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Increased urinary excretion of C-telopeptides of type II collagen (CTX-II) predicts cartilage loss over 21 months by MRI

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Summary

Objective: Osteoarthritis (OA) is characterized by increased bone and cartilage metabolism leading to joint damage. The urinary excretion of C-telopeptides of type II collagen (CTX-II) has earlier predicted progression in radiographic OA (ROA) – useful for participant selection in clinical studies of potential disease modifying OA drugs (DMOADs). We investigated the longitudinal interrelationship between CTX-II and knee cartilage volume quantified from magnetic resonance imaging (MRI).

Methods: We followed 158 subjects [48% females, 36 with knee ROA at baseline (BL)] for 21 months. The Kellgren and Lawrence (KL) index and joint space width were assessed from radiographs (acquired load-bearing, semi-flexed). MRI scans were acquired from a 0.18 T Esaote scanner (40° flip angle (FA), TR 50 ms, TE 16 ms, scan time 10 min, resolution 0.7 mm × 0.7 mm × 0.8 mm) and medial tibial and femoral cartilage volume was quantified. Radiographs and MRI were acquired at BL and follow-up. Fasting morning urine samples (second void) were collected for BL CTX-II measurement.

Results: CTX-II was 56% higher in ROA subjects ($P = 0.0001$). In addition, elevated BL CTX-II was associated with radiographic progression (by KL or joint space narrowing) although not statistically significant. Contrarily, elevated BL CTX-II predicted longitudinal cartilage loss by MRI (middle/high tertiles had odds ratios 4.0/3.9, $P < 0.01$) corresponding to 3.1% increased yearly cartilage loss.

Conclusion: Prognostic markers in study selection criteria must ensure that placebo-treated participants progress to enable efficacy demonstration. And efficacy markers must allow progression detection within the study period. Our results support applying CTX-II for selection of high risk subjects and applying the fully automatic MRI-based framework for quantification of cartilage loss.

Key words: Collagen type II, Cartilage, Volume, MRI, Prognostic.

Introduction

Osteoarthritis (OA) is a slow, chronic disease characterized by degradation of articular cartilage, leading to joint space narrowing (JSN), loss of joint mobility, pain, and eventually joint replacement. At present, no documented, effective treatment is available [recent trials of potential disease modifying osteoarthritis drugs (DMOADs) have shown no convincing results[1–4]], which may both be a result of lack of understanding of the disease but in addition lack of appropriate means of disease assessment[5–7].

Development of structure modifying drugs may be facilitated by the availability of appropriate biomarkers, which can be integrated into all steps in the development process[7,8]. The current gold standard JSN is slow and heterogeneous, and traditionally assessed from radiographs. However, the slow longitudinal progression in JSN – relative to the variation – does not allow for small sample sizes which are optimal for short term proof-of-concept phase II clinical trials. Therefore, to reduce both time and cost of the drug development process, there is a need for additional tools such as biochemical markers and advanced imaging technologies that will predict long term outcomes.

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Biochemical markers of protease degraded cartilage matrix constituents have been the subject of much attention from many groups of researchers[8]. Some biochemical markers respond to pathological activities resulting in increased turnover, e.g., matrix metallo-proteinase (MMP) mediated collagen type II degradation or aggrecanase mediated aggrecan degradation[9]. Levels of urinary C-telopeptides of type II collagen (CTX-II) have been reported to be associated with risk of radiographic disease[10,11] and elevated CTX-II has been associated with a more severe structural damage assessed by JSN[10].

Currently, structural joint damage is monitored by various radiograph techniques and other scoring systems (e.g., whole organ MRI score (WORMS)[12]). As cartilage is not visualized on radiographs, alternative imaging technologies are under validation and in particular magnetic resonance imaging (MRI) seems promising. First and foremost, cartilage is visible in MRI, and by using 3D scans morphometric analysis is possible. Several semi-automatic methods for cartilage quantification have been reported[13,14], and recently our group reported a fully automatic computer-based method for quantification of a range of morphometric parameters, including cartilage thickness, volume, curvature[15], and homogeneity[16].

The present study was undertaken to investigate the interrelationship between two biomarkers for assessment of joint damage in OA: (1) the urinary excretion of CTX-II, which is released during degradation of articular cartilage, and (2) a newly developed MRI-based, fully automated cartilage volume quantification procedure.

