High-Resolution X-Ray Microtomography Is a Sensitive Method to Detect Vascular Calcification in Living Rats With Chronic Renal Failure

Veerle Persy, Andrei Postnov, Ellen Neven, Geert Dams, Marc De Broe, Patrick D’Haese, Nora De Clerck

Objective—Chronic renal failure (CRF) is associated with a 10- to 20-fold increase in cardiovascular risk. Vascular calcification is a prominent feature of cardiovascular disease in patients with end-stage renal failure and contributes to the excess mortality in this population. In this study, we explored in vivo X-ray microtomography (micro-CT) as a tool to detect and follow-up vascular calcifications in the aorta of living rats with adenine-induced CRF.

Methods and Results—With in vivo micro-CT, calcification of the aorta in uremic rats was clearly discernible on transversal virtual cross-sections. Micro-CT findings correlated well with tissue calcium content and histology. Repetitive scans in animals with light, moderate, and severe vascular calcification showed good reproducibility with minimal interference of motion artifacts. Moreover, both calcified volume and area could be quantified with this method.

Conclusions—In vivo micro-CT scanning is a sensitive method to detect vascular calcifications in CRF rats, allowing follow-up and quantification of the development, and potential reversal during treatment, of vascular calcifications in living animals. (Arterioscler Thromb Vasc Biol. 2006;26:2110-2116.)

Key Words: ectopic calcification • imaging • micro-CT • renal failure • kidney

Chronic renal failure (CRF) is associated with a 10- to 20-fold increase in cardiovascular risk, which is responsible for ≈50% of the mortality in hemodialysis patients.1 Goodman et al demonstrated that vessel calcification is already present in young hemodialysis patients and shows rapid progression.2 In addition to increased calcification of atherosclerotic plaques, patients on dialysis also show characteristic calcifications of the vascular media,3 which were recently shown to contribute significantly to the excess cardiovascular mortality observed in uremic patients.4 Epidemiological studies identified elevated serum phosphate levels, increased calcium phosphate product and secondary hyperparathyroidism as risk factors for mortality in the hemodialysis population.5,6 The causative roles of phosphate and calcium in the calcification process were elegantly demonstrated by in vitro studies showing that blockage of the Na-phosphate co-transporter in cultured vascular smooth muscle cells inhibited the calcification process induced by either elevated phosphate7 or calcium levels.8 Katsumata et al recently described adenine-induced CRF as a suitable animal model for uremia-related vascular calcification.9 The availability of such a model allows for studies investigating how pharmacological agents, such as the recently developed alternative therapies to control hyperphosphatemia and secondary hyperparathyroidism, influence the CRF-related vascular calcification process and the mediators involved in it.

In the present study, we evaluated whether vascular calcifications in a rat model of CRF can be visualized by in vivo high-resolution X-ray microtomography (micro-CT). This technique has recently been introduced as a promising noninvasive imaging technique in living rats and mice. Micro-CT proved to be most suitable for the visualization of bones and other calcified tissues.10 Additionally, recent studies showed that noninvasive soft tissue imaging of the chest area was possible in living animals despite cardiac and respiratory movement, and allowed detection of lung tumors11 and lung emphysema.12 In the present study, the feasibility of detecting ectopic calcification in the aorta of uremic rats by in vivo micro-CT is demonstrated. This method proved to be reproducible and allowed quantification of the extent of calcification in living CRF rats, opening interesting perspectives for future interventional experimental studies.

Methods

Animal Procedures

Animals were housed in plastic cages, with a 12-hour light/dark cycle and with tap water and food available ad libitum. All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of...
Detected by In Vivo Micro-CT

Micro-CT Equipment and Scanning Parameters
A desktop in vivo X-ray micro-CT system was used (Skyscan 1076; Aartselaar, Belgium) without gating for cardiac or respiratory motion. With this system, both the polychromatic X-ray source (focal spot size 5 μm, energy range 20 to 100 keV) and the detector (charge-coupled device [CCD] camera 2.3k×4k) rotate around the animal. Details about the micro-CT equipment have been published elsewhere (www.skyscan.be).

