THE EFFECT OF DIETARY NUTRIENTS ON OSTEOCHONDROSIS IN SWINE AND EVALUATION OF SERUM BIOMARKERS TO PREDICT ITS OCCURRENCE.

by

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B.S., Tabor College, 2001
B.S., Kansas State University, 2002
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AN ABSTRACT OF A DISSERTATION

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ABSTRACT

Four experiments using 350 pigs were conducted to determine the effects of dietary nutrients on the incidence of osteochondrosis (OC) and to evaluate the use of biomarkers to predict its occurrence in growing-finishing pigs. The first experiment was conducted to evaluate the potential of dietary ingredients with known functions in cartilage and bone metabolism on incidence of OC in pigs (PIC 327 × 1050, initially 39 kg). Results suggest that pigs fed high levels of added copper and manganese, silicon, methionine and threonine, or proline and glycine had reduced OC severity scores. A second experiment evaluated other dietary ingredients that may impact OC as well as with or without ractopamine HCl (PIC 327 × 1050, initially 47 kg). Results suggest that feeding a combination of added methionine, manganese, proline, and glycine or added silicon can numerically reduce OC severity scores compared to pigs fed a standard corn-soybean meal based diet. Feeding ractopamine HCl did not affect the incidence or severity of OC. A third experiment was conducted to evaluate the effect of dietary lysine concentration with or without the addition of high methionine, manganese, and copper in a 3 × 2 factorial arrangement in growing-finishing pigs (PIC 327 × 1050, initially 41 kg). Results suggest that increasing dietary lysine concentrations increased the severity of OC. Furthermore, feeding additional methionine, manganese, and copper reduced OC severity scores. A fourth experiment was conducted to determine the usefulness of ten different biomarkers of cartilage and bone metabolism to predict the incidence of OC and the correlation of individual markers with the severity of OC. Results suggest that measuring serum C-propeptide of type II collagen (CPII) will predict animals with OC, as gilts with a two-fold increase in CPII are 97 times more likely to have OC. As well, serum collagen type II carboxy-terminal ¾ long fragment (C2C) explained 49% of the variation in OC severity scores. The results of these experiments suggest

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Thanks!

Nolan Frantz

DEDICATION

I would like to dedicate this work to my family. It was their encouragement and love that helped me get this far. I appreciate the opportunities that you provided me growing up and the support you've given me throughout the years. I'll never forget all the times my family has been there for me and will continue to be a huge part of my life.

CHAPTER I

THE POTENTIAL USE OF BIOMARKERS TO DIAGNOSE AND PREDICT OSTEOCHONDROSIS IN SWINE

Introduction

Current human disease research emphasizes identifying biological changes associated with specific disease pathology to allow early detection and possibly treatment intervention aimed at prevention. This focus has occurred because of the rapid advancement of assays for detection of specific proteins in tissues using biological markers and proteomic screening. Technology is currently available for identification of products of metabolism or proteins correlated with disease occurrence or changes resulting from treatment intervention. Biological markers for use in human and other animal models are under development. Because of technologies to diagnose and treat human disease, the swine industry has the opportunity to use these same biological markers to aid in disease detection.

Osteochondrosis (OC) in growing and mature swine as well as in several other animal species remains one of the most problematic diseases with no known treatment. Osteochondrosis involves the failure of endochondral ossification of cartilage which is then retained in the subchondral bone[1] (Figure 1 vs Figure 2). These lesions are thought to form at an early age in the underlying bone and can be aggravated by stress or trauma to the tissue. These changes result in pain and lameness as the animal continues to grow[2] (Figure 3). Many details of how OC disease progression occurs are unknown; however, several in-depth studies have been conducted to identify physiological changes that occur during its pathogenesis[3,4,5]. Characteristics observed include a decrease in proteoglycan content and collagen type II concentration, and an increase in chondrocyte necrosis and collagen type I concentration[5]. A low proteoglycan and collagen type II content would limit the ability of cartilage to absorb and distribute forces because of abnormal cartilage. Increased chondrocyte cell death would limit the ability of cartilage to repair lesions and may result in abnormal metabolism as chondrocytes synthesize collagen and extracellular components. These biological changes to load-bearing

joints leave the animal susceptible to stress or trauma which may result in lameness and decreased performance.

Lameness is a costly problem to the swine industry[6]. Currently, sow herd replacement rates are 50% or greater and maintaining an adequate number of replacement gilts represent a significant cost to producers[7]. Osteochondrosis is one of the main causes of lameness and can increase culling rates in sow herds[8]. Also, OC has also been shown to correlate with decreased meat yield of finishing pigs[9]. Several studies have observed that rough handling or trauma has a profound impact on the occurrence of OC[10]. Even so, strategies to limit OC either through handling techniques to minimize trauma or through nutritional intervention have had little success[11,12,13]. Genetics is the most likely contributor to the high prevalence of OC in pigs. As research has shown, estimates of OC heritability range from 0.1 to 0.5[9,14,15]. The prevalence of OC is correlated with specific sire lines whose progeny are more susceptible[16]. The most logical and potentially effective means of reducing OC is through genetic selection for structural soundness of females which may reduce OC occurrence[17]. Currently, several studies are being conducted in Europe to establish breeding values via magnetic resonance imaging (MRI) to aid in selection against lines with a high prevalence of OC. These studies using MRI to detect OC lesions are not practical for the commercial pork producer. Incorporating breeding values potentially could be an option, but because of technology requirements and difficulty in data collection, this method would prove expensive and challenging[18].

The recent mapping of the human genome has lead to the possibility of determining the gene or genes associated with a specific disease, but the swine genome is yet to be completed. Kadarmideen and Janss[18] reported that a major gene likely contributes to inheritance of OC; however, concerns exist regarding the usefulness of genetic markers, as selecting for a single

trait may also have negative influences on other traits. Proteomics is the study of the expression, structure, and function of proteins encoded by the genome and may have implications in predicting disease state. Proteomic screening is an alternative technology that is also available, developed around mass spectrometry to separate and detect a large number of specific proteins based on their mass from a tissue or biological sample[19]. Proteomics also may help identify specific proteins that can then be developed for biomarker assays.

Without knowing the specific gene or combination of genes involved in OC, visual evaluation of breeding animals and slaughter studies of related animals are tools that may or may not aid in selection against OC. With the use of biomarkers, the task of genetic selection against OC may become more effective. Blood samples could potentially indicate if an animal has a disease and which ones do not. This would allow selection against those animals that are predisposed or have OC to be withdrawn from the breeding herd. Using biomarkers offers the opportunity to reduce sow herd turnover rates, and may also help reduce the amount of chronic lameness seen in commercial finishing facilities that arise from OC.

What are biological markers?

Biological markers, or biomarkers, are products of metabolism from either synthesis or degradation in specific tissues that are released into the blood supply, present in the tissue of interest, or discarded in other biological fluids such as urine[20]. When an imbalance between the synthesis and degradative processes occurs the concentration of these metabolic products or biomarkers levels are abnormally altered[20,21]. Thus, biomarkers have the potential to directly correlate with the metabolic processes occurring within the joint or underlying bone.

Proteins can be products of specific tissues while others reflect systemic changes in metabolism (Table 1). This potentially offers two directions in relating a biological marker to disease pathology. First, markers from the tissue of interest, or direct markers, would allow

detailed information to be gathered about events occurring within that tissue [22]. Secondly, indirect markers or markers not originating in the tissue of interest, would reflect organism wide changes that occur before a disease or as a result of a disease process[22]. Even so, indirect markers are less specific in the information they provide and may be influenced by other factors other than the disease of interest. Thus, indirect markers must be used with caution and may have limited use as diagnostic indicators for specific disease pathology. Tissue specific markers are preferred because of their ability to be clearly identified with a disease process[20] and their relationship to the tissue of interest.

Biological markers of joint metabolism would be defined as those that are predictive of future joint deterioration, and thus treatment intervention before disease progression. Ideally, biomarkers also would allow measurement of treatment effectiveness in disease prevention[23]. Scientists have turned to animal models to determine biomarkers that can be correlated with actual changes in the joint[24]. For most human studies, biomarkers are related to some type of pain or mobility score as well as radiographic representations of joint width[20]. Although most of the data evaluating biomarkers have occurred in human models, animal models such as swine allow a direct relationship to be drawn between marker and disease state.

Detection of biochemical markers in body tissues

Biochemical markers can be determined by immunoassays conducted with antibodies to the protein of interest to allow detection and quantification in a tissue sample. This can be done using several different techniques including radio-immunoassay (RIA) or enzyme-linked immunosorbance assay (ELISA) that require a minimal amount of sample. In this process, antibodies to the protein of interest bind selectively to the protein of interest and can be quantified or expressed as a relative concentration to control proteins[25]. The ELISA method is rapidly becoming the preferred method to evaluate samples for biomarkers because of its speed

and accuracy. In addition, this assay requires less equipment to perform than other types of analysis[26]. In the case of OC, the options to analyze for biochemical markers include synovial fluid, serum, plasma, or urine.

Synovial fluid. Sampling of synovial fluid is the most likely to represent direct changes in metabolism of articular cartilage[27]. It also represents a single joint and thus the metabolism rate of that joint compared with blood or urine which would reflect several joints and other cartilgenous tissues in the body. In addition, markers measured in synovial fluid do not undergo further metabolism in the body as would either blood or urine, and thus are present in higher concentrations[27]. Even so, synovial fluid may be difficult to collect because of limited amounts, particularly in older animals. Markers of synovial fluid also may be influenced by changes in amount of fluid volume. If the goal is to determine the disease process occurring in a specific joint, analyzing synovial fluid should provide the best representation of changes occurring in that joint.

Urine. One of easiest and least invasive ways to obtain body fluids that reflect cartilage and bone metabolism is through urine. Urine is similar to blood in that it carries biological markers released from the joint and reflects changes that are occurring in all the joints of the body. Urine is different from blood in that it contains several markers that have been altered from their initial form due to the filtration process of the liver and kidneys. In the case of urine, some markers are more concentrated by further metabolism while others are degraded into products that are undetectable by standard assays[26]. In addition, several of the normal markers used to predict cartilage metabolism are absent from urine, and thus the usefulness of urine samples to predict cartilage metabolism events is limited. Markers of bone turnover are readily

determined from urine samples, and can provide an easy method to analyze for changes in bone metabolism when blood samples are not available.

Serum or plasma. Blood samples, like urine, indicate changes occurring systemically in the organism, but can be analyzed for a wider variety of metabolites. Compared to synovial fluid analysis, blood markers are easier to collect and may be a better indicator of systemic pathological processes occurring in the animal[28]. Another advantage blood markers provide is that blood is of a relatively consistent volume[29] while synovial fluid levels may fluctuate. The major disadvantage of blood markers is that some markers are present in very small amounts and are very difficult to detect[26]. This is due to processing before entering the blood stream as most of the biomarkers pass through the lymphatic system were they are further degraded. In addition, differences in filtration rates by the liver and kidneys can change the amount of a marker that is present. Blood markers appear to be the most logical choice in predicting disease occurrence because of the ease of collection and the range of markers that can be assessed.

Factors affecting sampling and concentrations of biomarkers

Determining what tissue to evaluate or is the best indicator can be challenging and may fluctuate with different diseases. Careful consideration needs to be taken before sample collection and processing as the concentration of specific markers can vary depending on several factors. The first factor to consider is which body fluid to sample. This will mainly depend on the response criteria selected and on which body fluid is an option for collection. In most circumstances, it is easier to collect a blood sample versus a urine sample in animal studies, with both being less involved than synovial fluid sampling. Secondly, blood marker concentrations may be affected by subjects with liver or kidney disorders due to their role in filtering and processing of these molecules[26]. Gender is also a factor that must be considered in selecting a marker as they may be influenced by differences in hormones during development and

maturity[30]. In a human study evaluating biomarkers, differences in gender and ethnicity have been found[31]. For swine, differences in markers may exist between gilts and barrows or boars; however, this has not been evaluated. Therefore it is probably best to use a sub-sample similar to the entire population. Marker concentrations are known to change with age and may be altered irrespective of disease state. Therefore, it is recommended to compare age-matched individuals of normal and diseased states[26]. For example, in swine, comparing older sows with gilts may not reflect a true difference in disease state. Another factor that affects body fluid markers is mechanical stress or movement. The effect of exercise[32] and type of exercise performed[33] has been shown to influence markers of cartilage and bone metabolism in humans. Along with this, dehydration can also alter the concentration of biomarkers in body fluids. This is primarily because of the involvement of motion in exchanging joint tissue materials and forces applied to the skeleton. In the case of swine, gilts housed in gestation crates may have different marker levels than loose-housed animals due to differences in movement or range of motion. Additionally, two considerations for blood sampling in particular may need to be addressed. Differences in the site of sampling may impact the level of markers detected, however, this problem can be easily solved. It is recommended to draw blood from the same location on each animal and a site that is representative of systemic blood flow rather than an individual joint[26]. Furthermore, the timing of collection may potentially have the biggest effect on marker levels. Diurnal variation may result in higher marker levels due to the build up of metabolites overnight with lack of movement to exchange fluid with the joint or clearance of bone markers[34,35]. Research has shown that blood samples should be taken at least two to three hours after fasted animals have awaken to allow exchange of fluids and account for differences in the half-life of certain markers[26]. Differences in the half-life of certain markers results in varying rates of

clearance from the blood and thus different markers have differing levels of day-to-day variability. Seasonal variation may also contribute to differences in marker levels but has not been demonstrated in all species[26]. Finally, samples should be processed as quickly as possible to avoid marker degradation. When analyzing for multiple markers, samples should be split into small aliquots to eliminate freeze-thaw cycles which may also cause unnecessary degradation of marker molecules. Most metabolites are stable if stored at -20º and this will prevent degradation until analysis. If all of these factors are taken into consideration, it is possible to collect samples that allow accurate determination of marker levels.

Application and limitations of biological markers to osteochondrosis

The available information regarding biomarkers that relate to OC in swine is limited. However, several studies using biomarker evaluation in horses and dogs that essentially involve the same disease process have been described. The majority of the information on biomarkers to predict disease has been acquired from human studies of OC or related diseases such as osteoarthritis (OA) or rheumatoid arthritis (RA)[36]. Both OA and RA are different from OC in that they are a result of inflammatory processes that degrade cartilage components, while OC results from an innate abnormality of cartilage ossification and occurs without inflammation. The implications drawn from these studies may or may not directly apply to OC in swine, but can provide a basis for an understanding of the biological events or changes that occur in joint diseases. The value of the individual data sets largely depends on the size of the study conducted. One of the limitations in advancing the understanding and interpretation of biomarkers in human studies has been the limited number of observations[37]. The inherent variation between individual subjects results in difficulty in correlating biomarker levels to a specific individual[38], but these studies provide useful information about predicting disease progression.

Markers of cartilage synthesis

C-propeptide of type II Collagen (CPII). Biomarkers of cartilage synthesis are a reflection of the production of collagen components. Of particular interest is the rate of collagen type II synthesis which represents approximately 90% of the collagen content of articular cartilage surrounding the end of long bones[39]. During synthesis of the type II procollagen molecule, the carboxy-terminal propeptide (CPII) and amino-terminal end of the propeptide are released into circulation before formation of the mature collagen fibril[40]. Thus, CPII can be interpreted as a marker of the type II collagen synthesis rate occurring in the body and is mainly analyzed in serum, but can also be analyzed from synovial fluid and cartilage tissue. During cartilage destruction, the articular cartilage initially tries to repair the damaged tissue by increasing synthesis of type II collagen and aggrecan[41]. A positive feedback loop may exist with degraded collagen type II stimulating collagen type II synthesis[42]. Degradation of type II collagen and proteoglycans have been determined as part of the early stages of arthritis[43]. In human OA studies, CPII has been shown to increase dramatically in diseased joints[40]; however, in rapidly progressing rheumatoid arthritis, CPII decreased relative to controls[41]. Bleasel et al.[44] found similar CPII concentrations in individuals with or without a collagen type II gene mutation that results in OA. The difference observed in CPII levels between diseases is thought to reflect differences in how each disease progresses, representing the difference between inflammatory diseases and OC. Additionally, several other studies have reported positive results when measuring synovial fluid CPII and relating it to progression of joint diseases[45]. In equine OC, CPII has been demonstrated to increase in animals with OC lesions versus those without[46,47,48]. These data suggest that CPII may also have the potential to be an indicator of OC in swine as the disease process has similar characteristics to OC in other species.

Chondroitin sulfate epitope 846 (CS846). Another major component of cartilage synthesis is the proteoglycan aggrecan which serves a structural function in the extracellular matrix[39]. Much like the collagen type II molecule, components from the synthesis of aggrecan are released before formation of the mature protein[49] and is absent in normal joints[50]. One of these molecules is known as the chondroitin sulfate 846 epitope (CS846) and is thought to reflect increased synthesis of aggrecan[44]. It detects a specific epitope that is released from the formation of new large aggrecan molecules. The CS846 molecule has been shown to increase in humans with RA[41] and in the serum and synovial fluid of individuals with OA[44,51]. It also parallels the CPII marker in OA and is thought increase at a similar proportion[50]. The CS846 epitope may be a unique marker of cartilage aggrecan synthesis rate with disease predicting potential.

Glycoprotein 39 (YKL-40). A non-collagenous product secreted by chondrocytes and synovial cells called glycoprotein-39 (YKL-40) may also be of interest in predicting cartilage synthesis rate[20]. For YKL-40, an increase in its presence is believed to reflect increased synthesis of cartilage components[52] but is also used as a marker of inflammation[53]. The YKL-40 marker has been used less extensively in human and animal studies than the previously mentioned markers and is less specific for its tissue of origin than CPII or CS846[54]. Although it has shown some success in predicting disease progression[55], it is probably less of a direct indicator of cartilage synthesis than CPII or CS846[37].

Markers of cartilage degradation

Collagen type II ¾ long fragment (C2C). During the process of cartilage degradation, matrix metalloproteinases (MMPs) breakdown collagen into two cleavage products (¾ and ¼ fragments) and a third alternative cleavage of the ¼ fragment. Because articular cartilage is almost entirely type II collagen, measurement of its components released during degradation potentially reflect the rate at which articular cartilage is being destroyed[56]. Four major variants

of type II collagen fragments and an indirect marker are currently available as measures of cartilage degradation. During collagen breakdown, collagen undergoes two main cleavage steps by collagenases resulting in a collagen ¾ fragment and a ¼ fragment[57]. Further processing of the ¾ long of both type I and II collagen results in two shorter fragments that can be measured in fluids[56]. The ¾ long fragment is denoted as the C-terminus of type II collagen ¾ long cleavage product (C2C) while the ¾ short is known as C1,2C because it results from additional cleavage of the ¾ long fragment resulting from either type I or II collagen. Measurement of C2C has shown that it is increased in the synovial fluid of both OA and RA disease states[43,58] indicating destruction of type II collagen. The C2C concentrations in an experimental model of arthritis also are elevated in serum and urine[59]. In addition to its use in determining cartilage degradation rate, it has also been used to successfully predict human OA progression[60] and thus may be a predictor of future joint damage. Although its use has not been extensively evaluated in animal models, it may provide an appropriate indication of cartilage type II degradation.

Collagen type I and II ¾ short fragment (C1,2C). The C1,2C molecule resulting from additional cleavage of the ¾ long fragment has also been implicated as a potential marker. Unlike the C2C marker, C1,2C is not specific for type II collagen but reflects degradation of either type I or II collagen. It is increased in cartilage samples[56] and serum of OA subjects[60,61]. Cerejo et al.[60] suggested that the ratio of C1,2C to C2C may be a better indicator of disease progression than either marker alone. Much like the C2C marker, C1,2C requires further research into its correlation with collagen degradation; however, because it is not specific for type II collagen, it may not be as predictive of articular cartilage destruction as the C2C marker.

C-terminal crosslinked telopeptide of type II collagen (CTX-II). One of the most recent and promising markers is an assay that recognizes the C-terminal cross linked ¼

telopeptide of type II collagen degradation (CTX-II). The CTX-II molecule is one that has received much attention recently because of its success as a prognostic indicator. Originally developed for detection in urine, it is now available as a serum assay. Detection of CTX-II in urine of subjects with OA or RA have shown increased levels relative to healthy controls[62] and are indicative of future disease progression[37,63,64]. The CTX-II assay has also been successful in predicting effectiveness of treatment strategies[65,66]. The research of Lohmander et al.[67] showed that CTX-II in synovial fluid is dramatically increased immediately after joint injury. With the initial success of correlating CTX-II with cartilage degradation, it appears to be one of the direct markers with the greatest potential to predict joint disease state.

