Quantitative *in vivo* CT arthrography of the human osteoarthritic knee to estimate cartilage sulphated glycosaminoglycan content: correlation with *ex-vivo* reference standards

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Abstract

Objective

Recently, computed tomography arthrography (CTa) was introduced as quantitative imaging biomarker to estimate cartilage sulphated glycosaminoglycan (sGAG) content in human cadaveric knees. Our aim was to assess the correlation between *in vivo* CTa in human osteoarthritis (OA) knees and *ex vivo* reference standards for sGAG and collagen content.

Design

In this prospective observational study 11 knee OA patients underwent CTa before total knee replacement (TKR). Cartilage X-ray attenuation was determined in 6 cartilage regions. Femoral and tibial cartilage specimens harvested during TKR were re-scanned using equilibrium partitioning of an ionic contrast agent with micro-CT (EPIC-μCT), which served as reference standard for sGAG. Next, cartilage sGAG and collagen content were determined using dimethylmethylene blue (DMMB) and hydroxyproline assays. The correlation between CTa X-ray attenuation, EPIC-μCT X-ray attenuation, sGAG content and collagen content was assessed.

Results

CTa X-ray attenuation correlated well with EPIC-μCT (r=0.76, 95% credibility interval (95%CI) 0.64 to 0.85). CTa correlated moderately with the DMMB assay (sGAG content) (r=-0.66, 95%CI -0.87 to -0.49) and to lesser extent with the hydroxyproline assay (collagen content) (r=-0.56, 95%CI -0.70 to -0.36).

Conclusions

Outcomes of *in vivo* CTa in human OA knees correlate well with sGAG content. Outcomes of CTa also slightly correlate with cartilage collagen content. Since outcomes of CTa are mainly sGAG dependent and despite the fact that further

validation using hyaline cartilage of other joints with different biochemical composition should be conducted, CTa may be suitable as quantitative imaging biomarker to estimate cartilage sGAG content in future clinical OA research.

Keywords:

CT arthrography; sulphated glycosaminoglycan content; knee osteoarthritis; articular cartilage; clinical research; translational study

Running title:

CT arthrography of human cartilage sGAG

Introduction

Knee osteoarthritis (OA) is the most common joint disease in middle-aged and elderly, causing serious morbidity and large socio-economic impact [1, 2]. Since no definitive treatment options other than joint replacement surgery in end stage OA are available, research focuses on development of disease modifying osteoarthritis drugs (DMOADs) which may be effective in early OA, for example by improving cartilage biochemical composition [3, 4].

To non-invasively monitor effectiveness of these novel interventions on cartilage biochemical composition, imaging techniques are essential. Therefore, quantitative imaging assessing important cartilage composites i.e. sulphated glycosaminoglycan (sGAG) and collagen, became of interest [5].

Most imaging techniques applied in clinical research are magnetic resonance imaging (MRI) based, e.g. delayed gadolinium-enhanced MRI of cartilage (dGEMRIC) for analyzing sGAG content [6] and T2-mapping for analyzing collagen

content [7]. Computed tomography (CT) based techniques have also been developed, but are mainly applied in *in vitro* or animal research. Examples are: equilibrium partitioning of an ionic contrast agent using micro-CT (EPIC-μCT) and μCT arthrography to estimate sGAG content [8-14].

Recently, a clinically applicable protocol for CT arthrography (CTa) was introduced as a potential alternative technique to MRI based estimate of cartilage biochemical composition [15]. Outcomes of *ex vivo* CTa applied in human cadaveric knee joints were shown to strongly correlate with cartilage sGAG content based on the inverse relation between the negatively charged sGAG and the ionic contrast agent used, similar to the working mechanism of dGEMRIC. [15]. However, outcomes of CTa were also dependent on integrity of the collagen network of cartilage, which influences the speed of contrast influx into cartilage [15]. Although CTa was already applied *in vivo* by comparing its outcomes to dGEMRIC and cartilage morphology observed during arthroscopy [16, 17], these studies did not assess the correlation between CTa and reference standards for cartilage biochemical composition and were not performed in knee OA patients which constitute an important target population for quantitative imaging techniques for cartilage composition.

The aim of this study was to assess the correlation between *in vivo* CTa in human knees with OA and *ex vivo* reference standards for sGAG and collagen content.

Methods

Study design and participants

For this prospective observational study, conducted between October 2012 and December 2013, all consecutive patients scheduled for total knee replacement (TKR) at our institution were approached.