Material and methods

STUDY POPULATION

The study population consisted of 159 subjects (48% females) above 21 years of age recruited to include individuals having OA according to American College of Rheumatology (ACR) criteria as well as healthy controls. The majority of the subjects were invited from address lists to ensure an even distribution across gender and ages, but the population also contained volunteers with known knee problems (and therefore likely to have OA). Individuals with other diseases affecting the joints (e.g., rheumatoid arthritis, Paget's disease, joint fractures, hyperparathyroidism, hyper- and hypothyroidism) were excluded as were individuals taking medication known to affect bone and/or cartilage (e.g., bisphosphonates, vitamin D, hormones, SERMs, prednisolone, anabolic androgens, and PTH). In addition, subjects with previous knee joint replacement or presenting any contraindication for MRI examination were excluded. All participants had radiographs and MRI of both knees at baseline (BL) visit, and fasting morning urine was collected and stored at -20°C until analysis. Participants were invited to attend a follow-up visit after 21 months with essentially the same investigations. For the present analysis, 158 participants were available excluding one where a urine sample was not obtained. For demographic details, see Table I.

The subjects signed informed consent forms. This single-center study was conducted in accordance with the Helsinki Declaration II and European Guidelines for Good Clinical Practice[17]. The study protocol was approved by the local ethical committee.

RADIOGRAPHIC ASSESSMENT

Digital radiographs of both knees were acquired simultaneously with subjects standing in a weight bearing position with knees slightly flexed and the feet rotated externally. The SynaFlex (Synarc, USA) was used to fix orientation and flexing[18]. Film distance was 1.0 m, and the tube was angulated 10° (the metatarsophalangeal view modified for fixed angle). Radiographs were acquired in the posterior–anterior (PA) position, while the central beam was displayed directly to the mid point of the line passing through both popliteal regions.

From the radiographs, the Kellgren and Lawrence (KL) score[19] was assessed by a trained radiologist. As the study was designed to potentially detect small differences, the BL and follow-up radiographs were examined pairwise blinded to the order. Furthermore, the joint space width (JSW) was measured by manually marking the minimal distance between tibia and femur within the medial tibial plateau. The inter-scan quantification imprecision for JSW (estimated from scan–rescan pairs with a week in-between) was coefficient of variation (CV) 3.3% (root mean squared (RMS) CV 6.2%).

MRI ASSESSMENT

The method has previously been described[15,20]. Briefly, MRI scans were acquired from an Esaote C-Span 0.18 T scanner using a modified Turbo 3D T1 sequence with near-isotropic voxels (40° FA, TR 50 ms, TE 16 ms, scan time 10 min, resolution 0.7 mm × 0.7 mm × 0.8 mm). During scanning, the test subjects were lying supine without loading.

Automatic segmentation of the tibial and femoral medial cartilage compartments was performed using a voxel classification method based on supervised learning and the total cartilage volume for the medial tibio-femoral compartment was quantified. The sensitivity and specificity of the automatic segmentation were 83.9 and 99.9%, respectively, compared to manual segmentation by a radiologist. The inter-scan quantification imprecision for total cartilage volume (estimated from scan–rescan pairs with a week in-between) was CV 2.6% (RMS CV 3.6%).

BIOCHEMICAL MEASUREMENT

Urinary excretion of CTX-II was quantified by the CartiLaps enzyme-linked immunosorbent assay (ELISA) (Nordic Bioscience, Denmark) using instructions from the manufacturer. Briefly, the competition ELISA employs monoclonal antibody MAb F46 recognizing the six amino acid sequence EKGPDP specific for the CTX-II. The intra- and inter-assay imprecision are 7.8 and 12.2% (RMS CV), respectively (according to the package insert). The fasting morning (second void) urinary CTX-II concentration was corrected for creatinine levels. To reduce the variability of the CTX-II measurements, BL values were calculated as the mean of two separate determinations.

