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# Assessment of the utility of biomarkers of osteoarthritis in the guinea pig[1]

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## Summary

*Objective*: To identify biochemical markers of osteoarthritis (OA) in the guinea pig, we characterized four biomarkers and 17 cytokines for age- and strain-related differences.

*Methods*: Two guinea pig strains were examined in this study: (1) the Hartley (OA-prone) and (2) Strain 13 (OA-resistant). Levels of synovial fluid keratan sulfate (KS) and cartilage oligomeric matrix protein (COMP), as well as levels of serum C2C, CPII, and a panel of cytokines and chemokines were quantified in both guinea pig strains. These included: IL-1$\alpha$, IL-1$\beta$, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-12p70, IL-17, G-CSF, GM-CSF, IFN-$\gamma$, KC, MIP-1$\alpha$, RANTES, and TNF-$\alpha$.

*Results*: Synovial fluid concentrations of KS and COMP increased coincident with histological OA and correlated positively with the severity of histological damage in both strains. Synovial fluid concentrations of these biomarkers were elevated in the knees of the Hartley compared to the Strain 13 animals, as early as 2 months of age. From as early as 4 months of age, the levels of serum C2C/CPII, representing the ratio of type II collagen degradation and synthesis, were elevated in the OA-prone Hartley compared with Strain 13 animals. Also, at 12 months of age, strain-related differences were apparent for 11 of the 16 cytokines and chemokines. Using multiple linear regression, serum IL-6 and TNF-$\alpha$ concentrations were each strongly associated with strain, weight, and their interaction ($r^2 = 0.80$, $P = 0.0002$ for IL-6; $r^2 = 0.55$, $P = 0.02$ for TNF-$\alpha$).

*Conclusions*: Biomarkers derived from synovial fluid are reflective of histological severity in the spontaneous model of OA in the guinea pig. The synovial fluid biomarker profiles indicated accelerated cartilage matrix turnover in the Hartley strain as early as 2 months of age, prior to evidence of histological damage. The Hartley strain also exemplified an imbalance in type II collagen metabolism and a serum cytokine/chemokine profile indicative of a pro-inflammatory state. These findings elucidate additional disease-related features in the guinea pig that have relevance to OA in humans.


*Key words*: Osteoarthritis, Biomarkers, Animal models, Guinea pig, Collagen, Cytokines, Chemokines.

## Introduction

Through both animal and human studies, efforts have continued to identify biomarkers that are specific for osteoarthritis (OA). OA-related biomarkers can complement, and maybe someday substitute for, histological and imaging assessments of disease progression or response to therapeutic interventions. In order for biomarkers to be useful, it is important that they be validated. Many factors influence biomarker concentrations[1] and animal models provide a less complex system than humans in which to validate and assess the utility of OA-related biomarkers.

The Hartley guinea pig is a well-characterized model of naturally occurring OA in which the development of knee joint pathology closely resembles that in humans[2–4]. We discovered previously that the inbred Strain 13 guinea pig is OA-resistant relative to the OA-prone Hartley guinea pig, and thus provides a valuable age-matched control to the Hartley strain[4]. In the current study, we characterized guinea pigs of both strains and of varying ages to evaluate the discriminating capabilities of four biomarkers and 17 cytokines for OA.

Among the four biomarkers, we evaluated keratan sulfate (KS) and cartilage oligomeric matrix protein (COMP) in synovial fluid to assess localized matrix turnover, disease progression, and association with histological OA. KS is a sulfated glycosaminoglycan component of aggrecan, the second most abundant protein in articular cartilage. COMP is a member of the thrombospondin family of extracellular proteins and is abundantly expressed in human cartilage[5]. The development of specific antibodies to KS and COMP has made it possible to assess cartilage metabolism at a molecular level. In this way, synovial fluid concentrations of KS and COMP have been validated as biomarkers of joint tissue turnover in animals[6–10], as well as in patients with arthritis[11–13]. C2C (Col2-3/4C long mono) is a marker of cartilage degradation which specifically recognizes the carboxy-terminus of the three-quarter length fragment of type II collagen[14]. This collagenase-generated cleavage epitope has been shown to be elevated in serum, synovial fluid, and urine in a model of experimental OA in the dog[15], suggesting its utility as a marker of joint tissue degradation that is capable of detecting early stages of type II collagen catabolism. CPII (C-propeptide) is a marker of type II collagen synthesis, measuring the carboxy-propeptide cleaved during processing of

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newly synthesized type II procollagen. This marker has been shown to be decreased in serum in OA[16]. A panel of 17 cytokines and chemokines in the serum was evaluated to quantify potential systemic indicators of OA. A predefined, commercially available mouse multiplex assay was utilized to permit the simultaneous analysis of OA-related analytes (IL-1, IL-6, IL-17, MIP-1$\alpha$, and TNF-$\alpha$) using a minimal volume of sera. To our knowledge, there have been no previous assessments of cytokines and chemokines in the guinea pig model of OA.

