Bone xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

# Development and optimization of a high-throughput micro-computed tomography imaging method incorporating a novel analysis technique to evaluate bone mineral density of arthritic joints in a rodent model of collagen induced arthritis

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ARTICLE INFO

*Article history:*
Received 4 April 2014
Revised 10 November 2014
Accepted 18 November 2014
Available online xxxx

Edited by: Sharmila Majumdar

*Keywords:*
Rheumatoid arthritis
CIA
Micro-CT
Bone mineral density
Automatic segmentation
Polynomial fitting

ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease resulting in joint inflammation, pain, and eventual bone loss. Bone loss and remodeling caused by symmetric polyarthritis, the hallmark of RA, is readily detectable by bone mineral density (BMD) measurement using micro-CT. Abnormalities in these measurements over time reflect the underlying pathophysiology of the bone. To evaluate the efficacy of anti-rheumatic agents in animal models of arthritis, we developed a high throughput knee and ankle joint imaging assay to measure BMD as a translational biomarker. A bone sample holder was custom designed for micro-CT scanning, which significantly increased assay throughput. Batch processing 3-dimensional image reconstruction, followed by automated image cropping, significantly reduced image processing time. In addition, we developed a novel, automated image analysis method to measure BMD and bone volume of knee and ankle joints. These improvements significantly increased the throughput of ex vivo bone sample analysis, reducing data turnaround from 5 days to 24 h for a study with 200 rat hind limbs. Taken together, our data demonstrate that BMD, as quantified by micro-CT, is a robust efficacy biomarker with a high degree of sensitivity. Our innovative approach toward evaluation of BMD using optimized image acquisition and novel image processing techniques in preclinical models of RA enables high throughput assessment of anti-rheumatic agents offering a powerful tool for drug discovery.



## Introduction

Rheumatoid arthritis (RA) is a chronic and progressive autoimmune disease in which the immune system triggers multiple inflammatory responses against self-antigens, resulting in erosion of cartilage and bone in and around the peripheral joints [1]. The pathogenesis of RA includes a complex inflammatory response involving innate and adaptive immune cells, proinflammatory cytokines and autoantibodies that infiltrate the synovia causing fluid accumulation, pain, and bone damage in the affected joints [2,3].

Animal models of RA have been used extensively to interrogate the distinct mechanisms of disease pathology and identify potential biological targets in pursuit of novel therapeutics. Collagen induced arthritis (CIA) is a widely used rodent model of induced arthritis. The rat CIA model exhibits multiple facets of human disease including profound cartilage degradation, dependence on complement immunity, periarticular inflammation, and bone resorption [4]. Following collagen injection, rats display a severe polyarthritic phenotype consisting of swollen extremities, cartilage degradation, and eventual loss of joint function, which is, in some aspects, similar to RA [5,6]. The reproducibility, low variability, and rapid disease onset of this model make it a

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http://dx.doi.org/10.1016/j.bone.2014.11.014

valuable tool for the assessment of novel compounds [4,7–9]. Hind limb paw thickness is a routinely used surrogate marker of inflammatory pathology in this model [5,8,9]. In the rat CIA model, approximately 80% of animals develop visually observable edema in the ankles and paws, while about 20% of animals develop visually observable edema in the knee [8–10]. However, even when edema is not present, cellular infiltration, cartilage degradation, and structural changes to bone occur at the knee and other affected joints [8,10]. While useful, paw thickness does not reveal the underlying structural changes to the bone caused by disease since paw thickness only directly measures edema. Assessment of bone morphology by imaging is a more sensitive and translational readout; damage to its structural integrity, and in particular, periarticular demineralization, points to hyper-activation of osteoclast activity, which is a critical component of RA [2].

Over the last two decades, imaging has made major advances in early diagnosis and therapeutic monitoring of RA [11]. In the clinic, X-ray radiography is the standard for imaging-assisted diagnosis of RA. Also based on X-ray technology, computed tomography (CT) has demonstrated higher sensitivity for quantifying bone erosions in RA patients, due to its 3-D visualization perspective [12]. It is ideal for bone measurements due to its high spatial resolution and the natural contrast between bone and soft tissue. A reduction in bone mineral density (BMD), an important biomarker of disease assessed by CT, indicates poor prognosis for patients with early stages of RA [13–15]. CT has also been demonstrated to be an excellent tool for assessment of bone damage in preclinical rodent models of RA due to its high reproducibility and inherently quantifiable properties [16,17]. Radiological examination has been applied successfully to monitor progression of RA and osteoarthritis in various rodent models to gain a deeper understanding of the pathophysiology of disease [18–25]. Many metrics are used to track disease progression, including bone volume changes, bone surface roughness, erosion scoring, 3-D tissue morphometry, and BMD measurements, as measured by X-ray, DEXA, or micro-CT [18–23,25]. Despite widespread use of both micro-CT and DEXA to gather BMD measurements, it has been demonstrated, in at least one animal model, that the volumetric BMD measured by micro-CT is more sensitive than the 2-D BMD measurements acquired by DEXA [24].