PROGRESSION DEFINITIONS

We used three alternative definitions for progression of OA:

Early radiographic progression: Those radiographically healthy at BL (KL ≤ 1) were divided into progressors/non-progressors depending on whether any knee had an increase in KL score at follow-up. Note that some of the progressors thereby remained radiographically healthy (KL 0 at BL and KL 1 at follow-up).
Early JSN: Again, we divided those healthy at BL. We defined JSN progressors to be above the median JSN of the radiographically healthy non-progressors (as defined above).
Early cartilage loss: Analogously, we defined increased cartilage loss to be above the median loss for the healthy non-progressors.

STATISTICAL ANALYSIS

The CTX-II values were logarithmically transformed to obtain normality and symmetry of variance. The unpaired two-tailed Student *t* test was used for the pair-wise comparison of BL characteristics between subjects with knee radiographic OA (ROA) and controls, for assessment of differences between genders, and for assessment of the difference in JSW and cartilage volume changes categorized according to the CTX-II levels. The Mantel-Haenszel estimate was used to test for significance of odds ratios (ORs).

The potential confounding effects of gender, age, and body mass index (BMI) were investigated and linear correction was applied where relevant (as stated below) to both BL and follow-up data.

For all tests $P < 0.05$ was considered significant. All statistical calculations were performed using the SAS software package (release 9.1, SAS Institute Inc., Cary, NC, USA) and the Matlab software package (version 7.4.0, Mathworks Inc., MA, USA).

Results

STUDY POPULATION

The demographic data for the study population demonstrated significant differences at BL among those individuals having radiographic knee OA (ROA) as compared to those without (radiographic disease is defined as having a KL > 1 in one or both knees). Females and males with ROA were about 14 years older, had slightly lower height of 3–4 cm, had about 6 kg higher body weight, increased BMI, and increased level of CTX-II (for details, see Table I).

Table I
Demographic characteristics of the study population at BL

	Females		Males	
	−ROA ($n = 60$)	+ROA ($n = 16$)	−ROA ($n = 62$)	+ROA ($n = 20$)
Age (years)	52 (17)	67 (10)**	53 (16)	67 (4)***
Height (cm)	166 (6)	163 (5)	178 (7)	174 (7)*
Weight (kg)	68.4 (12.7)	74.1 (7.9)	82.2 (11.9)	88.5 (12.5)*
BMI (kg/m^2)	24.8 (4.5)	27.9 (3.4)*	25.8 (3.6)	29.2 (3.7)***
CTX-II/Cr[a] (μg/mmol)	0.184 (0.099–0.342)	0.338** (0.167–0.679)	0.189 (0.109–0.329)	0.258* (0.150–0.444)
JSW (mm)	3.7 (0.7)	3.1 (1.5)*	4.2 (0.7)	2.7 (1.9)***

Values given are mean (SD), and [a]geometric mean (±1 SD range). The level of significance denotes for each gender the difference between −ROA and +ROA with *$P < 0.5$; **$P < 0.01$; ***$P < 0.001$.

Among the 158 subjects, 76 had a KL score of 0 in both knees, and 46 had a KL of 1 in one or both knees, 17 had a maximum KL of 2, 18 had maximum KL of 3, and one had KL 4.

Out of the 158 subjects being analyzed at BL, 138 (87%) attended a follow-up visit at 21 months. There were no significant differences in age, BMI, gender, JSW, OA state, or cartilage volume between those that did and did not attend follow-up.

At BL, we could not detect any associations between CTX-II and JSW by radiographs (e.g., no linear correlation: $r = -0.06$, $P = 0.5$) or between CTX-II and cartilage volume by MRI ($r = -0.05$, $P = 0.6$). There were also no associations between CTX-II and gender, age, or BMI.

However, at BL, gender-specific linear correlations were found between JSW and age and BMI, and gender-specific correlations between cartilage volume and BMI. For all results below, we therefore also provide the results where the data has been corrected accordingly (JSW corrected for gender, age and BMI; volume for gender and BMI).

BL ASSOCIATIONS OF KNEE ROA AND CTX-II LEVELS

First, it was observed that in this study population, individuals with radiographic disease in the knee had elevated urinary excretion of type II collagen fragments [Fig. 1(a)]. Specifically, CTX-II in the ROA group was 0.291 (0.262–0.323) μg/mmol creatinine (mean ± 1 S.E.M. range) compared to 0.187 (0.177–0.197) μg/mmol creatinine in the healthy population ($P = 0.0001$).

