The Potential of Multiple Synovial-Fluid Protein-Concentration Analyses in the Assessment of Knee Osteoarthritis

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Context: Joint trauma is a risk factor for osteoarthritis (OA), which is becoming an increasingly important orthopedic concern for athletes and nonathletes alike. For advances in OA prevention, diagnosis, and treatment to occur, a greater understanding of the biochemical environment of the affected joint is needed. Objective: To demonstrate the potential of a biochemical technique to enhance our understanding of and diagnostic capabilities for osteoarthritis. Design: Cross-sectional. Setting: Outpatient orthopedic practice. Participants: 8 subjects: 4 OA-knee participants (65 ± 6 y of age) and 4 normal-knee participants (54 ± 10 y) with no history of knee OA based on bilateral standing radiographs. Intervention: The independent variable was group (OA knee, normal knee). Main Outcome Measures: 16 knee synovial-protein concentrations categorized as follows: 4 as pro-inflammatory, or catabolic, cytokines; 5 as anti-inflammatory, or protective, cytokines; 3 as catabolic enzymes; 2 as tissue inhibitors of metalloproteinases [TIMPs]; and 2 as adipokines. Results: Two anti-inflammatory cytokines (interleukin [IL]-13 and osteoprotegerin) and a pro-inflammatory cytokine (IL-1β) were significantly lower in the OA knees. Two catabolic enzymes (matrix metalloproteinase [MMP]-2 and MMP-3) were significantly elevated in OA knees. TIMP-2, an inhibitor of MMPs, was significantly elevated in OA knees. Conclusions: Six of the 16 synovial-fluid proteins were significantly different between OA knees and normal knees in this study. Future research using a similar multiplex ELISA approach or other proteomic techniques may enable researchers and clinicians to develop more accurate biochemical profiles of synovial fluid to help diagnose OA, identify subsets of OA or individual characteristics, guide clinical decisions, and identify patients at risk for OA after knee injury.

Keywords: cytokines, matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), adipokines, osteoprotegerin
Osteoarthritis (OA), the most common form of arthritis, is a heterogeneous low-grade inflammatory disease characterized by multitissue organ failure in diarthrodial joints. OA is characterized by loss of joint space, articular cartilage degeneration and attrition, subchondral bone changes (eg, sclerosis, edema, and cysts), osteophyte formation, decreased synovial-fluid viscosity, and synovial-membrane thickening.\textsuperscript{1–4} Over 27 million U.S. citizens have clinically defined OA (symptoms with positive physical examination findings).\textsuperscript{5} Although extensive research has investigated OA, its etiology and pathophysiology are poorly understood. Joint trauma is a risk factor for OA, and it has been estimated that over 5.6 million individuals in the United States have lower extremity posttraumatic OA that requires intervention by an orthopedic surgeon.\textsuperscript{6} Reports of OA after an anterior cruciate ligament rupture, regardless of whether treated surgically or conservatively, range from 10% to 90% with a mean prevalence rate estimated at over 50%.\textsuperscript{7} It has been theorized that to prevent the onset of OA, disease-modifying interventions must be initiated before significant degenerative changes.\textsuperscript{8,9} There is a conundrum, however, in that there is no accepted method for diagnosing early, preradiologic OA or for identifying patients at risk for OA.

Radiographs are currently the gold standard for diagnosing and monitoring the progression of OA.\textsuperscript{10} Unfortunately, significant disease progression may occur before radiographic evidence of OA.\textsuperscript{10} Furthermore, radiographs are unable to detect subtle differences between joints and across time. These limitations may be hindering the development of disease-modifying OA interventions (eg, drugs) intended to slow, halt, or reverse the progression of OA. It has been theorized that interventions need to be initiated before the onset of gross degenerative changes and that sensitive measurements are necessary to detect subtle changes in the joint.

