Diagnostic criteria for comorbidities

Coronary artery disease was diagnosed on the basis of the clinical status (according to functional classification of the Canadian Cardiovascular Society) and invasive angiography (≥50% lumen diameter narrowing in the major epicardial coronary arteries). Patients were classified as having arterial hypertension if they met at least one of the following criteria: (1) diagnosis of hypertension in the medical history; (2) systolic or diastolic pressure ≥140mmHg or ≥90mmHg, respectively, on at least 2 different visits; (3) antihypertensive treatment before admission. Atrial fibrillation was diagnosed using 12 standard ECG leads. Diabetes was defined as history of diabetes regardless of the duration of disease, and/or treatment with hypoglycemic agents. Renal insufficiency was diagnosed on the basis of medical history or creatinine /eGFR level (> 124 mmol/l / < 90 ml/min/1.73 m² respectively).

Echocardiography

Prior to surgery all patients underwent transthoracic echocardiography; the measurements were performed by using conventional techniques in accordance with the European Society of Cardiology (ESC) guidelines. The transvalvular maximal and mean gradient was measured by Doppler echocardiography using the modified Bernoulli equation, with a 2.5MHz sector ultrasound transducer.
Assessment of bone metabolism and inflammatory biomarkers

The concentration of OPG, osteopontin (OPN), osteocalcin (OCN), TNFα, IL-1b and IL-6 in serum was measured by multiplex Luminex technology using Human Bone Panel and Cardiovascular Disease Panel kits (Millipore, USA), according to the manufacturer's instruction. Data were analyzed using a Flexmap 3D instrument (Luminex xPONENT® 4.0 Software) and Luminex Analyst Program (Luminex Corporation, Austin, Texas, USA). The levels of sRANKL were measured using enzyme-linked immunosorbent assay (ELISA) kit (BioVendor, Brno, Czech Republic), according to the manufacturer's instruction. Inter- and intra-assay coefficients of variation of both tests were between 7% and 11%.

Preparation of valve cusps for microscopy

The cusps of stenotic valves were dissected into two parts along the line passing from cusp base to its free margin through the most pronounced focal calcifications. One part/half was fixed for 24 h in 4% buffered paraformaldehyde, decalcified for 4 days in 10% EDTA and routinely embedded in paraffin. Six µm sections were mounted on polylysine-coated slides (Menzel-Gläser; Thermo Scientific, Germany). The other part/half was embedded in OCT (Jung, Nussloch, Germany) and frozen. Ten µm cryostat (Jung CM1800, Leica Instruments GmbH, Germany) sections were mounted on polylysine-coated slides, dried in air and fixed for 5 min in 4% buffered paraformaldehyde.

Histology and histochemistry

Deparaffinised sections were stained routinely with haematoxylin and eosin. Frozen sections were stained with Oil red O (ORO) to reveal lipids and with Alizarin red to visualize calcified areas.
**Immunohistochemistry**

Deparaffinised sections were first boiled in citrate buffer (pH 6.0) for antigen retrieval and then preincubated for 40 min with 5% normal goat serum (Vector, Burlingame, CA; # S-1000) in PBS containing 0.01% sodium azide, 0.05% thimerosal, 0.1% bovine serum albumin and 0.5% Triton X-100 to reduce nonspecific binding and to increase penetration of the antibodies. The sections were next incubated overnight with the primary antibodies against:

- Tartrate-resistant acid phosphatase (TRAP), dilution 1:50 (code no. NCL-TRAP; Novocastra, Newcastle, UK), and Cathepsin K dilution 1:100 (code no. ab19027; Abcam, Cambridge, UK), enzymes expressed by osteoclasts;

- CD68 antigen, dilution 1:1 (code no. IHCR2113-6; Chemicon, Temecula, CA), a monocyte/macrophage marker;

- CD163 antigen, dilution 1:100 (code no. ab 156769; Abcam, Cambridge, UK), a marker expressed by macrophages with M2 characteristics;

- CD34 antigen, dilution 1:50 (code no. NCL-END; Novocastra, Newcastle, UK), a marker of endothelial cells, to reveal blood vessels.

