Echocardiographic integrated backscatter for the differentiation between aortic valve calcification and valvular myxoid degeneration in rats

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Aims
Calcification is an independent predictor of mortality in aortic valve (AV) stenosis. Echocardiographic calibrated integrated backscatter (cIB) is a promising parameter for quantifying AV calcification. However, the ability of cIB to differentiate between calcification and valvular thickening has been questioned. Therefore, we aimed to use cIB to study AV calcification compared with non-calcified AV thickening in rats, with histology as reference.

Methods and results
Twenty male Wistar rats were studied. Group 1 (N = 6) received subcutaneous (SC) serotonin injections (60 mg/kg/day) for 12 weeks to induce myxoid non-calcified AV thickening. Group 2 (N = 7) received vitamin D₃ (25 000 UI/kg/day) SC to induce AV calcification, and Group 3 (N = 7) received only vehicle SC for 10 weeks. cIB of the AV was calculated at the end of the study, followed by measurement of the percentage of the histological AV calcification. At the end of the study, cIB values and calcification percentages were significantly higher in vitamin D₃-injected rats compared with serotonin-injected rats and controls. There was no significant difference in cIB values between serotonin-injected rats and controls (vitamin D₃: 21.5 ± 3.0 dB*; serotonin: 11.8 ± 3.1 dB; control: 10.3 ± 3.4 dB; *P < 0.05). The percentage of histological calcification was significantly higher in the vitamin D₃ group compared with the other groups. Serotonin-injected rats developed significant AV thickening.

Conclusion
Increased cIB values of the AV are related to increased calcification at histology and not to myxoid non-calcified valvular thickening. Therefore, cIB may be considered as a sensitive technique to quantify calcification of AV rather than for detecting non-calcified valvular thickening.

Keywords
Cardiovascular calcifications • Echocardiography • Integrated backscatter • Small animals

Introduction
Calcific aortic valve disease (CAVD) is the most frequent valvular heart disease in the western world.¹ The amount of calcification is an independent predictor of morbidity and mortality in CAVD.²,³ Computed tomography (CT) is currently considered as the reference technique to quantify and follow up aortic valve (AV) calcifications in patients with high cardiovascular risk.⁴ However, cardiac CT involves irradiation, which makes the technique less suitable for repetitive evaluations.⁵ Echocardiography, using ultrasound, is non-invasive and non-ionizing. When the ultrasound wave interacts with inhomogeneities smaller than the wavelength, scatter reflections occur that could be used to extract information about tissue composition. Integrated backscatter (IB) is the integrated average energy of the backscatter

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reflections originating from a certain region of interest within the tissue. We have recently validated echocardiographic IB as a non-invasive and non-ionizing technique for the quantification and detection of progression and regression of AV calcifications in rats. However, during the progression of CAVD, valvular thickening related to fibrosis may interfere with the IB measurements. Therefore, it is important to evaluate the respective contribution of non-calcified valvular thickening in this setting and to determine its effect on IB measurements. Administration of supraphysiologically vitamin D₃ has been validated as a reliable method to develop CAVD in rats. On the other hand, rats subcutaneously (SC) injected with high doses of serotonin are known to develop myxoid degeneration and AV thickening without calcification.

The aim of the present study was to evaluate the use of echocardiographic IB as a non-invasive and non-ionizing imaging technique for the differentiation between myxoid degeneration-related non-calcified AV thickening and AV calcification in rat models, compared with histology as a reference method.

Methods

Study design

Twenty male Wistar rats (Harlan, The Netherlands) were divided into three groups. Group 1 (N = 6) received SC serotonin injections of 60 mg/kg/day for 12 weeks. In order to decrease mortality (acute toxicity), the animals received 10 mg/kg at baseline that was progressively increased with 10 mg/kg each week until the maximal dose was reached. Group 2 (N = 7) received SC vitamin D₃ injections of 25 000 IU/kg/day, administered three times a week for 10 weeks. Group 3 (N = 7) of control rats received SC injections with the vehicle only for 10 weeks. Echocardiography was performed at baseline and at the end of the study (at 12 weeks for Group 1 and at 10 weeks for Groups 2 and 3). At the end of the study, all animals were killed with 100 mg/kg sodium pentobarbital intravenously. The hearts were excised within 30 min of death and fixed in neutral buffered formalin 4% for histopathological analyses.

Physiological parameters

Bodyweight (BW) was determined weekly. Physical condition of the animals was assessed daily.