Biochemical measurements may be more sensitive and capable of detecting early changes in the joint after an injury, diagnosing early OA, and monitoring disease progression. Biochemical measurements can be used to assess a variety of biomarkers, including (1) proinflammatory/catabolic mediators (eg, specific subsets of cytokines and chemokines including interleukin [IL]-1\textsubscript{β} and tumor-necrosis factor [TNF]-α), (2) anti-inflammatory/protective mediators (eg, specific subsets of cytokines and chemokines including IL-13), (3) catabolic enzymes (eg, matrix metalloproteinases [MMPs] and a disintegrin and metalloproteinase [ADAMs]), (4) tissue inhibitors of metalloproteinases [TIMP], (5) extracellular matrix fragments of articular cartilage (eg, cartilage oligomeric matrix protein [COMP], C-terminal cross-linking telopeptide of type II collagen [CTX-II]), and (6) markers of bone turnover (eg, C-terminal cross-linking telopeptide of type I collagen [CTX-I], N-terminal type I collagen telopeptide [NTX]). Previous research has analyzed protein concentrations in various tissues but has been limited to analyzing a few proteins per sample.\textsuperscript{11–20} For a more thorough understanding of OA and its progression, it would be ideal to measure multiple proteins in the same sample to determine a biochemical profile for a diseased joint.

Recently, new techniques (eg, mass spectrometry and multiplex enzyme-linked immunosorbent assay [ELISA]) have enabled simultaneous analysis of multiple proteins in small volumes of tissue samples (eg, synovial fluid or serum). The purpose of this study was to compare 16 proteins in synovial fluid from OA knees and normal knees using a multiplex ELISA. This is a preliminary study of a tech-
nique with a potential to enhance our understanding of and diagnostic capabilities for osteoarthritis.

Methods

Design

A cross-sectional design was used in this study. The independent variable was group: OA knee and normal knee, comprising nonosteoarthritic participants’ healthy knees. The dependent variables were 16 knee synovial-protein concentrations categorized as follows: (1) pro-inflammatory, or catabolic (ie, IL-1α, IL-1β, TNF-α, and receptor activator of nuclear factor-kappaB ligand [RANKL]); (2) anti-inflammatory, or protective (ie, IL-1 receptor antagonist [IL-1ra], IL-4, IL-10, IL-13, and osteoprotegerin [OPG]); (3) catabolic enzymes (ie, MMP-2, MMP-3, and MMP-13); (4) TIMPs (ie, TIMP-1 and TIMP-2); and (5) adipokines (ie, adiponectin and leptin). All of these proteins have been reported to be influential in the progression of OA.21–24

Participants

Eight participants were recruited to participate in this study. The 4 OA participants (2 men and 2 women, 65 ± 6 y of age, and body-mass index = 33.3 ± 5.9 kg/m²) were recruited from an outpatient orthopedic practice. They were selected based on a diagnosis of knee OA. The diagnosis was based on clinical symptoms of knee inflammation (eg, chronic joint effusion) and bilateral standing radiographic evidence, using the Kellgren-Lawrence grading system.25 The inclusion criterion was a Kellgren-Lawrence OA score of ≥3 (moderate to severe OA). Two of the OA participants had a history of cardiovascular disease, but none of them were current smokers.

The 4 normal-knee participants were recruited from a public university (3 men and 1 woman, 54 ± 10 y of age, and body-mass index = 26.7 ± 4.0 kg/m²). These participants had no history or evidence of knee OA, based on bilateral standing radiographs. Bilateral anteroposterior standing knee radiographs were used to select participants with bilateral Kellgren-Lawrence grading scores of <1 (no OA). Normal knees were arbitrarily selected. None of the normal-knee participants had a history of cardiovascular disease, but 1 of the normal-knee participants was a current smoker.

