Use of serum biomarkers to predict the development and severity of osteochondrosis lesions in the distal portion of the femur in pigs

Nolan. Z. Frantz, PhD; Kim G. Friesen, PhD; Gordon A. Andrews, DVM, PhD; Michael D. Tokach, PhD; Ryan M. Yamka, PhD; Tom L. Loughin, PhD; Jim L. Nelssen, PhD; Steve S. Dritz, DVM, PhD

Objective—To evaluate the use of serum biomarkers of cartilage and bone metabolism to predict the occurrence and severity of osteochondrosis (OC) lesions in the distal portion of the femur in growing swine.

Animals—71 gilts.

Procedures—At an abattoir, serum samples for analysis of 10 biomarkers indicative of cartilage and bone metabolism were obtained prior to processing of the pigs. The distal portion of each pig’s left femur was directly examined and cut into longitudinal sections to evaluate the number and severity of abnormalities on the external surface, articular cartilage, and growth plate. Each specimen was categorized as with (n = 56) or without (15) OC, and an overall OC severity score was assigned to affected pigs. Logistic and linear regression analyses were performed to predict odds of OC on the basis of biomarker concentrations and predict the severity of OC values in affected pigs, respectively.

Results—Compared with values in unaffected pigs, serum concentrations of C-propeptide of type II collagen (CPII) and cartilage oligomeric matrix protein were significantly increased and concentrations of carboxy-terminal telopeptide of type II collagen 3/4-length fragment (C2C) and pyridinoline cross-links were significantly decreased in affected pigs. A 2-fold increase in CPII concentration increased the odds of pigs having OC by a factor of 97 (95% confidence interval, 6 to infinity). Changes in serum C2C concentration accounted for 49% of the variation in overall OC severity score.

Conclusions and Clinical Relevance—Assessment of serum biomarker concentrations may be useful in the diagnosis of OC and aid in reduction of lameness in swine herds. (Am J Vet Res 2010;71:946–952)

Osteochondrosis involves the failure of endochondral ossification during bone formation, which results in cartilage retention in the subchondral bone. Osteochondrosis can be the cause of increased frequency of lameness in sow herds and reduce performance and meat yield in finishing pigs. Heritability estimates of OC in pigs range from 0.1 to 0.5 and may be affected by breed, growth rate, and trauma. Because the pathological processes that result in OC are not well understood and attempts at nutritional intervention have had limited success, the most likely opportunity to reduce the prevalence of OC in pigs is through genetic selection. Recently, a major gene that may relate to OC inheritance in pigs was identified. Thus, genetic selection strategies or assessment of biomarker proteins, which are the end result of gene expression, may enhance selection capabilities against OC and could reduce the economic consequences of this disease in pig populations.

For more than 10 years, much research has been conducted to determine the pathological processes and biological changes that occur during development of
OC, osteoarthritis, and rheumatoid arthritis in humans as well as in other animals.\textsuperscript{13,10} The changes identified in affected joints of pigs include increased chondrocyte necrosis, decreased amounts of type II collagen, and reduced concentrations of proteoglycans.\textsuperscript{17–19} Many immunohistological assays have been developed to determine biochemical changes in living animals via analysis of synovial fluid, blood, or urine samples.\textsuperscript{20} Biomarkers of OC are protein products of cartilage or bone metabolism that are released into circulation through events associated with their synthesis or degradation—processes that are altered as a result of loss of homeostasis during disease development.\textsuperscript{15,21} By use of antibody detection assays, differences in concentrations of these specific proteins in various samples from unaffected and affected animals have been detected.\textsuperscript{22} Cartilage and bone metabolism markers have been correlated with disease state with some success and have been used to determine treatment effectiveness in several different animal models of osteoarthritis\textsuperscript{23,24} and OC\textsuperscript{25–27}; however, only a few biomarkers of bone turnover have been evaluated in swine.\textsuperscript{28–30} Therefore, the objective of the study of this report was to evaluate the use of serum biomarkers of cartilage and bone metabolism to predict the occurrence and severity of OC lesions in the distal portion of femurs in growing pigs. We hypothesized that serum concentrations of biomarkers of cartilage and bone metabolism would correlate with OC development or severity and that those serum concentrations could potentially be used to identify pigs with OC. If so, we anticipated that prediction equations could be developed to potentially aid in gilt selection.

