

A study of the relationship between molecular biomarkers of joint degeneration and the magnetic resonance-measured characteristics of cartilage in 16 symptomatic knees

Karen B. King^{a,*}, Colleen T. Lindsey^b, Timothy C. Dunn^c, Michael D. Ries^d,
Lynne S. Steinbach^e, Sharmila Majumdar^b

^aDepartment of Medicine, University of California at San Francisco, Richmond, CA 94804, USA

^bMusculoskeletal and Quantitative Imaging, Department of Radiology, University of California at San Francisco, USA

^cJoint Bioengineering Graduate Group, University of California at San Francisco and Berkeley, USA

^dDepartment of Orthopaedic Surgery, University of California at San Francisco, USA

^eMusculoskeletal Section, Department of Radiology, University of California at San Francisco, USA

Received 22 July 2004; accepted 1 August 2004

Abstract

We used quantitative magnetic resonance (MR) imaging to determine if relationships exist between proposed molecular biomarkers for degenerative joint disease (DJD) and structural characteristics of articular cartilage. Subjects were eight male and eight female volunteers diagnosed with osteoarthritis. Magnetic resonance images of the symptomatic knee were taken and blood samples were drawn. Concentrations of serum cartilage oligomeric matrix protein (COMP) and cleaved collagen neoepitope were compared to cartilage volume and cartilage T2, respectively, in four compartments of the tibiofemoral joint. A significant, negative correlation was found between serum COMP and medial tibia volume in the male subject group ($\rho = -.738$, $P = .037$). A significant, positive correlation ($\rho = .881$, $P = .0039$) was found between serum COMP and lateral femur volume in the female subject group. In both groups, positive correlations were found between serum C2C and cartilage T2, which were significant in two compartments of the male group ($\rho = .714$, $P = .047$; $\rho = .738$, $P = .037$) and similarly strong, but not statistically significant ($\rho = .750$, $P = .052$), in one compartment of the female group. We identify strong and biologically relevant correlations between two proposed molecular biomarkers for DJD and MR measures of symptomatic knees of a small number of arthritic patients. Our findings support the hypothesis that cartilage molecular biomarkers reflect the molecular processes of cartilage degeneration and loss.

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Keywords: Human cartilage volume; Human cartilage T2; Biomarkers; COMP; Collagen neoepitope

1. Introduction

Degenerative joint diseases (DJD) such as osteoarthritis (OA) and rheumatoid arthritis (RA) have complicated etiologies but have in common degenerative changes in the tissues of articulated joints. These changes include disruption of the collagen network and eventual loss of cartilage

tissue. In many cases of DJD, cartilage degeneration begins before pain is felt, likely because cartilage is neither innervated nor vascularized. Diagnosis and/or prognosis of DJD at an early stage could be aided by molecular biomarkers that both reflect the molecular processes of degeneration and can be measured in human bodily fluids such as blood serum, synovial fluid or urine.

One proposed biomarker, cartilage oligomeric matrix protein (COMP), is found in high concentration in cartilage [1], and to a lesser degree in other joint tissues such as tendon [2] and synovium [3]. A cross-sectional study of a large population (the Johnston County cohort) has found a significant correlation between serum COMP and OA severity [4]. Longitudinal studies report higher serum COMP

Abbreviations: COMP, cartilage oligomeric matrix protein; DJD, degenerative joint disease; ELISA, enzyme-linked immunosorbent assay; K-L, Kellgren–Lawrence; MR, magnetic resonance; OA, osteoarthritis; RA, rheumatoid arthritis.

* Corresponding author. Tel.: +1 510 231 9448; fax: +1 510 231 5729.

E-mail address: kbking@itsa.ucsf.edu (K.B. King).

in patients who later demonstrate disease progression [5–7]. The potential for COMP as a diagnostic and prognostic indicator of RA [8–10] and chondromalacia patellae [11] has also been demonstrated.

