Monitoring of *Cryptococcus* lung infection with micro-CT and micro-MRI

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**Background**

*Cryptococcus* is an encapsulated yeast that causes life-threatening disease in both immunocompetent and immuno-suppressed individuals. The two predominant pathogenic strains of this yeast, *Cryptococcus neoformans* and *Cryptococcus gattii*, are generally found in soil, bird excrement and in the bark of certain trees and enter the host via inhalation. Cryptococcosis mostly affects the lung of a host and may spread to the brain, manifesting itself by cryptococcal meningitis and/or pseudocystic lesions (cryptococcoma) in the brain often with fatal outcome. It remains still unknown why, how and when the cryptococci are able to cross an apparently intact blood-brain barrier (BBB). Histological techniques will remain essential to confirm and unravel cellular and molecular interactions, but imaging techniques are indispensible to define the relevant time frames for histological analysis and this for each animal individually, to investigate crucial events in pathogenesis. As currently imaging tools to evaluate pneumonial cryptococcosis with good temporal and spatial resolution *in vivo* are lacking, advances made in lung µCT and µMRI techniques to follow-up disease progression non-invasively will greatly enhance the cryptococcosis research.

**Aims**

It is our objective to dynamically monitor cryptococcosis non-invasively in individual animals, in a mouse model for pneumonial and cerebral cryptococcosis. Thereby, we will establish the kinetics of cryptococcal lung infection with µCT and µMRI and the time profile of *Cryptococcus* spreading to the CNS, thereby defining the most critical time points for histological and immunological analysis of key events in the pathogenesis of cryptococcosis.

**Methods**

Balb/C mice were infected by inhalation of a *C. gattii* R265 cell suspension (500 cfu's) in PBS (n = 7) or with PBS alone (control, n = 3). Mice were scanned with MRI and CT at baseline and weekly until 45 days post infection. MRI images were acquired on a horizontal Bruker Biospec (9.4T, 20 cm) in combination with a 7cm quadrature coil using a retrospectively gated FLASH sequence IntraGate (Bruker Biospin, Ettlingen, Germany) with the following parameters: TR/TE = 30/1.26 ms, 17 deg flip angle, 5 slices covering the lung, slice thickness 1 mm and gap of 0.5 mm, FOV = 4 cm x 4 cm, matrix 256 x 256, in plane resolution of 156 μm, 80 repetitions resulting in a 10 min acquisition; the navigator slab was 1cm wide excited with a 0.8 ms sinc10H pulse with a 1.5 deg flip angle. For reconstruction, 70% of the respiration and ECG period was used (Paravision 5.1, Bruker). CT images were acquired on a dedicated small animal µCT scanner (SkyScan 1076, Kontich, Belgium) with the following parameters: 50 kV, 0.5 mm Al filter, 200 μA source current, 35 μm isotropic resolution, 120 ms exposure time, 9 projection images per 0.7° rotation step and...
retrospectively gated. Image analysis, segmentation and quantification of CT data were performed with custom written algorithms using SkyScan software. After the last time point, mice were sacrificed followed by sterile CNS removal and lung isolation for histological analysis (HE and PAS-stainings) and quantification of fungal load.

Results
Pneumonal cryptococcosis was successfully and reproducibly induced in immunocompetent mice. While the mice showed no phenotypical signs of cryptococcosis, the progression of the lung pathology could be non-invasively visualized using the here evaluated protocols for IntraGate MRI and µCT at different time points post infection (see figure 1). After optimization of quantification protocols, MRI and CT images were quantified (total lung volume, aerated lung volume ...) and the data correlated for cross-validation. Cryptococcal invasion of the lung and brain (meningitis) was confirmed by histochemical analysis (see figure 1, last panel), lung fungal load was quantified and compared with CT and MRI data.

Figure 1: Imaging cryptococcal lung infection with MRI and CT. MRI (first column), µCT (second column) and histology (PAS-staining, third column) images of a mouse 6 weeks post instillation of a Cryptococcus suspension (first row) or PBS (second row). White arrows point at cryptococci in the lung tissue.

Conclusions &Perspectives
To the best of our knowledge, this is the first study showing that non-invasive monitoring of pneumonal cryptococcosis is feasible with retrospectively gated µMRI (Intragate, Bruker) and µCT resulting in high resolution and contrast images. This imaging approach will allow longitudinal screening of animals, without radiotoxicity concerns when using the MRI, and with even better resolution when using the CT, thereby visualizing infection onset and progression on an individual basis and far before the appearance of any phenotypical signs of disease. We will further finetune the timing of disease onset and correlate this with the time of traversal of Cryptococcus cells to the CNS. MR imaging of cryptococcosis will greatly help unraveling the still enigmatic pathogenesis of this life-threatening disease.
References:
In vivo micro-computed tomography can visualize and quantify osteosarcoma primary tumor growth and pulmonary metastases over time.

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Aims
In osteosarcoma (OS), one important imaging tool to assess the extent of primary tumor growth, metastatic spread (mainly in the patients' lungs), and thus patient prognosis is computed tomography (CT). The earlier the metastases can be identified, the better the prognosis will be. In our laboratory, we established a method to visualize the presence of single OS tumor cells \textit{ex vivo}, by means of LacZ tagging\textsuperscript{1}. However, what exactly occurs during the course of the disease process remains largely unknown, since \textit{in vivo} visualization methods are not yet commonly employed in preclinical OS models. We therefore tested if \textit{in vivo} micro-CT can be used to monitor the growth of both the primary tumor as well as the pulmonary metastases in a preclinical OS model.

Method
Female SCID mice received an intratibial injection with LacZ-tagged osteoblastic SAOS-2 or osteolytic 143B OS cells. After growth of the primary tumor, mice were anesthetized and scanned in the Skyscan 1176 \textit{in vivo} microtomography system using the 35 µm setting (50 kV, 500 µA, 0.5 mm Alu filter, 0.5 degrees rotation angle, 80 ms exposure time, frame averaging 4, 180 degrees scan). Two separate scans were made, one of the chest and one of the hind limbs. Scan duration per scan was 8 minutes, with a dose of ~0.5 Gy. After two weeks, the scans were repeated. To verify the presence of the tumor nodules, mice were sacrificed immediately after the second scan, and their lungs were excised, X-Gal stained, air-dried, and scanned again using the 9 µm setting (30 kV, 415 µA, no filter, 0.3 degrees rotation angle, 1888 ms exposure time, frame averaging 4, 360 degrees scan, scan duration 3 hrs).

Results
In mice injected with SAOS-2 cells, mineralized foci could be observed inside the primary tumor mass, as well as in the pulmonary metastases (figure 1). The size of the smallest detectable metastasis was 0.5 mm. In the follow up scan two weeks after, the mineralized foci became more pronounced.

In 143B tumors, bone destruction at the proximal tibia could be visualized in detail. X-Gal stained and re-scanned lungs showed a perfect match between LacZ presence and micro-CT-detected metastatic sites \textit{ex vivo}.
Figure 1: Left panel: In vivo follow up of metastasizing osteoblastic SAOS-2 osteosarcoma cells in the proximal tibia (top) and in the lungs (bottom). Lung metastases are not observed in the first scan, but become apparent in the second follow up scan taken two weeks after the initial scan. Right panel: Both ex vivo microCT analysis and en bloc staining for LacZ presence (not shown) confirmed that the tissue spot shown in the in vivo scans was indeed a tumor nodule. L=left.

Conclusion
Micro-computed tomography can be used to monitor both primary and distal OS tumor growth in vivo, and reveals detailed 3D information of micro-metastasis distribution ex vivo. Future challenges will be to increase the contrast between tumor tissue and normal tissues, e.g. by using gold-labeled antibodies directed against specific tumor markers, and thus to be able to identify and monitor even smaller metastases over time.

References:
Detection of Lung Metastasis of Cancer Cells in Mice Using High Resolution Micro Computed Tomography

Tzu-Hung Lin, Chia-Hung Chien, Houng-Chi Liou, Wen-Mei Fu

Aims
For cancer research and chemotherapeutic drug development, non-invasive detection of the progress of cancer lung metastasis in live animal is needed. In this study, we compared wild-type (WT) mice and a certain gene knockout (KO) mice, which we supposed to be easier for the metastasis of cancer cells into lung tissue by using micro-CT.

Method
Animal and Cell culture
Female C57BL/6 mice (WT) or a certain gene KO mice were aged 5 to 6 weeks, weighing 20-25g. They were provided free access to food and water at room temperature. In cell culture, LL/2 murine lung carcinoma cells were maintained in the humidified incubator (5% CO2, 37°C) in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Invitrogen, Carlsbad, CA). To compare the metastasis of WT mice with that of knockout mice, LL/2 cells (6 × 10⁴ cells / mL) were injected into the femoral vein of mice. Two-Three weeks later, the mice were subjected to micro-CT scanning and then sacrificed for the count of lung nodules.

Micro-CT imaging of mice
The mice were anesthetized with isoflurane and scanned by Skyscan 1176 (Kontich, Belgium) at 35 μm resolution (50 KeV, 497 μA, 0.5 mm aluminum filter, 50 ms exposure, 0.8 per image, image averaging = 2) with respiratory synergism (5 ms after respiratory signal). At the end point of study, mice were sacrificed by overdose of trichloroacetaldehyde (TCA) and rapidly tracheotomised. 1 ml of air was injected into lung and the sacrificed mice were then scanned.

Results
To monitor the tumor metastasis into lung tissue in wild-type (WT) mice and gene knockout (KO) mice, living mice were scanned by Skyscan 1176 at week-3 after intravenous cell injection. Micro-CT imaging showed that the metastasis of LL/2 lung carcinoma is more prominent in gene KO mice than in wild-type mice (Fig. 1A). It was also found that there were more tumor nodules on lung surface in KO mice than in wild-type mice (Fig 1B). To gain better resolution of images, mice were sacrificed and scanned again. As shown in Fig.2, a gene KO mouse with moderate cancer lung metastasis was scanned representatively. Two tumor colonies were found at the bottom of lung space (Fig. 2A). Fig. 2B (left colony) and Fig. 2C (right colony) showed coronal and sagittal images of these tumor. The whole lung pictures were shown in Fig. 2D.
Figure 1: Severe cancer lung metastasis in gene KO mice
(A) Micro-CT imaging (Coronal and sagittal) in living animals showed that the lung space of gene KO mice was critically occupied by tumor tissues and less severe metastasis was found in wild-type mice. (B) More tumor nodules were found on the surface of lung in gene KO mice. Scale bar: 1 mm.

Figure 2: Micro-CT images of mice after sacrifice
(A) The transaxial image revealed two tumor colonies at the bottom of lung space in gene KO mice. (B and C) Micro-CT images showed the phenotype of metastatic tumor. (D) Representative images photographed using dissecting microscope. V: ventral view, D: dorsal view.

Conclusion
For the detection and monitoring of the progress of lung metastasis in living animals, micro-CT can provide imaging for studies such as cancer research and anti-cancer drug development. Micro-CT is also suitable for monitoring the spontaneous lung cancer or lung metastasis of cancer cells which are not labeled with luciferase or fluorescent dyes.

