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Longitudinal pQCT measurements can be used to evaluate bone loss in the ovariectomised rat model avoiding the use of sham animals

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Summary

In the past, osteoporotic bone loss in the ovariectomised (OVX) rat model was monitored with the use of ex vivo methods. Thus, sham animals were required. The purpose of this study was to determine if bone loss in the OVX rat model monitored in an in vivo longitudinal fashion using the peripheral quantitative tomography (pQCT) provides the same information eliminating the use of sham animals. Furthermore, parameters of bone loss measured with pQCT were directly compared to that conventionally measured with the micro-computed tomography (micro-CT). Bone mineral density (BMD) was measured at the distal femur of twenty-one 12 weeks old female Wistar rat with the pQCT. Thereafter, animals were either OVX or sham operated, pQCT measurements were repeated 4 weeks later and all rats were euthanized. Ex vivo BMD and BV/TV was measured with the use of micro-CT. Results showed that there was significant growth during the 4 weeks and that taking this into account, there was no difference between bone loss measured in a longitudinal fashion (pQCT) and that measured in comparison to a sham group (micro-CT). In addition, we could demonstrate that there was a strong linear correlation between BMD measured with pQCT and BV/TV (%) obtained from micro-CT. In conclusion, there is no need to use sham animals for monitoring bone loss in the OVX rat model and longitudinal pQCT measurements are sufficient, as long as the growth is known and adjusted for.

Keywords: pQCT; Micro-CT; sham animals; osteoporosis; in vivo; rat

Introduction

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue with a consequent increase of bone fragility and susceptibility to fractures [1]. Amongst the elderly, osteoporosis is a problem with great morbidity and high mortality. For older women, the risk to die from a hip fracture [2] is nearly as high as to die from a stroke [3]. In addition, the increase in the elderly population will lead to an increased prevalence of osteoporosis and subsequent costs to society will rise. In order to study and develop surgical treatments of osteoporotic fractures as well as new strategies for pharmaceutical therapies, animal models of osteoporosis are needed [4]. The ovariectomised (OVX) rat is the most widely used animal model for osteoporosis [5,6] as it shares many similar characteristics to postmenopausal bone loss in women, e.g. bone resorption exceeding bone formation, greater loss of cancellous bone than cortical bone as well as similar treatment response to drugs like biphosphonates, estrogen, parathyroid hormones and calcitonin [7,8,9,10].

To monitor bone loss in the OVX rat model conventional histology has been used in the past [11,12,13,14,15]. To overcome the limitations of histology, such as its manually intense nature, only two-dimensional morphometry and its destructive nature, the use of high-resolution micro-computer tomography (micro-CT) has become more and more popular[16,17,18]. However, despite the possibility to quickly assess mineral density and three-dimensional microarchitecture from bone samples in a repeatable, non-destructive manner, a major disadvantage of standard micro-CT is that, similar to conventional histology, it is used ex vivo making it impossible to assess longitudinal temporal changes in the same

animal. This is, amongst others, the reason why studies using these ex vivo methods depend on the use of sham operated animals to confirm the bone loss after OVX [19,12,13,20].

Peripheral computed tomography (pQCT) as well as a recently developed in vivo micro-CT (vivaCT40, Scanco Medical, Bassersdorf, Switzerland) is able to provide information from living animals in a longitudinal fashion. pQCT has been available for the detection of patients at high risk of osteoporosis. Serial examinations at different time points enable the individualisation of prophylaxis and treatment [21,22,23]. pQCT has been applied in rats [24,25,24,26], mice [27] and goats [28] for research purposes. Although the spatial resolution of the pQCT falls clearly short of that obtained by in vivo micro-CT, it provides a number of potential alternative advantages: (1) pQCT device can be used for patient assessment and for laboratory animals of different sizes, whereas in vivo micro-CT are limited to small laboratory animals; (2) although in vivo micro-CT offers a higher scanning speed than the previously developed micro-CT, depending on the resolution and sample size, the pQCT is normally still the faster system. This is important as the laboratory animals need to be anesthetized for the entire scanning duration and the longer the anesthesia, the burden for the animal in respect to its body circulation and inner body temperature regulation is increased; (3) radiation exposure is significantly lower in pQCT. The total radiation dose can be on the order of a few gray [29] with the use of in vivo micro-CT in typical longitudinal studies. The impact of radiation needs to be considered in such research, because intense radiation has been shown to have an effect on bone volume ratio [30].

Micro-CT with its high resolution is the gold-standard for evaluation of bone mass and there are two different outcome-parameters available to describe it. BMD is a value calculated by the average of all gray-values in the defined region-of-interest (ROI) and is presented in the

unit milligram hydroxyapatite/cm^3 (mg HA/cm^3). BV/TV is the bone volume, which has been defined with a specific threshold, divided by the total volume-of-interest (VOI) in percent and it is the parameter most widely used. The parameter to describe bone mass in the pQCT without adding further steps of calculations is BMD.

