Sex-Related Discordance Between Aortic Valve Calcification and Hemodynamic Severity of Aortic Stenosis
Is Valvular Fibrosis the Explanation?

Louis Simard, Nancy Côté, François Dagenais, Patrick Mathieu, Christian Couture, Sylvain Trahan, Yohan Bossé, Siamak Mohammadi, Sylvain Pagé, Philippe Joubert, Marie-Annick Clavel

Rationale: Calcific aortic stenosis (AS) is characterized by calcium deposition in valve leaflets. However, women present lower aortic valve calcification loads than men for the same AS hemodynamic severity.

Objective: We, thus, aimed to assess sex differences in aortic valve fibrocalcific remodeling.

Methods and Results: One hundred and twenty-five patients underwent Doppler echocardiography and multidetector computed tomography within 3 months before aortic valve replacement. Explanted stenotic tricuspid aortic valves were weighed, and fibrosis degree was determined. Sixty-four men and 39 women were frequency matched for age, body mass index, hypertension, renal disease, diabetes mellitus, and AS severity. Mean age (75±9 years), mean gradient (41±18 mm Hg), and indexed aortic valve area (0.41±0.12 cm²/m²) were similar between men and women (all P>0.18). Median aortic valve calcification (1973 [1124–3490] Agatston units) and mean valve weight (2.36±0.99 g) were lower in women compared with men (both P<0.0001). Aortic valve calcification density correlated better with valve weight in men (r²=0.57; P<0.0001) than in women (r²=0.26; P=0.008). After adjustment for age, body mass index, aortic valve calcification density, and aortic annulus diameter, female sex was an independent risk factor for higher fibrosis score in AS valves (P=0.003). Picrosirius red staining of explanted valves showed greater amount of collagen fibers (P=0.01), and Masson trichrome staining revealed a greater proportion of dense connective tissue (P=0.02) in women compared with men.

Conclusions: In this series of patients with tricuspid aortic valve and similar AS severity, women have less valvular calcification but more fibrosis compared with men. These findings suggest that the pathophysiology of AS and thus potential targets for drug development may be different according to sex. (Circ Res. 2017;120:681-691. DOI: 10.1161/CIRCRESAHA.116.309306.)

Key Words: aortic valve stenosis ■ fibrosis ■ pathophysiology ■ sex ■ valvular heart disease

Calcific aortic valve stenosis (AS) is the most frequent valvular heart disease in Western countries and the second most common indication for cardiac surgery after coronary artery bypass grafting surgery.1 The prevalence of AS increases markedly with age and affects 2% of the population ≥65 years of age and 4.6% of population ≥75 years of age.2,3 Noteworthy, 70% of AS cases are observed in male patients.1,4 To this day, there is still no efficient medical treatment for AS, and surgical or transcatheter aortic valve replacement remain the mainstay therapy for patients with severe and symptomatic AS.

Valve leaflet calcification is the main culprit lesion of AS. In addition to aortic valve calcification (AVC), lipid infiltration, aberrant extracellular matrix remodeling, and extensive valvular fibrosis may also contribute to the thickening and stiffening of aortic valve leaflets and thus to the development of AS.6,7 Previous studies have demonstrated that AVC load measured by multidetector computed tomography (MDCT) correlates well with hemodynamic severity of AS measured by Doppler echocardiography.9-11 However, recent studies showed that, for the same amount of AVC, women reach hemodynamically more severe AS compared with men.12,13 This difference remained significant even after adjusting for smaller body surface area and smaller aortic annulus area as typically observed in women.12,13 We hypothesized that this sex-related discrepancy between valvular calcification load and hemodynamic severity is explained by the fact that women have relatively

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more valvular fibrosis compared with men. Such differences in calcification/fibrosis ratio in men versus women could be of major importance in clinical practice, for the understanding of AS pathophysiology and the possible development of future individualized medical therapeutics targeting sex-specific pathways. We, thus, aimed to evaluate sex-specific fibrosis and calcification patterns in AS population.

Methods

Study Population

One hundred and twenty-five patients underwent a MDCT without contrast were performed using a 64-slice helical scanner (Somaton Definition, Siemens AG Medical Solution, Germany) with a tube potential at 120 kV and a tube current-time product at 60 to 80 mAs (Figure 1A and 1B). Operators blinded to patient data performed all MDCT examinations and analyses. The entire heart was assessed by 2.4- to 3-mm-thick transverse slices with a pitch of 0.15 to 0.25 mm during end-inspiration breath-hold. Acquisition was triggered by ECG at 60% to 70% of the R-to-R-wave interval. AVC scores were quantified with the Agatston scoring method using commercially available and validated software (Aquarius iNtuition from TeraRecon, Inc, San Mateo, CA), and all AVC data were expressed in Agatston units (AU).\(^{14}\) Calcification was defined as 4 adjacent pixels with a density >130 Hounsfield units. The summation of per-slice lesion scores was performed individually for every AVC score. To account for smaller heart size in women, we calculated the AVC density by dividing the AVC score by the cross-sectional aortic annulus area (\(\pi[aortic annulus diameter/2]^2\)) measured by echocardiography. AVC quantification assessment was performed by both the same and another investigator blinded to previous scoring and Doppler echocardiographic data and repeated at least 1 month after the original measurement in one third of the exams (ie, 30 exams) to evaluate intraobserver and interobserver variability. The variabilities were calculated by dividing the absolute value of the difference by the average of the 2 measurements. Intraobserver variability and interobserver variability were, respectively, 2.6±2.8% and 4.3±4.2%.

