Stenotic Bicuspid and Tricuspid Aortic Valves
— Micro-Computed Tomography and Biological Indices of Calcification —

Piotr Mazur, MD; Ewa Wypasek, PhD; Boguslaw Gawęda, MD; Dorota Sobczyk, MD, PhD; Przemyslaw Kapusta, PhD; Joanna Natorska, PhD; Krzysztof Piotr Malinowski; Jacek Tarasiuk, PhD; Maciej Bochenek, MD; Sebastian Wroński; Katarzyna Chmielewska; Boguslaw Kapelak, MD, PhD; Anetta Undas, MD, PhD

Background: Valve calcification is well estimated by ex-vivo micro-computed tomography (micro-CT). The objective of this study was to investigate the associations between micro-CT findings and biological indices of calcification in aortic stenosis (AS), as well as differences between bicuspid aortic valve (BAV) and tricuspid aortic valve (TAV).

Methods and Results: Aortic valves and plasma were obtained from patients undergoing valve surgery. Valves were dissected and underwent micro-CT, genetic analyses, and calcium content assessment. Plasma levels of calcification markers were measured.

Forty-two patients with isolated severe AS, including 22 with BAV, were studied. BAV patients had a lower median CT value (140.0 [130.0–152.0] vs. 157.0 [147.0–176.0], P=0.002) and high-density calcification (HDC) fraction (9.3 [5.7–23.3] % vs. 21.3 [14.3–31.2] %, P=0.01), as compared with TAV. Calcification fraction (CF) correlated with AS severity (measured as maximal transvalvular pressure gradient [r=0.34, P=0.03], maximal flow velocity [r=0.38, P=0.02], and indexed aortic valve area [r=−0.37, P=0.02]). For TAV patients only, mRNA expression of integrin-binding sialoprotein correlated with CF (r=0.45, P=0.048), and the receptor activator of the nuclear factor κ-B ligand transcript correlated with HDC corrugation (r=0.54, P=0.01).

Conclusions: TAV patients with AS present more mineralized calcifications in micro-CT than BAV subjects. The relative volume of calcifications increases with the AS severity. In TAV patients, upregulated expression of genes involved in osteoblastogenesis in AS correlates with leaflet mineralization in micro-CT.

Key Words: Aortic stenosis; Bicuspid aortic valve; Genes; Micro-computed tomography

Aortic stenosis (AS) is the most common acquired valvular disease, which develops more frequently and earlier in patients with bicuspid aortic valve (BAV). The gold standard in AS diagnosis is echocardiography; however, this method has limited application in assessment of calcium deposits. Computed tomography (CT) offers better objectivity in calcium assessment, and previous CT studies have related the degree of calcification to the AS severity, but the precision of in vivo CT images is limited as well. The high-quality structural evaluation of AS calcifications is made possible by the ex vivo micro-CT, a method providing an excellent estimation of tissue calcification. The micro-CT findings have been reported to correlate well with AS severity. Nevertheless, data on the relationship of valve calcification in AS and biological indices of calcification processes are lacking.

The pathobiology of calcification in AS shares similarities with atherosclerosis and, at later stages, with bone formation. In the interstitial aortic valve (AV) layer, valvular interstitial cells (VICs) have been found. Under pathological conditions, VICs can differentiate into myofibroblasts (causing fibrosis) and osteoblast-like cells (causing calcification), at least partially through epigenetic modifications. These activated cells are responsive to typical osteogenic media-
tors, such as transforming growth factor-β (TGF-β) super-family members, and bone morphogenetic proteins (BMPs). BMPs stimulate the valve calcification by activating Smad1/5/8 and Wnt/β-catenin signaling pathways, which leads to upregulation of master osteoblast transcription factor, Runx2/Cbfa1 (Runt-related transcription factor 2).
Runx2/Cbfα1 increases the expression of proteins directly associated with calcification and osteoblast differentiation; osteopontin (SPP1), bone sialoprotein (IBSP) and osteocalcin. Several studies confirmed the upregulation of these calcification markers in AS, both on the level of mRNA and protein. The late propagation phase of AS is driven by these pro-osteogenic and pro-calcific factors, resulting in a complex and regulated, self-perpetuating calcification process.

To the best of our knowledge, no study correlating the results of AS calcification assessment in micro-CT with biological determinants of this process has been published yet. Little is known about the differences in calcification between patients with BAV and tricuspid AV (TAV). We sought to correlate findings of micro-CT with genetic analysis and calcium content measurement, and to compare the findings in patients with normal anatomy and BAV patients.

