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**Contrast-Enhanced CT using a Cationic Contrast Agent Enables Non-Destructive
Assessment of the Biochemical and Biomechanical Properties of Mouse Tibial Plateau
Cartilage**

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RUNNING TITLE: CECT characterizes mouse tibial cartilage

AUTHOR CONTRIBUTIONS

B.A. Lakin designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript.

H. Patel performed data analysis, assisted with data interpretations, and reviewed the manuscript.

C. Holland performed data analysis, assisted with data interpretations, and reviewed the manuscript.

J.D. Freedman synthesized the CA4+ contrast agent, performed the initial diffusion experiment, and reviewed the manuscript.

J.S. Shelofsky performed data analysis, assisted with data interpretations, and reviewed the manuscript.

B.D. Snyder designed the experiments, interpreted the data, wrote the manuscript, and provided resources to perform the experiments.

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Abstract

Mouse models of osteoarthritis (OA) are commonly used to study the disease's pathogenesis and efficacy of potential treatments. However, measuring the biochemical and mechanical properties of articular cartilage in these models currently requires destructive and time-consuming histology and mechanical testing. Therefore, we examined the feasibility of using contrast-enhanced CT (CECT) to rapidly and non-destructively image and assess the

glycosaminoglycan (GAG) content. Using three ex vivo C57BL/6 mouse tibial plateaus, we determined the time required for the cationic contrast agent CA4⁺ to equilibrate in the cartilage. The whole-joint coefficient of friction (μ) of thirteen mouse knees (some digested with Chondroitinase ABC to introduce variation in GAG) was evaluated using a modified Stanton pendulum. For both the medial and lateral tibial plateau cartilage of these knees, linear regression was used to compare the equilibrium CECT attenuations to μ , as well as each side's indentation equilibrium modulus (E) and Safranin-O determined GAG content. CA4⁺ equilibrated in the cartilage in 30.9 ± 0.95 min (mean $\pm$ SD, tau value of 6.17 ± 0.19 min). The mean medial and lateral CECT attenuation was correlated with μ ($R^2=0.69$, $p<0.05$), and the individual medial and lateral CECT attenuations correlated with their respective GAG contents ($R^2\geq 0.63$, $p<0.05$) and E ($R^2\geq 0.63$, $p<0.05$). In conclusion, CECT using CA4⁺ is a simple, non-destructive technique for three-dimensional imaging of ex vivo mouse cartilage, and significant correlations between CECT attenuation and GAG, E, and μ are observed.

KEYWORDS: micro-computed tomography, compressive modulus, coefficient of friction, osteoarthritis, glycosaminoglycan

INTRODUCTION

Osteoarthritis (OA) is a common, non-inflammatory disease that alters synovial joint structure and performance. Articular cartilage provides a smooth, nearly frictionless surface that distributes applied loads to articulating joint surfaces. Unfortunately, this tissue breaks down as OA progresses, resulting in eventual cartilage erosion and bone-on-bone contact leading to patient pain. The mouse is an essential animal model to study cartilage degeneration mechanisms in OA. In this model, the disease can be induced via genetic knockouts (e.g., collagen IX [1, 2], osteopontin [3], ADAMTS4 [4], or ADAMTS5 [5] deficiencies), surgery (e.g., anterior cruciate

ligament transection (ACLT) [6] or destabilization of the medial meniscus (DMM) [6, 7]), or the disease can spontaneously develop in some murine strains (e.g. STR/ort) [8, 9]. Additionally, the mouse model is commonly used for the assessment of new OA therapies or treatments in pre-clinical development [10, 11].

During the early stages of OA, cartilage glycosaminoglycan (GAG) content decreases [12, 13]. Hence, GAG concentration is an important indicator of cartilage tissue health and is routinely studied in OA research. A reduction in GAG prohibits cartilage from maintaining a substantial interstitial fluid pressure, which diminishes its compressive stiffness and lubricating ability while distributing more of the tissue's internal load to the collagen matrix, resulting in eventual tissue damage. A common method to assess GAG content in mouse models is Safranin-O staining of histological sections [2, 14, 15]. Additionally, cartilage mechanical and lubricating properties are frequently examined for ex vivo mouse cartilage using indentation testing [2, 16, 17] and pendulum friction testing [18-20], respectively, as these properties are important indicators of cartilage functional performance. Although these techniques are common, histological and mechanical testing of mouse articular cartilage is challenging and limited. Specifically, the tests are time-consuming, require advanced skill, and cannot quickly provide a 3D map of the biochemical or mechanical properties of the cartilage (histology requires 2D sectioning, indentation tests analyze specific sites, and pendulum testing assesses whole-joint performance, not individual cartilage surfaces). Moreover, both histology and indentation are destructive and cannot be performed on intact joints or used to monitor disease progression or treatment efficacy in a single animal.

To generate more global measures of murine joint pathology, various imaging modalities are being explored to examine cartilage, including confocal scanning laser microscopy [7, 9], ,

phase-contrast computed tomography (CT) [21], and contrast-enhanced computed tomography (CECT) using an anionic contrast agent [22, 23]. However, these imaging techniques primarily assess morphological features of cartilage (e.g., thickness and volume). Thus, a facile, non-destructive technique that evaluates morphological features as well as provides information on the biochemical and mechanical properties of mouse articular cartilage is of significant interest. Herein, we report the application of CECT to examine ex vivo mouse articular cartilage GAG content and mechanical properties using the cationic contrast agent CA4⁺. Specifically, we report the diffusion profiles of CA4⁺ into cartilage as a function of time, 3D reconstructed images showing morphological features, and the subsequent correlations between CECT attenuation and cartilage GAG content, equilibrium compressive modulus, and coefficient of friction.

METHODS

Material/specimen preparation

The skin and other soft tissues were carefully removed from the hind limbs of seven, euthanized, 16 week-old, female C57BL/6 mice, leaving the knee capsules intact (n = 13). Three of the knees were set aside for the diffusion-in experiment, while the other knees were each flexed to approximately 90° and then randomly received a 20-μL infrapatellar, intra-articular injection of either Chondroitinase ABC (C3667, Sigma, St. Louis, MO) to deplete GAG at 0.1 U/mL (n=3) or 0.5 U/mL (n=3) or saline (control, n=4). These three groups created a larger range of cartilage GAG contents to mimic the range of GAG expected in common mouse OA models. The injected limbs were flexed and extended 30 times and then stored at 37 °C for 4 hrs. To facilitate easier gripping during imaging and mechanical testing, both the proximal femur and

the distal tibia of all knees were embedded in polymethylmethacrylate (PMMA) bone cement (Technovit 3040, Heraeus Kulzer GmbH, Wehrheim, Germany).

The cationic contrast agent CA4⁺ was synthesized as previously reported [24], and a CA4⁺ contrast agent solution was prepared at 12 mgI/mL by dissolving the dry compound in deionized water, balancing the pH to 7.4 using concentrated 4.0 M NaOH, and adjusting the osmolality to 400 mOsm/kg using sodium chloride to match the in situ osmolality of articular cartilage (350-450 mOsm/kg [25]). Preliminary pilot studies demonstrated that this CA4⁺ concentration provided cartilage CECT attenuation that was distinguishable from both the surrounding air and the subchondral bone. GIBCO Anti-Anti stock solution (Invitrogen, Grand Island, NY), 5mM of EDTA (Sigma, St. Louis, MO), and benzamidine HCl (Sigma B6506, St. Louis, MO) were included in all the contrast agent and saline solutions that were exposed to the cartilage to prevent nonspecific degradation of the cartilage during the study.