Type II collagen helical domain (HELIX-II). A new marker recognizing the helical domain of type II collagen (HELIX-II) has recently been developed[68]. This is the center portion of the collagen type II molecule remaining after cleavage rather than the telopeptide ends, but can only be detected in urine. In the first human study, the HELIX-II molecule has been shown to be elevated in both OA and RA diseases relative to healthy controls[68]. This biomarker may serve as a valuable indicator of cartilage degradation; however, further studies are needed to verify its correlation with disease state.

Cartilage oligomeric matrix protein (COMP). The final marker of cartilage degradation is an indirect marker called cartilage oligomeric matrix protein (COMP). It is a glycoprotein present in articular cartilage, tendons, and ligaments thought to be involved in maintaining collagen integrity, but its direct function is unknown[69]. The COMP molecule is probably the most researched marker available, and can be detected in serum and synovial fluid. Similar to the type II collagen fragments, COMP degradation may also result from activation of MMPs[70]. The use of COMP in both human and animal models of joint disease is because of its

consistent association with joint destruction. This is because cartilage breakdown increases with OA and correlates with radiographic progression of this joint disease[71]. In a study of individuals possessing a mutation in the type II collagen gene, COMP levels were abnormally elevated[44] and thought to reflect altered cartilage metabolism. Several studies using serum and synovial fluid have shown elevated levels of COMP in human OA and RA diseases[41,72,73]. Dodge et al.[74] and Di Cesare et al.[75] have also observed increased COMP levels in patients with OA and RA compared to controls. Some concern has been expressed about the diagnostic value of COMP as an individual marker[20,73] because of the range in values obtained in diseased and normal individuals[76]. Roux-Lombard et al.[77] did not find a correlation between COMP and joint disease progression in RA patients over a five year period. Even so, COMP may be most valuable as a prognostic indicator of joint disease progression[72,78,79]. The use of COMP should provide an indirect measure of cartilage destruction and may be beneficial to use in combination with other types of markers when trying to predict disease occurrence.

Markers of proteoglycan degradation

Keratan sulfate (KS) and hyaluron (HA). Two markers that represent proteoglycan and synovium turnover are keratan sulfate (KS) and hyaluronan (HA). Both have been used to predict joint destruction in human models[20]. The KS fragment is related to the CS846 molecule and is also part of aggrecan, but is released during degradation and measured in joint fluid as well as serum. Increased concentrations of serum and synovial fluid KS have been found in RA and OA disease states[80,81]. In synovial fluid of subjects with knee OA, KS is elevated compared to controls[51,82]. Also, KS levels are dramatically elevated in humans with the collagen type II gene mutation[44]. The HA marker is more of an indicator of synovial tissue processes and is measured in serum. Garnero et al.[37] described higher levels of HA in patients with knee OA, but it did not correlate with an index of joint damage. Mansson et al.[41] and Fex et al.[83] found

increased HA concentrations in aggressive RA. The major drawback in using HA is the large variation that occurs during the day because of build up in levels at night and decreasing levels later in the day[20]. Therefore, timing of serum collection for this marker is important to limit variation. Both of these markers offer alternative measures that may aid in prediction of joint diseases.

Markers of bone formation

Amino-terminal propeptide of type I collagen (PINP) and carboxy-terminal propeptide of type I collagen (PICP). Osteochondrosis involves both the articular cartilage and the underlying subchondral bone, thus markers related to bone formation or turnover may also prove to be valuable markers of this disease[84]. Most of the biomarkers for bone formation or turnover have been extensively evaluated for bone diseases. Several markers of bone formation have been developed for detection in blood or urine, but limited data from animal models is available[35]. Similar to articular cartilage, fragments from formation of collagen type I present in bone or other markers related to the formation of bone can be measured in body fluids. Two telopeptides of the type I procollagen molecule, both the amino-terminal (PINP) and carboxy-terminal (PICP), can be determined by immunoassay in serum; however, many other tissues besides bone contain type I collagen[85] which may limit their validity as markers. Hassager et al.[86] and Blumsohn et al.[87] observed evaluated PICP in women and found that its clearance from blood was not only influenced by hormone levels but may also be processed by a different mechanism than PINP. Even so, Parfiit et al.[88] found that PICP correlated with other measures of bone formation in subjects with a bone disease. Initial assays for the PINP molecule were unsuccessful in correlating with bone formation[89], but further research and development have resulted in assays that have been shown to closely follow other methods for evaluating bone

formation[90,91]. Because of differences in clearance or processing of PICP, it appears that PINP is a better indicator of bone formation[92,93].

Osteocalcin. One of the more recognized markers of bone formation is osteocalcin, also known as bone gla protein[35]. Osteocalcin is synthesized by osteoblasts and thought to directly reflect their activity, but its true function in bone is not clear[85]. Some researchers believe it is involved in crystal formation in bone. In the initial studies using osteocalcin from serum to predict bone formation, a large amount of variation was observed[94]. This is a result of the instability of the intact osteocalcin molecule. Therefore it is recommended to use an assay that detects both the intact and N-terminal fragment of osteocalcin[95]. Osteocalcin has been used as a successful marker in predicting OA disease progression with an increase in serum osteocalcin after a one year follow up from the initial evaluation[96]. Garnero et al.[37] on the other hand, observed a correlation of osteocalcin with a score of joint damage in which osteocalcin was significantly decreased relative to controls. Limited data is available using osteocalcin to predict joint disease, but it has been used to predict bone formation response to dietary calcium and phosphorus in swine[97]. The few studies that are available show that it may have potential to predict events in bone metabolism and disease progression.

Bone specific alkaline phosphatase (BAP). Serum bone specific alkaline phosphatase is a protein synthesized by bone cells and is considered a measure of osteoblast activity[35]. It provides the most accurate measure of bone formation among the alkaline phosphatases because of its origin in bone[98]. Increased concentrations of BAP are associated with excessive bone loss in several bone related diseases[99]. Decreases in BAP concentrations have been found after hormone treatment therapy was done to increase bone mass in women with osteopenia[100]. Peel et al.[101] observed decreased BAP concentrations in women with spinal osteoarthritis while

Mansell et al.[102] found an increase in BAP in women with hip OA. Even though BAP is an established marker of bone formation, the implications that can be drawn concerning joint diseases is limited. More data regarding the relationship between serum BAP concentrations and joint diseases is needed.

Markers of bone turnover or resorption

Amino-terminal and carboxy-terminal telopeptide of type I collagen (NTX and CTX-I).

Markers of bone turnover measure the rate of bone degradation. Much like type II collagen degradation, type I collagen of bone undergoes similar cleavage into NTX, CTX-I, and a secondary cleavage telopeptide molecule of CTX-I with cross-links attached (ICTP)[103]. Both NTX and CTX-I have been extensively evaluated in urine and more recently, serum assays have been developed[35]. In urinary assays, NTX and CTX-I need to be adjusted for creatinine because of variation in clearance rate; however, only serum can be used to measure ICTP[35]. High levels of NTX or CTX-I indicate excessive bone degradation by osteoclasts such as in osteoporosis. In a study of patients with OA, CTX-I was increased in subjects with erosive OA[104]. Garnero et al.[105] also found elevated levels of CTX-I that corresponded to joint destruction and reported that initial urinary CTX-I levels predicted RA progression; however, Garnero et al.[37] showed conflicting results of reduced serum and urinary CTX-I levels in subjects with OA. Treatment related changes in CTX-I have been observed as well[65]. Woitge et al.[106] observed similar responses from urinary or serum CTX-I after treatment of patients with various bone diseases. The NTX marker has not been used in studies of human joint diseases, but has been used in pigs to determine osteoclastic activity or bone turnover[107]. It is also considered a good marker for treatment responses to anti-resorptive therapy[108] and an excellent indicator of bone turnover[109]. The ICTP marker also has been used successfully to portray the excess bone turnover involved in

bone diseases[110]. All three markers (CTX-1, NTX, and ICTP) are good indicators of bone turnover, but more work to clarify their correlation to joint diseases is needed.

Pyridinoline and deoxypyridinoline (PYD and DPD). Like the CTX-1 and NTX molecules, the cross-links that hold collagen molecules together can also be measured in urine and serum as a result of collagen degradation. Hydroxypyridinoline cross-links originate from bone, cartilage, and tendons, whereas DPD crosslinks originate from bone and can only be measured in urine[103]. The PYD marker has potential as both a cartilage and bone turnover marker, but is not specific for either tissue type. Determining PYD and DPD in urine has limited potential as prognostic indicators because of further processing that makes determination of their tissue of origin difficult[20]. Even so, increases in urinary PYD are higher than DPD in RA patients and may reflect cartilage and bone degradation[111,112]. Garnero et al.[37] reported that PYD was elevated in patients with knee OA and was correlated with both joint surface area and an index of joint damage. In a study of markers and long-term knee osteoarthritis, Bruyere et al.[96] were not able to predict long-term changes with PYD or DPD. For prediction of joint destruction, PYD appears to be a better indicator of cartilage damage than DPD because of its origin in both cartilage and bone.

Tartrate-resistant acidic phosphatase and bone sialoprotein (TRAP and BSP). Tartrate-resistant acidic phosphatase is a bone specific marker of osteoclast activity[113]. Although several different isoforms of TRAP exist, assays recognizing a bone specific form have been developed[103]. It has been used as a measure of bone turnover in pigs[107] and to predict fracture risk in humans[106]. Bone sialoprotein is a non-collagenous protein that is localized to the junction of articular cartilage and subchondral bone[114] and may be an indicator of processes effecting their interaction. The BSP marker was shown to have a negative correlation with radiographic

bone changes in OA[115]. Saxne et al.[116] found increased concentrations of BSP in OA patients and was correlated with OA severity. Mansson et al.[41] and Petersson et al.[117] observed elevated BSP levels compared to controls in both RA and OA subjects, respectively. Both of these markers have implications to aid in the prediction of events associated with bone turnover, but more research is needed to clarify their role and specificity for joint diseases.

Markers of inflammation and cytokines

C-reactive protein (CRP). Measuring markers of inflammation or the cytokines that ultimately control the degradative processes may offer predictive potential of OC. Many of these cytokines have direct influences on gene expression and initiation of joint tissue destruction. They can also be measured in synovial fluid, cartilage samples, and blood; however, most of these are not specific to joint tissues and may be influenced by other processes or tissues in the body. One of the main markers used extensively to predict inflammation is C-reactive protein (CRP) because of its sensitivity[20]. The production of CRP is stimulated by cytokines like interleukin-1 and tumor necrosis α[26]. Increased levels of serum CRP have been shown in subjects with OA[118]. Garnero et al.[37,63] did not find a correlation of CRP to joint damage in OA or RA patients. As well, Sturmer et al.[119] found a significant relationship of CRP to pain but was unable to predict OA severity. Even though CRP is a consistent marker of inflammation, its use as an indicator of joint disease progression or early indicator of disease may be limited.

Interleukin-1 and tumor necrosis factor α (IL-1 and TNF-α). Measurement of the cytokines IL-1 and TNF-α in serum and synovial fluid have also been proposed as indirect markers of joint disease because of their direct effects in regulating collagen type II synthesis and proteolytic enzymes[120,121]. High levels of IL-1 and TNF-α would decrease expression of the collagen type II gene or the ability to repair, and increase the production of MMPs that degrade collagen. Both IL-1 and TNF-α have been measured in synovial fluid[122,123] and serum/plasma of

patients with joint diseases[124,125]. Wood et al.[122] found that IL-1 concentrations were elevated in synovial fluid of subjects with arthritis while Tetta et al.[124] described the presence of TNF-α in both serum and synovial fluid of patients with RA. In a study of 14 different markers in urine, Otterness et al.[126] found high correlations of increased TNF-α in patients with OA. The number of studies evaluating either IL-1 or TNF-α and their relationship to joint damage are small and mostly limited to their effects in vitro. They may be used as indirect markers but are probably better indicators if measured from synovial fluid samples.

Matrix metalloproteinases (MMPs). The main enzymes that breakdown collagen are the MMPs that regulate the cleavage of collagen during its degradation. Measurement of these molecules in cartilage samples, synovial fluid, and blood has been more extensively researched than cytokines because MMPs are the end product of inflammatory stimulators. The main MMPs involved in collagen breakdown are 1, 3, 8, and 13[26]. More recently, MMP-13 has been described as the primary collagenase of cartilage destruction[127]. The inhibitor of MMPs, tissue inhibitor of matrix metalloproteinases (TIMP-1) has also been measured and expressed relative to concentrations of the MMPs[20]. The TIMP-1 molecule binds to MMPs and regulates their activity, where an imbalance either prevents degradation or allows degradation. The MMPs are mainly measured in synovial fluid and serum, and elevated levels have been shown to correlate with RA disease[77,83]. Ishiguro et al.[128] demonstrated that both MMP-1 and MMP-3 were increased in patients with OA, and the ratio of these MMPs to TIMP-1 was drastically increased. Both Roux-Lombard et al.[77] and Garnero et al.[63] have demonstrated a positive correlation of MMP-3 with a score of joint erosion in RA patients. Increases in MMP-1 and -13 have been described in a guinea pig model of OA and concentrations were localized to lesion sites[129]. Alternatively, Hegemann et al.[130] reported a ratio of less than 1 for MMP-3/TIMP-1 in OA

subjects, which the authors suggest indicates a surplus of TIMP-1. In a study evaluating potential markers of OA, MMP-1 and -3 were not correlated with severity, but TIMP-1 showed marginal correlations with OA severity[131]. Other markers of the inflammatory process have also been proposed as markers for joint diseases, but many have not been evaluated in clinical studies.

As new markers become available, they will have to be evaluated in large, well controlled studies to determine their effectiveness in predicting joint diseases.

Conclusion

Biomarker technology either by antibody detection or proteomic screening offers the opportunity to predict disease occurrence and provide information regarding disease processes in living animals. This technology will further advance our understanding of the events involved in disease pathology, such as OC, the changes that occur as a result of the disease, and provide a measure of our ability to provide treatment intervention. Biomarkers will aid in selecting for animals free of diseases like OC where early detection is difficult on a live animal. Cost will be the main road block in implementing this technology for commercial applications; however, the expense of biomarker assays is decreasing. The potential to use biomarkers to predict OC in swine is rapidly becoming available; however, much research will be needed to validate assays and determine their predictive value in swine models. In the future, a combination of different biomarkers may provide an opportunity to aid in selection of animals that have not developed OC and potentially help reduce sow herd turnover rates.

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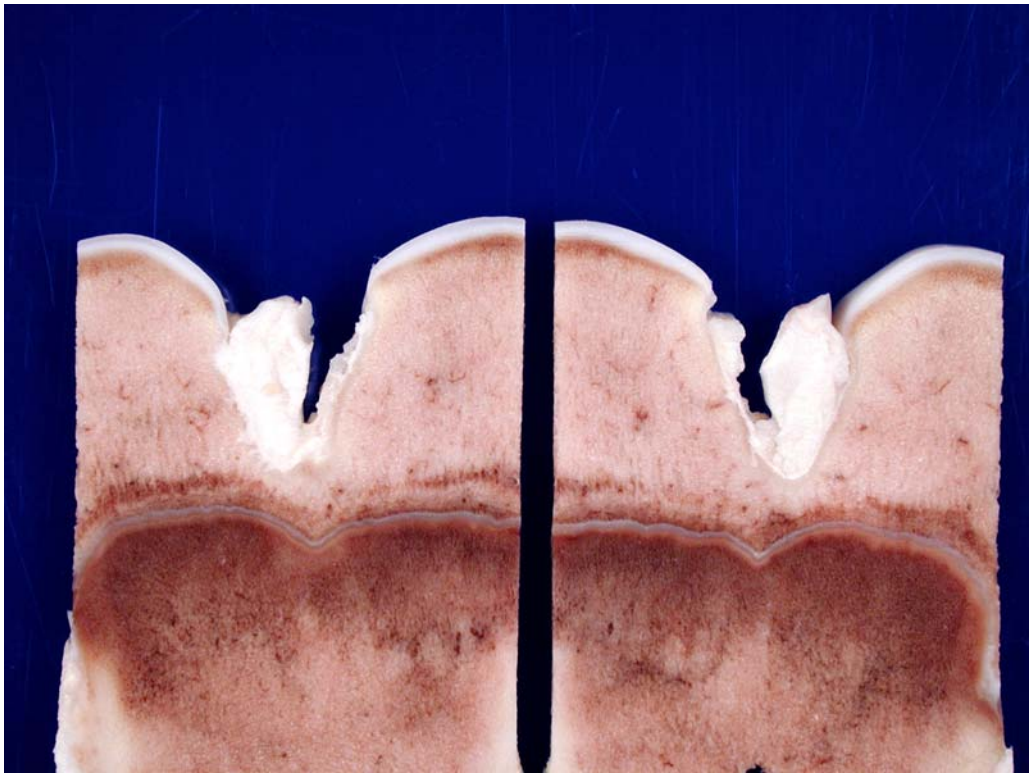

Figure 1. Normal physis (notice the even distribution of cartilage at the articular cartilage and growth plate with no evidence of osteochondrosis).

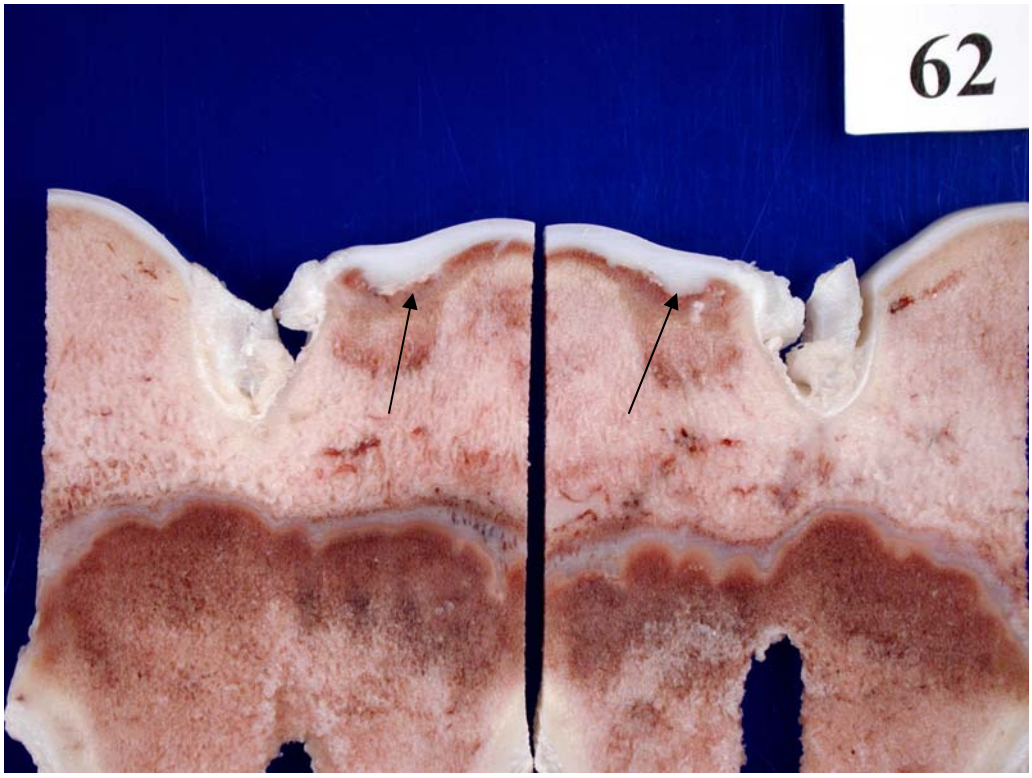

Figure 2. Osteochondrosis example (cartilage retained in subchondral bone).