The inclusion criteria were: age ≥ 18 years and radiographic knee OA with asymmetric distribution and a maximum of grade 1-2 (doubtful or definite osteophyte formation without definite joint space narrowing) according to the Kellgren & Lawrence (KL) grading system [18] in the least affected tibiofemoral compartment. We chose to include only these patients to be sure that we captured a relatively wide range of cartilage quality and therefore also sGAG content of the articular cartilage. Exclusion criteria were: glomerular filtration rate < 60 ml/min, previous reactions to CT contrast agent and co-morbidities in the ipsilateral lower extremity precluding exercise after contrast administration.

We performed a power analysis in which we used the Fisher transformation [19] to assess the number of measurements needed to establish a correlation coefficient of at least 0.7 (considered a good correlation [20]) with a predefined 95% confidence interval width of 0.5 - 0.9, and found that 25 measurements would be needed. Since six measurements are performed per participant, three participants would be enough for our study. Because we considered this number very low, we decided to include at least 10 participants (60 measurements for the correlation analyses) until the end date of the study (December 2013).

The study was approved by the institutional review board of our institution (MEC-2012-218) and written informed consent was obtained from all participants.

Acquisition of CT arthrography

CTa was performed four weeks before TKR. This time window was chosen to allow detection of infection caused by the intra-articular injection well before surgery. Patients were positioned in a supine position and after disinfection, 15 ml 30% ioxaglate (Hexabrix 320, Mallinckrodt, Hazelwood, USA) and 70% 1% phosphate buffered saline (PBS) solution was injected intra-articularly using a 21 gauge needle [15] and a superolateral approach [21]. We first aspirated synovial fluid from the knee in order to confirm that the needle was positioned in the knee joint and to ensure that we could inject the 15 ml of contrast dilution while minimizing further dilution due to extensive joint effusion. To promote contrast distribution throughout the joint, participants actively exercised their knee for two minutes over the full possible range of motion immediately after the injection.

Ten minutes post-injection, CT in the axial plane was acquired using a dual-source multidetector spiral CT scanner (SOMATOM Definition Flash, Siemens Healthcare AG, Germany). We used a tube voltage of 80 kV, units of current of 3140 mAs, pitch of 0.35 and collimation of 32 x 0.6 mm [15]. Scan time was approximately 30 seconds. These parameters resulted in an effective radiation dose of 0.4 millisievert (mSv) and an effective skin dose of 120 milligray (mGy) which is well below the threshold of 1000 mGy above which deterministic effects on the skin are expected [22].

All scans were reconstructed in the sagittal plane with an effective slice thickness of 0.75 mm and a sharp reconstruction kernel. Multiplanar reconstruction was performed resulting in an image voxel size of 0.265 by 0.265 mm, e.g. an in-plane resolution of 512 x 512 voxels.

Analysis of CT arthrography

Reconstructed datasets were segmented into binary datasets using a local attenuation threshold algorithm (3D-Calc, Skyscan, Kontich, Belgium) (**Figure 1A-D**) [10, 23]. These binary datasets (**Figure 1C-D**) were used to manually draw six cartilage regions of interest (ROIs): posterior non-weight-bearing femoral cartilage (pFC) (**Figure 1E**), weight-bearing femoral cartilage (wbFC) (**Figure 1F**) and weight-bearing tibial cartilage (wbTP) of the medial and lateral tibiofemoral compartment (**Figure 1G**). Each ROI consisted of 40 contiguous slices and was manually drawn by a researcher with four years of experience in musculoskeletal imaging (JvT) using CT Analyser software (Skyscan, Kontich, Belgium). In each ROI, mean cartilage X-ray attenuation was calculated using CT Analyser.

Harvesting of cartilage and acquisition of EPIC-µCT

During TKR, weight-bearing and non-weight-bearing femoral cartilage and weight-bearing tibial cartilage with adjacent subchondral bone were harvested, stored in saline and transported directly to the laboratory.

We used EPIC-µCT as reference standard for cartilage sGAG content since its outcomes have a good correlation with cartilage sGAG content [8, 9, 14]. Similar to CTa, EPIC-µCT provides information on sGAG distribution of cartilage within the entire cartilage volume, allowing analysis of articular cartilage regions exactly matching the cartilage ROIs analyzed with CTa.

Between 30 minutes and 1 hour after surgery, all specimens were removed from the saline and incubated in ioxaglate solution for 24 hours at room temperature [24-26]. A 20% ioxaglate with 80% PBS 1% solution was used since this results in optimal cartilage segmentation at the air/cartilage and bone/cartilage interfaces [15]. The contrast solution also contained Ethylenediaminetetraacetic acid (EDTA) (Sigma

Aldrich, St Louis, USA) and protease inhibitors (Roche, Basel, Switzerland) to prevent sGAG removal from the specimen during incubation.