References:
Animal positioning for high resolution *in vivo* bone scanning

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**Aims**

The Skyscan 1076 *in vivo* scanner allows high resolution scanning of mouse bone. For the analysis of mouse trabecular bone the maximum resolution of 9µm is required, as the lower 18 and 35 µm are not sufficient to properly image these structures which tend to have an average width of 40-60 µm. The best site for analysing trabecular bone in the mouse is the knee, as this is easily accessible and not surrounded by a large amount of soft tissue. For other types of analysis, lower resolution can be sufficient. We have successfully used 18µm scans to analyse relatively large bone lesions in an animal model of Paget’s disease of bone¹. As the exact site of the lesions can not be predicted in this model, it is essential to image as much of the leg as possible.

The main problem with *in vivo* scans is avoiding movement artefacts (see figure 1). our aim was to optimise methods for positioning and stabilising the mouse hind limbs for optimal *in vivo* scanning.

![Figure 1: Effects of leg movement during an in vivo scan on scan quality. Analysis of trabecular structure is not possible.](image)

**Method**

Mice were anaesthetised using isofluorane and scanned on a Skyscan 1076 *in vivo* µCT scanner. Scanner settings were 50 kV µA, 0.6° rotation angle, 0.5 mm Al filter. Several different beds and stabilisation methods were tested to obtain images with the least amount of movement. Scans were reconstructed using NRecon and analysed using CTAn and CTVol.

**Results**

To optimise the maximal amount of the leg to be imaged for the detection of Pagetic lesions, the legs were scanned in a bent position (Figure 2).
Figure 2: In vivo scanning of a mouse for maximal length scanning. The legs are in a bent position and the hind paws are supported by a block of Styrofoam. Next the knees are taped into position with masking tape. The imaging area includes both legs and the pelvic girdle.

This method of positioning allows both hind limbs to be imaged from the ankle to the hip joint with sufficient image quality at 18 \( \mu \)m to identify Pagetic lesions (Fig. 3).
Figure 3: In vivo scanning of a mouse: identification of lesions. An 18 µm in vivo scan was performed as described. A: This positioning allows for analysis of both hind legs and pelvic girdle in a single scan. B: Individual hind limb. C: A Pagetic lesion penetrating the cortex of the tibia (arrow).

For imaging the trabecular bone at the knee, it is imperative that movement is restricted to the absolute minimum. One of the sources of movement is abdominal breathing by the mice. To minimise this, the abdomen is taped fairly tightly, so that the mouse predominantly uses chest breathing (Fig. 4).

Figure 4: Positioning and stabilising the knee for high resolution In vivo scanning. The legs are straightened out and supported by a block of styrofoam below the knee. The feet and knees are fixed into position with masking tape. To minimise breathing movement, the abdomen is taped.
Using the method described above, we can reproducibly obtain good quality analysable scans (Fig. 5). The next step in the analysis is to find a good threshold to identify the bone. For mouse in vivo scans this can be slightly more difficult than in high resolution ex vivo scans, because of the limited resolution and slightly higher noise level. Standard global thresholding can be problematic, as too high a threshold will not identify thin structures, while a lower threshold will over emphasize the thickness of the structure (see fig 5). Adaptive thresholding tends to give better results in this respect. The thresholding method chosen will have a substantial effect on the values of the eventual 3D analysis.

As table 1 shows, at the same lower threshold of 45, Global thresholding results in a % bone volume of 34%, while two different adaptive threshold methods result in values of 28% and 23%. As can be seen from the table, this is mostly due to a much larger estimate of trabecular thickness (Tb.Th). Table 1 also shows that the values for trabecular pattern factor (Tb.Pf) and connectivity density (Conn.Dn) are extremely sensitive to the thresholding strategy.
Table 1. Effect of thresholding method on measurements. T1=lower threshold; upper threshold at 255 for all methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>T1</th>
<th>%</th>
<th>µm</th>
<th>µm</th>
<th>mm⁻¹</th>
<th>mm⁻¹</th>
<th>mm⁻³</th>
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<tr>
<td>Global</td>
<td>55</td>
<td>24.10</td>
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<td>271.15</td>
<td>2.72</td>
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<tr>
<td>Global</td>
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<td>34.48</td>
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<td>73.64</td>
<td>214.34</td>
<td>3.07</td>
<td>14.12</td>
<td>98.37</td>
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</tbody>
</table>

Conclusion

Good quality high resolution *in vivo* scans of mouse long bones can be obtained. However, proper positioning and stabilisation of the legs is essential to avoid movement artefacts. Controlling abdominal breathing can greatly improve the final quality of the scan.

References:

Monitoring anticancer therapies against lung tumor in experimental models using X-ray Micro Computed Tomography

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Background
Three-dimensional micro Computed Tomography (µCT) offers the opportunity to capture non-invasively images of lung structures and lesions in mice with a high spatial resolution allowing for accurate calculation of lung lesion volume. Longitudinal imaging overcomes the limitation of single time-point imaging because it enables tracking of the natural history of disease and provides quantitative assessments of the effects of an intervention in every single mouse.

ALK (Anaplastic Lymphoma Kinase) is a transmembrane protein and a member of the family of insulin receptor tyrosine kinases. Oncogenic fusion proteins harbouring the ALK kinase domain have been identified in different cancer types. In lung cancer, the most frequent chimeric protein involves fusion of the ALK kinase domain at the C-terminus to part of the EML4 (echinoderm microtubule-associated protein-like 4) protein at the N-terminus in approximately 3-13% of human non-small cell lung cancers (NSCLC). The pivotal role of EML4-ALK in the carcinogenesis of NSCLC was demonstrated in experimental models such as the transgenic mouse model described by Soda, expressing EML4-ALK specifically in lung alveolar epithelial cells under the control of the Surfactant Protein C (SPC) promoter (1). A similar transgenic model was developed internally using the lung specific Clara Cell Secretory Protein (CCSP) promoter.

A dual ALK/c-Met inhibitor (Crizotinib Xalkori®) was approved by FDA in September 2011 for non-small cell lung cancers (NSCLC) expressing ALK. Escape from Crizotinib treatment linked to mutations in the ALK kinase domain has been reported (2) and novel second generation Alk inhibitors active on Crizotinib resistant cancer are under development. Here we report the evaluation of an ALK inhibitor (hereafter compound-A) in comparison with Crizotinib, in the CCSP-EML4-ALK-TG16 transgenic mouse model using µCT.

Method
73 CCSP-EML4-ALK-TG16 transgenic mice were received at 6 weeks of age. For baseline imaging, mice were imaged twice at 1 week interval using µCT to identify growing lung tumor nodules. Three homogenous groups were randomized on the basis of the tumor size of one selected tumor nodule per mouse, and treated according to the following protocol:

<table>
<thead>
<tr>
<th>Group 1 (n=10)</th>
<th>Group 2 (n=10)</th>
<th>Group 3 (n=8)</th>
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<tr>
<td>Control</td>
<td>Compound-A</td>
<td>Crizotinib</td>
</tr>
<tr>
<td>µCT (Days 54, 61, 70, 77, 85)</td>
<td>µCT (Days 49-72)</td>
<td>µCT (Days 49-72)</td>
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<tr>
<td>Imaging</td>
<td>µCT (Days 49-72)</td>
<td>µCT (Days 49-72)</td>
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Mice were monitored longitudinally for 5 imaging sessions of 7 min each using the Skyscan 1076 ® scanner (35um, Ti 0.025mm, 72kV; 145uA, exposure 316ms, rot step 1°). During imaging, the mice were anesthetized with Aerane® (0.2%, O2:2l/min) and kept warm. Images
were reconstructed using NRecon® and Regions Of Interest (ROI) were manually drawn over the selected tumor nodule using the image analysis software CTAn®. Tumor volume was calculated by stacking 2-dimensional ROI. Statistical analysis was done on tumor volume from day 47 to day 77 using 2 way analysis of variance with repeated measures on one factor, with significance level set at p<0.05. Partial tumor Regression (PR) corresponds to reduction of 50% of the tumor size compared to baseline and Complete tumor Regression (CR) is declared when the tumor is no more visible on the scan.

Results
The figure below shows longitudinal μCT monitoring of the selected lung nodule in one representative mouse of control (top row) and compound-A treatment (bottom row) groups.

![Group Comparison](image)

Compound-A had a significant effect on tumor nodule volume vs control from day 54 (p=0.0098) to day 77 (p<.0001) with 96% tumor growth inhibition on day 77, 3/10 CR and 10/10 PR. Crizotinib had significant effect on tumor nodule volume vs control from day 61 (p=0.0191) to day 77 (p=0.0295) with 44% tumor growth inhibition on day 77, 0/10 CR and 3/10 PR. Compound-A was found significantly more active than Crizotinib on day 61 (p=0.013), day 70 (p=0.0025) and day 77 (p<.0001).

Conclusion
Longitudinal monitoring of lung tumor nodules using X-ray μCT in CCSP-EML4-ALK-TG16 transgenic mice developing ALK+ lung tumor nodules, revealed superior activity of the Alk Inhibitor compound-A compared to Crizotinib at 50 mg/kg given daily.

References:
Gold nanoparticle contrast agents for microCT applications

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Aims
Gold is an excellent material for generating X-ray contrast: it has an adsorption cross-section \( \sim 3 \) times that of iodine (at 20 and 100 keV),\(^{1,2} \) combined with a well-characterized chemistry that includes the formation of highly stable, biocompatible nanoparticles in a choice of sizes\(^3 \) that allows control over the biodistribution, blood pool lifetime and clearance pathway when used as injectable contrast agents. Furthermore, synthetic modification affords extraordinary solubility, very low toxicity, and conjugation to targeting agents for microCT imaging of specific organs or systems.\(^4,5 \)

Our goals are the development of gold nanoparticle contrast agents with extended residence times for blood pool imaging, agents for selectively imaging specific organs and systems, such as the kidney, and contrast agents targeted to biomarkers for selectively imaging tumors and other biological structures.

Method
Gold nanoparticles may be prepared in high yield in a choice of sizes. For this work, two sizes were prepared, in order to provide different blood pool residence characteristics: 1.9 nm, and 15 nm. These were stabilized with a layer of organic ligands structured to confer high stability combined with high solubility and minimal toxicity. Both sizes of particles were administered to mice via tail vein injection at a variety of concentrations.

To investigate targeting, the \( \text{HER2} \) oncoprotein, which is an important marker for aggressive malignant behavior in breast cancer,\(^6 \) was used as a test system, and gold particles were targeted using a monoclonal antibody against the \( \text{HER2} \) oncoprotein (Herceptin, Genentech). Mice were implanted with \( \text{HER2} \)-negative and \( \text{HER2} \)-positive tumors in opposite thighs. 15 nm particles were conjugated to antibody IgG molecules via the introduction of reactive functionalities after particle synthesis; these reactive particles were incubated with a monoclonal IgG antibody against the \( \text{HER2} \) oncoprotein (Herceptin), and conjugates were separated by centrifugation, pelleting and resuspension.

15 nm gold particles conjugated to Herceptin (monoclonal antibody against \( \text{HER2} \) oncoprotein) were then injected and the regions bearing the tumors were imaged by microCT.