Other potential molecular biomarkers of DJD include neopeptides of cartilage-specific molecules, that is, antigens resulting from peptide cleavage. Antibodies specific to the neopeptide of collagenase-cleaved type II collagen, the predominant collagen of cartilage, have been produced [12]. In animal models of DJD, the concentration of this collagen neopeptide, C2C antigen, increases in synovial fluid [13–15] and serum [15]. The C2C antigen has also been examined in the synovial fluid of OA, RA and psoriatic arthritis patients [16,17].

Published studies typically evaluate potential biomarkers of human DJD in relation to various scores of pathology such as the Kellgren–Lawrence (K-L) score [4,6,18] and the Larson score [8–10], or methods such as bone scintigraphy [5]. These scores and methods are useful in clinical diagnosis and prognosis and thus are relevant to the evaluation of biomarkers. However, these scores and methods are not very sensitive to specific cartilage molecular changes.

Recent advances in magnetic resonance (MR) imaging allow greater resolution and more detailed analysis of cartilage dimensions and structure than is possible with traditional radiography. Cartilage volume can be calculated from high-resolution MR images [19,20]. Quantitative cartilage proton MR (T2) can be used to infer cartilage structure, for example, collagen network organization and water content in vitro [21,22] and in vivo [23].

While others have made valuable investigations of the diagnostic and prognostic potential of DJD biomarkers, we choose to determine if two proposed biomarkers, COMP and C2C, reflect molecular processes in cartilage by testing the correlations of their respective concentrations to MR measures of cartilage. Our hypotheses are (1) an increase in serum COMP will correlate to a decrease in cartilage volume and (2) an increase in serum C2C will correlate to an increase in cartilage T2.

2. Methods

2.1. Study subjects

Eight male and eight female volunteers were recruited from the orthopedic clinic at the University of California,

Table 2
Descriptive volume data of the study

| Volume (cm ²) | Male subjects (n=8) | | | | Female subjects (n=8) | | | |
|---------------------------|---------------------|-------|--------|------|-----------------------|------|--------|------|
| | Min | Max | Median | S.D. | Min | Max | Median | S.D. |
| Medial tibia | 1.33 | 2.79 | 1.93 | 0.54 | 1.25 | 2.16 | 1.62 | 0.29 |
| Medial femur | 2.37 | 6.27 | 3.31 | 1.20 | 2.26 | 5.11 | 2.93 | 1.19 |
| Lateral tibia | 1.75 | 3.19 | 2.39 | 0.49 | 1.27 | 2.10 | 1.76 | 0.27 |
| Lateral femur | 6.02 | 10.60 | 7.59 | 1.64 | 4.30 | 6.10 | 5.08 | 0.70 |

Cartilage volume values were calculated from spoiled gradient MR data of the symptomatic knee.

San Francisco (UCSF). Descriptive details (age, body mass index, K-L score of the affected knee) of both groups are listed in Table 1. The nature of the procedure was explained to all subjects, and each subject granted an informed consent for the procedure. All portions of this study were approved by the UCSF Institutional Review Board.

2.2. Imaging protocol

Plain radiographs, MR scans and blood draws were taken at the Magnetic Resonance Science Center at UCSF. Symptomatic knees were radiographed using anterior–posterior and lateral projections. These radiographs were examined blind and scored for pathology by a radiologist (L.S.S.) using the K-L score [24,25].

Magnetic resonance images were obtained of the symptomatic knee of patients with a GE Signa 1.5 T clinical MR scanner (GE Medical Systems, Waukesha, WI) using a bilateral dual phased-array coil with four elements (USA Instruments, Cleveland, OH), as described previously [20]. Cartilage data were obtained as sagittal high-resolution MR images using a volumetric, fat-suppressed (using spectral inversion of lipids, SPECIAL), spoiled gradient echo pulse sequence (TE=3.3 ms, TR=30 ms, TI=8 ms, flip angle=30°, +15.6 kHz bandwidth) with in-plane resolution of 0.234 × 0.234 mm² and a 2-mm slice thickness. A total of 64 slices were acquired with a field of view of 12 cm (512 × 512 matrix) and a scan time of 9:31 min.