Doppler Echocardiography

Stroke volume was calculated by multiplying the left ventricular outflow tract area by the flow velocity–time integral and was indexed to body surface area. Left ventricular ejection fraction was measured by the biplane Simpson method. The hemodynamic severity of AS was assessed by transthoracic echocardiography using the standard parameters: \(V_{puls} \), MG, aortic valve area, and AVAi.\(^ {15-17}\) \(V_{puls} \) was measured from the transaortic jet continuous-wave Doppler. MG obtained with the use of the Bernoulli formula; aortic valve area was calculated by the standard continuity equation and indexed to body surface area.

Post surgery Histological Assessment of Aortic Valves

As part of an ongoing protocol (PROGRESSA study, ClinicalTrials.gov Identifier: NCT01679431), 2 leaflets of each valve excised at the time of the surgery were placed in a container filled with HEPEs solution, and the remaining leaflet was placed in RNA later (Ambion, Inc, Austin, TX) for subsequent analyses (Figure 1C and 1D). All valves

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Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AS</td>
<td>aortic stenosis</td>
</tr>
<tr>
<td>AVAi</td>
<td>indexed aortic valve area</td>
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<tr>
<td>AVC</td>
<td>aortic valve calcification</td>
</tr>
<tr>
<td>AVW</td>
<td>aortic valve weight</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>MDCT</td>
<td>multidetector computed tomography</td>
</tr>
<tr>
<td>MG</td>
<td>mean transvalvular gradient</td>
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<tr>
<td>(V_{puls} )</td>
<td>peak aortic jet velocity</td>
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</table>

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Novelty and Significance

**What Is Known?**

- Aortic valve calcification is the culprit lesion in aortic stenosis.
- For the same aortic stenosis severity, women have less aortic valve calcification than men.

**What New Information Does This Article Contribute?**

- There is a better correlation between valve weight and aortic valve calcification in men than in women.
- Women have more fibrosis and denser connective tissue than men for the same aortic stenosis severity.
- Histopathology and pathophysiology of aortic stenosis may differ between men and women.

No pharmacological treatment is currently available to alter the progression of aortic valve stenosis. Previous studies report that aortic valve calcification is the main culprit lesion of aortic valve stenosis. However, a majority of these studies have been performed in men or with a large majority of men. We recently reported that women present less aortic valve calcification than men for the same hemodynamic severity of aortic stenosis. In the present study, we confirm this finding and demonstrate that fibrosis and dense connective tissue were more abundant in women than in men for the same hemodynamic aortic valve stenosis. Thus, histopathology of aortic stenosis differs between sexes with a more calcific pattern in men and a more fibrotic pattern in women. These findings suggest that the pathophysiology of aortic valve stenosis differs between men and women and that a sex-specific approach should be considered in drug development for the treatment of aortic stenosis.

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 modeled using commercially available and validated software (Aquarius iNtuition from TeraRecon, Inc, San Mateo, CA), and all AVC data were expressed in Agatston units (AU).\(^ {14}\) Calcification was defined as 4 adjacent pixels with a density >130 Hounsfield units. The summation of per-slice lesion scores was performed individually for every AVC score. To account for smaller heart size in women, we calculated the AVC density by dividing the AVC score by the cross-sectional aortic annulus area (\(\pi[aortic annulus diameter/2]^2\)) measured by echocardiography. AVC quantification assessment was performed by both the same and another investigator blinded to previous scoring and Doppler echocardiographic data and repeated at least 1 month after the original measurement in one third of the exams (ie, 30 exams) to evaluate intraobserver and interobserver variability. The variabilities were calculated by dividing the absolute value of the difference by the average of the 2 measurements. Intraobserver variability and interobserver variability were, respectively, 2.6±2.8% and 4.3±4.2%.

Doppler Echocardiography

Stroke volume was calculated by multiplying the left ventricular outflow tract area by the flow velocity–time integral and was indexed to body surface area. Left ventricular ejection fraction was measured by the biplane Simpson method. The hemodynamic severity of AS was assessed by transthoracic echocardiography using the standard parameters: \(V_{puls} \), MG, aortic valve area, and AVAi.\(^ {15-17}\) \(V_{puls} \) was measured from the transaortic jet continuous-wave Doppler. MG obtained with the use of the Bernoulli formula; aortic valve area was calculated by the standard continuity equation and indexed to body surface area.
Aortic valve weight (AVW) was indexed by the cross-sectional aortic annulus area measured by echocardiography to obtain AVW density. Then, five 4-µm sections were obtained from each resected valvular leaflet and stained with hematoxylin–eosin (Figure 1E and 1F). Histological sections were analyzed, and the degree of valvular fibrosis was assessed using a validated semiquantitative score (0: absence of fibrosis; 1: mild fibrosis; 2: moderate fibrosis; and 3: severe fibrosis). A semiquantitative calcification score, as previously published by Warren and Yong, was assigned to each valve (1: absence of calcification; 2: mild valve thickening and early nodular calcification; 3: moderate thickening with many calcified nodules; and 4: severe thickening with many calcified nodules). All valves were scored by 4 pathologists with extensive experience in cardiovascular pathology (C.C., S.T., S.P., and P.J.) blinded to clinical data.