Materials and Methods

**Animals**—Seventy-one gilts (PIC line 327×L1050; mean weight, 114 kg; age range at completion of the study, 5 to 6 months) were used in the study. The study was conducted at the Kansas State University Swine Research and Teaching Center. Gilts were housed individually (2.31 m\(^2\)/pig) on totally slatted concrete flooring. Each pen was equipped with a dry self-feeder and an automatic nipple waterer to allow ad libitum access to feed and water. Gilts were offered enrichment toys and had daily opportunities for socialization with other pigs and people. The evening prior to sample collection, gilts were loaded in small groups onto a trailer for transport to the Kansas State University meat-processing facility, where data were collected. Two blood samples (approx 5 mL each) were collected via venipuncture from each pig just prior to electric stunning. The left hind limb was then collected for evaluation, and the remainder of the carcass was processed for human consumption. Sera from the blood samples for analysis. Procedures used in these experiments were approved by the Kansas State University Institutional Animal Care and Use Committee.

**Determination of OC lesion severity scores**—The left hind limb was severed at the midpoint of the femur and removed from each pig. The muscles and other tissues were carefully dissected from the stifle joint region. The femur was disarticulated from the tibia at the stifle joint so that the distal portion of the femur could be evaluated. The bone specimens were stored in neutral-buffered 10% formalin.

In each pig, the presence of OC lesions on the femoral condyles was assessed via gross examination by the same pathologist (GAA). The femoral condyles were also photographed for further visual evaluation of the external surface. After external evaluation, the distal end of each femur (regardless of whether lesions were or were not visible grossly) was sliced perpendicular to the long axis of the bone\textsuperscript{7} into 3-mm-thick sections by use of a band saw (12 to 14 surfaces/limb). The cut surfaces were directly evaluated and then photographed for further visual evaluation of the underlying articular cartilage—subchondral bone interface. Overall, each limb was evaluated for the number and severity of abnormalities on the external surface, the articular cartilage, and the growth plate. Severity of lesions in each region was scored on a scale of 0 to 4 (0 = apparently normal, 1 = mild, 2 = moderate, 3 = severe, and 4 = OC dissecans) on the basis of the extent of tissue involvement. The scoring system used was similar to systems described previously.\textsuperscript{31} From the scores, an overall OC severity score for the distal portion of the femur was calculated as follows: (number of external abnormalities × severity of those lesions) + (number of articular cartilage abnormalities × severity of those lesions) + (number of growth plate abnormalities × severity of those lesions). The distal portion of a pig’s femur was classified as without OC if the overall OC severity score was 0 after examination of gross specimens and photographs.

**Analysis of biomarkers**—Serum samples were stored at −20°C until evaluation. Samples were sent to a commercial laboratory\textsuperscript{b} for determination of the concentrations of 10 biomarkers. All samples were determined in duplicate by commercially available ELISAs that had previously been validated for swine at the commercial laboratory. C-propeptide of type II collagen\textsuperscript{c} is released during formation of the mature molecule of type II collagen and was measured as an indication of cartilage synthesis. Chondroitin sulfate epitope 846\textsuperscript{d} is an indicator of recent aggrecan synthesis and can be associated with cartilage repair. Both C2C\textsuperscript{e} and CTXI\textsuperscript{f} are cleaved from intact type II collagen molecules by matrix metalloproteinases during degradation and were measured as indicators of cartilage turnover. Cartilage oligomeric matrix protein,\textsuperscript{g} which is produced by chondrocytes and synovial cells, is considered an indirect marker of matrix remodeling or cartilage destruction. Osteocalcin\textsuperscript{h} and BAP are markers of bone formation or osteoblast activity. Concentrations of NTX,\textsuperscript{i} PYD,\textsuperscript{j} and ICTP\textsuperscript{k} were measured as bone turnover markers that are released during collagen type I degradation.

**Statistical analysis**—Logistic regression\textsuperscript{m} analysis was used to predict whether or not a pig had OC by a backward stepwise selection process to identify biomarkers that were significant in the model. Then, for pigs with OC, linear regression analysis was used to model the severity of OC by stepwise processes to determine the significance of biomarkers in the model. Because the biomarker data were not normally distributed, the individual markers were logarithmically transformed for evaluation in the model.
addition, the response variable or overall OC severity score was square root transformed because of increasing variation as scores increased. Normality was confirmed by adjusted residual evaluation. Data were first analyzed by use of computer software with the binary response (yes or no) to predict which pigs had OC. Next, another statistical procedure was used to predict overall severity scores by use of serum biomarker concentrations and final weight by forward selection for pigs with OC. The final model was determined on the basis of biomarkers that significantly contributed to the model at a level of P < 0.05. Differences in serum biomarker concentrations between pigs with or without OC were analyzed by use of ANOVA; differences were also considered significant at a value of P < 0.05. Simple correlations between biomarker concentrations and overall OC severity score were analyzed by use of the Spearman rank correlation coefficient procedure.