Echocardiography

The rats in Group 1 were anaesthetized with 50 mg/kg sodium pentobarbital (Nembutal, CEVA, Brussels, Belgium) intraperitoneally. The rats in Groups 2 and 3 were anaesthetized with 2% isoflurane (S.A. Abbott N.V., Ottignies, Belgium) with 2 L of O₂/min as carrier gas. The anterior chest wall was shaved and the rat was placed in lateral decubitus on a wooden bench in order to obtain optimal image quality and views, as previously described. Electrocardiogram electrodes were fixed on the paws. A Vivid 7 Pro system (GE Medical Systems, Milwaukee, WI, USA) with a 10-MHz neonatal probe (105S) was used. All echocardiographic imaging and analyses were performed by an experienced investigator. Settings of the ultrasound equipment were kept constant between all image recordings.

Histopathology

The excised and embedded hearts were cut in an axial plane (from base to apex). Three ranges of sections were made of ~100 µm between each range in order to visualize a maximum amount of valvular tissue. Each slice of 4–6 µm was stained with classic haematoxylin and eosin (H&E stain). Masson’s trichrome staining was used to evaluate collagen deposition. Glycosaminoglycan (GAG) deposits were analysed with Alcian Blue staining. An experienced pathologist performed tissue analysis with a PC digital image camera (Digital Sight DS-5 M, Nikon Corp., Japan) mounted on an Axioskop Zeiss light microscope (Carl Zeiss Corp., Germany).

The percentage of calcification was calculated using [image] 1.44 g (Wayne Rasband National Institutes of Health, USA). An AV calcified area was calculated for each rat, by summation of areas of deposits in the most calcified slice. The calcified area was divided by the total valvular area to obtain the percentage of calcification (Ca percentage, %).

Valvular thickness was measured on histological slices by using [image] 1.44 g (Wayne Rasband National Institutes of Health). The thickness of the valve leaflets was measured perpendicular on the long axis of the leaflet, at the most thickened part of the valve.

Product preparation and administration

For Group 1, serotonin (5-hydroxytryptamine creatinine sulphate complex, Sigma-Aldrich) was dissolved in physiological saline at the desired concentration. Solutions were made on a daily basis just before the injections. In order to avoid skin lesions like SC bleedings and traumatic wounds, the injection side was changed daily. In Group 2, vitamin D₃ (Sigma-Aldrich N.V., Bornem, Belgium) was prepared for SC injection as a 5% polysorbate 80 (emulsifier) (TWEEN 80, Sigma-Aldrich N.V.), 5% ethanol solution in saline, at a concentration of 0.625 mg/ml (25 000 IU/ml). These preparations were made fresh every 3 days and stored (in containers wrapped in foil) protected from light at a temperature of 2–8°C.

Statistical analysis

Data are expressed as mean ± standard error of the mean. Variables were tested for normality of distribution and homogeneity of variance by means of the Shapiro–Wilk and Levene tests. Comparisons between groups were performed by using the unpaired and paired Student’s t-test. The Mann–Whitney U-test and Wilcoxon test were used for variables approximating normality of distribution. The mean percent error ([absolute difference/average of both observations] × 100) and the Pearson correlation coefficient were calculated for intra-and interobserver variability. Correlations between continuous variables were evaluated by means of the Spearman correlation coefficient. All P-values were calculated two-tailed. A P-value < 0.05 was considered...
significant. Statistical analysis was done with IBM SPSS Statistics (version 22.0.0, SPSS Inc., IBM Company, Chicago, IL, USA).

**Results**

**Clinical parameters**

After 12 weeks, the mean BW was 422 ± 20 g in the serotonin group 1. Serotonin induced flushing, loose stools, drowsiness, and tachypnoea persisting for several hours after the injections.

At Week 10, physical signs of illness were evident in half of the animals of vitamin D3 group 2, like sitting in a hunched position, diarrhoea, teeth defects, poor grooming, and weight loss. The mean weight of animals in Group 2 was significantly lower than in Group 3 (BW 299 ± 48 vs. 513 ± 30 g, P < 0.001).

The control and serotonin groups had a significant increase in BW at the end of the study, compared with baseline (BW change of +199 ± 11 g, P < 0.001 and +84 ± 9 g, P < 0.001, respectively). In the vitamin D3 group 2, there was a decrease in BW; however, this decrease was not significant compared with baseline (−35 ± 16 g, P = 0.067).

BW changes for each group are summarized in Table 1.

**Echocardiography**

**Calibrated integrated backscatter analysis**

In Group 2 (vitamin D3), the mean cIB values of the AV were significantly increased at the end of the study compared with baseline [mean region of interest AV global (ROIAVGLOB) baseline: 13.7 ± 2.2 dB; end of the study: 21.5 ± 3.0 dB; P < 0.05]. On the other hand, the mean cIB values of Group 1 (serotonin) and Group 3 (controls) did not significantly increase at the end of the study compared with baseline.

