The use of biomarkers to determine the severity of osteoarthritis in the tarsus of an older horse population

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Dedication

I would like to dedicate this thesis to my family who has provided steadfast and unconditional support and encouragement through my educational journey. I would also like to dedicate this thesis to Dr. Troy Trumble, who refined my research skills throughout the development, execution, and analysis of this project.

ABSTRACT

Background: Osteoarthritis (OA) is a group of diseases of different causes that ultimately lead to synovitis, subchondral bone remodeling, and articular cartilage degeneration. OA commonly develops in the distal intertarsal (DIT) and tarsometatarsal (TMT) joints of performance horses. Currently, the most accurate method of identifying OA in these joints is a combination of thorough physical, lameness, and radiographic examinations. However, many horses may have pain attributed or localized to these joints with minimal radiographic changes present. A novel way to identify and classify the degree of OA is through the measurement of molecular biomarkers. Molecular biomarkers of OA reflect quantitative and dynamic variations associated with joint metabolism.

Objectives: (1) define the direct and indirect biomarker concentrations in synovial fluid from the tibiotarsal, distal intertarsal, and tarsometatarsal joints in horses with varying degrees of tarsal OA, and (2) validate/refute that the biomarker concentrations in these joints increase with severity of OA in the distal joints as determined by a radiographs (all joints), MRI (PIT, DIT, TMT joints), arthroscopic evaluation/ gross pathology (TT joint), and (3) determine if biomarker concentrations in the TT synovial fluid (SF) can be used to evaluated OA severity in the DIT and TMT joints.

Methods: A cohort study of 11 older horses (>8 years) with variable amounts of OA in the tarsal joints identified on radiographs were included. The TT joints were examined by arthroscopy/gross examination. The distal tarsal joints were examined by MRI. Biomarkers BAP, CPII, C2C, CTX II, CS846 were examined in the distal tarsal joints; additional biomarkers C1,2C, IL-1β, IL-6, IL-8, IL-10, and TNFα were examined

in the TT joint. Various statistical analyses were used to determine association between imaging modalities and biomarkers to degrees of OA severity.

Results: In the TT joint, C2C and IL-6 were the best biomarkers distinguishing OA severity. There was more pathology present in TT joints than could be seen on radiographs, suggesting that arthroscopic surgery is still the best method to evaluate TT joint OA. In the distal tarsal joints, radiographs were better at distinguishing OA and correlated to the corresponding MRIs, but underestimated the degree of SCB bone sclerosis, and number and size of osteophytes in many of the cases. MRI also provided information about cartilage damage and SCB hyperintensity. The severity of SCB sclerosis and presence of SCB hyperintensity on MRI was a good indicator for separating moderate/severe from mild OA. Of the biomarkers evaluated, the best at determining OA severity in the DIT joint were BAP, CPII, and C2C. These biomarkers also correlated to subchondral bone hyperintensity on MRI. In the TMT joint, CPII was the best biomarker to determine OA severity. No biomarker was identified in the SF in the TT joint capable of identifying OA in the distal tarsal joints.

Conclusions: Biomarkers have the potential to be a valuable source of information about the OA disease process in the tarsal joints. SF from the joint of interest must be collected. Further research is needed with more horses. To the author's knowledge, this is the first study examining biomarkers in an older horse population and hopes it provides a template for forthcoming research.

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Italicized number= half of the lowest standard concentration of the biomarker for concentrations that read below the lowest standard
Shaded = mild TT disease; Not shaded= moderate TT disease

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CHAPTER 1:

PRELIMINARY LITERATURE REVIEW

NORMAL EQUINE JOINT ANATOMY AND PHYSIOLOGY

A synovial joint consists of several components including: bone, ligaments, muscles, articular cartilage, joint capsule, and synovial fluid [1]. Proper function of these joint components is essential to create frictionless contact to allow pain-free mobility. Synovial joints are classified into three categories: synarthritic, amphiarthritic, and diarthritic. Synarthritic joints are non-mobile, amphiarthritic joints are slightly mobile, and diarthritic joints are mobile and the most numerous type in the body [2].

THE JOINT CAPSULE

The joint capsule is a bilayer structure surrounding the perimeter of each synovial joint. It consists of an outer fibrous layer and an inner synovial membrane. The outer layer provides support to the joint and the inner layer is secretory in nature. The inner synovial membrane is a bilayer coating arranged in a villous pattern for enhanced surface area and has direct contact with the synovial fluid. The coating consists of the intimal and subintimal layers [2].

The intimal layer of the synovial membrane is the thin inner layer that consists of one to four cell layers. It determines the make-up of synovial fluid within a joint by selective permeability, allowing the passage of small protein molecules less than 10 kDa, oxygen, carbon dioxide, and glucose [2]. The intimal layer has specialized cells termed synoviocytes. The three main types of synoviocytes are A, B, and C [2]. Type A

synoviocytes are macrophagic and regulate catabolic and anabolic processes through phagocytosis. Type B synoviocytes are fibroblastic and regulate protein secretion. They have abundant rough endoplasmic reticulum and dendritic processes that create a network within the cell membrane. Type B synoviocytes synthesize and secrete boundary lubricants, molecules that provide a coating on the synovial membrane that are dispersed into the synovial fluid and prevent joint surfaces from adhering together. Type C synoviocytes are cells that are transitioning from type A to type B [3].

The main boundary lubricant in synovial fluid is lubricin, made by type B synoviocytes. Lubricin is a 227 kDa O-linked mucinous glycoprotein that is the most potent boundary lubricant that is highly preserved across species. [4] It can reduce friction in cartilage up to 70 percent [5]. Lubricin is also produced by chondrocytes at the superficial and intermediate zone of articular cartilage (see below in articular cartilage section), as well as on the surface of the meniscus [6; 7].

Lubricin creates a brush like structure on cartilage surfaces. As cartilage is compressed through movement, lubricin molecules repel each other and create a frictionless surface [4]. Lubricin works in concert with hyaluronic acid (HA), present in the synovial fluid and articular cartilage. Type B synoviocytes also produce HA. It is a high molecular weight linear polysaccharide composed of D-glucuronic acid and N-acetylglucosamine. In the synovial fluid, HA is largely responsible for its viscoelastic and low shear rate characteristic [8]. HA allows lubricin to lubricate the joint surface at high pressures [4], and regulates the transport of substances by creating a diffusion barrier by steric hindrance. In the articular cartilage, HA links with aggrecan molecules to create osmotic pressure that resists joint compression [8].

Between the intimal layer of the synovial membrane and the fibrous joint capsule is the subintimal layer. This layer has an areolar arrangement and is composed of collagen, and adipose tissue. This layout allows the synovial villi to freely move [2]. In contrast to the intimal layer, the subintimal layer is highly vascularized and innervated. The veins and arteries within the subintimal layer anastomose with the vascular supply of the nearby periarticular bone [9].