**Results**

**Evidence of OC**—Of the 71 pigs, 15 had no evidence of OC in the distal portion of the left femur (ie, overall OC severity score was 0), as determined via gross visual examination and assessment of photographs. The other 56 pigs had OC of varying severity. Among the affected pigs, overall OC severity scores ranged from 2 to 27.

**Serum biomarker concentrations**—From the individual analysis of each biomarker as a function of pigs with OC versus pigs with no gross evidence of OC, serum concentrations of C2C and PYD decreased (P < 0.02 and P < 0.01, respectively) with development of OC (Table 1). Serum concentrations of biomarkers CPII and COMP were increased (P < 0.01 and P < 0.03, respectively) in pigs with OC, compared with findings in unaffected pigs. Serum concentrations of the other biomarkers did not differ (P > 0.20) between pigs with and without OC.

**Modeling for the development of OC**—By use of logistic regression analysis to predict the binary response (ie, yes or no, with regard to whether pigs did or did not have gross signs of OC) in all 71 pigs, the serum concentration of biomarker CPII was significant (P < 0.01) in the model. The equation was as follows: OC = −42.5748 + (4.5704 × log serum CPII concentration). All pigs with serum CPII concentration > 850 ng/mL had OC, and the odds ratio was 97 (95% confidence interval, 6 to infinity). This indicated that a 2-fold increase in serum CPII concentration increased the odds of having OC by a factor of 97. Serum concentrations of the other biomarkers were not significant (P > 0.14) in the model.

**Modeling for the severity of OC**—After the prediction of which pigs had OC, serum biomarker concentrations were evaluated in the 56 affected pigs for correlation with the overall OC severity score by use of linear regression. Only serum C2C concentration and the log value of C2C concentration were significant (P < 0.05) in the model. The equation was as follows: \[ \text{OC} = 18.62746 + (0.05507 \times \text{log serum C2C concentration}) + (-4.76606 \times \log \text{serum C2C concentration}) \]. On the basis of this single marker, C2C concentration and the log value of C2C concentration explained 44% and 49% (R² values) of the variation in overall OC severity scores, respectively.

**Associations between serum biomarker concentrations and overall OC severity score**—Spearman rank correlation coefficients were evaluated to determine whether there were any associations between serum biomarker concentrations and overall OC severity.

<table>
<thead>
<tr>
<th>Biomarker concentration (all pigs)</th>
<th>OC</th>
<th>Biomarker concentration (all pigs)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cartilage synthesis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPII (ng/mL)</td>
<td>648.1</td>
<td>1,110.5</td>
</tr>
<tr>
<td>CS946 (ng/mL)</td>
<td>977.4</td>
<td>857.8</td>
</tr>
<tr>
<td><strong>Cartilage degradation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2C (ng/mL)</td>
<td>163.0</td>
<td>123.9</td>
</tr>
<tr>
<td>CTXII (pg/mL)</td>
<td>213.6</td>
<td>235.8</td>
</tr>
<tr>
<td>COMP (U/L)</td>
<td>1.05</td>
<td>1.39</td>
</tr>
<tr>
<td><strong>Bone turnover</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTX (nM BCE)</td>
<td>215.3</td>
<td>234.9</td>
</tr>
<tr>
<td>ICTP (µg/L)</td>
<td>39.6</td>
<td>38.6</td>
</tr>
<tr>
<td>PYD (nmo/L)</td>
<td>8.1</td>
<td>6.6</td>
</tr>
<tr>
<td><strong>Bone formation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteocalcin (ng/mL)</td>
<td>22.2</td>
<td>23.6</td>
</tr>
<tr>
<td>BAP (U/L)</td>
<td>75.9</td>
<td>71.5</td>
</tr>
</tbody>
</table>

*The probability was the probability that the difference in concentration between pigs classified as no and yes was equal to 0, a value of P < 0.05 was considered significant.