Between groups

At baseline, no significant difference in cIB values was seen between groups.

At the end of the study, there was no significant difference in cIB values between Group 1 (serotonin) and Group 3 (controls). However, the cIB values of the AV for Group 2 (vitamin D3) were significantly higher, compared with Group 1 (serotonin) and Group 3 (control) animals (Figures 1 and 2; Table 1).

For the cIB values, there was a good inter- and intraobserver variability (Table 2).

**Histopathology**

**Calcification between groups**

At the end of the study, there was a significant increase of the calcification percentage in Group 2 (vitamin D3) with HE staining compared with Groups 1 and 3 (Figure 1 and Table 1). None of the valves in the serotonin-injected group or in the control group developed calcifications, whereas in the vitamin D3-injected group, all the animals developed calcifications.

Two animals developed minor calcifications with calcium percentage <25%, four animals had more severe calcification with calcium percentage between 25 and 50%, and one rat developed even very severe calcifications with calcium percentage of 62%.

There was a good positive exponential correlation between cIB values and Ca percentage ($r^2 = 0.7$ and $P < 0.001$) (Figure 3).

**Valvular thickening between groups**

At the end of the study, the thickness of the valvular leaflets was significantly increased in Group 1 (serotonin) compared with the control Group 3 (Figures 1 and 4; Table 1). The success rate of serotonin injections for inducing a valvular thickening with a thickness >205 μm was 100%, with a maximum thickness of 318 μm. In the control group, the valvular thickness was <205 μm in 85% of the animals.

There was a trend for a greater valvular thickness in the serotonin rats compared with Group 2 (vitamin D3), but this difference was not significant. There was no significant difference in valvular thickness between Group 2 (vitamin D3) and Group 3 (controls).

Trichrome staining showed no significant increase in collagen deposition between groups. However, GAG deposits were only observed, as previously described, in the serotonin group 1.

There was no significant correlation between cIB values and valvular thickness (Pearson’s $r = -0.08$, $P = 0.754$).

**Discussion**

In this study, we have demonstrated that cIB is a valuable, non-invasive, and non-ionizing technique for detecting and quantifying aortic valvular calcification. Moreover, we have shown that it

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**Table 1** Mean cIB values at baseline and end of the study, mean valvular thickness, mean calcification percentage, and BW change for the three groups

<table>
<thead>
<tr>
<th></th>
<th>Mean cIB baseline (dB)</th>
<th>Mean cIB end (dB)</th>
<th>Mean valvular thickness (μm)</th>
<th>Mean calcification percentage (%)</th>
<th>Bodyweight change (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (N = 6)</td>
<td>14.9 ± 3.2</td>
<td>11.5 ± 2.9</td>
<td>250 ± 49</td>
<td>0.0 ± 0.0</td>
<td>+83.8 ± 9.1</td>
</tr>
<tr>
<td>Group 2 (N = 7)</td>
<td>13.7 ± 2.2</td>
<td>21.5 ± 3.0*</td>
<td>207 ± 49</td>
<td>37.1 ± 15.7*</td>
<td>−35.0 ± 15.7*</td>
</tr>
<tr>
<td>Group 3 (N = 7)</td>
<td>13.7 ± 1.7</td>
<td>12.0 ± 4.0</td>
<td>186 ± 44</td>
<td>0.0 ± 0.0</td>
<td>+199.6 ± 11.0</td>
</tr>
</tbody>
</table>

Data are represented as mean values with standard deviation. Group 1: serotonin 60 mg/kg/day. Group 2: vitamin D3 25 000 U/kg/day. Group 3: controls.

cIB, calibrated integrated backscatter.

* vs. baseline and vs. Group 1 and Group 3, $P < 0.05$.

** vs. Group 1 and Group 3, $P < 0.001$.

*** vs. Group 3, $P < 0.05$.

**** vs. Group 1, $P < 0.05$. 

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allows the differentiation between AV calcifications and valvular thickening due to myxoid degeneration, compared with histology as a reference in these in vivo rat models.