## Materials and methods

### GUINEA PIG STRAINS

Two guinea pig strains were examined in this study: the Hartley strain guinea pigs (OA-prone) were obtained from Charles River Laboratories (Wilmington, MA) at 2 months of age and sacrificed at 2, 4, 7, 10, and 12 months of age ($n = 6$ per group). A different group of 2 ($n = 3$) and 12 ($n = 8$) month-old Hartley and Strain 13 animals were investigated to expand upon the characterization of strain-related differences in the development of OA, initially reported in a previous study[4]. The Strain 13 guinea pigs (OA-resistant) were obtained from Crest Caviary (Prunedale, CA) at 2 ($n = 3$) and 12 ($n = 8$) months of age. Animals were housed in solid bottom cages and fed water *ad libitum* and standard guinea pig chow (Purina Lab Diet 5025) containing Vitamin C (1 mg/g) and Vitamin $D_3$ (3.4 IU/g). All animals were acclimated to housing conditions for 1 week prior to sample collection. The Institutional Animal Care and Use Committee approved all aspects of this study.

### SAMPLE COLLECTION

Blood samples were obtained from guinea pigs under isoflurane anesthesia via cardiac puncture and collected in SST Brand Gel and Clot Activator Vacutainer tubes (VWR, Morrisville, NC). Blood samples were centrifuged at 3500 rpm for 10 min and serum stored in 1 ml aliquots at $-80°C$ until analyzed. Synovial fluid was collected from each knee joint as described previously[4] and stored at $-80°C$ until analyzed.

### HISTOLOGICAL ANALYSES

The knee joints were prepared for histological analyses as described previously[10]. Paraffinized sections (5 $\mu$M) of the central region of the joint were stained with toluidine blue and grading of serial sections of each knee was performed independently by two blinded observers (JLH, VBK) using a modified Mankin grading scheme[4]. Histological evidence of chondropathy was assessed by grading of articular cartilage structure (irregularities such as fibrillation, the presence of clefts, and loss of cartilage [0–8]), and proteoglycan loss (as determined by loss of toluidine blue staining [0–6]). The sum of articular cartilage structure and proteoglycan loss was tabulated for the tibial and femoral condyles for the medial compartment, the lateral compartment, and the whole joint (medial and lateral compartments). The possible total score for each compartment ranged from 0 (normal) to 14 (severe structural damage and complete loss of toluidine blue staining), hence the possible whole joint score ranged from 0 to 56.

### BIOMARKER ANALYSES

Synovial fluid concentrations of KS were measured using monoclonal antibody 5-D-4 (kindly provided by Dr Bruce Caterson, Cardiff, Wales) in a quantitative inhibition enzyme-linked immunosorbent assay (ELISA) as described previously[4]. This antibody reacts against several repeats of a highly sulfated heptasaccharide in KS, a glycosaminoglycan found in cartilage proteoglycans[17]. Levels of 5-D-4 KS epitope were reported in terms of equivalents of an international standard known as KS2 (kindly provided by Dr Eugene Thonar, Chicago, IL) and expressed in nanograms per milliliter. Intra- and inter-assay variabilities were 3.8% and 4.1%, respectively.

Synovial fluid concentrations of COMP were measured by competitive ELISA using monoclonal antibody 12C4 (kindly provided by Dr Vladimir Vilim, Prague, Czech Republic), which cross reacts to guinea pig COMP, as described previously[10]. This antibody recognizes an epitope at the beginning of the carboxy-terminal globular domain of human COMP[18]. Intra- and inter-assay variabilities were 2.3% and 5.9%, respectively.

Serum concentrations of C2C and CPII were quantified using commercially available kits from IBEX (Montreal, CAN) according to the manufacturer's instructions. The intra- and inter-assay variabilities for C2C were 3.3% and 16.3%, respectively, and for CPII, 3.3% and 8.1%, respectively. The ratio of C2C/CPII was calculated as an expression of the relative balance between type II collagen degradation and synthesis. This method has been shown to be more predictive of OA progression than either of the individual markers of synthesis and degradation[19].

Cytokines and chemokines in the serum were measured using the Bio-Plex Protein Array System with the Bio-Plex Mouse cytokine 18-Plex Panel including: interleukin-1alpha (IL-1$\alpha$), IL-1$\beta$, IL-2, IL-3, IL-4, IL-5, IL-6, KC (a murine IL-8 homologue), IL-10, IL-12p40, IL-12p70, IL-17, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-$\gamma$), macrophage inflammation protein 1 alpha (MIP-1$\alpha$), RANTES (regulated upon activation, normal T cell expressed and secreted), and tumor necrosis factor alpha (TNF-$\alpha$) (Bio-Rad, Hercules, CA). All samples were analyzed as recommended by the manufacturer using a standard range of 0–3200 pg/ml and a sample dilution of 1:2; utilizing a total of 30 $\mu$l of sera. For 16 of the 18 cytokines and chemokines measured, a measurable signal was obtained. Sera levels of both IL-1$\alpha$ and IL-4 were below the detection limit of the assay. Due to the limited quantities of guinea pig synovial fluid from knee joints, it was only possible to assess these cytokines and chemokines in the serum.

### STATISTICAL ANALYSES

Nonparametric Kruskal–Wallis test followed by Dunn's multiple comparison post test was used to assess for differences in biomarker concentrations in the Hartley strain at various ages. The nonparametric Mann–Whitney test was used to compare the two guinea pig strains at 2 and 12 months of age. Data analyses were performed using Graph Pad PRISM. A $P$-value of $\leq 0.05$ was considered statistically significant. Standard correlation analyses and linear regression were performed using JMP Discovery software (SAS, Cary, NC).

## Results

A cross-sectional analysis of the Hartley guinea pig strain revealed onset of histological knee OA by 4 months of age