In Vivo Scanning Parameters
For in vivo studies anesthetized rats were placed on their back in the animal bed of the scanner. A 25-μm titanium filter was used; tube voltage was set to 90 kV, with a 68-mm field of view. Scans were isotropic and a voxel size of 35×35×35 μm was chosen. These conditions allowed scanning of 36-mm axial length of the thorax in 50 minutes of exposure time (or 2 scans of 18 mm length, as indicated). Transversal virtual cross-sections were reconstructed from the raw dataset by Feldkamp cone beam algorithm.15

In Vitro Scanning Parameters
For in vitro studies, paraffin embedded aortic tissue was scanned with acquisition parameters chosen to optimize image quality instead of reducing scanning time and radiation dose. Extracted aortas were scanned for 50 minutes using a 500-μm aluminum filter and 100 keV energy, with a voxel size of 35×35×35 μm. In this case, beam hardening can be corrected by an appropriate polynomial to provide a linear dependence between absorption and gray values in the reconstructed cross-sections, as described previously.15 Virtual cross-sections were reconstructed by Feldkamp cone beam reconstruction algorithm, and images were analyzed as described.

Image Analysis and Comparison of 3-Dimensional Models
Density Histograms
In the reconstructed cross-sections, absorption of X-rays in each voxel was visualized in 8-bit bitmap format. Regardless of the scanning conditions, noncalcified aortas cannot be distinguished from the surrounding tissues because they have comparable X-ray absorption. On the contrary, calcified tissues become visible even if the calcification rate is slightly higher than in the healthy control animals (Figure 1A). Both in vivo and in vitro data analysis was represented by density histograms in which, the frequency of occurrence (expressed as the number of voxels in the dataset) for each gray value is plotted against the absolute gray values. These histograms of frequency distributions of absorption were created for the densities that are higher than those that are present in the healthy animals. A 256-color scheme was applied with gray values ranging between 0 (black) and 255 (white). Comparison of these histograms allows making conclusions about calcification rates in the animals. Histograms of extracted aortas also provide quantitative results. Lower limit for threshold was selected to exclude noncalcified tissue. Upper limit was corresponding to the densest calcified part. Gray value 229 represents the paraffin background.

3-Dimensional Models
Both in vivo and in vitro 3-dimensional (3D) models were created using Skyscan software packages “ANT” and “CT Analyser” (Skyscan, Aartselaar, Belgium). The 3D models built from the reconstructed cross-sections were analyzed to determine volume and surface of the calcifications. Reconstruction parameters for in vivo scans were selected to yield optimal visualization of the calcified inclusions in the aorta. Global density threshold was applied to visualize calcification rate in 3D models and for further quantitative analysis. 3D models were matched manually after visual inspection, as calcifications created unique patterns. Both the volume (in mm³) and area (in mm²) of the calcification were calculated.

Experimental Set-Up
The results of 2 different experiments are reported here. First, there was a feasibility study, in which a subset of animals from a larger study was subjected to in vivo micro-CT scanning to investigate whether aortic calcification can be visualized with this method. Second, there was a reproducibility study, in which repetitive scans of the same animals were used to assess the reproducibility of in vivo micro-CT to quantify vascular calcification in living uremic rats.

Persy et al Detection of Vascular Calcification by In Vivo Micro-CT
TABLE 1. Results From In Vivo Micro-CT Scanning Correlated Well With Tissue Calcium Content and Histological Evaluation

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal</th>
<th>Micro-CT</th>
<th>Tissue Ca mg/g Wet Tissue</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.03% P</td>
<td>1</td>
<td>+</td>
<td>1.29</td>
<td>4/6</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>0.22</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>0.26</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>1.2% P</td>
<td>4</td>
<td>+++</td>
<td>52.80</td>
<td>10/10</td>
</tr>
<tr>
<td>5</td>
<td>+++</td>
<td>26.07</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>0.18</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>0.23</td>
<td>0/9</td>
<td></td>
</tr>
</tbody>
</table>

Results of in vivo micro-CT scanning are expressed as: –, negative; +, focal calcification in some virtual cross-sections; and ++++, extensive aortic calcification, with dense, circumferential delineation of the aortic wall over the whole scanned area.

