical treatment that impedes the hemodynamic progression of aortic valve disease.

Clinical Perspective on p 1325

Inflammatory infiltration of activated macrophages and T-cells, as well as cytokine release, has been described in human stenotic aortic valves.11,16,17 Yet, the exact role of inflammation in the pathophysiology of aortic stenosis remains unknown. The aim of this study was to associate the local inflammatory response, exerted in aortic valves through the leukotriene (LT) pathway, with the clinical features of valvular function as determined echocardiographically. To this end, messenger RNA (mRNA) was extracted from human aortic valves derived from patients undergoing valve replacement surgery, and gene expression was measured by quantitative polymerase chain reaction (PCR). Through mac-

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Received May 18, 2010; accepted January 24, 2011.

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Circulation is available at http://circ.ahajournals.org

DOI: 10.1161/CIRCULATIONAHA.110.966846

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rscopic dissection, normal, thickened, and calcified areas of each valvular cusp were differentiated and analyzed separately, allowing us to obtain a pattern of gene expression that represents the entire disease spectrum in each individual, from early signs to severe morphological changes and the eventual destruction of the aortic valve architecture.

LTs are arachidonate-derived lipid metabolites and potent inflammatory mediators that are formed by 5-lipoxygenase (5-LO) and its activating protein (5-LO activating protein [FLAP]). LTs exert their effects by activating BLT1 and BLT2 receptors (BLT2R), the high- and low-affinity receptors for LTB4, respectively, and cysteine LT1 (CysLT1) and CysLT2 receptors for Leukotriene C4 (LTC4), D4, and E4. These LT receptors are expressed on inflammatory cells, such as macrophages, lymphocytes, and granulocytes and on structural cells within the vascular and airway walls. The function of LTs as inflammatory mediators was initially recognized through their robust effects as bronchoconstrictors; consequently, anti-LTs are used as treatments for asthma. LTs also have effects on the cardiovascular system, and recent studies have demonstrated their involvement in atherosclerosis and restenosis after percutaneous angioplasty. Despite beneficial effects of anti-LTs in experimental atherosclerosis, no study has explored the role of LTs in aortic valve stenosis. Nevertheless, because some data on atherosclerosis can be extrapolated to aortic stenosis, we studied the effects of LTs in human aortic valves.

Methods

Patients

Human aortic valves for RNA extraction were obtained from 79 patients undergoing aortic valve replacement surgery. After the exclusion of 11 patients because of insufficient RNA quality, 68 patients were included in the subsequent analysis (48 men and 20 women, mean age 65.9±13.4 years). For cell and organ culture experiments, aortic valves from an additional 12 patients were used. The study was approved by the local ethics committee (Karolinska Institutet 2008/630-32), and all patients gave informed consent. Only patients with a normal preoperative coronary angiogram and no history of rheumatic heart disease were included in the study.

Echocardiography

All patients underwent 2-dimensional transthoracic Doppler echocardiography using a Philips IE33 system (Philips Medical Systems, Andover, MA). The left ventricular function and aortic root dimensions were measured according to the recommendations of the American Society of Echocardiography. The severity of aortic valve stenosis was based on: (1) the ratio of the systolic velocity–time integral (VTI) in the left ventricle outflow tract to the VTI in the aortic valve; (2) the aortic valve area (AVA, cm²) calculated using the continuity equation; (3) AVA indexed for body surface area (AVA/BSA, cm²/m²); and (4) the mean transvalvular pressure gradient according to the current guidelines. The echocardiographic assessment of the aortic valve as bicuspid or tricuspid was confirmed intraoperatively. On the basis of the echocardiographic assessment, the study population was categorized into 5 groups: (1) normal aortic valve (pathology of the aortic root and/or the ascending aorta without valve dysfunction in which valve-sparing surgery could not be performed according to perioperative surgical decision; n=4); (2) combined aortic vitiitum (ie, increased transvalvular pressure gradient without fulfilling the criteria for severe aortic stenosis and the presence of aortic valve regurgitation; n=7); (3) isolated aortic regurgitation (n=11); and finally, isolated severe aortic stenosis with either a (4) bicuspid (n=23) or (5) tricuspid (n=23) valve.

Sample Preparation and Macroscopic Dissection

Immediately after surgical removal, valves were immersed in RNA Later (Qiagen) and stored at 4°C until transport to the laboratory. Macroscopic dissection was performed in the RNA Later solution, dividing each valvular cusp into: (1) normal areas, defined as noncalcified, smooth, pliable, and opalescent; (2) thickened areas; and (3) calcified areas. From each class, 1 sample from each cusp was frozen at −80°C until RNA extraction and another part of the same cusp was used for histological analysis. The macroscopic definitions were verified by histology in a subset of preparations (n=89) using eosin and hematoxylin, Masson trichrome, and Alizarin red as indicated in online-only Data Supplement Figure I. Furthermore, the degree of calcification detected macroscopically in each cusp was in agreement with preoperative echocardiographic scoring of calcification.

RNA Extraction and Quality Assessment

Total RNA was isolated from 431 preparations from 79 patients, representing the various portions (normal, thickened, and calcified) from each aortic valve cusp, using the RNeasy Lipid Tissue Mini kit (Qiagen). RNA concentrations were measured spectrophotometrically at 260 nm (A260/280 nm, Agilent Technologies, Palo Alto, CA). Then, the quality of the RNA was analyzed on a 2100 Bioanalyzer (Agilent, Palo Alto, CA) using RNA 6000 NANO chips to assess the RNA integrity number, as described. To ensure sufficient RNA integrity, a regression was computed between the RNA integrity number and PCR results for each gene, establishing a cutoff RNA integrity number score of 3.1, corresponding to the value at which the association between them was lost. Consequently, of the 79 valves that were collected for RNA analysis, 11 patients were excluded because if insufficient RNA quality, and the gene expression data for 311 preparations from 68 patients were used for further analysis. On average, 4 to 5 preparations were examined for each aortic valve. In subsequent analyses, a mean value of each tissue category (ie, normal, thickened, and calcified, respectively) was calculated for each individual.