BCE = Bone collagen equivalent. SED = Standard error of the difference. U = Arbitrary units. Blood samples were collected via venipuncture prior to slaughter; serum samples were analyzed by use of assays that were validated for swine. The distal portion of each pig’s left femur was directly examined after being cut into longitudinal sections to evaluate the numbers of abnormalities on the external surface, articular cartilage, and growth plate.
Table 2—Spearman rank correlation coefficients* determined between serum biomarker concentrations and overall OC severity score in pigs with (n = 56) and without (15) gross evidence of OC in the distal portion of their left femurs.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPII</td>
<td>0.430</td>
</tr>
<tr>
<td>CS846</td>
<td>0.311</td>
</tr>
<tr>
<td>COMP</td>
<td>-0.270</td>
</tr>
<tr>
<td>BAP</td>
<td>0.312</td>
</tr>
<tr>
<td>C2C</td>
<td>0.564</td>
</tr>
<tr>
<td>PYD</td>
<td>0.426</td>
</tr>
</tbody>
</table>

* Spearman rank correlation assigns ordered numbers to measure increasing or decreasing relationships between 2 variables without assuming a normal distribution; values shown were significant (P < 0.05). Each femur was evaluated for the numbers of abnormalities on the external surface, the articular cartilage, and the growth plate; severity of lesions in each region was scored on a scale of 0 to 4 (0 = apparently normal, 1 = mild, 2 = moderate, 3 = severe, and 4 = OC dissecans). An overall OC severity score for the distal portion of each femur was calculated as follows: (number of external abnormalities × severity of those lesions) + (number of articular cartilage abnormalities × severity of those lesions) + (number of growth plate abnormalities × severity of those lesions).

Discussion

The assessment of biomarker concentrations to diagnose joint diseases without invasive procedures has been a focus of human research for almost 20 years. Identification of humans and other animals with greater potential for joint deterioration from arthritis or OC would allow timely treatment interventions and, in pigs, for example, facilitate selection against genetic predisposition to OC. Radiographically, changes in progression of these diseases are difficult to evaluate over short periods and are typically only detectable in the later stages of osteoarthritis or OC. Assessment of circulating biomarker concentrations to detect disease and evaluate disease progression is promising, given that genetic markers are currently unavailable. The high prevalence of OC in swine is thought to correlate with increased selection pressure for lean growth rate. With the challenge of maintaining both highly prolific sow lines and maximum lean growth, increased selection pressure against OC is needed. Thus, in the present study, we hypothesized that serum concentrations of biomarkers of cartilage and bone metabolism would correlate with OC development or severity and that assessment of those biomarker concentrations could potentially be used as a diagnostic tool to identify pigs with OC.

Serum concentration of the biomarker of collagen type II synthesis (CPII) increases in humans with osteoarthritis and rabbits with ovalbumin-induced arthritis, can be used to predict radiographic progression of inflammatory arthritis in humans, and is high in horses with OC. This change in CPII concentration may be a programmed response to increased cartilage fragmentation through a potential feedback loop in which increased degradation stimulates CPII synthesis. The increased serum concentrations of CPII in pigs with OC, compared with findings in unaffected pigs, are indicative of an attempt to promote a repair process through synthesis of new type II collagen molecules. In addition, serum CPII concentration enabled prediction (with an odds ratio of 97) of which pigs had OC. All pigs with serum CPII concentration > 850 ng/mL had OC, and because of this grouping, the upper limit of the 95% confidence interval for the odds ratio was approaching infinity or complete separation in the data set. However, after pigs with OC had been identified, a negative correlation (ρ = -0.43) of serum CPII concentration with overall OC severity score was detected in that subgroup. Månsson et al observed lower serum CPII concentrations in humans with rapid erosive rheumatoid arthritis, compared with healthy controls. The response in pigs with OC may be a result of differences in the progression of lesions. Pigs with lower overall OC severity scores and higher serum CPII concentrations may have regressing or progressing lesions; however, more extensive lesions in pigs with lower serum CPII concentrations may be static or unable to repair through increased amounts of CPII. It appears that elevated serum CPII concentration is a good indicator of OC in swine.

A positive correlation (ρ = 0.311) of serum concentration of CS846, an aggrecan synthesis marker, with increased overall OC severity score was also identified in the present study. This may indicate that in addition to increased collagen type II synthesis in gilts with OC, the synthesis of aggrecan may also be increased in an attempt to repair damaged cartilage; however, the usefulness of this biomarker in relation to disease state has been debated. Serum concentrations of CS846 were increased in humans with rheumatoid arthritis and osteoarthritis, compared with findings in healthy controls. Similarly, changes in CS846 concentration were reflected in changes in CPII concentration in synovial fluid collected from knee joints of humans with various forms of knee injury or osteoarthritis. Markers of cartilage component synthesis may be valuable diagnostic indicators of the repair process and aid in identification of animals with OC.