**Masson Trichrome and Picrosirius Red Staining**
Among our cohort patients for whom valve leaflet samples were available (n=39), 24 patients (12 men and 12 women) were tightly matched (1:1) for AS severity based on MG (±2.5 mmHg), AVAi (±0.05 cm²/m²), and indexed stroke volume (±2 mL/m²). Both picrosirius red birefringence (Figure 1I and 1J) and Masson trichrome (Figure 1G and 1H) staining were performed on transversal sections of surgically excised valves of these matched men and women. Ten (5 men and 5 women) nonstenotic aortic valves harvested from hearts explanted for terminal heart failure after heart transplant were stained and stand for normal nonstenotic state with no echocardiographic detection of sclerosis on any of these valves. Aortic valve leaflets were embedded in OCT, and 5-µm sections were obtained from a skilled operator using a cryotome. All sections were fixed in acetone–methanol (60:40) at −20 °C for 10 minutes and washed with running tap water for 5 minutes. Picrosirius red staining and Masson trichrome staining kits were obtained from Sigma-Aldrich Corp (Ontario, Canada). Regarding picrosirius red staining, sections were immersed in a Weigert iron hematoxylin solution for 10 minutes and washed with running tap water for 5 minutes. Next, sections were incubated in picrosirius red dye for 60 minutes. They were then quickly washed in 2 consecutive acidified water baths (0.5%). Sections were rinsed and dehydrated through alcohol solutions, cleared in toluene solutions and mounted using a quick-hardening mounting medium (Eukitt, Sigma-Aldrich, Ontario, Canada). As for Masson trichrome staining, after the acetone–methanol fixation, sections were then immersed in a Weigert iron hematoxylin solution for 5 minutes and washed with running tap water for 5 minutes. Next, slides were incubated in Biebrich Scarlet–Acid Fuchsin for 5 minutes and briefly rinsed in deionized water. They were then placed in a phosphotungstic–phosphomolybdic acid solution (1:1) for 3 minutes. Slides were then directly immersed in an aniline blue solution for 2 minutes and 30 seconds and in 1% acetic acid for 2 minutes. Finally, slides were rinsed and dehydrated through alcohol solutions, cleared in tolune solutions, and mounted using a quick-hardening mounting medium (Eukitt, Sigma-Aldrich, Ontario, Canada). Tissue section images were acquired with a Zeiss Axio Observer Z1 widefield microscope for both picrosirius red and Masson trichrome staining and using polarized light for picrosirius red birefringence. They were later analyzed with MathWorks’s MATLAB software using an automatic algorithm for pixel intensities and pixel wavelengths for color differentiation (picrosirius red: only collagen fibers are revealed in red/orange color by polarized light; Masson trichrome: dense connective tissue, dark blue; loose connective tissue, light blue; mineralization, red/purple; and cell nuclei, black). Picrosirius red amount of collagen fibers were expressed as the ratio of polarized light pixels on the global brightfield tissue pixels. Masson trichrome staining data were expressed as the ratio of dense and loose connective tissue area on the global tissue area in brightfield.

**Statistical Analysis**
Results were expressed as mean±SD, median (percentile 25–75), or percentage as appropriate. Continuous variables were tested for normality by the Shapiro–Wilk test. AVC and AVC density were not normally distributed and, therefore, were normalized by square root.
transformation. Differences between men and women were assessed by Student t test for continuous normally distributed variables; the Wilcoxon rank-sum test for continuous non-normally distributed and ordinal variables; and categorical data were compared with the χ² or Fisher exact tests as appropriate. Correlation with valve weight or fibrosis score were identified by univariate and multivariate linear or Pearson regression, respectively. Differences in regressions between sexes were assessed by covariance analyses. Histological findings of Masson trichrome stains are presented as median (percentiles 25–75) and analyzed with the use of signed Wilcoxon rank-sum tests. A P value <0.05 was considered statistically significant. All statistics were performed with JMP 12 software and SPSS 20 software.

**Results**

**Population Characteristics**

Among the 125 patients eligible for this study, 64 men and 39 women were frequency matched and 22 patients were excluded because they did not meet matching criteria. Sex-related patient demographics and clinical characteristics are summarized in Table 1. Mean age of the study population was 75±9 years. Age, BMI, blood pressures, history of hypertension, diabetes mellitus, and renal failure were all similar between men and women (all P≥0.17). As expected, women had smaller body surface area (1.73±0.20 versus 2.00±0.19 m²; P<0.0001) and aortic annulus diameter (2.01±0.14 versus 2.27±0.17 cm; P<0.0001) and lower prevalence of coronary artery disease (38 versus 77%; P=0.0008) compared with men. Doppler echocardiographic parameters of AS severity were similar in women versus men (all P≥0.18). Although women presented higher left ventricular ejection fraction than men (65±8 versus 58±8%; P<0.0001), both groups had left ventricular ejection fractions within normal range and similar indexed stroke volume (P=0.93).

Tomographic and histological data of the 2 subgroups are summarized in Table 2. Women had much lower AVC load (1279 [882–1915] versus 2741 [1839–3858] AU; P<0.0001) and AVC density (400 [270–557] versus 670 [473–964] AU/cm²; P=0.0003; Figure 2A) compared with men. Women had significantly lighter A VW compared with men (1.87±0.58 versus 2.66±1.07 g; P<0.0001). However, this difference did not persist after indexation by the aortic annulus area (A VW density: 0.59±0.18 versus 0.66±0.25 g/cm²; P=0.28; Figure 2B).