**Methods**

**Patients**

From June 2014 to May 2015, we recruited 42 individuals with severe symptomatic AS scheduled for first-time elective surgical AV replacement (AVR) in the Department of Cardiovascular Surgery and Transplantology of the John Paul II Hospital, Krakow, Poland. The exclusion criteria were: rheumatic disease, any other significant valvular disease, concomitant coronary artery disease, acute cardiovascular event within 3 months prior to enrolment, left ventricular ejection fraction <30%, history of cancer or autoimmune disease, liver failure with an alanine aminotransferase above the 1.5-fold the normal value, and kidney failure with creatinine >177 µmol/L. Valve anatomy was left for intraoperative identification by a surgeon. The study was performed in accordance with the Declaration of Helsinki, and received approval from the Jagiellonian University Ethical Committee. All patients provided their written informed consent.

**AVR**

Standardized echocardiograms were obtained by an experienced echocardiographer using a Toshiba APLIO 80 (Toshiba, Tokyo, Japan) ultrasound machine. All patients underwent operation with cardiopulmonary bypass following standardized procedures. Native AV was meticulously excised with intention to remove the whole leaflets. In case of significant dilation of the ascending aorta, the Bentall procedure was performed.

**Laboratory Investigations**

Fasting blood samples were collected from an antecubital vein with minimal stasis at 06:00–08:00 h on the day of surgery. After centrifugé, the supernatant was stored at −20°C until assay. Plasma samples were centrifuged, frozen, and stored in aliquots at −80°C until assayed.

Concentration of markers responsible for AV calcification were determined in plasma using a commercially available ELISA tests as follows: soluble receptor activator for nuclear factor κ-B ligand (sRANKL) (RD193004200R; BioVendor, Brno, Czech Republic), osteopontin (SPP1) (SEA899Hu; Cloud-Clone Corp, Houston, TX, USA) and integrin-binding sialoprotein (IBSP) (SEB092Hu, Cloud-Clone Corp). All ELISA measurements were performed by technicians blinded to the sample status. Intra-assay and inter-assay coefficients of variation were <7%. Routine analyses (blood glucose, cholesterol levels, high-sensitivity C-reactive protein [hsCRP], creatinine) were measured using standard laboratory techniques.

**Valve Sample Preparation and Macroscopic Dissection**

To allow concomitant analyses by multiple methods, the valve was dissected in 3 parts in the operating room. In all cases, the non-coronary cusp (or in BAV, the cusp containing the non-coronary part; identification by the surgeon) was left for micro-CT, and stored in 10% formalin (at 18°C, for micro-CT). The remaining parts of each valve were stored in RNA-Later solution (Qiagen, Düsseldorf, Germany) for genetic analyses and calcium content assessment.

The RNA-Later fragments were macroscopically examined, dissected and divided into: (1) normal areas, defined as non-calcified, smooth and pliable; and (2) calcified areas. Chosen specimens were frozen at −80°C until future analysis. The 200–300 mg of frozen, stored samples were homogenized by Mikro Dismembrator S (LabWrench, Midland, Canada) in liquid nitrogen and calcium content assessment.

**Gene Expression Analysis**

Total RNA was isolated from approximately 30 mg of 84 preparations from 42 patients, representing the normal and calcified fragments from each AV, using a Universal RNA Purification Kit (Eurx, Gdańsk, Poland) according to manufacturer’s instructions. Total RNA concentrations and purity were determined using Picodrop 100 (Picodrop Ltd, Hinxton, United Kingdom) by measuring absorbance ratios: 260/230 nm and 260/280 nm, respectively.

First-strand complementary DNA was synthesized from 2 µg RNA by using a High Capacity RNA-to-cDNA Kit (Life Technologies, Carlsbad, USA) according to the manufacturer’s instructions. Quantitative TaqMan PCR was performed on a 7900HT Fast Real-Time PCR system (Life Technologies with prime/probe pairs that were obtained using inventoried or made-to-order assays (Hs00243522_m1, RANKL; Hs00765730_m1, nuclear factor kappa-B, NFκB; Hs00999010_m1, secreted phosphoprotein 1, SPP1; Hs00173720_m1, integrin-binding sialoprotein, IBSP; Hs00985639_m1, interleukin-6, IL-6; Hs00154192_m1, BMP 2, BMP2; Hs01047973_m1, runt-related transcription factor 2, Runx2/Cbfα1) from Life Technologies. The PCR reactions contained 10 ng diluted complementary DNA and Probe qPCR Master Mix (2x), plus a ROX Solution Kit (Eurx), according to the manufacturer’s instructions. Levels of measured mRNAs were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase. All samples were run in triplicate.