EPIC-μCT was performed using a Skyscan 1076 (Skyscan, Kontich, Belgium) with the following scan settings: isotropic voxel size of 35 μm; voltage of 95 kV; current of 100 mA; field of view 68 mm with a 1.0 mm aluminum / 0.25 mm copper filter over 198^{o} with a 0.36 degree rotation step. Plastic foil was wrapped around the specimen to avoid dehydration during scanning. Depending on the size of the specimen, scan time was 0.5 – 1.5 hours. The datasets were reconstructed identically using NRecon software (Skyscan, Kontich, Belgium).

Analysis of EPIC-μCT

To enable comparison of corresponding cartilage regions, EPIC-μCT datasets were registered to CTa datasets with Multimodality Image Registration using Information Theory (MIRIT, University of Leuven) [27]. This automated registration algorithm uses a rigid transformation model (translations and rotations) and uses mutual information as a similarity measure for the registration of the μCT datasets to the CT datasets. Next, using CT Analyser software, datasets were segmented into binary datasets using a previously determined fixed attenuation threshold (25 gray values for air and 120 gray values for subchondral bone) [15]. In the segmented μCT datasets, cartilage ROIs corresponding with ROIs of CTa were drawn and mean X-ray attenuation was calculated.

Biochemical cartilage analyses

After acquisition of EPIC-μCT, four (posterior femoral cartilage), six or eight (weight-bearing femoral and plateau cartilage based on the size) full thickness

cartilage explants of 6 mm diameter were taken using a biopsy punch from standardized locations corresponding with cartilage of the ROIs analyzed with CTa and EPIC-μCT. Location and number of cartilage explants were chosen to ensure representative cartilage samples in each ROI.

Since ioxaglate used for EPIC-μCT might interact with biochemical assays (pilot tests, data not shown), explants were washed at room temperature for 24 hours in 1% PBS. During washing, EDTA and protease inhibitors were added to prevent sGAG loss from cartilage. Next, explants were cut in halves and stored separately in airtight tubes at -20 °C together with the washing solution.

Before biochemical analyses were performed, explants were thawed at room temperature. One half of each explant was digested in papain solution containing 250 μg/ml papain and 5 MM l-cytein HCl overnight at 60 ºC. sGAG content in cartilage and in the washing solution of the matching explant was quantified with the 1,9dimethylmethylene blue (DMMB) dye binding assay at pH 3 described by Farndale *et al.* [28]. Absorption ratios at 540nm and 595 nm were used to calculate sGAG content using chondroitin sulphate (Sigma Aldrich, St Louis, USA) as standard. Total sGAG content in explant and washing solution was calculated to correct for possible loss of sGAG during washing.

The other half of each explant was used to quantify collagen content based on the hydroxyproline content according to Bank *et al.* [29]. Samples were digested with alpha-chymotrypsin followed by a papain solution and digests were hydrolyzed with equal volumes 12M HCl at 95 ºC overnight. Samples were then dried and re-dissolved in water. Hydroxyproline content was measured using a colorimetric method with chloramine-T and dimethylaminobenzaldehyde as reagents and hydroxyproline as

standerd (Merck, Darmstadt, Germany) at extinction 570 nm. Values of degraded and intact collagen content were summed, resulting in total collagen content per explant.

Next, for each ROI four to eight explants were used to calculate the mean sGAG and collagen content by averaging the content of the explants taken from that specific ROI. The mean sGAG and the mean collagen content of a specific cartilage ROI could then be correlated with the mean CT and μCT attenuation in the matching ROI.

Statistical analysis

To assess the correlation between CTa and reference tests (EPIC-μCT, sGAG content and collagen content), a four-dimensional multivariate mixed-effects model was applied. In this model, it is assumed that the CTa and the reference tests are multivariately normally distributed (i.e. $Y \sim N_4(\mu,\Sigma)$, where Y = (CTa, EPIC-μCT, sGAG content, collagen content); μ and Σ are the mean vector (i.e. $\mu = (\mu_1 = \text{CTa}, \mu_2 = \text{EPIC-}\mu\text{CT}, \mu_3 = \text{sGAG content}, \mu_4 = \text{collagen content})$) and covariance matrix of these variables, respectively. To take into account potential intrinsic correlation between outcomes of different ROIs within one participant, a random intercept was included in the model (e.g. $\mu_{1,i.j.} = \beta_1 + b_{1,I}$; i = 1, … , 11, j; j= 1, …, 62).