Results
1.9 nm (AuroVist™-1.9 nm) and 15 nm (AuroVist™-15 nm) gold nanoparticles were used in mice for imaging of the circulatory system. The 1.9 nm particles, which are cleared through the kidneys, can be followed as they pass from the blood through the kidneys, and ultimately into the bladder\(^7 \); this process is shown in Figure 1. These particles provide clear imaging of kidney fine structure about one hour after injection (Figure 1).
Both 1.9 nm and 15 nm also showed preferential accumulation in tumors, which was attributed to angiogenesis producing changes in permeability within tumors which allowed the gold particles to leave the blood vessels and accumulate in the tumor. Brain tumors could therefore be imaged (Figure 2).

Figure 2: Brain tumor in live mouse imaged using AuroVist™-15 nm particles.

The long residence times and high contrast obtained using AuroVist™-15 nm allow high resolution imaging over extended times up to hours, and this enables the clear delineation of blood vessels as small as 25 microns (Figure 3).
Figure 3: MicroCT of blood vessels in the legs and pelvic region of mouse after intravenous injection of AuroVist™-15 nm particles. MicroCT scanned for 3.5 hours at 41 kVp.

Herceptin-conjugated 15 nm gold particles, when injected into mice bearing HER2-positive and HER2-negative tumors, showed significantly greater uptake in the HER2-positive tumors (Figure 4).

Figure 4: MicroCT volume images from a mouse with HER2-negative (bottom) and HER2-positive (top) tumors implanted in opposite thighs. Imaged 20 h after injection of Herceptin-conjugated AuroVist™-15 nm particles. The tumor is 1.5 mm thick.

This new contrast agent can also be used to enhance x-ray dose to tumors\textsuperscript{8,9}.

**Conclusion**

Gold nanoparticles are a versatile contrast agent with several important advantages for microCT applications. With appropriate stabilization, they can achieve very high concentrations of up to 1.5 g/mL. When combined with their high X-ray cross-sections, this allows contrast up to ten times that available with iodine reagents. Their very low viscosity and osmolality allow injection into smaller blood vessels than conventional iodine reagents,
enabling local administration to specific organs under study. Control of particle size allows the blood pool residence to be adjusted for optimum microCT imaging, and particles of specific sizes can be used for high-resolution imaging of specific organs through which they clear, such as the kidney. In addition, we demonstrate that gold nanoparticles may be conjugated to antibodies to provide targeted imaging of cancers.

References:


Can combined CT and TOF-MRI assist in neuro-anatomical surgery planning in small animal models?

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\textbf{Aims}

Deep brain stimulation (DBS) for neurological and psychiatric disorders like Parkinson’s disease or major depression disorder requires the implantation of electrodes for the application of electrical pulses in deep brain anatomical locations. For such procedures, pre-operative or intra-operative multi-modality brain magnetic resonance imaging (MRI) or computed tomography angiography (CTA) images of the individual patient are extensively investigated to define the optimal trajectory for electrode insertion to avoid vasculature and functionally important brain areas. Unlike DBS in humans, planning of brain interventions in preclinical rodent models is typically restricted to defining the target and entry points in a generalized anatomical small animal brain atlas\textsuperscript{1} and transforming these onto the individual animal using a stereotactic reference frame. As current atlases provide limited or no blood vessel information, the outcome of neurosurgical small animal model experiments could be deleteriously influenced when a sub-optimal electrode trajectory ruptures the cerebral vasculature resulting in severe systemic effects. However, the feasibility of individual pre-operative imaging-based surgical path planning in animal studies is limited. Therefore, we aim to build a stereotactic (probabilistic) atlas based on anatomical (CT, MRI) and cerebral vasculature (TOF-MRI, CTA) information that can be used for neurosurgical planning (e.g. electrode implantation), without requiring the acquisition of vasculature and anatomical reference images for each individual animal. Here, we validate vasculature information from TOF-MRI with CT(A) and assess the intra-strain variability in skull reference points and cerebral vasculature for neurosurgery planning and subsequent (probabilistic) atlas building. Using this atlas, we aim to evaluate the risk of a user defined electrode trajectory damaging a blood vessel on its path. The use of such a method will be readily applicable to DBS in small animal models and also to a wide range of stereotactic surgeries like targeted injection of viral vectors, contrast agents, cells for the creation of neural disease models and \textit{in situ} cell labeling applications.

\textbf{Method}

\textit{In vivo} 3D anatomical MR brain images and 2D multi-slice MR angiography (MRA) time-of-flight cerebral angiography images (FLASH-TOF, isotropic resolution of 195 \(\mu\)m) were acquired for 10 male Wistar rats in a 9.4T Bruker small animal MRI scanner. \textit{In vivo} and \textit{ex vivo} CT images of the full rat head were acquired on a SkyScan1076 small animal CT scanner. \textit{In vivo} CT images of the full rat skull were acquired from isoflurane gas-anesthetized rats (3% for induction, 1.8 % for maintenance), positioned with bregma in the center of the FOV, using the following parameters: 35 \(\mu\)m isotropic resolution, 49 kV source voltage, 200 \(\mu\)A source current, 0.5 mm Al filter, 180 ms exposure time, 0.8\(^\circ\) rotation step, 2 averages, 2 connected scans to cover the complete rat skull length (this is important to include enough landmarks for coregistration such as lambda, bregma, nasal suture,\ldots). After the last \textit{in vivo} imaging time point, rats were sacrificed by administration of an overdose of anaesthesia (\textit{i.e.} nembutal, to which 20% heparine was added to avoid blood clotting) and
transcardially perfused, first with heparinized saline to flush the blood away, than with paraformaldehyde (4% in PBS) to fix the tissue, followed by a saline flush and in a last step with 30% BaSO₄ (in 2% gelatine) as a blood pool contrast agent for ex vivo CTA. We are currently evaluating a second perfusion protocol that would allow us to visualize the vessel tree on histological tissue sections. To this end, the BaSO₄ in the final perfusion step is mixed with liquid latex and waterproof black drawing ink. Ex vivo CTA images were acquired from the packed specimen with the following parameters: 35 µm isotropic resolution, 100 kV source voltage, 100 µA source current, 1 mm Al filter, 220 ms integration time, 0.7° rotation step, 3 averages, 2 connected scans to cover the complete rat skull length.

We used an in-house developed image analysis pipeline for image pre-processing (e.g. RF intensity inhomogeneity correction) and spatial normalization of MR and CT anatomical images, MRA and CTA vasculature images and reference atlas template images. A vasculature average image was constructed in atlas space (figure 1). We use ex vivo CTA for validation of the MRA-TOF. For the planning of stereotactic surgery, the targets are visualized in the Paxinos-MR template along with the multi-modality information of MR/CT anatomical and MRA images normalized to atlas space. The risk of the electrode damaging the vasculature is computed by representing vessels and electrodes in terms of Euclidean distance maps. A 2D automatic ray casting approach with potential trajectories radiating from the target point towards the skull is presented with associated information on the vasculature along each path. The coordinates of entry point, the angle of entry and the depth of incision along with the associated risk (maximum intensity and averaged sum-of-pixel intensities along trajectory) are presented to the user (figure 1, E).

Results
The information from multi-modality (MRI, CT and atlas) images of anatomy, vasculature and stereotactic coordinates was combined to realize an optimal 3D planning for stereotactic neurosurgery in rodents (figure 1).

Larger vessels are consistently visualized in all the TOF-MRI images that were processed and their geometric location is nearly identical. Depending on minor modifications in the animal position within the MRI scanner, some sections of the vessel tree were less visible for some animals compared to the others. An average vasculature template has been constructed from the MRA-TOF images in the atlas space. The CT data serves as ground truth for validation of this vasculature atlas (ex vivo CTA) and for evaluation of the variability of bregma (in vivo and ex vivo CT). Visual comparison of the MRA-TOF vasculature (major vessels) in individual animals indicates minimal variability. This could indicate the feasibility to use the vasculature template as a representative of the population for more precise planning of stereotactic surgeries like the DBS application. To validate this objective, we used the multi-modality information to plan stereotactic surgery using a potential risk path and an estimated safe trajectory (figure 1, E).

From these experiments we know that for optimal planning of stereotactic surgery (e.g. electrode implantation), coregistration of MRI/MRA images with CT data on bregma and lambda reference points on the skull is highly important. Therefore, information (CT) on the variability of bregma will be combined with the vasculature data in the atlas.
**Figure 1:** (A) Coregistration between MRI (top), MRA (middle) and MRI-MRA (bottom, red overlay). (B) 3D volume rendering of MRA images of the same 2 animals. Arrows indicate regions of similarity (pink) and variability (blue). (C) CT images providing information on bregma and lambda reference points and vasculature for validation of MRA. (D) Surgery planning in 2D (Paxinos) atlas space with possible trajectories at different angles. (E, a-c) Average vasculature images in atlas (Paxinos) space: the maximum-intensity MRA-TOF vasculature information constructed from 4 consecutive slices at the same location, with * marking the green target region. (d) Risk assessment: the averaged sum-of-pixel intensities along each of the trajectories determine the associated risk of the electrode traversing through hyperintense pixels (vasculature in TOF-MRI). Based on the averaged sum-of-pixel intensities along each of the trajectories, the paths are color-coded: high (red) - medium (cyan) – lower risk (green) (e).

**Conclusion**

For pre-clinical stereotactic surgeries, optimal trajectory planning is valuable input to avoid injuring vasculature. We address this issue by investigating the intra-strain variability in cerebral vasculature combined with the position of bregma for the Wistar rat strain. Provided that the intra-strain variability is small, a probabilistic vasculature atlas for the given strain could form a reference for brain surgery planning. We use image registration to spatially align multi-modal anatomical and vasculature information from age and weight matched animals from the same strain to the common reference frame in a standard atlas space. Excluding the acquisition effects, we observe consistency with vasculature for the test group. We demonstrate the methodology for building a vasculature template and subsequent use for planning neuromodulation experiments. We are currently extending this study with more animals, where validation of the proposed safe and risk paths through stereotactic surgeries, together with validation with CTA using contrast agents, are pursued not only for electrode insertion but also for the injection of cells and viral vectors.
References:

Quantitative multi-pinhole small-animal SPECT with U-SPECT-II/CT

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\textbf{Aims}

Small-animal SPECT plays an important role in biomedical research. Accurate and reliable quantitative imaging with small animal SPECT can help scientists with understanding the behavior of organs, tissues and pharmaceutical in vivo, which is a good motivation for developing accurate correction methods for photon attenuation. Previous phantom assessments\textsuperscript{1,2} show that photon attenuation reduces the measured activity concentration in the center of rat-sized and mouse-sized phantoms by up to 25\% and 18\% with Tc-99m, respectively. These studies lead to the conclusion that the attenuation compensation, together with correction for other image degrading effects, is required in order to consistently achieve accurate quantitative small-animal SPECT images. We here propose a CT-based modified non-uniform Chang\textsuperscript{3} method for attenuation correction and evaluate this method with phantom studies in a U-SPECT-II/CT system (with an integrated Skyscan 1178 single-source system)\textsuperscript{4}. The necessary information for calculating attenuation correction maps was derived from registered X-ray CT images of the phantom.