Contrast Agent Diffusion-In Experiment

The three non-injected knees were disarticulated, all the surrounding soft tissue was removed, and the tibias were rinsed three times in excess saline to remove any residual synovial fluid. The tibial plateau surfaces were then each immersed in 1 mL of CA4⁺ solution at room temperature. The samples were removed from the contrast agent for CT scanning after being immersed for the following total times: 0, 5, 10, 20, 30, 45, 60, and 90 min. When removed from the solution, the samples were gently blotted dry and individually positioned in a μ CT imaging system (μ CT40, Scanco Medical AG, Brüttisellen, Switzerland) using a custom, airtight holder that maintained a humid environment to prevent drying of the cartilage. Sequential transaxial μ CT images of the cartilage and subchondral bone were acquired at an isotropic voxel resolution of 6 μ m, 70-kVp tube voltage, 113- μ Amp current, and 300-ms integration time for all samples.

These diffusion-in μ CT data were converted to DICOM format using the proprietary software from Scanco Medical AG before being imported for post-processing using Analyze™ (AnalyzeDirect, Overland Park, KS). For each time point, the cartilage was segmented from the subchondral bone using a region-growing algorithm in the Volume Edit module of Analyze [26, 27]. Specifically, for each image slice, a seed point was placed in the cartilage, and then the upper and lower thresholds were manually adjusted until the grown region encompassed all of the cartilage, with care taken not to include any subchondral bone or surrounding air. All cartilage contours were manually reviewed and adjusted if necessary. This region-growing algorithm has been repeatable by each user in our group and reproducible between analyzers. The mean contrast-enhanced CT (CECT) attenuation value expressed in Hounsfield Units (HU) for the medial and lateral surfaces combined was obtained by averaging the x-ray attenuation over all transaxial μ CT slices corresponding to cartilage tissue. The attenuation values for each time point were then normalized by the 0-min time point (baseline/non-enhanced) attenuation.

Pendulum Coefficient of Friction (μ) Testing

Most reports examining the relationships between coefficient of friction (μ) and GAG utilized excised cartilage discs or osteochondral plugs. As a result of sample extraction, these sample types possess an exposed, circumferential edge that alters the mechanics and mass transfer of fluid and molecules in and out of the cartilage. Additionally, if using cartilage discs, the cartilage-bone interface is not preserved during testing. To overcome these limitations and preserve the native boundary conditions for the cartilage and subchondral bone, this study performed friction testing using a modified pendulum apparatus [18-20]. This approach also enables loading the cartilage surfaces in a physiologically relevant manner (cartilage-on-cartilage) with appropriate force and displacement trajectories. For the ten knees used in this

study, their femurs and tibias were gripped in a modified Stanton pendulum apparatus [20] with a 50-mg pendulum arm. The pendulum arm was offset to 15 degrees, released, and allowed to freely oscillate until coming to rest. Each knee was tested three times, and the pendulum arm motion was recorded with high-speed video. The video was analyzed to calculate a coefficient of friction (μ) for each knee joint using conservation of energy [18] and a custom MATLAB code (MATLAB R2010a, The MathWorks, Natick, MA).

Indentation Equilibrium Compressive Modulus (E) Testing

After pendulum testing, the knee joints were disarticulated, all the surrounding soft tissue was removed, and the tibias were rinsed three times in excess saline (to desorb any residual enzyme in the treated knees). The tibial plateau was selected for subsequent analyses, as it is a preferred tissue over the femoral surface for imaging and mechanical testing. Its cartilage surfaces are thicker than the opposing femoral surfaces and resemble a simpler 3D geometric shape, namely a convex, with relatively flat tissue surrounding the testing site (a circular area of at least 3 indenter tip radii) [28], while the femoral cartilage surfaces possess more complex surface shapes.

After rinsing, the tibial samples were then individually positioned in a μ CT imaging system (μ CT50, Scanco Medical AG, Brüttisellen, Switzerland) using a custom, airtight holder that maintained a humid environment. The samples were scanned at an isotropic voxel resolution of 2 μ m, 70-kVp tube voltage, 114- μ Amp current, 500-ms integration time, and 3x frame averaging. These settings on the μ CT50 scanner were used to generate baseline CT images with more voxels throughout the cartilage tissue depth to facilitate accurate thickness measuring. The baseline μ CT data were converted to DICOM format as previously described and imported for post-processing in Analyze. The mean cartilage thickness for each of the medial and lateral

cartilage volumes was then determined perpendicular to the cartilage articular surface at the indentation testing site by manually drawing 9 equally spaced lines from the articular surface to the subchondral bone on 7 equally spaced sagittal slices using the “Line Tool” in the Line Profile module in Analyze [27].

Both the medial and lateral volumes of each sample were then evaluated using a standard indentation testing procedure [29]. Briefly, the tibial diaphysis was secured in a fixture and positioned such that the central, weight-bearing region of the cartilage surface (a locally flat surface) was aligned perpendicular to a 0.3-mm diameter, plane-ended, glass indenter tip. After submerging the cartilage in saline, a 3 mN pre-load was applied. Each sample was then compressed using a 4-step indentation stress-relaxation regimen consisting of four 5% strain steps at a displacement rate of 60 $\mu\text{m}/\text{min}$ (Z005, Zwick/Roell GmbH, Ulm, Germany), each followed by a 200-sec relaxation period. The indentation, equilibrium compressive modulus (E) was then computed by fitting a linear regression line to the resulting equilibrium stress-strain data. Due to complications during testing, one lateral test was removed from subsequent analyses.

Contrast-Enhanced CT (CECT) Imaging

Following mechanical testing, the samples recovered in saline for at least 1 hr at room temperature before being immersed in 0.25 mL of CA4+ solution for 2 hrs at room temperature. Each sample was then gently blotted to remove excess contrast agent and scanned again using the same $\mu\text{CT}50$ scanner and settings. After scanning, each sample was rinsed in 1 mL of saline at 4°C overnight and then frozen. The CECT data were also converted to DICOM format, imported into Analyze, and the cartilage was segmented from the subchondral bone using a region-growing algorithm in the Volume Edit module [26, 27]. The mean CECT attenuation

value for each cartilage surface was obtained and is reported as grayscale intensities in Hounsfield Units (HU).

Determination of Glycosaminoglycan (GAG) Content

To determine cartilage GAG content, an optical density technique was applied to Safranin-O stained histological sections of the samples (see SI for details). Safranin-O is a red, cationic dye that binds to the negatively charged GAGs in cartilage histology sections, and the resulting red-stained regions indicate the distribution of the GAGs. Both the medial and lateral surfaces of each tibial plateau were embedded in paraffin, processed for histological sectioning as described in the SI, cut sagittally into 5- μ m thick sections, stained with Safranin-O, and imaged on a microscope at 10x magnification (Axio Imager 2, Zeiss Microscopy, Thornwood, NY) after using the software's white balance function to eliminate any red pixel bias from the background. Using Analyze's ROI and Image Calculator modules and a custom code (MATLAB 2010a), the cartilage region of each photomicrograph was analyzed to determine its mean red pixel intensity (expressed as mean I_p value) [30] by: 1) subtracting the average of the green and blue components from the red component of each pixel within both the cartilage region and a neighboring, background region in the same image that contained no tissue; 2) normalizing the resulting cartilage values by the max resulting background value to compute each cartilage pixel's I_p value (any pixel I_p values less than or equal to zero were ignored), and 3) averaging all the I_p values to compute the mean I_p value for the cartilage on each section. This approach resulted in the greatest dynamic range of I_p values across the spectrum of bovine GAG values obtained using the 1,9-dimethylmethylene blue (DMMB) assay for the standard curve described below. The global mean I_p value for each cartilage surface was then computed by averaging all the mean I_p values obtained for all sections across the width of the surface.