Immediately, a 2D dual echo spin echo sequence was used (TE₁/TE₂=10/45 ms, TR=1500 ms, 0.468 × 0.468 × 4 mm³ voxel size, scan time 5:24, FOV=12 cm, 256 × 256 matrix) to generate a sagittal T2 map using custom software (IDL, Research Systems, Boulder, CO), assuming a single exponential decay component. The cartilage morphological masks derived from the higher resolution images

Table 1
Descriptive data of the subjects

| Variable | Male subjects (n=8) | | | | Female subjects (n=8) | | | |
|--------------------------------------|---------------------|-------|--------|------|-----------------------|-------|--------|------|
| | Min | Max | Median | S.D. | Min | Max | Median | S.D. |
| Age (years) | 43 | 76 | 58.5 | 11.3 | 46 | 88 | 70 | 14.4 |
| Body mass index (kg/m ²) | 23.10 | 33.68 | 28.06 | 3.80 | 21.62 | 32.82 | 25.89 | 3.66 |
| K-L Score (0–4) | 1 | 4 | 3 | 0.9 | 1 | 4 | 2.5 | 0.9 |

Osteoarthritis score was determined using the K-L score where 0 is no pathology and 4 is severe pathology. All subjects were UCSF Orthopaedic Clinic patients.

Table 3
Descriptive T2 data of the study

| T2 (ms) | Male subjects (n=8) | | | | Female subjects (n=8) | | | |
|---------------|---------------------|-------|--------|------|-----------------------|-------|--------|------|
| | Min | Max | Median | S.D. | Min | Max | Median | S.D. |
| Medial tibia | 28.76 | 43.12 | 35.07 | 5.03 | 34.89 | 52.40 | 37.97 | 5.68 |
| Medial femur | 32.56 | 53.21 | 41.52 | 7.47 | 39.04 | 45.61 | 42.01 | 2.17 |
| Lateral tibia | 31.20 | 47.39 | 35.16 | 5.09 | 27.90 | 41.37 | 35.40 | 4.10 |
| Lateral femur | 35.75 | 47.32 | 41.39 | 3.97 | 38.34 | 57.75 | 40.08 | 6.46 |

Cartilage T2 values were calculated from spin echo MR data of the symptomatic knee.

(512 × 512 matrix) were superimposed on the cartilage T2 maps for analysis of structure, as described [26].

2.3. Serum analysis

Following the MR image collection, blood was collected from the subject by venous puncture and centrifuged to separate serum. Serum was stored at -80°C until enzyme-linked immunosorbent assays (ELISA) were performed.

Serum COMP was measured using a commercial sandwich-type ELISA according to the manufacturer's directions (AnaMar Medical, Uppsala, Sweden). Absorbance of the colorimetric reaction was measured at 450 nm using a multiwell plate reader (BioRad 550, BioRad Laboratories, Hercules, CA). Serum samples were tested in duplicate and in random order without knowledge of subject data. The results, expressed as units of COMP antigen, were determined from a five-point calibration curve. The manufacturer noted that 10 units COMP/ml is roughly equivalent to 1 $\mu\text{g}/\text{ml}$ but also noted that the relationship between units and mass is not linear due to the ability of the assay to measure pentameric, monomeric and fragmented COMP molecules. The detection limit of the assay was <0.1 unit/ml. The intra-assay coefficient of variation was 1.7–3.0%.

Serum C2C collagenase-generated neopeptide of type II collagen (also known as CIIC and also as Col 2-3/4_{long mono}) was measured using a commercial two-step competitive ELISA according to the manufacturer's directions (Ibex Diagnostics, Toronto, Canada). Absorbance of the colorimetric reaction was measured at 450 nm as above. In this ELISA, the absorbance was inversely proportional to the

amount of epitope present in the sample. Seven dilutions of standards (978 g/mol) from 0 to 1 mg/ml were used to prepare a standard curve. Two of three wells testing the serum of one (female) subject were out of range of the standard curve. Therefore, C2C ELISA and T2 data from that subject were not included in further analyses. Serum samples were tested in triplicate and in random order without knowledge of subject data. The detection limit of this assay was 6 ng/ml. The intra-assay coefficient of variation was 5.0–10.0%.