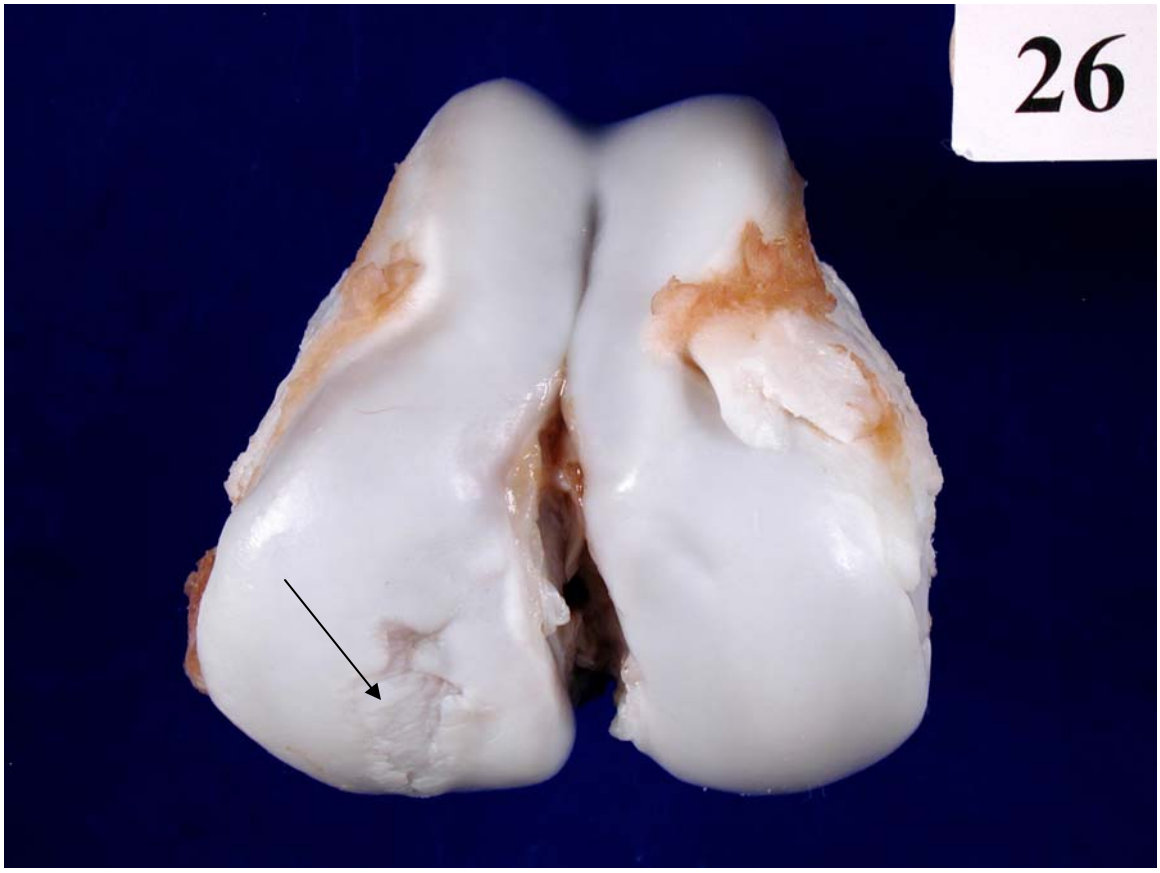

Figure 3. Osteochondrosis dissecans (lesion persisting through the articular surface causing fragmentation.

Table 1. Markers of cartilage and bone metabolism measured in synovial fluid, serum, and urine[a]

Direct markers	Metabolic process measured
Propeptide of type II collagen (CPII)	collagen type II synthesis rate
Collagen type II ¾ long fragment (C2C)	collagen type II degradation rate
Collagen type I and II short fragment (C1,2C)	collagen type I and II degradation
Collagen type II C-terminal crosslink fragment (CTX-II)	collagen type II degradation rate
Collagen type II helical domain (HELIX-II)	collagen type II degradation rate
Chondroitin sulfate epitope 846 (CS486)	aggrecan or CS chain synthesis rate
Keratan sulfate (KS)	aggrecan degradation
Pyridinoline crosslinks (PYD)	cartilage or bone turnover
Deoxypyridinoline crosslinks (DPD)	bone turnover or resorption
Glycoprotein 39 (YKL-40)	synovial tissue synthesis
Bone specific alkaline phoshphatase (BAP)	bone formation
Osteocalcin (OST)	bone formation or osteoblast activity
Bone sialoprotein (BSP)	bone turnover or resorption
tartrate-resistant acid phosphatase (TRAP)	bone turnover or resorption
Amino-terminal telopeptide of type I collagen (NTX)	collagen type I turnover
Carboxy-terminal telopeptide of type I collagen (CTX-I)	collagen type I turnover
Crosslinked carboxy-terminal telopeptide of type I collagen (ICTP)	collagen type I turnover
Amino-terminal procollagen type I propeptide (PINP)	collagen type I synthesis rate
Carboxy-terminal procollagen type I propeptide (PICP)	collagen type I synthesis rate

Indirect markers	Metabolic process measured
Cartilage Oligomeric matrix protein (COMP)	rate of cartilage destruction
Interleukin-1 (IL-1)	inflammatory cytokine secretion
tumor necrosis factor alpha (TNF-α)	inflammatory cytokine secretion
C-reactive protein (CRP)	inflammation or tissue degeneration
Hyaluron (HA)	synovial tissue synthesis
Matrix metalloproteinase-1 (MMP-1)	collagen degradation
Matrix metalloproteinase-3 (MMP-3)	Measure of collagen degradation
Matrix metalloproteinase-13 (MMP-13)	Measure of collagen degradation
Tissue inhibitor of matrix proteinase (TIMP-1)	inhibitor of MMPs

[a]Adapted from Garnero et al.[20] and Thonar et al.[26]

CHAPTER II

EFFECT OF DIETARY NUTRIENTS ON OSTEOCHONDROSIS LESIONS AND CARTILAGE PROPERTIES IN PIGS

ABSTRACT

Objective-To screen dietary ingredients involved in cartilage and bone metabolism on osteochondrosis (OC) in swine.

Animals-160 gilts (PIC 327×1050, 39 kg BW in Exp. 1 and 47 kg BW in Exp. 2) were used in two trials (80 per trial).

Procedures- Pigs were fed a corn-soy based diet or a diet containing additional minerals, vitamins, amino acids, or fatty acids involved in cartilage and bone metabolism. In Exp. 2, ractopamine HCl (RAC), a growth promoter, was added to half the pigs fed the control and methionine/proline/glycine/manganese (Met/Pro/Gly/Mn) diets. Upon completion of the feeding period, pigs were harvested and the distal femur collected for determination of OC lesions at the femoral condyle.

Results- In Exp. 1, feeding added proline/glycine, silicon, copper/manganese, methionine/threonine, or the combination of all additional ingredients reduced ($P < 0.03$) overall OC severity scores compared to controls. Additional methionine/threonine also tended ($P < 0.10$) to increase longissimus muscle area. In Exp. 2, pigs fed added silicon or dietary combination of additional Met/Pro/Gly/Mn tended ($P < 0.10$) to reduce the overall OC severity scores compared to pigs fed Met/Pro/Gly/Mn + RAC or arginine/glycine while the other treatments were intermediate. The addition of RAC to the control and Met/Pro/Gly/Mn diets compared to diets without RAC did not affect overall OC severity ($P > 0.10$).

Conclusions and Clinical Relevance- Feeding added dietary methionine/threonine, copper/manganese, or silicon decreased the severity of OC lesions compared to pigs fed a control diet, and further research on optimal concentrations and combinations is warranted.

(Key Words: Cartilage biology, Nutrition-swine, Osteochondrosis)

Introduction

Osteochondrosis (OC), a failure in the endochondral ossification of cartilage to bone[1,2], remains a common problem among growing swine that occurs in approximately 85 to 90% of all pigs[3,4]. Osteochondrosis lesions observed in pigs are similar to lesions in other animal species[5,6,7]. The main changes in cartilage that have been identified with OC are a loss of proteoglycans and collagen type II, with increased chondrocyte necrosis[8,9]. The presence of OC can reduce sow longevity because of lameness[10], and negatively affects carcass meat yield traits of finishing pigs[11]. Previously, OC was thought to result from a disruption in cartilage canal vessels that supply blood to the end of growing long bones[12,13,14], but OC more likely results from focalized disruption of endochondral ossification by mechanical stress[15]. Fast growth rate has been cited as a factor in manifestation of OC lesions[16,17]; however, several trials have observed no correlation between growth rate and OC occurrence[18,19]. Other factors, such as heredity[20,21] and trauma[22], have also been implicated in OC prevalence.

Several attempts have been made to determine the ability of dietary nutrients, such as protein[19,23,24], energy[25], calcium and phosphorus[23], Vitamin D[23,26], and Vitamin C[27,28], to influence OC occurrence and severity in swine with little success. In addition, several studies have evaluated the effect of growth promotants on OC[29,30,31] with mixed results. The use of glycosaminoglycans (GAGs), glucosamine and chondroitin sulfate, have been implicated for arthritic joint disorders[32], but have not been evaluated in swine OC models. Evaluation of several minerals involved in collagen and proteoglycan synthesis have suggested the importance of Cu[33], Mn[34], or Si [35,36,37]. Dietary Cu has been found to play a role in reducing the severity of OC lesions in horses[38,39]. Glucosamine[40] and n-3 fatty acids[41,42,43] have been implicated in minimizing cartilage degradation by blocking the production of matrix metalloproteinases (MMPs) which when present in high concentrations excessively degrade cartilage

components[44,45]. In addition, S-adenosylmethionine (SAMe), a metabolite of methionine, has been shown to increase synthesis of collagen and proteoglycan[46,47] components of cartilage. Collagen contains a high concentration of the non-essential amino acids proline and glycine[48], but the influence of feeding additional proline or glycine on OC or cartilage synthesis has not been evaluated. Although the role of minerals and fatty acids, such as Cu, Mn, silicon, and n-3 fatty acids, in cartilage and bone formation are relatively well known, their ability to prevent or aid in repair of joint diseases, such as OC, through dietary intervention have not been described.

Therefore, the objective of these experiments was to screen dietary ingredients involved in cartilage and bone metabolism for their influence on OC lesion occurrence and severity, other cartilage criteria, growth performance, and carcass characteristics in growing-finishing pigs.

Materials and Methods

General

Procedures used in these experiments were approved by the Kansas State University Animal Care and Use Committee. Two experiments were conducted at the Kansas State University Swine Research and Teaching Center finishing facility. The barn contains 80 pens with totally slatted concrete flooring (1.52 m^2), providing approximately 2.31 m^2/pig. Each pen was equipped with a one-hole dry self-feeder[a] and nipple waterer to allow ad libitum access to feed and water. In both experiments, each pen contained one pig for a total of ten replicates (pigs) per treatment for each trial.

Animals

In Exp. 1, 80 gilts (PIC line 327 × 1050; 39 kg initial BW) were blocked by weight for the 84-d growth assay and randomly allotted to one of eight dietary treatments. Experiment 2 was conducted similar to Exp. 1 with 80 gilts (PIC line 327 × 1050; 47 kg initial BW) that were again blocked by weight for a 70-d growth assay and randomly allotted to one of eight dietary

treatments. Pigs in Exp. 2 were heavier at the start of the experiment and were harvested at a lighter weight due to scheduling constraints.

Treatments

Minimum true ileal digestible (TID) amino acid ratios relative to lysine (Lys) were maintained in all diets with minimum ratios set at 30% for methionine, 60% for methionine and cystine, 65% for threonine, and 16.5% for tryptophan. All essential nutrients were supplied at or above National Research Council (NRC)[49] estimates. Diet samples from both experiments were analyzed for amino acid concentration and found to contain similar amounts compared to calculated values.

Experiment 1. Dietary treatments consisted of 1) control (standard corn-soy bean meal diet with 3.5% choice white grease) or the control diet with added, 2) fish oil (3.5%) replacing choice white grease, 3) proline and glycine (Pro/Gly, 300 and 200% of Lys, respectively), 4) leucine, isoleucine, and valine (BCAA; 200, 100, and 100% of Lys, respectively), 5) silicon (Si; 1,000 ppm), 6) copper and manganese (Cu/Mn, 250 ppm and 100 ppm, respectively), 7) methionine and threonine (Met/Thr, 110 and 100% of Lys, respectively), and 8) all ingredients in diets 2 through 7 combined into one diet (Table 2). The control diet contained amino acid levels of Pro (100% of lysine), Gly (65% of lysine), leucine (145 % of lysine), isoleucine (69 % of lysine), valine (76 % of lysine), Met (30% of lysine), and Thr (67 % of lysine) with mineral levels of Cu (16.5 and 14 mg/kg in phase I and phase II or III, respectively), Mn (40 and 33 mg/kg in phase I and phase II or III, respectively), and Si (0 mg/kg). Experimental diets were fed in meal form for 84 d in three 28-d phases. The phase I diets were formulated to contain 1.07% TID Lys and 3,457 Mcal of metabolizable energy (ME, Table 1), the phase II diets contained 0.94% TID Lys and 3,468 Mcal of ME, and phase III diets contained 0.80% TID Lys and 3,463 Mcal of ME. In each phase, all essential amino acids other than those used in dietary

treatments were provided at approximately 10% above the requirement for pigs in these weight ranges and added fat concentration varied slightly to maintain isocaloric diets.

Experiment 2. The eight dietary treatments were 1) control (standard corn-soybean meal diet, 3.4% added choice white grease); 2) control diet plus 20 ppm ractopamine HCl (RAC) as Paylean® to increase growth performance; 3) control diet plus methionine, proline, glycine (146, 173, and 174 % of Lys, respectively), and manganese (128 ppm; Met/Pro/Gly/Mn); 4) diet 3 plus RAC at 20 ppm; 5) control diet plus antioxidants (Vit. C and E 200 and 1,120 IU/kg, respectively), and fish oil at 0.45%); 6) control diet plus silicon (Si; 2,200 ppm); 7) control diet plus arginine and glycine (173 and 173% of Lys, respectively; Arg/Gly); and 8) glycosaminoglycans (GAGs), glucosamine at 460 ppm and chondroitin sulfate at 820 ppm, where chicken cartilage and poultry meal partially replaced soybean meal to provide the GAG concentrations (Table 3). Diets were formulated to contain 0.97 % total Lys and approximately 3,571 Mcal of ME (Table 1). The control diet contained amino acid levels of Pro (114% of lysine), Gly (79% of lysine), Met (30% of lysine), Arg (100 % of lysine), and vitamin and minerals levels of Vit. C (0 IU/kg), Vit. E (37 IU/kg), Mn (33 mg/kg), and Si (0 mg/kg). Pigs were fed the same treatment throughout the trial period. Extruder conditioned diets were fed as an extruded pellet from d 0 to 46 and from d 46 to 70 diets were fed in meal form. The two diets containing RAC were manufactured by taking half of the control and Met/Pro/Gly/Mn diets and remixing them with the addition of RAC at 20 ppm. The two diets containing RAC were analyzed for RAC concentration and found to contain levels similar to calculated amounts.

Growth performance and carcass data collection

Experiment 1 and 2. Pigs and feeders were weighed every 14 d to determine average daily gain (ADG), average daily feed intake (ADFI), and gain/feed (G/F). At the end of the trial, pigs were weighed and each pig marked with a distinctive tattoo before transport to the Kansas

State University Meats Laboratory, where the left hind leg was collected for determination of OC lesions and carcass data was collected. Pigs were loaded onto a trailer in small groups of 18-20 pigs and transported approximately 4 km to the processing facility. At the start of Exp. 1, all gilts were ultrasonically scanned to determine initial backfat depth and estimate fat-free lean. For carcass data, 10th rib backfat depth, longissimus muscle area, fat-free lean index, fat-free lean gain, and hot carcass weight were evaluated. Fat depth was measured with a ruler at the 10th rib, 6 cm off of the midline, while longissimus muscle area was traced on translucent paper and calculated using a grid. Fat-free lean index was calculated according to National Pork Producers Council (NPPC)[50] procedures and fat-free lean gain per day was calculated as the final fat-free lean minus initial fat-free lean divided by days on feed.

Collection of cartilage data and OC lesions scores

In both experiments, the left femur was collected and removed to visually determine the number of cartilage abnormalities and the occurrence of OC lesions by gross examination at the distal end of the femoral condyle. The joints were cleaned of excess tissue and then stored in 10% formalin until evaluation. Joints were photographed to allow visual evaluation of the external surface and the underlying articular cartilage/subchondral bone interface. After external evaluation, the distal end of the femur was cut into 3 mm thick sections perpendicular to the long axis of the bone using a bandsaw[21]. This resulted in 12 faces (cut surfaces) for evaluation of lesions. Each joint was evaluated for the number of external abnormalities (fissures or defects in the cartilage surface), and presence of OC lesions at the articular and growth plate cartilage. Lesions were given a severity score of 0 to 4 (0 = normal, 1 = mild, 2 = moderate, 3 = severe, and 4 = OC dissecans) based on the extent of tissue involvement. This scoring system is similar to those used previously[21,26]. The number of abnormalities at the external surface and faces or sections with lesions at the articular cartilage and growth plate were also noted. Each animal

was also given a "Yes" or "No" score for the presence or absence of OC lesions to determine the effect of treatment on OC occurrence.

A cartilage sample was cut from the patella for cartilage property analysis to determine the effect of dietary nutrients on mechanical properties of the cartilage. This is similar to the process used by Brama et al[51] (indenter system) but with force applied to the whole cartilage sample using an Instron machine[b]. Cartilage samples were weighed, measured for thickness and length using a caliper, and then tested for the ability to absorb compression or to resist shearing using an Instron machine. Cartilage samples were placed between two flat surfaces of the Instron to perform texture profile analysis and compressed half of the individual cartilage samples thickness. A second procedure was conducted in which the cartilage was cut using a Warner-Bratzler shear blade to determine the ability of the cartilage to withstand shearing force. Compression values and shear values were adjusted to a per gram of cartilage weight to equalize for differences in the actual cartilage weight.

Statistical analysis

Data were analyzed as a randomized complete block design using the PROC MIXED procedure of SAS[c] with pig as the experimental unit. The response criteria of growth performance, carcass composition, cartilage compression and shearing, and number of abnormalities were tested. Although scored categorically, severity scores were analyzed via PROC MIXED because low number of observations at some of the severity scores prevented categorical analysis. An overall score using the number of abnormalities at each location multiplied by the severity at each location and then summed was created to provide an overall severity score or indication of joint status. The 'Yes' or 'No' comparison of the presence of OC lesions was compared using the Cochran-Mantzel-Haenszel statistic of PROC FREQ. To evaluate the effect of amino acids or mineral containing diets relative to the other dietary

treatments single degree of freedom contrasts[52] were constructed. Values were considered significant at $P < 0.05$ and trends in the data at $P < 0.15$.

Results

Experiment 1

Growth and carcass data. Overall (d 0 to 84) growth performance was unaffected by dietary treatment ($P > 0.21$, Table 4). Pigs fed high Met/Thr tended to have increased ($P < 0.10$) longissimus muscle area compared to the other dietary treatments while pigs fed fish oil were intermediate; however, no other carcass differences were observed ($P > 0.84$).

Cartilage evaluation. Cartilage compression values were unaffected by dietary treatment ($P > 0.19$, Table 5), but pigs fed fish oil had lower ($P < 0.02$) shear energy values and a higher ($P < 0.03$) ratio for compression:shear energy compared to pigs fed the control diet, added Cu/Mn, or silicon with the other treatments intermediate.

Joint evaluation. No differences ($P > 0.52$) in the number of animals with OC were detected between treatments. Pigs fed diets containing fish oil or Si tended ($P < 0.07$, Table 6) to have a higher severity score for external joint abnormalities compared to pigs fed high BCAA, Met/Thr or a diet containing all additional ingredients with the other dietary treatments intermediate. Pigs fed high Met/Thr, Cu/Mn, or Si tended ($P < 0.08$) to have lower articular cartilage severity scores than pigs fed the control diet or BCAAs, with the other dietary treatments intermediate. The occurrence of OC lesions at the growth plate, total faces with lesions, or total number of abnormalities were not affected by dietary treatment ($P > 0.23$); however, there was a numerical trend ($P < 0.14$) for pigs fed diets containing high Met/Thr or the diet containing all additional ingredients to have lower total severity scores than pigs fed the control diet or fish oil with the other treatments intermediate. Finally, pigs fed diets containing additional Pro/Gly, Si, Cu/Mn, Met/Thr, or the diet containing all additional ingredients had

lower ($P < 0.03$) overall severity scores compared with pigs fed the control diet with the other treatments intermediate. Contrast statements also revealed that pigs fed the diets containing additional amino acids (Pro/Gly, BCAAs, Met/Thr) had lower external and total severity scores ($P < 0.05$) than pigs fed the other dietary treatments; however, pigs fed diets containing additional minerals (Si or Cu/Mn) tended to have lower articular cartilage severity scores ($P < 0.08$) and overall severity scores ($P < 0.02$).