Secondly, we determined if elevated urinary CTX-II was indicative of presence of radiographic disease at BL. For this investigation, the study population was stratified in tertiles according to the urinary CTX-II level [Fig. 1(b)]. The OR for having ROA in the high tertile compared to the low tertile was 4.8 ($P < 0.001$).

PREDICTION OF EARLY RADIOGRAPHIC PROGRESSION

Next, we investigated if BL measurements of CTX-II could predict structural degeneration during the 21 months follow-up period. Among the 109 subjects without knee ROA at BL, 19% had an increase in the KL score over the 21 months follow-up (Table II). Only three subjects in the diseased group had progression, corresponding to 10%.

We evaluated whether BL CTX-II was predictive of KL progression among those healthy at BL. Although elevated CTX-II implied a trend toward an elevated risk of longitudinal progression with an OR of 2.3 when comparing the low and high CTX-II tertile groups, this was not statistically significant (Fig. 2). The groups with low and elevated CTX-II were of similar ages (54 and 52 years, respectively, $P = 0.4$).

Likewise, elevated CTX-II at BL was associated with increased progression as determined by JSN (Fig. 3). Specifically, the OR for progression for the middle and high tertiles were 1.9 and 1.8 (with JSW corrected for gender, age, and BMI: 2.0 and 1.8, respectively). However, this trend did not reach statistical significance.

CTX-II WAS PREDICTIVE OF CARTILAGE LOSS DETERMINED BY MRI

Finally, we compared BL measurements of urinary CTX-II with changes over the 21 months in quantitative assessments of cartilage volume by MRI. The subjects with BL CTX-II in the middle and high tertiles had elevated risk for increased cartilage loss (Fig. 4). Specifically, the OR for the middle and high tertiles were 4.0 and 3.9 ($P < 0.01$ for both). When correcting cartilage volume for gender, age, and BMI, these ORs were 4.0 and 5.7 ($P < 0.01$ and $P < 0.001$, respectively). The groups with low and elevated cartilage loss were of similar ages (55 and 51 years, respectively, $P = 0.2$).

Further, the mean yearly cartilage loss in the middle/high CTX-II tertiles was 434 mm^3/year higher than in the low tertile ($P < 0.0001$) – corresponding to 3.1% of the mean cartilage volume. When cartilage volume was corrected as above, the increased cartilage loss was 437 mm^3/year or 3.2%.