Histological scoring was performed on microscopic sections stained for calcium precipitation with Von Kossa’s method and the results are expressed as the Number of cross-sections containing at least partially circumferential Von Kossa positivity (score 2)/the number of cross-sections evaluated.

Batch bulk content in vessels of normal animals is 0.16±0.04 mg/g tissue.

Feasibility Study

**In Vivo Micro-CT Scanning and Image Analysis**

The thoracic region of rats with adenine-induced CRF was scanned twice (after 8 and 10 weeks of CRF) in 4 animals maintained on a 1.2% phosphate diet and in 3 animals on 1.03% phosphate diet after 8 weeks of follow-up. Rats were anesthetized with 45 mg/kg sodium pentobarbital (Nembutal; CEVA Santé Animale, France) administered intraperitoneally and 36-mm axial length of the thorax was scanned, of which a 25-mm region of interest was selected. From this region of interest, 720 transversal virtual cross-sections were reconstructed.

The presence of calcification was scored by viewing the data sets with T View software (Skyscan, Aartselaar, Belgium). In 6 of 7 animals calcification was either apparent in all cross-sections, or absent from the entire dataset. In animal 1 (Table 1), however, some focal calcifications were observed. To further evaluate the degree of aortic calcification in this animal, all cross-sections from this dataset were reviewed in random order and scored for the presence of areas with increased density in the aortic region, indicative for calcification. The results are expressed as the percent of virtual cross-sections with calcification.

The 3D models were constructed as described from an 8.0-mm-long aortic region that was recognizable on both the 8- and 10-week scans of a rat with pronounced calcification (animal 4 in Table 1).

**Tissue Prelevation and Histology**

Animals were euthanized by exsanguination via the retro-orbital sinus after anesthesia with sodium pentobarbital 65 mg/kg intraperitoneally. Through a combined midline laparotomy and thoracotomy the vascular tree was exposed and the large vessels were removed en bloc. The thoracic aorta was divided in 2 pieces. The proximal half was fixed in neutral buffered formalin for histology; the distal part was used for determination of tissue calcium content.

Before embedding in paraffin, formalin-fixed aortic tissue was sectioned perpendicular to its length axis into 2- to 3-mm-long segments (6 to 10 per animal). All aortic segments from 1 animal were embedded upright in the same paraffin block. Four-micrometer sections were stained with Von Kossa’s method to visualize calcium-containing precipitates and counterstained with hematoxylin and eosin.

Vascular calcification was assessed by an observer blinded to the identity of the sections. The degree of Von Kossa positivity was scored semiquantitatively with scores ranging from 0 to 3. Score 0 indicated no Von Kossa positivity; score 1, focal Von Kossa positivity, larger than or not overlying a cell nucleus; score 2, partially circumferential Von Kossa positivity in the tunica media of the vessel; and score 3: Von Kossa positivity in the tunica media spanning the complete circumference of the vessel. To compare micro-CT data with histology, the number of cross-sections with score 2 or 3 were summed in Table 1 as cross-sections showing major calcification.

**Determination of Tissue Calcium Content**

Tissue samples were weighed with a precision balance and subsequently digested in 65% nitric acid at 65°C overnight. Subsequently, the tissue digests were adjusted to a final volume of 2.5 mL with 0.1% lanthanum nitrate in water to avoid chemical interferences. Tissue calcium content was measured by flame atomic absorption spectrometry (Model 3110; Perkin-Elmer) after appropriate further dilution of the samples in 0.1% lanthanum nitrate. Results are expressed as mg calcium per gram of wet tissue weight.