Experiment 2

Growth performace. Overall (d 0 to 70), there were no differences in ADG ($P > 0.36$, Table 7), ADFI ($P > 0.29$), or G/F ($P > 0.14$). Hot carcass weight (HCW) tended ($P < 0.06$) to increase in pigs fed the negative control + RAC diet compared to the other dietary treatments with pigs fed silicon intermediate. Dressing percentage also tended ($P < 0.08$) to increase in pigs fed the control + RAC diet compared to the other dietary treatments with pigs fed added Si, GAGs, or Met/Pro/Gly/Mn intermediate.

Cartilage evaluation. Instron measurements of cartilage compression and shear values were unaffected by dietary treatment ($P > 0.41$, Table 8).

Joint evaluation. No differences ($p > 0.54$) in the number of animals with OC were detected between treatments. From external evaluation of the joints, there were no treatment differences in either the number of abnormalities or severity score ($P > 0.46$, Table 9). There was a numerical trend ($P < 0.14$) for pigs fed additional Met/Pro/Gly/Mn to have lower severity scores at the articular cartilage than pigs fed added Met/Pro/Gly/Mn + RAC or Arg/Gly with the other dietary treatments intermediate. There were no differences in the number of faces with lesions or severity score at the physeal growth plate ($P > 0.23$). The overall evaluation revealed that there was a numerical trend ($P < 0.11$) for pigs fed added Si to have a reduced total number of faces with lesions compared to pigs fed added GAGs, Arg/Gly, or Met/Pro/Gly/Mn + RAC

with the other dietary treatments intermediate. The total number of abnormalities was unaffected by dietary treatment ($P > 0.17$). The total severity score of all three locations was unaffected by dietary treatment ($P > 0.63$); however, the overall severity scores (abnormalities × severity) of OC tended ($P < 0.10$) to be reduced in pigs fed added Si or Met/Pro/Gly/Mn compared to pigs fed added Met/Pro/Gly/Mn + RAC or Arg/Gly with the other dietary treatments intermediate. The comparison of the control and Met/Pro/Gly/Mn diets with RAC versus all other treatments without RAC by orthogonal contrast did not show an affect on the overall severity score ($P > 0.40$).

Discussion

The goal of this research was to screen and possibly identify nutrient strategies to reduce the severity or prevalence of OC lesions in swine. The incidence of OC is high in swine and is similar to OC in other species[3,5]. One of the main concerns with OC and lameness in swine is the negative effects it may have on sow longevity[10,54]. Sow herd turnover rates approaching greater than 50% have been noted[55] with the largest reasons for turnover including reproductive failure and lameness[56]. In the first study, dietary ingredients were screened with structural and functional roles in cartilage and bone metabolism to determine their ability to impact the occurrence and severity of OC lesions. The second study was conducted to confirm the results found in Exp. 1 and to explore other dietary nutrients that could have an impact on OC lesions.

No differences in growth performance were observed in either experiment as expected except for the response to RAC; however, growth performance in both studies was similar to other research studies and slightly higher than growth rates found in commercial facilities.

The results from Exp. 1 suggest that the minerals Cu and Mn provided in excess of requirements[49] may reduce the severity of OC lesions at the articular cartilage and overall severity score. In Exp. 2, Mn fed in combination with Met, Pro, and Gly numerically reduce the

overall severity score of OC. This data would suggest that both Cu and Mn may reduce the severity of OC lesions either by maximizing lysyl oxidase activity[57,58], stimulating proper collagen crosslinking[59,60], improving the integrity of vascular walls of cartilage canal vessels, or increased formation of proteoglycans[34]. Swine diets are often supplemented with Cu for its positive effects on growth and efficiency[61] and thought to have antimicrobial activity. Previous research with added Cu fed to rats demonstrated that a Cu deficient diet or a diet with additional Cu did not effect the level of lysyl oxidase mRNA, but additional Cu supplementation increased the activity of the enzyme[60]. Heraud et al.[33] showed that the addition of Cu to human articular chondrocytes resulted in a dose and time dependent increase in collagen synthesis determined by [3H]-proline incorporation. Pasqualicchio et al.[62] reported similar benefits with the addition of Cu to porcine articular cartilage cell culture preventing depletion of proteoglycans or stimulating proteoglycan synthesis. Supplementation of swine diets with high levels of zinc (5,000 ppm) have been proposed to inhibit Cu absorption and result in an increased occurrence of OC[63,64]. Aballi and Austbo[65] also reported that sows fed 100 ppm of additional Cu had offspring with less severe OC lesions than sows fed 15 ppm. Similar to previous trials, added Cu decreased the severity of OC; however, additional research is required to verify the mechanism.

Manganese fed in combination with Cu in Exp. 1 and with added Met/Pro/Gly in Exp. 2 reduced overall severity scores. Manganese is involved in proteoglycan metabolism through glycosyltransferases which are abundant in cartilage[66], and serves a structural role in linking chondroitin sulfate molecules. Manganese is also critically important in mitochondrial superoxide dismutase to control free radical production by oxidation reactions in the mitochondria[67]. Feeding rats a Mn deficienct diet resulted in decreased bone formation[68], and

has been shown to negatively impact proteoglycan metabolism in chickens[34]; however, studies on the influence of manganese on joint diseases are limited.

Feeding pigs a diet with added Si, a mineral with no established requirement for swine[49], reduced the severity of OC lesions at the articular cartilage and overall severity scores in Exp. 1, and numerically reduced overall severity scores in Exp. 2. In both studies, Si was supplied as Zeolite A (silica acid) which has been shown to significantly increase serum concentrations of Si in horses[69]. It has been speculated that Si is required for proper cartilage and bone metabolism due to its role in collagen formation and bone mineralization[70], and is found in relatively large quantities in the proteoglycan matrix[35]. Silicon is required for maximal prolyl hydroxylase in the synthesis of hydroxyproline, a rate limiting step in collagen formation[71], and a deficiency is associated with decreased collagen formation[72]. Supplementation of Si to growing chicks resulted in a greater concentration of glycosaminoglycans and water content in the cartilage[36], while Calomme and Vanden Berghe[37] showed a positive correlation between serum Si and collagen concentrations in bovine cartilage explants. Increasing dietary intake of Si has also been shown to increase bone mineral density in humans[73]. Thus, the positive results observed on OC overall severity score in Exp. 1 and overall severity score in Exp. 2 may be due to the positive role Si has in collagen formation and stabilizing the proteoglycan matrix.

The addition of high levels of Met/Thr reduced the severity of OC at the articular cartilage and the overall severity score in Exp. 1. In Exp. 2, even though the incidence of OC was dramatically lower than Exp. 1, numerical improvements in the overall severity score were observed when Met was fed in combination with Mn, Pro, and Gly compared to pigs fed the control diet. Previous research with a metabolite of Met, S-adenosylmethionine (SAMe), indicated SAMe has positive effects on collagen synthesis and proteoglycan formation[46,47]. In

addition, sulfur is required for the formation of proteoglycan chains that extend from the hyaluronic acid backbone and give cartilage its absorptive properties[74]. More recently, it has been proposed that the protective effects of nutrients containing sulfur on cartilage may be a result of overcoming a deficiency of sulfur in the extracellular matrix and emphasized the role of sulfur amino acids for glutathione formation (antioxidant) and in cartilage metabolism[75,76]. However, it is highly unlikely that the positive effects seen in these experiments are due to the antioxidant activity of Met since neither fish oil nor other antioxidant strategies provided any benefit. The amino acid Thr can be metabolized to Gly, a component of collagen, by threonine dehydrogenase[77]; however, we believe the effects seen in Exp. 1 and 2 are primarily due to methionine's role as SAMe or as a sulfur donor. The positive effects SAMe has on proteoglycan and collagen metabolism may help offset the loss of these two cartilage components during OC.

In Exp. 1, pigs fed high Met/Thr had increased LMA. This is similar to the response observed by Knowles et al.[78] on increased lean:fat ratio with increasing total sulfur amino acid concentrations in finishing pig diets. The increase in LMA may be due to the methyl donor properties of Met which may protect DNA in proteins from degradation[79] and thus reduce protein turnover. Additionally, certain genes involved in protein synthesis or inhibitors of protein synthesis also may increase or be suppressed by methylation[80].

Supplementing swine diets with additional fish oil (Exp. 1) or in combination with added Vit. C and Vit. E (Exp. 2) did not affect the occurrence or severity of OC lesions. Conversely, fish oil added to diets in Exp. 1 caused cartilage to require less energy to shear and had the highest ratio of compression:shear. This suggests that the cartilage samples from pigs fed fish oil required more energy to compress while at the same time were more brittle and easier to shear into two pieces, demonstrating less ability to distribute mechanical forces and resist tearing. Fish

oil may have prevented the proper turnover of cartilage components by inhibiting MMPs and resulted in cartilage with inferior mechanical properties. Excessive free radicals may signal increased activation of inflammatory cytokines that stimulate cartilage degradation by MMPs[63,81,82,83,84,85]. Increasing dietary n-3 fatty acids have shown positive results in arthritic joint disease because of their ability to reduce the production of inflammatory intermediates [41,42,43,86]. Vitamin C also has a role in collagen formation and is required for the hydroxylation reaction that produces hydroxylysine and hydroxyproline for cartilage[87], while both Vit. C and Vit. E function as antioxidants within the cell and lipid membranes[88], respectively. However, pigs are able to synthesize Vit. C in sufficient quantity[49] for proper cartilage hydroxylation. Several attempts have been made to determine the ability of vitamin C to influence OC and bone characteristics in swine because of this role. Similar to our results, Grondalen and Hansen[27] found no effect of Vit. C on OC occurrence in growing pigs, while Nakano et al.[28] reported no benefit of supplementation with 350 or 700 ppm on hydroxyproline concentrations in cartilage samples from pigs fed these levels from weaning till slaughter. Armocida et al.[89] did not find a relationship between plasma Vit. C and OC in four litters of pigs. The inflammatory process known to be involved in arthritic joint diseases may not be of major importance in the pathology leading to OC as arthritic conditions are often secondary to OC in pigs[5].

In Exp. 2, feeding pigs GAGs did not affect OC severity or occurrence. Glucosaminoglycans are thought to possess a similar ability to n-3 fatty acids and antioxidants to inhibit production of inflammatory mediators that signal the degradation of cartilage components[39,90]. Both glucosamine and chondroitin sulfate (GAGs) are components of proteoglycans in the extra cellular matrix and have been implicated as effective pain relief treatments in arthritis[32], but their ability to positively affect cartilage or proteoglycan metabolism

is less well determined[40]. The positive effect of GAGs on cartilage may work to a greater extent on older animals as demonstrated in bovine articular cartilage explants from older animals compared to younger animals[91]. Again, it appears that ingredients with positive effects on inflammatory mediators, such as GAGs or antioxidants, in osteoarthritis diseases may not be directly involved in OC pathogenesis.

In Exp.1, feeding high levels of Pro and Gly had intermediary effects on overall severity of OC lesions. In Exp. 2, Pro and Gly were included in combination with additional Met and Mn and numerically reduced OC overall severity scores. Proline and Gly are two non-essential amino acids highly concentrated in collagen[47]. Supplementing additional dietary Pro and Gly has not been evaluated; however, we theorized that supplying large quantities of these amino acids may have a positive influence on collagen formation. An additional dietary treatment combination of Arg and Gly was formulated to determine if the same results could be observed using Arg, which can be converted to Pro through several reactions[92]. However, pigs fed additional Arg and Gly had a greater number of abnormalities and numerically higher overall severity scores compared to the control diet. We do not have an explanation as to why high levels of Arg and Gly increased abnormalities and overall severity score in Exp. 2, but it may be because of excessive conversion of arginine to nitric oxide (NO). Nitric oxide is an inflammatory mediator with downstream activation of MMPs and is involved in cell signaling of apoptosis[93]. High levels of Arg may increase NO and thus increase signaling of inflammatory pathways that increase collagen breakdown or control chondrocyte apoptosis.

Ractopamine HCl (RAC), a beta-agonist that enhances protein deposition and growth rate[94], was added to both the control and Met/Mn/Pro/Gly diets in Exp. 2 to increase growth performance. In Exp. 2, the addition of RAC to the diet with added Met/Pro/Gly/Mn increased

overall severity, but did not affect any of the OC response measures when comparing the two diets containing RAC to the other dietary treatments without RAC, suggesting no negative implications of RAC feeding on OC severity or occurrence in this study. Previously, it has been proposed that a hormonal imbalance may cause OC, particularly growth hormone and insulin-like growth factor; however, mixed results have been shown on the effects of growth enhancers on OC. Similar to our experiment, Hill and Dalrymple[29] found no effect of adding cimaterol to the diet of pigs on OC. However, He et al.[31] injected pigs with somatotropin and found a higher incidence of OC independent of growth rate compared to pigs injected with saline. Evock et al.[30] also noted an increased incidence of OC in pigs injected with recombinant growth hormone compared to controls.

In conclusion, feeding dietary ingredients involved in cartilage and bone metabolism may offer potential to reduce the severity of OC lesions, but only fish oil in Exp. 1 negatively affected cartilage mechanical properties. The minerals Cu, Mn, and Si appear to play a role in either preventing the cartilage matrix from degradation or increasing the ability of the tissue to repair lesions, particularly at the articular-epiphyseal cartilage. Similarly, adding high levels of the amino acids Met, Thr, Pro, and Gly may also positively influence cartilage metabolism and reduce the severity of OC lesions. However, the limited effects of these nutrients on growth plate lesions may be because they are resolved before reaching slaughter weights. More research will be required to allow a better understanding of the influence these minerals and amino acids have on OC and to further evaluate combinations of these dietary ingredients.

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[a] Farmweld, Tuetopolis, IL.

[b] Instron model 4201.

[c] SAS, version 8.0, SAS Institute, Cary, NC.

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Table 1. Diet composition (As-fed)

	Experiment 1[abc]			Experiment 2[de]
Item	Phase I	Phase II	Phase III	Control
Ingredient				
Corn	62.65	68.60	74.05	69.96
Soybean meal (49% CP)	-	-	-	18.79
Soybean meal (46.5% CP)	30.45	24.95	19.50	-
Choice white grease[f]	3.50	3.50	3.50	3.41
Monocalcium phosphate (21 % P)	1.50	1.25	1.25	-
Dicalcium phosphate (18.5 % P)	-	-	-	2.17
Limestone	1.05	1.00	1.00	0.87
Salt	0.35	0.35	0.35	0.63
Vitamin premix[g]	0.15	0.13	0.13	0.13
Trace mineral premix[h]	0.15	0.13	0.13	0.13
Choline chloride	-	-	-	0.11
L-lysine HCl	0.15	0.15	0.15	0.17
DL-methionine	0.06	0.03	-	0.04
L-threonine	0.06	0.05	0.05	-
L-proline	-	-	-	-
L-glycine	-	-	-	-
Manganese sulfate	-	-	-	-
Cornstarch[d]	-	-	-	3.60
Total	100.00	100.00	100.00	100.00

Table 1 Continued (Calculated analysis)

Total lysine, %	1.20	1.05	0.90	0.97
True ileal digestible amino acids				
Lysine, %	1.07	0.94	0.80	0.87
Isoleucine:lysine ratio, %	69	69	70	67
Leucine:lysine ratio, %	145	154	164	156
Methionine:lysine ratio, %	32	31	29	31
Met & Cys:lysine ratio, %	60	60	60	60
Threonine:lysine ratio, %	65	66	68	68
Tryptophan:lysine ratio, %	20	19	19	21
Valine:lysine ratio, %	76	78	80	78
ME, kcal/kg	3,457	3,468	3,461	3,567
CP, %	19.5	17.4	15.4	16.7
Ca, %	0.80	0.72	0.72	0.91
P, %	0.70	0.62	0.62	0.74
Lysine:calorie ratio, g/mcal	3.47	3.03	2.60	2.72

[a]Diets fed in meal form in three 28-d phases.
[b]Dietary treatments were created by substituting ingredients for corn or CWG in Exp. 1.
[c]Analyzed values for lysine and other amino acids were found to contain similar levels relative to the calculated values.
[d]Additional dietary treatments for Exp. 2 were created by replacing cornstarch or soybean meal in the negative control diet.
[e]Analyzed values for lysine, other amino acids, and vitamins/minerals were found to contain similar levels relative to the calculated values.
[f]CWG varied in the diet slightly to maintain isocaloric diets.
[g]Vitamin premix contributed per kilogram of complete diet in phase I: vitamin A, 6,613 IU; D_3, 992 IU; vitamin E, 26 IU; vitamin K, 2.7 mg; vitamin B_{12}, 0.03 mg; riboflavin, 6 mg; pantothenic acid, 20 mg; niacin, 33 mg, and in the phase II or III diet: vitamin A, 5,512 IU; D_3, 827 IU; vitamin E, 22 IU; vitamin K, 2.2 mg; vitamin B_{12}, 0.02 mg; riboflavin, 5 mg; pantothenic acid, 16 mg; niacin, 27 mg.
[h]Trace mineral premix provided per kilogram of complete diet in phase I: copper (from copper sulfate), 16.5 mg; iodine (from calcium iodate), 0.3 mg; iron (from ferrous sulfate), 165 mg; manganese (from manganese oxide), 40 mg; selenium (sodium

selenite), 0.3 mg; zinc (from zinc oxide), 165 mg, and in phase II or phase III: copper (from copper sulfate), 14 mg; iodine (from calcium iodate), 0.25 mg; iron (from ferrous sulfate), 138 mg; manganese (from manganese oxide), 33 mg; selenium (sodium selenite), 0.25 mg; zinc (from zinc oxide), 138 mg.

Table 2. Ingredients added to form dietary treatments (Exp. 1)[ab]

Treatment	
Control	Standard corn-soybean meal based diet.
Fish oil	3.5% fish oil replaced choice white grease resulting in an n-6 to n-3 ratio of 2:1.
Pro/Gly	L-proline was added at 2.55% and L-glycine at 1.70% to create a ratio of proline:lysine of 300% and a glycine:lysine ratio of 200%.
BCAA	L-leucine was added at 0.60%, L-Isoleucine at 0.35%, and L-Valine at 0.29% to create a leucine:lysine ratio of 200%, isoleucine:lysine ratio of 100%, and valine:lysine ratio of 100%.
Silicon	Silicon was added at 0.80% (Zeolite A) to create the Si diet (1,000 ppm).
Cu/Mn	Copper was added at 0.1% (250 ppm) and manganese was added at 0.02% (100 ppm).
Met/Thr	DL-methionine was added at 1.05% to create a methionine:lysine ratio of 110% while L-threonine was added at 0.45% to create a threonine:lysine ratio of 100%.
Combination	Contained all additional dietary ingredients at the expense of corn and choice white grease.

[a]All dietary treatments were fed in meal form and maintained throughout the three 28 d feeding phases.
[b]Control diet contained amino acid levels of Pro (100% of lysine), Gly (65% of lysine), leucine (145 % of lysine), isoleucine (69 % of lysine), valine (76 % of lysine), Met (30% of lysine), and Thr (67 % of lysine) with minerals levels of Cu (16.5 and14 mg/kg in phase I and phase II or III, respectively), Mn (40 and 33 mg/kg in phase I and phase II or III, respectively), and Si (0 mg/kg).