The question addressed in this work is whether bone loss in the osteoporotic rat model monitored in a longitudinal fashion (i.e. between the time of OVX and the beginning of the specific treatment) with the use of pQCT is similar to bone loss detected with micro-CT in comparison to a sham group so that sham animals could be avoided. Furthermore, in order to translate studies using pQCT measurements of bone density in the OVX rat model to those using micro-CT measurements, the correlations between pQCT BMD and micro-CT BV/TV was explored.

Materials and Methods

Animals

Twenty-one 8 weeks old female Wistar rats (RCC, Basel, Switzerland) were acclimatised for four weeks, housed in Makro Type 4 cages in groups of four and fed with standard rodent chow (Provimi, Provimi Kliba AG, Kaiseraugst, Switzerland) and water ad libidum. At 12 weeks of age, they were randomly divided into two groups: one group underwent bilateral ovariectomy (n=13, larger number for a subsequent study), while the other group received a sham operation (n=8). During the surgical procedures, animals were premedicated with buprenorphine (Temgesic®, Essex Chemie AG, Lucerne, Switzerland) and anesthetized with isoflurane (Isofluran Baxter ad us. vet.®, Baxter AG, Volketswil, Switzerland). Buprenorphine was given for two days post-operatively for analgesia. Each animal received a pQCT scan prior to surgical intervention; thereafter, they were kept under standard housing

conditions for four weeks to induce osteoporosis. The pQCT scan was repeated and then animals were euthanized by CO2 inhalation. The left femurs were harvested, freed from soft tissue and immediately fixed in 70% ethanol for high-resolution micro-CT. The procedure for animal care, experimental protocol and euthanasia in the study was approved by the Animal Experimentation Commission of the Veterinary Office of the Canton of Grison, Switzerland and followed the guidelines of the Swiss Federal Veterinary Office for the use and care of laboratory animals.

pQCT

pQCT was performed using 3D XtremeCT (Scanco Medical, Bassersdorf, Switzerland), which is used for patient examination[23] (Figure 1).

Figure 1: Peripheral quantitative computed tomography (XtremeCT, Scanco Medical, Bassersdorf, Switzerland).

This scanning system consists of a two-dimensional detector array with 3072x255 elements in combination with a microfocus X-Ray-tube having a spot size of 70 μm, enabling simultaneous acquisition of a stack of parallel CT slices. The rat was positioned on an in-house custom made holder made of plexiglass, which allowed consistent and precise positioning of the rat leg (Figure 2). The longitudinal axis of the left leg was aligned perpendicular to the scanner beam. Fifty-three cross-sectional slices (corresponding to a

length of 2 mm) were collected at the distal femoral metaphysis with an isotropic voxel resolution of 41 µm and a pixel matrix of 3072x3072, using an effective energy of 60 keV and a current intensity of 900 µA. The total examination time was 10 minutes. The resulting gray-scale images were then processed using a Gaussian low-pass filter (sigma=0.7; support=1) to remove noise, and apparent bone mineral density (BMD) was calculated.