TaqMan Real-Time PCR

First-strand complementary DNA was synthesized from 0.5 µg RNA (Superscript II, Invitrogen, Carlsbad, CA) with random hexamers according to the manufacturer’s instructions. Quantitative TaqMan PCR was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems) with primer/probe pairs that were obtained using Assay-on-Demand from Applied Biosystems (online-only Data Supplement Table I); the reactions contained 5 µL complementary DNA that was diluted to 1.5 ng/µL and 5 µL TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA).

Immunohistochemistry

Transversal cryosections (10 µm thick) were cut, oriented from the cusp base to the free edge, and fixed in acetone. Double immunofluorescent stainings were performed using polyclonal rabbit antihuman CysLT1 receptor (Cayman Chemical Company) or 5-LO (Life Science Bioscience) and monoclonal mouse antihuman vimentin or CD68 (Dako) as primary antibodies. Isotype-specific either Texas Red or Alexa Fluor 488-conjugated secondary antibodies (Abcam) were used, and the nuclei were counterstained with 4′,6-diamino-2-phenylindol (DAPI, Vector). Images were viewed in Leica fluorescence microscope and captured with confocal microscope Leica TCS SP5.

Valve Conditioned Media

Conditioned media from stenotic valves was obtained as previously described. In summary, 72 preparations of healthy, thickened, and calcified parts of stenotic aortic valves derived from 5 patients were incubated at 1 mL/g tissue (wet weight) for 24 hours at 37°C in serum-free Dulbecco’s modified Eagle’s medium supplemented with 100 IU/mL streptomycin, 50 IU/mL penicillin, and 2.5 µg/mL.
Fungizone. Concentrations of CysLTs were measured using an enzyme immunoassay kit from Cayman Chemicals with a monoclonal antibody recognizing the total of all 3 CysLTs (LTC₄, LTD₄, and LTE₄), as previously described.²⁹

**Myofibroblast Isolation**

Aortic valve samples from 7 patients were transported immediately in cell culture medium to the laboratory where the valves were cut into small cubes (1 to 2 mm in each dimension) using a scalpel and placed onto culture dishes to dry for 15 minutes under sterile conditions. Then, the valvular pieces were covered with Dulbecco’s Modified Eagle’s Medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics at 37°C and 5% CO₂. The culture medium was changed 3 times per week, and the outgrowth of valvular myofibroblasts was evaluated during 2 to 3 weeks. At this point, outgrown myofibroblasts were detached and reseeded for measurement of intracellular calcium concentrations, mitochondrial membrane potential, reactive oxygen species (ROS), calcified nodule formation, and transcriptional changes as described in the online-only Data Supplement (Methods).

**Data Analysis**

Clinical parameters and gene expression data are expressed as median and ranges. A nonparametric Mann-Whitney U test was used for comparisons between 2 groups, and a Kruskall-Wallis 1-Way ANOVA on ranks followed by all-pairwise Dunn post hoc testing for multiple comparisons. For myofibroblast experiments, data are expressed as mean±SEM, and paired analysis was performed using either a Wilcoxon signed-rank test or a repeated measures ANOVA. Correlations between the quantitative gene expression data and echocardiographic parameters were established by Spearman correlation. P<0.05 was considered significant. A multiple stepwise linear regression was performed to evaluate the impact of the components of the 5-LO pathway on AVA/BSA. Analyses were performed using SigmaPlot version 11 (Systat Software Inc).

**Results**

**Gene Expression Analysis**

Patient characteristics are shown in Table 1 according to diagnosis group. Gene expression in the macroscopically normal area of the valve did not differ significantly between groups (online-only Data Supplement Table II). Thus, the normal area, according to our macroscopic classification,³ served as the control in subsequent analyses of mRNA levels in stenotic valves.

The inflammation-associated genes 5-LO, FLAP, LTA₄H, CD68, CD8, and interleukin (IL) 1β increased gradually in thickened areas of the valves from stenotic valves compared with the control area of each individual valve (Figure 1). Further increases in the transcription of 5-LO, FLAP, CD68, CD8, and IL-1β were observed in the calcified areas, but the differences in FLAP and LTA₄H expression did not reach statistical significance. With regard to the 2 matrix metalloproteinases (MMPs) that we examined, MMP-9 levels increased in calcified tissues whereas MMP-2 remained constant between the 3 categories of valvular tissue (Figure 1). Conditioned media derived from different parts of stenotic valves exhibited gradually increased CysLT concentrations (healthy: 28.7±2.9 pg/mL; thickened: 40.9±2.9 pg/mL; calcified: 47.0±2.3 pg/mL; P<0.05).

**Correlation of Gene Expression Data With Severity of Aortic Stenosis**

Within the aortic stenosis group, the gene expression data obtained in the normal area of the valve exhibited no correlation with echocardiographic measures of stenosis severity, apart from the finding that MMP-9 correlated significantly with the mean transvalvular pressure gradient (online-only Data Supplement Table III). In contrast, in the thickened areas, 5-LO, FLAP, and LTA₄H correlated inversely with VTI ratio. In multiple linear stepwise regression, FLAP remained significantly correlated with VTI ratio (P=0.025). In addition, the transcript levels for LTA₄H, IL-1β, and MMP-9 correlated inversely with AVA, indexed for BSA (Table 2). Furthermore, mRNA levels for LTC₄S correlated significantly with the mean transvalvular pressure gradient. There was a highly significant positive correlation between the expression levels for the components of the LT pathway (5-LO, FLAP, and LTA₄H), as well as between these transcript and MMP-9.
expression in thickened areas of stenotic aortic valves (data not shown). In addition, the expression levels of 5-LO were significantly correlated with those of CD68 ($r = 0.409$, $P = 0.016$, $n = 34$). In the calcified areas, no significant correlations were found between the quantitative echocardiographic parameters and the gene expression data indicated in online-only Data Supplement Table IV). However, the expression levels of several components of the 5-LO pathway in the calcified areas

![Figure 1. Relative gene expression data (normalized to the normal area of the stenotic aortic valve cusps) for (A) 5-LO (5-lipoxygenase), (B) FLAP (5-LO-activating protein), (C) LTA$_4$H (leukotriene $A_4$ hydrolase), (D) CD68 (macrophage marker), (E) CD8 (T-lymphocyte marker), (F) IL-1$\beta$ (interleukin 1$\beta$), (G) MMP9 (matrix metalloproteinase 9), and (H) MMP2 (matrix metalloproteinase 2). mRNA indicates messenger RNA. *$P < 0.05$.