**Reproducibility Study**

**Study Set-Up**

To study the reproducibility of the technique, the impact of motion artifacts on the micro-CT images and finally to develop a quantification method, 14 male Wistar rats were subjected to the adenine model of CRF as detailed and maintained on 1.03% phosphate diet after adenine withdrawal. In this study, rats were anesthetized by intravenous administration of 35 mg/kg sodium pentobarbital. After 7 weeks of CRF, a micro-CT screening (36-mm axial length of the thorax) was performed to assess the number of animals with calcification. One week later, scans were repeated in a shorter region (18 mm), so that the same region could be scanned twice during the same anesthesia period. Subsequently, animals were euthanized by a Nembutal overdose, and dead animals were left to stabilize at room temperature for 1 hour and scanned again with the same parameters. Animals in which the micro-CT screening at week 7 revealed no calcification were followed-up until week 9, after which the abdominal aorta was collected for determination of the tissue calcium content. Image analysis, 3D reconstruction, and subsequent quantification were performed as described above.

**Results**

**Renal Function**

In both studies, feeding rats with an adenine-rich diet (0.75% w/w of adenine) for 4 weeks induced stable, moderate to severe chronic renal failure, with serum creatinine values rising from baseline values of 0.45±0.05 mg/dL and stabilizing around 2.0 to 2.5 mg/dL from week 2 onwards (data not shown).

**Feasibility Study: In Vivo Micro-CT Scanning**

Eight weeks after induction of CRF, rats of the 1.2% phosphate group were scanned for the first time. In 2 out of 4 animals, prominent calcifications were detected in the aorta. These calcifications presented as dense rings (Figure 1A) delineating the vessel wall over the whole length of the scan. The animals were then maintained on 1.2% phosphate diet for another 2 weeks and micro-CT scans were repeated after 10 weeks of CRF, with comparable results.

Three animals fed a 1.03% phosphate diet were scanned after 10 weeks of follow-up. In 1 out of 3 rats modest, focal calcifications were detected by micro-CT. In the 4 remaining animals, the aorta was not visible on micro-CT scans, because of the absence of calcification, which was confirmed by light microscopy and calcium bulk analysis (Table 1).
Correlation of In Vivo Micro-CT With Histology and Tissue Calcium Content

At euthanization, the vascular tree showed macroscopically visible calcification in both animals that showed prominent vascular calcification on micro-CT scan. The thickening of the aortic wall at the ventral side of the micro-CT virtual cross section (Figure 1A) correlated well with the presence of calcification rings visible by macroscopic examination of the excised aorta (Figure 1B).

To validate micro-CT findings, the proximal half of the thoracic aorta was embedded in paraffin and stained with Von Kossa’s method for histological evaluation of calcification, whereas the distal part was used for determination of tissue calcium content by atomic absorption spectrometry.

As summarized in Table 1, micro-CT data correlated well with histological findings and aortic calcium content. In animals without detectable calcification on micro-CT, no Von Kossa positivity was observed histologically and bulk calcium content of the tissue was low, comparable with that of controls with normal renal function (0.17±0.04 and 0.16±0.03 mg calcium/g wet tissue in controls of the 1.03% phosphate and 1.2% phosphate group, respectively). In contrast, in animals with strong calcification on micro-CT, aortic calcium content was elevated to values of 52.80 and 26.07 mg calcium per gram wet tissue, respectively, and major Von Kossa positivity was present in every cross-section.

The sensitivity of the in vivo micro-CT method to detect vascular calcifications is demonstrated by the results of animal 1, in which only a modest increase in tissue calcium content was measured (1.29 mg calcium/g wet tissue) and only partially circumferential Von Kossa positivity was observed in 4 out of 6 microscopic cross-sections. Nevertheless, careful revision of the micro-CT dataset revealed focal calcifications in the aortic wall in 19.6% of the virtual cross-sections.

In Vitro Scanning and Image Analysis

To compare micro-CT findings with the gold standard of histological evaluation, ex vivo micro-CT scanning of the paraffin blocks was performed, with the same scanner. After reconstruction of the dataset, the distribution of the gray values in the virtual cross-sections was analyzed for each rat. Subsequently, 3D models were reconstructed from the virtual cross-sections. Figure 2A illustrates the calcified parts of the aorta the way they are embedded in paraffin.
Figure 2B shows the same parts of the aorta after histological processing, sectioning, and Von Kossa staining, showing good correlation between micro-CT and microscopic aspect of the calcifications.