2.4. MR image analysis of cartilage volume

The MR images were transferred to a computer workstation (Sun Microsystems, Mountain View, CA). All images were subjected to a preprocessing step involving a 3D low-pass filter-based coil correction algorithm [27]. The cartilage was segmented using an in-house algorithm created using Matlab software (Mathworks, Natick, MA) in which defined regions of interest were outlined and segmented on the original image for the following four cartilage compartments: medial tibia, medial femur, lateral tibia and lateral femur [20]. Guidelines for determining the cartilage boundaries were defined and observed for each data set. Mean cartilage thickness was computed using an iterative radius minimization technique as described [20]. Cartilage compartment areas were computed for each slice. Cartilage volumes were computed from the segmented regions of interest for each compartment. For this study, volumes were not normalized to epicondylar distance because the mass of molecular turnover is expected to be proportional to subject size. In other words, the larger the patient, the more molecules of biomarker would be available for release.

2.5. Analysis of cartilage structure using MR image T2 maps

Using the dual echo images at 10 and 45 ms, T2 (spin–spin relaxation time) maps were computed for each subject [26]. The cartilage boundary segmented for the

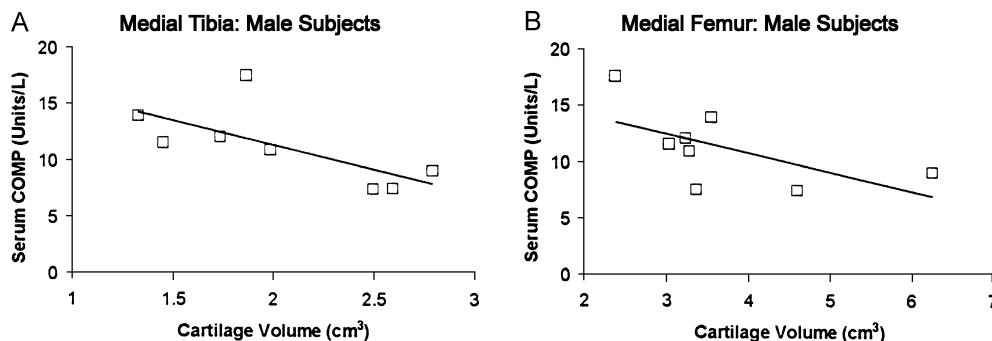


Fig. 1. Scattergrams plotting individual serum COMP concentration against cartilage volume. For eight male subjects, there was a linear relationship between COMP and cartilage volume in the medial tibia volume (A, $R^2=0.47$) and in the medial femoral volume (B, $R^2=0.35$). Concentration of 10 units COMP/ml was determined by the assay manufacturer to be roughly equivalent to 1 $\mu\text{g}/\text{ml}$; however, the relationship between units and mass was not linear due to the ability of the assay to measure pentameric, monomeric and fragmented COMP molecules.

Table 4

Spearman's rank order correlation coefficients (ρ) of the relationships between serum COMP and cartilage volume

| COMP vs. volume | Male subjects ($n=8$) | | Female subjects ($n=8$) | |
|-----------------|-------------------------|------|---------------------------|-------|
| | ρ | P | ρ | P |
| Medial tibia | -.738 | .037 | .238 | .57 |
| Medial femur | -.643 | .086 | .476 | .23 |
| Lateral tibia | .357 | .39 | .357 | .39 |
| Lateral femur | -.238 | .57 | .881 | .0039 |

Negative ρ values indicated an inverse relationship.

volume determination defined the outer limits of a binary mask, or an image, where cartilage is represented by ones and otherwise the image has a value of zero. The in-plane mask resolution was degraded (by a factor of 2) to match the resolution of the T2 map, and the union of adjacent slices was used to adjust for the differing voxel sizes between the T2 map and spoiled gradient scans. The mask image was then multiplied pixel by pixel with the T2 map to provide the four cartilage regions of interest.