\textbf{Method}

First a SPECT scan was made in the U-SPECT-II/CT system of a small drop of a solution containing 57.2 MBq Tc-99m to obtain the calibration factor of the SPECT system. Later on a 30-mm NEMA-small-animal phantom filled with 8.66 MBq/ml Tc-99m solution was scanned with both the SPECT and CT modalities. List-mode SPECT data were acquired and a scatter-corrected SPECT image was reconstructed by using the pixel-based ordered subset expectation maximization (POSEM\textsuperscript{5}) algorithm combined with a triple-energy-window (TEW\textsuperscript{6})-based scatter correction. A CT image was also reconstructed in Hounsfield unit (HU) and registered to the SPECT image.

The attenuation coefficient ($\mu$) at the location of each voxel was derived from the registered CT image, by employing a linear scaling of the HU numbers:

$$\mu = \mu_0 \left( \frac{\text{HU}}{1000} + 1 \right),$$

where $\mu_0$ is the attenuation coefficient associated with water and the energy of the photons used in SPECT. In the case of Tc-99m studies, $\mu_0$ equals 0.151 cm\(^{-1}\).

The amount of attenuation was quantified by the transmitted fraction (TF) which is the ratio of detected counts with attenuation to the counts in an ideal attenuation-free situation. In the modified non-uniform Chang\textsuperscript{3} method, the overall TF of each voxel in a SPECT image is
treated as the average of TFs along different projection trajectories starting from that voxel. Along each projection line, the TF is simply computed as the line integral of the attenuation coefficient on that line. The calculation above is represented as the following equation:

$$\text{TF} = \frac{1}{M} \sum_{m=1}^{M} \exp \left( -\int_{L_m} \mu(l) \, dl \right),$$

in which M is the number of projections in acquisition for a certain voxel, $L_m$ denotes the m-th projection path of gamma photons, and $\mu(l)$ is the attenuation coefficient as a function of location l on that projection line $L_m$.

With the scatter-corrected image voxels (SC), the calibration factor (CF) and the overall transmitted fraction (TF), the attenuation-compensated image (AC) was given by:

$$AC = \frac{SC \cdot CF}{TF}.$$ 

**Results**

Figure 1(a) shows uniform and non-uniform slices of the NEMA phantom from un-corrected and attenuation-corrected SPECT images. Figure 1(b) shows their corresponded CT slices, and the derived attenuation correction maps (slices of transmitted fraction values) are illustrated in Figure 1(c).

![Figure 1](image)

Figure 1: Slices and attenuation correction maps. (a) un-corrected (UC) and attenuation-corrected (AC) SPECT slices. (b) CT slices. (c) attenuation correction map derived from the CT slices.

The transmitted fraction values in the attenuation correction maps demonstrated about 20~23% attenuation in the center area of the phantom, which is consistent with literature\(^1-^2\), and indicating that the attenuation correction is important for quantification even in small-animal SPECT. The quantitative errors are visualized more clearly by using line profiles in Figure 2. The average quantification error of the entire phantom volume was $-16.2\%$ without attenuation correction, and was reduced to 4.7% with attenuation correction.
Figure 2: Line profiles in (a) uniform slice and (b) non-uniform slice. NC: without attenuation correction. AC: with attenuation correction. GS: gold standard obtained with dose calibrator.

**Conclusion**

The effects of attenuation in small-animal SPECT can be corrected by using the CT-based non-uniform Chang method in the U-SPECT-II/CT system, with a good accuracy of less than 5% error on average.

**References:**

The Development of Micro-CT for Imaging and Measuring Experimental Pulmonary Fibrosis

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\textbf{Aims}

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive lung disease for which there is currently no effective treatment. After instillation of Bleomycin into the mouse lung fibrosis develops, which models pulmonary fibrosis, and may be used to test new therapies. The ability to view and measure structures in 3D throughout the lung, and the use of CT analysis in the clinic, has led us to test micro-CT analysis of fibrosis in this model, as an alternative to histological or biochemical measurement of collagen deposition.

\textbf{Method}

Bleomycin or saline was instilled into the mouse lung through either the intra-tracheal or oropharyngeal routes. Four days to 3 months later small groups of 1-3 mice were killed at different timepoints and the lungs prepared for ex-vivo micro-CT scanning.

The lungs were fixed by inflation with paraformaldehyde fixative. They were then dehydrated and chemically dried for micro-CT scanning using a method kindly given to us by Jeroen Hostens, SkyScan. The lungs were enclosed in a tightly fitting expanded polystyrene plastic container and scanned in the SkyScan 1072 scanner at 40kV 100mA without filtration and with 11\textmu m voxel resolution, and 1,100 sections per lung were reconstructed with the SkyScan NRecon software.

For the analysis of the micro-CT sections we tested a new method using pattern recognition to identify different structures within the images without having to define regions of interest. Inform software (CRI Inc.) was trained to recognize fibrotic and normal lung parenchyma, large airways and section background, by defining a few training regions in 3 sections from each lung. After checking the accuracy of the segmentation, the trained software was used to automatically find and measure the volume and density of fibrotic and normal lung tissue throughout all the sections of all lungs in the study.

\textbf{Results}

The fibrotic lung showed large regions of increased density around bronchioles (arrowed in the example micro-CT sections shown below) and in the peripheral lung parenchyma, and these dense regions correlated well with mature collagen staining by histology. They often also contained enlarged airspaces, particularly at 3 months after Bleomycin. In contrast the only dense structures found in the saline control lung were airway walls and vessels.
Example micro-CT sections at mid-lung level, 21 days after Bleomycin or saline.

3D reconstructions of the lung were made using CTvox from the micro-CT sections and were rotated in order to show the localisation of fibrosis. (examples shown: 21 days after Bleomycin). The 3D images show fibrosis located mainly in the central lung bordering the larger airways with smaller patches of fibrosis in the periphery of the lung, which models the focal distribution found in the clinic.
Measurements of relative volume of dense fibrotic lung tissue taken at different times after Bleomycin instillation into the lung (above) showed that fibrosis developed rapidly and persists through 3 months after bleomycin in 20% of the total lung volume, without resolution, in contrast to the common perception of this as a resolving model. A large window of measurement was found between the bleomycin and control lungs at the 21 days and 3 months timepoints.

**Conclusion**

These initial pilot studies showed that micro-CT followed by pattern recognition is a good method for imaging and objective measurement of fibrosis. The micro-CT analysis of fibrosis has been so successful that we have been able to justify the purchase of an in-vivo micro-CT scanner which will refine the experiments by allowing longitudinal studies of the effects of therapy and reduce animal numbers. Experiments are now underway using micro-CT to measure the effects of therapeutic compounds and also to compare micro-CT measurements with those made by the gold standard hydroxyproline assay for collagen.
Micro-CT analysis of human lung presenting Idiopathic Pulmonary Fibrosis

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Aims
Idiopathic Pulmonary Fibrosis (IPF) is a fibrotic lung disease, characterized by progressive heterozygous fibrosis leading to architectural deformation of the parenchyma. Patients have a restrictive decline of pulmonary function with a survival prognosis of 50% 3-5 years after diagnosis with no adequate therapy available yet. Because diagnosis is very complex we want to use micro-CT as a bridge between radiologic and histological data and gain knowledge on the impact of this disorder on the airways and alveolar tissue.

Method
When endstage IPF patients underwent a lung transplantation, the explant lung was inflated up to total lung capacity at 30 cm H2O pressure and frozen at -192°C. The frozen lung was cut in 2cm slices with a bandsaw and cylinders of 1.5 cm were pinched with a hammer drill. As control, an unused donor lung has been processed in the same manner.

These frozen samples were fixed with glutaraldehyde-aceton, dehydrated with ethanol and hexamethyldisalazane followed by air drying. Tissue was scanned with a resolution of 8.4 µm in the SkyScan 1072 scanner at 40kV 250mA without filtration. Raw data was reconstructed with the SkyScan Nrecon software. Analysis was performed with SkyScan CTan software.

Results
From one explant IPF lung 2 regions were selected (Fig. 1 B and C), which represent an early and late stage of architectural deformation in end stage IPF. These regions were compared to control (Fig. 1 A).

Fig. 1 Reconstructed micro-CT image of A) Control, B) IPF early stage and C) IPF late stage
Fig. 2 Representation of Total Tissue or Alveolar Tissue over Total Volume

An increase of total tissue per volume was found for both IPF in early stage and late stage compared to control (Fig 2). Surprisingly there was almost no difference between IPF early stage and IPF late stage sample in total tissue. When specifically looking at alveolar tissue volume, almost no decrease was found in the control. In early stage IPF however there was a slight decrease, which was even more pronounced in late stage IPF.

Fig. 3 Distribution curves of A) Structure Thickness and B) Structure Separation
The distribution of the structure thickness of tissue (Fig 3A) showed a similar curve for Control (average of 80µm) and early stage IPF (average of 102 µm). For late stage IPF the curve has shifted to the right (average of 151 µm). Tissue from early stage IPF (average of 218 µm) showed an increased distribution of tissue with small structure separation, while the distribution curve from late stage IPF (average of 551µm) spread more towards higher separation with values higher then 900µm when compared to control (average of 328µm) (Fig 3B).

**Conclusion**
As expected on basis of histology we found an increase of tissue volume in samples with early and late stage IPF. Endstage IPF is heterozygous in its manifestation and shows different stages of fibrosis spread over the parenchyma. In early stages of fibrosis there is an increase of tissue with a slight decrease of alveoli. Due to neighboring fibrosis, alveoli in an early stage of IPF may be compressed resulting in a lower structure separation distribution compared to control. Fibrosis starts to form as is visible by looking at structure thickness.

In late stage fibrosis the parenchyma is severely deformed, resulting in decreased alveolar tissue. This is also measured by increase of structure thickness and an increase of structure separation as the alveoli are presumably disappeared by collapse and fibrosis with traction on the airways.

These preliminary data showed the possibility to use micro-CT imaging as a crossover between histology and conventional radiology. Being able to asses our samples in 3 dimensions brings more information of the ultrastructure of the airways and parenchyma.
In vivo vs. ex vivo scanning in rat osteoporotic model

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Aims
Osteoporosis is a disease characterized by low bone mass and loss of bone tissue which advances through time. The importance of microarchitecture in the pathogenesis of bone fragility must be recognized to follow this process µCT scans in different time points need to be performed. In this experiment we wanted to explore end point of ex vivo analysis compared with data obtained from in vivo scans which will be useful for further analysis.

Method
Female Sprague-Dawley rats (four months old, weighing around 300g) were assigned in two experimental groups: SHAM - ovaries were exteriorized but remained intact (n=10), and OVX - bilaterally ovariectomized rats (n=10). Animals were anesthetized with an intraperitoneal injection of thiopenthal at doses of 4 mg/kg body weight. After operation animals were left without therapy for a period of 8 weeks for development of osteoporosis.

After 8 weeks distal rat femurs were scanned in vivo and following the termination of experiment we performed ex vivo µCT scanning.

Trabecular bone analysis was performed in CTAn software using bridge between growth plates as a reference point with 100 slices offset and 250 slices of region of interest¹.

Statistical analysis was performed with one way ANOVA.

Results
The lack of estrogen and progesterone in OVX rats leads to progressive trabecular bone loss in distal femur² which was evident when we compared trabecular bone analysis between SHAM and OVX groups. During eight weeks ovariectomized animals lost 75% of trabecular bone volume. Trabecular thickness and number also decreased in the same manner (Figure 1A,B.).
Figure 1. Parameter indicating bone loss in OVX animals. No difference in tissue volume (TV), profound loss of bone volume (BV) and bone volume percentage (BV/TV) is observed in OVX animals (A). Trabecular thickness (Tb.Th) and trabecular number (Tb.No) decreased while trabecular separation (Tb.Sp) values increased (B).