**Calcium Content Assessment**

Quantitative calcium content in homogenized AV fragments was determined as previously described, with modification. Briefly, 100–150 mg of homogenized tissue was incubated with 1 mL of 1 mol/L HCl in 4°C for 24 h to release calcium ions. Then, samples were centrifuged at 12,000 g for 5 min. The calcium content in the supernatant was measured by a Calcium Colorimetric Assay Kit (Genetex, Hsinchu City, Taiwan) according to the manufacturer’s instructions. All samples were run in triplicate.

**Micro-CT**

The micro-CT was performed using a Nanotom 180N tomo-
Morphological Micro-CT Parameters  

The following parameters were included in the analysis:  
- Average diameter of the calcification particles;  
- Calcification fraction (CF) (calculated as \( \frac{\text{total calcification volume}}{\text{total valve volume}} \); reflecting the percentage of calcifications in studied valve sample volume);  
- HDC fraction (calculated as \( \frac{\text{HDC volume}}{\text{total calcification volume}} \); reflecting the percentage of heavy fraction in calcifications);  
- HDC corrugation (estimated as the ratio: \( \frac{\text{HDC volume}}{\text{HDC surface}} \); an object with the larger surface from 2 objects of identical volume is more corrugated); and  
- Median CT value (reflecting median radiation absorption, and thus valve mineralization).

### Table 1. Baseline Characteristics of the Patients  

<table>
<thead>
<tr>
<th>Variable</th>
<th>All patients (n=42)</th>
<th>Bicuspid (n=22)</th>
<th>Tricuspid (n=20)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>66.0 (58.8–74.0)</td>
<td>62.50 (56.5–71.0)</td>
<td>71.0 (60.3–76.8)</td>
<td>0.03</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>20 (48)</td>
<td>13 (59)</td>
<td>7 (35)</td>
<td>0.14</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>29.2±5.4</td>
<td>27.7±4.3</td>
<td>29.6±6.1</td>
<td>0.53</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>8 (19)</td>
<td>2 (9)</td>
<td>6 (30)</td>
<td>0.11</td>
</tr>
<tr>
<td>Arterial hypertension, n (%)</td>
<td>32 (76)</td>
<td>16 (72)</td>
<td>16 (80)</td>
<td>0.50</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>35 (83)</td>
<td>18 (81)</td>
<td>17 (85)</td>
<td>0.48</td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>2 (5)</td>
<td>0 (0)</td>
<td>2 (10)</td>
<td>0.16</td>
</tr>
<tr>
<td>History of smoking, n (%)</td>
<td>13 (31)</td>
<td>9 (41)</td>
<td>4 (20)</td>
<td>0.16</td>
</tr>
<tr>
<td>Previous MI, n (%)</td>
<td>1 (2)</td>
<td>1 (5)</td>
<td>0 (0)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Medication  

- Aspirin, n (%) | 29 (69) | 13 (59) | 16 (80) | 0.11 |
- ACEI, n (%) | 24 (57) | 12 (55) | 12 (60) | 0.56 |
- β-blocker, n (%) | 32 (76) | 17 (77) | 15 (75) | 1.00 |
- Statin, n (%) | 30 (71) | 14 (64) | 16 (80) | 0.19 |

Preoperative echocardiography  

- Mean transvalvular gradient, mmHg | 54.1±15.9 | 57.1±17.2 | 51.5±13.2 | 0.25 |
- Maximal transvalvular gradient, mmHg | 87.5±23.9 | 93.7±24.9 | 81.37±20.6 | 0.09 |
- Maximal flow velocity, m/s | 4.4 (4.2–4.8) | 4.7 (4.2–5.1) | 4.4 (4.2–4.6) | 0.10 |
- Aortic valve area, cm² | 0.67±0.19 | 0.63±0.17 | 0.70±0.17 | 0.26 |
- Aortic valve area index, cm²/m² | 0.37±0.11 | 0.35±0.11 | 0.38±0.11 | 0.47 |
- Aortic insufficiency | 0.29 |

- None, n (%) | 17 (40) | 11 (50) | 6 (30) | 0.39 |
- I°, n (%) | 20 (48) | 7 (32) | 13 (65) | 1.00 |
- II°, n (%) | 5 (12) | 4 (18) | 1 (5) | 0.73 |

LVEF, % | 58.7±7.2 | 57.75±8.78 | 59.80±4.79 | 0.59 |

Data are shown as mean ± SD or median (IQR) or number (percentage). ACEI, angiotensin-converting enzyme inhibitors; BMI, body mass index; LVEF, left ventricular ejection fraction; MI, myocardial infarction.