ARTICULAR CARTILAGE

Articular cartilage is specialized connective tissue that lines weight-bearing surfaces of the subchondral bones that surround each joint. The function of articular cartilage is to provide a smooth surface with a low coefficient of friction, allowing smooth motion during loaded movement of the body [10]. Fluid movement is extremely important in maintaining a healthy joint because the motion between cartilage and synovial fluid aids in cell nutrition and molecular diffusion [11]. Articular cartilage is made up of 1) specialized cells called chondrocytes that produce the extracellular matrix and 2) extracellular matrix (ECM) containing mostly water, collagen, and proteoglycans, with minimal amounts of noncollagenous proteins, and glycoproteins. There is no vascular, neural, or lymphatic supply to the articular cartilage [10].

Chondrocytes make up 2% of the articular cartilage volume [12]. Each cell has its own microenvironment and creates individual extracellular matrices, preventing direct signal transduction [10]. Chondrocytes respond to hydrostatic pressure, growth factors, and mechanical loads placed upon them [13]. They have non-motile primary cilia that sense mechanical load [9]. In horses, the primary cilia were investigated in the weight

bearing lateral femoral condyle and the cranial non-weight bearing aspect of the condyle. The study found that the primary cilia were organized in areas of high weight bearing cartilage and disorganized in non-weight bearing cartilage, suggesting an important role in mechanoreception and subsequent chondrocyte metabolism [14]. Chondrocytes require specific microenvironments to survive and have limited ability to replicate after injury [1].

The extracellular matrix is 80% fluid and 20% dry matter. The breakdown of these components is available in Figure 1.1 Water makes up 80% of the total volume. The dry component of the extracellular matrix is predominately collagen. While several types of collagen are present within the extracellular matrix (types I, II, IV, V, VI, IX, XI), type II makes up the majority, comprising up to 95% of the collagen fibrils and fibers [10]. Collagen creates a scaffold around which the proteoglycans are arranged in the articular cartilage and gives it high tensile strength [15]. Collagen also helps keep the form of articular cartilage by resisting the pressure placed upon the matrix as water is aggregated to the negatively charge glycosaminoglycans [16]. Each collagen molecule is composed of 3 identical alpha chains that are wound into a triple helix. Several helices combine into a quarter staggered array formation to create the collagen fibrils [17].

The extracellular matrix is classified into three zones: pericellular, territorial, and interterritorial [10]. The pericellular zone is the part of the extracellular matrix that surrounds the chondrocytes and is composed primarily of proteoglycans. The territorial matrix encompasses the pericellular matrix and creates a woven network of collagen fibrils that surround and protect the chondrocytes. The interterritorial region is composed

of large collagen fibrils arranged in different orientations related to the joint surface depending on the zone of the articular cartilage (Figure 1.2) [10].

Articular cartilage is made up of largely non-calcified components with a small area that is calcified. There are four zones of articular cartilage (Figure 1.2): the superficial/ tangential zone, the intermediate/transitional/middle zone, the deep/ radiate zone, and the calcified zone [2]. The superficial zone represents 10-20% of the articular cartilage and has the largest number of chondrocytes with the collagen fibrils oriented parallel to the joint surface and being the narrowest in diameter at this location [18]. This layer protects the underlying layers from compressive, sheer, and tensile forces induced by movement and bearing weight. The integrity of this layer is crucial to the health of the deeper layers [10]. The intermediate zone represents 40-60% of the articular cartilage and the chondrocytes in this zone are larger and more oval in shape [10]. The collagen fibrils are loosely arranged in this zone and have an increased diameter compared to the superficial zone and also help absorb forces of compression [10]. The deep zone represents 30% of the articular cartilage and contains the largest chondrocytes [10]. The collagen fibers are oriented perpendicular to the joint surface and have the greatest resistance to compressive forces of all the layers. [10]. Under the deep zone is a layer of calcified cartilage. The calcified cartilage is the interface between the articular cartilage and underlying subchondral bone. This layer helps attach the collagen to the subchondral bone [10]. The junction of the non-mineralized and mineralized portions of cartilage can be identified histopathologically and is known as the "tidemark" region [19]. As animals age, blood vessels from the subchondral bone can infiltrate into to calcified cartilage [20]. Proteoglycans are the second most abundant dry matter molecules present within the

extracellular matrix, comprising up to 15% of the total dry matter weight (Figure 1.1). Proteoglycans are protein monomers consisting of a core protein with glycosaminoglycan side chains attached by covalent bonds. They are categorized as aggregating and non-aggregating molecules. The most common proteoglycans in articular cartilage are aggrecan, decorin, biglycan, and fibromodulin [10].

Aggrecan is the most prevalent and largest aggregating proteoglycan in articular cartilage [2]. Each core protein of an aggrecan molecule consists of over 100 side chains of the following glycosaminoglycans (GAGs): chondroitin-4 sulfate, chondroitin-6 sulfate, and keratan sulfate (Figure 1.3). The chondroitin sulfates attach at the carboxy end of the aggrecan molecule and keratan sulfate attaches to the N- terminus, creating chondroitin rich and keratan rich areas. The aggrecan molecule is separated into three globular domains: G1, G2, and G3 (Figure 1.3). The G1 region interacts with hylauronan, the G2 region is on the N- terminal side at the location of keratan sulfate aggregation, and the G3 region is on the carboxy side of the molecule [2].

The function of aggrecan is to help a joint withstand load by binding to HA via a link protein (Figure 1.3). The aggrecan core proteins accumulate on the HA molecule, which arrange in close proximity. The negative charges of the GAGs attract water into the intrafibrillar space, while simultaneously repelling each other [2]. This interaction allows the cartilage to withstand compressive forces during movement [10].

JOINT STABILITY

Joint stability is defined as "the ability of a joint to maintain an appropriate functional position throughout its range of motion" [21]. Stability is provided to the joint

by surrounding ligamentous, tendinous, and osseous structures. Ligaments are fibrous structures connecting bone to other bones. In horses, most diarthritic joints in the limbs are surrounded by collateral ligaments that provide medial and lateral stability. Ligaments are mostly composed of type I collagen fibrils arranged parallel to the longitudinal axis of the joint capsule they surround and they have a high elastin to collagen ratio, allowing the structure to stretch under normal movement [22].

Tendons are attachments between muscle and bone. Tendons are also mainly composed of type I collagen fibrils in a proteoglycan matrix [22]. Joints in the proximal equine limb rely heavily on tendinous and muscular stability [2].

Subchondral and epiphyseal bone provide stability and shape to the cartilage. Subchondral bone is defined as the bone just below the articular cartilage. It is highly dynamic in nature and adapts to mechanical forces placed upon it. The subchondral bone has two anatomical parts: the subchondral bone plate and the subchondral trabecular bone [23]. The subchondral bone plate is located under the calcified portion of articular cartilage. It is porous in composition and has channels extending into the cartilage. Vessels and nerves run through the channels and supply the calcified cartilage [23]. The arteries, veins, and nerves are highly susceptible to damage from increasing age and compressive forces [23]. The channels are abundant in areas of joints where the bone plate is thicker and under high mechanical load and more scarce in areas where the bone plate is thinner and under less of a mechanical load [24]. The subchondral trabecular (cancellous) bone located beneath the subchondral bone plate functions as a shock absorber during movement. The cancellous bone is organized into trabeculae that

organize into a honeycomb appearance and is extremely porous. This area is highly vascularized and innervated [23].