Pearson's correlation coefficients of CTa and each reference test were extracted from the results of this model. For each Pearson's correlation coefficient the 95% credibility interval (95%CI) was calculated. To assess goodness-of-fit, we used an omnibus posterior predictive check (PPC) [30]. We computed a Bayesian p-value with extreme values of this p-value (e.g., < 0:05 or > 0:95) indicating a poor fit of the model to the data [30].

To assess if the correlation coefficients calculated within the model were significantly different, we calculated the contour probability of the correlations. For these values, similar to the Bayesian p-value, extreme values, i.e. <0.05 or >0.95, indicate that there is a statistically significant difference between two correlation coefficients [31].

An additional univariate mixed-effects regression analysis was performed to estimate the capability of *in vivo* CTa to predict outcomes of *ex vivo* EPIC-µCT (thus sGAG content). In this analysis, we modeled EPIC-µCT outcomes based on CTa measurements, using random effects to capture heterogeneity between patients, and predicted the EPIC-µCT outcomes and their 95%CI for all cartilage regions.

All analyses were performed using a Bayesian approach with Markov chain Monte Carlo (McMC) sampling using WinBugs [32].

Results

Participants

Fourteen patients were included. Two participants were excluded because their TKR was postponed after inclusion, in one participant ioxaglate was injected extra-articularly and four cartilage specimens (two weight-bearing cartilage specimens of the medial tibial plateau, one posterior non-weight-bearing cartilage specimen of the lateral femoral condyle and one weight-bearing cartilage specimen of the medial femoral condyle) were severely damaged during surgery and were therefore excluded from the analysis. Therefore, results are based on data of 11 participants (5 women and 6 men, 7 left and 4 right knees).

The mean age with standard deviation was 64 ± 7 years and their mean body mass index with standard deviation was 33 ± 6 kg/m^2. The KL grades in the medial

tibiofemoral compartments were 3 or 4 in seven participants and 1 or 2 in four participants. KL grades in the lateral tibiofemoral compartments were 1 or 2 in nine participants and 3 in two participants. We did not observe any adverse reactions related to the intra-articular contrast injections.

Correlation of CTa, EPIC-µCT and biochemical cartilage analyses

For the applied four-dimensional mixed-effects model, the Bayesian p-value of the PPC was 0.52, which indicates that the model assumptions appear to be satisfied.

Mean CTa X-ray attenuation in all femoral and tibial cartilage ROIs correlated well with attenuation of EPIC-µCT (r=0.76, 95%CI 0.64 to 0.85; **Figure 2A**). When each ROI was analyzed separately, the range of correlation coefficients between outcomes of CTa and EPIC-µCT was 0.75 to 0.80.

The correlation between CTa and sGAG content measured using the DMMB assay was moderate (r=-0.66, 95%CI -0.87 to -0.49; **Figure 2B**). A range of -0.75 to -0.60 was observed for the correlation coefficients between X-ray attenuation of CTa and sGAG content in all separate cartilage ROIs.

The correlation between outcomes of CTa and collagen content measured using the hydroxyproline assay was also moderate (r=-0.56, 95%CI -0.70 to -0.36; **Figure 2C**). Here, a range of correlation coefficients from -0.56 to -0.51 was obtained for each separate ROI.

Mean EPIC-µCT outcomes and sGAG content measured using the DMMB assay correlated well (r=-0.81, 95%CI -0.87 to -0.69; **Figure 2D**). The range of correlation coefficients for each separate ROI was -0.82 to -0.75.

By calculating the p-values of the contour probability of the different correlations we observed that the correlation between CTa and EPIC-µCT was

significantly different from the correlation between CTa and sGAG or collagen content (contour probability > 0.99). The correlation between EPIC-μCT and sGAG content was significantly different from the correlation between EPIC-μCT and collagen content (contour probability = 0.002). The other correlation coefficients did not differ significantly from each other.

The matched images of CTa, EPIC-μCT and histology (visual representation of sGAG content using Safranin-O staining) representing cartilage with relatively high and low sGAG content shown in **Figure 3** confirmed the good correlation between CTa and EPIC-μCT and cartilage sGAG content.

The additional univariate mixed-effects regression analysis to estimate the capability of CTa to predict EPIC-μCT showed that the 95%CIs of the predicted EPIC-μCT outcomes overlap with all of the observed outcomes of CTa, indicating good predictive performance (**Figure 4**).

Discussion

Quantitative imaging biomarkers that non-invasively estimate cartilage biochemical composition are essential for development and monitoring of DMOADs in OA. This study was performed to assess the correlation between *in vivo* CTa in human OA knees and *ex vivo* reference standards for sGAG and collagen content.