**Correlation Between Aortic Valve Weight and Aortic Valve Calcification**

AVC density strongly correlated with AVW density in men (r²=0.57; P<0.0001), but this correlation was much weaker in women (r²=0.26; P=0.0008; Figure 2C). For a given AVW density, women invariably had lesser amount of calcification in their valve leaflets. Furthermore, women presented a much higher AVW/AVC ratio than men (1.71±1.24 versus
1.14±0.82 mg/AU; \( P=0.0002 \); Figure 2D), indicating an excess of noncalcified tissue in women. There was the same \( P=1.00 \) absence of relationship, between the AVW/AVC ratio and native valve size, assessed by the aortic annulus diameter, in men \( (r^2=0.01; \ P=0.38) \) and women \( (r^2=0.004; \ P=0.71) \). However, the ratio was constantly higher in women than in men for a given aortic annulus size. In a multivariate regression analysis adjusted for age, BMI, and aortic annulus diameter, female sex was found to be an independent predictor for increased noncalcified tissue amount in the aortic valve as documented by the AVW/AVC ratio \( (P=0.02; \ Table 3) \). Further adjustments for AS severity improved these results \( (V_{peak}, \ MG, \text{ and } AVAi; \ P=0.001; \ P=0.005; \text{ and } P=0.01, \text{ respectively}) \).

### Table 2. Tomographic and Histological Data of the Study Population

<table>
<thead>
<tr>
<th>Variables</th>
<th>Whole Cohort (n=103)</th>
<th>Men (n=64, 62%)</th>
<th>Women (n=39, 38%)</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MDCT data</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVC, AU</td>
<td>1973 (1124–3490)</td>
<td>2741 (1839–3858)</td>
<td>1279 (882–1915)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AVC density, AU/cm²</td>
<td>548 (334–833)</td>
<td>670 (473–964)</td>
<td>400 (270–557)</td>
<td>0.0003</td>
</tr>
<tr>
<td><strong>Histological data</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AW, g</td>
<td>2.36±0.99</td>
<td>2.66±1.07</td>
<td>1.87±0.58</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AW/aortic annulus area, g/cm²</td>
<td>0.63±0.23</td>
<td>0.66±0.25</td>
<td>0.59±0.18</td>
<td>0.28</td>
</tr>
<tr>
<td>AVW/AVC, mg/AU</td>
<td>1.36±1.03</td>
<td>1.14±0.82</td>
<td>1.71±1.24</td>
<td>0.0002</td>
</tr>
<tr>
<td>Fibrosis score, ( n ) (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>31 (30)</td>
<td>22 (34)</td>
<td>9 (23)</td>
<td>0.43</td>
</tr>
<tr>
<td>2</td>
<td>57 (55)</td>
<td>34 (53)</td>
<td>23 (59)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>15 (15)</td>
<td>8 (13)</td>
<td>7 (18)</td>
<td></td>
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<tr>
<td>Warren–Yong score, ( n ) (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>1</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>19 (19)</td>
<td>10 (16)</td>
<td>9 (23)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>63 (61)</td>
<td>36 (56)</td>
<td>27 (69)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>21 (20)</td>
<td>18 (28)</td>
<td>3 (8)</td>
<td></td>
</tr>
</tbody>
</table>

AVC indicates aortic valve calcification; AU, Agatston units; AVW, aortic valve weight; and MDCT, multidetector computed tomography.

### Histological Assessment of Fibrous Tissue

Twelve men and 12 women were tightly matched for AS severity and major clinical baseline characteristics. Matched patients characteristics are summarized in Table 4. Picrosirius red birefringence staining was performed and Masson trichrome staining on all 12 matched valve pairs. Picrosirius red staining analyses of these paired stenotic aortic valves \( (SAV) \) showed significantly more collagen fibers in women than men \( (50.3 [42.3–65.8] \text{ versus } 39.9 [27.5–47.2]%; \ P=0.01; \ Figure 4A) \). Moreover, SAV of men showed a significant decrease in relative collagen fiber amounts compared with nonstenotic aortic valves \( (NSAV) \); \( 39.9 [27.5–47.2] \text{ versus } 66.4 [56.0–76.8]%; \ P=0.003 \), whereas women did not \( (50.3 [42.3–65.8] \text{ versus } 73.0 [52.3–82.5]%; \ P=0.13) \). As for Masson trichrome staining, we measured the proportion of the valve area occupied by dense connective tissue and loose connective tissue. The total proportion of fibrous tissue, being dense and loose connective tissue altogether, was significantly greater in women \( (95.0 [94.0–95.7] \text{ versus } 91.2 [76.8–95.5]%; \ P=0.06) \) than in men \( (92.0 [88.8–93.3] \text{ versus } 91.2 [76.8–95.5]%; \ P=0.86) \). Looking at dense and loose connective tissue separately, SAV of women presented a greater proportion of dense connective tissue than men \( (77.3 [69.8–82.8] \text{ versus } 66.0 [58.6–80.8]%; \ P=0.02; \ Figure 4B) \). Moreover, SAV of women showed a substantial increase in dense connective tissue compared with NSAV \( (77.3 [69.8–82.8] \text{ versus } 58.6 [40.1–61.2]%; \ P=0.0002) \), whereas SAV of men did not \( (66.0 [58.6–80.8] \text{ versus } 65.8 [55.0–77.5]%; \ P=0.68) \).