Table 3. Ingredients added to form dietary treatments (Exp. 2)[abc]

Treatment	
Control	Standard corn-soybean meal based diet.
Control + RAC	Ractopamine HCl (RAC) supplied as Paylean® was supplemented at 0.02% (20 ppm) and replaced corn starch.
Met/Mn/Pro/Gly	Corn-soybean meal based diet containing additional DL-methionine (1.17%), L-proline (0.55%, L-glycine (0.91%), and manganese sulfate (0.025%).
Met/Mn/Pro/Gly + RAC	Ractopamine HCl supplied as Paylean® was supplemented at 0.02% (20 ppm) and replaced corn starch in the Met/Pro/Gly/Mn diet.
Antioxidants	Added Vitamin C (200 IU/kg), Vitamin E (1120 IU/kg) and fish oil (0.45%) replaced cornstarch in the control diet.
Silicon	Silicon was added at 2.0% (Zeolite A) replacing corn starch in the control diet to create the Si diet (2,200 ppm).
Arginine/Glycine	L-arginine (0.79%) and L-glycine (0.91%) replaced cornstarch to form the Arg/Gly treatment.
GAGs	Poultry meal (13.06%) and chicken cartilage (2.15%) replaced cornstarch and soybean meal to provide 460 ppm glucosamine and 820 ppm chondroitin sulfate.

[a]Dietary treatments were fed as an extruded pellet from d 0 to 46 and in meal form from d 46 to 70 to accommodate the feeding of ractopamine HCl (RAC).

[b]Diets were fed in one phase throughout the trial period.

[c]Control diet contained amino acid levels of Pro (114% of lysine), Gly (79% of lysine), Met (30% of lysine), Arg (100 % of lysine), and vitamin and minerals levels of Vit. C (0 IU/kg), Vit. E (37 IU/kg), Mn (33 mg/kg), and Si (0 mg/kg).

Table 4. Effect of different nutrients on growth performance and carcass composition (Exp. 1)

	Dietary treatment									Probability[a], P <
	Control	Fish Oil	Pro/Gly	BCAA	Si	Cu/Mn	Met/Thr	All ingredients[b]	SED	Treatment
Growth, d 0 to 84										
ADG, kg	1.09	1.06	1.08	1.12	1.11	1.13	1.03	1.04	0.047	0.21
ADFI, kg	2.78	2.65	2.71	2.80	2.79	2.74	2.64	2.58	0.110	0.26
Gain/Feed	0.39	0.40	0.40	0.40	0.40	0.41	0.39	0.40	0.013	0.91
Final weight, kg	133.6	128.0	132.2	133.1	135.5	130.3	128.9	129.5	4.64	0.70
Carcass data										
Initial backfat, mm	5.3	5.4	5.1	5.5	5.1	5.1	5.4	5.1	0.02	0.83
Hot Carcass Weight[c], kg	95.2	93.4	93.0	94.9	96.5	93.8	91.5	89.4	3.28	0.43
Final backfat, mm	15.6	15.7	15.1	14.3	14.2	15.5	15.9	16.1	0.06	0.92
LMA, cm^{2}[d]	49.5[gh]	51.2[hi]	48.3[gh]	48.8[gh]	48.4[gh]	49.0[gh]	53.5[i]	47.1[g]	2.24	0.10
Fat free lean index[e]	55.4	55.7	55.3	55.5	55.7	55.4	56.1	54.5	1.09	0.92
Fat-free lean gain kg/d[f]	0.436	0.432	0.428	0.454	0.450	0.431	0.424	0.400	0.020	0.19

[a]Each value is the mean of 9 or 10 replications with pigs initially 39 kg and average final weight of 131.5 kg.
[b]Diet contained all additional ingredients added into one diet.
[c]Hot carcass weight was used as a covariate in analysis except for fat-free lean gain.
[d]Longissimus muscle area.
[e]Fat free lean index calculated according to the procedures of the NPPC[50].
[f]Calculated as the final fat-free lean minus initial fat-free lean divided by days on feed.
[g,h,i] Treatments with different superscripts differ (P < 0.05).

Table 5. Effect of different nutrients on cartilage properties (Exp. 1)

	Dietary treatment									Probability, P <
Item	Control	Fish Oil	Proline/ Glycine	BCAA	Si	Cu/Mn	Met/Thr	All Ingredients[b]	SED	Treatment
Instron measures										
Compression energy, n/g[c]	85.4	126.9	144.7	102.5	86.0	59.8	116.4	110.7	39.22	0.59
Shear energy, n/g[d]	518.1[gh]	371.4[g]	444.7[gh]	491.8[gh]	527.1[gh]	601.5[h]	498.8[gh]	540.9[gh]	61.32	0.02
Total energy, n/g[2e]	1271.4	1226.8	976.5	1303.3	1342.3	1401.9	1326.9	1539.6	291.54	0.73
Ratio of CE/SE[f]	0.15[h]	0.41[g]	0.31[gh]	0.25[gh]	0.17[h]	0.15[h]	0.25[gh]	0.21[gh]	0.081	0.03

[a]Each value is the mean of 9 or 10 replications with pigs initially 39 kg and a final weight of 131.5 kg.

[b]Diet contained all additional ingredients added into one diet.

[c]Amount of energy in newtons per gram of cartilage to compress the cartilage half its thickness.

[d]Amount of peak energy in newtons per gram of cartilage to shear the cartilage into two pieces.

[e]The total amount of energy required to shear the cartilage into two pieces.

[f]The ratio of compression energy to shear energy in which lower values would indicate more desirable characteristics.

[g,h]Treatments with different superscripts differ ($P < 0.05$).

Table 6. Effect of dietary nutrients on the occurrence of osteochondrosis (Exp. 1)[a]

	Dietary treatment									Probability, P<		
											Contrasts	
Item	Control	Fish Oil	Pro/ Gly	BCAA	Si	Cu/ Mn	Met/ Thr	All Ingredients[b]	SED	Treatment	Minerals vs others	AAs vs others
Total animals/trt[c]	10	10	10	10	10	9	10	10	-	-	-	-
Animals with lesions[d]	9	9	9	9	6	7	7	7	1.5	0.52	-	-
External												
Abnormalities[e]	1.9^{op}	2.6^{o}	2.0^{op}	1.4^{p}	2.5^{o}	1.8^{op}	1.3^{p}	1.4^{p}	0.56	0.13	0.86	0.02
Severity score[f]	2.1^{op}	2.5^{o}	1.9^{op}	1.4^{p}	2.4^{o}	1.8^{op}	1.3^{p}	1.4^{p}	0.48	0.07	0.94	0.01
Articular cartilage												
Number of faces[g]	5.0	4.5	2.4	5.0	2.2	2.3	2.6	4.1	1.44	0.16	0.19	0.87
Severity score[h]	2.0^{o}	1.3^{opq}	1.2^{opq}	1.6^{op}	0.7^{q}	0.8^{pq}	0.7^{q}	1.3^{opq}	0.48	0.08	0.08	0.98
Growth plate												
Number of faces[i]	0.9	1.7	1.2	0.6	1.2	1.6	1.8	0.2	0.68	0.23	0.47	0.25
Severity score[j]	0.6	1.1	0.9	0.6	0.8	0.8	1.0	0.1	0.43	0.38	0.21	0.43
Overall												
Total faces[k]	5.9	6.2	3.6	5.6	3.4	3.9	4.4	4.3	1.81	0.63	0.17	0.68
Total abnormalities[l]	8.7	8.8	5.6	7.0	5.9	5.7	5.7	5.7	1.89	0.54	0.21	0.27
Total severity[m]	4.7^{o}	4.9^{o}	4.0^{op}	3.6^{op}	3.9^{op}	3.3^{op}	3.0^{p}	2.8^{p}	0.85	0.14	0.12	0.05
Overall score[n]	17.1^{o}	15.0^{op}	8.8^{pq}	12.4^{opq}	8.4^{pq}	6.4^{q}	6.6^{q}	7.0^{q}	3.76	0.03	0.02	0.11

[a]Each value is the mean of 9 or 10 replications with one pig per pen initially 39 kg and a final weight of 131.5 kg.

[b]Diet contained all additional dietary ingredients added into one diet.

[c]Total animals evaluated per treatment.

[d]The number of animals with OC lesions (Cochran-Mantzel-Haenszel test).

[e]Number of abnormalities noted upon visual evaluation of the external surface.

[f]Lesion severity score (0 - 4 with 0 being normal, 1 mild, 2 moderate, 3 severe, and 4 OC dissecans) of the external surface of the intact joint.

[g]The number of faces showing lesions at the articular cartilage evaluating 12 cut surfaces.

[h]Lesion severity score (0 - 4 with 0 being normal, 1 mild, 2 moderate, 3 severe, and 4 OC dissecans) at the articular cartilage.

[i]The number of faces showing lesions in the growth plate evaluating 12 cut surfaces.

[j]Lesion severity score (0 - 4 with 0 being normal, 1 mild, 2 moderate, 3 severe, and 4 OC dissecans) at the growth plate.

[k]Total faces showing lesions at the articular cartilage and growth plate evaluating 12 cut surfaces.

[l]Sum of external abnormalities, articular faces, and growth plate faces.

[m]Sum of severity scores for external, articular cartilage, and growth plate faces.

[n]Calculated as the number of abnormalities multiplied by the severity for each location and then summed.

[o,p,q] Treatments with different superscripts differ ($P < 0.05$).

Table 7. Effect of dietary nutrients on growth performance (Exp. 2)[a]

	Dietary treatment									
Item	Control	Control+ RAC[b]	Met/Mn/ Pro/Gly	Met/Mn/ Pro/Gly + RAC[b]	Anti-oxidants[c]	Si	Arg/Gly	GAGs[d]	SED	Probability, P < Treatment
Day 0 to 70										
ADG, kg	0.99	1.10	1.01	1.02	1.02	1.03	1.02	0.97	0.053	0.36
ADFI, kg	2.25	2.47	2.22	2.23	2.25	2.20	2.24	2.26	0.114	0.29
Gain/Feed	0.44	0.45	0.46	0.46	0.45	0.47	0.45	0.43	0.014	0.14
Final weight, kg	111.3	120.4	114.1	113.6	115.0	115.4	114.5	111.6	3.44	0.25
HCW[e], kg	80.1[f]	88.6[g]	82.5[f]	81.8[f]	82.3[f]	83.8[fg]	81.9[f]	80.8[f]	2.54	0.06
Dressing %	72.0[f]	73.5[g]	72.3[fg]	72.0[f]	71.6[f]	72.6[fg]	71.6[f]	72.4[fg]	0.007	0.08

[a]Each value is the mean of 10 replications with pigs initially 47 kg and average final weight of 114.3 kg.

[b]Fed same diet as other controls until d 46 and then were fed RAC (Paylean® 20 ppm) from d 46 to 70.

[c]Antioxidant diet contained Vit. C (200 IU/kg), Vit. E (1,120 IU/kg) and 0.45% fish oil.

[d]Glucosaminoglycans derived from chicken cartilage and poultry meal (glucosamine, 420 ppm, and chondroitin sulfate, 860 ppm).

[e]Hot carcass weight (HCW).

[f,g]Treatments with different superscripts differ ($P < 0.05$).

Table 8. Effect of dietary nutrients on cartilage properties (Exp. 2)[a]

	Dietary treatment									Probability, P <
Item	Control	Control +RAC[b]	Met/Mn/ Pro/Gly	Met/Mn/ Pro/Gly +RAC[b]	Anti-oxidants[c]	Si	Arg/Gly	GAGs[d]	SED	Treatment
Instron measurements										
Compression energy n/g[e]	43.6	61.5	43.2	30.7	17.8	28.2	45.6	70.5	25.53	0.49
Shear peak energy n/g[f]	533.7	373.1	520.5	458.9	457.1	474.3	480.6	492.7	84.35	0.80
Total energy n/g[2g]	847.3	715.6	769.8	819.8	826.2	737.4	742.1	598.3	163.66	0.62
Ratio of CE/SE[h]	0.10	0.09	0.07	0.10	0.05	0.06	0.12	0.14	0.059	0.76

[a]Each value is the mean of 10 replications with pigs initially 47 kg and a final weight of 114.3 kg.

[b]Fed same diet as other controls until d 46 and then were fed ractopamine HCl (Paylean® 20 ppm) from d 46 to 70.

[c]Antioxidant diet contained Vit. C (200 IU/kg), Vit. E (1,120 IU/kg) and 0.45% fish oil.

[d]Glucosaminoglycans derived from chicken cartilage and poultry meal (glucosamine, 420 ppm, and chondroitin sulfate, 860 ppm).

[e]Amount of energy in newtons per gram of cartilage to compress the cartilage half its thickness.

[f]Amount of peak energy in newtons per gram of cartilage to shear the cartilage into two pieces.

[g]The total amount of energy required to shear the cartilage into two pieces.

[h]The ratio of compression energy to shear energy in which lower values would indicate more desirable characteristics.

Table 9. Effect of dietary nutrients on the occurrence of osteochondrosis (Exp. 2)[a]

	Dietary treatment									Probability, P <	
Item	Control	Control +RAC[b]	Met/Mn Pro/Gly	Met/Mn/ Pro/Gly RAC[b]	Anti-oxidants[c]	Si	Arg/Gly	GAGs[d]	SED	Treatment	Contrast RAC vs others
Total animals/trt[e]	10	10	10	10	10	10	10	10	-	-	-
Animals with lesions[f]	6	6	6	9	8	7	9	8	1.49	0.54	-
External											
Number abnormalities[g]	0.5	0.5	0.6	0.1	0.5	0.5	0.7	0.9	0.32	0.46	0.10
Severity score[h]	0.7	0.9	0.9	0.1	0.6	0.5	0.9	1.0	0.44	0.50	0.30
Articular Cartilage											
Number of faces[i]	2.5	2.7	2.3	3.9	3.2	2.0	3.8	3.7	1.06	0.38	0.41
Severity score[j]	0.9^{pq}	0.8^{pq}	0.6^{q}	1.4^{p}	1.2^{pq}	0.9^{pq}	1.4^{p}	0.9^{pq}	0.32	0.14	0.30
Growth plate											
Number faces[k]	0.4	0.6	0.6	1.7	0.7	0.2	1.0	0.8	0.57	0.29	0.11
Severity score[l]	0.2	0.3	0.4	0.6	0.3	0.1	0.5	0.4	0.24	0.50	0.34
Overall											
Total faces	2.9^{qr}	3.3^{qr}	2.9^{qr}	5.9^{p}	3.9^{pqr}	2.2^{r}	4.8^{pq}	4.5^{pq}	1.28	0.11	0.16
Total abnormalities[m]	3.4	3.8	3.5	6.0	4.4	2.7	5.5	5.4	1.34	0.17	0.34
Total severity[n]	1.9	2.3	2.1	2.3	2.1	1.5	3.1	2.5	0.76	0.63	0.82
Overall score[o]	6.0^{pq}	4.9^{pq}	4.2^{q}	9.4^{p}	6.7^{pq}	3.1^{q}	9.6^{p}	6.0^{pq}	2.44	0.10	0.40

[a]Each value is the mean of 10 replications with one pig per pen initially 47 kg and a final weight of 114.3 kg.

[b]Fed same diet as other controls until d 46 and then were fed ractopamine HCl (Paylean® 20 ppm) from d 46 to 70.

[c]Antioxidant diet contained Vit. C (200 IU/kg), Vit. E (1,120 IU/kg) and 0.45% fish oil.

[d]Glucosaminoglycans derived from chicken cartilage and poultry meal (glucosamine, 420 ppm, and chondroitin sulfate, 860 ppm).

[e]Total animals evaluated per treatment.

[f]Number of animals with OC lesions (Cochran-Mantzel-Haenszel test).

[g]Number of abnormalities noted upon visual evaluation of the intact external joint.

[h]Lesion severity score (0 - 4 with 0 being perfect, 1 mild, 2 moderate, 3 severe, and 4 OC dissecans) of the external surface of the intact joint.

[i]The number of faces showing lesions at the articular cartilage evaluating 12 cut surfaces.

[j]Lesion severity score (0 - 4 with 0 being normal, 1 mild, 2 moderate, 3 severe, and 4 OC dissecans) at the articular cartilage.

[k]The number of faces showing lesions in the growth plate evaluating 12 cut surfaces.

[l]Lesion severity score (0 - 4 with 0 being normal, 1 mild, 2 moderate, 3 severe, and 4 OC dissecans) at the growth plate.

[m]Sum of external abnormalities, articular faces, and growth plate faces.

[n]Sum of severity scores for external surface, articular, and growth plate.

[o]Calculated as the number of abnormalities multiplied by the severity score at each location and then summed over the three locations.

[p,q,r]Treatments with different superscripts differ ($P < 0.05$).

CHAPTER III

THE EFFECT OF DIETARY LYSINE OR METHIONINE AND COPPER/MANGANESE ON OSTEOCHONDROSIS LESIONS AND CARTILAGE PROPERTIES IN PIGS

ABSTRACT

Objective-Evaluate the influence of dietary lysine concentration and added methionine, copper, and manganese on osteochondrosis occurrence in swine.

Animals-120 gilts (PIC 327 × 1050; 40.5 kg initial BW) were used.

Procedures-Gilts were fed below (0.71% phase I and 0.53% phase II), at (0.89% phase I and 0.71% phase II), or above (1.16% phase I and 0.98% phase II) their requirement for true ileal digestible (TID) lysine (Lys) with standard concentrations or high added methionine(1%)/ copper(250 ppm)/manganese(220 ppm) in a 3 × 2 factorial, 84-d study. The effects on growth performance, visual soundness, carcass traits, the occurrence and severity of osteochondrosis (OC) lesions, and cartilage properties were measured. Upon completion of the feeding period, 60 gilts were harvested and the distal aspect of the left humerus and femur were evaluated by gross examination for OC lesions.

Results- Increasing dietary Lys increased ($P < 0.01$) ADG, but feeding high Met/Cu/Mn decreased ADG ($P < 0.02$). Overall severity score did not correlate with ADG (R^2 0.03) or weight (R^2 0.015). Increasing dietary Lys concentration ($P > 0.64$) did not effect the overall severity score (abnormalities × severity); however, the addition of high Met/Cu/Mn tended ($P < 0.09$) to reduce the overall severity score of OC compared to pigs fed diets with normal Met/Cu/Mn.

Conclusions and Clinical Relevance-Feeding growing gilts to maximize growth performance with high dietary Lys may increase the severity of OC lesions, while a diet with additional Met/Cu/Mn above requirements may aid in the reduction of OC abnormalities and severity.

(Key Words: Cartilage biology, Nutrition-swine, Osteochondrosis)

Introduction

Osteochondrosis (OC) is the focalized disruption in the endochondral ossification of cartilage at the end of growing long bones leaving areas of retained cartilage in the subchondral bone or defects in the cartilage surface[1,2,3]. It occurs in 85-90% of all swine[4], decreases meat yield in finishing pigs[5], and is associated with reduce longevity of sows[6]. The changes that occur in the joint from OC include decreased type II collagen concentrations, decreased proteoglycans, increased chondrocyte necrosis, and increased type I collagen[7,8] due to the loss of balance between synthesis and degradative processes. Osteochondrosis is thought to be a result of degeneration of cartilage canal vessels[9,10,11] that supply blood to the end of growing long bones; however, it is not know whether this is the cause or a result of OC[12]. Previously, it has been thought that OC is associated with pigs or other animals with fast growth rates[13,14] which may be due to the increased mechanical stress relative to the maturity of the joint; however several studies have shown no correlation of growth rate with OC in swine[15,16,17]. Dietary intervention to prevent or aid in the repair of joint disorders such as OC in swine through supplementation with vitamins and minerals have not shown reductions in OC occurrence or severity[18,19,20,21,22]. Dietary ingredients such as copper, manganese, and methionine, however, have shown positive effects on cartilage or proteoglycan synthesis in vitro[23,24,25,26,27] as well as reduced OC and osteoarthritis occurrence in several animal studies[28,29,30]. These nutrients may provide a means to reduce the occurrence of OC or aid in the repair of lesions. Previous research from our lab[31] observed positive effects of these dietary ingredients on OC severity in pigs, but to varying degrees among studies. Differences in the starting weights, length of the trial period, and dietary lysine concentrations may have affected our ability to influence OC or lowered OC occurrence. We hypothesized that the addition of methionine, copper, and manganese could reduce the

severity of OC lesions, and that feeding different levels of dietary lysine may affect the occurrence of OC.