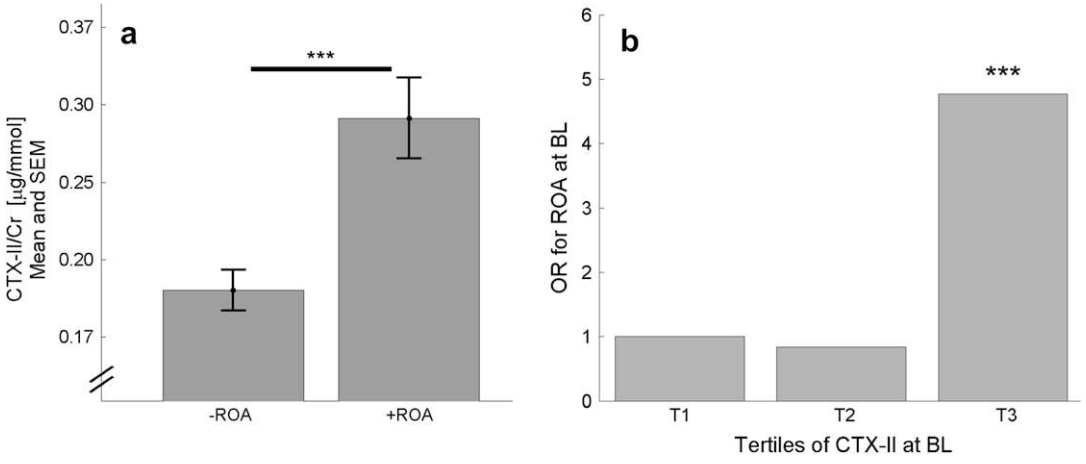

Fig. 1. Relationship between CTX-II and ROA at BL: urinary excretion of CTX-II in study participants stratified according to the presence ($n = 36$) or the absence ($n = 122$) of radiographic knee OA (ROA) at BL [Fig. 1(a)]. Individuals with a maximum KL score of 1 in either knee were designated −ROA, the remaining grouped in +ROA. Subjects with ROA of the knee had BL urinary CTX-II levels of 0.291 (0.262–0.323) μg/mmol creatinine (mean ± 1 S.E.M. range) compared to 0.187 (0.177–0.197) μg/mmol creatinine in the healthy population ($P = 0.0001$). The inverse relation was analyzed by the OR for having ROA (KL > 1 in at least one knee) at BL when the population was stratified based on the BL urinary CTX-II level [Fig. 1(b)]. The low tertile was used as a fiducial reference with OR = 1. The OR for ROA at BL for the high tertile was 4.8 ($P < 0.001$).

Table II
Radiographic progression in the study population

Disease status	BL (*n*)	Follow-up (*n*)	Progression (*n*)
−ROA	122	109 (89%)	21 (19%)
+ROA	36	29 (81%)	3 (10%)

Radiographic disease progression in 138 subjects attending the follow-up visit at 21 months. Radiographic progression was defined as having an increase in KL score in one or both knees.

Discussion

We investigated the diagnostic and prognostic properties of the CTX-II biomarker targeting urinary excretion of CTX-II. Firstly, we evaluated the diagnostic ability to distinguish the group with ROA. Secondly, we evaluated the prognostic ability by investigating whether the BL CTX-II values predicted the longitudinal progression in KL score, JSW, and cartilage volume.

CROSS-SECTIONAL DATA

In this study population, we observed a significant association between the presence of radiographic disease and urinary CTX-II. This is in agreement with several previous reports[10,11,21–24] (for a review of additional collagen type II markers, see Ref. 25). In patients with ROA of the knee we found the urinary CTX-II to be 0.291 μg/mmol creatinine, corresponding to a 56% increase above the healthy controls. Previously, increases of 37[10] and 126%[21] have been reported.

In contrast, we did not find an association between BL urinary CTX-II and cartilage volume by MRI. Not even in the healthy population. This suggests that the inter-individual variation in metabolic activity of the articular cartilage is significant. Interestingly, in the healthy sub-group of this population with females and males having near-identical mean ages of 52.5 and 52.9 years, we did not detect any gender difference in CTX-II levels. In contrast, cartilage volume by MRI was significantly higher in men than women (data not shown). This discrepancy could be caused by the correction of the biochemical index with creatinine, which will correct for kidney function and muscle mass, in which muscle mass is the larger determinant in between genders.

Fig. 3. The OR for predicting increased longitudinal JSN when the population was stratified based on the BL urinary CTX-II level. Increased JSN was defined as a JSN above the mean JSN for the group without no ROA at BL (KL $\leq$ 1) and no longitudinal progression. The low CTX-II tertile was used as a fiducial reference with OR = 1. The OR for longitudinal progression for the middle and high tertiles were 1.9 and 1.8 (not significant).

PROGRESSION DATA, RADIOGRAPHS

Some longitudinal studies have investigated using BL urinary CTX-II for prediction of long term radiographic progression in OA[10,26–30]. In particular, for the Rotterdam Study, Reijman and coworkers[10] found that subjects in the highest

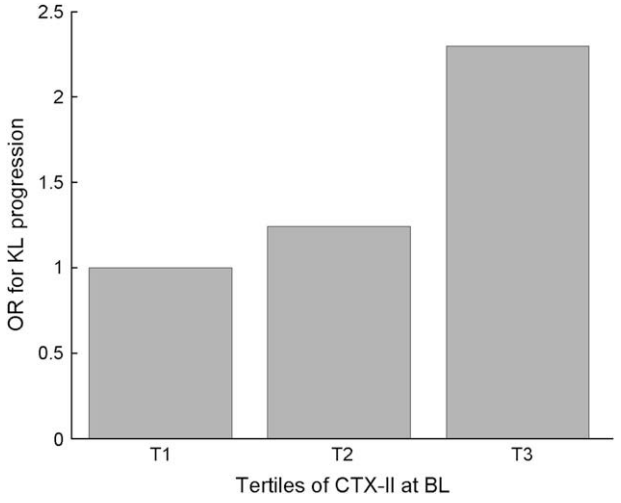

Fig. 2. The OR for longitudinal progression in the level of ROA when the population was stratified based on the BL urinary CTX-II level. We included the group of healthy at BL and defined progression as an increase in KL in either knee. The low CTX-II tertile was used as a fiducial reference with OR = 1. The OR for longitudinal progression for the high tertile was 2.3 (not significant).