#### Semiquantitative Fibrosis Score Analysis

To compare fibrosis levels in equivalently calcified valves, we stratified the fibrosis score distribution by the Warren–Yong score in men and women (Figure 3). Interestingly, the fibrosis score tightly correlated with the Warren–Yong score described in men \( (P=0.001) \) but not in women \( (P=0.66) \). Moreover, there was a higher score of fibrosis in women compared with men \( (P=0.04) \). After further adjustment for age, BMI, and aortic annulus diameter, female sex remained an independent predictor of higher fibrosis score \( (P=0.008; \ Table 3, \text{ model 1}) \). Finally, when using the AVC density measured by MDCT in the previous model instead of the Warren–Yong score, female sex was also found to be an independent predictor of higher fibrotic levels \( (P=0.003; \ Table 3, \text{ model 2}) \). Female sex remained significantly associated with increased valvular fibrosis after further adjustment for AS severity \( (V_{peak}, \ MG, \text{ and } AVAi; \ P=0.02; \ P=0.02 \text{ and } P=0.004, \text{ respectively}) \).
the average increase in dense connective tissue from NSA V to SA V was 23.8±8.5% in women, whereas only 2.7±11.9% in men ($P=0.02$; Figure 4D). Finally, there was no difference in the proportion of loose connective tissue between women and men SA V (16.8 [11.9–22.6] versus 14.8 [9.0–22.9]% respectively; $P=0.62$). When compared with NSA V, there was a significant decrease of loose connective tissue in women SA V (35.5 [31.4–51.3] versus 16.8 [11.9–22.6]%; $P<0.0001$), and the same trend, although not statistically significant, was found in men SA V (21.4 [15.7–36.2] versus 14.8 [9.0–22.9]%; $P=0.13$).

The Discussion section outlines the findings of the study, noting that women are prone to have greater amounts of valvular fibrosis, localized in dense connective tissue, than men for the same hemodynamic AS severity and same valve weight density. Furthermore, the extent of fibrosis correlated well with the amount of calcification in men but not in women. Thus, pathogenesis of AS seems to be sex specific with predominance of valvular calcification in men versus predominance of valvular fibrosis in women. These findings demonstrate that valvular fibrosis is likely the main factor explaining the previously reported AVC-hemodynamic severity disproportion in women compared with men.12,13

### Table 3. Multivariable Analyses of Aortic Valve Fibrosis

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variables</th>
<th>Estimate (95% CI) or estimate ±SEM</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium weight ratio</td>
<td>Female sex</td>
<td>0.30 (0.04 to 0.55)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>$-0.04$ ($-0.06$ to $-0.01$)</td>
<td>0.003</td>
</tr>
<tr>
<td>Fibrosis score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1*</td>
<td>Warren–Yong score</td>
<td>1.51±0.40</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>3 vs 2</td>
<td>1.20±0.38</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>4 vs 3</td>
<td>0.74±0.29</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>Female sex</td>
<td>0.84±0.29</td>
<td>0.003</td>
</tr>
<tr>
<td>Model 2*</td>
<td>Square root AVC density</td>
<td>0.09±0.03</td>
<td>0.0007</td>
</tr>
<tr>
<td></td>
<td>Female sex</td>
<td>0.84±0.29</td>
<td>0.003</td>
</tr>
</tbody>
</table>

AVC indicates aortic valve calcification.

*Models adjusted for age, body mass index, left ventricular outflow tract diameter.
In our population, the only clinically significant difference between men and women was the history of coronary artery disease given that male sex is an important and independent risk factor for atherosclerotic diseases. Nevertheless, the frequency matching of sexes performed in this study allows direct comparison of the different valvular tissue components (calcification versus fibrosis) contributing to AS with exclusion of major confounders.

Recent studies reported that women, with similar AS severity, present lower AVIC loads than men even after taking into account the effect of smaller body size, heart, and, thus, aortic annulus size. We confirmed the presence of greater aortic valve calcification loads in men compared with women in our population even after normalization for aortic annulus size, which further supports the concept that this gap in calcification cannot be solely explained by valve size. In contrast, the weight of the excised aortic valves, which was initially statistically different between the 2 sexes, became similar when indexed to the cross-sectional aortic annulus area. Yet, we showed for the first time that women reached a valve weight density equivalent to men but with a significantly lower calcium density.

The differences between sexes in the linear regression curves between valve weight and calcification densities further emphasized this point. The calcification gaps combined with the poor correlation strength in women suggest that noncalcified tissue would have a more important contribution in the development of AS in women compared with men. Moreover, the slope of the valve weight-calcium curve was different between sexes, meaning that increment in valve weight is mainly driven by calcium gain in men but not in women. The obvious separation between aortic valve weight-calcification curves represents the contribution of noncalcific tissue, that is, fibrosis. The histopathologic data obtained on the excised aortic valves further confirm that the gap between valve weight and valve calcification is actually fibrosis. Indeed, a multimodality assessment approach of valvular fibrosis, including semiquantitative calcification and fibrosis scores and hematoxylin–eosin, picrosirius red, and Masson trichrome staining, helped to establish that women experiencing AS have more collagen-rich sections in their valve leaflets than men. Furthermore, the meaningful augmentation in dense connective tissue observed in women and not in men could suggest a stronger activation and more