IB quantifies the reflectivity of the ultrasound tissue Doppler signal. Previous studies suggest that an increase in IB reflects valvular thickening, fibrosis, and calcification in AV sclerosis. IB was initially validated to characterize the tissue of atherosclerotic lesions and to differentiate between fibrotic lesions and calcifications in arterial plaques in humans. Valvular calcifications bring about an increase in IB values, but less is known about the contribution of fibrosis to IB measurements of the AV. A previous study in human patients with AV sclerosis suggests that a cIB value of 16.3 ± 4.4 dB in the AV could be considered as a cut-off value for the presence of AV sclerosis. However, the presence of calcifications and fibrosis and their effect on the IB values was not evaluated in these sclerotic valves. In our animal study, we have shown that serotonin-induced valvular thickening does not lead to an increase in cIB values.
whereas calcifications significantly contribute to higher cIB values, as confirmed by histopathology. The distribution of the calcification percentage in the vitamin D group ranged from mild to severe calcification (16–62%). We can conclude that cIB is useful for the detection and evaluation of mild to very severe AV calcifications in rats. Further studies should be conducted to evaluate the sensitivity of cIB to detect lower amounts of calcification in CAVD.

In humans, the serotonin receptor 5-HT\textsubscript{2B} is associated with heart valve fibrosis.\textsuperscript{18} In rats, however, serotonin is known to induce valvular thickening by GAG deposition rather than through an increased collagen content.\textsuperscript{11} This is confirmed by a negative trichrome staining in all the three groups. This animal model is a model of non-calcified and GAG-related AV thickening. Consequently, this study cannot exclude that increased collagen deposition may alter IB measurements. This needs to be addressed in future studies.

The advantage of animal models is to provide a pure model of either valvular thickening or calcification. In clinical practice, both conditions may be present at the same time and act as confounding factors.

Subcutaneous tissues cause diffraction of the ultrasound waves, leading to background reverberation, so animal weight and age could be important when comparing IB values.\textsuperscript{16} Therefore, we corrected IB values for the background noise signal. There is also an influence of angle-dependent scattering of ultrasound. These issues could limit quantitative diagnosis.\textsuperscript{16,19} However, we eliminated such interference by keeping any diffraction similar in each specimen. We used the same transducer and the same settings for each animal and calculated cIB values by subtracting the background signal of the adjacent blood pool. Moreover, by considering the mean cIB value of different ROIs for each structure, more diffracted ROIs get cancelled out.

Echocardiography is used to investigate valve morphology and function through measuring specular reflections and Doppler ultrasound. IB can extract information related to the valvular composition by analysing the backscattered reflections that occur when the ultrasound signal interacts with tissue structures and inhomogeneities. It therefore has great potential for the evaluation of structural changes during calcifying AV disease progression. Backscatter scores of the AV correlated well with subjective sclerosis scoring and transvalvular pressure gradients in patients.\textsuperscript{3} However, there are some difficulties regarding the translation to clinical practice. Echocardiography and more specifically IB of ultrasound is susceptible to the ultrasound system settings. It therefore could be difficult to use IB for retrospective evaluation of patient databases where settings are not kept constant. The ultrasound probe, the frequency, frame rate, and depth, for example, determine the ultrasound signal and reflection and therefore may influence the IB values. Also, differences in patients’ morphology such as BW and pericardial fat may have an impact on the results.

We used a 10-MHz neonatal probe to obtain echocardiographic images of good quality in rats. Other probes and frequencies are used in clinical practice. Absolute cIB values may therefore be difficult to extrapolate to humans.

Future studies should be conducted to determine the effect of different ultrasound system settings and ultrasound probes used in clinical practice on the IB measurements. Nevertheless, when settings are kept equal, in the same patient sequential IB measurements could be of great interest to evaluate AV disease progression in a non-invasive, non-ionizing way. This has big potential clinical implications, given that calcification is an independent predictor of mortality in AV stenosis (AS). More specifically, in patients with low-flow low-gradient AS, a higher valvular calcium amount is associated with a higher long-term mortality.\textsuperscript{20} The degree of AV calcification is also of predictive value for post-procedural complications in patients who underwent transcatheter aortic valve implantation.\textsuperscript{21} For those reasons, detection of valvular calcium is important in the decision-making for AV replacement strategies.

This study confirms the potential of cIB for quantifying the amount of calcifications in calcific AV disease. It may be useful for prognostic assessment, for serial follow-up during medical intervention, and for pre-intervention (like percutaneous valve replacement) evaluation.
Limitations
In clinical practice, echocardiographic settings may vary overtime. For IB studies, standardization of these settings is mandatory. Although in the present animal study, the settings were maintained constant, this would be a limitation for a retrospective analysis of existing databases in patients. However, the other non-invasive modalities for imaging calcifications like CT are facing the same issues.22

Conclusions
AV calcification is an important prognostic factor of morbidity and mortality in cardiovascular disease. This in vivo rat study shows that cIB is related to the amount of calcification at histology and is not related to the presence of valvular thickening of the AV. Therefore, IB of ultrasound may be considered a sensitive, non-invasive, and non-ionising technique to quantify calcification of the AV rather than detecting valvular thickening not related to calcification.

Conflict of interest: none declared.

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