To compare results from in vivo and ex vivo scans we had to align the in vivo scans due to non ideal position of the femur during scanning. Observing the cross sections from ex vivo, in vivo and aligned in vivo scan we saw that in vivo scans are elongated and do not have a proper shape which was the reason why the alignment was done (Figure 2.).

Figure 2. µCT cross sections compared for SHAM and OVX groups for ex vivo, in vivo and in vivo scans aligned by Data Viewer software.

Comparing the data for the SHAM group we saw very similar values of measured parameters for ex vivo and aligned scans in vivo (Figure 3A). In OVX group the values varied slightly more between ex vivo and aligned in vivo data (Figure 3B). Trabecular parameters showed expanding range of variation.
Figure 3. Data comparison between ex vivo and aligned in vivo scans. Comparison between SHAM group data (A, B) and OVX group data (C, D).

Conclusion

The factors of limitation in in vivo scanning were determined with animal movement despite anesthesia and the surrounding tissue which have negative effect on measuring delicate structures such as trabecular bone.

Bone volume (BV) and bone volume percentage (BV/TV) data can be useful for comparison of SHAM and OVX groups ex vivo and aligned in vivo data because variation were small without statistical significance.

Trabecular parameters like trabecular thickness (Tb.Th) and trabecular number (Tb.No) are more sensitive so variations between same groups were higher and statistically significant.

MicroCT offers the unique possibility to visualize in 3D the microarchitectural changes occurring in the various types of osteoporosis. Our results showed that integrating data from ex vivo and in vivo scans is a possible but only for robust parameters such as bone volume data. More delicate parameters like trabecular thickness and number are not reliable due to different values obtained.

References:

1. P. Salmon - Method for ex-vivo micro-CT analysis of mouse bone (proximal tibia, distal femur), SkyScan
Phenotyping the fetoplacental vasculature of a pre-pregnancy smoking mouse model using micro-computed tomography and automated vascular segmentation

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Aims
Maternal cigarette smoking is associated with numerous reproductive abnormalities including placental disorders and low birth weight infants. The main toxicants found in cigarettes are a group of carcinogens known as polycyclic aromatic hydrocarbons (PAHs). Importantly, these toxicants can accumulate in adipose and mammary tissue and therefore can slowly be released into the blood stream during pregnancy, even when a woman ceases smoking upon learning she is pregnant. In a mouse model, prepregnancy injections of PAHs lead to growth restricted fetuses and reduced surface area and volume of the fetal arterial vasculature of the placentas. The purpose of this study was to apply advances in micro-computed tomography (micro-CT) imaging and together with vascular segmentation analysis to phenotype the branching pattern of the fetoplacental arterial tree and to quantify the effect of prepregnancy PAH exposure on this vasculature. We subsequently used these data to predict the influence of such changes on fetoplacental vasculature resistance.

Method
Two groups of C57BI6/J virgin female mice were randomly separated into vehicle control and PAH-treated groups. They were subcutaneously injected with vehicle (corn oil) or PAHs for a 9 week period at a total cumulative dose of 12 mg/kg, a dosage equivalent to \~7 cigarettes/day for 9 week in humans. Females were mated with no further injections given during pregnancy. At day 15.5 of gestation pregnant mice were sacrificed and the fetuses and placentas were removed from the uterus. A radio-opaque silicone rubber contrast agent (Microfil, Flow Tech, Carver, MA) was injected into umbilical artery using established methods \cite{1}. After being fixed in 10\% buffered formalin phosphate for 24-48 hours, the perfused specimens (n=10/group) were scanned using an eXplore Locus SP micro-CT scanner (GE Healthcare, London, ON, Canada). Three-dimensional (3D) datasets were acquired for each specimen with a voxel size of 13 microns. Individual isosurface rendering images of the arterial fetoplacental tree were generated using the Amira visualization package (Visage Imaging, San Diego, CA). Umbilical artery diameters and the span and depth of the fetoplacental arterial tree were measured directly from these surface renderings. We applied an automated vascular segmentation process that transformed the data into a tubular model for which diameters and lengths are known to identify vessel-like structures in the image \cite{2}. The process is illustrated in Fig. 1. Vascular resistance was calculated based on vessel architecture through use of standard formulas for pipe flow and for resistances in parallel and in series \cite{3}.
Fig. 1 Automated vascular segmentation methodology. A: Isointensity surface rendering of the fetoplacental arterial tree of a control specimen. B: Vessel center lines generated by the vascular segmentation algorithm. The initial seed was placed in the umbilical artery and tracked the vessels shown in white; subsequent seeds were placed in vessels visible in A but missed by the algorithm, these seeds yielded the vessels shown in red; a final group of seeds yielded the vessels shown in cyan. C: A tubular model of the data is generated for which diameter, length, and connectivity of each vessel segment is known.

Results
In comparison with the control group, PAH embryo weight was reduced by 23% (p<0.0001). There was a 27% decrease in the number of arteriole-sized (50-100 um) vessels (p<0.01). No change was observed in the number of large, chorionic plate vessels. However, PAH exposure increased curvature of the chorionic plate vessels as shown by a significantly increased tortuosity ratio of the tree (p=0.001) (Fig. 2). No changes were observed in the depth or span of the tree, the diameter scaling coefficient, or the segment length-to-diameter ratio. Arterial vascular resistance was increased by 30% (p=0.015). Assuming equal pressure in both groups, blood flow would be 19% lower in PAH-exposed placentas (p=0.01) (Fig. 3).

Fig. 2 Vascular tortuosity. A: Isosurface rendering of control specimen. B: PAH-exposed specimen, vessel tortuosity was quantified as the ratio of vascular path length (red line) to the beeline distance (green arrow) from the umbilical artery to each terminal vessel. C: A histogram of the vessel tortuosity ratio for controls (black bars) and PAH-exposed specimens (red bars) demonstrates a shift towards larger ratios in the PAH-treated group.
Fig. 3 Vascular hemodynamics. A: Total vascular resistance of the fetoplacental arterial tree of PAH-exposed (black bar) and control (gray bar) placentas. *p = 0.015. B and C: Isosurfaces were color rendered to illustrate blood flow magnitude in control (B) and PAH-treated (C) placentas. The trees of PAH-exposed placentas had lower flows throughout. This was noticeable at the terminal vessels, which display more blue coloring compared with controls. Scale bar = 1 mm.

**Conclusion**

We used Micro-CT technology together with automated vascular segmentation to detect quantitative differences between control and PAH-exposed fetoplacental vessels, including vascular tortuosity, branching pattern, and distribution of vessel diameters. Computational flow calculations were used to estimate vascular resistance. Our findings show that PAH-exposed mice had a significant reduction in the number of small diameter intraplacental vessels, but not in the large chorionic plate vessels. An increase in vascular tortuosity was also found in PAH-exposed vascular tree. These changes in vascular geometry were predicted to increase arterial vascular resistance by 30%, and decrease blood flow by 19%. Low flow may contribute to the 23% reduction observed in fetal weight.

**References**


Imaging of the Tumor Vascularization using µCT

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Aims
µCT provides spatial resolution in the micrometer range to image even the smallest capillaries with diameters of approximately 4 µm [1]. Because different types of soft tissue exhibit similar absorption leading to reduced density resolution in the tomograms, preparation protocols for contrast enhancement have been proposed [3]. One of the most powerful preparation procedures is the corrosion casting [2]. Such specimens provide perfect contrast for imaging and subsequent intensity-based segmentation. The tomographic data can be used to validate computer simulations of tumor angiogenesis to develop strategies against cancer [4].

Materials and Methods
C51 tumor cells were injected in mouse models (nude mice balb/c) and extracted after 7 days. The corrosion cast of the whole vascular system of the mice was performed at the Institute of Zoology, University of Zurich, Switzerland.

After anesthesia, the blood was exchanged by para-formaldehyde (PFA) 4% solution via the left heart ventricle to avoid vessel decomposition. Subsequently, the PFA solution was substituted by perfusing the mice with PU4ii resin and hardener-L [2], added to the resin just before injection. Blue ink was added to verify the perfusion process in situ. Two hours after injection, the vascular system was dissected. The mice were stored for five days at room temperature for final curing of the cast. The obtained casts are highly elastic and resistant against rupture. The tumors were extracted from the casts and coated with an osmium compound to increase their X-ray attenuation.

Tomography data of the tumors were acquired using a SkyScan™ 1174 (SkyScan, Kontich, Belgium) at an acceleration voltage of 35 kV and beam current of 800 µA. The elastic casts were fixed on the sample holder with wax pellets.

Data reconstruction was carried out with the NRecon software (SkyScan, Kontich, Belgium) using a modified Feldkamp algorithm. The reconstruction of the 1800 projections per scan produced volumetric datasets with 12 µm-wide isotropic voxels. Total acquisition and reconstruction time amounted to about 2 h.

The same specimens were measured at the beamline TOMCAT (SLS, Switzerland) with pixel sizes of 5.92 and 0.74 µm using photon energy of 15 keV. Because the tumor dimensions exceeded the available field of view, local tomography measurements were performed. The data were reconstructed using a filtered back-projection algorithm available at the beamline. The VGStudioMAX software (Volume Graphics GmbH, Heidelberg, Germany) was used for the 3D visualization of the tomography data. Because of the high contrast between elastomere (vessels) and air no additional filtering was necessary.

Results
The diameter of the smallest distinguishable vessels on the scans acquired with the SkyScan system was around 50 µm (see Fig. 1). The high resolution tomography setup at the
TOMCAT beamline permits to resolve vessels down to a diameter of 2.5 µm (see Fig. 2). Both kinds of tomography data clearly allow for the visual discrimination between healthy and cancerous tissues [4]. Many vessels in the cancerous section appear unshaped or twisted [5, 6], while they are better ordered in the healthy tissue. The casting elastomere penetrated into the cancerous tissue outside the vessels at several locations (Fig. 2), presumably as the result of vessel wall damages in the necrotic part of the tumor.

Figure 1: 3D image of the tumor vessel system measured with a SkyScan 1174 (a) using a detector pixel size of 12 µm and the synchrotron radiation source at the beamline TOMCAT (b) (SLS, Switzerland) using a detector pixel size of 5.92 µm.

Figure 2: Shows a cropped region from the tumor cast (highlighted in Fig. 1 (b)) measured at TOMCAT beamline using 5.92 µm (a) and 0.74 µm (b) detector pixel size.
Discussion and Conclusions
Both conventional and synchrotron radiation-based µCT allow visualizing the 3D structure of an appropriately prepared cast from the vascular network of tumors. The SkyScan™ 1174 scanner has the advantage of availability combined with relatively short acquisition time (below 1 h) and easy operation. The SkyScan 1174 offers availability for overview scans, where moderate spatial resolution is sufficient. For high-resolution imaging down to the smallest capillaries, synchrotron radiation sources are better suited. The improvement in the spatial resolution by almost 2 orders of magnitude, however, comes at the cost of a significantly increased acquisition time. The tomography data can be converted into vector-data format for quantifying the network bifurcations and vessel shapes. Although the vessel diameters are included in the tomography data, their exact diameter is not directly accessible, because the casting elastomere undergoes shrinkage during the curing process.