![Figure 3. Level of valvular fibrosis according to calcification and sex. Bar graphs represent proportion of patients according to semiquantitative score of fibrosis stratified for Warren-Yong score in men (A) and women (B).](http://circres.ahajournals.org/)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Men (n=12, 50%)</th>
<th>Women (n=12, 50%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical data</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>77±6</td>
<td>77±5</td>
<td>0.34</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27.7±5.9</td>
<td>26.4±4.5</td>
<td>0.55</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>129±20</td>
<td>135±18</td>
<td>0.54</td>
</tr>
<tr>
<td>Diastolic</td>
<td>72±8</td>
<td>71±13</td>
<td>0.93</td>
</tr>
<tr>
<td>Coronary artery disease, n (%)</td>
<td>7 (58)</td>
<td>5 (42)</td>
<td>0.41</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>8 (67)</td>
<td>8 (67)</td>
<td>1.00</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>5 (42)</td>
<td>3 (25)</td>
<td>0.73</td>
</tr>
<tr>
<td>Renal failure, n (%)</td>
<td>2 (17)</td>
<td>2 (17)</td>
<td>1.00</td>
</tr>
<tr>
<td>Echocardiographic data</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak aortic jet velocity, m/s</td>
<td>3.9±0.6</td>
<td>4.0±0.7</td>
<td>0.65</td>
</tr>
<tr>
<td>Mean gradient, mmHg</td>
<td>38±15</td>
<td>38±13</td>
<td>0.90</td>
</tr>
<tr>
<td>Aortic valve area, cm²</td>
<td>0.79±0.19</td>
<td>0.67±0.19</td>
<td>0.16</td>
</tr>
<tr>
<td>Indexed aortic valve area, cm²/m²</td>
<td>0.41±0.09</td>
<td>0.41±0.12</td>
<td>1.00</td>
</tr>
<tr>
<td>Stroke volume, mL</td>
<td>71±17</td>
<td>63±15</td>
<td>0.27</td>
</tr>
<tr>
<td>Indexed stroke volume, mL/m²</td>
<td>36±8</td>
<td>38±8</td>
<td>0.61</td>
</tr>
</tbody>
</table>
weighty implications of profibrotic signalization pathways in women.

Despite constantly increasing knowledge on the disease, to date, important blanks remain about the understanding of the complex interactions between fibrocollagen deposition and osteoblastic–chondrocytic transformations in the valve tissue and the time dependency of these processes. As it was previously demonstrated, the initial histopathologic response in AS is fairly similar with vascular atherosclerosis, which is characterized by lipid-mediated infiltration and inflammation, increased valvular fibrosis, and mineralization.²¹ We should bear in mind that there is a latent interval between transformation of fibrotic tissue to more intense calcification. According to this study inference, we could say that one of the most important independent factor concerning calcification delay or even as a player in the complex interaction between fibrogenesis and calcification is the female sex.

One question, still, unresolved, is why the content of collagen and the magnitude of mineralization differ according to sex in AS? On the one hand, compared with men, women tend to develop heart diseases, such as AS, later in life.²²,²³ This difference may be attributable to the loss of estrogen during the menopause; however, the biological explanation for sexual dimorphism in AS (more fibrosis and less mineral in women for a given AS severity) is likely more complex and may not rely entirely on estrogen levels. On the other hand, male sex has been shown to be a risk factor for AS.²⁴ Androgens have been reported to play a major regulatory role in bone formation, having significant

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**Figure 4.** Histological assessment of collagen fiber content, dense and loose connective tissue proportion in matched stenotic valves, and nonstenotic aortic valve stained with picrosirius red and Masson trichrome staining. Picrosirius red stain showed significantly greater relative amount of collagen fibers in women’s stenotic aortic valve (SAV) than men’s SAV (A). Global fibrous tissue was also more abundant in SAV of women than of men after Masson trichrome staining (B). Stenotic aortic valves of women presented higher proportions of dense connective tissue than men (C) and the increase of dense connective tissue from nonstenotic aortic valves (NSAV) to SAV was greater in women than in men (D).
effects on osteoclast and osteoblast activity and function.\textsuperscript{25} Of interest, the administration of testosterone to apolipoprotein E knockout mice, resulted, regardless to the sex, into higher level of mineral into the aortic sinus and innominate artery.\textsuperscript{26} In the same line, a recent study demonstrated that testosterone plays a role in promoting the calcification of vascular smooth muscle cell cultures.\textsuperscript{27} Also, women with polycystic ovary syndrome tend to develop vascular calcification, which could be related to higher levels of androgen hormones.\textsuperscript{28,29} Moreover, studies emphasized that regardless of the hormonal status, the behavior of cell culture is influenced by sex.\textsuperscript{30} To this effect, McCoy et al\textsuperscript{31} showed in porcine valve interstitial cells that sex has a significant effect on the gene expression pattern. For instance, the expression of stanniocalcin 1 precursor (STC1) and natriuretic peptide precursor C (NPPC), which may play a role in the regulation of mineralization, were increased in porcine male valve interstitial cells.\textsuperscript{31} Whether these differences play a significant role in sex-dependent fibrosis mineralization of the aortic valve remains to be determined.