Automated analysis can dramatically increase throughput, so special attention has been given to performance tuning the analysis procedure by developing new automation methods. For example, Barck et al. describe an automated method to measure BMD of the paw joints in mice and Huber et al. describe automated BMD measurements of femur in human [23,26]. However, other biomarkers, such as changes in bone architecture, are more heavily emphasized in the literature on automated bone analysis. There are examples of automation techniques that can separate trabecular bone from cortical bone or segment trabecular bone using high resolution images [27,28]. An in vivo method using registration techniques to detect differences in bone lesion volume in a rat model using magnetic resonance has also been described [29]. Segmenting specific bone components, such as trabecular or cortical bone, and then measuring shape and attributes such as 2-D area and 3-D volume, or bone erosion are the main analysis endpoints in these publications. Of note, the automation techniques in these studies were not able to avoid manually separating their regions of interest (ROIs) from their samples; instead, ROIs were either prepared prior to imaging or were manually segmented from a larger area after imaging and before automated image analysis was used to extract measurements.

Herein, we describe a high-throughput method to evaluate BMD and track volume changes of peripheral joints for pharmacological assessment of drug candidates to support discovery of novel therapeutics. Our objective was to apply BMD measurements of the knee and ankle joints in the rat CIA model as a rapid and efficient biomarker for drug screening and optimization. The methods we detail had to replicate the sensitivity and accuracy of slower acquisition processes, while speeding up work-flow. The automated analysis method also needed to correlate with manual analysis and show high reproducibility from study to study. To achieve this goal, we developed a streamlined process to image eight bone samples simultaneously and perform batch image reconstruction and automated image cropping. In addition, we demonstrate a novel automated method of combining image processing techniques, such as intensity thresholding and skeletonization, with mathematical techniques in curve fitting and curvature calculations, to find and place a bounding box around the ROIs in CT images quickly and consistently. The algorithm can process individual images or entire data sets and provides various metrics of interest including volume and mean intensity of Hounsfield units of the bone ROI within the bounding box. This manuscript further expounds new methods of data acquisition and analysis that utilize the predictive potential of BMD assessment as it relates to RA outcome and therapeutic treatment in the rat CIA model.

## Materials and methods

### *Development*

#### *Micro-CT: acquisition, image reconstruction, and cropping*

Conical tubes containing fixed rat hind limbs stored in 70% ethanol solution were loaded into the micro-CT holder. Samples were positioned symmetrically on the perimeter of the holder for equal X-ray beam exposure, with a phantom located in the middle of the holder (Fig. 1B). CT scans were acquired as described in Haines et al. (2009) with the exception that the three dimensions of the image data set (X, Y, and Z) were adjusted to 1000 × 900 × 1300 slices at 100 μm cubic voxel dimensions and scaled to Hounsfield units (HU) [30]. The reconstructed data were then cropped into images containing a single bone sample, using MATLAB (MathWorks, Inc., Natick, MA, USA) software to automate this process.

#### *Automated image analysis and manual correction*

The CT images were downsampled and thresholded to 300 HU. We chose this range to be consistent between studies and because it showed the best dynamic range while still excluding soft tissue. Importantly, the bone samples on which this threshold is applied undergo rapid formalin fixation and are then stored in ethanol filled conical tubes. This threshold is designed to take the tubes, scanning holder, and ethanol into account. After thresholding, a morphological closing operation was applied to the bone mask to fill the holes, and this resultant mask was skeletonized to obtain the main shape of the hind limb bone. A 6th degree polynomial curve was fitted using a Chebyshev polynomial, as detailed previously [31]. The curvature of the skeleton was calculated and the peaks of maximum curvature were found, using a previously published equation [32]. Using the location of maximum curvature, segmentation of the knees and ankles was performed by placing predefined boxes of sizes [X, Y, Z] around each ROI. The two knee and ankle joint regions of interest at X, Y, and Z were set to 15 × 15 × 15 mm and 13 × 13 × 13 mm, respectively. These bounding boxes were placed by aligning the center point of the turning point of the joint (Figs. 2A, B, C). After box placement, the bone inside is quantified using thresholding to obtain volume and HU data for our desired final output: BMD of knee and ankle regions. By thresholding the box, only the bone is selected as our ROI; air and other materials are excluded from our measurements. We evaluate BMD as bone mineral content (mg)/bone region (ROI in $cm^3$) and track volume of the ROI as well. After automated analysis was complete, cross-sectional views of the boxes were exported as 2-D image files for visual inspection to ensure that the algorithm worked properly (Fig. 2D) and a master Excel sheet was generated containing the volumes and average HUs for the ROIs (Fig. 2E). Bounding box placement was visually reviewed and samples were manually re-analyzed if the boxes were misplaced or if samples were not analyzed correctly using Amira® 5.4.2 image analysis software (Mercury Computer Systems, Inc., Chelmsford, MA). Detailed methods are provided in the Supplementary methods section.



Please cite this article as: Sevilla RS, et al, Development and optimization of a high-throughput micro-computed tomography imaging method incorporating a novel analysis technique..., Bone (2013), http://dx.doi.org/10.1016/j.bone.2014.11.014