In conclusion, the diarthritic joint is made up of many components that must work in unity to create and maintain a healthy joint environment to allow normal motion. A balance of normal movement biomechanics, catabolic and anabolic processes, and selective diffusion are required to maintain a healthy joint environment. When the normal metabolism of the joint is altered, osteoarthritis may develop.

PATHOPHYSIOLOGY OF OSTEOARTHRITIS IN THE EQUINE JOINT

Osteoarthritis (OA) is a group of diseases of different causes that ultimately lead to synovitis, subchondral bone remodeling, and articular cartilage degeneration [1]. The origin of osteoarthritis is multifactorial including: synovitis, changes in subchondral bone, abnormal biomechanical properties, joint instability, ageing, and metabolic disorders [9; 25; 26]. Joint instability occurs when surrounding tissues are damaged from either a traumatic event or chronic microdamage from irregular weight bearing, such as abnormal conformation. Simultaneous changes in the articular cartilage, subchondral bone, and synovial membrane contribute to the irreversible and perpetuating disease process of osteoarthritis. The effects of osteoarthritis on the synovial membrane, articular cartilage, and subchondral bone are outlined below.

CHANGES OF THE SYNOVIAL MEMBRANE IN OSTEOARTHRITIS

The synovial membrane can be damaged primarily, termed primary synovitis, or secondarily, termed secondary synovitis [2]. Initially, as inflammation occurs within a

joint and the cartilage is damaged, there is a buildup of debris within the synovial fluid. To clear the debris, more type A (macrophagic) synoviocytes are required. Keeping up with increased production needs, the synovial villi undergo hyperplasia and subsequent hypertrophy. More synovial fluid is synthesized to provide nutrients for the new synoviocytes. The combination of these events creates the characteristic synovial joint effusion seen in osteoarthritis. Additionally, the joint is unable to clear some of the debris and it becomes embedded within the synovial membrane, creating additional areas of local synovitis [27].

As OA progresses, the permeability of the synovial membrane is altered, allowing larger molecules to traverse the membrane. Lubricin and hyaluronan exit more readily across the membrane [28]. The synovial fluid composition becomes higher in water content, decreasing its viscosity [29]. In osteoarthritis, lubricin levels are decreased from reduced expression in type B synoviocytes and chondrocytes in the superficial zone, causing a subsequent increase in friction between cartilages surfaces [5]. As the joint experiences an increase in friction, articular cartilage becomes damaged and undergoes degradation, leading to further cartilage damage, subchondral bone sclerosis, and osteophyte formation [30]. In horses with osteoarthritis, it has been shown that there is a reduced sialation of lubricin that may decrease its boundary lubricating capability [31].

Once the synovial membrane is damaged, it heals with fibrous tissue. The fibrous tissue has decreased elasticity compared to healthy tissue and creates limited range of motion to the joint. Limited range of motion is followed by atypical biomechanics, causing abnormal loading to the joint, and the disease process is perpetuated. The fibrous

tissue also has altered permeability, adding to the amount of abnormal debris trapped within a joint, and the cycle repeats [2].

CHANGES OF ARTICULAR CARTILAGE IN OSTEOARTHRITIS

After the process of osteoarthritis has been incited within a joint, inflammation occurs and causes changes within the joint environment. Cartilage changes start at areas of increased sheer stress, that could be induced primarily, or may occur secondary to changes in the underlying subchondral bone [32]. Chondrocytes alter their metabolism in response to the changes in the surrounding environment and become active [9]. Chondrocytes have limited ability to regenerate and the activation of them can lead to changes in the ECM and increased maturation and calcification of the calcified zone [9]. An increase in vascularity of the calcified cartilage from subchondral bone vessels is observed [1]. The vascular channels also have nerves that can be a nidus for pain [33].

As chondrocytes detect environmental change, they are stimulated to release regulatory proteins termed cytokines. Cytokines are classified as anabolic, modulatory, or catabolic [34]. Anabolic cytokines are up-regulated in early OA then down-regulated in late stage OA [35]. Major anabolic cytokines include insulin-like growth factor (IGF) and transforming growth factor (TGF). These cytokines stimulate the production of chondrocytes, proteoglycans, and type II collagen [2]. Modulatory cytokines, such as IL-6 can be both pro- or anti-inflammatory by up-regulating other anabolic or catabolic cytokines [36]. Major catabolic cytokines produced in response to osteoarthritis are interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) [37]. When IL-1 and TNFα are increased, there is an up-regulation and production of matrix

metalloproteinases (MMPs), aggrecanases, nitric oxide (NO); and a down-regulation of synthesis of aggrecan, type II collagen, and MMP inhibitors [2; 9]. Chondrocytes also release specific cytokines called chemokines. Chemokines act as attracting agents for inflammatory cells. Examples of chemokines released by chondrocytes are IL-8 and CXCR-3, 4, 5, and 6 [9]. When inflammation occurs in a joint, the chemokines can be up-regulated in an exaggerated manner and provoke further inflammation [38].

MMPs are enzymes that are capable of degrading components of the extracellular matrix. They require zinc for binding sites to be active and are named both by numerical standards and by the substrate they degrade [2]. The main MMPs seen with osteoarthritis are: MMP-1 (collagenase 1), MMP-8 (collagenase 2), MMP-13 (collagenase 3), MMP-2 (gelatinase A), MMP-9 (gelatinase B), and MMP-3 (stromelysin 1). The three collagenases cleave the type II collagen triple helix into 1/4 and 3/4 length fragments, with MMP-1 being the most predominant [16]. . MMP-8 is usually only present in times of great inflammation or sepsis as it is released from neutrophils. MMP-13 (up-regulated by IL-1 and TNF) is the most potent collagenase as it cleaves type II collagen 10 times as quickly as MMP-1 [2]. Gelatinases degrade collagen by unwinding the triple helix formation after cleavage and stromelysins break down proteoglycans and collagen. [1; 2; 9]. As the cartilage matrix is degraded, there is an increase in water content in the extracellular matrix that makes the joint less effective at withstanding loads [16]. In addition, when cartilage is injured, damage-associated molecular patterns (DAMPs) attach to Toll-like receptors (TLR) on chondrocytes. The expression of TLRs in chondrocytes increases in joints with OA [39]. Up-regulation of TLRs (specifically TLR 2 and TLR 4) cause an increased expression of MMPs and NO [9]. Other enzymes that

contribute to cartilage breakdown include: a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) [2]. ADAMTS enzymes are also called aggrecanases and they break down and deplete the proteoglycan core protein. There are natural inhibitors of MMPs and aggrecanases, called tissue inhibitors of metalloproteinases (TIMPs) that bind to MMPs and aggrecanases, making them inactive [2].