Our results show a good correlation between X-ray attenuation of CTa and EPIC-μCT, a good predictive performance of CTa for EPIC-μCT outcomes, and a somewhat less pronounced correlation between CTa and cartilage sGAG content determined by the DMMB assay. These results are in agreement with previous research showing a good correlation between outcomes of CTa acquired in *ex vivo* human cadaveric knee joints and EPIC-μCT [15]. The results are also consistent with several previous *in vitro*

studies examining the correlation between contrast-enhanced (micro)CT and the sGAG content of articular cartilage [8, 9, 14]. Therefore, we believe that CTa X-ray attenuation may be used as a quantitative estimate for sGAG content of articular cartilage in future clinical OA research.

The difference in strength of correlation between CTa and sGAG content measured using EPIC-μCT versus DMMB assay might be caused by the fact that the attenuation of EPIC-μCT and cartilage sGAG content are well correlated, but not by a linear relationship. This indicates that, although not fully specific, EPIC-μCT is good reference test for cartilage sGAG content. Another explanation for the difference in strength of correlation may be that the ROIs in CTa and EPIC-μCT were matched exactly by image registration while the DMMB assay was limited to assessment of sGAG content in representative cartilage explants that did not correspond exactly with the cartilage volume of the imaging ROIs. We chose this approach since we considered it to be reliable to analyze representative focal cartilage explants taken from standardized locations out of the cartilage ROIs analyzed using CTa and EPIC-μCT. Since there were no large spatial differences in sGAG distribution within cartilage ROIs in EPIC-μCT (data not shown), we are convinced that this did not influence our results compared to analyzing total cartilage ROIs using the DMMB assay.

An import remark with regard to the interpretation our results is the fact that the observed good correlation between CTa and EPIC-μCT does not automatically imply that both tests have equal or comparable diagnostic capacity. Calculation of diagnostic performance statistics such as sensitivity, specificity, positive predictive value and negative predictive value requires the availability of threshold values that are indicative for disease (in our study OA). Although sGAG content is diminished in

OA, no threshold values exist for sGAG content in relation to diagnosis of OA. Despite the absence of these analyses, but because of the moderate to strong correlation between outcomes of CTa and cartilage sGAG content and the good predictive performance of CTa for EPIC-μCT (thus sGAG) outcomes, we consider CTa as a worthwhile quantitative estimator of cartilage sGAG content in future clinical research.

We found a moderate correlation between outcomes of CTa and collagen content of cartilage measured using the hydroxyproline assay. This result could potentially be explained by a strong relation between collagen and sGAG content of cartilage and a concomitant loss of sGAG and collagen in the OA process. Cartilage sGAG and collagen content were, however, only weakly correlated in our study (r=0.40, data not shown). This indicates that, in addition to sGAG content, the integrity of the collagen network also influences contrast influx into cartilage as suggested in previous *ex vivo* research [15]. It is important to note that in CTa, there is no equilibrium between cartilage sGAG content and the contrast agent because CTa images are acquired already 10 minutes after contrast administration. This is unlike EPIC-μCT in which cartilage is incubated in contrast agent for 24 hours [8, 9, 14]. Therefore, measurements from non-equilibrium CTa are also influenced by other factors than sGAG content alone [24-26]. In particular, the collagen network of the extracellular matrix of the cartilage, which determines the permeability of the cartilage, influences the diffusion rate of contrast agent into the cartilage besides its sGAG content [33, 34]. Contrast diffusion goes slowly in healthy cartilage, in which an intact collagen network and densely packed collagen parallel to the cartilage surface result a relative low permeability of the cartilage [35, 36]. When the collagen network is impaired, e.g. in case of loss of collagen content, cartilage permeability increases,

resulting in higher diffusion rate of contrast into the cartilage. The influence of collagen content of cartilage on CTa outcomes, however, is less pronounced compared to the influence of cartilage sGAG content since the correlations were significantly different. This suggests that, although not totally sGAG specific, CTa may be considered a useful imaging biomarker to estimate cartilage sGAG content in future human OA research.

CTa might be a worthwhile quantitative biochemical cartilage imaging technique in future clinical research additional to contrast-enhanced MRI based techniques used for the same purpose. CTa has a relatively fast acquisition time and can be acquired already ten minutes after contrast administration, while the delay between intravenous contrast administration in knee dGEMRIC is at least 1.5 hours [(37)]. This makes CTa more patient friendly and clinically feasible than MRI. Moreover, in the generally middle-aged or elderly OA population, the relative long acquisition time of MRI compared to CT (minutes versus seconds) and the number of patients with possible contra-indication for MRI (for example non MRI compatible implants) may favor CTa as an alternative to MRI in clinical OA research [(38)]. CTa might also be applicable as imaging biomarker for cartilage biochemical composition in large cohort studies since it is relatively cheap and widely available [(39)].