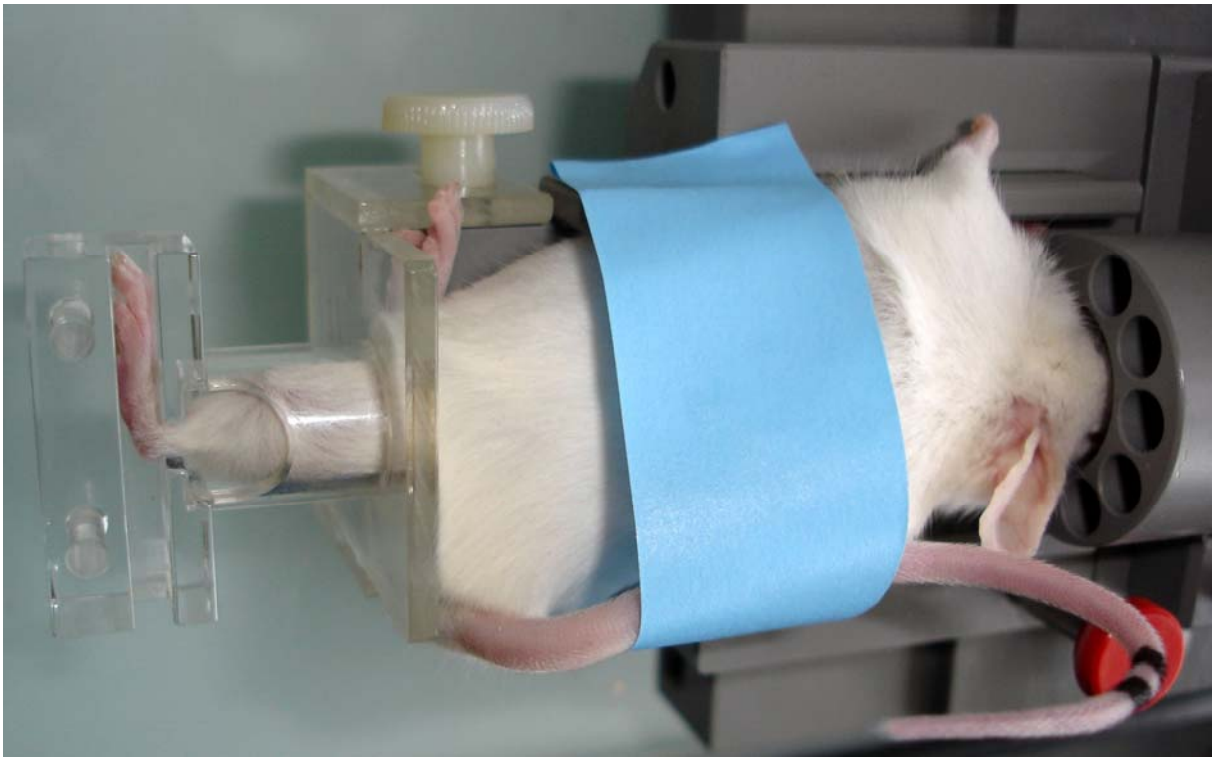

Figure 2: In vivo analysis of distal rat femur with pQCT scanner using a plexiglass holder.

Micro-CT

The femurs were also examined with a fan-beam micro-CT system (µCT40, Scanco Medical, Bassersdorf, Switzerland; Figure 3). This scanner was equipped with a microfocus X-ray tube (5-µm spot size) and a two-dimensional detector array with 2048x256 elements. The post-mortem harvested left femur was placed longitudinally into a cylindrical holder with a diameter of 16 mm filled with 70% ethanol. Analogous to the pQCT measurements, the distal

femoral metaphysis was scanned, whereby in this case with a higher isotropic voxel resolution of 16 µm. One-hundred and thirty high-resolution slices (= 2 mm length, same length as chosen for the pQCT) with a pixel matrix of 2048x2048 were measured using an effective energy of 70 keV and a current intensity of 114 µA. After pre-processing the images with the same Gaussian filter (sigma=0.7; support=1), a threshold was selected at 28 % of the maximal gray scale value, which corresponds to the peak for bone tissue in the histogram of the gray value distribution in the image. BMD as well as BV/TV were evaluated.

Figure 3: High-resolution micro-computed tomography (µCT 40, Scanco Medical, Bassersdorf, Switzerland)

Region of interest (ROI)

For accurate matching of the 2-mm-long ROI between pQCT and micro-CT measurements in the axial direction, an anatomical reference point was defined, which could easily be determined by visual inspection of both the micro-CT as well as the pQCT images (Figure 4). For each sample, the most proximal aspect of the distal femoral growth plate was chosen for this landmark. Analysis was performed for the total cross-sectional area (including cortical and trabecular bone; defined as "out") as well as for trabecular bone only. For the selection of trabecular bone, two different algorithms were used. With the first algorithm the central 50%

cross-sectional area was defined based on an automatic contouring procedure available from the built-in computer software of the manufacturer (defined as "d50"). In the second algorithm, the trabecular region was precisely contoured in each single cross-section manually (defined as "man_in").