All participants were screened for general exclusion factors. Exclusion criteria were (1) any joint pathology other than OA (eg, rheumatoid arthritis, gout, pseudogout), (2) uncontrolled metabolic or immunological disorders, (3) cancer, (4) skin irritation or dermatological conditions (eg, abscess, cellulitis) in the vicinity of the knee from which samples would be obtained, (5) prior history of a fracture within 12 months of entering the study, (6) consistent use of an anti-inflammatory medication for 14 to 90 days, (7) use of an antibiotic within 1 month before the study, and (8) prior history of injury within 3 months of the study. Potential participants were excluded if they presented any additional contraindications for arthrocentesis: bacteremia, knee prosthesis, or bleeding disorders. Potential participants were also excluded if the study physician observed any pathological conditions (eg, crystals,
blood, sepsis) other than OA in the synovial fluid. If they were found, the sample was not used for the study but underwent a separate analysis for diagnostic purposes. Study participation was not based on gender, ethnicity, anthropometric variables, or demographic variables. The study was approved by a university institutional review board, and the Health Insurance Portability and Accountability Act guidelines were adhered to throughout testing. Participants signed an informed-consent form before inclusion.

**Procedures**

OA patients were recruited after their clinical examination during a follow-up visit. Each patient underwent a standard orthopedic evaluation (eg, functional assessment) with a board-certified orthopedic surgeon with over 25 years of clinical experience with knee OA. All participants had had X-rays within 6 months before the study. The study physician evaluated the standing bilateral anteroposterior knee X-rays to determine the Kellgren-Lawrence score. Patients who met the radiographic criteria (moderate to severe OA) were informed of and asked to participate in the study. Those willing to participate signed an informed consent, completed health-history questionnaires, and had their height and weight measured and recorded. The OA participants who met all the eligibility criteria progressed to the synovial analyses.

Normal-knee participants received an orthopedic assessment and radiograph to ensure that they had no significant signs of OA or prevailing joint conditions. They completed the informed consent and the health-history questionnaires before being evaluated by the orthopedic surgeon. Their height and weight were measured and recorded.

Participants who met all the inclusion criteria were x-rayed for a standing anteroposterior film. Only 1 bilateral standing anteroposterior X-ray was taken, minimizing participants’ exposure to X-rays. Once the X-rays were digitized, the study physician evaluated each to ensure that the radiographic criterion of the study was met.

After the participants were cleared for study participation by the primary investigator and study physician, a hypodermic needle was inserted into the knee and a 20-mL syringe was placed on the hypodermic needle to remove a sample of synovial fluid. Approximately 0.5 to 1.5 mL of synovial fluid was extracted from the joint by the study physician. A greater amount of synovial fluid was withdrawn, if present, to reduce the patient’s discomfort. The control participants’ synovial fluid was extracted by injecting 7 to 14 mL of sterile saline into the joint. The amount of saline injected was individualized by the study physician, based on the ability to aspirate synovial fluid after every 2 mL of saline. Once the synovial fluid was extracted, the study physician performed a visual analysis of the fluid to check for the aforementioned pathological conditions. If the sample appeared yellow and translucent, the sample was placed in a sterile test tube, which was then placed in an insulated container with ice for transport. At the conclusion of each data-collection session, the synovial-fluid sample was flash frozen and stored at −80°C. After all the samples were collected, an aliquot of the stored synovial fluid was shipped to Thermo Scientific SearchLight’s laboratory (Pierce Biotechnology, Rockford, IL, USA). The laboratory used a custom multiplex ELISA to analyze the 16 knee synovial-protein concentrations. Another portion of each sample was
analyzed for total protein content, using a bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL, USA). Synovial-protein concentrations were normalized to total protein content.

**Statistical Analyses**

Data were analyzed with descriptive and inferential statistics using SPSS 15.0 statistical package (Chicago, IL). Independent-sample t tests were used to analyze the 16 synovial-protein concentrations between OA knees and normal knees. SPSS-corrected test statistics were reported if Levene’s test for equality of variances revealed the 2 groups did not have equal variance. Statistical significance was based on $P \leq .05$. Three of the OA participants had bilateral OA data recorded. Only the more effused knee was included in the independent t tests. Corrections for multiple t tests were not performed because our emphasis was on identifying synovial-protein concentrations to pursue in future research rather than identifying group differences. However, Cohen effect sizes ($d$), a standardized measure of the magnitude of differences between groups, and effect-size 95% confidence intervals were manually calculated for each t test. Cohen effect sizes were defined as low ($d = .20$), medium ($d = .50$), and large ($d = .80$).