The objective of this experiment was to determine the effect of dietary lysine level and the combination of additional methionine, copper sulfate, and manganese sulfate on OC lesions, growth performance, carcass composition, and several cartilage criteria in growing-finishing pigs.

Materials and Methods

General

Procedures used in these experiments were approved by the Kansas State University Animal Care and Use Committee. The experiment was conducted at the Kansas State University Swine Research and Teaching Center. A total of 120 gilts (PIC line 327 × 1050; 40.5 kg initial BW) were blocked by weight in an 84-d growth assay. Each pen contained two pigs per pen and there were ten replicates (pens) per treatment. The barn contains 80 pens with totally slatted concrete flooring (1.52 m^2) and provided approximately 1.15 m^2/pig. Each pen was equipped with a one-hole dry self-feeder[a] and nipple waterer to allow ad libitum access to feed and water.

Treatments

Experimental diets were fed in meal form for 84 d in two 42 d phases. Dietary treatments were arranged in a 3 × 2 factorial consisting of three true ileal digestible (TID) lysine (Lys) levels and two levels of supplemental methionine, copper, and manganese (Met/Cu/Mn). The TID Lys levels were formulated below the requirement (0.71% phase I, 0.53% phase II), at the requirement,(0.89% phase I, 0.71% phase II), or above their requirement (1.16% phase I, 0.98% phase II). The requirement was estimated from earlier titrations conducted within these facilities. The Met/Cu/Mn treatments were either at standard inclusion typical of swine diets (no

added methionine, 9 ppm of Cu and 20 ppm of Mn) or high added methionine (1% added DL-methionine), 250 ppm Cu, and 220 ppm Mn). Copper sulfate and manganese sulfate were the Cu and Mn sources, respectively. Added Met/Cu/Mn replaced sand in each of the Lys diets to form the other dietary treatments. The values used in diet formulation and TID digestibilities were based on those published in the National Research Council (NRC)[31]. Diet samples were analyzed for amino acid content and contained levels similar to calculated concentrations.

Growth performance and carcass composition

Pigs and feeders were weighed every 14 d to determine average daily gain (ADG), average daily feed intake (ADFI), and gain/feed (G/F). At the end of the trial, pigs were weighed and the heaviest pig from each pen was marked with a distinctive tattoo before transport to the Kansas State University Meats Laboratory, where the left humerus (elbow joint) and left femur (knee joint) were collected for determination of OC lesions for one pig in each pen. Pigs were loaded onto a trailer in small groups (15 pigs) and transported approximately 4 km to the processing facility. For carcass data, 10^{th} rib backfat depth, longissimus muscle area (LMA), percentage lean, and hot carcass weight were evaluated. Fat depth was measured with a ruler at the 10^{th} rib, 6 cm off of the midline, while LMA was traced on translucent paper and calculated using a grid. Percentage lean was calculated using an equation from the National Pork Producers Council[32] (NPPC).

Visual soundness scores

Prior to harvest, the heaviest pig from each pen was scored by two evaluators for the front leg and rear leg (1-5 where 1 = poor and 5 = excellent) based on angle and conformation, and for locomotion (1-5 where 1 = poor and 5 = excellent) as an indication of mobility. The front and rear legs scores were added together to form the total score according to the National

Swine Improvement Federation[33] (NSIF) system (2-3 = poor or unsuitable for breeding purposes, 4-7 = average, and 8-10 = excellent or desirable for breeding purposes).

Collection of cartilage data and OC lesions scores

The left humerus (elbow joint) and femur (knee joint) were collected and removed to visually determine the number of surface cartilage abnormalities and the occurrence of OC lesions by gross examination of the humerus and femoral condyles for one pig from each pen. The joints were cleaned of excess tissue and then stored in 10% formalin until evaluation. After external evaluation, the distal end of the humerus and femur were cut into 3 mm thick sections perpendicular to the long axis of the bone using a bandsaw. This resulted in 12 to 14 cut surfaces for evaluation, and the number of abnormalities was adjusted for 12 evaluated faces. Each joint was evaluated for the number of external abnormalities at the femoral and humerus condyles, OC lesions at the articular and growth plate cartilage of the distal femur, and humerus articular cartilage. Lesions were given a severity score (0-4); with 0 = normal, 1 = mild, 2 = moderate, 3 = severe, and 4 = OC dissecans based on the extent of tissue involvement. This scoring system is similar to that used by Ytrehus et al.[17] and Jefferies et al.[20] but slightly modified. All pigs had OC lesions at one of the locations evaluated, so we were unable to analyze for differences in OC occurrence (number of animals with OC).

In addition, a cartilage sample was cut from the patella for cartilage property analysis. Cartilage samples were weighed, measured for thickness and length using a caliper, and then tested for the ability to absorb compression force or to resist shearing force. The process was similar to that used by Brama et al[34] (indenter system) but with force applied to the whole cartilage sample using an Instron testing machine[b] as a measure of mechanical properties. Cartilage samples were placed between two flat surfaces of the Instron to perform texture profile analysis and compressed half of the thickness to measure the ability of the cartilage to resist

compression force. A second measure was conducted in which the cartilage was cut using a Warner-Bratzler shear blade to determine the ability of the cartilage to withstand shearing force. Compression and shear values were adjusted to a per gram of cartilage weight to equalize for differences in the actual cartilage sample weight.

Relationship between growth rate, weight, visual soundness, and overall severity score

Because there were differences in growth rate among our dietary treatments, a correlation between growth rate or weight and the overall severity score was conducted. Visual evaluation of soundness or leg conformation was also evaluated for correlattion with the overall severity score. Each prediction variable was plotted by the overall severity score and a linear regression line fitted to determine how much of the variation in overall severity score could be explained by the variables (R^2 value).

Statistical analysis

Data were analyzed as a randomized complete block design using the PROC MIXED procedure of SAS[c] with pig as the experimental unit to determine the main effect of treatment. The response criteria of growth performance, carcass composition, cartilage compression and shear energy, and number of abnormalities were tested. Although scored categorically, soundness and OC severity scores were analyzed via PROC MIXED because low number of observations at some of the severity scores prevented categorical analysis. Linear and quadratic effects of increasing dietary Lys were determined using single degree of freedom contrasts[35].

Results

Growth performance. Overall, d 0 to 84, a Lys × Met/Cu/Mn interaction was observed for ADG ($P < 0.02$; Table 2), thus the interactive means are presented. In pigs fed standard concentrations of Met/Cu/Mn, increasing dietary Lys concentration increased (quadratic, $P < 0.01$) ADG from below to the pigs estimated requirement with no improvements thereafter.

Added high Met/Cu/Mn reduced ADG ($P < 0.01$) compared to pigs fed diets with standard Met/Cu/Mn, particularly in the diets with Lys fed below or at the requirement. Increasing dietary Lys tended (linear, $P < 0.09$) to increase ADFI, while added high Met/Cu/Mn reduced ADFI ($P < 0.01$) compared to pigs fed diets with standard Met/Cu/Mn. Increasing dietary Lys up to the requirement improved F/G (quadratic, $P < 0.01$), and feeding diets containing added high Met/Cu/Mn had no effect on F/G ($P > 0.57$).

Carcass data. No interaction between Lys × Met/Cu/Mn was detected for carcass traits ($P > 0.49$; Table 3). The addition of high Met/Cu/Mn tended ($P < 0.07$) to reduce backfat thickness compared to pigs fed diets with standard Met/Cu/Mn. Pigs fed increasing dietary Lys from below to the requirement increased LMA (quadratic, $P < 0.04$) with no improvements thereafter, but the addition of high Met/Cu/Mn did not affect LMA ($P > 0.61$). Increasing dietary Lys improved (linear, $P < 0.01$) percentage lean.

Leg scoring. No Lys × Met/Cu/Mn interactions were observed for leg scores ($P > 0.21$; Table 4). Visual soundness scores were unaffected by dietary Lys ($P > 0.26$); however, the addition of high Met/Cu/Mn to the diet tended ($P < 0.07$) to negatively reduce front leg scores and locomotion ($P < 0.06$) compared to pigs fed standard Met/Cu/Mn.

Cartilage properties. No Lys × Met/Cu/Mn interactions for instron measurements were detected ($P > 0.17$; Table 5). In pigs fed standard Met/Cu/Mn, increasing dietary Lys decreased cartilage shear energy (quadratic, $P < 0.01$); however, no other instron measurements were affected by Lys ($P > 0.24$). The addition of high Met/Cu/Mn had no effect on any cartilage instron measurements ($P > 0.23$).

Osteochondrosis evaluation. No Lys × Met/Cu/Mn interactions were observed for OC measures ($P > 0.12$; Table 6). All animals had OC lesions at either the humerus or femur. Thus,

we could not test for differences in the number of animals with OC between treatments. At the external surface of the femur, increasing dietary Lys concentration tended (linear, $P < 0.08$) to increase the number of abnormalities and there was a numerical trend ($P < 0.13$) to increase the external severity score. The addition of high Met/Cu/Mn to the diet reduced the number of abnormalities ($P < 0.02$) and severity score ($P < 0.01$) at the external surface of the femur compared to pigs fed diets with standard Met/Cu/Mn. At the external humerus, increasing dietary Lys increased both the number of abnormalities (linear, $P < 0.01$) and severity score (linear, $P < 0.01$). The addition of high Met/Cu/Mn to the diet reduced ($P < 0.03$) the number of abnormalities and severity score ($P < 0.03$) for the external humerus compared to pigs fed standard Met/Cu/Mn.

At the femoral articular cartilage, neither increasing dietary Lys nor the addition of high Met/Cu/Mn affected either the number of faces with lesions ($P > 0.35$) or the severity score ($P > 0.36$). The number of faces with lesions and the severity score at the femoral growth plate was unaffected by increasing dietary Lys concentration ($P > 0.52$) or the addition of high Met/Cu/Mn to the diet ($P > 0.16$).

The number of faces with lesions and severity score at the humerus articular cartilage was unaffected by increasing dietary Lys concentration ($P > 0.16$) or the addition of high Met/Cu/Mn to the diet ($P > 0.37$).

Overall, the total faces with lesions were not affected by increasing dietary Lys concentration ($P > 0.78$) or the addition of high Met/Cu/Mn ($P > 0.86$). There was a numerical trend (linear, $P < 0.12$) for the total abnormalities (external abnormalities and the number of faces with lesions) to increase with increasing dietary Lys. The addition of high Met/Cu/Mn did not affect the total number of abnormalities ($P > 0.16$). The total severity score (sum of

severities at the humerus and femur) increased with increasing dietary Lys concentration (linear, $P < 0.01$). The addition of high Met/Cu/Mn decreased the total severity score ($P < 0.02$) compared to pigs fed diets with standard Met/Cu/Mn. Finally, increasing dietary Lys concentration did not effect the overall severity score (abnormalities × severity, $P > 0.64$), but the addition of high Met/Cu/Mn tended ($P < 0.09$) to reduce the overall severity score compared to pigs fed diets with standard Met/Cu/Mn.

Correlation of growth rate and visual evaluation with overall severity score. The correlations were low between overall severity score and ADG (R^2= 0.0316; Figure 1), weight (R^2=0.0262; Figure 2), total leg score (R^2= 0.0153; Figure 3), or locomotion score (R^2= 0.0197; Figure 4).

Discussion

Osteochondrosis is a multi-factorial disease that has increased in occurrence with the selection of lean, high growth genetics in swine[36]. One of the main concerns with this increase in OC is to the welfare and longevity of sows[6,37]. The resulting lameness from OC can decrease mobility, reproductive performance, and increase sow herd turnover rates[38]. This increase in sow turnover rate represents a substantial economic loss[39] to replace these animals as the average parity per sow has dropped to less than three[6], resulting in failure of sows to reach their most productive parities.

Much of the research for joint disorders in humans and other animal models have focused on dietary intervention involving glucosamine, chondroitin sulfate, and polyunsaturated fatty acids which have been shown to decrease production of inflammatory mediators that in turn regulate cartilage degradation[40,41,42,43]. However, the results from these studies are based on reductions in pain or indirect measures of osteoarthritis. Limited data exists on the ability of these anti-inflammatory ingredients to alter synthesis of matrix components or influence OC[44].

Previous research from our lab[45] studied the effects of different dietary nutrients and their effects on OC occurrence and severity. In these previous experiments, feeding added dietary n-3 fatty acids or glucosamine and chondroitin sulfate did not affect OC in swine, and may reflect a difference between OC and osteoarthritis pathology. However, several minerals and amino acids were identified that indicated potential in aiding the repair process or preventing OC lesions from manifesting into more severe cases. The differences in dietary Lys concentrations between the previous two experiments may account for the some of the difference in severities and magnitude of response observed. Thus, two minerals and an amino acid that showed positive effects on OC as well as dietary Lys concentrations on OC were tested.

Lysine is the first limiting amino acid in swine diets and is used as the basis in diet formulation[31]. Increasing dietary Lys from below requirements to the animal's requirement will improve growth performance, efficiency, and protein deposition[46]. As expected, increasing dietary Lys from below to the pigs requirement increased ADG, G/F, LMA, percentage lean, and decreased backfat depth with minimal improvements in those pigs fed above their requirement; however, feeding high Met/Cu/Mn reduced growth rate and backfat with no affect on LMA or percentage lean.

Growth rate, which is dramatically affected by dietary Lys concentration, has been shown to increase OC occurrence in swine[13,14]; however, mixed results have been reported. Limiting energy consumption and thus growth rate in dogs also has been shown to reduce the occurrence of hip dysplasia and osteoarthritis[47,48]. In the current experiment, no correlation between overall severity score and ADG, weight, or visual soundness scores were observed. Nankano et al.[15] and Woodard et al.[16] showed no correlation of growth rate with OC in swine. Similarly, Ytrehus et al.[12] found no correlation between OC and weight, growth rate, or femoral shape of pigs. In

addition, feeding ractopamine HCl, a beta-agonist which increases growth rate and protein deposition by 20%[49], did not increase overall severity of OC[45].

An increase in the number of abnormalities and external severity scores along with higher total severity scores were observed with increasing dietary Lys, but Lys did not affect overall severity score or visual soundness evaluation. Previous experiments demonstrated that pigs fed dietary Lys above their requirement had a greater severity of OC than pigs fed closer to their requirement[45] although similar growth performance was observed. Because of the linearity of the response to increasing dietary Lys concentrations on OC severity, it appears that providing excess dietary Lys and thus other amino acids relative to Lys increases external and total OC severity scores. We do not have a explanation as to why overfeeding dietary Lys has negative implications on OC; however, because we supplied additional Lys through increased soybean meal in the diet, crude protein (CP) concentrations were allowed to vary. Supplying a combination of non-essential amino acids arginine and glycine negatively impacted OC severity scores[45]. On the other hand, Woodard et al.[16] and Jorgenson et al.[19] evaluated the effect of dietary CP and energy density on OC and found no effect. It does not seem logical that dietary Lys by itself can influence OC severity to this degree, but rather may be due to changes in arginine or non-essential amino acids concentrations when dietary Lys is increased. Arginine is metabolized to nitric oxide, one of the key mediators of the inflammatory response and can stimulate the breakdown collagen and signal chondrocyte apoptosis[50]. Providing dietary lysine and other amino acids in excess of requirements may negatively impact OC severity

The addition of Met/Cu/Mn at high levels in this study resulted in a reduction in the number of external abnormalities and severity scores as well as lower total and overall severity of OC. Similarly, in previous experiments, a combination of Met and threonine or a combination

of Cu and Mn reduced OC severity scores[45]. A positive response to dietary intervention with a combination of added Met, proline, glycine, and Mn or by feeding silicon also reduced the overall severity score. This effect may be a result of the positive influence Met has on cartilage metabolism[25,26], Cu in crosslinking of collagen molecules or vascular stability, and Mn on proteoglycans within the extracellular matrix. Also, inadequate sulfur in the extracellular matrix leads to the formation of under-sulfated proteoglycans[51,52,53]. Copper has been shown to stimulate cartilage synthesis in vitro[23], reduce OC severity in young horses[27,28], and decrease the incidence of OC in offspring from copper supplemented sows[30]; however, deficiencies in Cu do not inhibit the function of the lysyl oxidase enzyme involved in crosslinking[54]. Manganese is important for proteoglycan synthesis[55], bone formation, and may also serve a structural role in linking chondroitin sulfate molecules[24]. These dietary ingredients may stimulate collagen or proteoglycan synthesis. The loss of proteoglycan content and decreased collagen type II content are the primary changes seen in cartilage of swine with OC[8]. Thus, the positive effects these ingredients have shown on in vitro collagen synthesis and proteoglycan synthesis may also be taking place in this animal model; however, further research into the affect of these nutrients on cartilage concentrations or gene expression will be needed to verify this response.

The addition of high Met/Cu/Mn tended to have adverse affects on visual front leg and locomotion scores compared to pigs not fed added Met/Cu/Mn. This may have been mainly due to the high level of Mn fed in these diets as excessive Mn is known to result in limb stiffness and reductions in mobility in swine[56]. Also, the limited differences observed from the analysis of cartilage properties was due to high variability between samples and suggests that the sample taken may not accurately reflect events occurring within the joint.

In conclusion, increasing dietary Lys concentration increased external abnormalities and total severity scores and appears to be independent of growth rate, while the addition of high Met/Cu/Mn above requirements reduced abnormalities and severity scores. This study suggests that the addition of high Met/Cu/Mn to the diets of growing gilts may reduce the severity of OC in articular joints. The increase in external abnormalities seen at both the femur and humerus with increasing dietary Lys concentration may be due to a greater supply of arginine or other non-essential amino acids when fed at or above their requirement for Lys. Furthermore, the reduction in external abnormalities that were seen with the addition of high Met/Cu/Mn is similar to results from our previous experiments and may be due to the positive influence of Met on cartilage metabolism and Cu/Mn in stabilizing the extracellular matrix. This combination of ingredients may allow articular cartilage a greater ability to repair or prevent OC lesions and provide a potential treatment strategy to reduce OC in swine and other animal species.

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[a] Farmweld, Tuetopolis, IL.
[b] Instron model 4201.
[c] SAS, version 8.0, SAS Institute, Cary, NC.

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Table 1. Diet composition (as-fed)

	Phase I[a]			Phase II[b]		
Item	Below	Requirement	Above	Below	Requirement	Above
Ingredient						
Corn	76.20	69.05	58.25	83.90	76.75	65.95
Soybean meal (46.5% CP)	16.05	23.25	34.15	8.75	16.00	26.85
Soy oil	3.00	3.00	3.00	3.00	3.00	3.00
Monocalcium phosphate (21 % P)	1.85	1.80	1.75	1.50	1.45	1.40
Limestone	1.03	0.98	0.90	1.03	0.98	0.90
Salt	0.35	0.35	0.35	0.35	0.35	0.35
Vitamin premix[c]	0.15	0.15	0.15	0.13	0.13	0.13
Trace mineral premix[d]	0.08	0.08	0.08	0.08	0.08	0.08
L-lysine HCl	0.15	0.15	0.15	0.15	0.15	0.15
DL-methionine	-	0.02	0.09	-	-	0.04
L-threonine	0.02	0.04	0.07	-	0.03	0.05
Manganese sulfate	-	-	-	-	-	-
Copper sulfate	-	-	-	-	-	-
Sand[e]	1.15	1.15	1.15	1.15	1.15	1.15
Total	100.00	100.00	100.00	100.00	100.00	100.00

Calculated analysis
(Table 1 continued)

Total lysine, %	0.80	1.00	1.30	0.60	0.80	1.10
True ileal digestible amino acids						
Lysine, %	0.71	0.89	1.16	0.53	0.71	0.98
Isoleucine:lysine ratio, %	70	70	69	71	70	69
Leucine:lysine ratio, %	168	153	140	192	168	149
Methionine:lysine ratio, %	30	30	32	34	30	30
Met & Cys:lysine ratio, %	63	60	60	71	63	60
Threonine:lysine ratio, %	67	67	67	68	68	67
Tryptophan:lysine ratio, %	19	19	20	18	19	19
Valine:lysine ratio, %	68	66	64	88	83	79
ME, kcal/kg	3,408	3,408	3,408	3,424	3,424	3,424
CP, %	13.9	16.7	20.8	11.2	14.0	18.1
Ca, %	0.81	0.80	0.80	0.71	0.71	0.70
P, %	0.71	0.73	0.77	0.61	0.63	0.66
Available P equiv.	0.52	0.52	0.52	0.43	0.43	0.43
Lysine:calorie ratio, g/mcal	2.3	2.9	3.8	1.7	2.3	3.2

[a]Phase I fed in meal form from d 0 to 42.
[b]Phase II fed in meal form from d 42 to 84.
[c]Vitamin premix contributed per kilogram of complete diet in phase I: vitamin A, 6,613 IU; D_3, 992 IU; vitamin E, 26 IU; vitamin K, 2.7 mg; vitamin B_{12}, 0.03 mg; riboflavin, 6 mg; pantothenic acid, 20 mg; niacin, 33 mg, and in the phase II diet: vitamin A, 5,512 IU; D_3, 827 IU; vitamin E, 22 IU; vitamin K, 2.2 mg; vitamin B_{12}, 0.02 mg; riboflavin, 5 mg; pantothenic acid, 16 mg; niacin, 27 mg.
[d]Trace mineral premix provided per kilogram of complete diet in phase I and II: copper (from copper sulfate), 9 mg; iodine (from calcium iodate), 0.15 mg; iron (from ferrous sulfate), 83 mg; manganese (from manganese oxide), 20 mg; selenium (sodium selenite), 0.15 mg; zinc (from zinc oxide), 83 mg.
[e]DL-methionine (1%), manganese sulfate (0.05%), and copper sulfate (0.1%) replaced sand to form the other dietary treatments.