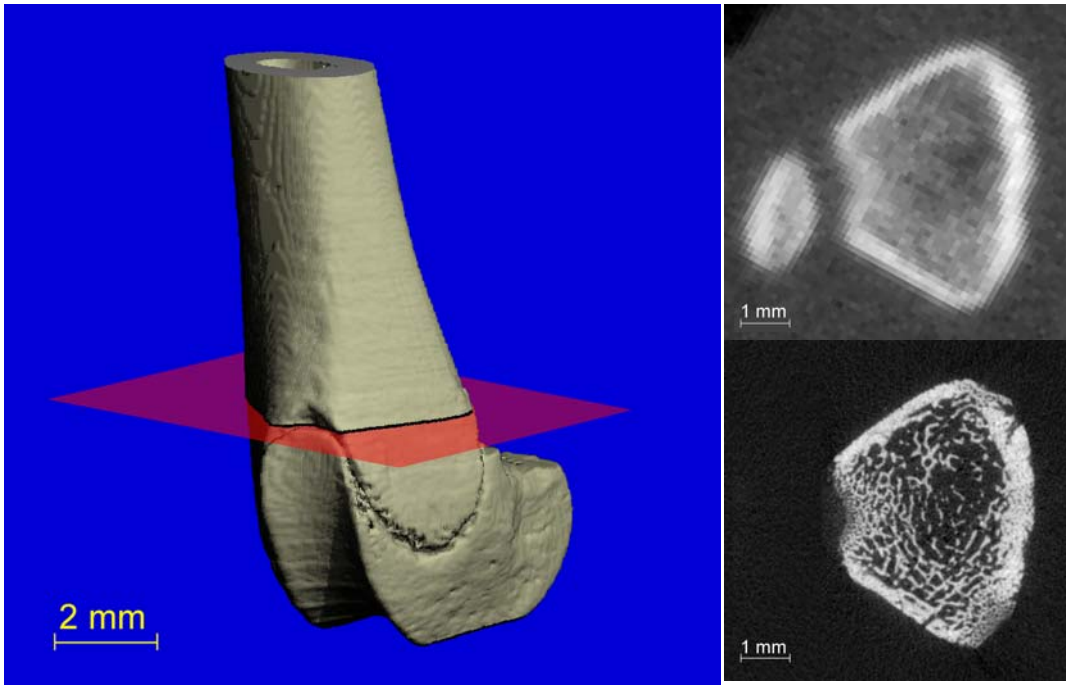

Figure 4: a) The distal half of the rat femur as imaged with the micro-CT. The square plane indicates the distal most cross-section included in the ROI. b) The corresponding pQCT cross-section image taken at the distal most aspect of the analysed ROI and c) the corresponding micro-CT image. Note the difference in resolution.

Statistics

Analyses were carried out using SPSS software (SPSS Science, 14.0 for Windows Chicago, IL, USA) and the significance threshold of $p<0.05$ was applied. A paired samples t-test was used to detect differences between the baseline and the follow-up measurement of the sham group and to evaluate differences between values assessed using the d50 and man_in. Instead, a repeated measures general linear model to compare differences in bone loss obtained with out, d50 and man_in was applied. A Tukey HSD post-hoc test to examine differences within a

group was used. An analysis of variance (ANOVA) was carried out to determine whether there were significant differences between bone loss measured in a longitudinal fashion (pQCT) versus the bone loss analysed with the use of a sham group (pQCT and micro-CT). If significant differences were detected, a Tukey HSD post-hoc test was used to examine direct inter-group differences. A linear regression was also applied to evaluate the correlation between pQCT and micro-CT measurements as well as between the automatic (i.e. d50) and the manually defined areas of interest (i.e. man_in).

Results

All animals survived the entire study period. The mean bone changes in percent measured in a longitudinal fashion (pQCT) as well as with the use of a sham group (pQCT and micro-CT) are showed in figure 5. There was a significant increase in BMD from the baseline and follow-up pQCT in the sham animals in all measured ROIs (out= 10.3%, p=0.000; d50= 11.2%, p=0.001; man_in= 9.9%, p=0.007), as a consequence of bone growth.

The BMD loss between the baseline and follow-up pQCT in the OVX animals was 11%, 35.1%, 32.1% for out, d50 and man_in, respectively. This was in contrast to bone loss measured between OVX and sham animals with the pQCT in terms of BMD (20%, 42%, 37.9%), with the micro-CT in terms of BMD (21.3%, 40.5%, 36,0%) and with the micro-CT in terms of BV/TV (22,5%, --, 44,5%; d50, an algorithm provided by the manufacturer does not include calculation of BV/TV). As the bone loss between the baseline and follow-up pQCT in the OVX animals does not take into account the significant normal bone growth detected in the longitudinal measurements of the sham animals, the longitudinal OVX bone loss was adjusted by adding the mean bone growth of the sham animals. No significant differences were detected between the “growth-corrected” longitudinal bone loss of the OVX

animals (pQCT/BMD) in comparison to bone loss measured with the help of sham animals (pQCT/BMD, micro-CT/BMD and micro-CT/(BV/TV) in any of the characterised ROI (out: p=0.492; d50: p=0.312; man_in: p=0.126, Figure 6).