### Statistical Analysis  

Continuous variables were checked for normal distribution with the Shapiro-Wilk test. Data are expressed as mean±standard deviation or median (interquartile range) unless otherwise stated. To assess the differences between 2 continuous variables, the Student’s t-test (for normally distributed values), or the Mann-Whitney U-test (for non-normally distributed values) were applied. Categorical variables were compared by using a Chi-squared test or Fisher’s exact test, as appropriate. The Pearson’s correlation coefficient (Pearson’s r) or Spearman’s rank correlation coefficient were calculated to assess the linear correlations between variables with a normal or non-normal distribution, respectively. Multivariable linear regression was used to identify relationships between maximal transvalvular...
pressure gradient (PGmax) and sRANKL, and other variables selected in univariable analyses as having P<0.1. The covariable with the weakest semi-partial correlation was removed at each step, until all remaining covariables reached statistical significance. Predictors of being in the highest CF quartile were identified in logistic regression analysis (variables with P<0.1 in univariable analysis [PGmax and IBSP mRNA expression] entered the multiple model). A strong significant correlation between any 2 parameters (r>0.5) excluded one of the parameters from the multiple linear and logistic models. All final multiple parameters (r>0.5) excluded one of the parameters from the multiple linear and logistic models. All final multiple models were adjusted for age, sex, body mass index (BMI) and BAV status.

Results

A total of 42 patients with isolated severe AS (52% with BAV) were analyzed (Table 1). Patients with BAV were younger and tended to have a higher PGmax than patients with TAV (Table 1). Most patients received AVR through median sternotomy (mini-sternotomy was performed in 19% of cases). Due to post-stenotic dilation of the ascending aorta, 2 (5%) patients with BAV received Bentall procedure. There was 1 death (2%) and 3 (7%) re-do sternotomies due to bleeding.

Women more often had diabetes mellitus than men (30% vs. 5%, P=0.048) and were older (70±8 vs. 60±13 years, respectively, P=0.006), but there were no sex-related differences in pharmacotherapy, BAV incidence and disease severity.

Micro-CT

The diameter of calcification particles averaged 0.19 mm, and calcifications represented approximately 19% of the investigated tissue, 16.5% of which was of high density (Table 2). BAV patients had a lower median CT value and HDC fraction, as compared with TAV patients (Table 2). There were no differences whatsoever between patients with a PGmean ≥50 mmHg, as compared with a PGmean <50 mmHg, also in subgroup analysis in BAV and TAV. However, when we compared BAV patients with a PGmean ≥50 mmHg with TAV patients with a PGmean ≥50 mmHg, the BAV group had a lower median CT value (lower by 16% [P=0.02]).

The quartile analysis of the CF revealed that individuals with CF in the highest quartile had a higher PGmax (103.5±25.3 mmHg vs. 78.9±22.8 mmHg, P=0.03), and a higher Vmax (4.8 [4.4–5.7] m/s vs. 4.2 [3.9–4.4] m/s, P=0.01) but a lower HDC fraction (14.6±12.6% vs. 30.1±9.1%, P=0.004), when compared with the lowest quartile. There were no differences in BAV incidence between the extreme quartiles of CF.

When we compared the individuals with a HDC fraction in the highest quartile with those in the lowest quartile, the highest quartile group was older (72±10 vs. 62±8 years, P=0.02), but had a lower PGmax (78.7±11.3 vs. 101.5±23.3 mmHg, P=0.01), PGmean (50.7±6.3 vs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>All patients (n=42)</th>
<th>Bicuspid (n=22)</th>
<th>Tricuspid (n=20)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro-CT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average diameter of the calcification particles, mm</td>
<td>0.19 (0.16–0.28)</td>
<td>0.18 (0.16–0.45)</td>
<td>0.19 (0.16–0.24)</td>
<td>0.87</td>
</tr>
<tr>
<td>CF, %</td>
<td>19.1±10.3</td>
<td>18.4±9.7</td>
<td>18.8±11.1</td>
<td>0.86</td>
</tr>
<tr>
<td>HDC fraction, %</td>
<td>16.5 (7.8–25.5)</td>
<td>9.3 (5.7–23.3)</td>
<td>21.3 (14.3–31.2)</td>
<td>0.01</td>
</tr>
<tr>
<td>HDC corrugation, mm⁻¹</td>
<td>13.9 (12.6–15.1)</td>
<td>14.3 (12.5–15.3)</td>
<td>13.3 (12.5–15.5)</td>
<td>0.28</td>
</tr>
<tr>
<td>Median CT value</td>
<td>147 (134.0–161.3)</td>
<td>140.0 (130.0–152)</td>
<td>157.0 (147.0–176.0)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 2. Micro-CT Analysis, Gene Expression and Tissue Calcification