Table 2. Correlations Between Echocardiographic Parameters and Gene Expression Levels in Thickened Areas of Stenotic Aortic Valves, Including Bicuspid and Tricuspid Valves

<table>
<thead>
<tr>
<th></th>
<th>5-LO</th>
<th>FLAP</th>
<th>LTA$_4$H</th>
<th>LTA$_4$S</th>
<th>CD68</th>
<th>CD8</th>
<th>IL-1$\beta$</th>
<th>MMP-9</th>
<th>MMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>0.01</td>
<td>0.14</td>
<td>0.12</td>
<td>0.08</td>
<td>0.31</td>
<td>0.27</td>
<td>0.24</td>
<td>0.13</td>
<td>-0.29</td>
</tr>
<tr>
<td>Correlation coeff</td>
<td>0.93</td>
<td>0.41</td>
<td>0.57</td>
<td>0.65</td>
<td>0.07</td>
<td>0.19</td>
<td>0.19</td>
<td>0.49</td>
<td>0.09</td>
</tr>
<tr>
<td>$P$</td>
<td>-0.31</td>
<td>-0.3</td>
<td>-0.52</td>
<td>-0.24</td>
<td>-0.31</td>
<td>-0.17</td>
<td>-0.4</td>
<td>-0.44</td>
<td>-0.29</td>
</tr>
<tr>
<td>AVA/BSA, cm$^2$/m$^2$</td>
<td>0.07</td>
<td>0.07</td>
<td>0.00729</td>
<td>0.17</td>
<td>0.07</td>
<td>0.41</td>
<td>0.0249</td>
<td>0.0183</td>
<td>0.09</td>
</tr>
<tr>
<td>Correlation coeff</td>
<td>0.07</td>
<td>0.0205</td>
<td>0.0152</td>
<td>0.08</td>
<td>0.18</td>
<td>0.35</td>
<td>0.09</td>
<td>0.00297</td>
<td>0.13</td>
</tr>
<tr>
<td>$P$</td>
<td>-0.35</td>
<td>-0.385</td>
<td>-0.48</td>
<td>-0.3</td>
<td>-0.23</td>
<td>-0.2</td>
<td>-0.3</td>
<td>-0.53</td>
<td>-0.26</td>
</tr>
<tr>
<td>VTI ratio</td>
<td>0.0357</td>
<td>0.0205</td>
<td>0.0152</td>
<td>0.08</td>
<td>0.18</td>
<td>0.35</td>
<td>0.09</td>
<td>0.00297</td>
<td>0.13</td>
</tr>
<tr>
<td>Correlation coeff</td>
<td>0.9</td>
<td>0.55</td>
<td>0.19</td>
<td>0.0446</td>
<td>0.32</td>
<td>0.44</td>
<td>0.3</td>
<td>0.19</td>
<td>0.18</td>
</tr>
<tr>
<td>$P$</td>
<td>-0.02</td>
<td>0.1</td>
<td>0.27</td>
<td>0.35</td>
<td>0.17</td>
<td>-0.16</td>
<td>0.19</td>
<td>0.25</td>
<td>0.23</td>
</tr>
</tbody>
</table>

FLAP indicates 5-lipoxygenase-activating protein; 5-LO, 5-lipoxygenase; LTA$_4$H, Leukotriene $A_4$ hydrolase; CD68, macrophage marker; CD8, T-lymphocyte marker; IL-1$\beta$, interleukin 1$\beta$; MMP-9, matrix metalloproteinase 9; MMP-2, matrix metalloproteinase 2; coeff, coefficient; AVA/BSA, aortic valve area indexed for body surface area; VTI ratio, velocity–time integral ratio; and P-mean, mean transvalvular pressure.
were significantly correlated with markers of osteogenic pathways, such as bone morphogenic proteins (BMPs) and runt-related transcription factor 2 (Cbfa1/Runx2), as shown in Table 3.

**LT Receptor Expression in Aortic Valve Stenosis**

On the basis of the upregulation of LT-synthesizing enzymes in stenotic valves and their correlation with the severity of aortic stenosis, we sought to measure the expression of LT receptors. BLT₁ receptor (BLT₁R) expression levels differed significantly between control and thickened areas of stenotic valves whereas BLT₂R receptor mRNA levels were unchanged between normal, thickened, and calcified areas in stenotic valves. CysLT₁ receptor was upregulated in thickened areas of stenotic valves compared with normal parts but downregulated in calcified areas of the same valves; these changes, however, were not statistically significant (Figure 2). CysLT₂ receptor transcripts were downregulated in different stages of valvular disease without any significant changes.

### Immunohistochemical Analysis

Double immunofluorescent staining of stenotic valves revealed that 5-LO colocalized with CD68-positive macrophages (Figure 3A through 3C) and with vimentin-positive myofibroblasts (Figure 3D through 3F). Likewise, CysLT₁ receptor protein was detected in colocalization with CD68 (Figure 3G through 3I) and vimentin (Figure 3J through 3L). The latter finding was, in addition, confirmed in primary myofibroblast cultures (Figure 3N through 3O) in which the CysLT₁ receptor exhibited a perinuclear localization.