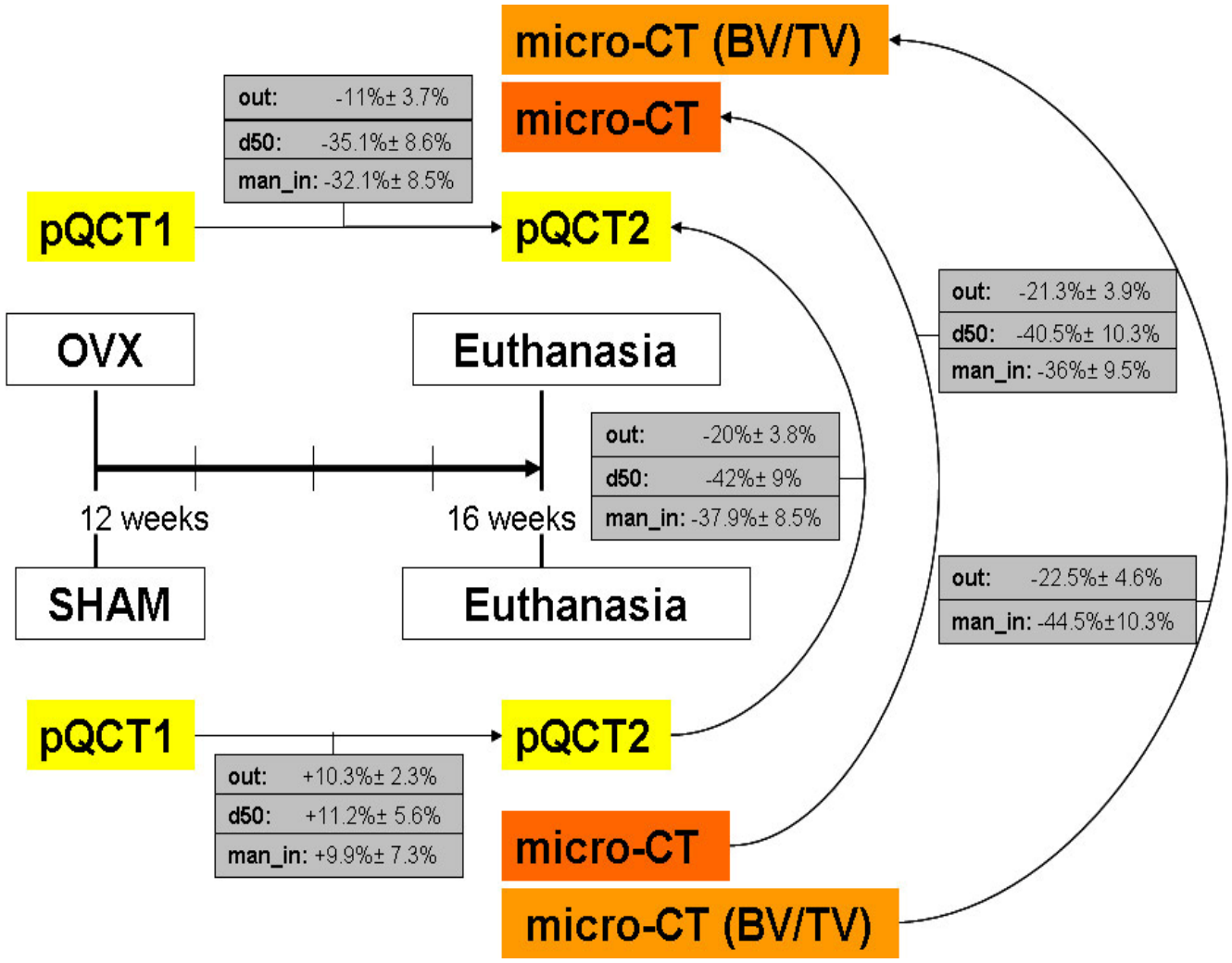

Figure 5: Experimental design and relative bone quality changes. The relative changes for the different ROIs are expressed as paired or independent mean changes ± S.D. All parameters are BMD unless labelled differently. The time in weeks is the age of the rats.