### 3D Model and Quantification of Vascular Calcification

To assess whether the degree of aorta calcification can be followed-up over time, 3D reconstructions were made from the CT data sets at 8 and 10 weeks in a representative rat with prominent vascular calcification (animal 4 in Table 1). To build the 3D models of a densely calcified part of the aorta shown in Figure 3, calcified tissue was selected by density thresholding in combination with manual matching of the models. Volume analysis of these models revealed an overall increase in calcification of 2% (from 15.4 to 15.7 mm³) in the 2-week interval between both scans. Newly formed calcified foci, as well as calcified regions that have grown further during the interval between both scans, are discernible on the 3D models (Figure 3).

### Reproducibility Study: In Vivo Micro-CT Screening

CRF rats were scanned for the first time after 7 weeks of CRF. Calcification was detected by in vivo micro-CT in 8 of 14 animals (57%). The results of micro-CT screening corresponded well with vessel calcium accumulation: bulk calcium values of calcifying animals averaged 41.5±8.9 mg/g tissue (range, 18.7 to 60.5 mg/g tissue), whereas in noncalcifying animals an average tissue calcium content of 0.35±0.06 mg/g tissue (range 0.22 to 0.60 mg/g tissue) was observed, indicating that in vivo micro-CT is suitable to discriminate between calcifying and noncalcifying animals.

### Quantification and Reproducibility of Micro-CT Measurements

Five animals with varying extent of vascular calcification were scanned again a week later. In animals with moderate and severe calcification, the extent of calcification showed only minor changes during this week (Figure 4A), corresponding with the histological finding that the extent of vascular calcification in this model was near maximal after 6 weeks of CRF. In 2 animals with limited calcification after 7 weeks of CRF, the degree of calcification had substantially increased by week 8 (with 372% and 61%, respectively), indicating ongoing calcification in these animals (Table 2).

To assess the reproducibility of the measurement, the same region was scanned twice at the same time point in 3 animals with different degrees of calcification. Both the calcified surface and the calcified volume proved to allow reliable quantification of the calcification extent, with coefficient of variation values limited to a maximum 10% for both parameters (Table 2), although coefficient of variation values tended to be higher in the volume measurements.

In vivo analysis was complicated by unavoidable movement artifacts. Breathing and heart beating somewhat blurred the images, as is apparent from panels C and D in Figure 4. However, in heavily calcified aortas the increased stiffness caused much more limited movement artifacts than in slightly calcified animals. Unfortunately, postmortem distortion (Figure 4C versus 4D) prevented direct comparison of in vivo and ex vivo scans and therefore also quantitative assessment of the impact of movement artifacts.

### Discussion

Excess adenine feeding is a model of moderate to severe chronic renal failure,13,16 which also induces uremia-related vascular calcification.9 Dietary administration of 0.75% adenine for 4 weeks resulted in stable renal function impairment from 2 to 10 weeks after the start of adenine administration,
with serum creatinine values comparable to those previously published for this model. Although there is considerable biological variation in the development of vascular calcifications, the adenine model has clear advantages over the biological variation in the development of vascular calcifications, which can be detected and followed noninvasively, is of crucial importance to study the effect of both novel and already widely used pharmacological agents on the development of vascular calcification. Such experimental studies can give substantial direction to future clinical studies and help to limit cardiovascular mortality in the growing dialysis population.

In this study, we demonstrated that ectopic calcification in the aorta of uremic rats can be detected by in vivo micro-CT. The findings obtained with this technique correlated well with other parameters of aortic calcification, such as tissue calcium content and histological evaluation on Von Kossa staining. Additionally, 3D rendering of the calcified vessel wall allowed quantitative follow-up of the calcification process in successive scans.