### Comparison of Quantitative Gene Expression Data Between Tricuspid and Bicuspid Valves

Tricuspid and bicuspid valves did not differ with regard to the expression of any of the genes that we examined in normal or calcified tissue (data not shown). In contrast, the thickened areas of stenotic valves with tricuspid morphology expressed significantly higher levels of CD68, CD8, BLT₁R, and BLT₂R mRNA compared with thickened parts of bicuspid stenotic valves (Figure 4). The transcription of other components of the LT pathway and MMPs did not differ significantly between thickened parts from tricuspid and bicuspid valves (data not shown).

### LTC₄-Induced Effects in Human Valvular Myofibroblasts

Because the CysLT₁R was expressed on valvular myofibroblasts (Figure 3), the effects of one of its agonists, LTC₄, was examined. Exposure of myofibroblasts to LTC₄ (1 nmol/L) for 15 to 30 minutes caused a significant rise in 

\[ \text{Ca}^{2+}/H_{2}O \]

with the most pronounced increase detected in the nuclear and perinuclear region of the cell (Figure 5A). However, in the absence of extracellular 

\[ \text{Ca}^{2+}/H_{2}O \]

no LTC₄-induced increase in intracellular 

\[ \text{Ca}^{2+}/H_{2}O \]

was detected (Figure 5B). Moreover, LTC₄ caused morphological changes to the myofibroblasts with an increase in the formation of vacuoles within the cytoplasm (Figure 5D). These changes were not evident when LTC₄ was applied in conjunction with the Ca²⁺-free Tyrode solution (Figure 5E). Because the latter changes in morphology are seen in cell death, 2 cellular signals that are involved in cell death were further examined,

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**Table 3. Correlations Between Quantitative Gene Expression Data Obtained in the Calcified Part of the Valvular Tissue Derived From Stenotic Valves**

<table>
<thead>
<tr>
<th></th>
<th>BMP-2 (Correlation coeff)</th>
<th>BMP-6 (Correlation coeff)</th>
<th>Runx2 (Correlation coeff)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-LO (Correlation coeff)</td>
<td>0.23</td>
<td>0.15</td>
<td>0.38</td>
</tr>
<tr>
<td>( P )</td>
<td>0.11</td>
<td>0.41</td>
<td>0.0074</td>
</tr>
<tr>
<td>FLAP (Correlation coeff)</td>
<td>0.33</td>
<td>0.44</td>
<td>0.24</td>
</tr>
<tr>
<td>( P )</td>
<td>0.0258</td>
<td>0.0034</td>
<td>0.1</td>
</tr>
<tr>
<td>LTA₄H (Correlation coeff)</td>
<td>0.2</td>
<td>0.5</td>
<td>0.07</td>
</tr>
<tr>
<td>( P )</td>
<td>0.24</td>
<td>0.0033</td>
<td>0.66</td>
</tr>
<tr>
<td>LTC₄S (Correlation coeff)</td>
<td>0.28</td>
<td>0.79</td>
<td>0.05</td>
</tr>
<tr>
<td>( P )</td>
<td>0.0756</td>
<td>0.001</td>
<td>0.74</td>
</tr>
</tbody>
</table>

BMP indicates bone morphogenic protein; Runx2, runt-related transcription factor 2 (Runx2/Cbfa1); 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase-activating protein; and LTA₄H, leukotriene A₄ hydrolase.
the increase in ROS and the dissipation of the mitochondrial membrane potential \( \Delta \Psi_m \). Interestingly, exposing myofibroblasts to LTC\(_4\) increased mitochondrial ROS levels \((110\pm26\%, n=20, P<0.001)\), as measured by MitoSOX Red. Moreover, a time-dependent decrease in \( \Delta \Psi_m \) was observed in the cells on exposure to LTC\(_4\) (1 nmol/L; Figure 6).

Prolonged exposure of myofibroblast cultures to LTC\(_4\) induced calcium nodule formation, as demonstrated by representative micrographs of Alizarin red stained cell cultures in online-only Data Supplement Figure IIA. Furthermore, after 24 hours of LTC\(_4\) stimulation, myofibroblasts exhibited significantly increased mRNA levels of BMP-2 and BMP-6, whereas no significant differences were observed for Cbfa1/Runx2 (online-only Data Supplement Figure IIB). Finally, the levels of 5-LO expression were significantly higher in adherent monocytes compared with valvular myofibroblasts (expressed in \( 2^{-\Delta \Delta CT} \): \( 3.2\times10^{-3}\pm1.1\times10^{-3} \) (n=6) versus \( 2.9\times10^{-5}\pm4.3\times10^{-6} \) (n=4) for monocytes and valvular myofibroblasts, respectively (\( P=0.010 \)). Furthermore, after calcium-ionophore stimulation, monocyte supernatants contained significantly higher LT concentrations (\( 1.6\pm0.08 \) ng/mL; n=3) compared with supernatants derived from valvular myofibroblasts (\( 0.31\pm0.01 \) ng/mL; n=3; \( P<0.001 \)).

**Discussion**

Our results point to an important role of LTs in aortic valve stenosis. LT-producing enzymes and LT receptors were upregulated in thickened valve tissue. Further, 5-LO, FLAP, LTC\(_4\)S and LTA\(_4\)H expression levels correlated significantly with the severity of aortic valve stenosis. Also, LTC\(_4\) induced
nuclear calcium signaling, morphological changes, calcification, and mitochondrial ROS production in valvular myofibroblasts. Collectively, these data suggest an important role of the LT pathway in aortic stenosis; consequently, the inhibition of LT signaling may represent a therapeutic strategy for this disease.

In this study, the expression levels of several genes from various pathways that regulate aortic valve stenosis were compared between differentially affected areas of aortic valves, normal, leaflet thickening, and calcified aortic valve tissue. Notably, the mRNA levels in morphologically normal areas of the valve did not differ significantly between diagnosis groups with different aortic valve conditions. The latter finding suggests that the division of aortic valves, based on macroscopic appearance, represents a model of the development of aortic stenosis over time.