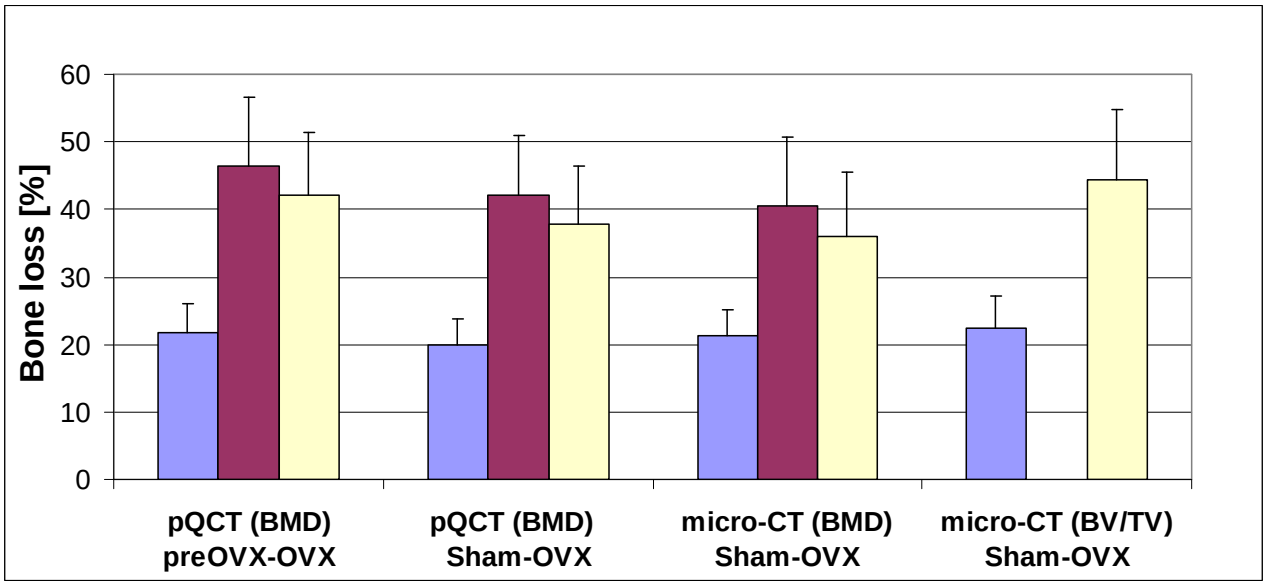

Figure 6: Post-ovariectomy bone loss as calculated by various methods, in percent: mean ± S.D. The bars are grouped according to imaging machine type and parameter as well as comparison type (blue = out, red = d50, yellow = man_in). pQCT (BMD) preOVX-OVX has been growth adjusted.

The BMD values obtained from high-resolution micro-CT measurements showed a strong correlation with those measured by lower-resolution pQCT (out: R^2=0.99, p=0.000; d50: R^2=0.96, p=0.000; man_in: R^2=0.95, p=0.000). The slope of the correlation curve was slightly lower for out (0.82) than for d50 (0.93) and man_in (0.93) (Figure 7). Moreover, BV/TV measured with micro-CT also correlated positively with BMD measured by the pQCT (out: R^2=0.96, p=0.000; man_in: R^2=0.95, p=0.000) (Figure 8).

As expected, the osteoporotic bone loss was always significantly more pronounced when analysing the trabecular region only (d50, man_in) than when examining the full cross-section (out) (p=0.000). There was also a significant difference in the evaluation of the trabecular region using d50 or man_in (p=0.000) with man_in exceeding d50 measurements. However, data showed a strong correlation to each other (R^2=0.98, p=0.000).

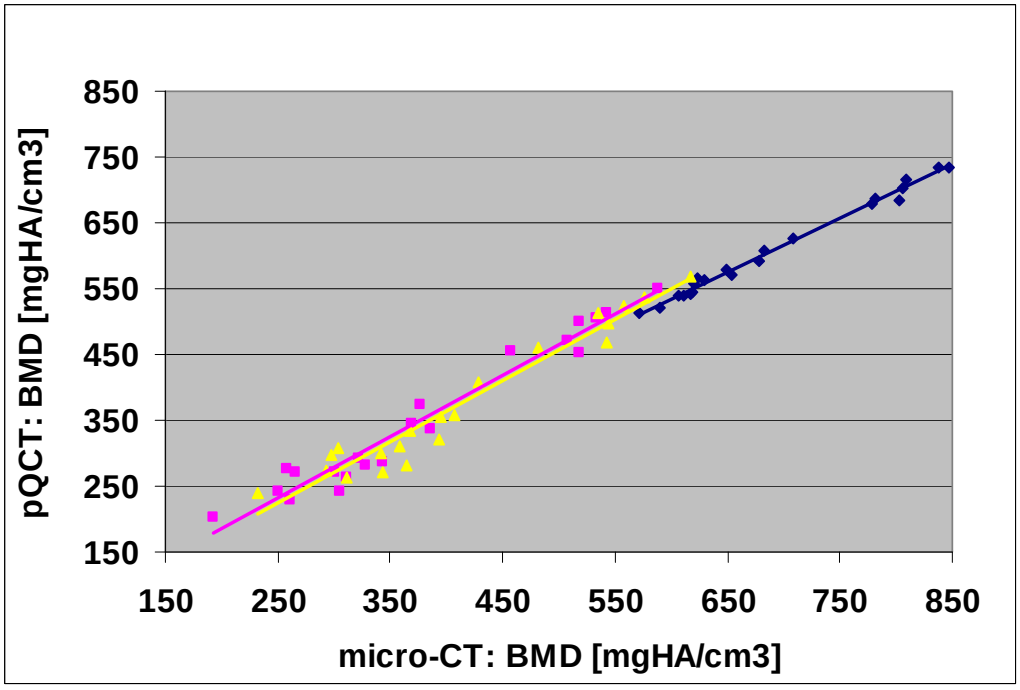

Figure 7: Correlation between micro-CT (BMD) and pQCT (BMD); blue: out (R^2= 0.99; y= 0.82+ 44.1); pink: d50 (R^2= 0.99; y= 0.93- 1.1812); yellow: man_in (R^2= 0.95; y= 0.93- 9.29).