The change in mRNA expression is shown as median factor (interquartile range), by which the gene expression was increased in calcified, as compared to soft tissue (all, P<0.05). P values shown refer to a comparison of bicuspid vs. tricuspid valves.
Aortic Stenosis in Micro-CT: Biological Links

Calcification-Related mRNA Expression, Valve Calcium Content and Serum Markers

The expression of studied genes was increased in calcified parts of the valve, as compared with the non-calcified soft valvular tissue, from 79% (RANKL) to 486% (ISBP), with no differences between BAV and TAV (Table 2). Patients with diabetes had a higher increase in calcium content in calcified tissue than non-diabetics (2.95 ± 1.90 vs. 1.84 ± 0.90 µg/mg, P=0.002).

The mRNA levels of SPP1 correlated with mRNA levels of IBSP (r=0.47, P=0.002), and was also the case for the BAV and TAV subgroups (r=0.5, P=0.02 and r=0.4, P=0.06). The SPP1 and IBSP transcripts correlated inversely with BMP2 in both BAV (r=−0.6, P=0.001; r=−0.5, P=0.01, respectively) and TAV (r=−0.6, P=0.01; r=−0.5, P=0.02, respectively) groups. In the entire study group, the calcium content in heavily calcified valvular parts correlated with the upregulation of mRNA for BMP2 expression (r=0.32, P=0.04).

TAV, but not BAV, patients showed the correlation of mRNA expression of Runx2/Cbfa1 with IBSP transcript (r=0.52, P=0.02). Also, only in the TAV group did mRNA expression of IBSP correlate with CF (r=0.45, P=0.048), and the RANKL transcript correlate with HDC corrugation (r=0.54, P=0.01).

There were no sex- or valve anatomy-related differences in plasma SPP1, IBSP and sRANKL levels (Table 2).

However, there was a positive correlation between serum sRANKL and median CT value (r=0.45, P=0.048), and the RANKL transcript correlate with HDC corrugation (r=0.54, P=0.01).

Table 3 delineates correlations between the micro-CT parameters. The positive association between the HDC fraction and median CT value (Figure B) was confirmed in TAV (r=0.69, P=0.001) and BAV (r=0.92, P<0.0001). The CF correlated with PGmax (r=0.34, P=0.03; Figure A) and Vmax (r=0.38, P=0.02), and inversely with AIA (r=−0.37, P=0.02).

Calcification-Related mRNA Expression, Valve Calcium Content and Serum Markers

The expression of studied genes was increased in calcified parts of the valve, as compared with the non-calcified soft valvular tissue, from 79% (RANKL) to 486% (ISBP), with no differences between BAV and TAV (Table 2). Patients with diabetes had a higher increase in calcium content in calcified tissue than non-diabetics (2.95 ± 1.90 vs. 1.84 ± 0.90 µg/mg, P=0.002).

The mRNA levels of SPP1 correlated with mRNA levels of IBSP (r=0.47, P=0.002), and was also the case for the BAV and TAV subgroups (r=0.5, P=0.02 and r=0.4, P=0.06). The SPP1 and IBSP transcripts correlated inversely with BMP2 in both BAV (r=−0.6, P=0.001; r=−0.5, P=0.01, respectively) and TAV (r=−0.6, P=0.01; r=−0.5, P=0.02, respectively) groups. In the entire study group, the calcium content in heavily calcified valvular parts correlated with the upregulation of mRNA for BMP2 expression (r=0.32, P=0.04).

TAV, but not BAV, patients showed the correlation of mRNA expression of Runx2/Cbfa1 with IBSP transcript (r=0.52, P=0.02). Also, only in the TAV group did mRNA expression of IBSP correlate with CF (r=0.45, P=0.048), and the RANKL transcript correlate with HDC corrugation (r=0.54, P=0.01).

