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Quantitative Investigation of Bone Microvascularization from 3D Synchrotron Micro-Computed Tomography in a Rat Model

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***Abstract*—A new method for simultaneous 3D imaging and analysis of microvascularization and bone microstructure in rat bone is developed. The method is based on the use of quantitative synchrotron micro-computed tomography (SR-μCT) coupled to an automatic image analysis procedure. Analysis of bone microvascularization is generally performed from 2D histology. The proposed method enables for the first time the simultaneous 3D analysis of microvascularization and bone microstructure in a rat model. It was applied to investigate the effect of intermittent parathyroid hormone (PTH) administration on angiogenesis and osteogenesis in rats. Rats were posthumously injected with a contrast agent and subsequently imaged . The algorithm allowed the reconstruction and the extraction of 3D quantitative parameters both on bone microstructure and microvascularization. Due to the short acquisition times of SR-μCT and the efficiency of the image analysis algorithm, a large data set was analyzed, which permitted statistical analysis of the measured parameters. Statistical analysis confirmed that treatment with PTH significantly increased bone volume and thickness, but decreased bone mineralization. It was further revealed that treatment with PTH significantly increased average vessel thickness.**

I. Introduction

BONE vascularization plays a major role in many physiological events such as fracture healing and bone growth, and pathological processes such as metastasis, Paget's disease and hematopoïetic disorders [1]. Moreover, bone blood supply has been recently shown to be involved in osteoporosis, the most frequent metabolic bone disease [2]. Further, the effects of anti-osteoporotic treatment on bone vascularization have yet to be investigated. The anatomical characterization of the bone vascular network was performed many years ago using 2D X-Ray imaging after intravascular opacification with contrast products in animal models [3]. Other data were provided by histological analyses of vessels in bone [4]. However, these methods did not permit precise quantification and imaging of the vascular network in three dimensions (3D), nor did they permit a full analysis of the spatial relationships between bone and vascular system. These studies focused on analyzing either bone microstructure or microvascularization separately.

The purpose of this work was to develop a new method in order to quantify simultaneously the 3D organization of bone microstructure and microvascularization in a rat model. To this end, we used synchrotron radiation 3D micro-computed tomography (SR-μCT) at the European Synchrotron Radiation Facility (ESRF), Grenoble, France [5]. Due to the high flux of third generation synchrotron sources, SR-μCT is a quantitative CT technique allowing to reach micrometric spatial resolution [6]. Imaging was performed on rat samples posthumously injected with a contrast agent at a high spatial resolution (voxel size 2.8μm) to correctly resolve the microvasculature. An automatic image analysis method was then developed to identify cortical and trabecular bone envelopes, and bone and vascular structure within each envelope, Characteristic 3D quantitative parameters were then extracted from both microstructures.

The method was applied to investigate the effect of the potentially anti-osteoporotic agent parathyroid hormone (PTH) on the bone and vascular networks in rats. Quantitative parameters were computed from 28 3D images corresponding to 14 rat femur samples Statistical analysis revealed that treatment with PTH significantly increased bone volume and thickness, decreased bone mineralization and increased average vessel thickness. This method allowed for the first time to analyze quantitatively both microvasculature and bone tissue.

II. Materials and Methods

A. Sample preparation

Rats were given either PTH (sc, 100 μg/kg/day) for 5 days per week for 4 weeks or placebo (0.9 % saline). Following euthanization, the rats were subsequently infused with a barium sulfate solution (contrast agent). The femora were dissected, fixed in 10 % paraformaldehyde for 3 days, then transfered to and stored in 100 % acetone, and subsequently embedded in methylmethacrylate (MMA). Of these embedded femora small sub-samples (parallelepiped side ~4mm) were cut for imaging.