High serum concentrations of COMP (a noncollagenous protein synthesized by chondrocytes and synovial cells) are thought to indicate cartilage destruction or matrix remodeling that is occurring within joints. Several human studies have revealed positive correlation of serum COMP concentration with severity of osteoarthritis and its use as a predictor of future joint deterioration. Similarly, higher concentrations of serum COMP concentration were detected in pigs with OC, compared with unaffected pigs, in the present study. This suggests increased matrix remodeling or turnover in pigs with OC. However, a negative correlation (ρ = -0.270) between serum COMP concentration...
and increasing overall OC severity score was detected. Similar to the results found with serum CPII concentration, although a higher mean concentration of COMP was detected in pigs with OC (compared with those without OC), the negative correlation identified in the pigs with OC could indicate that higher concentrations of COMP may be present with newer, more active but smaller lesions versus larger and more chronic lesions.

Direct markers of cartilage and bone turnover also have been shown to correlate with specific disease states. In the present study, serum PYD and C2C concentrations were significantly decreased in pigs with OC, compared with findings in pigs with no gross OC lesions; however, when used to predict the overall OC severity score, both serum concentrations of PYD and C2C had strong positive correlation (r = 0.426 and 0.564, respectively). In humans with rheumatoid arthritis or osteoarthritis, serum and urinary PYD concentrations are increased, compared with healthy controls, and correlate with disease progression; however, this may be more closely associated with alterations in bone metabolism. Similarly, increases in serum or synovial fluid C2C concentrations with increasing severity of osteoarthritis in dogs, rabbits, and humans have also been described.23,55,57 As have increases in synovial fluid in horses with increasing severity of OC.27,49 In the present study, the pigs with OC unexpectedly had lower PYD and C2C mean serum concentrations, compared with those that did not have evidence of OC. Even so, in pigs with OC, there was a positive correlation of C2C and PYD with increasing severity score. The physiologic explanation for this observation is not known.

Measurements of serum CTXII concentration (a marker of cartilage turnover) in synovial fluid and urine21,50,51 correlate with joint deterioration in humans and other animals, but serum concentrations were not altered in pigs with OC and did not correlate significantly with overall OC severity score in the present study. These findings may be attributable to the inherited variation among individual animals.

Serum concentrations of other biomarkers of bone turnover or formation were not significantly altered in pigs with OC in the present study, but concentrations were similar to previously reported values for swine.26–30 Nevertheless, the positive correlation (r = 0.312) of serum BAP concentration with increasing overall OC severity score suggests that a potential use of this biomarker might become evident if larger population samples are evaluated. In horses, BAP concentrations in synovial fluid samples from osteoarthritic joints are greater than concentrations in samples from the contralateral unaffected joints.35 Although NTX-I, osteocalcin, and BAP have been successfully used as biomarkers of bone turnover in swine, as has ICTP in various animal species,35 the findings of the present study suggest that bone remodeling does not have a significant role in prediction of OC in gilts. A positive correlation of osteocalcin concentration with macroscopic lesion score at 5 months of age in foals has been identified36; however, biomarkers of bone turnover or formation had limited usefulness for predicting OC severity in older foals. The latter finding may be mirrored by results of the present study because some lesions in the pigs may have resolved as they aged.

The results of the present study indicated that serum concentrations of biomarkers of cartilage synthesis and turnover were altered in pigs with OC, compared with findings in unaffected pigs, and were predictive for OC and lesion severity. However, serum concentrations of biomarkers of bone metabolism did not correlate with presence of OC or lesion severity. Logistic regression analysis revealed that gilts with a high risk of OC could be identified on the basis of serum CPII concentration. Although serum C2C concentration explained as much as 49% of the variation in overall OC severity scores, individual marker variation appears to limit the ability of other biomarkers to predict OC severity scores with higher accuracy, and that limited ability may be influenced by other factors such as growth rate. Evaluation of biomarkers of cartilage metabolism, in particular CPII and C2C, may be of use in estimating the presence of OC in replacement gilts if measurements are performed prior to introduction into the breeding herd. By selection of gilts based on serum CPII and C2C concentrations, increased selection pressure may be placed against breeding stock with a greater incidence of OC, thereby reducing the incidence in future generations. Currently, we are unaware of any tools or procedures that are available for providing genetic selection pressure in live animals. Biomarker analysis of a serum sample obtained just prior to selection of replacement gilts may be 1 such quantitative selection tool. However, further studies will be required to test and refine the equations formulated in the present study for practical applications. Additionally, studies evaluating changes in serum concentrations of these biomarkers at earlier stages in the growth period and over time will be needed to further refine their usefulness in a commercial gilt selection program.
References

42. Clark AG, Jordan JM, Vilim V, et al. Serum cartilage oligomeric