Potential limitations of CTa include concerns of ionizing radiation. The effective radiation dose used for CTa as presented in this paper (0.4 mSv) is four times higher than a regular CT of the knee [(40)]. However, it has been shown that CTa acquired using only 10% of this dose also has a good correlation with cartilage sGAG content *ex vivo* in cadaveric knees [(41)]. Besides, active knee flexion and extension may be impossible for the full range of motion for OA patients, resulting in variations in contrast concentration across the knee joint. Recent research by Silvast *et al.*, however, shows that

differences in contrast concentration do not influence the speed of contrast influx into cartilage and would therefore not influence the reliability of CTa outcomes [42]. Finally, although not observed in our study and also not reported in other studies applying CTa *in vivo* in humans [16, 17], the intra-articular injection introduces the risk of infection and increases the risk of knee pain after injection. It may be worthwhile to perform fluoroscopic-guided intra-articular injections in future research with CTa since this may overcome the problem of extra-articular contrast agent deposition, which happened in one of our study participants, however against increased costs and logistic complexity of the procedure.

Based on our results and despite the potential drawbacks we propose that CTa may be applicable in future clinical OA research as an estimate for cartilage sGAG content in cross-sectional study designs. Of course, more research is needed, particularly to assess reproducibility in OA patients before CTa could be applied in longitudinal studies. Such a reproducibility study might also benefit from including more participants and different delays between contrast administration and CT acquisition to assess if this influences the correlation between CTa and cartilage composition in full thickness ROIs. In addition, a depth-wise analysis to assess the effect of different concentrations of cartilage composites throughout the extracellular matrix and across the cartilage layer would be interesting, possibly include patellar cartilage, which is thicker and has been shown to have a different composition than femoral and tibial cartilage [43]. Further studies will also need to be performed to assess the capability of CTa to serve as a predictive tool, for example for OA progression or clinical OA symptoms. Also, assessing the capability of CTa to estimate cartilage sGAG and collagen content in fibrocartilage or cartilage of other joints could be of interest to assess the influence of the differences in cartilage composition on the diffusion of contrast agent into the cartilage. Nowadays, OA

is considered a whole joint disease in which not only cartilage, but also subchondral bone, menisci and synovium play important roles in disease development and progression [44]. The simultaneous analysis of cartilage and subchondral bone was also described previously in *in vitro* studies using contrast-enhanced μCT [45, 46]. It would also be worthwhile to assess the ability of CTa to evaluate cartilage and meniscus composition within one examination. Simultaneous analysis of cartilage and meniscus composition has recently been described for contrast-enhanced MRI [47, 48] and contrast-enhanced CT *in vitro* [49, 50]. Finally, future research might assess the possibility of injecting the contrast agent intravenously instead of intra-articularly to make the technique more patient friendly. This dGEMRIC like approach will, however, be challenging because the intra-articular contrast is also used for the purpose of cartilage segmentation. Moreover, an intravenous approach requires a longer delay between contrast administration and acquisition of the CT scan.

In conclusion, our study shows that when applied *in vivo* in human OA knees, X-ray attenuation of CTa correlates well with sGAG content. Outcomes of CTa also slightly correlate with cartilage collagen content. Since outcomes of CTa are mainly sGAG dependent and despite the fact that further validation using hyaline cartilage of other joint with different biochemical composition should be conducted, CTa may be suitable as quantitative imaging biomarker to estimate cartilage sGAG content in future clinical OA research.

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Contributions

All authors have made substantial contributions to (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Specific contributions are:

(1) The conception and design of the study: JvT, MS, MR, PKB, JHW, JV, HW, EO

(2) Acquisition of data: JvT, PKB

(3) Analysis and interpretation of data: JvT, MS, MR, JHW, KN, EO

(4) Drafting the article: JvT, MS, EO

(5) Revising the article critically for important intellectual content: JvT, MS, MR, PKB, JHW, AMZ, KN, GvO, JV, GPK, HW, EO

(6) Final approval of the version submitted: JvT, MS, MR, PKB, JHW, AMZ, KN, GvO, JV, GPK, HW, EO

(7) Statistical expertise: JvT, MR, JHW, KN

(8) Obtaining of funding: JvT, JV, GPK, HW, EO

(9) Administrative, technical, or logistic support: JvT, MS, JHW, KN,

(10) Collection and assembly of data: JvT, MS, PKB, JHW, AMZ, KN

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Competing interests

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647 **Figure Legends**

648 Figure 1: Regions of interest in CTa and EPIC-μCT datasets

649 Representative sagittally reconstructed images of a medial and lateral compartment of
650 a knee joint which underwent CTa (A-B). After segmentation into a binary datasets,
651 the different regions of interest are shown in 2D (C-D) and in a 3D representation: the
652 posterior non-weight-bearing cartilage of the femoral condyles (pFC) (E), the weight-
653 bearing cartilage of the femoral condyles (wbFC) (F) and the weight-bearing cartilage
654 of the tibial plateaus (wbTP) (G). After image registration, the same ROIs were
655 analyzed in EPIC-μCT datasets.