In support of this model, the mRNA levels of markers for macrophages (CD68) and T-lymphocytes (CD8) were upregulated gradually as the valve progressed from healthy to thickened and calcified tissue, corroborating previous histological classifications of aortic valve stenosis and evidence of the steady infiltration of these inflammatory cells. Similarly, transcripts of IL-1β, MMP-9, and the LT-synthesizing enzyme 5-LO rose, correlating significantly with CD68 expression, supporting the hypothesis that activated macrophages are a major source of inflammatory mediators in stenotic aortic valves. In addition, valvular myofibroblasts also exhibited 5-LO expression and LT synthesis, albeit at lower levels compared with leukocytes, suggesting structural valvular cells as an additional source of inflammation in aortic stenosis.

On the basis of this classification, the early thickening of valve cusps before calcification represents an intermediate

![Figure 4](image_url)

**Figure 4.** Comparison between TaqMan real-time polymerase chain reaction (PCR) data in the thickened areas of tricuspid and bicuspid valves. mRNA indicates messenger RNA; BLT1R, BLT1 receptor; BLT2R, BLT2 receptor; CD8, leukocyte marker; and CD68, macrophage marker. *P<0.05.

![Figure 5](image_url)

**Figure 5.** LTC4-mediated increase in nuclear and perinuclear Ca2+. Representative confocal images of human myofibroblasts loaded with the Ca2+ indicator Fluo-3. A, Images before and after treatment with LTC4 (1 nmol/L; 30 minutes) in Ca2+-containing medium. B, Effects of LTC4 on cellular Ca2+ in Ca2+-deficient buffer. C, Average and individual fluorescence of nuclear and perinuclear cell regions, with and without extracellular Ca2+. The y values indicate relative changes in Fluo-3 fluorescence before and after LTC4 treatment. D and E display the number of vacuoles found in each individual cell before and after exposure to LTC4. Arrows in A indicate examples of vacuoles after LTC4 treatment. Group with extracellular Ca2+: n=23; group without extracellular Ca2+: n=12. **P<0.01, *** P<0.001. AU indicates fluorescence arbitrary units; LTC4, Leukotriene C4; and ∆[Ca2+]i, the change in intracellular [Ca2+]i measured as the change in Fluo-3 florescence.
stage of aortic valve stenosis progression at which one can intervene and reverse the pathology. It is therefore interesting that our univariate correlation analysis in thickened areas of stenotic aortic valves revealed that, in addition to the already explored mediators of aortic stenosis, IL-1β and MMP-9, the mRNA levels for 5-LO, its activating protein FLAP, and the downstream enzymes LTC₄S and LTA₄H correlated significantly with severity of stenosis. When comparing thickened valve tissue between tricuspid and bicuspid stenotic aortic valves, only transcript levels for CD68, CD8, and the 2 BLT receptors increased significantly. The latter enzyme leads to the formation of LTC₄, which has bronchoconstrictor properties, and induces contraction of human atherosclerotic coronary arteries. LTC₄-mediated contractility of smooth muscle cells has been demonstrated to be caused by influx of Ca²⁺ via opening of plasma membrane Ca²⁺ channels. Valvular myofibroblasts are a mesenchymal cell type that share phenotypic similarities with vascular smooth muscle cells. Earlier investigations showed that vasoactive mediators, such as serotonin and angiotensin II, increased cytoplasmic (Ca²⁺) was most pronounced in the nuclear and perinuclear region of the cell, associated with mitochondrial permeability transition. Furthermore, in the presence of LTC₄, myofibroblasts underwent time-dependent changes in cell morphology with overt formation of vacuoles in the cytoplasm. Taken together, these LTC₄-mediated processes may be associated with activation cell death pathways, which have been implicated in valve pathology and calcification. In addition, LTC₄ induced an increased ROS production, which has been associated with calcification in situ in human aortic valves and also with osteoblast differentiation through induction of BMP-2. In line with those findings, LTC₄ upregulated BMP-2 and BMP-6 mRNA in myofibroblasts in the present study, suggesting also a LT-induced stimulation of osteogenic pathways in aortic stenosis. The latter was further supported by the significant correlation between the components of the 5-LO and osteogenic pathways in calcified stenotic valves.

In summary, we provide evidence that LT-synthesizing enzymes are upregulated in thickened tissue from stenotic...
aortic valves and that their local expression levels correlate significantly with severity of stenosis. In addition, LTC₄ induced perinuclear calcium overload, increased ROS production and cytoplasmic vacuolization, calcified nodule formation, and induced osteogenic pathways in myofibroblasts. In conclusion, upregulation of the LT pathway in calcified aortic valve stenosis and the LTC₄-induced effects on myofibroblast link inflammation and calcification, a finding that provide a first suggestion of therapeutic interventions with anti-LTs at the potentially modifiable, thickened aortic valve to prevent the development of aortic stenosis.

Acknowledgments
The authors would like to thank Ingrid Törnberg, Linda Haglund, and Therese Olsson for excellent technical assistance.

Sources of Funding
This study was supported by the Swedish Heart and Lung Foundation and with funds from the Karolinska Institutet. Dr Andersson was supported by the Lars Hierta and Golje Foundation. Dr Franco-Cereceda was supported by a donation from Fredrik Lundberg. Drs Caidahl and Hansson were supported by the Swedish Research Council (grant Nos. 114231 and 06816).

Disclosures
None.