The mean I_p values were then converted to GAG content using a standard curve generated from the articular cartilage of 6 neighboring pairs of bovine osteochondral plugs [30] (see SI for details), as performing the standard DMMB assay directly on small mouse samples is unreliable due to difficulty removing the cartilage tissue without also excising subchondral bone fragments or surrounding soft tissue pieces. The GAG content of several 100- μ m thick, depth-wise layers from one plug of each pair was measured using the DMMB assay [31], while mean I_p values of corresponding layers from the other plugs of each pair were determined as described for the murine samples. The standard curve between mean I_p value and GAG content was generated using linear regression and was used to convert from mean I_p value to GAG content for the mouse tibial cartilage samples.

Statistics

Univariate linear regression analysis (SPSS 17.0, Chicago, IL) was applied to examine relationships between E , μ , CECT attenuations, and GAG content. The coefficient of determination (R^2) was used to assess the strength of the correlations, and significance was defined as two-tailed P -value < 0.05 .

RESULTS

CA4+ Diffusion into Mouse Tibial Plateau Cartilage

The cationic contrast agent CA4+ rapidly diffused into the mouse tibial plateau cartilage tissue and reached equilibrium after approximately 30 min (**Figure 1**). After fitting the data for each sample with an equation of the form $CECT\ Attenuation = a * e^{-b(time)} + c$, tau values (calculated as $1/b$) were computed for each sample, and the mean tau value was found to be 6.17

± 0.19 min (mean $\pm$ SD). Multiplying the tau value by 5 yields 99% of the fitted equilibrium attenuation, hence the calculated mean equilibrium time was $5 \times \tau = 30.9 \pm 0.95$ min.

Quantifying Cartilage GAG Content from Safranin-O Stained Histological Sections

Using 42 depth-wise layers obtained from six neighboring bovine cartilage plug pairs, a standard curve correlating mean Ip Value (a measure of the red intensity from Safranin-O stained histology sections) to GAG content determined via the DMMB assay was developed (**Figure 2**). Since the correlation was $R^2 = 0.93$ and statistically significant ($p < 0.05$), the red content from Safranin-O stained histology sections reflected the GAG content of the cartilage tissue. Therefore, this standard curve was used to convert from mean Ip value to GAG content for the mouse cartilage samples.

Relationships between CECT Attenuation, GAG Content, and Equilibrium Compressive Modulus

The CA4+ enhanced CT attenuation was significantly correlated with GAG content (determined via Safranin-O staining) for both the medial and lateral (**Figure 3a**) samples, with coefficients of variation greater than or equal to 0.64. Positive correlations were observed between indentation, equilibrium compressive modulus (E), and GAG content for both the medial and lateral surfaces (**Figure 3b**, $R^2 \geq 0.63$, $p < 0.05$). Additionally, accounting for up to 78% of the variation in E, the CECT attenuation was positively correlated with E for both surfaces (**Figure 3c**).

Relationships between CECT Attenuation, GAG Content, and Coefficient of Friction

As shown in **Figure 4a**, pendulum coefficient of friction (μ) was negatively correlated with GAG content ($R^2 = 0.59$, $p < 0.05$). And, CECT attenuation was negatively correlated with μ (**Figure 4b**), with μ accounting for 69% of the variation in CECT attenuation.

DISCUSSION

Contrast-enhanced CT (CECT) of cartilage relies on the partitioning of a mobile contrast agent probe in the tissue [32]. If the probe is charged, it will equilibrate according to Donnan Equilibrium Theory in proportion to the negative fixed charge density arising from the GAGs in the cartilage extracellular matrix (ECM) [33-40]. For example, anionic contrast agents partition in inverse proportion to the GAGs, and the resulting CECT attenuations correlate with cartilage GAG content [33, 37-40] and compressive stiffness [33]. Alternatively, cationic contrast agents (such as CA4⁺ [24]) partition in direct proportion to GAG content [34, 35], and the resulting CECT attenuations strongly correlate with cartilage GAG content [34, 35, 41], equilibrium compressive modulus [41] and torsional coefficient of friction [41]. To evaluate the potential of CECT using the CA4⁺ contrast agent to image mouse articular cartilage, we first measured the diffusion kinetics of CA4⁺ into the cartilage of three murine tibial plateaus. The CECT attenuation reached a mean value of 1118 ± 69 HU [mean $\pm$ SD] after 30 min of diffusion (**Figure 1a**) with a corresponding mean tau value of 6.17 ± 0.19 min, representing the time at which 63.2% of the final attenuation was reached. Ioxaglate (an anionic CT contrast agent with similar molecular size and structure used at 80-160 mgI/mL [24]) also attains equilibrium within 30 min in mouse femoral (tau value of 6.7 min) [22] and tibial [23] articular cartilage specimens, indicating similar diffusion kinetics for both agents. The similar tau value for CA4⁺ and Ioxaglate likely reflects the thin mouse cartilage. Longer equilibration periods appear necessary for phosphotungstic acid (PTA) [42]; however, early diffusion time points were not examined. Both the tau value and equilibrium immersion time for CA4⁺ are less than previously reported for this contrast agent when diffused into bovine cartilage tissue [35] and are less than those

reported for two cationic near infrared fluorescent dipicolylamine probes diffused into human cartilage [43], again due to the smaller volume and thickness of mouse cartilage.

The positive correlation obtained between the mean Safranin-O stained Ip Value of the histology sections and GAG content determined via the DMMB assay (**Figure 2**) agrees with previous reports using a similar procedure for bovine [44] and human [30] cartilage. This relationship is essential for quantifying GAG content of small rodent cartilage, as performing the DMMB assay directly on such samples is unreliable due to difficulty removing the cartilage tissue without also excising subchondral bone fragments or surrounding soft tissue pieces.

The significant correlations between CA4+ enhanced CT attenuation and GAG content for both the medial and lateral cartilage surfaces used in this study ($R^2 = 0.74$ and 0.64 , respectively, **Figure 3a**) agree with previous reports for bovine cartilage. Specifically, using the same CA4+ concentration, Lakin et al [41] reported positive and significant correlations between CECT and GAG using bovine cartilage with coefficients of variation (R^2) of 0.74 for a study using native tissue, and 0.87 for a study that used both native and GAG-depleted tissue. Additionally, this study's correlations agree with those published with CA4+ at lower and higher concentrations, 8 mgI/mL [35] and 27 mgI/mL [34], respectively. Our positive relationship between CECT and GAG content agrees with the correlation reported for human cartilage and the trend reported for murine cartilage when both tissues were immersed in a cationic near infrared fluorescent dipicolylamine probe [43]. Although the GAG ranges are the same, the medial CECT attenuations tended to be greater than those of the lateral surface, resulting in a slightly greater y-intercept for the medial samples, despite the similar slopes. There are a few potential reasons that could explain the difference in equilibrium CECT values. First, the medial samples were significantly thicker than the lateral samples, which would result in more voxels of

data from the GAG-rich middle and deep cartilage zones contributing to the mean CECT attenuation values. Although it was not possible to assess porosity or water content with our methods, the lateral samples could also be less porous or have lower water contents than the medial samples, which would result in reduced equilibrium contrast agent concentration in the tissue yet a similar sensitivity to GAG. Further, the slight difference in the correlations emphasizes that future studies should investigate the need for surface-specific phantoms to effectively predict cartilage GAG.