2.6. Statistical analysis

Since the medial side of the knee is more commonly affected than the lateral side [28,29], we divided the images into four compartments prior to calculating volume and average T2. Since a recent report concludes that sex differences affect serum levels of COMP [18], we analyzed the data in two groups: male and female subjects. Differences in biomarker concentration between the two groups

were tested with two-tailed unpaired t tests. The relationships between serum COMP and cartilage volume and between serum C2C and cartilage T2 were assessed with Spearman's rank order correlation coefficient, ρ [30], using Stata, version 8.2 (Stata, College Station, TX). The assumptions of the Spearman's test were met. The probability (P value) tested the null hypothesis that the "true" ρ is equal to zero (i.e., no linear relationship between two variables). The threshold of statistical significance (alpha level) was set at $P=.05$.

3. Results

The median cartilage volumes of all four compartments were greater in the male subject group than the female group (Table 2). Median volume of the male group medial tibia was 19% greater, the medial femur 13%, the lateral tibia 36, and the lateral femur 54% greater than the respective median volumes of the female group. The median cartilage T2 values of all four compartments for both groups were within 10% of each other (Table 3). Serum COMP concentration was similar in the two groups ($P=.75$). The male group's serum COMP ranged from 7.42 to 17.57 units/L (median=11.22, S.D.=3.41). The female group's serum COMP range was 5.03–14.91 units/L (median=10.73, S.D.=3.83). These ranges are within that obtained by the manufacturer from the serum of 336 blood donors from Sweden. In a published study using the same assay, the serum COMP concentration of 62 RA patients was 5.2–29.7 units/L (mean=11.7) [9].

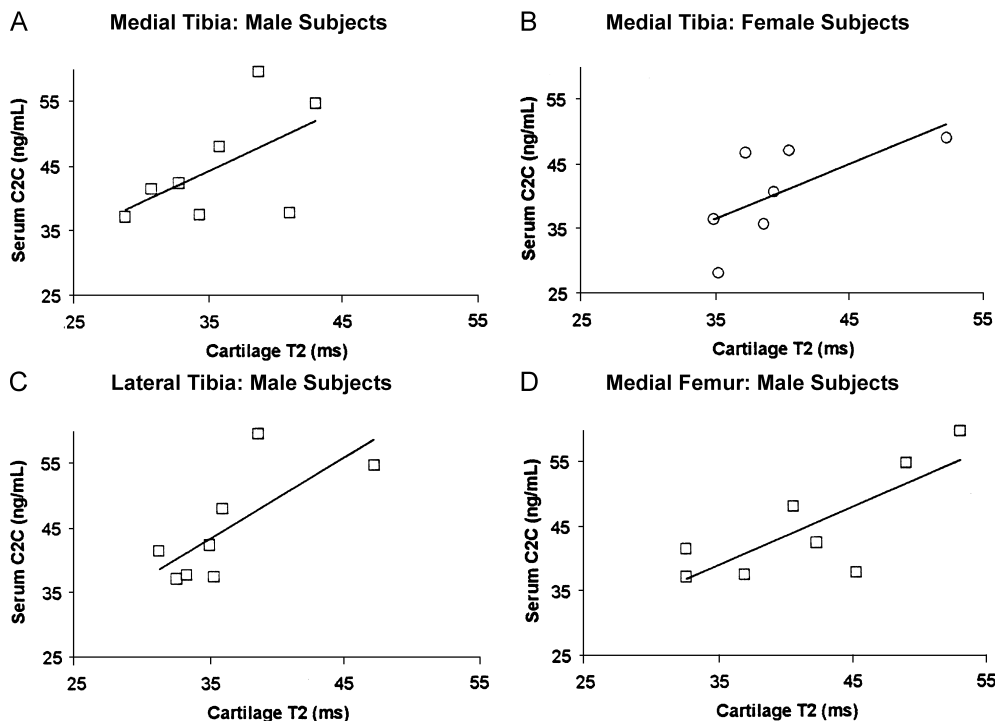


Fig. 2. Scattergrams plotting individual serum C2C concentration against cartilage T2 values. There was a linear relationship between C2C and medial tibia cartilage T2 for eight male subjects (A, $R^2=.32$) and for seven female subjects (B, $R^2=.43$). For eight male subjects, there was a linear relationship between C2C and lateral tibia cartilage T2 (C, $R^2=.54$) and medial femur cartilage T2 (D, $R^2=.61$).

Table 5
Spearman's rank order correlation coefficients (ρ) of the relationships between serum C2C and cartilage T2

| C2C vs. T2 | Male subjects (n=8) | | Female subjects (n=7) | |
|---------------|---------------------|------|-----------------------|------|
| | ρ | P | ρ | P |
| Medial tibia | .595 | .12 | .750 | .052 |
| Medial femur | .714 | .047 | .143 | .76 |
| Lateral tibia | .738 | .037 | -.054 | .91 |
| Lateral femur | .310 | .46 | .451 | .31 |

Negative ρ values indicated an inverse relationship.

There was a negative, linear relationship between serum COMP and cartilage volume in both medial compartments of the male subject group (Fig. 1A and B). As the serum COMP concentration increased, the cartilage volume decreased. There was no such relationship in the medial compartments of the female subject group (not shown). Statistical significance of the correlations between the serum COMP and the volumes of specific cartilage compartments was tested using Spearman's rank correlation coefficient (Table 4). The correlation between serum COMP and medial tibial cartilage volume was statistically significant for the male subject group. The only other statistically significant correlation was between serum COMP and the cartilage volume of the lateral femur compartment in the female subject group. However, this was a positive, linear relationship. As the serum COMP concentration decreased, the cartilage volume also decreased.

Serum C2C concentration was similar between the two groups of our study ($P=.33$). The male group's serum C2C ranged from 37.12 to 59.57 ng/ml (median=41.89, S.D.=8.50) while the female group's serum C2C from 28.15 to 48.88 ng/ml (median=40.74, S.D.=7.59). Communication with the manufacturer suggests that C2C antigen concentration in normal human serum is about 20–30 ng/ml. Human serum C2C has not been reported in the literature.

The R^2 values of scatter plots of serum C2C and cartilage T2 showed a linear relationship between C2C and T2 in the medial tibia of both male and female subject groups (Fig. 2A and B). As the serum C2C increased, the cartilage T2 increased. This relationship was also observed in the lateral tibia and medial femur compartments of the male subject group (Fig. 2C and D). A similar, though weaker, relationship in the lateral femur compartment of both male and female subject groups was observed (not shown).

Statistical significance of the correlations between the serum C2C and the T2 of the four cartilage compartments was tested using Spearman's rank correlation coefficient (Table 5). The positive correlations between serum C2C and both lateral tibia and medial femur cartilage T2 were statistically significant for the male subject group. The positive correlations between serum C2C and medial tibial cartilage T2 were not statistically significant for the male and female subject groups. However, at $P=.052$, the

correlation for the female group (C2C vs. medial tibia T2) was close to the a priori alpha.

4. Discussion

To our knowledge, this is the first study to correlate potential serum biomarkers to quantitative MR measures of cartilage volume and structure. We find that the male subject group data support the specific COMP hypothesis. We find a strong, inverse relationship between serum COMP and medial tibia cartilage volume of our male group (Fig. 1A). The correlation is high and statistically significant ($\rho=-.738$, $P=.04$). The correlation in the medial femur (Fig. 1B) is not statistically significant ($\rho=-.643$, $P=.09$). Based on the present data, 11 observations are the detectable effect size estimated to achieve statistical significance in the medial femur provided the ρ remains at least .643. In addition, it has been reported that measurements of the femoral compartment are less reliable than those of the tibial compartment [31]. Indeed, the standard deviations of volume measurements in this study are greater in the femoral compared to the tibial compartments for both medial and lateral sides in both subject groups (Table 2).

In contradiction to the male group data, we find that the female group data do not support the specific COMP hypothesis. In this group, we did find a strong and statistically significant correlation between serum COMP and lateral femur volume ($\rho=+.881$, $P=.0039$). A positive ρ indicates that the higher the serum COMP concentration, the greater the cartilage volume, which is contrary to the specific COMP hypothesis. The disparity of the COMP–cartilage relationship between male and female groups may be due to differences in pathology between our two subject groups.