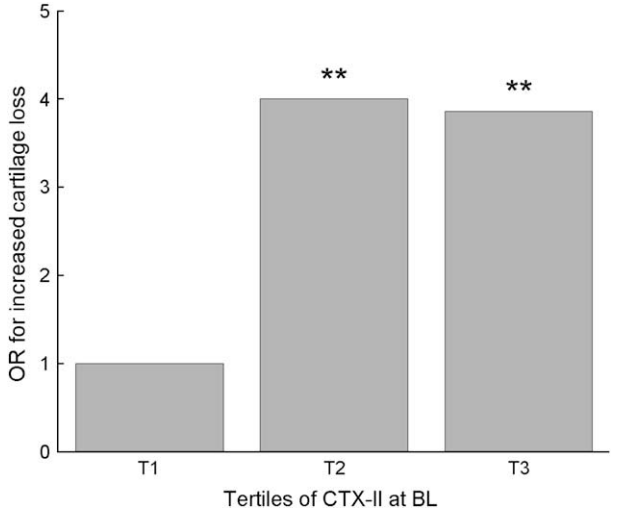

Fig. 4. Prediction of longitudinal cartilage loss was quantified by the OR for elevated cartilage loss when the population is stratified into tertiles by the BL CTX-II level. Only subjects without ROA at BL were included. The low CTX-II tertile was used as a fiducial reference with OR = 1. The OR for longitudinal progression for the middle and high tertiles were 4.0 and 3.9 ($P < 0.01$ for both).

quartile of CTX-II values had an increased risk of disease progression in knee OA (defined as a JSN $\geq$ 2 mm) of OR 6.2 ($n = 1235$). Two of the other studies, both investigating the associations for subjects in the upper tertile of CTX-II values, reported a 23% decreased JSW for hip OA subjects ($n = 376$, Echodiah[30]) and an OR of 6.7 for rapidly progressive hip OA ($n = 115$[29]). Finally, other studies have also investigated associations between CTX-II and JSN, e.g., Mazières *et al.*[26] ($n = 333$, also Echodiah, elevated CTX-II gives twofold risk of JSN and hip replacement) and Sharif *et al.*[27] ($n = 135$, mild-to-moderate OA, CTX-II above mean gives 3.4-fold risk of JSN or knee surgery) supporting the relationship and Mazzuca *et al.*[28] ($n = 120$, obese women with OA) finding no relationship.

In the present study, the association between elevated CTX-II and radiographic disease progression did not reach statistical significance. Direct comparisons with the results from the Echodiah[30] and Garnero[29] studies are problematic since they investigated hip OA. However, one of the structural endpoints in the reports from the Rotterdam[10] and Sharif[27] studies was JSN in the knee − similar to this study. In the present study, the study period was relatively short and the number of individuals having radiographic progression of knee OA was relatively small, i.e., only 19 individuals without knee ROA at BL, which could explain the lack of statistical significance (compared to the Reijman report where they followed 1235 subjects over 6.6 years and the Sharif report following 135 subjects for 5 years).

PROGRESSION DATA, MRI

We wanted to investigate if an alternative measure of structural damage in knee OA could be predicted by elevation in the urinary CTX-II levels. To our knowledge, investigations of the association of CTX-II scores and longitudinal cartilage loss from MRI are rare. The first known was a study by Bruyere *et al.*[31], where they found no prediction of 1-year cartilage loss by BL CTX-II values. However, they did find a marginal association between 3-month CTX-II elevation and 1-year cartilage thinning (comparing highest and lowest quartiles, $P = 0.04$, 62 OA subjects).