Finally, female sex is not to be considered as an independent factor for lower amounts of calcification load in a global cardiovascular phenomenon as previous studies showed higher mitral annular calcification in women than in men as so did our cohort.\textsuperscript{32,33} Instead, we should bear in mind that sex-specific pathophysiology of valve diseases could differentially affect patient, and individualized drug development should be dichotomized accordingly.

**Limitations**

The major limitation of this study resides in the lack of quantification of fibrotic tissue in the entire series of calcified aortic valves. Indeed, the measurement of fibrosis, by quantitative method, was available in a subset of histological sections. However, it is worth highlighting that different methods used to measure fibrosis in aortic valve sections (semiquantitative scoring, quantitative assessment with Masson trichrome, and picrosirius red) concurred and showed a higher level of fibrosis in female valves for a given AS severity. In the future, other imaging techniques (ie, microcomputed tomography and magnetic resonance imaging) could bring valuable information to support the findings presented in this study.

The sample size of this study is relatively small. However, results in AVC were highly consistent with those of previous studies in larger number of patients,\textsuperscript{12,13,34} and the results on valvular fibrosis were based on robust histological analyses performed in carefully matched subsets of male and female patients. We used the Agatston method to measure AVC. The volumetric may potentially be more accurate than the Agatston method. However, the Agatston method has been well validated by previous studies, with the calcium weight measured on explanted valves,\textsuperscript{9} AS hemodynamic severity measured by Doppler echocardiography\textsuperscript{12,23} and clinical outcomes,\textsuperscript{14} and has excellent interscan, interobserver, and intraobserver reproducibility.\textsuperscript{9,12}

Given that male sex is an independent risk factor for AS,\textsuperscript{4,24} two third of our primary cohort were men, and our matching reflected this point. Nevertheless, despite this over-representation of men, our cohorts (frequency matched and 1:1 matched) were well balanced according to match criteria and thus allowed a good comparison of aortic valves. Also, we did not take into account the native valve weight or the weight of lipid that infiltrates in the valve leaflets. However, given that the native valve weight is negligible and that lips weight is ≤0.06 g,\textsuperscript{35} it is highly improbable that these omissions may have significantly impacted the results of our study.

**Conclusions**

In this series of AS patients, women presented higher levels of valvular fibrosis and dense connective tissue than men for a same hemodynamic stenosis severity and similar indexed aortic valve weight independently of confounding factors. These results may explain the previously reported intersex discrepancy in the levels of aortic valve calcification density required to achieve hemodynamically severe AS.\textsuperscript{1,2,3} These findings also suggest that the pathobiological processes underlying the development and progression of AS may be different or at least differently modulated between sexes. Further studies are needed to confirm these results and elucidate these differential pathobiological processes in women versus men. At term, we hope that new insights concerning the pathophysiology of AS according to sex will help for future medical treatment development of AS.

**Clinical Implications**

The findings of this study are important in understanding the pathology of AS and have crucial clinical implications because they tend to demonstrate a sex-specific pathogenesis or modulation of the disease. AS was identified as a calcific disease with presence of valvular fibrosis; we hereby propose a potential fibrotic pathogenesis with presence of calcification that would be more specific to women. Thus, women will present with symptomatic severe AS with less calcium but more fibrosis than men. This discrepancy in calcification/fibrosis ratio may explain in part the better results after transcatheter aortic valve implantation demonstrated in women versus men,\textsuperscript{36,37} given that higher AVC was associated with higher degree of paravalvular regurgitation\textsuperscript{38,39} and aortic annular rupture.\textsuperscript{40} Effective pharmacological treatment that would target the pro-fibrotic pathways implicated in the development of AS would be beneficial especially in women.

Furthermore, as previously published, ≤30% of AS patients have echocardiographic discordant markers of AS severity (ie, aortic valve area <1.0 cm\textsuperscript{2}; mean gradient <40 mm Hg).\textsuperscript{13,41} In this population, aortic valve calcium scoring by MDCT has been proposed as a complementary diagnosis tool to confirm AS severity. However, fibrotic tissues cannot be visualized by MDCT and different thresholds of AVC to identify severe AS have been proposed in women versus men to overcome this limitation (AVC ≥1200 AU in women and AVC ≥2000 AU in men).\textsuperscript{13} However, given the limited correlation between aortic valve calcification and fibrosis in women, some women may end up with underestimated AS severity by MDCT. Hence, women with significant calcification (ie, AVC ≥800 AU)\textsuperscript{13} having symptoms and significant LV hypertrophy
should receive a particular attention. However, there is a need for new development of imaging modalities that would measure not only aortic valve calcification but also valvular fibrosis. Some recent studies suggest that this may be accomplished in the future with cardiac magnetic resonance. Nonetheless, further research is needed to understand key underpinning molecular processes that drive the development of AS in a procalcifying/profibrotic sex-specific manner. In a perspective of individualized medical intervention, deeper insights on AS pathogenesis would allow further research oriented toward sex-specific therapeutic targets (fibrosis in women and calcification in men).

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Disclosures

None.

References


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Supplemental Material

for

Sex-Related Discordance between Aortic Valve Calcification and Hemodynamic Severity of Aortic Stenosis: Is Valvular Fibrosis the Explanation?