As shown in **Figures 3b-c**, the indentation equilibrium compressive modulus (E) was positively correlated with GAG content and mean CECT attenuation for the medial ($R^2 = 0.78$ for both) and lateral ($R^2 = 0.63$ for both) surfaces (all $p < 0.05$). The correlations between E and GAG content and CECT and E both agree with previous results using bovine cartilage [41]. The specific correlation comparisons are: E vs. GAG: $R^2 = 0.90$ (bovine femoral condyle) vs. $R^2 = 0.78$ and $R^2 = 0.63$ (murine medial and lateral tibial plateau, respectively). With CA4+, the electrostatic attraction between the contrast agent and the negatively charged GAGs again results in high contrast agent uptake in mouse cartilage and positive linear correlations between CECT attenuation and both GAG content and E. The relationship between CECT and GAG appears consistent between the medial and lateral volumes, while the relationships between E and both GAG and CECT have different slopes. The similar slopes for CECT vs. GAG are likely a result of CA4+ equilibrium partitioning being primarily influenced by GAG alone, while E is known to vary between joint surfaces [45], likely as a result of differences in other tissue constituents, including collagen density, water content, and porosity. Since our CECT technique is sensitive to GAG, it is not surprising that both CECT attenuation and GAG content have comparable correlations with E for each tibial plateau surface. However, these other tissue constituents could

explain the differences in the correlations between the medial and lateral tibial plateau surfaces for both GAG and CECT. Thus, to determine the ability of our imaging technique to predict GAG-related cartilage mechanical properties, additional predictive data sets will be required for each articular cartilage surface of interest in the desired species.

In addition to affecting the compressive stiffness of cartilage, GAG content is also known to influence cartilage coefficient of friction (μ) [41, 46-48]. GAGs contribute to the frictional performance of cartilage through both hydrostatic and elastohydrodynamic lubrication [45, 49]. Herein, negative correlations are observed between the pendulum coefficient of friction (μ) and both GAG content and CECT attenuation ($R^2 = 0.59$ and 0.69 , respectively; **Figures 4a & b**). These relationships agree with those reported using bovine cartilage plugs with three torsional coefficients of friction [41]. Specifically, for the bovine cartilage samples, the coefficient of variation ranged between 0.49 to 0.78 for GAG vs. μ (torsional) and between 0.69 and 0.79 for CECT vs. μ , while the R^2 values obtained in this study were 0.59 for GAG vs. μ (pendulum) and 0.69 for CECT vs. μ . However, in this study, the pendulum μ values ranged from 0.00025 to 0.00075, which is 2-3 orders of magnitude lower than those reported for the torsional samples (range: 0.05 to 0.4). The discrepancy in the ranges is likely due the fact that coefficients of friction are highly dependent on testing conditions [50]. The coefficients of friction reported herein are not significantly different than those reported for a murine study by Jay et al [18] (range: 0.0005 to 0.003), in which a 20-mg pendulum arm was used (compared to the 50-mg pendulum arm in this study). Of note, this study is the first report of a significant correlation between CECT attenuation and pendulum coefficient of friction, further demonstrating the potential feasibility of CECT as a useful tool for assessing cartilage GAG and, through that relationship, GAG-related mechanical properties.

Compared to indentation, pendulum, and histological evaluation, CECT provides several substantial advantages. To generate a compressive stiffness map of a sample using indentation testing, the sample must be positioned and tested for each discrete location on its surface, requiring many hours. Additionally, to compute a compressive modulus, the sample thickness at each testing site must be measured. This is commonly performed with needle-puncture [16], which is a destructive technique. Regarding pendulum testing, this technique is less destructive than indentation testing, however it would be challenging to apply in vivo, as the surrounding muscle tissue would influence joint articulation. Additionally, reliably performing the pendulum testing requires developing an apparatus and properly gripping both the femur and tibia such that the natural axis of pivot for the knee is properly maintained during artificial articulation. One CECT scan following contrast agent immersion to equilibrium yields relevant information non-destructively and faster. For histology, there are some considerable challenges and pitfalls associated with using this technique to obtain information about cartilage biochemical content. These include: 1) decalcifying the samples, which requires several days; 2) machine or personnel processing glitches that can cause irreversible sample damage; 3) difficulty sectioning samples in the correct orientation (also irreversible); 4) the tissue can tear during sectioning or fold or wash off during mounting, both resulting in loss of data; and 5) once stained, a different staining cannot be performed (in CECT, contrast agents can be washed out of samples so the samples can be subsequently immersed in a different agent). If quantitative measures (e.g., optical density) are desired from histological sections, debris on the slides can affect optical measurements, and day-to-day variability in microscope lighting and staining batches (even with the same settings or recipes) necessitate that all tissue sections be stained in the same batch and imaged in an uninterrupted sequence. Such constraints can be problematic if re-staining is required. On the

contrary, one rapid, facile CECT scan post-contrast immersion generates related information faster (~2 hrs vs. many days for histology), non-destructively, and without the risk of sample damage/loss. Additionally, histology only permits 2D sections to be obtained from samples, which cannot easily be viewed from a different orientation, while CECT enables the same 2D views (**Figure 5a & b**) analogous to Safranin-O sections (**Figure 5c & d**) in any desired plane and 3D reconstructions of samples, which can be visually sectioned in real-time in one to three planes simultaneously (**Figure 5e-h**). Therefore, the same information that quantitatively agreed with GAG content, equilibrium compressive modulus, and coefficient of friction in **Figures 3-4** can easily be viewed in any arbitrary plane or as a whole 3D structure. The 3D and non-destructive nature of CECT coupled with the encouraging results obtained in this experiment set the stage for additional CECT studies using CA4+ such as: 1) examining OA induced mouse models (e.g., surgical instability or knockout models), and 2) performing longitudinal assessment of in vivo mouse knee cartilage during OA progression.

In summary, we demonstrated the feasibility of CA4+ enhanced CT as a simple, non-destructive technique for imaging ex vivo mouse cartilage and for assessment of GAG content, and the significant correlations between CECT attenuation and GAG content, equilibrium compressive modulus, and coefficient of friction. The significant correlations observed between CECT attenuation and GAG content, equilibrium compressive modulus, and coefficient of friction agree with previous correlation results obtained for bovine cartilage GAG content [33, 35-40], equilibrium compressive modulus [33, 41], and coefficient of friction [41]. With the development of adequate species- and surface-specific phantoms and additional predictive data sets, CECT may become a valuable tool for assessing cartilage GAG composition and GAG-dependent mechanical properties. Additionally, CECT is faster, easier to perform, more flexible,

and non-destructive compared to common mechanical testing and histological methods for evaluating cartilage. Given the continued development of mouse genetic and surgical OA models, the mouse model is and will continue to be a preferred OA model. However, the practical limits of mouse knee size and cartilage volume, as well as the use of time-consuming and destructive assessment techniques, necessitate the development of new non-destructive and quantitative techniques, such as the one described herein, for evaluation of key cartilage properties of relevance to researchers and clinicians.

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COMPETING INTERESTS

The authors have nothing to disclose.