Acknowledgement
We kindly acknowledge Alexandra Ulmann and Eric P. Meyer for the corrosion cast of the vessels and Marco Stampanoni and Federica Marone for their support during data acquisition at TOMCAT.

References:
A *bona fide* model for age-related osteoporosis in accelerated aging trichothyiodystrophy (TTD) mice

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**Aims**

Osteoporosis typically manifests itself at old age. One potential mechanism by which aging can occur is through the accumulation of DNA damage, and premature aging can occur when repair of these damages is distorted [1]. By using DNA repair deficient mouse models as a tool, research in our laboratory focuses on the mechanism of aging and the etiology of aging related pathology. In this study, we used a mouse model that closely mimics the human premature aging syndrome trichothiodystrophy (TTD). Much like human TTD patients, these mice show accelerated onset and progression of age-related diseases [2]. The goal of this study was to assess if TTD mice can be used as a model for spontaneous, age-related osteoporosis that mimics the human situation more closely.

**Method**

From a cohort of 120 female wild type (WT) C57Bl/6 and 120 TTD animals groups of mice (n=10/group) were sacrificed at defined time points (13, 26, 39, 45, 52 and 65 weeks of age), followed by dissection of femur and tibia. Separate groups of TTD mice (aged 26-65 weeks) were injected with ALN and PTH, to investigate if these drugs would have the same bone preserving effect in TTD mice as in human osteoporosis patients. Of each mouse, the bone phenotype was determined using micro-CT analysis (right femur), using the Skyscan 1076 scanner at a voxelsize of 9 µm. Bone strength was assessed using break tests (left femur), and transcriptional profiling was performed using micro-arrays (of tibia’s, n=50). Serum and plasma was collected for future biomarker approaches.

**Results**

At 13 weeks of age, the bone phenotype of WT and TTD animals was not significantly different, but from 26 weeks onwards TTD animals had a faster age-related decline both in trabecular and cortical bone (BV/TV at 65 wks: WT: 12.5±0.5%; TTD: 9.1±0.6%, Ct.Th.: 218.3±3.9µm, TTD: 194.1±5.9µm). Bone strength was significantly lower at 65 weeks of age (WT: 97.1 ± 5.7 N/mm, TTD: 53.4 ± 3.6 N/mm). Both ALN and PTH were able to overcome the bone loss and maintain bone strength. Micro array analysis of bone tissue from untreated TTD animals showed that typical bone markers (SOST, Bglap, Coll-1, ALP, Periostin, Runx2 and TRAP) had a similar pattern of expressional change.
Conclusion
TTD mice showed accelerated bone loss during aging which could be reversed with drug treatment. At young age their bone phenotype did not differ from WT animals. This makes the TTD mouse model a suitable screening tool for determining bone-preserving qualities of both existing and novel treatments. In addition, its spontaneous development allows the discovery of biomarkers that may predict osteoporosis onset.

References:
Evaluation of new obesity-related phenotype: a correlation between fat and bone morphological characteristics

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Aims. Previous study showed that highly positive correlation between volume of whole body and volume of partial region, as well as mass and volume of whole body fat. Also this study showed that fat volume of partial region measured by in-vivo micro-CT was correlated with fat pad weight [1]. From these results, we consider that in-vivo micro-CT might be effective to investigate correlation between fat volume of partial region and bone morphological characteristics.

Although the obesity-related gene regulates fat mass, the effects of it on bone remain unclear. The aim of this study was to investigate a correlation between fat volume and bone morphological characteristics using in-vivo micro-CT for evaluation of new phenotype with deficiency of obesity-related gene. In the present study, lengths of femur and dimensions of skull were measured to investigate the site specific effects of obesity-related gene on bone growth and development. In addition, structural parameters of femur trabecular bone were measured to investigate the effects of obesity-related gene on trabecular bone microarchitectures.

Method. 7-week-old (7wks) and 42-week-old (42wks) normal mice (WT) and knockout mice (KO) were used in the experiment. The torso, especially around the 2nd lumbar to the 5th lumbar, and the head of each mouse were scanned at an isotropic voxel size of 35μm and the femur of each mouse was scanned at an isotropic voxel size of 18μm by a SkyScan 1076. Three dimensional models on fat (Fig. 1 (a)), femur and skull (mandible and maxilla, Fig. 1 (b)) of each mouse were reconstructed by mimics 12.1 (Materialise). The volume (mm³) of each fat and the length of femur (mm) were measured. Dimensions of skull (mm) were measured. 13 and 9 references were decided to measure dimension of skull, mandible and maxilla respectively, of each mouse. Structural parameters and BMD (g/cm³) for trabecular bone of each femur were measured by CTAn 1.8.

Results. Fat volumes in WT (54.33mm³ on 7wks, 986.96mm³ on 42 wks) were bigger than those in KO (23.11mm³ on 7wks, 467.66mm³ on 42 wks) regardless of age. Lengths of femur in WT (15.31mm on 7wks, 16.78mm on 42 wks) were longer than those in KO (13.62mm on 7wks, 16.17mm on 42 wks) regardless of age. Quantity and quality for trabecular bone were better in WT than in KO regardless of age (Table 1). Most dimensions of mandible were bigger in WT than in KO regardless of age (Fig. 2). Most dimensions of maxilla were bigger in WT than in KO for 7-week-old mice, although this trend was reversed for 42-week-old mice (Fig. 1).

Conclusion. The results of present study showed that KO had smaller volume of fat and shorter length of femur and dimension of mandible, as well as worse quantity and quality for
trabecular bone, compared with WT. These resulted indicated that obesity-related gene positively regulated not only volume of fat, but also growth, development and microarchitectures of bone.

Table 1. Structural parameters and BMD for trabecular bone, mean values

<table>
<thead>
<tr>
<th></th>
<th>BV/TV(%)</th>
<th>Tb.Th(mm)</th>
<th>Tb.Sp(mm)</th>
<th>Tb.N(1/mm)</th>
<th>Tb.Pf(1/mm)</th>
<th>SMI</th>
<th>BMD(g/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7wks 42wks</td>
<td>7wks 42wks</td>
<td>7wks 42wks</td>
<td>7wks 42wks</td>
<td>7wks 42wks</td>
<td>7wks 42wks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>11.47</td>
<td>11.16</td>
<td>0.09</td>
<td>0.09</td>
<td>1.30</td>
<td>1.23</td>
<td>24.37</td>
</tr>
<tr>
<td>KO</td>
<td>4.30</td>
<td>3.92</td>
<td>0.07</td>
<td>0.08</td>
<td>0.45</td>
<td>0.48</td>
<td>34.61</td>
</tr>
</tbody>
</table>

Fig. 1 3D architectures of torso and skull

(a) torso

(b) skull

Fig. 2 Dimensions of skull; mean ± standard deviation

Small animal imaging methods for *in-vivo* assessment of biochemical composition and volume of cartilage


Erasmus University Medical Center, the Netherlands

Aims. In-vivo imaging of cartilage in small animal models for osteoarthritis would be highly valuable for monitoring therapeutic interventions. Glycosaminoglycan content of cartilage can be measured by negatively charged radiopaque contrast agents, such as ioxaglate; which penetrates degenerated cartilage and can be visualized with micro-CT.

Purpose: Enable *in-vivo* imaging of both cartilage biochemical content and cartilage morphology using µCT in combination with a negatively charged radiopaque dye and a nanometer-scale contrast agent.

Method. 1 Cartilage biochemical composition: 4, 16 and 44 days after induction of experimental knee OA in rats, the knees were assessed with ioxaglate enhanced µCT. 2. Cartilage morphology: at day 44, the knees were additionally assessed with a larger contrast agent. This latter contrast agent is too large to penetrate the cartilage matrix and enables measurement of cartilage matrix volume.

Results. Cartilage biochemical composition: the cartilage in the degenerated knees was not clearly distinct from its surrounding contrast fluid in the joint cavity; indicating diffusion of the contrast agent into the GAG-depleted cartilage matrix, which was more profound in time. Cartilage volume: The cartilage obtained by nanometer-scale contrast enhanced µCT-arthrography was visible as a well-defined band, indicating no contrast leakage into the cartilage matrix. The actual cartilage matrix volume was less in the experimental OA induced knees compared to the left counterparts.

Conclusion. µCT-arthrography makes in-vivo monitoring of cartilage biochemical composition as well as volume possible. It proved to be highly sensitive for cartilage biochemical composition measurement. By combining the two contrast agents, information about cartilage morphology and the biochemical composition of the cartilage can be obtained. These findings make µCT-arthrography very valuable for longitudinal monitoring of the effect of possible therapeutic interventions for OA on cartilage in small animal models and possible interesting for highly sensitive assessment of cartilage alterations in humans.
Follow-up of osteoporosis and vascular calcifications in rats with chronic kidney disease

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Introduction. Impaired bone mineralization and vascular calcification (VC) are frequently seen together in patients with chronic kidney disease (CKD) or postmenopausal women. Moreover, in CKD patients osteoporosis often is seen superimposed upon the presence of renal osteodystrophy. A close relationship exists between osteoporosis and VC, the latter being generally accepted as an active and strictly regulated process with close relation to bone mineralization. Apparently, patients with the highest amount of bone loss have the greatest progression of VC¹,². Additionally, medications used to treat osteoporosis, such as bisphosphonates, seem to prevent VC development³. This is the so called calcification paradox.

The present study focused on osteoporosis complicated with VC in CKD-rats. To induce chronic kidney disease and VC, the adenine-low protein (AD) diet was administered to rats. Since it is known that adenine-induced CKD animals develop a high bone turnover caused by hyperparathyroidism, we included also the ‘remnant kidney rat’ (RK) model. In this model, CKD is induced by a 5/6th nephrectomy and bone turnover will stay rather normal. This was combined with either ovariectomy (OVX,) to induce osteoporosis, or sham operation.

Method. 50 female Wistar rats (ca. 250 g) were divided into 6 groups: OVX (ovariectomy, n=8), CKD combined with OVX (AD+OVX, n=12) or sham operated (AD+sham, n=8). CKD and VC were induced by administration of the 0.75% adenine-2.5% low protein diet during 4 weeks. Two additional groups were added to the study: ‘remnant kidney’ combined with either OVX (RK+OVX, n=12) or sham operated (RK+sham, n=10). Two weeks before starting and throughout the study, rats were fed a 1.03% phosphate diet.

Bone status and aortic calcification (AC) were evaluated in vivo at week -2, 2, 4, 5, 6, 8 and 10 by a SkyScan 1076 micro-CT scanner. A thoracal scan was made to evaluate AC, at 35 µm, with Ti filter and 90 kV/110 µA source energy. Scan lasted about 19 min. Subsequently, a short scan of the tibia was performed after fixation of the leg, at 35 µm, with Al 0.5 mm filter and source energy of 100 kV/100 µA. Bone loss was calculated using image analysis software CTAnalyzer (SkyScan) of the metaphyseal trabecular and cortical bone of the tibia. Urine and blood samples were collected at regular time points to determine Ca, P and creatinine.