References
Clinical Perspective

Aortic valve stenosis is a complex potentially modifiable inflammatory process with a spectrum of disease ranging from aortic sclerosis to severe destroyed valvular architecture, akin to atherosclerosis, and has become the most common indication for surgical valve replacement. The diseased valve is characterized by pathological remodeling and pronounced calcification leading to obstruction of the left ventricle outflow tract. Factors predicting the transition from early potentially modifiable stages of the valvular disease to manifest stenosis have not yet been fully elucidated. Echocardiography is the key tool for assessment of stenosis severity, and clinical decision making is based on its results. The inflammatory environment within the affected aortic valve stimulates the 5-lipoxygenase pathway leading to production of potent inflammatory mediators, leukotrienes. In the present study, a unique macroscopic dissection technique was used to model in vivo disease development, representing the entire disease spectrum from early signs to more advanced morphological changes. In addition, correlation analyses between echocardiographic parameters quantifying the stenosis severity and the quantitative gene expression data obtained from the thickened part revealed significant influence of several downstream components of the leukotriene pathway on stenosis severity in an early potentially modifiable stage of the valve disease. The translational implication of our data is that pharmacological intervention using leukotriene receptor antagonists could potentially retard the hemodynamic progression.
Upregulation of the 5-Lipoxygenase Pathway in Human Aortic Valves Correlates With Severity of Stenosis and Leads to Leukotriene-Induced Effects on Valvular Myofibroblasts
Edit Nagy, Daniel C. Andersson, Kenneth Caidahl, Maria J. Eriksson, Per Eriksson, Anders Franco-Cereceda, Göran K. Hansson and Magnus Bäck

Circulation. 2011;123:1316-1325; originally published online March 14, 2011; doi: 10.1161/CIRCULATIONAHA.110.966846
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Calcium measurements
Free cytoplasmic [Ca^{2+}] was measured using the fluorescent Ca^{2+} indicator Fluo-3 (Molecular Probes/Invitrogen) and confocal microscopy (Bio-Rad MRC 1024, attached to a Nikon Diaphot inverted microscope with a Nikon Plan Apo 40× oil immersion objective (NA 1.3)). The myofibroblasts were loaded with the cell-permeant acetoxyethyl ester (AM) form of Fluo-3 (Fluo-3 AM; 5 µm) for 30 minutes at 37°C, followed by washout with CO_{2}/O_{2} (5/95%)-bubbled Tyrode solution with the following (in mmol/l): 121 NaCl, 5.0 KCl, 1.8 CaCl_{2}, 0.5 MgCl_{2}, 0.4 NaH_{2}PO_{4}, 24 NaHCO_{3}, 0.1 EDTA, and 5.5 glucose. In some experiments, Ca^{2+}-free Tyrode solution was used, wherein CaCl_{2} was replaced with 5 mM EGTA.

Fluo-3 fluorescence was stimulated by excitation with 488 nm laser light while measuring the emitted light through a filter that allowed wavelengths >515 nm to pass. A stack (z-dimension) of 15-20 confocal images, each approximately 0.5 µm thick, was acquired at regular intervals during the experiment. Fluorescence intensity was measured in the same cell regions at each time point. All experiments were performed at room temperature (~22°C).

Measurement of mitochondrial membrane potential and reactive oxygen species (ROS)
To measure mitochondrial membrane potential (∆Ψ_{m}), the fluorescent indicator tetramethylrhodamine ethyl ester (TMRE; Invitrogen/Molecular Probes) was used. Because TMRE accumulates in the negatively charged mitochondrial matrix in proportion to the electrical potential over the inner mitochondrial membrane, the change in fluorescence reflects the changes in ∆Ψ_{m}.

The myofibroblasts were loaded with TMRE (~1 µM) for 20 min at 37°C, followed by washout. Confocal images of TMRE fluorescence were obtained at 568 nm excitation/585 nm emission.
At the end of the experiment, the myofibroblasts were exposed to the mitochondrial uncoupling agent carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 1 μM), which depolarizes the mitochondria, allowing the dynamic range of the dye to be measured. Fluorescence was measured in the same cell regions at each time point.

Changes in mitochondrial ROS (superoxide (O₂⁻)) production were monitored using the fluorescent indicator MitoSOX Red (Invitrogen/Molecular Probes), which accumulates preferentially in mitochondria and specifically detects the formation of O₂⁻. Cells were incubated with MitoSOX Red (5 μM) for ~30 min at 37°C, and experiments were started after the cells were washed with Tyrode solution. MitoSOX Red was measured at 488 nm excitation/>585 nm emission.

Calcified nodule formation in myofibroblast cultures

For studies of calcification, primary cultures of human myofibroblasts were plated into 6-well cell culture plates with a cell density of 2x10⁵ cells/well in DMEM supplemented with 10% heat inactivated fetal bovine serum and antibiotics. In addition, β-glycerophospat (10 mmol/L), CaCl₂ (1.5 mmol/L) and ascorbic acid (50μg/mL) were added to the cell culture medium to enhance calcification, a modification of the osteogenic medium as previously described. The cell culture medium was changed every second day with LTC₄ added at different concentrations (1-100 nM). After two weeks, calcified nodule formation was demonstrated by Alizarin red staining.

Transcriptional changes in myofibroblasts

To evaluate transcriptional changes induced by LTC₄, myofibroblasts (passages 2-3) were seeded in six-well plates (10⁵ cells per well). After 24 h serum-starvation, myofibroblasts were incubated in the absence or presence of LTC₄ at either 1 or 10 nM and collected for RNA
extraction and determination of expression levels of bone morphogenic protein (BMP) -2, BMP-6, and the runt-related transcription factor 2 (Cbfa1/Runx2) using TaqMan real time PCR (see Suppl Table 1 for primer/probe references).

**Leukotriene synthesis in myofibroblasts and monocytes**

Monocytes were isolated from 6 healthy volunteers using standard procedure\(^2\). Mononuclear leukocytes were resuspended in DMEM supplemented with 10% heat inactivated fetal bovine serum and antibiotics and left to adhere for 24 hours. Adherent monocytes were then harvested for RNA extraction and determination of 5-LO expression levels. In addition, myofibroblasts and monocytes were stimulated with the calcium ionophore A23187 (10 \(\mu\)M) \(^3\) and supernatants assayed for cysteinylic-LT concentrations by EIA.

**Supplemental Table 1.**

Assay-on-demand™ from Applied Biosystems, used for real-time TaqMan PCR.