There were no sex- or valve anatomy-related differences in plasma SPP1, IBSP and sRANKL levels (Table 2).

However, there was a positive correlation between serum sRANKL and median CT value (r=0.45, P=0.048), and the RANKL transcript correlate with HDC corrugation (r=0.54, P=0.01).

Table 3 delineates correlations between the micro-CT parameters. The positive association between the HDC fraction and median CT value (Figure B) was confirmed in TAV (r=0.69, P=0.001) and BAV (r=0.92, P<0.0001). The CF correlated with PGmax (r=0.34, P=0.03; Figure A) and Vmax (r=0.38, P=0.02), and inversely with AIA (r=−0.37, P=0.02).

Calcification-Related mRNA Expression, Valve Calcium Content and Serum Markers

The expression of studied genes was increased in calcified parts of the valve, as compared with the non-calcified soft valvular tissue, from 79% (RANKL) to 486% (ISBP), with no differences between BAV and TAV (Table 2). Patients with diabetes had a higher increase in calcium content in calcified tissue than non-diabetics (2.95 ± 1.90 vs. 1.84 ± 0.90 µg/mg, P=0.002).

The mRNA levels of SPP1 correlated with mRNA levels of IBSP (r=0.47, P=0.002), and was also the case for the BAV and TAV subgroups (r=0.5, P=0.02 and r=0.4, P=0.06). The SPP1 and IBSP transcripts correlated inversely with BMP2 in both BAV (r=−0.6, P=0.001; r=−0.5, P=0.01, respectively) and TAV (r=−0.6, P=0.01; r=−0.5, P=0.02, respectively) groups. In the entire study group, the calcium content in heavily calcified valvular parts correlated with the upregulation of mRNA for BMP2 expression (r=0.32, P=0.04).

TAV, but not BAV, patients showed the correlation of mRNA expression of Runx2/Cbfa1 with IBSP transcript (r=0.52, P=0.02). Also, only in the TAV group did mRNA expression of IBSP correlate with CF (r=0.45, P=0.048), and the RANKL transcript correlate with HDC corrugation (r=0.54, P=0.01).

There were no sex- or valve anatomy-related differences in plasma SPP1, IBSP and sRANKL levels (Table 2).

However, there was a positive correlation between serum sRANKL and median CT value (r=0.45, P=0.048), and the RANKL transcript correlate with HDC corrugation (r=0.54, P=0.01).

Table 3 delineates correlations between the micro-CT parameters. The positive association between the HDC fraction and median CT value (Figure B) was confirmed in TAV (r=0.69, P=0.001) and BAV (r=0.92, P<0.0001). The CF correlated with PGmax (r=0.34, P=0.03; Figure A) and Vmax (r=0.38, P=0.02), and inversely with AIA (r=−0.37, P=0.02).

Calcification-Related mRNA Expression, Valve Calcium Content and Serum Markers

The expression of studied genes was increased in calcified parts of the valve, as compared with the non-calcified soft valvular tissue, from 79% (RANKL) to 486% (ISBP), with no differences between BAV and TAV (Table 2). Patients with diabetes had a higher increase in calcium content in calcified tissue than non-diabetics (2.95 ± 1.90 vs. 1.84 ± 0.90 µg/mg, P=0.002).

The mRNA levels of SPP1 correlated with mRNA levels of IBSP (r=0.47, P=0.002), and was also the case for the BAV and TAV subgroups (r=0.5, P=0.02 and r=0.4, P=0.06). The SPP1 and IBSP transcripts correlated inversely with BMP2 in both BAV (r=−0.6, P=0.001; r=−0.5, P=0.01, respectively) and TAV (r=−0.6, P=0.01; r=−0.5, P=0.02, respectively) groups. In the entire study group, the calcium content in heavily calcified valvular parts correlated with the upregulation of mRNA for BMP2 expression (r=0.32, P=0.04).

TAV, but not BAV, patients showed the correlation of mRNA expression of Runx2/Cbfa1 with IBSP transcript (r=0.52, P=0.02). Also, only in the TAV group did mRNA expression of IBSP correlate with CF (r=0.45, P=0.048), and the RANKL transcript correlate with HDC corrugation (r=0.54, P=0.01).

There were no sex- or valve anatomy-related differences in plasma SPP1, IBSP and sRANKL levels (Table 2). However, there was a positive correlation between serum sRANKL and median CT value (r=0.31, P=0.047), observed in patients with a PGmean ≥50 mmHg (r=0.44, P=0.035) but not in those with lower gradients. Moreover, in patients with a PGmean ≥50 mmHg, the calcium content in heavily calcified parts of the valve correlated with plasma SPP1 levels (r=0.51, P=0.01), and CF correlated with plasma IBSP levels (r=0.42, P=0.04).