References

1. Fässler R, Schnegelsberg P, Dausman J, Shinya T, Muragaki Y, McCarthy MT, et al. Mice lacking alpha 1 (IX) collagen develop noninflammatory degenerative joint disease. *Proceedings of the National Academy of Sciences* 1994; 91: 5070-5074.
2. Hu K, Xu L, Cao L, Flahiff CM, Brussiau J, Ho K, et al. Pathogenesis of osteoarthritis-like changes in the joints of mice deficient in type IX collagen. *Arthritis and rheumatism* 2006; 54: 2891-2900.
3. Matsui Y, Iwasaki N, Kon S, Takahashi D, Morimoto J, Matsui Y, et al. Accelerated development of aging-associated and instability-induced osteoarthritis in osteopontin-deficient mice. *Arthritis and rheumatism* 2009; 60: 2362-2371.
4. Glasson SS, Askew R, Sheppard B, Carito BA, Blanchet T, Ma H-L, et al. Characterization of and osteoarthritis susceptibility in ADAMTS-4-knockout mice. *Arthritis and rheumatism* 2004; 50: 2547-2558.
5. Li J, Anemaet W, Diaz MA, Buchanan S, Tortorella M, Malfait AM, et al. Knockout of ADAMTS5 does not eliminate cartilage aggrecanase activity but abrogates joint fibrosis and promotes cartilage aggrecan deposition in murine osteoarthritis models. *Journal of Orthopaedic Research* 2011; 29: 516-522.
6. Glasson SS, Blanchet TJ, Morris EA. The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2007; 15: 1061-1069.
7. Moodie JP, Stok KS, Müller R, Vincent TL, Shefelbine SJ. Multimodal imaging demonstrates concomitant changes in bone and cartilage after destabilisation of the medial meniscus and increased joint laxity. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2011; 19: 163-170.
8. Mason RM, Chambers MG, Flannelly J, Gaffen JD, Dudhia J, Bayliss MT. The STR/ort mouse and its use as a model of osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2001; 9: 85-91.
9. Stok KS, Pelled G, Zilberman Y, Kallai I, Goldhahn J, Gazit D, et al. Revealing the interplay of bone and cartilage in osteoarthritis through multimodal imaging of murine joints. *Bone* 2009; 45: 414-422.
10. Li J, Gorski DJ, Anemaet W, Velasco J, Takeuchi J, Sandy J, et al. Hyaluronan injection in murine osteoarthritis prevents TGFbeta 1-induced synovial neovascularization and fibrosis and maintains articular cartilage integrity by a CD44-dependent mechanism. *Arthritis research & therapy* 2012; 14: R151.
11. Takamatsu A, Ohkawara B, Ito M, Masuda A, Sakai T, Ishiguro N, et al. Verapamil Protects against Cartilage Degradation in Osteoarthritis by Inhibiting Wnt/ β -Catenin Signaling. *PloS one* 2014; 9: e92699.
12. Felson DT, Lawrence RC, Dieppe PA, Hirsch R, Helmick CG, Jordan JM, et al. Osteoarthritis: new insights. Part 1: the disease and its risk factors. *Annals of Internal Medicine* 2000; 133: 635-646.
13. Maroudas A, Leaback D, Stockwell R. Glycosaminoglycan turn-over in articular cartilage [and discussion]. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences* 1975; 271: 293-313.

14. Xu L, Flahiff CM, Waldman BA, Wu D, Olsen BR, Setton LA, et al. Osteoarthritis-like changes and decreased mechanical function of articular cartilage in the joints of mice with the chondrodysplasia gene (cho). *Arthritis and rheumatism* 2003; 48: 2509-2518.
15. Glasson S, Chambers M, Van Den Berg W, Little C. The OARSI histopathology initiative—recommendations for histological assessments of osteoarthritis in the mouse. *Osteoarthritis and Cartilage* 2010; 18: S17-S23.
16. Hyttinen MM, Töyräs J, Lapveteläinen T, Lindblom J, Prockop DJ, Li SW, et al. Inactivation of one allele of the type II collagen gene alters the collagen network in murine articular cartilage and makes cartilage softer. *Annals of the rheumatic diseases* 2001; 60: 262-268.
17. Cao L, Youn I, Guilak F, Setton LA. Compressive properties of mouse articular cartilage determined in a novel micro-indentation test method and biphasic finite element model. *Journal of biomechanical engineering* 2006; 128: 766-771.
18. Jay GD, Torres JR, Rhee DK, Helminen HJ, Hyttinen MM, Cha C-J, et al. Association between friction and wear in diarthrodial joints lacking lubricin. *Arthritis and rheumatism* 2007; 56: 3662-3669.
19. Drewniak EI, Jay GD, Fleming BC, Crisco JJ. Comparison of two methods for calculating the frictional properties of articular cartilage using a simple pendulum and intact mouse knee joints. *Journal of biomechanics* 2009; 42: 1996-1999.
20. Drewniak EI, Jay GD, Fleming BC, Zhang L, Warman ML, Crisco JJ. Cyclic loading increases friction and changes cartilage surface integrity in lubricin-mutant mouse knees. *Arthritis and rheumatism* 2012; 64: 465-473.
21. Ruan MZC, Dawson B, Jiang M-M, Gannon F, Heggeness M, Lee BHL. Quantitative imaging of murine osteoarthritic cartilage by phase-contrast micro-computed tomography. *Arthritis and rheumatism* 2013; 65: 388-396.
22. Kotwal N, Li J, Sandy J, Plaas A, Sumner DR. Initial application of EPIC- μ CT to assess mouse articular cartilage morphology and composition: effects of aging and treadmill running. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2012; 20: 887-895.
23. Kerckhofs G, Sainz J, Wevers M, Van de Putte T, Schrooten J. Contrast-enhanced nanofocus computed tomography images the cartilage subtissue architecture in three dimensions. *European cells & materials* 2013; 25: 179-189.
24. Joshi NS, Bansal PN, Stewart RC, Snyder BD, Grinstaff MW. Effect of contrast agent charge on visualization of articular cartilage using computed tomography: exploiting electrostatic interactions for improved sensitivity. *Journal of the American Chemical Society* 2009; 131: 13234-13235.
25. Baumgarten M, Bloebaum RD, Ross SD, Campbell P, Sarmiento A. Normal human synovial fluid: osmolality and exercise-induced changes. *The Journal of bone and joint surgery. American volume* 1985; 67: 1336-1339.
26. Stewart RC, Bansal PN, Entezari V, Lusic H, Nazarian RM, Snyder BD, et al. Contrast-enhanced CT with a high-affinity cationic contrast agent for imaging ex vivo bovine, intact ex vivo rabbit, and in vivo rabbit cartilage. *Radiology* 2013; 266: 141-150.
27. Lakin BA, Ellis DJ, Shelofsky JS, Freedman JD, Grinstaff MW, Snyder BD. Contrast-Enhanced CT Facilitates Rapid, Non-Destructive Assessment of Cartilage and Bone Properties of the Human Metacarpal. *Osteoarthritis and Cartilage* 2015.