<table>
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<tr>
<th>Gene of interest</th>
<th>Abbreviation</th>
<th>Assay on Demand</th>
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<tr>
<td>5-lipoxygenase</td>
<td>5-LO</td>
<td>Hs00167536_m1</td>
</tr>
<tr>
<td>5-LO activating protein</td>
<td>FLAP</td>
<td>Hs00233463_m1</td>
</tr>
<tr>
<td>LTA(_4) hydrolase</td>
<td>LT(_{A4})</td>
<td>Hs00168505_m1</td>
</tr>
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<td>BLT(_1) receptor</td>
<td>BLT(_{1R})</td>
<td>Hs00609525_m1</td>
</tr>
<tr>
<td>BLT(_2) receptor</td>
<td>BLT(_{2R})</td>
<td>Hs00251973_s1</td>
</tr>
<tr>
<td>CysLT(_\alpha) receptor</td>
<td>CysLT(_{\alpha})</td>
<td>Hs00272624_s1</td>
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<tr>
<td>CysLT(_{\beta}) receptor</td>
<td>CysLT(_{\beta})</td>
<td>Hs00252658_s1</td>
</tr>
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<td>LTC(_1) synthase</td>
<td>LTC(_{1})</td>
<td>Hs00168529_m1</td>
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<td>Macrophage marker</td>
<td>CD68</td>
<td>Hs00154355_m1</td>
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<td>Lymphocyte marker</td>
<td>CD8</td>
<td>Hs01555800_m1</td>
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<tr>
<td>Interleukin1(\beta)</td>
<td>IL-1(\beta)</td>
<td>Hs00174097_m1</td>
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<td>MMP9</td>
<td>Hs002344579_m1</td>
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<td>Matrix metalloproteinase-2</td>
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<td>Bone morphogenetic protein 2</td>
<td>BMP-2</td>
<td>Hs01055564_m1</td>
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<td>Bone morphogenetic protein 6</td>
<td>BMP-6</td>
<td>Hs01099594_m1</td>
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<tr>
<td>Runt-related transcription factor 2</td>
<td>Runx2/Cbfa1</td>
<td>Hs00231692_m1</td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>PPIA</td>
<td>Hs99999904_m1</td>
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Supplemental Table 2.

The quantitative gene expression data (median values) obtained in the normal part in each diagnosis group.

<table>
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<tr>
<th>Diagnosis Group</th>
<th>5-LO</th>
<th>FLAP</th>
<th>LAT,$\alpha$H</th>
<th>LTC,$\alpha$S</th>
<th>BLT,$\alpha$R</th>
<th>BLT,$\beta$R</th>
<th>CysLT,$\alpha$R</th>
<th>CysLT,$\beta$R</th>
<th>CD68</th>
<th>CD8</th>
<th>IL-1$\beta$</th>
<th>MMP-9</th>
<th>MMP-2</th>
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<tbody>
<tr>
<td>Aortic root pathology without aortic valve dysfunction</td>
<td>0.08</td>
<td>0.40</td>
<td>0.32</td>
<td>0.08</td>
<td>0.01</td>
<td>0.01</td>
<td>0.009</td>
<td>0.11</td>
<td>0.14</td>
<td>0.05</td>
<td>0.020</td>
<td>0.002</td>
<td>2.28</td>
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<tr>
<td>Combined aortic vitium</td>
<td>0.18</td>
<td>0.36</td>
<td>0.36</td>
<td>0.01</td>
<td>0.03</td>
<td>0.03</td>
<td>0.009</td>
<td>0.12</td>
<td>0.27</td>
<td>0.01</td>
<td>0.006</td>
<td>0.001</td>
<td>3.96</td>
</tr>
<tr>
<td>Aortic valve regurgitation</td>
<td>0.09</td>
<td>0.31</td>
<td>0.44</td>
<td>0.16</td>
<td>0.01</td>
<td>0.06</td>
<td>0.006</td>
<td>0.13</td>
<td>0.23</td>
<td>0.04</td>
<td>0.003</td>
<td>0.003</td>
<td>3.30</td>
</tr>
<tr>
<td>Aortic valve stenosis with bicuspid anatomy</td>
<td>0.05</td>
<td>0.29</td>
<td>0.30</td>
<td>0.26</td>
<td>0.01</td>
<td>0.01</td>
<td>0.009</td>
<td>0.18</td>
<td>0.13</td>
<td>0.01</td>
<td>0.002</td>
<td>0.005</td>
<td>4.40</td>
</tr>
<tr>
<td>Aortic valve stenosis with tricuspid anatomy</td>
<td>0.09</td>
<td>0.29</td>
<td>0.25</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.009</td>
<td>0.10</td>
<td>0.19</td>
<td>0.02</td>
<td>0.006</td>
<td>0.002</td>
<td>2.90</td>
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<tr>
<td>P-values</td>
<td>0.17</td>
<td>0.67</td>
<td>0.45</td>
<td>0.49</td>
<td>0.24</td>
<td>0.12</td>
<td>0.49</td>
<td>0.86</td>
<td>0.80</td>
<td>0.41</td>
<td>0.09</td>
<td>0.35</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Supplemental Table 3.

Correlations between echocardiographic parameters and gene expression levels in normal areas of stenotic aortic valves, including bicuspid and tricuspid valves.