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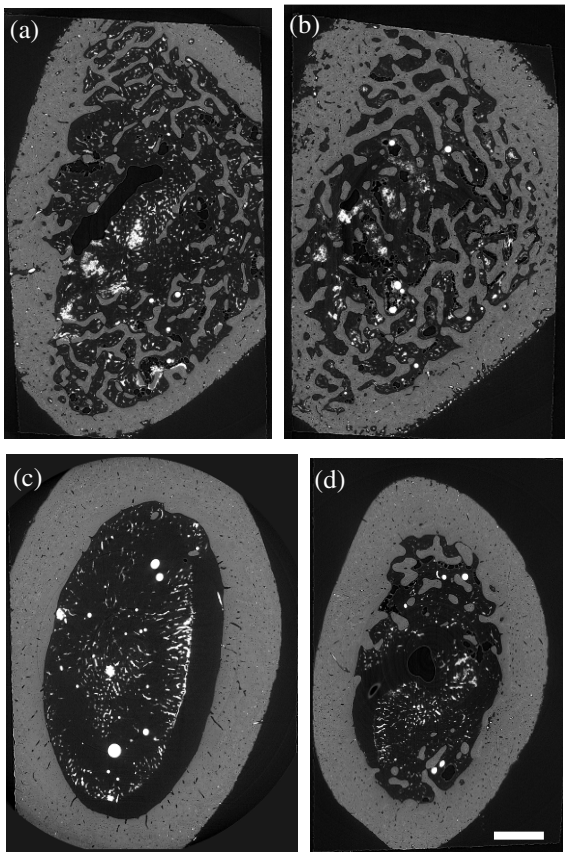

Fig. 1. Tomographic slice through a femur of a rat injected with contrast agent and embedded in methylmethacrylate (MMA): (a) control sample, ROI1, (b) PTH sample, ROI2, (c) control sample, ROI2, (d) PTH sample, ROI2 All phases are visible on the slice: vessels brightest (due to the contrast agent), bone as light grey, acetate as dark gray and air as black. The scale bar corresponds to 1 mm.

B. *Image acquisition*

3D SR-µCT imaging was performed on beamline ID19 at the ESRF, where a parallel beam 3D µCT setup has been developed [5]. It consisted in recording 2000 radiographs of the sample under different angles of view over 360° using a 2048x2048 pixel CCD-based detector [7]. The imaging system was set up to give a pixel size of 2.8 µm.on the detector, yielding a cylindrical field of view (FOV) of diameter 5.6 mm. The energy was set to 25 keV, giving a beam height of approximately 2 mm. Exposure time was set to 0.25 s per image to ensure a large dynamic range (the detector provides 14 bits). The acquisition time for one scan was about 18 minutes.

For each sample, two images corresponding respectively to a region of interest in the metaphysis (ROI1) and diaphysis (ROI2) were acquired. The ROI1 was scanned in the IISP. Since at this level the sample did not fit the FOV, we used a non-conventional acquisition procedure, recording images over 360° with the axis of rotation displaced to the edge of the FOV, thus enabling the reconstruction of a larger FOV which encompasses the sample. This procedure yielded reconstructed 3D images of 2500x2500x700 voxels.