**Results**

Independent t tests demonstrated that IL-13, OPG, and IL-1β concentrations were significantly greater in the normal- than in the OA-knee group (500%, 733%, and 200%, respectively; see Table 1). TIMP-2, MMP-2, and MMP-3 concentrations were significantly greater in the OA- than in the normal-knee group (1460%, 1450%, and 1206%, respectively; see Table 1). No other synovial-protein concentrations were statistically significant (see Table 1). Fourteen synovial-protein concentrations had large effect sizes between the 2 groups. IL-1ra and TNF-α had effect-size 95% confidence intervals that included medium effects (0.71 to 1.19 and 0.61 to 0.62, respectively).

**Discussion**

OA research can no longer solely rely on radiographs, because significant disease progression may occur before radiographic onset and radiographs are not able to detect subtle changes across time. To develop disease-modifying osteoarthritis drugs, researchers and clinicians need highly sensitive measures that can diagnose early OA, detect subtle joint changes, and expand our understanding of OA pathology. Biochemical analyses may be capable of satisfying these conditions, but previous research has been limited to analyzing a few proteins per sample. For a more thorough understanding of the disease, it would be ideal to measure numerous proteins in the same sample for the purpose of eventually determining a biochemical profile for a diseased joint. This pilot study was novel because it analyzed 16 synovial-protein concentrations simultaneously in small samples of synovial fluid using a multiplex ELISA protein assay to determine whether there were intra-articular biochemical differences between an OA- and a normal-knee
Table 1  Synovial-Protein Concentrations for Osteoarthritic and Normal Knees

<table>
<thead>
<tr>
<th>Protein</th>
<th>Osteoarthritic knees (n = 4)</th>
<th>Normal knees (n = 4)</th>
<th>P (95% CI)</th>
<th>Effect size</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>0.00002 ± 0.0001</td>
<td>0.00006 ± 0.0001</td>
<td>.001*</td>
<td>4.00 (4.00, 4.00)</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>0.034.6271 ± 12.6397</td>
<td>0.022194 ± 1.6396</td>
<td>.014*</td>
<td>3.60 (1.99, 5.20)</td>
</tr>
<tr>
<td>MMP-2</td>
<td>102.3384 ± 41.0003</td>
<td>06.6046 ± 2.5433</td>
<td>.018*</td>
<td>3.30 (0.80, 5.79)</td>
</tr>
<tr>
<td>MMP-3</td>
<td>120.4708 ± 59.5160</td>
<td>05.0544 ± 4.0930</td>
<td>.030*</td>
<td>2.74 (1.28, 6.75)</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.00001 ± 0.0000</td>
<td>0.00006 ± 0.0002</td>
<td>.005*</td>
<td>2.50 (2.50, 2.50)</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.00002 ± 0.0001</td>
<td>0.00009 ± 0.0004</td>
<td>.053</td>
<td>2.40 (2.40, 2.40)</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.00002 ± 0.0001</td>
<td>0.00009 ± 0.0004</td>
<td>.052</td>
<td>2.40 (2.40, 2.40)</td>
</tr>
<tr>
<td>OPG</td>
<td>0.00006 ± 0.0004</td>
<td>0.00050 ± 0.0022</td>
<td>.008*</td>
<td>2.00 (2.00, 2.00)</td>
</tr>
<tr>
<td>RANKL</td>
<td>0.00001 ± 0.0000</td>
<td>0.00005 ± 0.0003</td>
<td>.160</td>
<td>1.89 (1.89, 1.89)</td>
</tr>
<tr>
<td>Leptin</td>
<td>0.000.5910 ± 0.3866</td>
<td>0.00980 ± 0.0142</td>
<td>.084</td>
<td>1.80 (1.79, 1.82)</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>257.7233 ± 222.9196</td>
<td>03.1310 ± 1.7974</td>
<td>.107</td>
<td>1.62 (–0.15, 3.38)</td>
</tr>
<tr>
<td>IL-1α</td>
<td>0.00001 ± 0.0001</td>
<td>0.00003 ± 0.0002</td>
<td>.077</td>
<td>1.26 (1.26, 1.27)</td>
</tr>
<tr>
<td>Acrp</td>
<td>095.1944 ± 76.8100</td>
<td>15.0323 ± 9.7633</td>
<td>.084</td>
<td>1.04 (–8.52, 10.61)</td>
</tr>
<tr>
<td>MMP-13</td>
<td>00.0188 ± 0.0097</td>
<td>00.0347 ± 0.0164</td>
<td>.146</td>
<td>0.97 (0.95, 0.99)</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>00.0236 ± 0.0078</td>
<td>00.1878 ± 0.2445</td>
<td>.272</td>
<td>0.95 (0.71, 1.19)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.0007 ± 0.0005</td>
<td>0.0024 ± 0.0039</td>
<td>.466</td>
<td>0.61 (0.61, 0.62)</td>
</tr>
</tbody>
</table>