<table>
<thead>
<tr>
<th></th>
<th>5-LO</th>
<th>FLAP</th>
<th>LAT,$\alpha$H</th>
<th>LTC,$\alpha$S</th>
<th>CD68</th>
<th>CD8</th>
<th>IL-1$\beta$</th>
<th>MMP-9</th>
<th>MMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Correlation coeff.</td>
<td>0.483</td>
<td>0.432</td>
<td>0.468</td>
<td>-0.081</td>
<td>0.12</td>
<td>0.14</td>
<td>0.274</td>
<td>-0.283</td>
<td>-0.057</td>
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<tr>
<td>P-value</td>
<td>0.017</td>
<td>0.035</td>
<td>0.066</td>
<td>0.737</td>
<td>0.589</td>
<td>0.616</td>
<td>0.252</td>
<td>0.223</td>
<td>0.792</td>
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<tr>
<td>AVA/BSA, cm$^2$/m$^2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coeff.</td>
<td>-0.073</td>
<td>-0.037</td>
<td>-0.394</td>
<td>0.300</td>
<td>-0.420</td>
<td>-0.170</td>
<td>-0.339</td>
<td>-0.362</td>
<td>-0.121</td>
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<tr>
<td>P-value</td>
<td>0.731</td>
<td>0.863</td>
<td>0.127</td>
<td>0.207</td>
<td>0.051</td>
<td>0.565</td>
<td>0.153</td>
<td>0.114</td>
<td>0.579</td>
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<tr>
<td>VTI-ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coeff.</td>
<td>0.309</td>
<td>0.213</td>
<td>-0.094</td>
<td>0.009</td>
<td>-0.181</td>
<td>0.214</td>
<td>-0.007</td>
<td>-0.302</td>
<td>-0.263</td>
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<tr>
<td>P-value</td>
<td>0.140</td>
<td>0.313</td>
<td>0.721</td>
<td>0.968</td>
<td>0.414</td>
<td>0.469</td>
<td>0.974</td>
<td>0.191</td>
<td>0.222</td>
</tr>
<tr>
<td>P-mean, mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coeff.</td>
<td>-0.269</td>
<td>-0.233</td>
<td>0.085</td>
<td>-0.023</td>
<td>0.148</td>
<td>0.179</td>
<td>0.120</td>
<td>0.498</td>
<td>0.200</td>
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<tr>
<td>P-value</td>
<td>0.201</td>
<td>0.269</td>
<td>0.746</td>
<td>0.922</td>
<td>0.508</td>
<td>0.541</td>
<td>0.620</td>
<td>0.025</td>
<td>0.357</td>
</tr>
</tbody>
</table>

AVA/BSA: aortic valve area indexed for body surface area; VTI-ratio: velocity time integral ratio; P-mean: mean transvalvular pressure.
Supplemental Table 4.

Correlations between echocardiographic parameters and gene expression levels in calcified areas of stenotic aortic valves, including bicuspid and tricuspid valves.

<table>
<thead>
<tr>
<th></th>
<th>5-LO</th>
<th>FLAP</th>
<th>LTA_H</th>
<th>LTC6S</th>
<th>CD68</th>
<th>CD8</th>
<th>IL-1β</th>
<th>MMP-9</th>
<th>MMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, y</strong></td>
<td>0.109</td>
<td>0.106</td>
<td>0.145</td>
<td>0.120</td>
<td>0.298</td>
<td>-0.020</td>
<td>0.232</td>
<td>-0.0858</td>
<td>-0.002</td>
</tr>
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<td><strong>P-value</strong></td>
<td>0.481</td>
<td>0.495</td>
<td>0.419</td>
<td>0.449</td>
<td><strong>0.049</strong></td>
<td>0.912</td>
<td>0.148</td>
<td>0.601</td>
<td>0.989</td>
</tr>
<tr>
<td><strong>AVA/BSA, cm²/m²</strong></td>
<td>-0.227</td>
<td>-0.157</td>
<td>0.032</td>
<td>0.076</td>
<td>-0.265</td>
<td>-0.083</td>
<td>-0.148</td>
<td>-0.0231</td>
<td>-0.048</td>
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<tr>
<td><strong>Correlation coeff.</strong></td>
<td>0.138</td>
<td>0.312</td>
<td>0.858</td>
<td>0.631</td>
<td>0.082</td>
<td>0.643</td>
<td>0.361</td>
<td>0.888</td>
<td>0.755</td>
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<tr>
<td><strong>P-value</strong></td>
<td>-0.222</td>
<td>-0.187</td>
<td>-0.110</td>
<td>-0.101</td>
<td>-0.070</td>
<td>0.068</td>
<td>-0.169</td>
<td>-0.137</td>
<td>-0.017</td>
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<tr>
<td><strong>VTI-ratio</strong></td>
<td>0.147</td>
<td>0.229</td>
<td>0.540</td>
<td>0.521</td>
<td>0.652</td>
<td>0.704</td>
<td>0.296</td>
<td>0.403</td>
<td>0.912</td>
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<td><strong>Correlation coeff.</strong></td>
<td>-0.057</td>
<td>-0.154</td>
<td>0.229</td>
<td>0.134</td>
<td>0.046</td>
<td>-0.130</td>
<td>0.140</td>
<td>0.188</td>
<td>-0.025</td>
</tr>
<tr>
<td><strong>P-value</strong></td>
<td>0.710</td>
<td>0.321</td>
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<td>0.764</td>
<td>0.469</td>
<td>0.388</td>
<td>0.250</td>
<td>0.874</td>
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</table>

AVA/BSA: aortic valve area indexed for body surface area; VTI-ratio: velocity time integral ratio; P-mean: mean transvalvular pressure.
Representative micrographs of sections from human stenotic aortic valves stained with eosin and hematoxylin (Panels A-C), Masson trichrome (Panels D-F) and Alizarin red (Panels G-I) based on the macroscopic classification of tissue as being healthy (Panels A, D, and G), thickened (Panels B, E, and H) or calcified (Panels C, F, and I).
Supplemental Fig 2

A: Representative micrographs of Alizarin Red stained human aortic valve myofibroblasts cultured with β-glycerophosphat (10 mmol/L), CaCl₂ (1.5 mmol/L) and ascorbic acid (50μg/mL) in the absence or presence of LTC₄ (100 nM) for 2 weeks. B: mRNA levels for BMP-2, BMP-6 and Cbfa1/Runx2 in myofibroblasts after 24h exposure to LTC4 (1-10nM). *P<0.05.

Supplemental References:

2. Fuss IJ, Kanof ME, Smith PD, Zola H. Isolation of whole mononuclear cells from peripheral blood and cord blood. Curr Protoc Immunol. 2009;Chapter 7:Unit7 1