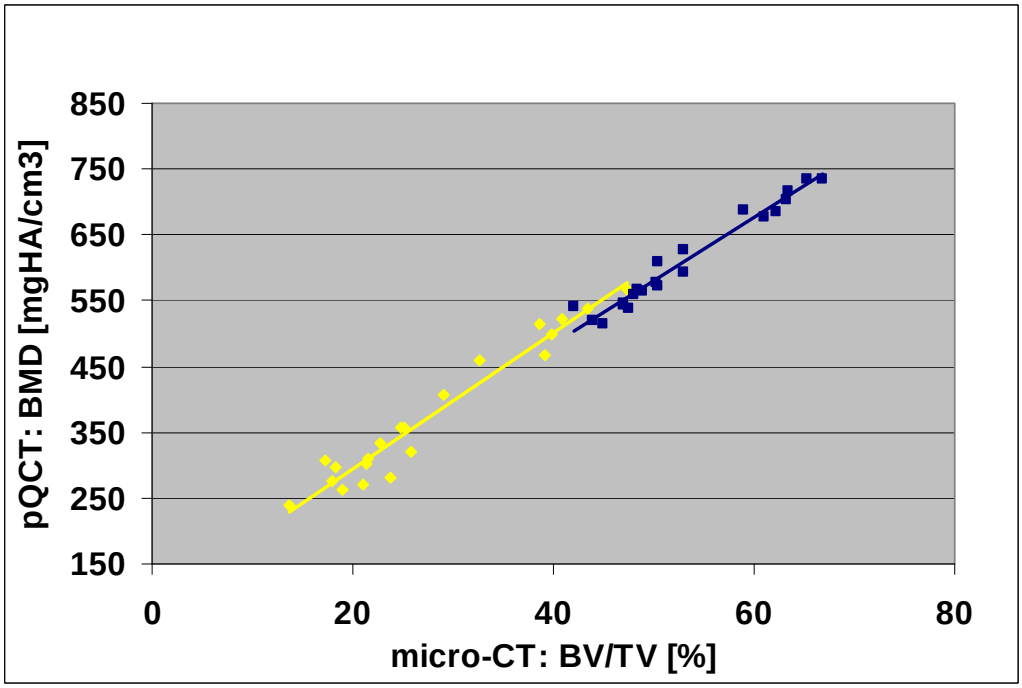

Figure 8: Correlation between micro-CT (BV/TV %) and pQCT (BMD); blue: out (R^2= 0.96); yellow: man_in (R^2= 0.95).

Discussion

The OVX rat is a well described model for osteoporosis [7,4]. In the past, osteoporotic bone loss was monitored with the use of ex-vivo methods such as histology and micro-CT [31,32]. Sham animals were essential for the control of induced bone loss as well as to evaluate changes which may have been caused by applied treatment, e.g. biphosphonates or calcitonin. Fortunately in vivo systems such as in vivo micro-CT and pQCT are available nowadays to offer the advantage of longitudinal measurements of one and the same animal. Although in vivo micro-CT provides the highest resolution data, these systems are not readily available due to their high cost and limited use for only small animals. Furthermore, the radiation dosages required for high resolution imaging may affect the bone volume ratio when used repeatedly in a longitudinal fashion [30]. In contrast, the pQCT is more widely available because it can also be used for larger animals as well as clinically and the radiation dosages are up to one-hundred-fold smaller [33]. The main difference between these two systems is the resolution of the image set. Although it was shown in studies using other animal models (mouse and goat) [28,27] or in a study using human specimen [34] that there is a good correlation between data received from the micro-CT and pQCT, it was not known for the OVX rat model whether the resolution differences would allow the bone loss to be monitored in a longitudinal fashion, eliminating the need to use sham animals.

The findings of this study indicate that bone loss monitored longitudinally and in vivo by pQCT corresponds to bone loss measured by high-resolution micro-CT using a sham group of the same age for comparison. The only difference between OVX induced bone loss measured in a longitudinal fashion to bone loss detected in a comparative way to a sham group is represented by the factor “growth” which we evaluated with longitudinal measurements of the

sham animals. The same outcome was observed in each defined ROI (out, d50 or man_in) and was also true for different outcome parameters (pQCT BMD, micro-CT BMD and BV/TV). By adding the amount of bone growth to the bone loss measured longitudinally in the OVX group, the growth adjusted bone loss was not different to that measured in comparison to sham operated animals (figure 6). Moreover, the calculated values for BMD and BV/TV also correlated very closely. Therefore, in the future, the use of sham animals in the OVX rat model can be avoided when the animals growth rate is known. If normal bone changes are not expected during the duration of the experiment, this additional adjustment is probably not required. This finding is especially important for this particular rat model as there are many studies in which several time points are needed to evaluate changes induced by a specific treatment. With increased number of time-points, more animals are needed to improve the power of the analysis. This is true for bone of the treated and the untreated or sham control groups. Hence, we hope that now only one group of animals is actually needed independent on how many time-points are of interest and the large number of rats used in such studies can be substantially reduced.