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Institut Universitaire de Cardiologie et de Pneumologie de Québec, Université Laval, Québec, Canada.
**DETAILED METHODS:**

**Doppler-echocardiography:**

Doppler-echocardiography images were performed on an IE33 ultrasound system (Philips, Andover, MA, USA). The left ventricular (LV) dimensions and LV ejection fraction were measured according to recommendations of the American Society of Echocardiography. Briefly, linear internal measurements of the LV and its walls were performed in the parasternal long-axis view. LV ejection fraction was calculated by the biplane Simpson method. Doppler echocardiographic left ventricular outflow tract, Vmax, and time velocity integral allowed calculation of mean transvalvular pressure gradient by modified Bernoulli formula, dimension less velocity index, stroke volume, and aortic valve area by continuity equation. The aortic valve area was also indexed to body surface area.

**MDCT scan:**

The non-contrast computed tomography was performed with multidetector scanners (SOMATOM, Siemens Medical Systems, Fordheim, Germany or ICT-256, Philips, Andover, MA, USA). The entire heart was assessed by 3 mm thick axial slices with a pitch of 0.35 and B35f kernel during held inspiration. Acquisitions were obtained with a tube potential at 120 kV and a tube current-time product at 80 mAS. No contrast was needed, nor was beta-blocker administered for the purpose of the examination. Measurements of AVC were performed offline on dedicated workstations with validated software (Aquarius, TeraRecon Inc, San Mateo, California, USA) with the use of the Agatston method. Briefly, calcification was defined as 4 adjacent pixels with density >130 Hounsfield units. The aortic valve was visualized in multiple planes, and careful measurement section by section aimed to accurately exclude contiguous calcium in coronary arteries, mitral valve annulus, or aortic wall.

**Histology:**

**Masson Trichrome staining:** Staining was done with a Masson Trichrome kit (Sigma-Aldrich, ON, Canada) on aortic valve sections embedded in OCT. Sections were fixed with acetone-methanol (60:40) at -20°C for 10 minutes and then rinsed in running tap water for 5 minutes. Then sections were immersed in Weigert’s iron hematoxylin staining for 5 minutes. Slides were once again rinsed in running tap water for 5 minutes. Sections were then stained in Scarlet-Acid fuchsin for 5 minutes and rinsed by 10 successive wettings in distilled water. They were then incubated in phosphotungstic/phosphomolybdic acid solution for 3 minutes. Slides were then placed in aniline blue solution for 2 minutes and 30 seconds and then rinsed in 1% acetic acid for 2 minutes. Finally, slides were dehydrated through alcohol solutions, cleared in toluene solutions: 25 wetting in ethanol 95%, 75 in ethanol 100%, 25 in ethanol-toluene 1:1, 50 in toluene 100%. Then slide were mounted using a quick-hardening mounting medium (Eukitt, Sigma-Aldrich, ON, Canada) and dried 12 hours on flat surface. Images were acquired using a Zeiss Axio Observer microscope using the Zen software (Zeiss, ON, Canada), with a LD A-Plan 20x/0.25 Ph1 objective (Zeiss) with mosaic mode in bright field light.
Images were processed and quantifications were performed with MathWorks’s MATLAB® software using an automatic algorithm for pixel intensities and pixel wavelengths for color differentiation (dense connective tissue = dark blue, loose connective tissue = light blue, mineralization = red/purple, cell nuclei = black).

**Picrosirius red staining:** Staining was done with Picrosirius red staining kit (Sigma-Aldrich, ON, Canada) on aortic valve sections embedded in OCT. Sections were fixed with acetone-methanol (60:40) at -20°C for 10 minutes and then rinsed in running tap water for 5 minutes. Sections were immersed in a Weigert’s iron hematoxylin solution for 10 minutes and washed with running tap water for 10 minutes. Next, sections were incubated in picrosirius red dye for 60 minutes. Picrosirus red solution was obtained by dilution of 0.5g of Direct Red 80 in 500ml of picric acid. Then slides were quickly washed in two consecutive acidified water baths (0.5%). Finally, slides were dehydrated through alcohol solutions, cleared in toluene solutions: 25 wetting in ethanol 95%, 75 in ethanol 100%, 25 in ethanol-toluene 1:1, 50 in toluene 100%. Then slide were mounted using a quick-hardening mounting medium (Eukitt, Sigma-Aldrich, ON, Canada) and dried 12 hours on flat surface. Images were acquired using a Zeiss Axio Observer microscope using the Zen software (Zeiss, ON, Canada), with a LD A-Plan 20x/0.25 Ph1 objective (Zeiss) using polarized light for picrosirius red birefringence. Images were processed and quantifications were performed with MathWorks’s MATLAB® software using an automatic algorithm for pixel intensities and pixel wavelengths for color differentiation (only collagen fibers are revealed in red/orange color by polarized light).
Online Figure I: Flow chart of the study population

328 Surgical AVR + CT pre-AVR (<3months) without contrast

- 49 Moderate to Severe AR
- 27 Previous AVR (redo)
- 13 Endocarditis
- 9 Radiotherapy-induced AS
- 5 Rheumatic AS

225 AVR for Calcific AS + CT<3months without contrast

- 45 Bicuspid
- 38 LVEF<50%
- 17 Incomplete CT/Echo

125 Eligible Patients

78 Men

- Matching for Age, BMI, Hypertension, Diabetes, Renal failure and AS severity (V_{peak}, MG, AVAi)

64 Men

47 Women

39 Women
REFERENCES LIST:

