Protein Modification by Deamidation Indicates Variations in Joint Extracellular Matrix Turnover

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As extracellular proteins age, they undergo and accumulate nonenzymatic post-translational modifications that cannot be repaired. We hypothesized that these could be used to systemically monitor loss of extracellular matrix due to chronic arthritic diseases such as osteoarthritis (OA). To test this, we predicted sites of deamidation in cartilage oligomeric matrix protein (COMP) and confirmed, by mass spectroscopy, the presence of deamidated (Asp64) and native (Asn64) COMP epitopes (mean 0.95% deamidated COMP relative to native COMP) in cartilage. An Asp64, D-COMP-specific ELISA was developed using a newly created monoclonal antibody 6-1A12. In a joint replacement study, serum D-COMP (p = 0.017), but not total COMP (p = 0.5), declined significantly after replacement demonstrating a joint tissue source for D-COMP. In analyses of 450 participants from the Johnston County Osteoarthritis Project controlled for age, gender, and race, D-COMP was associated with radiographic hip (p < 0.0001) but not knee (p = 0.95) OA severity. In contrast, total COMP was associated with radiographic knee (p < 0.0001) but not hip (p = 0.47) OA severity. D-COMP was higher in soluble proteins extracted from hip cartilage proximal to OA lesions compared with remote from lesions (p = 0.007) or lesional and remote OA knee (p < 0.01) cartilage. Total COMP in cartilage did not vary by joint site or proximity to the lesion. This study demonstrates the presence of D-COMP in articular cartilage and the systemic circulation, and to our knowledge, it is the first biomarker to show specificity for a particular joint site. We believe that enrichment of deamidated epitope in hip OA cartilage indicates a lesser repair response of hip OA compared with knee OA cartilage.

As proteins age they undergo nonenzymatic post-translational modifications. When aged proteins are intracellular, they can be repaired or the protein replaced (1); however, in the extracellular milieu, where no repair mechanisms exist, nonenzymatic modifications can accumulate in a time-dependent manner in proteins whose turnover is slow. Accumulation of these modifications in long lived proteins can potentially alter both their structural and functional properties and provide insights into turnover rates at which proteins are replaced through net synthesis and degradation processes.

One form of nonenzymatic protein modification, deamidation, is believed to be a mechanism of amino acid damage and aging in numerous proteins (2) and a variety of tissues (2, 3). Protein deamidation has been demonstrated to interfere with protein function of interleukin-1β (4), soluble CD4 (5), angio- genin (6), and Bcl-xL (4–7) and may also incite autoimmunity (8). Nonenzymatic deamidation involves the conversion of Asn to Asp or the conversion of Gln to Glu in a spontaneous manner without enzymes (3). During nonenzymatic Asn deamidation, the side chain amine group is lost, causing formation of a succinimide ring. The succinimide ring is unstable and susceptible to hydrolysis at the imide to form Asp in either the α-Asp or β-Asp (isomerized) form depending upon which side of the imide hydrolysis occurs. Things are further complicated as the α-carbon atom in the succinimide ring can undergo racemization allowing the formation of both levorotatory and dextrorotatory optical isomers after hydrolysis. Similarly, Gln deamidation involves formation of a ring structure (glutarimide), where loss of the functional amine group leads to the formation of the acidic residue Glu in again both the levorotatory and dextrorotatory forms of α- or β-Glu. Asn deamidation occurs roughly twice as fast as Gln deamidation because of the less favorable 6-membered glutarimide ring structure. Particular hot spots for deamidation are predicted to exist based upon factors such as steric hindrance and protein context (3), as well as peptide sequence (for instance, dipeptides that deamidate more readily than others include Gly–Asn, Asn–Gly, and Gln–Gly (3)).

One of the tissues in the body most susceptible to accumulation of nonenzymatic protein modifications is cartilage. This is due to the slow turnover rate of many cartilage proteins; for instance, cartilage aggrecan has a predicted half-life of 25 years (9), whereas cartilage collagens have predicted half-lives of at least 120 years (10, 11). Nothing at present is known of the biological effects of these amino acid changes in cartilage, thus representing a significant knowledge gap. We hypothesized that the nonenzymatic modifications that accumulate with cartilage aging could be used to systemically monitor onset and...
degree of extracellular matrix loss during osteoarthritis (OA). To test this hypothesis, we identified a novel protein modification due to deamidation in cartilage oligomeric matrix protein (COMP). COMP is a noncollagenous glycoprotein and a member of the thrombospondin family of extracellular calcium-binding proteins that was initially isolated from cartilage (12). Although primarily expressed in cartilage, COMP is also expressed in tendons, meniscus, and synovial membranes. The carboxyl-terminal globular domain of COMP binds to collagen I and II (13). The amino terminus of COMP oligomerizes to form a pentamer of five identical subunits of 110 kDa (14), creating a pore that is believed to bind chloride and vitamin D₃ (15).

We raised novel monoclonal antibodies (mAb) and developed an ELISA that specifically distinguished and quantified the deamidated form of the epitope from the total amount of the epitope (amidated and deamidated). We further demonstrated the presence of deamidated COMP (D-COMP) in articular cartilage and the systemic circulation, an enrichment of this epitope specifically in hip articular cartilage, and a correlation of this epitope in the systemic circulation with hip OA severity.

**EXPERIMENTAL PROCEDURES**

*Patient Samples*—The Biomarker and Joint Arthroplasty (BAJA) cohort provided serum samples drawn before or 6 months after either total hip or knee arthroplasty. A total of 14 subjects (9 male and 5 female) underwent total knee replacement (n = 10, six male and four female) or total hip replacement (n = 4, three male and one female) (Table 1). The overall mean age was 63.4 ± 13.2 years (59.6 ± 13.8 years for men, 70.2 ± 9.8 for women). The population was overweight with a mean body mass index of 26.8 ± 5.8 (27.8 ± 5.6 for men and 25.0 ± 6.6 for women). Samples were collected under approval of the Institutional Review Board of Duke University.

The Johnston County OA Project (JoCo OA) provided selected serum samples from 450 individuals at baseline evaluation. The JoCo OA is an ongoing, community-based study of knee and hip OA in African American and Caucasian residents in a rural county in North Carolina. Details of this study have been reported previously (16), and demographics are summarized in Table 1. Briefly, this study involved civilian, noninstitutionalized adults aged 45 years and older who resided in six townships in Johnston County. Participants were recruited by probability sampling, with oversampling of African Americans. A total of 3,187 individuals completed a baseline clinical evaluation from 1991 to 1997. Serum was collected for all participants at baseline. To allow for analyses of biomarkers in a sample balanced for gender and age, 450 participants were selected with complete radiographic data at baseline to represent roughly equal proportions of women (54%) and men (46%) across a range of ages (see Table 1). A total of 39% of participants were African American. Individuals having radiographic evidence of rheumatoid arthritis or other inflammatory arthropathies in the knees or hips were not included in the subsample. Participants completed bilateral anteroposterior weight-bearing radiography of the knees with foot mat placement and supine anteroposterior hip radiographs. Radiographs were read, without knowledge of participant clinical or biomarker status by a single musculoskeletal radiologist (J. B. R.), for overall radiographic severity by Kellgren-Lawrence (17) (KL, score 0–4) of the knees and hips and for knee osteophyte (score 0–3) and joint space narrowing (score 0–3) based on the standardized Burnett atlas (18). Inter-rater reliability (comparison of radiograph readings between J. B. R. and another radiologist) and intra-rater reliability (comparison of radiograph readings completed by J. B. R. at two separate times) were high (weighted κ for inter-rater reliability 0.9; κ for intra-rater reliability 0.9) (19). The study was conducted under approval of the Institutional Review Boards of the University of North Carolina, Chapel Hill, and the Centers for Disease Control and Prevention.

Waste articular cartilage specimens were obtained from randomly selected total knee (n = 15, mean age 66.5 ± 8.9, 54–88 years) and total hip (n = 11, mean age 77.3 ± 11.3, 57–90+ years) arthroplasties performed at Duke University Medical Center to alleviate symptoms of OA. Patient characteristics are summarized in Table 1. From each arthritic joint, cartilage was harvested from around the lesion (lesion cartilage) and for comparison cartilage remote from the lesion (remote cartilage). Nonarthritic control samples (n = 11) were obtained from National Disease Research Interchange (Philadelphia) or at the time of reconstructive surgery for trauma from patients without evidence of OA as determined by the surgeon and macroscopic inspection of the specimens. Samples were collected under Duke Institutional Review Board approval as waste surgical specimens.

*Monoclonal Antibody Generation and Screening*—To generate novel specific mAbs to investigate the predicted deamidated epitopes in COMP, a COMP peptide, TFLKD₆⁴TVMEC, specific for the deamidated epitope was used to immunize mice (A&G

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**TABLE 1**

Cohort demographics

<table>
<thead>
<tr>
<th></th>
<th>BAJA</th>
<th>JoCo OA</th>
<th>Human cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (% female)</td>
<td>14 (36%)</td>
<td>450 (54%)</td>
<td>26 (42%)</td>
</tr>
<tr>
<td>Age mean ± S.D. years (range)</td>
<td>63.4 ± 13.2 (44–81)</td>
<td>58.88 ± 9.62 (44–86)</td>
<td>71.3 ± 10.0 (52–90+)</td>
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<tr>
<td>No OA (N)</td>
<td>0</td>
<td>164</td>
<td>11</td>
</tr>
<tr>
<td>Hip OA without knee OA (N)</td>
<td>4</td>
<td>143</td>
<td>11</td>
</tr>
<tr>
<td>Knee OA without hip OA (N)</td>
<td>10</td>
<td>79</td>
<td>15</td>
</tr>
<tr>
<td>Hip and knee OA (N)</td>
<td>0</td>
<td>64</td>
<td>N/A</td>
</tr>
</tbody>
</table>

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2 The abbreviations used are: OA, osteoarthritis; BAJA, Biomarkers and Joint Arthroplasty study; COMP, cartilage oligomeric matrix protein; Gdn-HCl, guanidine HCl; JoCo OA, Johnston County Osteoarthritis Project; KL grade, Kellgren-Lawrence grade of OA severity; mAb, monoclonal antibody; OPD, o-phenylenediamine dihydrochloride; D-COMP, deamidated COMP.
Protein Modification by Deamidation

Pharmaceutical, Columbia, MD). Antibody specificity was confirmed using a direct ELISA. Briefly, 96-well ELISA plates were coated with COMP purified from hip cartilage (a gift from Dr. V. Vilim) or BSA conjugated to the deamidation-specific COMP peptide (EITFLKD$_{64}$TVMEC) or the nondeamidated native COMP peptide (EITFLKN$_{64}$TVMEC). For later screening experiments, was pulverized under liquid nitrogen and extracted in 4M guanidine HCl (Gdn-HCl) in sodium acetate buffer, pH 4.0, with protease inhibitor mixture (Sigma) at a ratio of 2 ml of Gdn-HCl extraction buffer per 1 g of wet weight cartilage. Cartilage was extracted for 24 h at 4 °C with gentle mixing, followed by centrifugation and collection of the supernatant. The remaining cartilage was extracted a second time with Gdn-HCl extraction buffer for a further 24 h. Both the first and second Gdn-HCl extractions were combined and stored at −80 °C until needed. A 200-µl aliquot of the extract was buffer-exchanged on an AKTA purifier 10 (GE Healthcare) using a HiTrap desalting column (GE Healthcare) into PBS and stored at −80 °C. Proteoglycan content was determined using the dimethylmethene blue assay as described previously (20). Protein concentrations were determined using the Bradford protein assay (Thermo Scientific) to allow normalization of values.

To confirm the efficiency of our Gdn-HCl extraction protocol, we performed five sequential Gdn-HCl extractions in triplicate with brief water washes between two specimens, a hip (non-OA, female, 69 years) and a knee (non-OA, male, 77 years). These analyses showed that the method we used routinely, that of two serial Gdn-HCl extractions, was highly efficient and extracted between 99.3% (as estimated by glycosaminoglycan) and 100.0% (as estimated by protein) of the soluble proteins from cartilage (supplemental Table 1).

Mass Spectroscopic Identification of Deamidated COMP—To prevent interference with downstream liquid chromatography-mass spectroscopy (LC-MS/MS), residual Gdn-HCl was removed by buffer exchanges into 50 mM ammonium bicarbonate, pH 7.8, using a HiTrap desalting column on an AKTA HPLC system (GE Healthcare) followed by trypsin digestion. The insoluble residue left after Gdn-HCl extraction was prepared for LC-MS/MS by extensive washing of the cartilage with 50 mM ammonium bicarbonate, pH 7.8, for 3 days with five buffer changes per day. After these extensive washes, the residual cartilage was weighed before direct trypsin digestion. After trypsin digestion, all samples were subjected to shotgun proteomics using LC-MS/MS as described previously (21). Briefly, 0.1% v/v rapigest (Waters) was added before reduction with 10 mM dithiothreitol at 80 °C for 15 min, alkylated with 20 mM iodoacetamide for 30 min at room temperature, and digested with proteomics grade trypsin (Promega, Madison, WI) overnight at 37 °C. Prior to LC-MS/MS analysis, all samples were resuspended in 20 µl of 2% acetonitrile, 0.1% formic acid, pH 3.0. Chromatographic separation of peptide mixtures was performed on a Waters NanoAcquity UPLC equipped with a 1.7-µm BEH130 C$_{18}$ 75-µm inner diameter × 250-mm reversed-phase column. The mobile phase was 0.1% formic acid in water (buffer A) and 0.1% formic acid in acetonitrile (buffer B). MS analysis was performed on a Waters Synapt G2 mass spectrometer, and label-free quantification and integration of qualitative peptide identifications were performed using Rosetta Elucidator (Version 3.3, Rosetta Inpharmatics, Seattle). All raw LC-MS/MS data were subjected to chromatographic retention time alignment using the PeakTeller® algorithm. Quantification of all signals in the precursor MS spectra was performed by Elucidator by calculating peak volume (area under curve). Data were searched with Mascot (Matrix Science, Boston) against a human protein data base downloaded from SwissProt appended with yeast alcohol dehydrogenase.

D-COMP Competitive ELISA—A competitive ELISA was developed to determine whether the D-COMP epitope could be measured using our mAbs in biological samples. Briefly, coating concentrations were optimized (data not shown), and binding plates were prepared by coating 96-well ELISA plates with 6.5 µg/ml D-COMP-specific BSA-peptide (BSA-EITFLKD$_{64}$TVMEC) in 0.02 M sodium carbonate coating.
buffer, pH 9.6, overnight at 4 °C. Plates were blocked for at least 2 h with 5% w/v BSA in PBS, pH 7.4, at 37 °C prior to use. Protein G-purified 6-1A12 was diluted to 2 μg/ml in protein diluents (0.1% w/v BSA in PBS, pH 7.4) before addition of 80 μl of mAb to 80 μl of either sample or standard in a low protein binding mixing plate for 30 min at room temperature with gentle mixing. Excess antibody was discarded, and the membrane was washed before signal development with ECL Plus reagent (GE Healthcare) using Hyperfilm ECL x-ray film (GE Healthcare).

**Statistical Methods**—Because of the small sample size, the BAJA study data were analyzed using the Wilcoxon signed rank nonparametric paired t test using GraphPad Prism Version 5 (La Jolla, CA). The COMP concentrations from the JoCo OA cohort were log transformed to achieve a normal distribution before analysis using a generalized linear model, the cumulative logit model, controlling for age, gender, and race. As radiographic data from the left and right joints of a person were likely to correlate, the intra-individual correlation was estimated in the model as a variance component. The JoCo OA data represented in Fig. 4 were log transformed and analyzed by one-way analysis of variance with a Bonferroni correction for multiple comparisons. The cartilage extract data were analyzed either with the Wilcoxon signed rank nonparametric paired t test for paired data or using the nonparametric Mann-Whitney U test for unpaired data.

**RESULTS**

**Epitope Selection**—We identified putative deamidation hot spots within COMP using the algorithm developed by Robinson and Robinson (3). This algorithm used inputted crystal structure and amino acid composition of COMP to predict Asn residues susceptible to deamidation and gave an estimate of the deamidation half-life for each individual Asn residue (CD value × 100 days) and half-life for net deamidation value (ID value × 100 days). As the crystal structure for human COMP was not available at the time of this project, we used the two partial rat Protein Data Bank structural files 1fbm (24) and 1vdf (25). Both crystal structures span the amino–terminal region of rat COMP between Gly27 and Gly72. Using the two rat terminal structural files, we identified two Asn residues (amino acids 41 and 63) susceptible to deamidation, in human COMP designated Asn41 and Asn63, respectively (Table 2). Neither of these residues has a particularly fast deamidation rate, with Asn41 predicted to have a deamidation half-life between 21 and 26 years and Asn63 between 39 and 50 years, making them ideal markers for monitoring degradation of a long lived tissue such as cartilage matrix degradation. We compared the sequence alignment for a range of different species available in the NCBI database (www.ncbi.nlm.nih.gov) and confirmed that the predicted Asn deamidation hot spots were contained in human COMP and conserved in a wide range of other animals (Table 2).

To determine whether the predicted deamidated sequences occur in vivo, we performed LC-MS/MS analysis of Gdn-HCl extracts from five articular cartilage specimens from three subjects. We were unable to confirm the presence of the predicted deamidation event at Asn42 in these samples. However, we were able to confirm the presence of the native Asn63 tryptic peptide (63NTVMECDACGMQQSVCR) and the deamidated Asp64 peptide (64DTVMECDAGMQQSVCR) in normal hip, OA hip, and OA knee cartilage (both remote and lesioned regions). As we were able to determine peak intensities for both the D-COMP ELISA sensitivity was increased using avidin poly-
Asp$^{64}$ and Asn$^{64}$ peptides within the same MS run, we calculated within sample ratios of D-COMP to native COMP. In all five specimens, the native Asn$^{64}$ peptide was more abundant than the Asp$^{64}$ deamidated peptide (by 83–330-fold) based on intensities of mass spectroscopic traces, with a mean (S.D.) intensity of 145,178/1100686,144 for the Asn$^{64}$ peptide compared with an intensity of 1,382/110061,092 for the Asp$^{64}$ deamidated peptide (representing a mean 0.95% relative level of D-COMP to native COMP).

Antibody Generation and Validation—To generate mAbs to deamidated epitopes in COMP, mice were immunized with a peptide containing Asp$^{64}$ (TFLKD$^{64}$TVMEC) found in deamidated COMP. Using this approach, we expected to generate two classes of mAbs, those that recognized the deamidated Asp$^{64}$ residue and mAbs that were deamidation-independent, recognizing both the amidated and deamidated sequence. We identified 21 mAb clones that recognized the immunogen. To determine the specificities of these 21 clones, we compared their immunoreactivity to four different BSA-conjugated COMP peptides as follows: the deamidated Asp$^{64}$ (TFLKD$^{64}$TVMEC) found in deamidated COMP, native Asn$^{64}$ (TFLKN$^{64}$TVMEC), deamidated Asp$^{42}$ (CELOQETD$^{42}$AALQ), and native Asn$^{42}$ (CELQETN$^{42}$AALQ) (Fig. 1a). As anticipated, we identified the following two types of mAbs using this approach: mAbs that appeared to be deamidation-independent as they recognized both the Asn$^{64}$ and Asp$^{64}$ containing BSA constructs (clones 6-1A10, 6-3B3, 6-3B1, 6-3A1, 6-1G7, 6-1H12, 6-1H7, and 6-2A9), and deamidation-specific clones (clones 6-1A12, 6-1A6, 6-1A8, 6-1A9, 6-1F9, 6-1B4, 6-3B5, 6-1H3, 6-3A5, 6-3B10, 6-1F8, 6-1H1, and 6-1C12). None of the 21 clones raised to the Asp$^{64}$ peptide bound the BSA-coupled Asn$^{42}$ or Asp$^{42}$ control peptides; the immunoreactivity of these control peptides was confirmed by reactivity with the deamidation-independent mAb (5-3D4) raised specifically to the Asp$^{42}$-containing peptide (Fig. 1a).

To confirm that the mAbs recognized COMP from human cartilage and not just peptides, a subset of these mAbs was screened by direct ELISA against COMP purified from human hip cartilage pooled from two subjects (Fig. 1b) (generous gift from V. Vilim (see Ref. 26)). All mAbs tested displayed higher than background immunoreactivity to cartilage COMP. Of note, the mAb preparations used in these validation experiments were not purified so the apparent differences in immunoreactivity could reflect mAb concentration rather than mAb affinity. In addition, the fact that the deamidation-specific mAbs reacted to cartilage COMP suggested sufficient sensitivity on the part of these mAbs to identify the presence of deamidated Asp$^{64}$ in COMP purified from human articular cartilage.

For the further experiments in this study, we selected the Asp$^{64}$ deamidation-specific 6-1A12 mAb because it grew well in culture and produced a high yield of mAb. The specificity and affinity of the 6-1A12 mAb was confirmed through evaluation of its binding to different coating concentrations of both the native and deamidated BSA peptides (Fig. 1c). The 6-1A12 mAb had no affinity for the native Asn$^{64}$-containing sequence but instead had a high specificity for only the deamidated Asp$^{64}$ COMP-containing peptide. To further confirm that the 6-1A12 mAb recognized COMP extracted from cartilage and not just a COMP-specific peptide, we performed a reduced Western blot of OA hip cartilage Gdn-HCl extract (Fig. 2). The deamidation specific 6-1A12 mAb detected a 110-kDa protein that corre-
sponded to a band of similar molecular mass detected by the established anti-COMP mAb 17-C10 (27) and that corresponded to the size of monomeric COMP.

Decline in Serum Concentrations of D-COMP but Not Total COMP after Joint Arthroplasty—To determine whether the D-COMP epitope could be measured in biological fluid, we

![Diagram](image_url)

**FIGURE 1.** Monoclonal antibody specificity of mAbs raised to the COMP-deamidated epitope Asp\(^{64}\). a, screening of mAbs against deamidated and native COMP peptides. Two native COMP BSA-peptide constructs, Asn\(^{42}\) (BSA-CELQET\(^{42}\)AALQ) and Asn\(^{64}\) (TFLK\(^{64}\)TVMEC-BSA), and the corresponding two deamidated BSA constructs, Asp\(^{42}\) (BSA-CELQET\(^{42}\)AALQ) and Asp\(^{64}\) (TFLK\(^{64}\)TVMEC-BSA), were coated onto 96-well plates. Hybridoma culture media were incubated with the coated plates for 2 h before addition of goat anti-mouse alkaline phosphatase secondary antibody and ELISA development using OPD substrate. A negative control containing no primary antibody was included, and a mAb raised against CELQET\(^{42}\)AALQ was used to confirm immunoreactivity of the BSA-CELQET\(^{42}\)AALQ control peptides. Screening yielded a total of eight deamidation-independent (immunoreactivity to Asn\(^{64}\) and Asp\(^{64}\)) and 13 deamidation-dependent mAbs (preferential immunoreactivity to Asp\(^{64}\)). b, screening of mAbs against purified cartilage COMP. Purified COMP protein (a generous gift from V. Vilim) was coated onto 96-well plates. Hybridoma culture media were incubated with the coated plates for 2 h before addition of goat anti-mouse alkaline phosphatase secondary antibody and ELISA development using OPD substrate. A negative control containing no primary antibody and a positive control using the anti-COMP 17-C10 mAb were included. c, mAb 6-1A12 preferentially reacted against deamidated COMP peptide and not the native COMP sequence. To test the affinity of the 6-1A12 mAb for both native and deamidated COMP, different concentrations of either the D-COMP Asp\(^{64}\) specific TFLK\(^{64}\)TVMEC-BSA or the native COMP-specific Asn\(^{64}\) TFLK\(^{64}\)TVMEC-BSA were coated onto a 96-well plate in a direct ELISA, performed as for a. A standard curve with an appropriate dose response is generated for 6-1A12 and the deamidated COMP Asp\(^{64}\)BSA construct; 6-1A12 did not recognize the native Asn\(^{64}\)BSA construct.
developed a competitive ELISA using the 6-1A12 mAb and the BSA-coupled deamidated COMP-specific (TFLKD<sup>64</sup>TVMEC) construct as both the coating antigen and the assay standard. In the BAJA study, serum (n = 14) was collected before joint replacement (pre-joint arthroplasty samples) and 6 months after either hip (n = 4) or knee (n = 10) total arthroplasty. We expected to observe a decrease in the serum concentrations of biomarker after joint arthroplasty, thereby supporting in principle a joint tissue source for the measured biomarker in the serum. Six months after joint replacement, serum total COMP concentration (1,513 ± 457 ng/ml) was not significantly changed (p = 0.502) from baseline prior to joint replacement (1,600 ± 655 ng/ml) (Fig. 3a). However, we did observe a small but significant (p = 0.017) decrease in the serum concentrations of D-COMP 6 months after joint replacement (578.6 ± 154 pg/ml) compared with baseline prior to joint replacement (726 ± 158 pg/ml) (Fig. 3b). Stratified by joint site, we observed a small but nonsignificant decreasing trend for D-COMP following knee replacement (13% mean decline from 732 ± 48 pg/ml prior replacement to 638 ± 34 pg/ml 6 months post-replacement) and a larger decreasing trend for D-COMP following hip replacement (39% mean decline from 711 ± 108 pg/ml to 431 ± 83 pg/ml post-replacement). There was minimal change in total COMP stratified by joint site for knee (5% mean decline from 1680 ± 225 to 1593 ± 166 ng/ml post-replacement) or hip (6% mean decline from 1400 ± 260 to 1316 ± 26 ng/ml post-replacement).

Serum concentrations of D-COMP and total COMP were reported on hip and knee OA severity, respectively. To further investigate the utility of D-COMP as a novel biomarker for OA, we measured D-COMP and total COMP in the JoCo OA subsample of patients. This sample subset was derived from the population-based JoCo study (16). The total subsample of 450 individuals included 898 nonreplaced knees and 896 nonreplaced hips, graded for radiographic OA severity by KL grade. For improved sensitivity, we developed and optimized (data not shown) a sandwich ELISA using our deamidation-specific 6-1A12 mAb for capture and the 17-C10 total COMP mAb for detection. Significant differences were determined using the paired nonparametric Wilcoxon signed rank test. Total COMP was unchanged after joint replacement (a), and D-COMP declined significantly (p = 0.017) following joint replacement (b).
To better understand the turnover of COMP at the different sites, we evaluated the ratio of aged D-COMP/total COMP (Fig. 5c). The D-COMP/total COMP ratio was highest in hip OA.
lesional cartilage and higher at the hip lesion versus any of the other regions, namely versus the hip remote \( (p = 0.001) \), knee remote \( (p < 0.0001) \), or knee lesion \( (p = 0.0003) \) cartilage (mean ± S.D., 29.96 ± 38.18 hip lesion, 9.58 ± 18.59 hip remote, 1.30 ± 0.78 knee remote, and 2.47 ± 2.84 knee lesion). We also observed a significantly higher D-COMP/total COMP ratio between hip remote and knee remote cartilage (mean ± S.D., 9.58 ± 18.59 and 1.30 ± 0.78, respectively, \( p = 0.017 \)).

Relative to non-OA hip cartilage, hip lesion OA cartilage had significantly higher \( (p = 0.012) \) extractable concentrations of D-COMP as a proportion of total COMP (mean ± S.D., 1.84 ± 0.5 and 2.47 ± 2.1 respectively, Fig. 5c). As D-COMP has a long residence time in cartilage, it is conceivable that the differences observed between hip and knee OA D-COMP levels could be due to differential cross-linking of D-COMP to the collagen matrix preventing release from the cartilage in knee OA. To test this, we performed LC-MS/MS analysis of extracts from lesional regions of three age-matched cartilage samples as follows: an OA hip (72 years), an OA knee (75 years), and a normal hip (76 years). Gdn-HCl extraction of the cartilage samples yielded a soluble protein fraction and a paired insoluble fraction that was subjected to trypsin digestion and mass spectroscopic analysis. The tryptic peptide \( (^6\text{NTVMECDACGMQQSVR}) \) corresponding to the native COMP sequence was present in all samples. As expected, more native COMP peptide was found in the Gdn-HCl-soluble fraction than in the insoluble cartilage residua (see supplemental Table 2). However, we were only able to identify the deamidated peptide \( (^6\text{DTVMECDACGMQQSVR}) \), corresponding to the D-COMP epitope, in the Gdn-HCl soluble fraction and not in the insoluble fraction. Although this result does not absolutely exclude its presence in the insoluble fraction, it suggests that the deamidated epitope is not preferentially tethered in cartilage. The ratios of the intensity of the deamidated/native COMP peptide in the soluble protein fractions confirmed the abundance of extractable deamidated COMP from hip compared with knee cartilage (supplemental Table 2).

As we have no evidence to suggest preferential cross-linking in the OA knee cartilage, we believe that these results suggest lower turnover and older proteins in hip OA lesions. Interestingly, we observed the reverse in knee OA. Relative to non-OA knee cartilage (mean ± S.D., 6.73 ± 4.9), knee OA had significantly lower concentrations of D-COMP as a proportion of total COMP at both remote \( (p = 0.004, 2.47 ± 2.8) \) and lesion \( (p = 0.009, 1.30 ± 0.9) \) sites. These results suggest that the COMP in knee OA cartilage is newer and turning over faster. Non-OA hip and knee cartilages were statistically similar with respect to D-COMP as a proportion of total COMP suggesting that non-OA hip and knee cartilages have similar turnover and that the joint response to OA is quite different by hip versus knee joint site. Taken together, these data are consistent with a
model positing lower rates of cartilage COMP synthetic repair in hip OA, and particularly at hip OA lesions, compared with normal hip and non-OA and OA knee.

**DISCUSSION**

To our knowledge, this is the first demonstration that a cartilage extracellular matrix component, measured systemically, demonstrated specificity for OA severity at a particular joint site. We believe this reflects a fundamental underlying difference between hip and knee cartilage turnover and repair responses in OA. We observed no association between D-COMP serum levels and chronological age in non-OA subjects. This is not surprising as the half-life for generating our epitope is predicted by the Robinson and Robinson algorithm (3) to be between 39 and 50 years. Assuming COMP has a turnover rate similar to the predicted half-life of 25 years for aggregan (9), then accumulation of D-COMP with age in healthy cartilage would be prevented by normal cartilage turnover.

We demonstrated in our post-joint arthroplasty BAJA study of 14 patients, a significant decrease in the concentrations of our new and novel D-COMP biomarker after the removal of the affected arthritic joints. The greatest decrease in D-COMP was observed for the hip, although the knee showed a much more modest decrease in D-COMP levels. As OA does not usually affect only a single joint, and even normal joint turnover would be expected to contribute to the overall serum D-COMP levels, replacement of a single joint led, as expected, to a decline but not a disappearance of D-COMP from the serum. In contrast, we did not observe any significant decrease in total COMP levels after joint arthroplasty. These results with total COMP are consistent with observations made previously by Sharif *et al.* (28) who demonstrated that total COMP actually increased, rather than decreased, for at least 6 months following joint replacement. Other reports have demonstrated total COMP production by osteoblasts (29) and the potential for elevated total COMP during the period of reactive bone repair following joint arthroplasty. We believe that D-COMP measured systematically reflects degradation of mature aged cartilage; this is in contrast to native or total (the majority of which is native non-deamidated) COMP epitope in the systemic circulation that we posit reflects, in part, high turnover of newly synthesized tissue, or so-called “frustrated repair” (30), or new COMP production from a reparative bone response occurring after joint replacement.

In the much larger JoCo OA cohort, we were better able to refine our understanding of the utility of D-COMP as a biomarker. In this cohort, we observed that D-COMP was highly significantly correlated with hip OA severity but not with knee OA severity. We also observed that total COMP was strongly associated with knee OA but not with hip OA severity. Of note, in the JOCO cohort sample, the greatest increase in serum D-COMP occurred with the transition from the KL0-1 to the KL2 level of hip OA severity. This would suggest that D-COMP may be of particular value as an early indicator of hip pathology and OA.

The association of total COMP with knee OA progression is well established (31–35). A few studies have investigated total COMP in the setting of hip OA (36–39). Three studies observed that higher levels of baseline COMP predicted the development of hip radiographic OA (36, 38, 39). One of these studies suggested that high baseline COMP levels were associated with a reduction in radiographic progression (36). One small study found an association between hip JSN and increasing serum COMP levels over a 1-year follow up period (37), and another larger study found no association of total serum COMP with hip OA but a significant association with knee OA osteophyte (40). We believe the unique ability of our D-COMP biomarker to correlate with hip but not knee OA severity demonstrates the utility and value of studying post-translational modifications in cartilage proteins as biomarkers of OA.

To better understand the underlying biological basis for D-COMP as a hip OA but not knee OA biomarker, we investigated the concentrations of D-COMP in cartilage extracts from OA hips and knees. We hypothesized that D-COMP would be increased in cartilage with a low turnover rate and that biologically older tissues would have a higher D-COMP/total COMP ratio. We observed a significantly higher mean D-COMP/total COMP ratio in OA hip cartilages compared with OA knee cartilages. Total COMP concentrations between hip and knee cartilage were not significantly different confirming that the differences observed in the ratios were due to a higher proportion of D-COMP in hip cartilage. These data strongly suggest that COMP, and by inference the cartilage extracellular matrix in an OA hip joint, is turned over (net of catabolism and anabolism) at a much lower rate than cartilage in an OA knee joint. Conversely, this would indicate that knees are more robust at repairing ongoing degradation than hips.

In the hip, we also observed significantly higher concentrations of D-COMP and D-COMP/total COMP ratio in lesional OA cartilage when compared with remote regions. The elevated concentrations of D-COMP at hip OA lesions further support the serum biomarker observation that D-COMP was associated with hip OA. Moreover, this suggests that lesional cartilage is biologically older than the cartilage remote from the lesion and is consistent with lower rates of COMP synthesis at sites of hip OA lesions. For comparison, we included non-OA and age-matched cartilage collected as cadaveric tissue or shortly after trauma at the time of surgical repair. We found no significant difference between non-OA hip and knee cartilage for either D-COMP or total COMP. However, the D-COMP/total COMP ratio of OA lesional hip cartilage was significantly higher than non-OA cartilage suggesting that cartilage at the lesion is older. In contrast, we observed the opposite in the knee, with significantly lower than normal D-COMP/total COMP ratios in the OA tissue. As we were unable to find any evidence of enhanced cross-linking of older COMP in the knee OA cartilage, we believe these data support the hypothesis that serum D-COMP reflects cartilage turnover and that hip OA tissue is older (low repair response) than non-OA hip, whereas the knee OA tissue is younger (high repair response) than non-OA.

Although higher concentrations of D-COMP at hip lesions could potentially be explained by accelerated D-COMP production, we believe this to be unlikely as there was no evidence for accelerated production of D-COMP at knee OA lesions, which would be expected to be exposed to very similar catabolic...
conditions during cartilage loss. Radiolabeled proteoglycan studies in cartilage have shown that cartilage matrix turnover is greatest in the superficial zone whereas the matrix becomes older and the protein turnover rate decreases deeper into the cartilage (41). Therefore, we favor a model wherein gradual erosion of the cartilage at hip OA lesions leads, in the relative absence of synthetic repair, to the loss of the superficial and mediat cartilage layers, leaving behind the older deeper zones.

In summary, we were able to predict a deamidation event in the cartilage matrix molecule COMP, demonstrate its presence in human cartilage, produce specific mAbs to this modification, and develop an ELISA to study its utility as a biomarker in OA. We identified a D-COMP modification that is not only a novel biomarker in OA but also a biomarker with specificity for hip OA, which, to the best of our knowledge, is the first OA biomarker specific to a particular joint site. Based upon our studies of COMP in cartilage, we believe we can explain the specificity of our D-COMP biomarker through a lower COMP protein synthesis in hip OA when compared with knee OA and higher rate of knee cartilage extracellular matrix turnover. The clear differences in D-COMP concentrations at remote OA hip and knee cartilage are consistent with different biological aging rates in these two large joint sites in OA due in part to less COMP synthesis in hips than knees. Hip OA cartilage lesions, in particular, were differentially enriched for D-COMP. This is compatible both with loss of the more rapidly regenerating superficial cartilage layers and inadequate COMP synthesis, resulting in biologically older remaining deep layers of hip OA cartilage. We believe that D-COMP warrants further study as a marker of hip disease to determine more clearly its utility in a patient setting, although currently we would envision its use as a longitudinal marker to follow disease progression within a subject. Examination is also warranted to determine its ability to detect occult or pre-radiographic hip disease.

Acknowledgments—We thank David Quintana for assistance with the BAJA sample collection. We also thank the Duke Proteomics Core Facility (Drs. Erik Soderblom and Arthur Moseley) for their expert assistance and Dr. Vladimir Vilim for the gift of 17-C10 antibody and purified COMP from human cartilage.

REFERENCES


### Supplemental Table 1. Data from cartilage extraction optimization experiments demonstrating highly efficient extraction of soluble proteins from cartilage with a single Gu-HCl incubation.

Two non-OA cartilage samples were extracted in triplicate with five sequential Gu-HCl extractions to determine the efficiency of the extraction protocol. Total protein was determined using the Bradford protein assay and total glycosaminoglycan (GAG) was determined using the dimethylmethylene blue (DMMB assay). Results were normalized to cartilage wet weight.

<table>
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<tr>
<th>Joint</th>
<th>Age</th>
<th>Gender</th>
<th>Total Extracted</th>
<th>Gu-HCl Extraction 1</th>
<th>Gu-HCl Extraction 2</th>
<th>Gu-HCl Extraction 3</th>
<th>Gu-HCl Extraction 4</th>
<th>Gu-HCl Extraction 5</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>µg/mg</td>
<td>%</td>
<td>µg/mg</td>
<td>%</td>
<td>µg/mg</td>
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<tr>
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<td>259.5</td>
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<td></td>
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<td>297.5</td>
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Supplemental Table 2. Native COMP but not deamidated COMP is detected in the insoluble fraction of cartilage after extraction with Gu-HCl. Three cartilage samples [hip OA lesional (72 years), knee OA lesional (75 years) and non-OA hip (76 years)] were extracted with Gu-HCl as per our standard protocol to yield soluble proteins (Gu-HCl extractable) and insoluble residual proteins. The soluble protein fractions were prepared for LC-MS/MS by extensive dialysis over 3 days at 4°C with 5 changes of 50 mM ammonium bicarbonate, pH7.8, per day. The insoluble cartilage protein fractions were also similarly washed to remove the Gu-HCl. Samples were trypsinized using standard protocols and separated by LC-MS/MS on a Waters Synapt G2 mass spectrometer. Data were aligned and label-free quantification and integration of qualitative peptide identifications was performed using Rosetta Elucidator (v 3.3, Rosetta Inpharmatics, Seattle, WA). Peptides corresponding to native (non-deamidated) COMP (N<sub>64</sub>TVMECDACGMQSVR) and deamidated D-COMP (D<sub>64</sub>TVMECDACGMQSVR) were identified in the soluble fractions but only native COMP was identified in the non-soluble cartilage protein fraction. The proportion of native COMP remaining in cartilage after extraction was estimated based on the total yield of the soluble and insoluble fractions from each sample. The ratios of the intensity of the Deamidated/Native COMP peptide in the soluble protein fractions confirmed the abundance of extractable deamidated COMP from hip compared with knee cartilage.