CHANGES OF SUBCHONDRAL BONE IN OSTEOARTHRITIS

There is evidence that suggests initial changes in OA occur in the subchondral bone [32; 40-42]. The subchondral bone is traumatized either by an injury or chronic microdamage. As the bone remodels, the subchondral bone plate thickens. The changes in thickness between normal and abnormal bone cause irregular shear forces on the joint. The adjacent subchondral bone spongiosa is subjected to the abnormal forces and experiences inflammation, bone edema, and bone marrow necrosis [43]. The appearance of the changes in the spongiosa and bone marrow can be visualized on MRI using T2 fat suppressed images and are consistent with early OA [43]. The articular cartilage can also experience abnormal shear forces from changes of the subchondral bone plate that result in areas of fibrillation [32]. It is also hypothesized that forces placed upon the subchondral bone cause sclerosis at weight bearing areas causing the tidemark region to thicken and the articular cartilage to thin [32]. The cartilage eventually ossifies at the articular margins, leading to osteophyte formation [29]. Conversely, as the cartilage erodes subchondral bone is eventually exposed. Loading the subchondral bone without the protection of the articular cartilage causes it to undergo eburnation and become

thickened. The subchondral bone also undergoes lysis and can have cystic formation from chronic bone-to-bone contact and synovial fluid invasion [32].

CLINICAL MANIFESTATION OF OSTEOARTHRITIS IN THE TARSUS OF THE EQUINE ATHLETE

The equine tarsus consists of five joints: tibiotarsal, talocalcaneal, proximal intertarsal, distal intertarsal, and tarsometatarsal (Figure 1.4). OA commonly develops in the distal intertarsal (DIT) and tarsometatarsal (TMT) joints of performance horses (collectively termed "the distal tarsal joints"). OA in the distal tarsal joints is one of the most common causes of pelvic limb lameness in performance horses [44]. Proximal intertarsal (PIT) OA is rare, and it can be associated with DIT and TMT OA with clinical signs often manifesting in the tibiotarsal (TT) joint [45; 46]. OA of the TT joint is less commonly reported than in the distal joints, but may be under reported because OA is often not visible on radiographs.

Currently, the most accurate method of identifying OA in the distal tarsal joints is the combination of thorough physical, lameness, and radiographic examinations [47-49]. Physical examination often relies on conformation assessment, presence of a positive Churchill test (the application of digital pressure on the distal intertarsal joint and assessing pain response), combined with bilateral hind limb lameness, and positive response to upper limb flexion [47]. Intra-articular anesthesia can be administered to help localize lameness. Radiography is the most commonly used imaging modality but lameness often does not correlate with radiographic changes; several studies have noted both severe radiographic changes with mild lameness in horses, or very little radiographic

changes with severe lameness [48-54]. Radiographic evidence of OA (narrowed joint space, sclerotic bone, osteophytes, and bony bridging) takes time to develop, which is likely why radiographic findings are highly variable compared to clinical findings and the degree of lameness. For example, up to a 50% decrease in bone density can occur before changes are visualized on radiographs [55]. Additionally, radiology gives little information about cartilage and soft tissue integrity [48].

In the TT joint, primary OA usually presents as effusion and lameness that responds to flexion with no detectable radiographic abnormalities [45; 46; 56]; many aged horses have this clinical presentation that is usually complicated by radiographic OA in the distal joints. It has even been pointed out that radiographs could not identify subchondral cysts in TT joints due to summation of opacities and overlapping contours [57; 58]. Because of the lack of radiographic findings in TT joints and presence of distal joint changes, the distal joints are often treated, while the TT joint effusion is ignored. It is not clear how the disease in the distal joints affects the TT joint, or whether OA is under-diagnosed in TT joints of older horses.

Diagnosing OA in the tarsal joints in horses presents a clinical challenge because readily attainable and reliable diagnostics are not available. Arthroscopic surgery is the gold standard for evaluating the TT joint [45; 46]. Arthroscopy allows direct visualization of the articular cartilage and joint environment. Inflammation can be visualized by the presence of hyperemia, thickened synovial villi, new villi formation, and villus atrophy. Lesions can be categorized based on size and number, presence of a kissing lesion, and extent of cartilage damage [59]. However, arthroscopy provides little information about the underlying subchondral bone. Additionally, arthroscopy is a

surgical procedure requiring general anesthesia, a known risk factor in aged horses [60], while potentially being cost prohibitive.

In the distal tarsal joints, arthroscopy is not an option. The small size of these joints prevents the use of this diagnostic tool. Since direct visualization of the distal tarsal joints is not possible, Magnetic Resonance Imaging (MRI) is considered the gold standard for evaluating them. MRI is a superior imaging modality in identifying specific anatomic lesions affecting cartilage, subchondral bone, synovium and surrounding soft tissues when compared to radiology, ultrasonography, and nuclear scintigraphy [61-64]. An important finding visualized on MRI and not radiographs is subchondral bone hyperintensity. As bone marrow necrosis and bone edema forms in early OA, the changes in the spongiosa and marrow of the bone cause a hyperintense area to appear on T2 weighted fat suppressed MRI [63]. In humans, it has been noted that SCB hyperintensity viewed on T2 STIR images could be indicative of reactive sclerosis with newly formed fibrovascular tissue, versus mature sclerosis that often appears hypointense [65]. If horses act in a similar manner, identification of SCB hyperintensity could be a good indicator of early and active disease since it has been stated that SCB hyperintensity of the distal tarsal joints may correlate with clinical lameness [63].

Unfortunately, MRI of the tarsus in a live horse in a high field magnet is often unattainable due to financial limitations, anatomical restraints of fitting the leg into magnets with smaller bores, and the need for general anesthesia. The lack of a diagnostic tool able to not only determine the presence of OA in the distal hock joints but to also track disease progression and response to treatment, often leads to over-treatment of these joints. In fact, it is common clinical practice to inject the distal tarsal joints of horses

with intra-articular corticosteroids up to every 6 months throughout horses' careers even though there is plenty of suggestion in the literature that corticosteroid treatment into a joint is not benign [66-68]. Therefore, there is an urgent need for minimally invasive, reliable, quantitative diagnostic test in horses that can detect early OA in these joints; ideally one that can then monitor the progression of OA such that the timing of treatment can be appropriately controlled.

A novel way to detect early OA changes in the joint is through the measurement of biomarkers from the synovial fluid (SF) from the joint of interest [35]. To the authors' knowledge, there are no studies examining biomarkers from the TT, DIT, and TMT joints in an aged horse population.

MOLECULAR BIOMARKERS OF OSTEOARTHRITIS

There is an urgent need for minimally invasive, reliable, quantitative tests for detection of early OA changes in joints. Molecular biomarkers of OA are some of the most minimally invasive tests used at this time. They reflect quantitative and dynamic variations associated with joint metabolism [59]. Two basic types of molecular biomarkers have been studied: direct and indirect biomarkers. Since the concentrations of these biomarkers often drive joint metabolism, they may provide an earlier indication of disease status.

DIRECT BIOMARKERS:

Direct biomarkers are a result of changes in joint metabolism [69]. There are many commercially available direct biomarkers that have been analyzed in the horse. This study will focus on those that represent collagen synthesis: CPII[a]; collagen degradation: C12C[a], C2C[a], and CTXII[b]; aggrecan turnover: CS846[a]; and osteoblastic turnover: BAP[c]. The premise behind each of these biomarkers is listed below.