656

657 Figure 2: Correlation plots of CTa, EPIC-μCT and ex vivo reference standards for
658 sGAG and collagen content of articular cartilage

659 Correlation plots of mean attenuation of CTa in all anatomical ROIs with EPIC-μCT
660 attenuation (A), sGAG content of the cartilage measured with DMMB assay (B),
661 collagen content of the cartilage measured with hydroxyproline assay (C) and mean

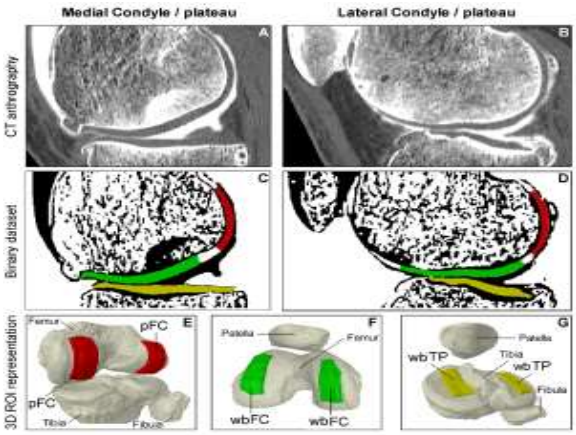
attenuation of EPIC-μCT and sGAG content measured with DMMB assay (D). The dashed lines indicate the 95% credibility interval of the Pearson's correlation coefficient.

Figure 3: Cartilage sGAG content estimated using CTa, EPIC-μCT and histology

Representative images of matching sagittal slides of CTa, EPIC-μCT and histology (Appendix 1, which is available online, provides the methods used for preparation and staining of the bone-cartilage specimen with safranin-O). The attenuation of cartilage is visualized in color: A high attenuation represents a low sGAG content of cartilage and a low attenuation represents a high sGAG content. A high intensity of safranin-O staining on histology represents a high sGAG content and a low intensity or discoloration represents a low or absent sGAG content. The top row shows visual agreement in high cartilage sGAG content and the bottom row shows visual agreement for low cartilage sGAG content.

Figure 4: Capability of in vivo CTa to predict outcomes of ex vivo EPIC-μCT.

Filled circles are observed outcomes of EPIC-μCT and the non-filled circles are predicted EPIC-μCT outcomes based on CTa outcomes. It is clearly visible that the 95%CI of the predicted EPIC-μCT outcomes overlap with all of the observed outcomes of CTa, which indicates that CTa is able to predict outcomes of EPIC-μCT and therefore cartilage sGAG content.


Medial Condyle / plateau
Lateral Condyle / plateau
CT arthrography
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3D ROI representation
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Patella
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Tibia
wb TP
Fibula

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687 Fig.1

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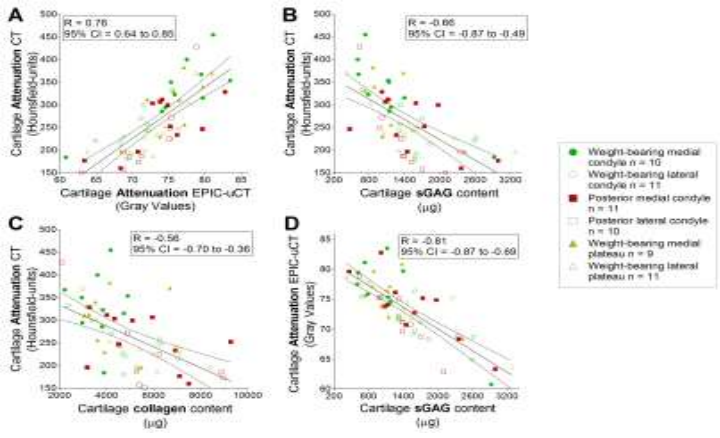