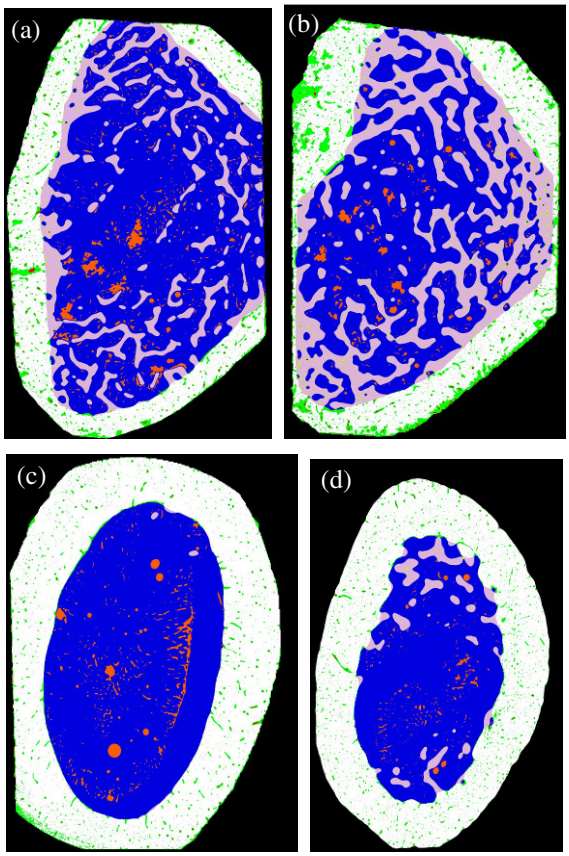

Fig. 2. Labelled volume consisting of the different segmented volumes: (a) control sample, ROI1, (b) PTH sample, ROI2, (c) control sample, ROI2, (d) PTH sample, ROI2. All desired bone parameters can be calculated based on this volume.

C. *Image segmentation*

The segmentation step requires identifying vessels, bone and back-ground. In addition, since it was desired to separately analyze trabecular and cortical bone, trabecular and cortical bone envelopes had to be labeled.

Segmentation of bone and vessels was performed using 3D region growing based on a gray level criterion. Separating the trabecular and cortical bone compartments automatically was not straightforward, since there was no measurable difference in grayscale between the two types of bone. In ROI2, it was observed that the cortical bone generally has a thicker cross-section. Segmentation of cortical bone could therefore be performed by first filling pores in the bone volume with a median filter, then calculating and thresholding the 3D local thickness map [8][9]. This yields the envelope of the cortical bone. This volume can then be used to separate bone into trabecular and cortical bone, and vessels into cortical and internal vessels by arithmetical operations. The total volume is calculated by filling in pores in the bone volume with a median filter, then filling in the empty space spanned by the bone.

There are specific problems in this study that needed to be addressed. The high resolution enables imaging of the vascularization, but also allows to resolve pores (lacunae) in both cortical and trabecular bone. The recorded volumes to

be treated are very large due to the large detector size. This puts stringent requirements on time and memory complexity of the image processing algorithm.

After segmentation, each volume was partitioned into seven sub-volumes (Fig. 2): Cortical volume, Trabecular volume, Pore volume in cortical bone, Pore volume in trabecular bone, Vessel volume in cortical volume, Vessel volume in Inner volume, Marrow Volume. 3D renderings of bone and vessel volumes are shown in Fig. 3.

D. Extraction of quantitative parameters

From this splitting, it was possible to recover all other desired subvolumes. For instance the cortical bone envelope is the union of the cortical volume, pore volume in cortical bone and vessel volume in cortical volume.