Collagen synthesis is up-regulated in early OA and the carboxy propeptide of type II collagen (CPII) is an epitope of protein fragments released during collagen formation. After the procollagen molecule is released by a chondrocyte, the carboxy-propeptide is cleaved and the collagen molecule joins the fibril. The cleaved portion of the procollagen, CPII, can be measured in synovial fluid [17; 70]. In humans, CPII has been shown to increase in OA until synthesis cannot keep up with degradation and then concentrations decrease [71]. A study in humans demonstrated CPII was able to predict future radiographic changes in women with tibiofemoral joint osteoarthritis [17]. This could be extremely important clinically in horses, as radiographic changes often lag behind the disease process. In horses with intra-articular fractures, CPII was elevated compared to normal horses [72]. Another study in horses demonstrated that CPII is affected by age with yearlings having significantly higher concentrations compared to adult horses [73]. In horses with OCD lesions on the distal intermediate ridge of the tibia, CPII values were not found to be increased compared to non-affected joints [74].

C1,2C concentrations represent degradation products of both type I and type II collagen by identifying the neoepitope created after collagenase cleavage of the triple helix at the ¾ length carboxy (C) terminus region. The neoepitope created after MMP

cleavage is relatively long so it identifies both type I and II collagen [16]. After the triple helix is cleaved, the pieces are released into synovial fluid and can be measured [75]. C1,2C has been demonstrated to increase in horses with induced middle carpal joint OA compared to horses without OA [76] and in synovial fluid from equine carpal and fetlock joints with osteochondral injury, and in yearlings [73].

C2C also represents collagen degradation by identifying the neoepitope created after collagenase cleavage of the triple helix. The difference is that the neoepitope is shorter than that of C1,2C making it more specific to type II collagen [16] . After the triple helix is cleaved, the pieces are released into synovial fluid and can be measured [75]. A specific equine antibody for this neoepitope, 234CEQ, has been described and utilized [75] but is not readily available; thus a human assay (C2C[a]) has been used by most equine researchers. Studies evaluating C2C concentrations in horses have found that concentrations are affected by age and are elevated in joints with OA and OCD. It has been shown that osteochondral injury of Thoroughbred horses increases the concentration of C2C in the synovial fluid of carpal and fetlock joints compared to uninjured cartilage of rested and exercised horses [77]. One study showed that age, presence of osteochondral injury and joint affected all influenced C2C concentrations [73]; yearling horses were found to have higher concentrations of C2C compared to adults. In horses with osteochondral injury in the fetlock and carpus, higher C2C concentrations were found in the carpus [73]. In horses with OCD lesions on the distal intermediate ridge of the tibia, C2C values were not found to be increased compared to non-affected joints in one study [74], but were increased compared to normal horses in another study [72].

Cross-linked C-telopeptide fragments of type II collagen (CTX II) measures degradation of type II collagen (close to the bone cartilage interface) via identification of a type II collagen C-telopeptide epitope [69]. In horses, CTX II concentrations have been shown to increase in horses with OA, and are also affected by age. Synovial fluid concentrations of CTX II in adult horses with osteochondral injury in the middle carpal and fetlock joints were significantly higher compared to radiographically normal joints of horses before and after exercise [78]. CTX II values were increased in yearlings compared to adult horses and in osteochondral injured middle carpal joints compared to injured metacarpophalangeal joints [73]. In Standardbred horses with post-traumatic osteoarthritis there was an increase in CTX II concentrations over a 4 year time period [79].

The chondroitin sulfate 846 (CS846) epitope is located on the chondroitin sulfate side chains near the G3 domain and as such represents newly synthesized aggrecan molecules; it is released from the extracellular matrix into the synovial fluid once it is cleaved from the aggrecan protein. These large fetal forms of aggrecan (intact protein cores out to G3 domain) are naturally present in young animals, but then decrease in cartilage with age. Concentrations increase again in older animals with osteoarthritis suggesting that cartilage is undergoing aggrecan core protein turnover more readily when pathology is present [80]. In horses, CS846 concentrations were elevated in joints with OA compared to those without, and have been used to predict OA in working horses. In polo ponies, CS846 increased during the working season and were significantly higher in ponies that later developed osteoarthritis [81]. CS846 was also elevated in the middle carpal joints in 2 year old horses with induced OA undergoing exercise [76].

Bone alkaline phosphatase (BAP), is an isoenzyme of alkaline phosphatase, which is a marker of early osteoblastic activity [82]. It locally increases the concentration of extracellular inorganic phosphate, which increases mineral formation and decreases extracellular pyrophosphate, a mineral inhibitor [82]. BAP is a biomarker that could potentially provide information about the subchondral bone status in joints. In horses, BAP concentrations have been shown to increase in joints with OA and OCD lesions. In a study of racehorses in training, carpal and fetlock joint synovial fluid BAP concentration was positively correlated with cartilage damage [83]. In horses with osteochondral fragments in the carpus, BAP in synovial fluid was significantly elevated [59].

INDIRECT BIOMARKERS

Indirect biomarkers, by definition, indirectly change the metabolism in the joint [35]. Commercially available indirect biomarkers that will be focused on in this research include cytokines interleukins (IL) -1β[d], -6[d], -8[d], -10[d], and tumor necrosis factor alpha (TNFα[d]). IL-1β and TNFα are catabolic cytokines. IL-6 is a modulatory cytokine, IL-8 is a chemokine, and IL-10 is an anti-catabolic cytokine [36].

IL-1β is a strong pro-inflammatory cytokine that stimulates cartilage matrix degeneration [36]. It was found to be a good identifier of acute severe joint disease, but not good as a screening tool for chronic or less severe joint disease in horses with naturally occurring OA [84]. In horses, IL-1β concentrations have been shown to elevate acutely after joint injury then fluctuate afterwards, and are also affected by age. In Standardbred racehorses with post traumatic OA, IL-1β levels peaked acutely, declined

over the first year after injury, than gradually increased again over the following three years [79]. Trumble et al [85] identified elevated levels of IL-1β in younger horses with OCD lesions.

TNFα is another pro-inflammatory cytokine that works synergistically with IL-1β [86]. Studies evaluating TNFα have demonstrated that this cytokine is beneficial to identify severe OA. A study examining joints of horses with naturally occurring OA found that TNFα was a good predictor of acute severe joint disease, but not in chronic disease [84]. Another study found TNFα levels elevated in horses with severe OA in both acute and chronic cases [87]. A couple of studies have found no correlation between macroscopic cartilage damage and SF TNFα concentrations in the joints of horses [88; 89]. One study identified increased SF concentrations in horses with severe carpal fractures but not in horses with smaller OCD fragments [90] In contrast, another study did find increased SF concentrations of TNFα in horses with OCD lesions and in joints after acute joint trauma [85]. The results of these studies indicate that TNFα is predominately elevated in acute and severe traumatic OA and in some horses with OCD lesions.