A major advantage of in vivo micro-CT is its noninvasive character, allowing longitudinal studies. Repeated in vivo scans make it possible to use each animal as its own control. This way, the number of animals needed to reach statistically significant conclusions can be reduced.

As a tool to evaluate vascular calcification, both in vivo and ex vivo, micro-CT scanning is much less time consuming than equally detailed histological analysis. Moreover, micro-CT analysis allows taking into account the density, as well as the amount of calcification, whereas Von Kossa staining is a sensitive staining method for calcium precipitates, but it does not discriminate for the density of the calcium precipitation.

The reproducibility of the quantification method we used is more than acceptable, with coefficient of variation values below or limited to 10%. Especially measurement of calcified volume suffers from somewhat higher variability, which can be explained by the fact that blurring can easily increase models by an additional 1-pixel surface. In this case, total surface of the model changes to a lesser extent than the volume.

Limitations of the technique include the requirement of general anesthesia during image acquisition. This can, especially with cardiovascularily unstable animals such as calcifying uremic rats, cause loss of animals. Another harmful effect to the experimental animals is the application of X-rays where the ionizing effect can result in immediate radiation damage and in long-term genetic damage. The question of radiation dose during in vivo scanning is still undergoing debate. However, experience with in vivo scanning learns that single and repetitive scans after a time interval do not seem to have harmful effects. The number of scans that can be performed in the same animal and the minimum time interval between scans that has to be respected to avoid deleterious effects of the radiation dose is still controversial. In the present study, 2 scans with 50 minutes of radiation exposure time were performed with an interval of 1 week (reproducibility study) or 2 weeks (feasibility study), without apparent negative effects on the rats.

We chose to scan the thoracic region because the functional implications of vessel calcification and the concurrent arterial stiffening are most pronounced at this site: increased rigidity of the proximal part of the aorta leads to loss of vessel compliance, causing increased pulse pressure and left ventricular hypertrophy, and ultimately results in increased stroke work of the heart and insufficient coronary perfusion. Moreover, the descending aorta is more or less fixed to the vertebral column and shows limited movement with respect to other anatomic structures. In the abdominal region image quality would be low because of intestinal movement.

In vivo scans were performed without gating for cardiac or respiratory motion, and although image quality was good, some movement artifacts are inevitable and limit the maximal spatial resolution that can be obtained. Resolution is determined by the voxel size, which was set at $35 \times 35 \times 35 \, \mu m$. The equipment used in this study can provide a pixel size down to $9 \, \mu m$; however, the true resolution achieved depends on the properties of the scanned material as well as the maximal exposure time allowable with living animals. With a smaller voxel size, the scan time to obtain similar signal-to-
noise ratio to scan a region of the same size increases, accompanied by a proportional increase in the radiation dose to the animals.\textsuperscript{19}

The micro-CT scanning parameters used provide a relatively fast scan with the highest possible sensitivity for slightly calcified material, allowing the detection of small calcifications with a density lower than that of normal bone. Unfortunately, at the same time the use of soft X-rays does not allow accurate correction for beam hardening, which is necessary for direct calculation of the Ca content from in vivo scans. However, the isotropic scans enabled the reconstruction of 3D models, in which the extent of calcification in successive scans from the same animal could be measured based on a density threshold.

Movement artifacts may interfere with the manual matching of structures in successive scans. Additionally, movement of the vascular wall caused by the pumping action of the heart may artificially increase the wall thickness in the reconstructed images. Therefore, the 3D models were generated from the descending aorta, where movement is restricted by fixation to the vertebral column. Moreover, the compliance in heavily calcified vessels is low, so that elastic extension of the aorta during systole is expected to be rather limited.

In summary, in vivo micro-CT scanning is a sensitive method to detect calcifications in the thoracic aorta of CRF rats. Micro-CT data showed good correlation with histological analysis of vascular calcification and bulk calcium content of the tissue. In the future, this promising technique will allow to follow-up and quantify the development, and potential reversal under particular treatment conditions, of vascular calcifications in living animals.

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Disclosures
None.

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