Table 2. Effect of dietary lysine and methionine/copper/manganese on growth performance[ab]

Item	TID Lysine[c]			Added Met/Cu/Mn[d] TID Lysine[c]			SED	Probability, P < Lys × Met/Cu/Mn	Lysine		Met/Cu/Mn
	Below	Require-ment	Above	Below	Require-ment	Above			Linear	Quadratic	
Day 0 to 84											
Initial weight, kg	40.4	40.5	40.6	40.3	40.4	40.5	0.25	0.99	0.18	0.76	0.55
ADG, kg	0.89[f]	1.01[g]	0.99[g]	0.76[e]	0.93[f]	0.98[g]	0.027	0.02	0.01	0.01	0.01
ADFI, kg	2.57[g]	2.56[g]	2.56[g]	2.29[e]	2.35[ef]	2.50[fg]	0.077	0.12	0.09	0.64	0.01
Gain/Feed	0.35[e]	0.39[f]	0.39[f]	0.34[e]	0.39[f]	0.40[f]	0.009	0.68	0.01	0.01	0.57
Final weight, kg	119.3[f]	129.6[g]	128.4[g]	107.7[e]	122.4[f]	128.7[g]	2.53	0.06	0.01	0.01	0.01

[a]Each value is the mean of 10 replications with two pigs per pen initially 40.5 kg and an average final weight of 120.5 kg.

[b]Pigs were fed meal diets in two 42 d phases.

[c]Diets contained 0.71, 0.89, and 1.16 % TID lysine during phase I and 0.53, 0.71, and 0.98 TID lysine during phase II, respectively.

[d]Diet contained added methionine (1%), copper (250 ppm), and manganese (220 ppm).

[e,f,g]Means with different superscripts differ (P < 0.05).

Table 3. Main effect of lysine level and additional methionine/copper/manganese on carcass characteristics[a]

	TID Lysine[b]			Met/Cu/Mn[c]			Probability, P<			
								Lysine		
Item	Below	Require-ment	Above	Standard	Added	SED	Lys × Met/Cu/Mn	Linear	Quadratic	Met/ Cu/Mn
HCW, kg[d]	80.9	90.5	91.3	89.7	85.5	1.84	0.01	0.01	0.01	0.01
Backfat, mm[d]	15.6	14.6	13.6	15.5	13.7	1.35	0.80	0.16	0.99	0.07
Longissimus area, cm^{2}[d]	48.3	61.9	65.3	57.9	59.1	3.19	0.49	0.01	0.04	0.61
Lean, %[d]	55.8	59.4	60.6	58.0	59.2	1.10	0.92	0.01	0.14	0.14

[a]Each mean represents 20 replications for lysine treatments and 30 replications for Met/Cu/Mn with the heaviest pig from each pen initially 40.5 kg and an average final wt of 127 kg..

[b]Diets contained 0.71, 0.89, and 1.16 % TID lysine during phase I and 0.53, 0.71, and 0.98 % TID lysine during phase II.

[c]Diet contained 1% added methionine, copper (250 ppm), and manganese (220 ppm).

[d]Hot carcass weight (HCW) used as a covariate in analysis.

Table 4. Main effect of lysine level and additional methionine/copper/manganese on visual soundness scores[ab]

	TID Lysine[c]			Met/Cu/Mn[d]			Probability, P <			
								Lysine		
Item	Below	Require-ment	Above	Standard	Added	SED	Lys x Met/Cu/Mn	Linear	Quadratic	Met/ Cu/Mn
Front legs	2.7	2.4	2.5	2.7	2.4	0.13	0.37	0.45	0.36	0.07
Rear legs	2.8	2.4	2.6	2.6	2.6	0.12	0.28	0.39	0.18	0.69
Total[e]	5.4	4.8	5.1	5.3	4.9	0.21	0.21	0.34	0.18	0.17
Locomotion[f]	2.9	2.6	2.8	3.0	2.6	0.14	0.34	0.68	0.28	0.06

[a]Each mean represents 20 replications for lysine treatments and 30 replications for Met/Cu/Mn with the heaviest pig from each pen initially 40.5 kg and an average final wt of 127 kg.

[b]Front, Rear, and Locomotion scores are the mean of two evaluators for each animal given a score of 1-5, where 1=poor and 5=excellent according to NSIF system.

[c]Diets contained 0.71, 0.89, and 1.16 % TID lysine during phase I and 0.53, 0.71, and 0.98 TID lysine during phase II, respectively.

[d]Diet contained 1% added methionine, copper (250 ppm), and manganese (220 ppm).

[e]Sum of front and rear scores according to NSIF system (7-10, excellent, 4-6, average, 2-3, poor)

[f]An independent measure of mobility scored from 1-5 (1=poor and 5=excellent) according to NSIF system.

Table 5. Main effect of lysine level and additional methionine/copper/manganese on cartilage properties[a]

	TID Lysine[b]			Met/Cu/Mn[c]			Probability, P <			
								Lysine		
Item	Below	Require-ment	Above	Stand-ard	Added	SED	Lys x Met/Cu/Mn	Linear	Quadratic	Met/ Cu/Mn
Instron measurements[d]										
Compression energy, newtons/g[e]	13.2	9.5	19.0	12.7	15.1	6.74	0.35	0.40	0.27	0.67
Shear energy, newtons/g[f]	558.1	432.2	591.1	538.1	516.2	59.06	0.34	0.58	0.01	0.66
Total energy, newtons/g[2g]	572.9	611.4	716.2	696.2	570.6	297.11	0.61	0.30	0.75	0.23
Ratio CE/SE[h]	0.025	0.025	0.033	0.024	0.031	0.012	0.51	0.49	0.72	0.52

[a]Each mean represents 20 replications for Lys treatments and 30 for Met/Cu/Mn with the heaviest pig from each pen initially 40.5 kg and an average final wt of 127 kg.

[b]Diets contained 0.71, 0.89, and 1.16 % TID lysine during phase I and 0.53, 0.71, and 0.98 TID lysine during phase II.

[c]Diet contained added DL-methionine (1%), copper (250 ppm), and manganese (220 ppm).

[d]Instron measurements were conducted on model 4201 Instron.

[e]Amount of force required in newtons per gram of cartilage to compress the cartilage half its thickness.

[f]Amount of peak force required to shear the cartilage into two pieces in newtons per gram of cartilage.

[g]Total amount of energy required to shear cartilage into two pieces in newtons per gram of cartilage.

[h]Ratio of compression energy to shear energy, respectively.

Table 6. Main effect of lysine level and additional methionine/copper/manganese on osteochondrosis evaluation

	TID Lysine[c]			Met/Cu/Mn[d]			Probability, P <			
								Lysine		
Item	Below	Require-ment	Above	Standard	Added	SED	Lys × Met/Cu/Mn	Linear	Quadratic	Met/ Cu/Mn
External femur										
# abnormalities	0.7	1.4	1.3	1.5	0.8	0.33	0.73	0.08	0.23	0.02
Severity score	0.8	1.4	1.3	1.5	0.8	0.32	0.24	0.18	0.26	0.01
External humerus										
# abnormalities	0.8	1.4	1.9	1.6	1.1	0.30	0.52	0.01	0.99	0.03
Severity score	0.7	1.3	1.7	1.5	1.0	0.26	0.61	0.01	0.83	0.03
Femur articular cartilage										
# of faces	4.4	4.4	3.6	4.3	3.9	0.84	0.78	0.35	0.59	0.57
Severity score	1.4	1.4	1.2	1.3	1.3	0.28	0.83	0.29	0.68	0.99
Femur growth plate										
# of faces	0.5	0.4	0.6	0.3	0.7	0.32	0.13	0.88	0.55	0.16
Severity score	0.2	0.3	0.3	0.2	0.3	0.16	0.12	0.52	0.96	0.19
Humerus articular cartilage										
# of faces	0.9	1.6	1.9	1.5	1.4	0.70	0.61	0.16	0.81	0.86
Severity score	0.5	0.6	0.8	0.7	0.5	0.28	0.77	0.23	0.92	0.32
Overall										
Total faces[e]	5.8	6.2	6.1	6.1	5.9	1.13	0.45	0.83	0.78	0.86
Total abnormalities[f]	7.3	8.9	9.3	9.2	7.8	1.23	0.65	0.12	0.56	0.16
Total severity[g]	3.6	4.8	5.2	5.2	3.9	0.59	0.32	0.01	0.44	0.01
Overall severity[h]	15.2	16.5	16.9	18.7	13.7	3.56	0.43	0.64	0.88	0.09

[a]Each mean represents 20 replications for Lys treatments and 30 replications for the Met/Cu/Mn treatment with the heaviest pig from each pen initially 40.5 kg and an average final wt of 127 kg.

[b]Joints were scored on a scale of 0-4 (0=normal,1=mild, 2=moderate, 3= severe, and 4=osteochondrosis dissecans) for each location.

[c]Diets contained 0.71, 0.89, and 1.16 % TID lysine in phase I and 0.53, 0.71, and 0.98 % TID lysine in phase II, respectively.

[d]Diet contained additional methionine (1%), copper (250 ppm), and manganese (220 ppm).

[e]Total faces showing lesions at the humeral articular cartilage, femoral articular cartilage, and growth plate.

[f]Total number of external abnormalities and faces with lesions.

[g]Sum of severity scores for the external, articular, and growth plate for both the humerus and femur.

[g]Calculated as abnormalities multiplied by severity score for each location and then summed for all locations.

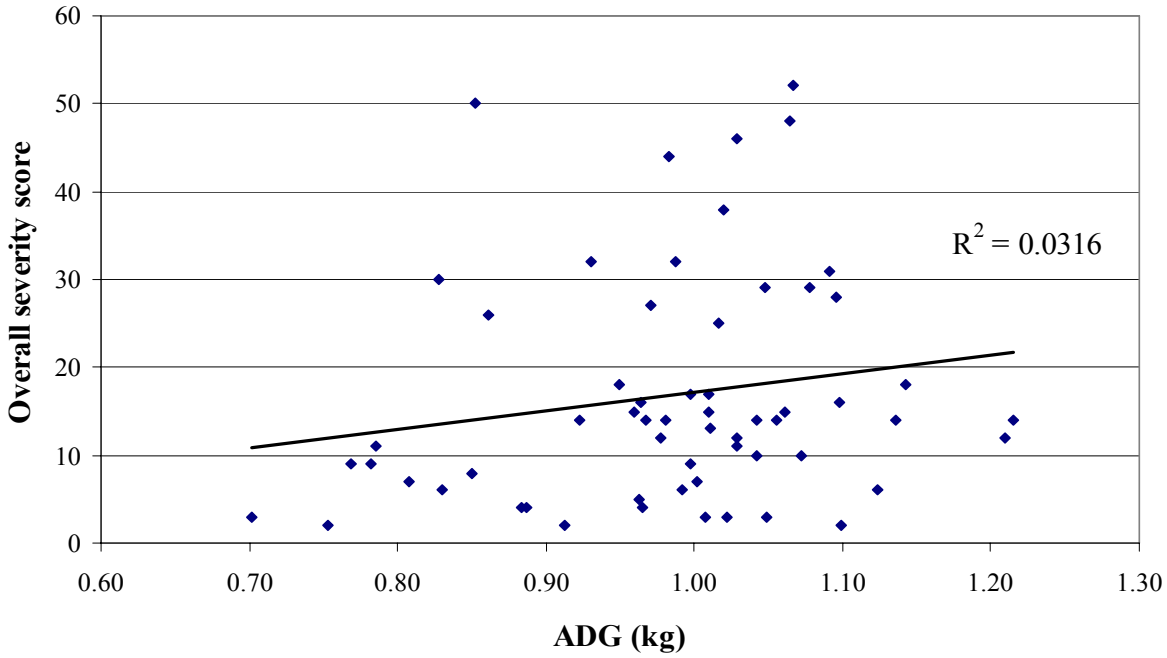

Figure 1. Average daily gain (ADG) versus overall severity score of osteochondrosis using 60 gilts. A linear line was fitted to the data to determine the amount of variation in overall severity score that could be explained by ADG.

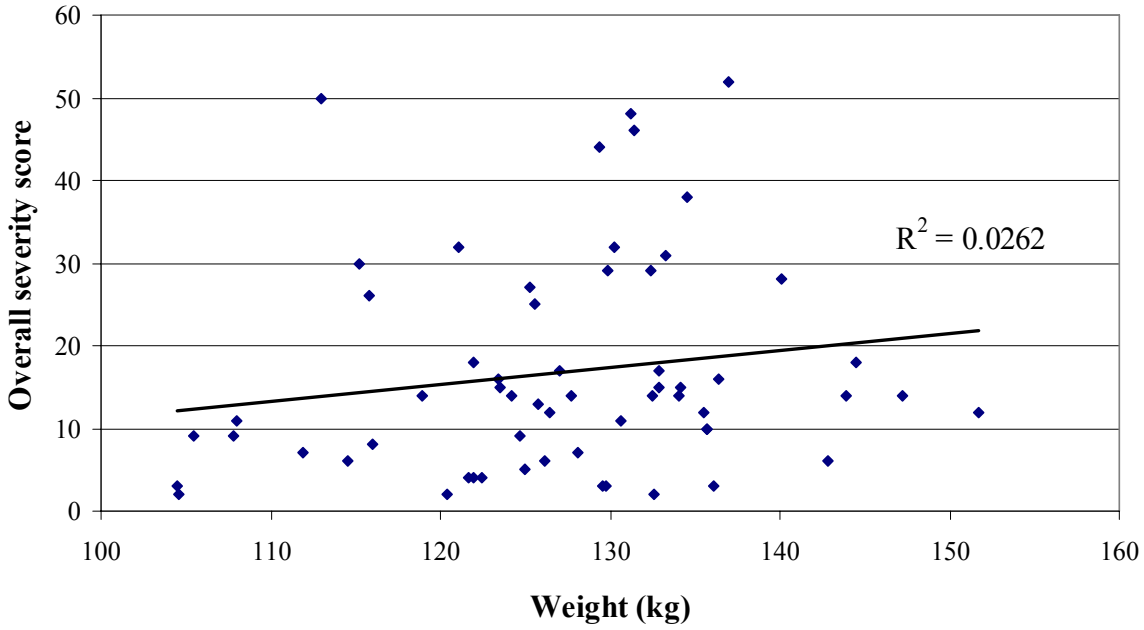

Figure 2. Weight versus overall severity score of osteochondrosis using 60 gilts. A linear line was fitted to the data to determine the amount of variation in overall severity score that could be explained by weight.

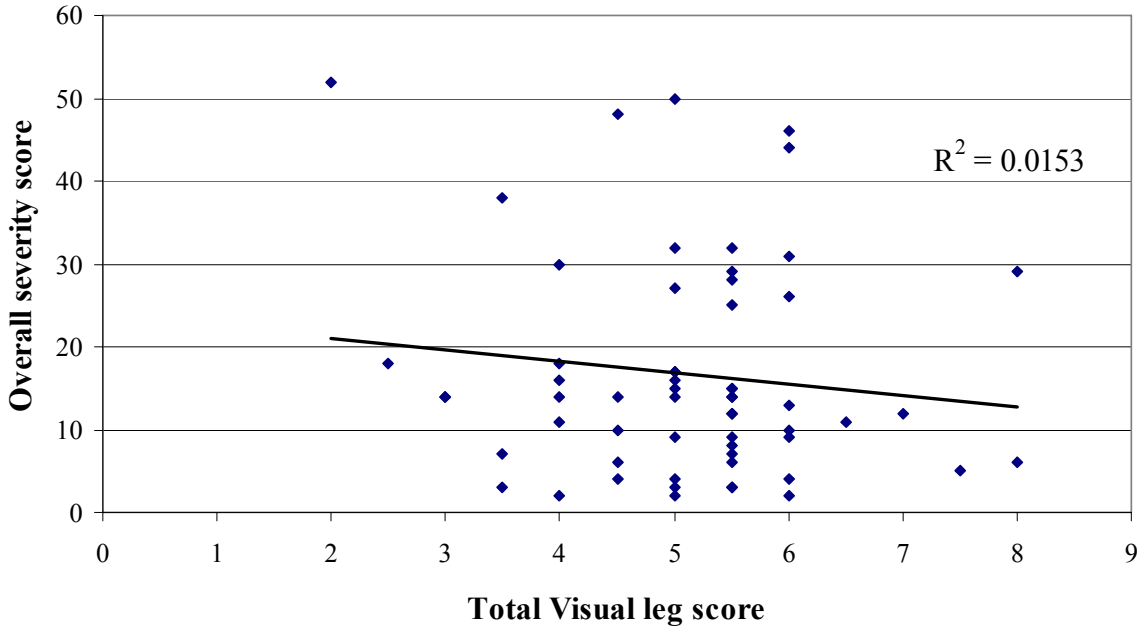

Figure 3. Total visual leg score versus overall severity score of osteochondrosis. Total leg score is the sum of the front and rear leg scores which were scored from 1-5 where 1=poor and 5=excellent and then summed to form the total score according to the NSIF system on 60 gilts.

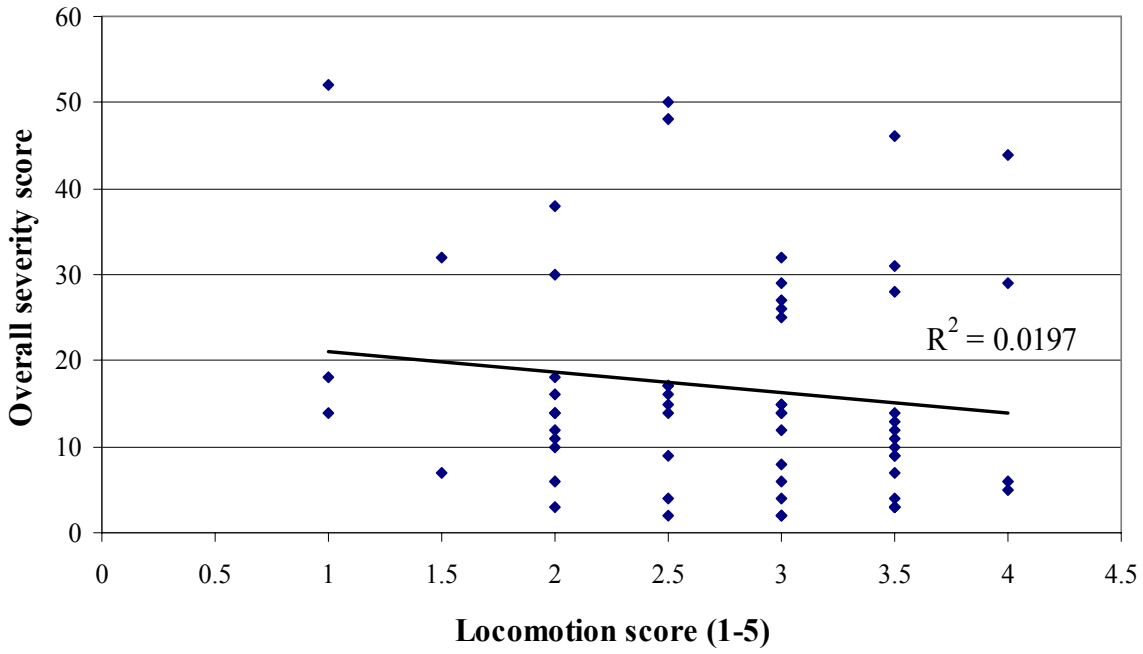

Figure 4. Locomotion score versus overall severity score of osteochondrosis. Locomotion (measure of mobility) was scored from 1-5 where 1=poor and 5=excellent according to the NSIF system and is the average of two evaluators using 60 gilts.