A modulatory cytokine such as IL-6 can be either pro- or anti-inflammatory [91]. IL-6 has been shown to increase in carpal joints of horses with both induced synovitis [92; 93] and in naturally occurring disease [84; 88; 90]. One study had the highest levels of IL-6 in horses with carpal osteochondral fragments and synovitis [88]. A single sample of IL-6 from an injured joint was found to be an excellent predictor of OA; Il-6 was not found in any normal joints, suggesting that finding any levels of IL-6 may be

relevant [84]. The results of these studies demonstrate that the source of IL-6 could be from the synovial cells and/or osteoclasts to induce bone resorption.

IL-8 is a chemokine and as such recruits neutrophils and stimulate their secretion [94]. It has also been shown to stimulate the release of enzymes that degrade the ECM [95]. IL-8 is also angiogenic [96]. IL-8 was up-regulated in vitro in equine chondrocytes that were stimulated by human recombinant IL-1β [97]. IL-8 may have an important role in equine OA.

IL-10 is an anti-catabolic cytokine. This cytokine can inhibit cartilage degeneration directly and indirectly. Directly, IL-10 degrades proteinases and decreases the synthesis of pro-inflammatory cytokines [36]. Indirectly, IL-10 has been shown to increase interleukin-1 receptor antagonist (IL-1ra) concentrations, inhibiting IL-1 [34]. In horses, IL-10 concentrations have been shown to increase within one week of intra-articular mesenchymal stem cell injection into fetlock joints [98].

In conclusion, OA is a multifactorial disease with many anatomical structures involved. Biomarkers have been shown to have clinical value in identifying OA. To the author's knowledge, no study has yet looked at biomarkers in the tarsus specifically in an aged horse population.

CHAPTER 2:

CLINICAL CHALLENGE OF DETERMINING OSTEOARTHRITIS IN TIBIOTARSAL JOINTS OF OLDER HORSES: USE OF DIRECT AND INDIRECT BIOMARKERS TO DETERMINE THE PRESENCE AND SEVERITY OF DISEASE

Osteoarthritis (OA) is a multifactorial disease characterized by synovitis, subchondral bone remodeling, and articular cartilage degeneration. In horses, OA clinically manifests as lameness [99], with the tarsus being a common location. The distal joints (distal intertarsal [DIT] and tarsometatarsal [TMT]) are the most commonly affected, often showing mild to marked radiographic changes of OA. Proximal intertarsal (PIT) OA is rare, and it can be associated with DIT and TMT OA with clinical signs often manifesting in the tibiotarsal (TT) joint [45; 46]. Primary OA of the TT joint usually presents as effusion and lameness that responds to flexion with no detectable radiographic abnormalities [45; 46; 56]; many older horses have this clinical presentation that is usually complicated by radiographic OA in the distal joints. Because of the lack of radiographic findings in TT joints and presence of distal joint changes, the distal joints are often treated, while the TT joint effusion is ignored. It is not clear how the disease in the distal joints affects the TT joint, or whether OA is under-diagnosed in TT joints of older horses. Arthroscopic surgery is the gold standard for OA diagnosis in TT joints [45; 46] but is rarely performed in older horses with distal tarsal OA.

Another way to identify the presence of TT joint OA is through measurement of molecular biomarkers from synovial fluid (SF). Biomarkers reflect quantitative and dynamic variations of joint metabolism, and are classified as direct (result of changes in metabolism) or indirect (alters metabolism) [35]. Biomarkers have been successfully used

in horses to identify mainly carpal and/or fetlock OA [59; 73; 76-79; 83; 84; 88]. The TT joint has been examined using biomarkers, but the focus has been on OCD lesions in young horses [72; 74; 100-104]. To the authors' knowledge, there is only one biomarker study within the tarsus that has focused on OA (specific to TMT joint) [105]; no study has examined biomarkers in the TT joint from older horses that also have radiographic OA in distal tarsal joints.

The objective of this research was to measure SF biomarker concentrations from TT joints of older horses with distal tarsal joint OA to determine if they correlate to radiographic, clinical, arthroscopic, and/or gross evidence of OA in the TT, PIT, DIT, and/or TMT joints. We hypothesized that TT OA would be underestimated on radiographs and clinical examination compared to arthroscopic and gross findings, and that TT joint biomarker concentrations would correlate to TT and PIT pathologic findings, but would not be influenced by the presence of DIT and TMT OA.

Materials and methods

Horses were enrolled from a population of animals donated to the University (IACUC Approval #1411-32001A) for educational purposes (Table 2.1). The inclusion criterion was radiographic evidence of tarsal OA in any or all of the tarsal joints (TT, PIT, DIT, TMT). Horses with OCD lesions in TT joints were excluded. Radiographic changes could range from mild to severe joint space narrowing, soft tissue swelling, subchondral bone sclerosis/lucency, and/or presence of osteophytes/enthesophytes. Controls were selected from an additional group of 13 horses donated to the University (IACUC Approval #1201A08201) that lacked specific musculoskeletal abnormalities.

No radiographic, clinical, or arthroscopic data were available for control horses. Control horses had TT joint SF collected and processed as described below with a subsequent post-mortem gross exam that was documented via digital photographs. To be included as controls, cartilage lesions were graded as described below and had to have a score ≤2. For both cases and controls, Phenylbutazone was discontinued 48 hours prior to evaluation since it can alter biomarker concentrations [106].

Both tarsi of each case horse were radiographed (lateromedial, dorsoplantar, and dorsolateral to plantaromedial and dorsomedial to plantarolateral obliques). The tarsus with the most overall radiographic changes was chosen as the limb of interest (Table 2.2), and radiographic scores were individually determined for each joint (TT, PIT, DIT, and TMT) using a modified scoring system [59] for a total possible score of 18 (Table 2.3). Radiographs were scored by a blinded surgeon and radiologist and mean scores were recorded.

Each case horse received a musculoskeletal examination. Churchill responses, TT joint effusion was evaluated and circumferential measurements were performed by the same investigator and were scored as listed in Table 2.2. Two blinded surgeons graded videotapes of a walk and trot in a straight line (hard surface), and in circles in each direction on a soft surface using the AAEP scale [107]. Full limb flexion tests were also scored (Table 2.2). Mean scores were recorded. No attempt was made to isolate the cause of lameness.

Horses were then euthanized and TT joint SF was harvested within 30 minutes. Samples were collected from the dorsomedial pouch, and were centrifuged, aliquoted, and stored at -80C° until further analyses.

Contrast radiography was performed on the limb of interest of case horses to determine presence of direct communication between tarsal joints. Three mL of iohexol (Omnipaque)[e] were injected into TMT joints, with needle placement verified by radiographs. A lateromedial radiograph was obtained to assess and record communication. This was repeated for the DIT joint.

Tibiotarsal joints of each limb of interest in case horses were examined arthroscopically using standard dorsal and plantar portals [108]. By modifying a published scoring system [59], arthroscopic findings were scored for synovial inflammation, adhesion formation, presence of synovialized debris, number, length, and depth of non-linear cartilage lesions, and number and depth of linear score lines (Table 2.4). Scores were summated to yield an overall score that could range from 0-45.