28. Spilker RL, Suh JK, Mow VC. A finite element analysis of the indentation stress-relaxation response of linear biphasic articular cartilage. *Journal of biomechanical engineering* 1992; 114: 191-201.
29. Stok KS, Lisignoli G, Cristino S, Facchini A, Müller R. Mechano-functional assessment of human mesenchymal stem cells grown in three-dimensional hyaluronan-based scaffolds for cartilage tissue engineering. *Journal of Biomedical Materials Research Part A* 2010; 93A: 37-45.
30. Flahiff CM, Narmoneva DA, Huebner JL, Kraus VB, Guilak F, Setton LA. Osmotic loading to determine the intrinsic material properties of guinea pig knee cartilage. *Journal of biomechanics* 2002; 35: 1285-1290.
31. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochimica et Biophysica Acta* 1986; 883: 173-177.
32. Lusic H, Grinstaff MW. X-ray-Computed Tomography Contrast Agents. *Chemical Reviews* 2013; 113: 1641-1666.
33. Bansal PN, Joshi NS, Entezari V, Grinstaff MW, Snyder BD. Contrast enhanced computed tomography can predict the glycosaminoglycan content and biomechanical properties of articular cartilage. *Osteoarthritis and Cartilage* 2010; 18: 184-191.
34. Bansal PN, Stewart RC, Entezari V, Snyder BD, Grinstaff MW. Contrast agent electrostatic attraction rather than repulsion to glycosaminoglycans affords a greater contrast uptake ratio and improved quantitative CT imaging in cartilage. *Osteoarthritis and Cartilage* 2011; 19: 970-976.
35. Bansal PN, Joshi NS, Entezari V, Malone BC, Stewart RC, Snyder BD, et al. Cationic contrast agents improve quantification of glycosaminoglycan (GAG) content by contrast enhanced CT imaging of cartilage. *Journal of Orthopaedic Research* 2011; 29: 704-709.
36. Kallioniemi AS, Jurvelin JS, Nieminen MT, Lammi MJ, Toyras J. Contrast agent enhanced pQCT of articular cartilage. *Physics in Medicine and Biology* 2007; 52: 1209-1219.
37. Palmer AW, Guldborg RE, Levenston ME. Analysis of cartilage matrix fixed charge density and three-dimensional morphology via contrast-enhanced microcomputed tomography. *Proceedings of the National Academy of Sciences of the United States of America* 2006; 103: 19255-19260.
38. Siebelt M, van Tiel J, Waarsing JH, Pijpers TM, van Straten M, Booijs R, et al. Clinically applied CT arthrography to measure the sulphated glycosaminoglycan content of cartilage. *Osteoarthritis and Cartilage* 2011; 19: 1183-1189.
39. Silvast TS, Jurvelin JS, Aula AS, Lammi MJ, Toyras J. Contrast agent-enhanced computed tomography of articular cartilage: association with tissue composition and properties. *Acta Radiologica* 2009; 50: 78-85.
40. Silvast TS, Jurvelin JS, Lammi MJ, Toyras J. pQCT study on diffusion and equilibrium distribution of iodinated anionic contrast agent in human articular cartilage - associations to matrix composition and integrity. *Osteoarthritis and Cartilage* 2009; 17: 26-32.
41. Lakin BA, Grasso DJ, Shah SS, Stewart RC, Bansal PN, Freedman JD, et al. Cationic agent contrast-enhanced computed tomography imaging of cartilage correlates with the compressive modulus and coefficient of friction. *Osteoarthritis and Cartilage* 2013; 21: 60-68.

42. Das Neves Borges P, Forte AE, Vincent TL, Dini D, Marenzana M. Rapid, automated imaging of mouse articular cartilage by microCT for early detection of osteoarthritis and finite element modelling of joint mechanics. *Osteoarthritis and Cartilage* 2014; 22: 1419-1428.
43. Hu X, Wang Q, Liu Y, Liu H, Qin C, Cheng K, et al. Optical imaging of articular cartilage degeneration using near-infrared dipicolylamine probes. *Biomaterials* 2014.
44. Martin I, Obradovic B, Freed LE, Vunjak-Novakovic G. Method for Quantitative Analysis of Glycosaminoglycan Distribution in Cultured Natural and Engineered Cartilage. *Annals of Biomedical Engineering* 1999; 27: 656-662.
45. Mow VC, Huiskes R. *Basic Orthopedic Biomechanics and Mechanobiology*. 3rd Edition. Philadelphia, Lippincott Williams & Wilkins 2005.
46. Katta J, Stapleton T, Ingham E, Jin ZM, Fisher J. The effect of glycosaminoglycan depletion on the friction and deformation of articular cartilage. *Proceedings of the Institution of Mechanical Engineers. Part H, Journal of engineering in medicine* 2008; 222: 1-11.
47. Krishnan R, Kopacz M, Ateshian GA. Experimental verification of the role of interstitial fluid pressurization in cartilage lubrication. *Journal of Orthopaedic Research* 2004; 22: 565-570.
48. Basalo IM, Raj D, Krishnan R, Chen FH, Hung CT, Ateshian GA. Effects of enzymatic degradation on the frictional response of articular cartilage in stress relaxation. *Journal of biomechanics* 2005; 38: 1343-1349.
49. Schmidt TA, Sah RL. Effect of synovial fluid on boundary lubrication of articular cartilage. *Osteoarthritis and Cartilage* 2007; 15: 35-47.
50. Gleghorn JP, Bonassar LJ. Lubrication mode analysis of articular cartilage using Stribeck surfaces. *Journal of Biomechanics* 2008; 41: 1910-1918.

FIGURE LEGENDS

Figure 1: Diffusion-in data for CA4+ into the cartilage of three mouse tibial plateaus. Data is plotted as the mean CECT attenuation of both the medial and lateral tibial plateau cartilage normalized by the mean baseline (not contrast-enhanced) attenuations (lines are for visualization purposes only). By fitting the data for each sample with an equation of the form $\text{CECT Atten.} = a * e^{-b(\text{time})} + c$, tau values (calculated as $1/b$) can be computed for each sample, and the mean tau value was found to be 6.17 ± 0.19 min (mean $\pm$ SD).

Figure 2: Standard curve correlating mean Ip Value (a measure of the red intensity from Safranin-O stained histology sections) to GAG content determined via the DMMB assay for 42 depth-wise layers obtained from six neighboring bovine cartilage plug pairs. The correlation was $R^2 = 0.93$, and statistically significant ($p < 0.05$), indicating that quantifying the red content from Safranin-O stained histology sections reflects the GAG content of the cartilage tissue.

Figure 3: Correlations for mouse medial (♦) and lateral (■) tibial plateau surfaces between: **a)** mean CECT attenuation and GAG content, **b)** indentation equilibrium modulus (E) and GAG content, and **c)** mean CECT attenuation and E. All correlations had $R^2 > 0.6$ and were statistically significant ($p < 0.05$) for both surfaces.

Figure 4: Correlations between **a)** pendulum coefficient of friction (μ) and average tibial plateau GAG content and **b)** mean tibial plateau cartilage CECT attenuation and μ for mouse knees. Both correlations had coefficients of variation greater than or equal to 0.59, and were statistically significant ($p < 0.05$).

Figure 5: Using contrast-enhanced CT, color maps can be generated in any orientation for visualization of depth-wise cartilage attenuation with the underlying bone morphology and attenuation also visible. For example, traditional sagittal CECT color maps can be generated for the medial tibial plateau samples with **a)** the highest GAG content and **b)** the lowest GAG

content found in this study, which are analogous to their corresponding Safranin-O stained histological sections (**c & d**, respectively). CECT also enables 3D color map generation that can be visually sectioned in any view independently or up to three planes simultaneously, as demonstrated by the 3D color maps for the same **e**) highest GAG and **f**) lowest GAG samples. By zooming into the regions encompassed in the red squares, it is easier to distinguish the difference in depth-wise attenuation patterns for the same **g**) high GAG and **h**) low GAG samples. Note that the dotted lines in **g** and **h** separating the articular cartilage surface (Cart. Surface), cartilage (Cart.), and bone are manually drawn for visualization purposes only, and the actual cartilage-bone and cartilage-air boundaries were found quantitatively using the region-growing algorithm in the Analyze software “Volume Edit” module. The color scale bar indicates the corresponding CECT attenuation in Hounsfield Units (HU) for all CECT color maps. The distribution of the CA4+ contrast agent matches the typical distribution of Safranin-O stain in histological sections, with a greater attenuation in the middle and deep zone for the sample with high GAG and a fairly uniform distribution for the sample with low GAG.