When we compared the patients in the highest quartile of CF with those with CF in lowest quartile, the former group had a higher increase of IBSP mRNA expression (10.5 [5.5–63.6] vs. 4.7 [3.4–5.4], P=0.04).

There were no differences in mRNA expression, calcium content and serum calcification markers between the extreme quartiles of HDC fraction.
Inflammation
The median hsCRP level was 1.46 (0.96–2.54) mg/L, and levels >3 mg/L were observed in 7 (17%) cases. The hsCRP level was higher in patients with TAV, as compared to those with BAV (2.36 [1.26–4.31] vs. 1.01 [0.75–1.92] mg/L, P=0.008).

Furthermore, in only the TAV group, a positive correlation between hsCRP and Runx2/Cbfal expression (r=0.52, P=0.02) was found. In the same group of patients, mRNA expression of interleukin-6 (IL-6) correlated with mRNA levels for Runx2/Cbfal (r=0.43, P=0.05).

Regression Analyses
A multivariable linear regression (adjusted R²=0.31, P<0.008) identified CF as the only predictor of PG_max (b=0.47, P=0.005). A logistic regression model identified PG_max as the independent predictor of being in the highest CF quartile (OR per 1 unit increase [95% CI], 1.06 [1.01–1.12]; P=0.02). From mRNA expression variables and serum calcification markers, a regression model could be built only for sRANKL (adjusted R²=0.18, P<0.03), showing that a median CT value is a predictor of sRANKL levels (b=0.58, P=0.001).

Discussion
This study demonstrates for the first time that TAV patients with AS have more mineralized valvular calcifications than BAV subjects, although the relative calcification volume is similar. We confirmed in a very homogenous AS population that the burden of calcifications imaged in micro-CT correlates with AS severity. Our findings indicate that upregulated expression of genes involved in osteoblastogenesis in AS is associated with valve mineralization measured by micro-CT in TAV patients.

Recently, it has been shown that AS patients with BAV have a higher AV calcification volume, as measured in ECG-gated, contrast-enhanced multi-slice CT (MSCT) in 67 AS patients (11 had BAV). In another study, Hope et al. retrospectively studied MSCT scans of 35 patients with BAV, and compared them with 573 TAV patients. BAV subjects were more likely to have severe calcification, based on visual evaluation by 2 radiologists. On the contrary, Ferda et al performed a prospective calcium scoring study of 37 patients (13/37 with BAV) using 64-detector row non-enhanced CT with prospective ECG triggering, and using the Agatston method found no differences in calcium scores between BAV and TAV patients (P=0.14; however, BAV patients had higher calcium volumes [3.810 vs. 2.818 µL]). Although our study was not intended to compare the absolute volumes of calcifications between BAV and TAV patients (which are apparently higher in BAV patients, as based on in vivo studies), using the most precise method available, we found more mineralized calcifications, as reflected by a higher HDC fraction and higher median CT values, in older patients with tricuspid anatomy. In a micro-CT study on 35 AS patients (6/35 with BAV), Chitsaz et al reported a higher median “ratio of calcium volume” (corresponding to CF; 33% vs. 19%). The highest CF values in micro-CT (40%) were presented by Orzechowska et al; however, valves were vacuum-dried prior to imaging, which might have reduced the volume of the soft tissue. However, our finding of CF association with echocardiographic parameters corroborates the findings from the study by Chitsaz et al, and points to a relationship between calcification volume and AS severity at this late stage of disease. Neither group investigated the internal structure of calcifications in micro-CT. In our study, patients with a lower HDC fraction (a parameter strongly correlated with a median CT value and reflecting the internal mineralization of calcifications) belonged more often to the BAV group, thus the clinically important conclusion that can be drawn from our experimental study is that in BAV, the valvular calcifications are less mineralized. This information may potentially be important in the context of percutaneous valvular interventions. In the era of younger and lower-risk patients undergoing transcatheter AV implantation (TAVI), BAV incidence rises among subjects undergoing this procedure. Paravalvular leak (PVL) in patients undergoing TAVI is more frequent in BAV than in TAV. The degree of calcification mineralization (as demonstrated by the current study, where it was lower in BAV than in TAV) probably does not contribute to PVL in BAV, and other causes (such as calcification volume and distribution and device characteristics) may play the primary role. The finding of slightly lower transvalvular pressure gradients in TAV patients, when compared with BAV patients, is in line with previous reports.