Tibiotarsal joints were then disarticulated and examined grossly. Lesions identified by arthroscopy were confirmed, and were not given a gross score. An additional gross score was calculated for case horses using the arthroscopic scoring system (Table 2.4) to account for lesions that could only be identified after joint disarticulation. The intertrochlear groove (ITG) was only evaluated and scored grossly because its entirety could only be evaluated after disarticulation. If synovial fossae on the proximal and distal aspects connected (Figure 2.1), then an ITG score of 1 was recorded. For case horses, arthroscopy and additional gross scores were summated to derive a total arthroscopy/gross score for TT joint pathology. Control horses did not have any arthroscopic examination but were assigned gross scores based on arthroscopic scoring for cartilage lesions (Table 2.4) and ITG gross scoring described above.

Tibiotarsal joint SF was analyzed using commercially available ELISAs. For case horses, the following direct biomarkers were measured for type II collagen synthesis (carboxy-terminal propeptide of type II collagen: CPII[a] and degradation (neoepitope generated by collagenase cleavage of type II collagen: C2C[a] and types I and II collagen: C12C[a] as well as the C-terminal crosslinked telopeptide of type II collagen: CTX II[b]. In addition, aggrecan turnover was assessed using the chondroitin sulfate epitope 846: CS846[a] and early osteoblastic turnover was assessed using bone alkaline phosphatase: BAP[c] Indirect biomarkers examined represented different equine specific cytokines including: catabolic (interleukin [IL]-1 beta [IL-1β][d]and tumor necrosis factor alpha [TNFα][d], modulatory (IL-6)[d]anti-catabolic (IL-10)[d] and chemokines (IL-8)[d] For control horses, previously analyzed direct (C2C, CTXII, CPII, CS846, and BAP) and indirect (IL-1β, IL-6, and IL-10) biomarkers were available for comparison. Biomarkers were analyzed according to manufacturer instructions at appropriate dilutions. Concentrations determined to be less than the lowest standard were assigned a value of ½ the concentration of the lowest standard so that samples could be represented graphically and statistically.

Gaussian distribution could not be assumed and no transformation was appropriate for all data. Spearman correlations were used to determine correlations amongst all horses (controls and cases) for: age, sum of cartilage arthroscopy and gross scores, and biomarker concentrations. Spearman correlations were also performed on cases only for: age, Churchill test, TT joint effusion and circumference, lameness, radiographic scores, sum of synovial arthroscopy scores, sum of cartilage arthroscopy scores, total arthroscopy score, combined total arthroscopy/gross scores, and biomarker

concentrations. Based on total arthroscopic/gross scores, a clear line was evident to separate case horses using 15 as the cut-off into subgroups of mild (<15) and moderate (≥15) OA severity. Kruskal-Wallis test with Dunn's multiple comparisons was used to determine differences among control, mild, and moderate OA severity groups for C2C, CPII, CS846, CTXII, BAP, IL-1β, IL-6, and IL-10. Unpaired Mann-Whitney tests were used to compare mild and moderate OA severity groups for clinical and radiographic findings as well as C12C, IL-8 and TNFα (no control values available). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and likelihood ratio of SF C2C concentration to discriminate between presence and absence of OA between controls and case horses were determined using a ROC analysis and Fisher's exact test. P values of ≤0.05 were considered significant using statistical software[f,g].

Results

Eleven case horses, ranging in age from 9-35 years old (mean=21), and 6 control horses, ranging in age from 7-26 years old (mean=16) were included in the study. Signalment and clinical findings for case horses are summarized in Table 2.1 and 2.2. Radiographic scores for the TT, PIT, DIT, and TMT joints are listed in Table 2.3. The TT joint radiographic scores ranged from normal to mild disease (0-2.5). Radiographic scores for the PIT, DIT, and TMT ranged from normal to severe disease, with DIT disease scores being higher than all PIT scores and most TMT scores. There were no significant correlations of TT joint radiographic scores and PIT, DIT, or TMT. DIT and TMT radiographic scores positively correlated (R=0.740; P=0.009). Contrast radiography

identified direct communication between DIT and TMT in 4 horses (36%), with one (9%) also communicating with PIT and TT joints (Table 2.3).

Musculoskeletal and lameness examination findings are reported in Table 2.2. Effusion scores did not correlate with circumference measurements, but did positively correlate with Churchill responses (R=0.800; P=0.003). There were no other significant correlations of clinical parameters to themselves or radiographic scores. Seven horses had baseline lameness in the chosen limb; however, one of these horses (horse 2) had a more pronounced forelimb lameness. Of the 10 horses that were able to trot, 9 were positive to full limb flexion of the chosen limb, with most being mild. Two horses (horses 3 and 8) donated specifically for tarsal OA demonstrated baseline lameness grades of 2/5, moderate flexion and Churchill responses, and moderate to severe TT joint effusion.

Most of the pathology present within TT joints could be identified via arthroscopic examination (Table 2.4 and Figure 2.2). All but one horse had changes present in the synovial membrane (Figure 2.2A), with age positively correlating to synovial membrane changes (R=0.630; P=0.040). All horses had cartilage lesions in TT joints, with the most common locations on medial (Figure 2.2B), and lateral trochlear ridges (Figure 2.2C) of the talus. Six horses had deep cartilage lesions present, with exposure of subchondral bone in 4 (Figure 2.2D). When linear score lines were present (Figure 2.3A), they extended the majority of proximal to distal distance of the trochlear ridges. Arthroscopic synovial and cartilage scores were positively correlated (R=0.640; P=0.030). Additional gross finding scores ranged from 0-8 (Table 2.5). The cochlea of the distal tibia had cartilage lesions present in 7 horses including lesions surrounding the

synovial fossa and/or multiple focal lesions or score lines distant from the fossa (Figure 2.3). Total arthroscopic/gross scores negatively correlated with DIT radiographic scores (R=-0.640; P=0.040), with no other significant correlations with remaining radiographic scores or clinical findings.

Based on total arthroscopic/gross scores, case horses were divided into two OA subgroups for TT joints (Table 2.4): ≤ 15=mild OA (n=6) and >15=moderate OA (n=5). For clinical and radiographic findings there were no significant differences between mild and moderate severity groups. There were also no significant differences between controls and/or mild and moderate severity groups for any direct (Figure 2.4) or indirect (Figure 2.5) biomarker concentrations. Complete data for all biomarkers with all horses combined, as well as for each individual horse are summarized in Tables 2.6-2.8. IL-6 was the only biomarker to positively correlate to amount of disease identified on arthroscopy and/or gross examination when only looking at the case horses (total arthroscopy score R=0.683; P=0.020, and combined total arthroscopy/gross scores R=0.650; P=0.030). However, when controls were combined with case horses, C2C and IL-6 concentrations both positively correlated with cartilage arthroscopy/gross scores (R=0.540; P=0.030 and R=0.530; P=.030, respectively). Based on ROC analysis, a C2C concentration of 187.44 pmol/mL was used to determine predictive value (P=0.03) for discriminating OA in the TT joint (OA>187.44), yielding: sensitivity=91%, specificity=67%, PPV=83%, NPV=80%, and likelihood ratio=3. In addition, when all horses were combined, age positively correlated with cartilage arthroscopy/gross scores (R=0.580; P=0.020), IL-1β (R=0.540; P=0.030), and IL-10 (R=0.570; P=0.030). In horse 6 who had direct communication of all joints, indirect biomarker concentrations were

elevated compared to other horses (denoted by triangle in Figures 2.4 and 2.5). All cytokines were positively correlated to each other.