CI, confidence interval; IL, interleukin; TIMP, tissue inhibitor of metalloproteinase; MMP, matrix metalloproteinase; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-kappaB ligand; Acrp, adiponectin; ra, receptor antagonist; TNF, tumor-necrosis factor. All data are reported in pg/μg. All values are reported as mean ± SD. Effect sizes are reported in absolute values.

*P ≤ .05.
group. Most OA research has been limited to a few proteins and 1 or 2 classifications of proteins (eg, cytokines, chemokines, MMPs, or TIMPs). This study assessed a variety of proteins from various functional categories (eg, proinflammatory/catabolic, anti-inflammatory/protective, enzymatic) using a multiplex ELISA technique.

Although the small sample size limits our interpretation of the results, the biochemical analyses of knee synovial fluid revealed that IL-1β (a proinflammatory/catabolic cytokine) was 200% greater (a large effect size) in the normal-knee group than in the OA-knee group. This differs from previous studies that demonstrated elevated IL-1β in OA synovial fluid.14,23,26 The contradiction may be related to the normalization of IL-1β to total protein content. Previous OA research did not normalize synovial-fluid concentrations of IL-1β to total protein content.14,23,26 In this study, the normal-knee samples were diluted by saline to recover the synovial-fluid sample. To correct for the dilution, the data were normalized to total protein content. Synovial-fluid total protein content has been reported to be greater in OA joints than in normal joints.19,23,27 IL-1β may not increase proportionately to total protein content and may account for a smaller concentration of total protein content in OA knees. Further studies using this normalization method are needed to determine its appropriateness.

Two synovial-protein concentrations associated with anti-inflammatory/protective roles in joints were elevated with a large effect size in the normal knees (ie, IL-13, OPG). OPG, an inhibitor of RANKL, has been reported to be lower in OA joints than in normal joints.20 IL-13 has been reported to be elevated in OA. However, in relation to other proteins (eg, proinflammatory cytokines), IL-13 does not maintain a concentration sufficient for a homeostatic balance of pro- versus anti-inflammatory proteins.21 When these synovial-protein concentrations were normalized to total protein concentrations in this study, they were statistically significantly higher in the normal knees.

Two MMPs (ie, MMP-2 and MMP-3, each a catabolic enzyme) and 1 MMP inhibitor (TIMP-2) were elevated with a large effect size in OA knees. All 3 proteins have been previously demonstrated to be elevated in OA joints.23,26 MMP-2 is associated with denaturing of collagen, and MMP-3 has been implicated in proteoglycan degradation.28,29 TIMP-2 is a natural inhibitor of MMPs, and its elevation in OA joints signifies the chondrocytes’ failed attempt to maintain a homeostatic balance between catabolic and anabolic processes.

It is also important to note that significant differences in biochemical markers may be influenced by age. As humans age, proinflammatory/catabolic mediators increase,30 as does the risk of OA.31,32 The association between OA and age creates a challenge to find healthy age-matched controls. In the current study, there was a mean difference of 10 years between the normal- and OA-knee groups (range: normal knees = 39.5–64.3 y, OA knees = 59.5–71.1 y). Future studies should include a larger sample to enable statistical corrections for mediating factors like age.