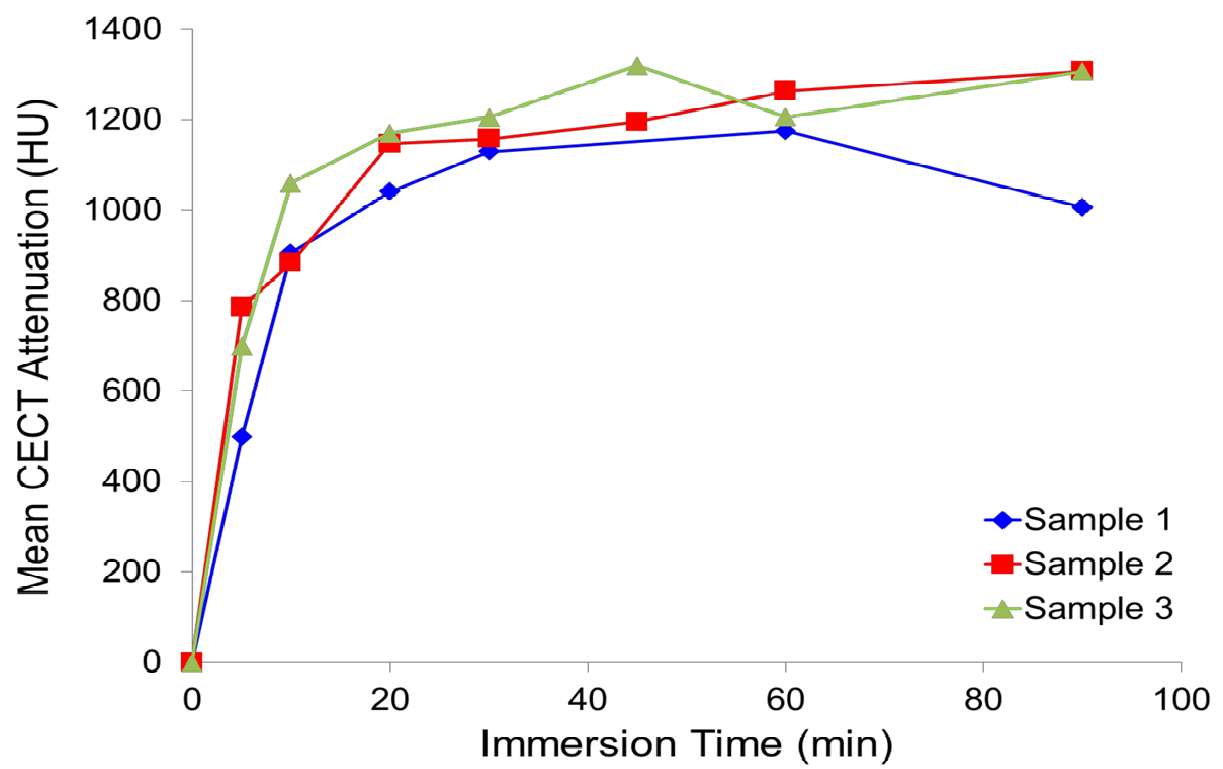


Figure1 .

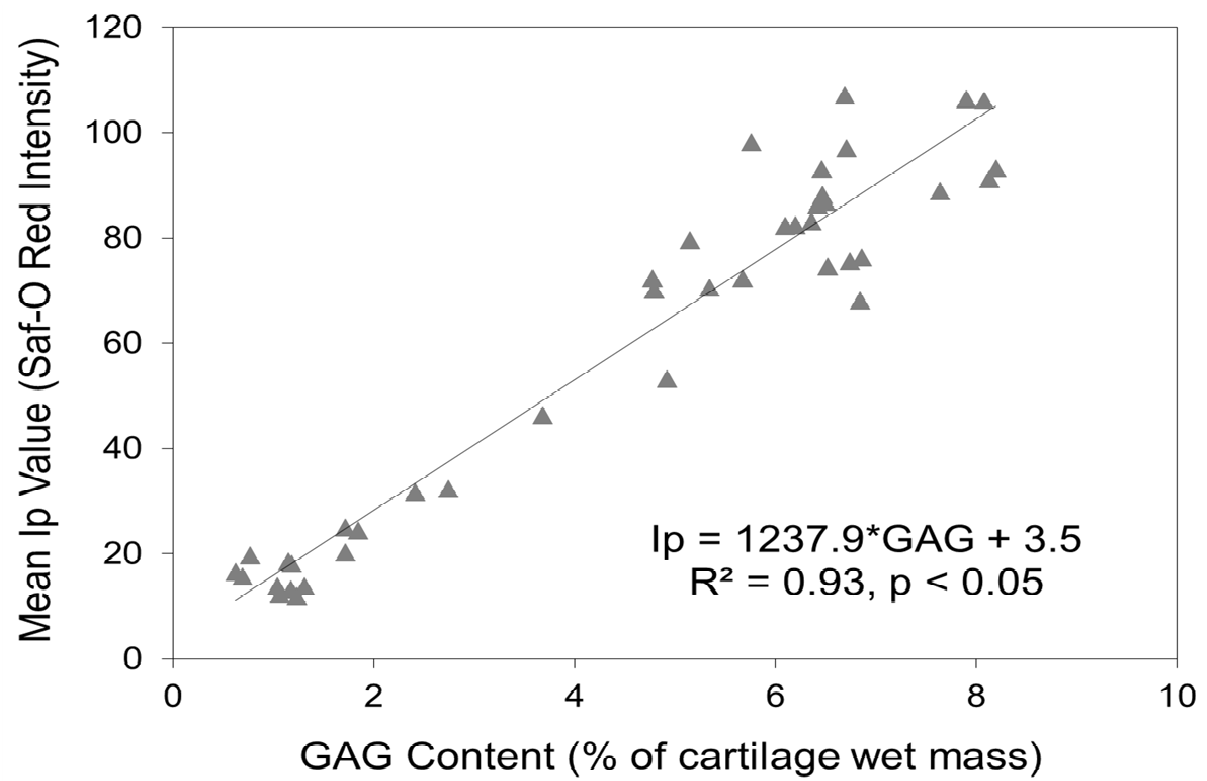


Figure2 .