CHAPTER IV

THE USE OF SERUM BIOMARKERS TO PREDICT THE OCCURRENCE AND SEVERITY OF OSTEOCHONDROSIS LESIONS IN PIGS

ABSTRACT

Objective- Evaluate the use of serum biomarkers of cartilage and bone metabolism to predict the occurrence and severity of OC at the distal left femur in swine.

Animals- 71 gilts (PIC 327×1050) were used.

Procedures- Serum blood samples were collected prior to harvest by venipuncture. Samples were centrifuged and separated into one ml aliquots for each animal and sent to a commercial lab for analysis of ten different markers involved in cartilage and bone metabolism. Gilts were categorized as with or without OC. Logistic regression analysis was then performed to predict risk of OC based on biomarker concentration. After determining which animals had OC, we used linear regression to predict the severity of the animals with OC.

Results- Serum C-propeptide of type II collagen (CPII, $P< 0.01$) and cartilage oligomeric matrix protein (COMP, $P< 0.01$) were increased in gilts with OC compared to normal animals. Carboxy-terminal collagen type II ¾ long fragment (C2C, $P< 0.01$) and pyridinoline crosslinks (PYD, $P< 0.01$) were decreased in gilts with OC compared to normal gilts. A two-fold increase in CPII level increased the odds of having OC 97 times (95% CI, 6-infinity). Using serum C2C, we were able to explain 49% (R^2) of the variation in overall severity score.

Conclusions and Clinical Relevance- Serum biomarkers, CPII and COMP, are significantly increased while C2C and PYD were decreased with OC occurrence. Thus, biomarkers may be a valuable selection tool to diagnose OC and aid in the reduction of lameness in sow herds.

(Key Words: Animal models, Cartilage biology, Osteochondrosis)

Introduction

Osteochondrosis (OC) involves the failure of endochondral ossification occurring during bone formation that results in cartilage retained in the subchondral bone[1,2]. Osteochondrosis may increase lameness in sow herds[3,4] and reduce performance and meat yield in finishing pigs[5]. Heritability estimates of OC range from 0.1-0.5[6,7] and also may be affected by breed[8], growth rate[9], and trauma[10]. Because the pathology that results in OC is not well understood and attempts at nutritional intervention have had limited success[11,12,13], the most likely opportunity to reduce its prevalence is through genetic selection. Recently, Kadermideen and Janss[14] identified a major gene that may relate to OC inheritance in pigs. Thus, genetic strategies or biomarker proteins, which are the end result of gene expression, may enhance our selection capabilities against OC and could reduce the economic consequences of this disease.

Over the past ten years, much research has been conducted to determine the pathological process and biological changes that occur during osteochondrosis, osteoarthritis, and rheumatoid arthritis in humans as well as animal models[15,16]. These include increased chondrocyte necrosis, decreased collagen type II, and reduced proteoglycan concentrations[17,18,19]. As a result, many immunoassays have been developed to help in determining biochemical changes in living animals from synovial fluid, blood, or urine samples[20]. Because of the limited ability of scientists to obtain tissue samples and conduct experiments with human subjects, their research has focused on alternative methods to diagnose disease and measure treatment effectiveness. Biomarkers are products of cartilage or bone metabolism that are released into circulation from events associated with their synthesis or degradation that are altered due to loss of homeostasis during disease[15,21]. These specific proteins can be measured in samples by antibody detection assays and may reflect changes between normal and diseased animals[22]. Cartilage and bone metabolism markers have been used with some success in correlating with disease state and

determining treatment effectiveness in several different animal models of osteoarthritis[23,24] and OC[25,26,27]; however, only a few biomarkers of bone turnover have been evaluated in swine[28,29,30].

Therefore, the objective of this experiment was to determine the ability of serum biomarkers of cartilage and bone metabolism to predict the occurrence and severity of OC lesions in growing gilts and develop prediction equations that potentially can aid in gilt selection.

Materials and Methods

General

Procedures used in these experiments were approved by the Kansas State University Animal Care and Use Committee. A total of 71 gilts (PIC line 327 × L1050, 114 kg) were used for serum collection and determination of OC severity. The experiment was conducted at the Kansas State University Swine Research and Teaching Center. Gilts were housed individually and had ad libitum access to feed and water. The barn contains 80 pens with totally slatted concrete flooring (1.52 m^2), providing 2.31 m^2/pig. Each pen was equipped with a one-hole dry self-feeder[a] and nipple waterer. Pigs were loaded in small groups onto a trailer for transport 4 km to the Kansas State meat processing facility.

Osteochondrosis lesions severity scores

The distal left femur was collected and removed to visually determine the number of cartilage abnormalities and the occurrence of OC lesions at the femoral condyle by gross examination. The joints were stored in 10% formalin until evaluation. Joints were photographed to allow visual evaluation of the external surface and the underlying articular cartilage/ subchondral bone interface. After external evaluation, the distal end of the femur was sliced perpendicular to the long axis of the bone[7] into 3 mm sections using a bandsaw. Samples were evaluated for the number of abnormalities, then lesions were given a severity score (0-4), where

0 = normal, 1 = mild, 2= moderate, 3= severe, and 4=OC dissecans for the external surface, articular cartilage, and growth plate based on the extent of tissue involvement. The scoring system used is similar to those described previously[7,31]. From these scores, a prediction response of number of external abnormalities multiplied by the severity plus the number of articular cartilage abnormalities multiplied by the severity plus the number of growth plate abnormalities multiplied by the severity was used as the overall severity score for the joint.

Analysis of biomarkers

Serum samples were collected and stored at -20° C until evaluation. Samples were sent to a commercial laboratory[b] for determination of the concentration of ten biomarkers. All samples were determined in duplicate by commercially available enzyme-linked immunosorbance assays (ELISA) that had previously been validated for swine at this commercial lab. The C-propeptide of type II collagen (CPII) was measured an indication of cartilage synthesis and is released during formation of the mature molecule. Chondroitin sulfate epitope 846 (CS846) is released during the formation of mature aggrecan and was measured an indication of aggrecan synthesis. The carboxy-terminal telopeptide of type II collagen ¾ long fragment (C2C) and carboxy-terminal crosslinked telopeptide fragment of type II collagen (CTXII) are cleaved from the intact collagen molecule by matrix metalloproteinases during degradation and were measured as indicators of cartilage destruction. Cartilage oligomeric matrix protein (COMP), produced by chondrocytes and synovial cells, is considered an indirect marker of cartilage destruction. Osteocalcin (OST) and bone specific alkaline phosphatase (BAP) are markers of bone formation or osteoblast activity. The amino-terminal telopeptide of type I collagen (NTX), pyridinoline crosslinks (Pyd), and carboxy-terminal crosslinked telopeptide of type I collagen (ICTP) were measured as markers of bone turnover that are released during collagen type I degradation.

Statistical analysis

Logistic regression was used to predict whether or not an animal had OC by backward step-wise parameter selection to identify biomarkers that were significant in the model. Then in animals with OC, linear regression was used to model the severity of OC by step-wise analysis to determine the significance of biomarkers in the model.

Because the biomarker data was not normally distributed, the individual markers were log transformed for evaluation in the model. In addition, the response variable or overall score was square root transformed due to increasing variation as scores increased[32]. Normality was confirmed by adjusted residual evaluation. Data were first analyzed using the PROC LOGISTIC procedure of SAS[c] with the binary response YES or NO to predict which animals had OC. Next, PROC REG was used to predict overall severity scores using biomarkers and final weight by forward selection for animals with OC. The final model was determined as the biomarkers that significantly contributed to the model at the $P < 0.05$ level. Differences in biomarker concentrations between animals with or without OC were analyzed by ANOVA using PROC MIXED with these differences also considered significant at $P < 0.05$. Simple correlations between markers and severity of OC were analyzed using Spearman's rank correlation coefficient procedure of PROC CORR in SAS.

Results

Individual markers. Of the 71 gilts, 15 had no gross evidence of OC, while the remaining 56 gilts had varying OC severity. For the individual analysis of each marker as a function of gilts with OC versus gilts with no gross evidence of OC, serum C2C ($P< 0.02$, Table 1) and PYD ($P< 0.01$) decreased with the occurrence of OC. Serum biomarkers CPII ($P< 0.01$) and COMP ($P< 0.03$) were increased in gilts with OC compared to gilts with no gross evidence

of OC. No other biomarkers were different ($P > 0.20$) in animals with OC versus those without OC.

Modeling the occurrence of OC. Using logistic regression analysis to predict the binary response of YES or NO (whether or not an animal has any gross sign of OC), the biomarker CPII was significant in the model ($P < 0.01$, Table 2) and resulted in an odds ratio of 97 (95% CI, 6 - infinity). This indicates that a two-fold increase in the CPII marker would increase the odds of having OC by 97 times. No other biomarkers were significant in the model ($P > 0.14$).

Modeling the severity of OC. After predicting which animals had OC, biomarkers were evaluated for correlation with the overall severity score using linear regression. Only C2C and the log of C2C were significant ($P < 0.05$) in the model. Using this single marker, C2C and the log of C2C explained 49% (R-square, Table 3) of the variation in overall severity scores.

Spearman's rank correlation coefficients. Spearman's rank correlation coefficients were evaluated to determine if any associations exist between biomarker concentrations and overall severity score. This analysis assigns rankings to both the response and indicator variables and ignores the actual values. Thus, it gives an indication of increasing or decreasing associations that are present between our response and biomarker values. From this analysis, correlations ($P < 0.05$) between six markers and the overall severity score were observed. The CPII (-0.430, Table 4) and COMP (-0.270) markers showed negative correlations with increasing severity score, while BAP (0.312), CS846 (0.311), Pyd (0.426), and C2C (0.564) demonstrated positive correlations with increasing severity score.

Discussion

The use of biomarkers to diagnose joint diseases without invasive procedures has been a focus of human research for almost twenty years. Identifying subjects with greater potential for joint destruction from arthritis or OC would allow treatment intervention in humans and

companion animals, as well as selection against animals that are genetically predisposed to OC in swine. Using radiographic procedures to measure changes in progression of these diseases is difficult to evaluate over short periods of time and are only detectable in the later stages[20] of OA or OC. The potential to use biomarkers to detect and correlate with disease progression holds the most promise as genetic markers are currently unavailable. The high incidence of OC in swine is thought to correlate with increased selection pressure for lean growth rate[33]. With the challenge of maintaining both highly prolific sow lines and maximum lean growth, increased selection pressure against OC is needed. Thus, we hypothesized that serum biomarkers of cartilage and bone metabolism would correlate with OC occurrence or severity and potentially could be used as a diagnostic tool to identify animals with OC.

The serum CPII marker of collagen type II synthesis has been shown to increase with osteoarthritis[34,35,36] and is elevated in horses with OC[23,24,25]. This may be a programmed response to increased cartilage fragmentation through a potential feedback loop[37]. The increased serum concentrations of CPII in gilts with OC compared to normal gilts demonstrates an increased repair process by trying to synthesize new type II collagen molecules. In addition, serum CPII predicted which animals had OC with an odds ratio of 97. All animals with greater than 850 ng/ml of CPII had OC, and because of this the upper limit of the 95% confidence interval for the odds ratio is approaching infinity or complete separation in the data set. However, once gilts with OC had been determined, a negative correlation (-0.43) of CPII in those animals with OC as severity score increased was observed. Mannsson et al.[38] observed lower CPII levels in patients with rapid rheumatoid arthritis compared to controls. The response in animals with OC may be a result of differences in the progression of lesions. Gilts with lower severity scores and higher CPII concentrations may indicate regressing lesions; however, more

involved lesions with lower CPII concentrations may be stagnant or unable to repair through increased CPII. It appears that elevated CPII is a good indicator of the presence of OC in swine.

A positive spearman correlation of CS846 (0.311), aggrecan synthesis marker[39], with increased severity score was also found. This may indicate that in addition to increased collagen type II synthesis in gilts with OC, the synthesis of aggrecan may also be elevated; however, the success of this marker in correlating with disease state has been debated. The CS846 biomarker was increased in humans with rheumatoid arthritis[39] and osteoarthritis[40] compared to controls. As well, Lohmander et al.[41] found parallel changes in CS846 with CPII in a human osteoarthritis model. Synthesis markers of cartilage components may be valuable diagnostic indicators of the repair process and aid in identification of those animals with OC.

Elevated serum COMP concentrations, a non-collagenous protein synthesized by chondrocytes and synovial cells[20], are thought to indicate cartilage destruction occurring within joints. Several human studies have demonstrated positive correlations of COMP with severity of osteoarthritis[42,43,44] and as a predictor of future joint deteriation[45,46]. Similarly, higher concentrations of serum COMP were observed in animals with OC compared to those without. This would indicate increased cartilage destruction occurring in animals with OC. However, a negative Spearman correlation of COMP (-0.27) was observed with increasing severity score. This may be similar to the results found with the CPII marker, and thus could indicate more active or regressing lesions versus larger and stagnant lesions in animals with OC.

Direct markers of cartilage degradation also have been shown to correlate with specific disease states. In the present study, serum Pyd and C2C were significantly decreased in gilts with OC compared to those with no gross lesions; however, when used to predict the OC severity score, both Pyd (0.426) and C2C (0.564) showed strong positive spearman correlations. In

comparison, several osteoarthritis models have shown increased serum and urinary Pyd concentrations and correlations to disease progression[47,48,49]. Similarly, increased C2C concentrations have also been described with severity of osteoarthritis[23,35,50] and in equine OC[27]. In the current study, the change in the direction of correlation between diseased and normal animals may indicate a difference in the pathology between normal cartilage turnover and those animals with varying severities of OC. In gilts with OC, the decrease in both PYD and C2C may represent an altered turnover of cartilage when compared to gilts with no OC but still showed a positive correlation with increasing severity score. The physiological explanation for this observation is not known.

Measuring serum CTX-II, a newer marker of cartilage destruction, has been shown to correlate with joint deterioration in synovial fluid[51] and urine[48,52,53,54] in human and animal models, but serum concentrations were not altered or did not significantly correlate with OC severity score in this study. The reason a correlation or difference in CTX-II was not observed with the incidence of OC or severity may be due to the inherent variation between individual animals.

Markers of bone turnover or formation were not significantly altered with disease state in this study, but concentrations were similar to previously reported values in swine[28,29,30]. However, a positive spearman correlation of BAP (0.312) with increasing severity score indicates the potential for this marker given larger population samples. Fuller et al.[55] also demonstrated elevated BAP levels in an equine osteoarthritis model. Although NTX-I, OST, Pyd, and BAP have been successfully used as markers of bone turnover in swine[28,29,30] as well as ICTP in other animal models[56], it does not appear that bone remodeling is significantly altered with OC occurrence in gilts. Billinghurst et al.[26] found a positive correlation of OST with

macroscopic lesion score at five months of age; however, they described limited use of bone turnover or formation markers in predicting OC severity in older foals which may be similar to the results from the current experiment as some lesions may have had time to resolve.

In summary, biomarkers of cartilage synthesis and destruction were altered with the presence of OC compared to gilts without OC, and predictive of OC occurrence and severity. Biomarkers of bone metabolism did not correlate with OC occurrence or severity. Using logistic regression, gilts with a high risk of OC (odds ratio of 97) could be determined by measuring only serum CPII. Once gilts with OC had been determined by measuring serum CPII, linear regression was used to explain 49% (R-square) of the variation in OC severity with C2C. However, the individual marker variation appears to limit the ability of other biomarkers to predict OC severity scores with a higher accuracy. Biomarkers of cartilage metabolism, in particular CPII and C2C, may be of use in determining the occurrence of OC in replacement gilts and to potentially help in selection against animals with OC; however, further work will be required to test and refine these equations for practical applications.

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[a]Farmweld, Tuetopolis, IL.

[b]MD Biosciences, Minneapolis, Minnesota.

[c]SAS, version 8.0, SAS Institute Inc., Cary, NC.

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Table 1. Effect of disease state on serum biomarker concentrations[abcd]

	Osteochondrosis				Biomarker concentration	
	No	Yes	SED	Probability, P <	Minimum	Maximum
Cartilage synthesis						
CPII, ng/mL	648.1	1,110.5	85.06	0.01	537.5	1,723.1
CS846, ng/mL	977.4	857.8	145.4	0.42	228.5	2,960.1
Cartilage degradation						
C2C, ng/mL	163.0	123.9	10.42	0.01	60.1	202.9
CTXII, pg/mL	213.6	235.8	43.37	0.66	110.7	480.4
COMP, U/L[e]	1.05	1.39	0.101	0.01	0.51	2.16
Pyd, nmol/L	8.1	6.6	0.38	0.01	4.5	9.8
Bone turnover						
NTX, nM BCE[e]	215.3	234.9	21.61	0.37	100.0	411.0
ICTP, µg/L	39.6	38.6	1.55	0.56	24.9	48.5
Bone formation						
Osteocalcin, ng/mL	22.2	23.6	1.03	0.20	11.5	34.8
BAP, U/L[e]	75.9	71.5	5.04	0.39	21.6	123.8

[a]Blood samples were collected by venipuncture prior to slaughter using gilts (113.4 kg).
[b]Assays were validated for swine and conducted at a commercial lab.
[c]Included 15 gilts without gross OC and 56 gilts with gross OC.
[d]CPII= collagen type II C-propeptide, CS846=chondroitin sulfate epitope 846, C2C= collagen type II ¾ long fragment, CTXII= C-terminal crosslinked fragment of type II collagen, COMP= cartilage oligomeric matrix protein, Pyd= Pyridinoline crosslinks, NTX= amino-terminal telopeptide of type I collagen, ICTP= carboxy-terminal crosslinked telopeptide of type I collagen, BAP= bone specific alkaline phosphatase.
[e]BCE= Bone equivalency units, U= arbitrary units.

Table 2. Modeling of biomarkers to predict osteochondrosis occurrence in pigs[a]

Logistic regression to predict OC (Yes or No)				
Marker	Equation	Odds ratio[b]	95% CI	Probability, P <
$\log^2$ CPII	OC = -42.5748 + (4.5704 * $\log^2$ CPII)	97	6 - infinity	0.01

[a]Logistic regression was conducted using all 71 animals to determine the Yes or No presence of OC lesions.
[b]The odds ratio indicates that a two-fold increase in the collagen type II C-propeptide biomarker (CP II)would be 97 times more likely to have OC.

Table 3. Linear regression to predict severity score in gilts with osteochondrosis[a]

Marker	Equation	Model R^2
C2C[b]		0.44
log C2C[b]	OC = 18.62746 + (0.05507*C2C) + (-4.76606*logC2C)	0.49

[a]Linear regression was conducted on 56 animals with varying levels of OC severity to predict the overall severity score.

[b]Collagen type II C-terminal ¾ long fragment (C2C) and the log transformed C2C.

Table 4. Spearman rank correlation coefficients between biomarkers and osteochondrosis severity score[ab]

Biomarker	Correlation coefficient value
Collagen C-propeptide of type II (CP II)	-0.430
Chondroitin sulfate epitope 846 (CS846)	0.311
Cartilage oligomeric matrix protein (COMP)	-0.270
Bone specific alkaline phosphatase (BAP)	0.312
Collagen type II C-terminal 3/4 long fragment (C2C)	0.564
Pyridinoline crosslinks (Pyd)	0.426

[a]Values shown were significant ($P < 0.05$).
[b]Spearman's rank correlation assigns ordered numbers to measure increasing or decreasing relationships between two variables without assuming a normal distribution.