Results. At sacrifice, renal function was decreased in CKD rats compared to OVX (serum creatinine OVX 0.47 mg/dl vs. AD+OVX 1.19 mg/dl and AD+sham 1.11 mg/dl, RK+OVX 1.07 mg/dl and RK+sham 1.08 mg/dl, p<0.01) and serum phosphate was not significantly higher in CKD rats compared to OVX. Excretion of phosphate in urine was decreased (OVX 576.35 mg/24h vs. AD+OVX 224.66 mg/24h, RK+OVX 286.42 mg/24h and RK+sham 290.95 mg/24h, p<0.001).
According to micro-CT bone analysis, trabecular bone volume (TBV) of all rats declined during the first 4 weeks, probably induced by disturbances in Ca and P metabolism after onset of renal failure. Subsequently, TBV of adenine animals dramatically increased to initial values between week 4 and 6, coinciding with the switch to high P diet and appearance of AC. This is confirmed by histomorphometry. In contrast, a clear deterioration of the cortical bone could be detected from week 4 in the in vivo scans (fig. 1). There was no clear effect of OVX between both groups with adenine induced CKD. This suggests that the impairment of the renal function exceeded the effect of OVX on the bone. Also for RK+sham, TBV increased from week 4 to 10 but not for RK+OVX. Since renal function was only moderate in RK rats compared to adenine rats, the effect of OVX was visible.

Figure 13: Methaphysis of the tibia of a severe calcified animal. A: week -2, B: week 4 and C: week 6.

Half of the adenine rats manifested AC from week 5 as detected by in vivo micro-CT and only 2 RK+sham rats. Ca bulk analysis revealed 2 more AD+OVX animals with mild calcifications, probably below the detection limit of in vivo micro-CT whereas ex vivo micro-CT did detect these calcifications. No AC could be distinguished by micro-CT or Ca bulk analysis in OVX and RK+OVX animals.

Direct correlations were found between the Ca content in the aorta and TBV (r = 0.510, p < 0.01) and cortical bone (r = -0.482, p < 0.05).

Table 3: Vascular calcification. p<0.05 versus a control with normal renal function (NRF), b none – CKD, c mild, d moderate calcifications.

<table>
<thead>
<tr>
<th>Vascular calcification</th>
<th>Number animals</th>
<th>Area % calcification</th>
<th>mg/g wet tissue</th>
<th>Calcified volume (mm²)</th>
<th>Trabecular bone volume (%)</th>
<th>Bone area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe</td>
<td>5</td>
<td>26.70ᵇᵇ</td>
<td>63.73ᵃᵇᵇᵈᵈ</td>
<td>48.38ᵃᵇᵇ</td>
<td>14.68ᵇᵇ</td>
<td>0.95ᵃᵇᵇ</td>
</tr>
<tr>
<td>Moderate</td>
<td>4</td>
<td>8.84ᵃᵇᵇᵈᵈ</td>
<td>21.91ᵃᵇᵇᵈᵈ</td>
<td>12.94ᵃᵇᵇᵈᵈ</td>
<td>12.44ᵃᵇᵇ</td>
<td>0.85ᵃᵇᵇ</td>
</tr>
<tr>
<td>Mild</td>
<td>2</td>
<td>0.87ᵃᵇᵇ</td>
<td>7.39ᵃᵇᵇ</td>
<td>0.70ᵇᵇ</td>
<td>15.87ᵃᵇᵇ</td>
<td>0.93ᵃᵇᵇ</td>
</tr>
<tr>
<td>None – CKD</td>
<td>17</td>
<td>0.04</td>
<td>0.52</td>
<td>0.02</td>
<td>6.03</td>
<td>0.50</td>
</tr>
<tr>
<td>None – NRF</td>
<td>8</td>
<td>0.03</td>
<td>0.47</td>
<td>0.02</td>
<td>4.02</td>
<td>0.45</td>
</tr>
</tbody>
</table>

**Conclusion.** In uremia, VC development seems to be accompanied by inverse disturbances in the cortical and the trabecular bone. Cortical rather than trabecular bone loss is determining for VC accession. Animals establish a high bone turnover, probably due to secondary hyperparathyroidism, and seem to be predestined to develop VC.

**References.**
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3. Price PA et al., Kidney Int., 2006
Correlations between fat volume and bone architecture

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**Aim.** The objective of the present study was to evaluate the correlation between fat and bone architecture in high fat diet (HFD) mouse compared with normal diet (ND) mouse.

**Method.** 8 Balb/c mice were used and randomly allocated into 2 group, HFD group and ND group. ND group and HFD group fed a normal diet and a high fat diet, respectively. The torso (the 2nd lumbar to the 5th lumbar) and the femur of each mouse were scanned at a resolution of 35μm and 18 μm, respectively, Skyscan 1076. Three dimensional models of fat were reconstructed and the volume (mm³) of each fat were measured by mimics 12.1 (Materialise, Belgium). Structural parameters for trabecular and cortical bone of femur were measured by CTAn 1.8.

**Results.** The fat volume (5185.04mm³) in HFD was significant smaller than that (896.39mm³) in ND (p<0.05, Fig. 1). The BV/TV (5.94%), Tb.Th (0.08mm) and Tb.N (0.74mm⁻¹) in HFD were significant smaller than those (11.34%, 0.09mm, 1.19mm⁻¹) in ND, whereas the Tb.Pf (25.10mm⁻¹) and SMI (2.73) in HFD were significant bigger than those (16.78mm⁻¹, 2.39) in ND (p<0.05) (Fig. 2). For structural parameters of cortical bone, there were no significant differences between HFD and ND. There were negative correlation between fat volume and BV/TV (r=0.85), Tb.Th (r=0.90) and Tb.N (r=0.86), whereas there were positive correlation between fat volume and Tb.Pf (r=0.88) and SMI (r=0.86) (p<0.01). In addition to negative correlation between fat volume and MMI (r=0.71) was shown (p<0.05).
Conclusion.
The results of present study showed that high fat volume caused bone loss, especially trabecular bone. Additionally, negative correlations between fat volume and bone quality and quantity on both trabecular and cortical bone were shown.
Acute spinal cord injury increases callus formation during fracture healing in an experimental rat model

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Aims
In patients who have sustained traumatic brain or acute spinal cord injuries associated with fractures of extremities the rate of new bone formation around the fracture site is increased. Moreover heterotopic ossification is a fairly common complication in spinal cord trauma and brain injuries¹,². The knowledge about the background mechanisms affecting bone formation after trauma of the central nervous system is limited³. Upgrading understanding of the pathomechanisms might give treatment options for heterotrophic ossifications and in addition for delayed bone union. The objective of this study was to establish a reproducible animal model in order to study the influence of acute spinal cord injury on fracture metabolism.

Method
A mid diaphyseal femur osteotomy was created in 20 female Sprague-Dawley rats. Therefore the right femur was exposed and a PEEK plate with angular stable fixation (AO Development Institute, Switzerland) was fixed. A transverse osteotomy was performed using a gigli wire saw resulting in a 2 mm gap. The animals were randomly assigned into intact control and spinal cord injury. The spinal cord was injured at the day of osteotomy using a balloon compression technique⁴. After laminectomy of the T10 arch, a catheter is inserted into the epidural space, advanced cranial to the T8-9 spinal level, inflated and held for 20 min. Neurological deficits were classified using BBB score (0-21 Points). Callus formation was analyzed at 14 days after surgery using µCT (1076, SkyScan, Belgium) and histology (decalcification, paraffin section, HE-stain).

Results
The radiological scout view of the femurs showed increased callus formation in animals with spinal cord injury at 14 days after surgery. In contrast to control group the callus formation was not restricted to the gap and extensive bone formation was observed at periosteal sites (0 out of 10 in control, 8 out of 10 in injury group) (Fig. 1). The newly formed bone volume within the gap was BV/TV 0.11 ± 0.19 (mean ± SD) for control and BV/TV 0.51 ± 0.43 for injury group (Tabl.1). The neurological deficit after spinal cord injury was incomplete and characterized using BBB-score with mean values of 3.4 points at day 1 increasing to 11.4 points at day 14. No correlation was found between newly formed callus volume and BBB score. Histology revealed that mature woven bone was formed 14 days after spinal trauma at periosteal sites.
**Conclusion**

To our knowledge this is the first reproducible animal model to study the increased bone formation in acute spinal cord injury. The femur osteotomy was fixed with a rigid angular stable screw-plate-device that allows maximal standardization of the bone injury and decreases the influence of body motion on the process of bone healing. Increased periosteal callus formation was found at 14 days after spinal cord injury. The model seems to simulate the clinical findings in patients with spinal trauma. Factors that are responsible for the stimulation of bone formation are to be characterized using this new rat model.

**References:**


Vanici I, a simple and reproducible model of spinal cord injury by epidural balloon infiltration in the rat, J of Neurotrauma 2001,Vol18,Nr.12
X-Ray Imaging of Intraplaque Hemorrhage in Aortas of ApoE\(^{-/-}\)/LDL\(^{-/-}\) Double Knockout Mice

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2 Department of Pathology, University of Giessen, Germany
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**Aims:**
Pathologic neovascularization is a consistent and well-known feature of plaque development and progression (1-4). In the event of a plaque hemorrhage it is presumed that ectopic neovascularization as well as vasa vasorum might be the source of the hemorrhage and the reason for the accumulation of erythrocytes within the plaque (5). The accumulation of erythrocytes after intraplaque hemorrhage is considered as an important factor in the transition from a stable to an unstable atherosclerotic plaque (6). Damaged erythrocytes generate conglomerations of iron within the atherosclerotic lesion. Due to the different x-ray photon energy-dependant attenuation coefficients of iron, calcium and the surrounding tissue, localization of iron deposits and calcium within an atherosclerotic lesion should generally be possible using high-resolution x-ray imaging. The relation of fibro-calcified lesions, as determined by CT using the Agatston score, and cardio-vascular events has been demonstrated in the past, but imaging of advanced, vulnerable lesions with clinical imaging modalities like CT or MRI has remained difficult (7, 8). Therefore, this study explores the possibility of 3D Nano-Computed Tomography imaging to directly detect hemorrhage in the arterial wall by virtue of iron accumulation and to differentiate intraplaque hemorrhage from calcified lesions.

The present study was designed to demonstrate nano-CT’s technical feasibility for imaging iron deposits as a marker of intraplaque hemorrhage and for imaging calcified lesions as a consequence of intraplaque hemorrhage in the aortas of apoE\(^{-/-}\)/LDL\(^{-/-}\) double knockout mice.

**Methods:**

**Experimental Design**
Animal studies were performed according to the „German Animal Protection Law“ (1993). Approval of the institutional animal care and use committee was obtained before the start of this study.

Five apoE\(^{-/-}\)/LDL\(^{-/-}\) double knockout mice (80 weeks) were infused in situ with Microfil\textsuperscript{\textregistered} contrast agent. After removing the heart and aorta en bloc from the animals, samples were segmented (n=12) and scanned with nano-CT between 900 nm \(\pm\) 2.5 µm isotropic voxel size.