Recently, a fully automatic knee cartilage volume quantification framework, including assessment of cartilage volume, was developed by our group[15,20], which was reported to be highly correlated to manual assessment of cartilage volume, able to discriminate groups of healthy and OA subjects, associated with disease severity as determined by KL score, and quite precise with a mean inter-scan CV of 2.6%. We therefore wanted to determine if prediction of joint damage by BL CTX-II was improved using MRI-based assessment of cartilage loss as the study endpoint. We found that high BL CTX-II reflecting cartilage degradation was indeed significantly associated with a more accelerated loss of cartilage volume − specifically, the middle/high tertiles had ORs of 4.0/3.9 compared to the low tertile ($P < 0.01$, results not reduced by correction for gender, age, and BMI). It is noteworthy, that in the current study of only 158 subjects of which 21 had early radiographic progression (13%) it was possible to show a statistical association between the biochemical index at BL and the change in structural readout in just 21 months.

Intuitively, the urinary excretion of the type II collagen fragments should be more related to cartilage volume than the radiographic observations of KL score and JSW. KL score reflects both bone and cartilage morphology of the joint and JSW is confounded by the 3D to 2D projection and meniscus influence. This is confirmed by our results. Despite a clear trend, the association between BL CTX-II scores and longitudinal radiographic progression were not statistically significant contrary to what larger, longer studies have demonstrated; but still our results showed a strong association with cartilage loss quantified from MRI. This supports that MRI may indeed be a more sensitive modality than radiographs for quantifying progression of OA.

LIMITATIONS OF THE STUDY

We focused the investigation of progression of OA to the early stages. Specifically, we analyzed the sub-population without radiographic signs of OA at BL (KL $\leq$ 1). Therefore, the conclusions are only valid for progression during the early stages of OA. A study population with progressed OA would be needed to validate the findings at later stages of OA. Furthermore, the relatively small number of subjects in this study also implies that the findings need to be validated on other populations.

The cartilage volume measurements were based on an MRI scanner with a 0.18 T magnet. The use of low-field MRI is sparsely validated compared to high-field MRI[32]. In particular, high-field MRI may allow cartilage volume measurements with higher accuracy and precision (implying that studies may be conducted with a smaller population). However, low-field MRI is much cheaper to install, maintain, and operate. Future studies are needed to evaluate whether low-field MRI can be a cost-effective alternative to conventional high-field MRI for clinical studies.

MEDICAL INTERVENTION, CLINICAL STUDIES

It is a prerequisite for successful clinical investigations of chondroprotective drugs, that further structural damage in the study participants receiving placebo exceeds the level of detection. Therefore it is essential to identify individuals who in the follow-up period have accelerated structural damage in the joint. A recent phase III clinical trial failed to demonstrate a treatment effect of risedronate to reduce radiographic progression (decrease in JSW) in patients with knee OA[2]. In the study placebo group, 13% had radiographic progression in the 2 year follow-up similar to the 13% with early radiographic progression in our population. We could therefore speculate that use of the methodology presented here − a combination of selection of high risk subjects using CTX-II with quantification of efficacy using our MRI-based quantification of cartilage loss − would potentially have facilitated a different study outcome. Interestingly, in a sub-group analysis of the risedronate trial, the biochemical responders actually had significantly reduced radiographic disease[33].

In addition to the relatively strong association between CTX-II and the longitudinal cartilage loss by MRI demonstrated in this study, the independence of operator interaction of this fully automatic imaging procedure may facilitate large scale studies without a potentially problematic single-reader bottleneck or alternatively added measurement variation implied by multiple readers.

CONCLUSION

Previous reports have shown that CTX-II is associated with both the prevalence and the progression of ROA at the knee; however, this is the first study to show that elevated BL urinary CTX-II is predictive of cartilage loss

determined by MRI. The demonstrated ability to predict a large sub-group (the two upper tertiles) with an increased cartilage loss of 3.1% (comparable with the precision of CV 2.6%) appears very applicable for study population selection. Further studies are warranted to investigate the usefulness of applying both the fully automatic MRI-based quantification of cartilage volume as well as a biochemical index, e.g., urinary CTX-II, in the selection and monitoring of study populations with elevated risk of cartilage damage.

Conflict of interest

Erik B. Dam and Inger Byrjalsen are employees of Nordic Bioscience. Morten A. Karsdal, Per Qvist, and Claus Christiansen are employees and shareholders of Nordic Bioscience. The study was sponsored by the Center for Clinical and Basic Research (Ballerup, Denmark) that was previously affiliated with Nordic Bioscience.

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