Next, sections were washed extensively in PBS and incubated for 90 min with goat anti-mouse Alexa555-conjugated antibody, dilution 1:200 (code no. A-21424; Molecular Probes, Eugene, OR) and/or goat anti-rabbit Alexa488-conjugated antibody, dilution 1:200 (code no. A-11008; Molecular Probes, Eugene, OR). Cell nuclei were counterstained with DAPI (2-(4-aminophenyl)-1H-indole-6-carboxamidine) (Sigma, Saint Louis, MO, USA). Sections were washed three times in PBS and mounted in glycerol/PBS solution (pH = 8.6). Negative controls were performed by omitting the primary antibodies during the first incubation.

**Microscopy, confocal microscopy and morphometry**
Sections were examined under Olympus BX50 bright field/fluorescence microscope (Olympus, Japan). Images were recorded using DP-71 digital CCD camera (Olympus, Tokyo, Japan) coupled to PC-class computer equipped with AnalySIS-FIVE® (Soft Imaging System GmbH, Münster, Germany) image analysis system. Sections of valves containing TRAP+/cathepsin K+ cells were also examined in a laser scanning (confocal) fluorescence microscope (FluoView FV1200, Olympus, Tokyo, Japan) to verify their mono- or multinucleated character. Only multinucleated TRAP+/cathepsin K+ cells were considered as fully differentiated osteoclasts. Images were collected using the FV10-ASW v.4.1a software (Olympus, Tokyo, Japan) and then visualized and analysed with the use of Imaris software v. 7.7.1. (Bitplane AG, Zurich, Switzerland).

To assess the total macrophage population (CD68+) and M2 macrophages (CD163+), the cells were counted in five representative high power field areas per case, and a mean value was calculated to express the respective cell number per high power field. The same method was employed to assess microvessel density expressed as the number of vascular profiles per high power field. We also calculated the percentage of M2 macrophages (number of M2 macrophages/number of total macrophages) x 100.

The areas occupied by ORO-stained lipids were measured using the image analysis system and expressed as the percentage of the total cusp section area.

**Statistical analysis**

Categorical variables were expressed as percentages. Continuous variables were expressed as mean ± SD or median [lower-upper quartile values] depending on their type and distribution. The statistical analysis included (in compliance with the specific category of collected data) t-Student test for unpaired data or Mann-Whitney U test, and χ² or Fisher’s exact tests. Correlation between continuous variables were analyzed using Pearson test or the Spearman's
rank test, as appropriate. Non-linear regression models were fitted for correlation between OPG and age in all patients: $Y = 1/(11.0511 - 0.111271 \times X)$, in osteoclastic group: $Y = \exp(-2.5588 + 0.000227274 \times X^2)$ and in nonosteoclastic group: $Y = \exp(-1.79528 + 0.000164119 \times X^2)$. Stepwise backward multivariate logistic regression analysis was performed to assess independent predictors of the osteoclastogenesis. Included were variables with $p < 0.1$ in univariate analysis (i.e. OPG, TNFα, IL-6 and diabetes). Model was adjusted for age, gender and aortic valve type (bicuspid/tricuspid) and all continuous variables were dichotomized by their medians. Statistical analyses were performed using Statgraphics Centurion XVI (StatPoint Technologies INC, Warrenton, USA) software. All tests were two-tailed with $p < 0.05$ considered statistically significant.
Table S1 Correlation of biochemical parameters with OPG serum concentration in stenotic patients

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPN</td>
<td>0.31 (0.04391 - 0.5286)</td>
<td>0.02</td>
</tr>
<tr>
<td>OCN</td>
<td>-0.03 (-0.2950 - 0.2358)</td>
<td>0.8</td>
</tr>
<tr>
<td>Ca</td>
<td>0.09 (-0.1844 - 0.3477)</td>
<td>0.5</td>
</tr>
<tr>
<td>P</td>
<td>-0.12 (-0.3711 - 0.1534)</td>
<td>0.4</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.64 (0.2801 - 0.6843)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.55 (0.3321 - 0.7136)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>IL-1b</td>
<td>0.13 (-0.1436 - 0.3840)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*assessed by Spearman’s correlation coefficient

OPG = osteoprotegerin; OPN = osteopontin; OCN = osteocalcin;