Although radiographs remain the current diagnostic gold standard for OA, proteomic studies are emerging and expanding the understanding of OA.33–37 Future research may determine that proteomic techniques, such as multiple protein arrays, represent a new method for diagnosing preradiographic OA.35 Furthermore, these techniques have identified unique subsets of OA that are not dependent on stage of disease progression, age, sex, ethnicity, or medications.34 OA is described as a
disease or syndrome defined by a group of subsets, conditions, or diseases with a common end point. Subsets of OA should not be clinically accepted, however, until they are defined by unique biochemical (or genetic) markers and therapeutic responses (or outcomes). It is highly likely that future research will use proteomic techniques (eg, multiplex ELISA) to assess biochemical differences between therapeutic responders and nonresponders. Data from these studies may indicate protein concentrations that identify therapeutic responders and enable clinicians to use baseline biochemical markers to optimize treatments.

Rather than by stratifying patients, OA may be more accurately described with a continuous-gradient model. In this model a unique patient–pathology interaction defines the pathologic joint within a continuous disease model rather than a discrete classification (eg, OA vs no OA or effused vs noneffused). Patient–pathology interactions may be characterized by subsets or variables that remain constant throughout the progression of disease (eg, mechanism of onset) or fluctuate across time (eg, progression rate, effusion, disease stage, symptom severity). These subsets and variables are influenced by intrinsic (eg, age, weight, limb alignment, genetic variations) and extrinsic (eg, activities of daily living, environment) factors. Proteomic techniques may be ideal for evaluating the continuous-gradient model because they can assess large numbers of biochemical markers with an array of physiological functions and, therefore, provide a more thorough description of an individual’s unique patient–pathology interaction. Understanding patient–pathology interactions may lead to new strategies to develop disease-modifying interventions and provide them to a targeted set of patients.

Proteomic techniques offer a sensitive and continuous measure of potential diagnostic markers that can detect individual differences across time and between patients. For example, Carp et al used ELISA methods to examine patients with early-onset overuse-related musculoskeletal disorders of the upper extremity. They found that serum proinflammatory markers differentiated patients from controls and correlated with severity of upper extremity signs and symptoms, as well as predicting overuse-injury severity. Various OA studies, assessing a limited number of proteins, have found unique biochemical changes in early OA and after joint trauma. A proteomic study of incident knee OA that evaluated 169 serum protein concentrations related to inflammation, cell growth, activation, and metabolism identified 10 proteins that were significantly different from controls before radiographic evidence of OA. More proteomic research will be needed to follow up on this study and to assess longitudinal biochemical changes after joint trauma.

As diagnostic techniques and our understanding of pathophysiology improve, our preconceptions about diseases like OA will be challenged. Until the late 18th century, all arthritis was diagnosed as gout, but distinct pathologies emerged (eg, rheumatoid arthritis, osteoarthritis) as our understanding of arthritic conditions advanced. Current literature suggests that OA may be stratified into subsets. However, with the introduction of proteomics and other diagnostic methods that enable the development of profiles or continuous diagnostic scores, our perceptions of diseases may shift from discrete definitions to continuous-gradient models of pathologies. More research is needed to determine whether proteomic diagnostic strategies are appropriate and to explore the clinical implications of subsets of OA, as well as a continuous-gradient model.
Conclusions

Proteomic techniques (eg, multiplex ELISAs and mass spectrometry) offer new insights into diseases. In this study, a multiplex ELISA was used to assess synovial-fluid proteins with various properties. Although this was a small sample, we observed group differences between OA knees and normal knees. We found significantly lower levels of select anti-inflammatory/protective cytokines (IL-13 and OPG) and lower levels of a key proinflammatory/catabolic cytokine (IL-1β) but significantly higher levels of 2 key catabolic enzymes (ie, MMP-2 and MMP-3) and 1 MMP inhibitor (TIMP-2) in OA knees than in normal knees. Further research using proteomic techniques may eventually lead to new diagnostic profiles or tests capable of early diagnosis, guiding clinical decisions, monitoring disease progression or response to interventions, and helping clinicians, including certified athletic trainers, identify injured athletes who may be at elevated risk for OA.

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References