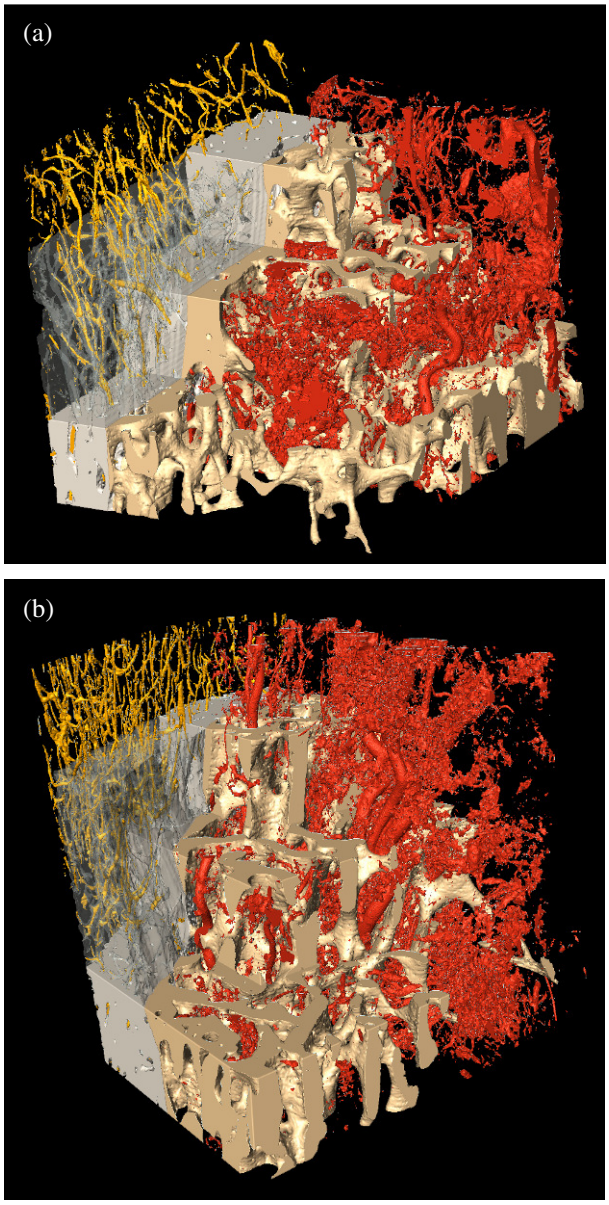

Fig. 3. Volume rendering showing bone and vessel compartments in ROI1. (a) control sample, (b) PTH sample. Note the apparent increase of vessel and trabecular thickness in the PTH sample. This is supported by the quantitative findings (Fig. 4, Tab. I).

Several parameters were extracted from the segmented volumes. The volume of each compartment was directly measured by counting voxels. The measured volumes are

- Total volume (TV): volume spanned by the outer contour of the cortical bone
- Inner volume (IV): volume spanned by the inner contour of the cortical bone
- Bone volume (BV)
- Cortical volume (CtV)
- Cortical envelope volume (CtMV): volume of cortical bone with porosity filled in
- Trabecular volume (TbV)
- Trabecular envelope volume (TbMV): volume of trabecular bone with porosity filled in
- Total vessel volume (VV)
- Vessel volume inside cortical envelope (VcV)
- Vessel volume inside inner volume (ViV)
- Marrow volume (MaV)
- Pores in cortical bone (PoCtV)
- Pores in trabecular bone (PoTbV)

To allow for comparison between samples, normalized ratios are calculated:

- Bone ratios: BV/TV, CtV/TV, CtMV/TV, TbV/IV, TbMV/IV
- Vessel ratios: VV/TV, VcV/CtV, ViV/IV, ViV/MaV
- Lacunar ratios: PoCtV/CtMV, PoTbV/TbMV
- Marrow ratio: MaV/TV

Based on the segmented volumes, more complex parameters have also been extracted. Local thickness of bone and vessel compartments are calculated, yielding

- Mean cortical thickness (Ct.Th)
- Mean trabecular thickness (Tb.Th)
- Mean vessel thickness (V.Th)
- Mean thickness of internal vessels (Vi.Th)
- Mean thickness of cortical vessels (Vc.Th)

By multiplying the segmented bone compartment with the grayscale volumes, only the bone part of the grayscale images is extracted. This gives access to the bone mineralization levels, which yields the mean degree of mineralization of bone (DMB)

E. Statistical analysis

Statistical testing was performed between the two groups on all measured parameter, both in each ROI separately and the two ROIs combined. The two-sample unpooled t-test was used in all cases. Differences between the two groups were considered statistically significant at the 5 % level ($p<0.05$).

III. Results

In total, 28 samples were analyzed, 13 controls and 15 from the PTH group. Significant differences between the two are reported in Tab. I. Box plots of a selection of important parameters are shown in Fig. 4.The following significant differences could be observed between the two groups