To the best of our knowledge, the biological association of micro-CT parameters was never studied. Valvular calcification in AS is associated with the presence of osteoblast-like cells, developing an osteogenic phenotype. Increased valvular expression of osteoblast-specific proteins, including Runx2/Cbfal transcription factor (essential for osteoblastic differentiation and regulation of osteoblast function), was previously observed. We observed a 3-fold increase in Runx2/Cbfal mRNA expression between non-calcified and calcified parts of the valves. The AV tissue gene expression analysis revealed increased activity of all studied genes in calcified parts, as compared with soft fragments, but the most significant increase was observed for ISBP and SPP1 expression, genes associated with calcification pathways. We found that a larger relative volume of calcifications (CF) was related to a more pronounced increase of ISBP mRNA levels, a finding confirmed in the CF quartile analysis. Of note, the positive association of ISBP mRNA expression and CF was present in TAV, but not in BAV. Expression levels of ISBP and SPP1 correlated inversely with BMP-2, a protein known to act as an activator of calcification, whose increased expression was associated with higher calcium content in the current study. This phenomenon may be explained by the fact that ISBP and SPP1 may act, on the one hand, as a positive regulator of cardiovascular calcification, but on the other, may be involved in its inhibition. Local environment and the types of responding cells may play critical roles in the function of these proteins, and are postulated as an explanation to the dual nature of ISBP and SPP1. Additionally, Pohjolainen et al postulated that ISBP and SPP1 might be in the control of valve calcification rather than its genesis, as both of them were significantly associated with the degree of calcification at all stages of the AS development.

In the current study, we show that upregulated expression of genes involved in osteoblastogenesis in AS may affect the calcification process mostly in TAV patients. In TAV individuals with upregulated RANKL mRNA expression, the calcification process is more active, as depicted by the more corrugated shape of dense calcifications. Also in the TAV group, a central transcriptional regulator of osteoblasts, Runx2/Cbfal, correlated with ISBP and IL-6 tran-
scripts, as well as with hsCRP levels. These facts may indicate the role of inflammation in valve calcification processes, leading to heavily mineralized valves in this subset of patients. The role of inflammation in AS has been previously studied. Shavelle et al reported in a study investigating the relationship of different inflammatory markers with AV calcification in a large multi-ethnic cohort of asymptomatic patients that soluble intercellular adhesion molecule-1 (ICAM-1) is associated with more severe AV calcification. In 2014, Abdelbaky et al showed that inflammation may, in fact, precede and predispose to AV sclerosis, using 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) in correlation with non-gated, non-contrast-enhanced CT. CT scans were repeated after a median of 1.75 years, revealing that patients with an initial high inflammatory FDG-PET/CT signal have an increased likelihood of subsequent valvular calcification.

Hence, the role of inflammation in AS seems to be established; however, the differences between BAV and TAV were not studied.

We noticed that patients with more mineralized valves (higher median CT value) have higher levels of sRANKL, especially in cases of more progressed disease (PG\text{mean} ≥50 mmHg). This is in line with a previous report by Nagy et al, where osteoclastic differentiation mediators correlated with AS severity. Moreover, in this subset of patients with a PG\text{mean} ≥50 mmHg, we found associations of plasma SPPI levels and calcium content in calcified tissue. Plasma IBSP levels, in contrast, correlated with CF, reflecting larger volumes of calcified tissue.

Interestingly, lipoprotein (a) (Lp[a]), a lipoprotein subclass, was associated with AV calcification as well. In a genome-wide association study, single nucleotide polymorphism rs10455872 in the Lp(a) gene was strongly associated with AS severity.

The study limitations are our results may be influenced by uneven distribution of calcifications within the valve. The sample size was small, and the interpretation of multivariable analyses must be cautious. Subanalysis should be interpreted with caution.

The micro-CT analysis is unfeasible in a living patient. In cardiac surgery, scanning the excised valve has experimental value; however, micro-CT has been used for in vivo imaging of bicuspid and tricuspid valves by micro-computed tomography with severity of aortic stenosis. The authors declare that they have no conflicts of interest.

Acknowledgments
This work was supported by a grant from the National Science Centre (UMO-2012/05/N/NZ5/00846 to Piotr Mazur).

Disclosure
The authors declare that they have no conflicts of interest.

References
1050

MAZUR P et al.