**Nano-Computed Tomography**
Samples were scanned using a nano-computed tomograph (Nano-CT_2011), manufactured and developed by SkyScan\textsuperscript{\textregistered} (Kontich, Belgium). The microfocus X-ray source is a pumped type source (open type x-ray source) with a LaB6 cathode. The electron beam is focused by two electromagnetic lenses onto the surface of an x-ray target. The x-ray target (Au) contains a thin tungsten film plated on the surface of the beryllium window producing x-ray emission reaching a minimum spot size of < 400nm. At this small spot size, small-angle scattering enhances object details down to 150 nm isotropic voxels size. The X-ray detector consists of a 12-bit digital, water-cooled CCD high-resolution (1280 x1024 pixel) camera with fibre optic 3.7:1 coupling to an X-ray scintillator and digital frame-grabber. In our experimental setting, samples were positioned on a computer controlled rotation stage and
scanned 180° around the vertical axis in rotation steps of 0.25 degrees at 40 kV. Acquisition time for each view was 2.4 seconds. Relative position of the object to the source determines geometric magnification and thus the pixel size defined by the cone-beam geometry of the system. Maximum possible magnification is also limited by the specimen size, which has to be within the cone-beam in its horizontal diameter. Raw data were reconstructed with a modified Feldkamp cone-beam reconstruction modus resulting in two dimensional 8-bit gray-scale images consisting of cubic voxels.

**Histology**

After nano-CT scanning, the entire tissue block was embedded in paraffin wax and sectioned. The 6µm-sections were stained with hematoxylin and eosin. Contiguous serial sections within each sample were prepared to detect iron by staining with Perls’ Prussian blue reaction with 3,3′-diaminobenzidine (DAB) intensification as described previously (9).

**Results:**

Two types of opacities within the atherosclerotic lesions were identified. One type of opacities manifested as clusters of randomly distributed punctuate deposits, being predominantly localized in the descending aorta (Figure 1). Histology confirmed these deposits as being iron deposits after intraplaque hemorrhage (Figure 2). The other opacities detected in nano-CT scans of the aortic samples were confluent accumulations and primarily located in the aortic arch. These opacities were exclusively found as being calcium (Figure 3). Vasa Vasorum neovascularization was present and related to advanced atherosclerotic lesions in the descending aorta.

**Conclusion:**

The advanced technology and high resolution of nano-CT allows localizing iron deposits within advanced atherosclerotic lesions. These findings enable us to detect intraplaque hemorrhage by high-resolution x-ray imaging. Differentiation of iron deposits and calcified lesions is only possible by estimating the size of the opacities. Measuring the density of the two different sized opacities within the atherosclerotic lesions does not allow any statement concerning its substance.
Figure 1. A+B Maximum intensity projection of a nano-CT scan (coronal view) demonstrating iron deposits along the descending aorta. C Maximum intensity projection (axial view) of a nano-CT scan. The arrows pointing at accumulations of iron within the atherosclerotic lesion. * marking the lumen of the descending aorta. D Single slice of a nano-CT image. The arrows pointing at iron deposits within the atherosclerotic plaque. * marking the lumen of the descending aorta.

Figure 2. A Histological slice (HE) of a atherosclerotic lesion demonstrating vasa vasorum in the aortic wall (black arrows). B Perls’ Prussian staining. Blue arrows pointing at iron deposits, black arrows hinting at vasa vasorum in the aortic wall.
**Figure 3**  
A Maximum intensity projection (coronal view) of a nano-CT scan. Arrows pointing at Calcium within the atherosclerotic lesions of the aorta. IVC describing the inferior vena cava. B Axial nano-CT single slice image showing calcifications along the lumen of the aorta.

**References**

In-vivo µCT analysis of the rabbit radius critical size defect by SkyScan 1076 Hasitom

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Aims.
Micro-CT analyses are first choice for the investigation of bone relevant parameters like bone volume and density as well as visualisation of the bone profile, porosity and trabecular structure. High resolution analyses are eminent for acquisition of data in experimental bone healing using tissue engineering approaches in order to quantify even minor changes in new built bone mass. Particularly with regard to small animals like rabbits a high resolution is a crucial precondition. The rabbit affords best premises for a critical size defect (CSD) model because of physiological distinctions in the anatomy of radius and ulna which are adnated in their proximal and distal endings enclosing a spatial separation. A 15 mm bone fragment can be resected from the radius to receive a non-self healing fracture model without affecting functionality of the legs. In order to evaluate time dependent progression within the same animal and to reduce the number of animals needed, in-vivo µCT analyses could be the prime solution. Aim of the study was to establish a method to scan forelegs of vital rabbits regarding particularly the separation of radius and ulna. Therefore a SkyScan 1076 was modified and a bedding solution was made up because there was no µCT equipment for rabbit in-vivo analyses available.

Method.
We examined forelegs of 5 anesthetised hale New Zealand White Rabbits (NZW) in a customised SkyScan 1076 Hasitom. We tested different beddings for the rabbits to guarantee full vitality and breath monitoring while unlimited highlighting of the leg’s interesting part is achieved. Furthermore different filters, voltages (kv) and energies (µA) were tested and the relevant parameters were compared to examine interindividual varieties. Subsequently we assayed 8 NZW rabbits directly after setting a CSD as well as 4 weeks postoperatively. Therefore rabbits were laired on their backs under injection anaesthesia associated with controlled narcosis monitoring.

Results.
A positioning was found which allowed a complete exposition of the rabbit foreleg and particularly of the area of physiological spatial separation of radius and ulna. Furthermore a reliable narcosis monitoring was ensured in the enclosed system which allows in-vivo µCT analyses even with a resolution of 9µm/pixel. Application of an aluminium filter provides reproducible outcomes displaying the CSD and relevant criteria of the new built calcified matrix.

Conclusion.
The system SkyScan 1076 Hasitom based µCT analyses of NZW rabbit radius CSDs should offer an optimal system for explorations of bone regenerating therapies.
Organ approximation in μCT data with low soft tissue contrast using an articulated whole-body atlas

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Aims.
The availability of small animal whole-body imaging modalities (μCT and μMRI) adds a new dimension to animal experiments. It enables monitoring dynamic processes in-vivo, within a population and at subsequent time points, i.e. cross-sectional and longitudinal studies. To be able to quantify disease and developmental processes in whole-body scans, it is necessary to capture and match the body as a whole. Since the animal skeleton is the rigid frame of a body and is the main determinant of whole-body shape we earlier presented a method to perform atlas-based (MOBY mouse atlas [Segars et al. 2004]) skeleton registration [Baiker et al. 2007]. Using μCT data for this purpose enables robust and automated segmentation of the skeleton but has the disadvantage that it lacks soft tissue contrast. As a result, intensity feature-based registration between an atlas and the subject is not possible for most soft tissues. However, using an atlas that contains all major organs allows approximating the missing soft tissue parts.

Method.
We present a method to approximate major organs in μCT. To account for potentially large posture and shape variations among animals, we apply a non-rigid mapping from an atlas to the subject, based on a set of corresponding anatomical landmarks (Thin-Plate-Spline mapping [Bookstein et al. 1989]). To properly constrain the mapping, correspondences are determined on the registered skeleton and subsequently on the lung and the skin.

Results.
The feasibility of the method has been tested by means of 26 μCT mouse datasets and a different whole-body mouse atlas (Digimouse [Dodgas et al. 2007]). Proper mapping of the lung and the skin as well as major organs could be achieved in all cases yielding a mean Euclidean distance between surface nodes of 0.42 ± 0.068 mm for the lung and 0.34 ± 0.036 mm for the skin. The performance of the organ interpolation has been assessed on the basis of manual segmentations of two μCT datasets of mice with injected contrast agent and the Digimouse. The calculated dice indices of volume overlap show significant improvement compared to earlier studies in literature.

Conclusion.
The presented method is applicable for referencing of internal processes in molecular imaging research or whole-body segmentation (e.g. to provide a heterogeneous tissue model for bioluminescence tomography). Furthermore, the approximation result could serve to initialize a subsequent highly accurate registration of a specific bone or organ of interest, as long as the image data shows sufficient contrast. For CT data this might be realized by using a suitable contrast agent. This way, the approximation could be improved using organ registration results of e.g. the kidneys.
3-D Rendering of the Vascular of the Pig Kidney

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Aims. The vasculature of the adult kidney is a most complex structure from the very entrance of the renal artery into the kidney and the multiple subsequent divisions ending in distinct capillary beds in the cortex and medulla. In this study we attempted to view the entire vascular network from the largest artery to the capillary beds in an intact kidney so as to maintain all structural relationships.

Methods. Young adult female farm pigs were anesthetized and the kidneys perfusion fixed in situ with a solution of 2.5% glutaraldehyde in a 0.1 M Na cacodylate buffer. After one week of post-fixation, the renal vasculature was filled with radiopaque yellow Microfil, which contained no diluent and allowed to cure. Subsequently a single kidney was embedded in paraffin and placed in the specimen chamber of an 1172 SkyScan high resolution micro-CT unit. A 3-D reconstruction of the entire renal vasculature was rendered from oversized scans.

Results. For the first time we were able to visualize the renal vasculature from the level of the main renal artery to single glomerular tufts in a pattern that allows one to sample the entire kidney. We clearly were able to visualize and follow a more complex branching pattern at the level of the arcuate vessels than we had ever previously observed. The detailed 3-D reconstruction images allowed us to examine the interrelationships of the cortical and medullary vascular networks quickly and precisely.

Conclusions. The high throughput of samples and high resolution capabilities of the 1172 SkyScan system allowed us for the first time to collect detailed images of an entire pig kidney that were easily used to generate a 3-D reconstruction of the complete renal vasculature.
Validation of new potential drug targets for osteoporosis and fracture repair by *in vivo* microCT

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**Aims.** At the Target Discovery Department of Organon N.V., part of Schering Plough Corporation, we use the Skyscan 1076 microCT to validate new potential drug target genes for osteoporosis and fracture repair.

**Method.** For this purpose we have set up several models in mice including (i) *ex vivo* microCT measurement of the femur, tibia and LV5, (ii) *in vivo* microCT measurement of the distal femur, and (iii) *in vivo* microCT measurement of the tail vertebrae in a “drill hole injury” model.

**Results.** *In vivo* microCT measurement of the femur is currently used for studying the effectiveness of osteoporosis therapies in a mouse model of ovariectomy (OVX) induced bone loss, and for studying changes in bone mass in transgenic mouse models over time. With the *in vivo* microCT, the number of animals used in an experiment can be reduced as the same animal can be measured at different time points. The first time point can then be used as control instead of using a separate SHAM control group. Using this *in vivo* microCT model we demonstrated that in C57Bl/6 mice treated with compound X the bone mass increased significantly compared with the control group.

Bone injury is an important clinical issue and studying the process of bone regeneration can help to find a potential drug target for bone repair. For studying the process of bone regeneration in mice, we adapted a “drill hole injury” model that allows us to introduce a reproducible injury with a clearly defined boundary in the cortical midsection of a tail vertebra. The Skyscan microCT was used for measuring the rate of healing of cortical bone after drill-hole injury of 0.5 mm at different time points. Our results demonstrated that in C57Bl/6 mice, the tail vertebra was healed after 60 days.

**Conclusion.** The ability to determine changes in bone mass, bone mineral density, and bone restoration over time by *in vivo* microCT measurement is a valuable additional tool for the validation of osteoporosis and fracture repair drug targets, which can also reduce the number of mice used in an experiment.