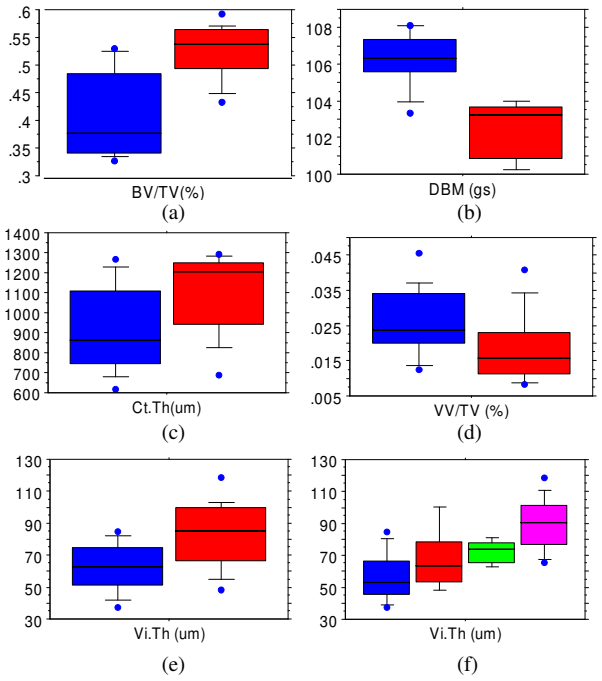

Fig. 4. Box plots of selected bone parameters (control in blue, PTH in red). Several observations can be made, which are shown to be significant with a non-paired t-test. (a) PTH increases BV/TV (b) PTH reduces DBM in ROI1 (c) PTH increases Ct.Th (d) PTH decreases VV/TV (e) PTH increases ViV.Th and (f) PTH increases Vi.Th in ROI2 but not significantly in ROI1 (ROI1 in blue/red, ROI2 in green/magenta).

Bone volume. PTH treated rats had 30 % higher bone ratio ($p<0.0001$), 40 % higher in ROI1 ($p<0.0001$) and 10 % higher in ROI2 ($p=0.0364$). PTH treated rats had 70 % greater trabecular volume ratio ($p<0.0001$) and trabecular envelope ratio ($p<0.0001$), both in ROI1. PTH treated rats also had a slightly higher cortical envelope ratio in ROI2 ($p=0.0455$).

Bone thickness: Rats treated with PTH showed a 50 % increase in cortical thickness ($p<0.0001$) and 30 % increase in trabecular thickness in ROI1 ($p<0.0001$).

Bone mineralization: the control group showed 10 % higher average mineralization in ROI1 ($p=0.0003$).

Marrow volume: PTH treated rats showed a 20 % decrease of marrow volume ratio ($p<0.0001$)

Total Vessel Volume: PTH treated rats showed a decrease in vessel volume by 27 % ($p=0.0451$).

Vessel thickness: PTH treated rats showed an increase in thickness of the internal vessels by 30 % ($p=0.0040$).

IV. Discussion and Conclusion

SR-µCT allowed for the first time to study simultaneously bone microstructure and microvascularization at very high spatial resolution. Due to adapted image analysis, it was possible to extract from the data a large number of 3D quantitative parameters.

The method was applied to study the effect of PTH treatment on bone microvascularization. The results demonstrated that PTH modulated both bone and vascular parameters (e.g., BV/TV, trabecular and cortical bone volume, blood vessel thickness, etc.) The increases in bone volume and trabecular and cortical thicknesses are in agreement with previous studies. Further, the 3D vessel thicknesses were also significantly increased with PTH treatment. SR micro-CT also permitted the analysis of the degree of bone mineralization in 3D, which was diminished with intermittent PTH administration.

TABLE I
HYPOTHESES SIGNIFICANT AT 5% LEVEL

Hypothesis	p-number
PTH increases BV/TV	0.0001
PTH increases CtMV/TV in ROI2	0.0455
PTH increases TbMV/IV in ROI1	<0.0001
PTH increases TbV/IV in ROI1	<0.0001
PTH increases Ct.Th	<0.0001
PTH increases Tb.Th in ROI1	<0.0001
PTH decreases MaV/TV	<0.0001
PTH decreases DMB in ROI1	0.0003
PTH decreases VV/TV	0.0451
PTH increases Vi.Th	0.0040

We acknowledge that the number of samples analyzed is limited at present. Further data has been acquired and will be analyzed with the present methods to augment the number of samples and make the statistical analysis more robust.

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