There is a slight difference between the disease scores of the two groups. While the ranges are identical, the median score for the female group (K-L=2.5) is lower than that of the male group (K-L=3). The difference in the score definitions is that 2="minimal," while a score of 3="moderate" knee pathology. Specifically, Grade 2 is defined by osteophytes and possible joint space narrowing, while Grade 3 is defined by multiple osteophytes, definite joint space narrowing, some subchondral sclerosis and possible deformity of bone ends [25]. The (less severe) female subject group may exhibit residual anabolic characteristics of early-stage tissue repair prior to cartilage destruction [32], that is, that there is increased cartilage COMP production and overflow to the serum. A similar time course is observed in a mouse model of DJD in which an increase in knee cartilage COMP mRNA at 3 months precedes cartilage degeneration observed by histology at 4 months [33]. This experiment cannot be replicated in humans. However, COMP synthesis is higher in "early" degenerative than in normal human knee cartilage explants as seen by ^3H -leucine fluorography (Lorenzo, Bayliss and Heinegård, personal communication). Furthermore, high

serum COMP prior to joint destruction in RA patients has been demonstrated by Skoumal et al. [9].

An alternative explanation is the effect of sex differences in serum COMP concentration. Serum COMP concentration in Caucasian men is significantly higher than in Caucasian women [18]. This difference suggests the possibility of differences between men and women in the pathogenesis of DJD and/or differences in COMP clearance. Additionally, the incidence of OA of the knee is higher in women than in men [34]. How this would affect our data is not clear.

For the second potential molecular biomarker, we find that both male and female groups support the specific C2C hypothesis with a strong, positive linear relationship between C2C antigen and T2 values. The C2C neoepitope concentration is a surrogate measure for type II collagen cleavage [12]. This cleavage allows the further proteolysis of the normally protease-resistant triple helical molecule. The degradation of type II collagen, as the major collagen type of cartilage, will result in collagen network disruption. Previous studies have demonstrated spatial variation of T2 relaxation in cartilage explants [35], in normal subjects [36], with aging [37], and in humans with OA [26]. Lüsse et al. [22] have shown a positive correlation between T2 and cartilage water content in ex vivo cartilage from OA patients undergoing total knee replacement surgery.

Two limitations of this study should be discussed. A small number of subjects had been recruited for serum ELISAs, cartilage volume and cartilage structure analyses. A much larger population study is required to determine if these findings can be expected of the general DJD patient population. Of particular interest would be a test of sex differences as first suggested by Jordan and also supported, to some degree, by our data [18]. A second limitation is that analysis of blood serum reflects the systemic circulation including other joints, and in the case of COMP, other tissues of the affected joint. Subject recruitment was limited to those without symptoms of the other joints; however, recent evidence suggests that unilateral knee OA is less common than previously believed, and that many subjects develop bilateral degeneration [38]. Finally, diurnal variations may affect cartilage volume and/or biomarker concentration. The MR scan for 10 subjects began between 11:14 a.m. and 11:46 a.m.; 3 subject began between 12:37 p.m. and 2:41 p.m.; and the remainder at 9:42 a.m., 5:42 p.m., and 6:04 p.m. For each subject, blood was drawn after the subject had been motionless in the scanner for the full study.

This study was limited to the tibiofemoral joint, and other joints, for example, hip, may exhibit different characteristics. This study did not examine the relationship between COMP and C2C concentrations and morbidity. Disease progression over time was not examined in this study. These data validate neither these specific antigens nor these specific MR techniques for clinical diagnosis or prognosis of DJD in patients.

In conclusion, this is the first report to identify significant correlations between potential molecular biomarkers for joint disease and quantitative MR measures of symptomatic knees. Our findings support the hypothesis that molecular biomarkers can reflect the molecular processes of cartilage degeneration and loss. Furthermore, our findings support the continued development of both COMP and C2C antigen as biomarkers for DJD.

Acknowledgments

The authors wish to acknowledge the support of NIH grants AR046905 and AG17726.

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