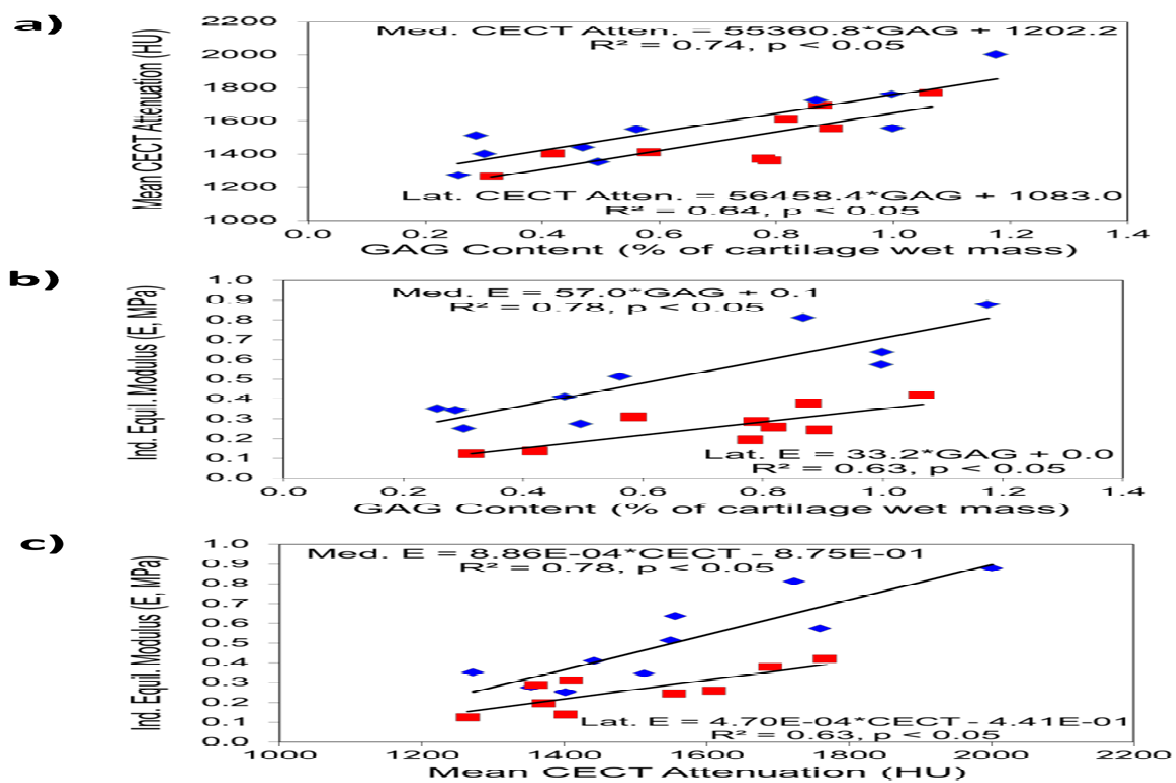


Figure3 .

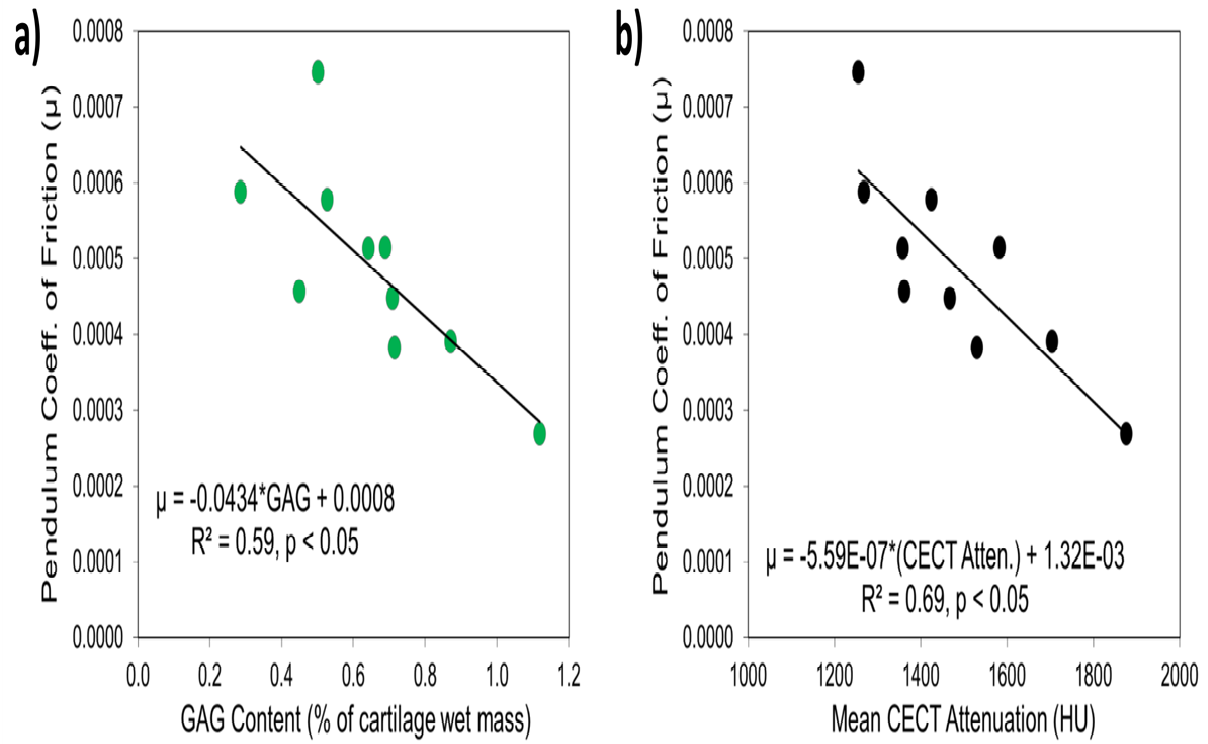


Figure4 .

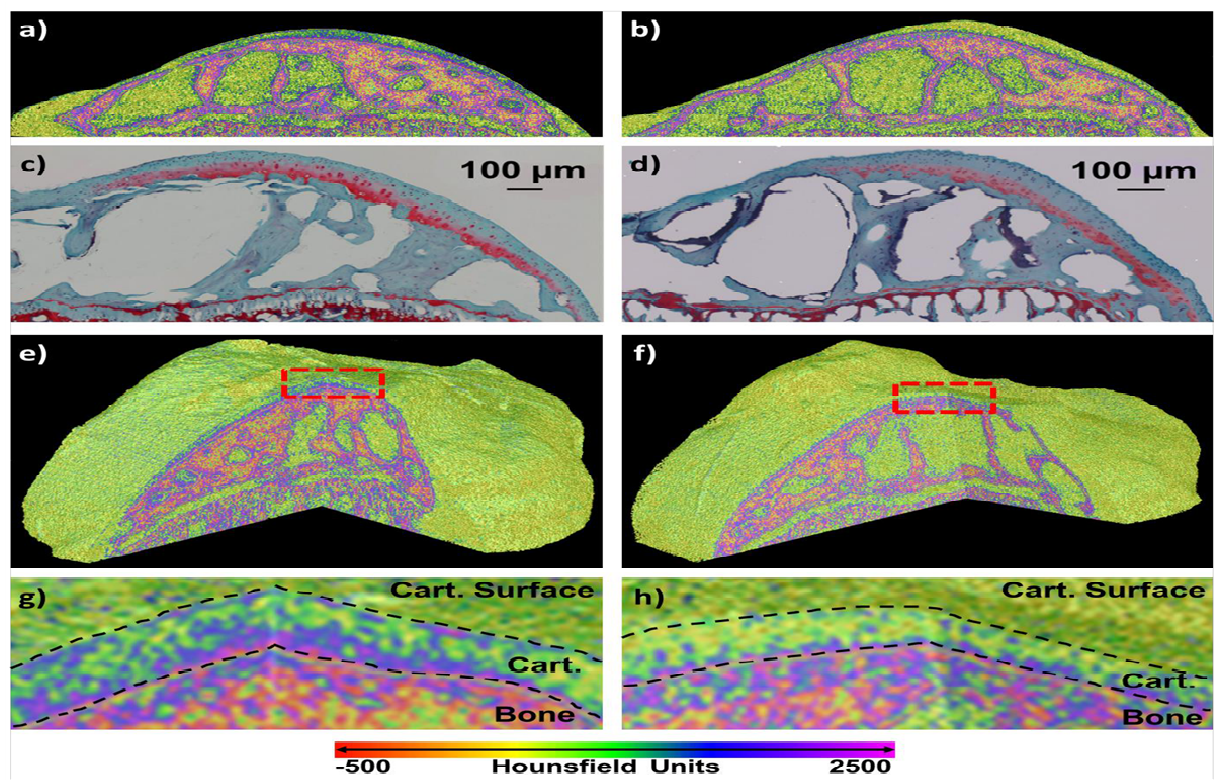


Figure5 .