In the literature, bone loss in the OVX rat model has been measured via the gold-standards of micro-CT [17] or histologically [13] in terms of BV/TV. Previous studies demonstrated in other animal models (mouse and goat) [28,27] and human [34] specimens that there is a good correlation between micro-CT and pQCT evaluations of bone density. The results of our study showed that this is also true for the OVX rat model. There was a strong correlation ($R^2 > 0.95$) between the two machines, independent from the chosen correlation parameter. When looking at only the trabecular region, there was even a stronger correlation ($R^2 > 0.95$) compared to the results of a goat study with an R^2 of 0.7 [28]. The reason for this is probably the more accurate and very consistent definition of the region of interest in our study whereby

the exact same volume size and location were chosen. It can be generally said that bone density measured with pQCT will always provide slightly different values than obtained with micro-CT because effective energy, current intensity as well as calibration files are device-dependent. The offset resulting in the correlation between BV/TV and BMD is due to the background density of bone marrow, which is not reflected when calculating bone ratio based on a segmentation threshold. However, there is a strong correlation between the two machines and the resulting percentual bone loss will be equal.

Bone density values of the trabecular region evaluated with man_in significantly exceeded d50 values. This is due to the fact that with manual contouring the entire trabecular region was precisely selected, while the automatic procedure selected a smaller central area within the trabecular region. This can become an issue when the ROI is adjacent to the formation of the medullary canal where endosteal bone thinning has a stronger effect. However, the two evaluation systems showed again a very strong relation to each other (R^2=0.98), which makes both systems appropriate for the evaluation of bone loss in the trabecular bone region. The advantage of d50 is that being a full-automatic procedure, it is less labor intensive and can clearly be faster than the man_in definition.

According to the literature, osteoporotic bone loss was more pronounced in the trabecular region than in the cortical region in this study [35,36]. Moreover, the detected bone loss in the trabecular region of approximately 40 % within 4 weeks in this specific model stands in good agreement to previous publications (figure 5) [17].

In conclusion, this study showed that it is possible to eliminate the use of sham animals to monitor bone loss in the OVX rat model when using longitudinal pQCT measurements, as long as bone quality changes due to growth is known and adjusted for. The strong correlation

between BMD measured with pQCT and BMD as well as BV/TV (%) obtained from micro-CT was confirmed in OVX rat bones. pQCT appears to be an accurate tool to measure bone loss (%) in the OVX rat model offering the same information than the gold-standard micro-CT.

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EDUCATION – Degrees, diplomas

University

09/05	Diploma for a rotating Small Animal Internship
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09/98	**Candidatus Medicinae Veterinariae**,
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Matura (A-level Certificate)

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LANGUAGE SKILLS AND EXPERIENCES

German: native language
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PREVIOUS PROFESSIONAL EXPERIENCE

Since 11/05 **Research assistant, AO Foundation, Davos, Switzerland**
Dr.med.vet degree Research Projects:
Longitudinal pQCT measurements can be used to evaluate bone loss in the ovariectomised rat model avoiding the use of sham animals;
Effects of hydroxyapatit on implant osseointegration in osteoporotic bone;
Responsible for transactions of own projects from planning phase, experimental phase including surgical procedures to publishing phase
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10/04 – 09/05 **Rotating Internship in Small Animal Medicine and Surgery**
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PUBLICATIONS

Ein XX Sex Reversal bei einem Mischlingshund: Ein klinischer Fall
M. Pucher, J. Handler, H. Korb, B. Mayr, A. Holzmann. Kleintierpraxis 2006; 51; 9, 464-469

In Progress:

Longitudinal pQCT measurements can be used to evaluated the bone loss in the ovariectomised rat model avoiding the use of sham animals
M. Pucher, A. Tami, P.Montavon, K. Ito. BONE

Effects of hydroxyapatite ceramic particles on implant osseointegration in osteoporotic trabecular bone evaluated with micro-computed tomography, mechanical testing and histology
M. Pucher, A. Tami, H. van Lenthe, R. Müller, P. Montavon, K. Ito. JBMR

INTERESTS

Jogging, mountain climbing, ski-touring, cross country skiing, mountain biking, snowboarding