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691 fig.2

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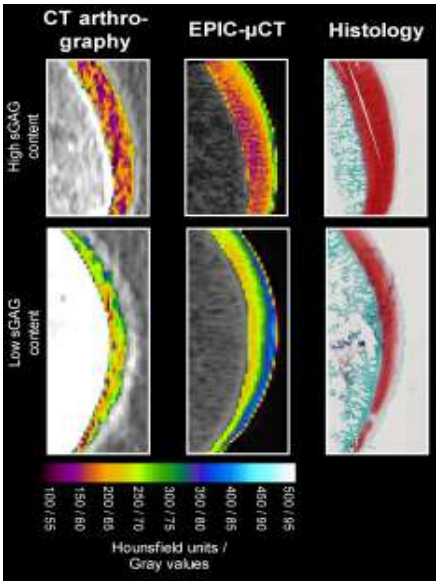

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695 fig.3

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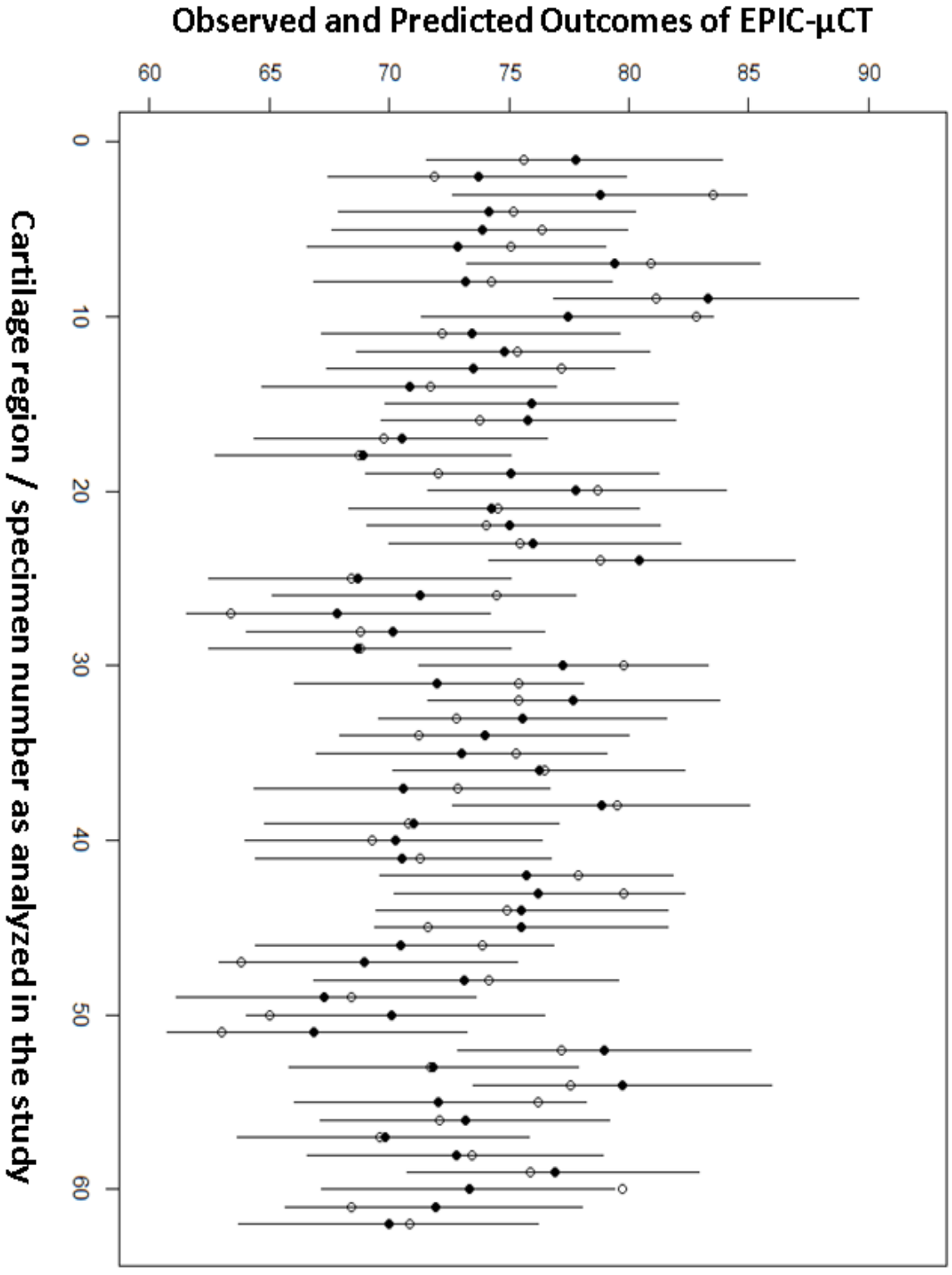
Observed and Predicted Outcomes of EPIC-µCT
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Cartilage region / specimen number as analyzed in the study

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699 fig.4