Discussion

Mature horses with evidence of radiographic OA present in distal joints (DIT and TMT joints) were enrolled into our study to evaluate whether radiographic changes in these joints correlated to biomarker concentrations and pathologic changes present within TT joints. To the authors' knowledge, this is the first study to examine biomarkers in TT joints of older horses, and hope that it provides the template for future studies. Our results suggest that C2C and IL-6 are the best biomarkers of those studied at distinguishing OA in TT joints. In addition, more pathology was present in TT joints than expected based on radiographic examination, with no correlation to the PIT, DIT, and TMT disease processes.

While radiography is a commonly performed diagnostic test for tarsal OA, TT joint OA is rarely associated with radiographic changes [46; 56]. It has even been pointed out that radiographs could not identify subchondral cysts in TT joints due to summation of opacities and overlapping contours [57; 58]. Our study supports these observations because 8 horses had normal TT joints on radiographs with the remaining 3 only having mild changes. Radiographs demonstrated low sensitivity because most TT joint pathology involved the synovial membrane and cartilage. It is important to point out though that the 3 horses (horses 3, 8, and 9) with mild TT joint radiographic changes were grouped into the moderate disease subgroup based on total arthroscopic/gross score, suggesting that any TT joint abnormalities on radiographs, even if subtle, could be indicative of more severe pathology. In addition, PIT joint radiographs did not correlate

to radiographic or arthroscopic/gross changes in TT joints, in contrast to previously suggested interconnections [45]. Therefore, radiographic changes in PIT joints could not be used to predict disease in TT joints. This finding is most likely from the difference in function between these joints, despite direct communication. Nonetheless, radiographic changes in PIT joints also did not correlate to the similar functioning DIT or TMT joints, contradicting previous suggestions [46].

The fact that radiographs do not adequately demonstrate presence or severity of TT joint OA is a problem because clinicians may miss clinically relevant disease. This is especially important in older horses that have TT joint effusion, but also have a Churchill response and radiographic evidence of distal tarsal joint OA, as the assumption will be that the lameness originates from the distal joints. This common clinical scenario was demonstrated in our case population. These findings did not help delineate whether the cause of lameness was from TT joints, distal joints, or both, emphasizing the need for thorough diagnostic analgesia in these cases. Interestingly, the 2 horses donated for tarsal OA were part of the moderate TT joint OA subgroup and had grade 2 hindlimb lameness with at least moderate effusion, Churchill response, and flexion responses (Table 2.2) with variable radiographic OA of both TT and distal joints (Table 2.3). This demonstrates that neither clinical nor radiographic signs of these horses could completely identify where lameness originated from in the tarsus. Unfortunately, the way our study had to be set up, we were unable to perform diagnostic analgesia; thus we could not prove the origin of hindlimb lameness. In addition, effusion grades, circumferential measurement, Churchill responses, lameness examination, and full limb flexion tests in case horses did not correlate to TT joint disease severity found on arthroscopic and gross

examination, making it difficult to draw conclusions from our clinical data. Future studies that identify the exact source of lameness within the tarsus are needed to further delineate the usefulness of particular clinical signs in determining the relevance of TT joint OA with concurrent distal tarsal joint OA.

Lack of information about TT joint OA from radiographs and clinical signs makes it important to investigate other diagnostics. Arthroscopy is the gold standard diagnostic for determining cartilage and synovial changes within TT joints [45; 46; 108], but arthroscopic evaluation is not a practical screening tool in older horses. Biomarkers of OA from SF of other joints have been successfully used to determine presence and severity of joint disease in horses [59; 73; 76-79; 83; 84; 88]. They have also been used to distinguish presence and severity of inflammation and cartilage damage related to TT joint OCD in young horses [72; 74; 100-104]. Therefore, using biomarkers to identify OA in TT joints is appealing, especially considering there is ample SF that can be collected.

We could not identify significant differences between control and/or mild to moderate OA subgroups in any of the biomarkers studied, likely due to variability of the disease, combined with a low number of horses. Since this was the first biomarker study of TT joint OA in older horses, we were unsure of the number of horses needed to obtain significance between groups. Therefore, in an effort to promote future studies, we have supplied all of our biomarker data (Figures 2.4 and 2.5; Tables 2.6-2.8) because our study demonstrates potentially useful biomarkers for identifying OA in TT joints. It is our hope that it will help researchers decide which biomarkers to examine and provide a basis for power calculations for future studies.

Our results highlight two biomarkers that may warrant future studies in larger populations: C2C and IL-6. IL-6 was the only biomarker to correlate to multiple pathology scores in case horses, and when the controls and case horses were examined together, IL-6 and C2C were both positively correlated with cartilage arthroscopy/gross scores. The control group was not examined arthroscopically, but their scores would be comparable to cartilage arthroscopy/gross scores performed on case horses since all arthroscopic lesions in case horses were confirmed grossly. Even though there was no significant difference among groups (control, mild, and moderate) for C2C (P=0.080) and IL-6 (P=0.260), these correlations show that C2C and IL-6 concentrations increase with the severity of OA. In fact, when looking at C2C data, ROC analysis demonstrated that 187.44 pmol/ml could be used to distinguish disease, with horses being 3 times more likely to have disease when concentrations were >187.44 pmol/ml. This is comparable to a young control horse population where the mean C2C concentration from TT joint SF was 170.9 ng/ml (equivalent to 176 pmol/ml) [74]. C2C concentrations for mild (mean±SD; 295.2±85.6 pmol/ml) and moderate (302.9±106.6 pmol/ml) subgroups in our study were more comparable to the presence of osteochondral fracture in carpi or fetlocks [72; 73] than those from TT joints with OCD in young horses [72; 74]. This makes sense since cartilage changes would likely be more widespread and deeper in older horses with OA than younger horses with OCD. IL-6 is considered a modulatory cytokine because it can exhibit both pro- and anti-inflammatory properties. Equine studies have shown that IL-6 is rarely present in SF in measurable concentration when joints are normal, but concentrations increase with worsening OA [84; 88], making it a good predictor of joint disease in horses [84]. Our results are comparable in that we had positive correlations

with pathology scores and had 6/11 of our TT joint concentrations below the lowest assay standard in control and mild groups compared with only 1 for the moderate group (Figure 2.5; Table 2.7).

Age has been demonstrated to affect biomarker concentrations [73; 100]. In most equine studies, biomarker levels were elevated in young populations in response to the developing/modelling musculoskeletal system. This is clearly not the case in this study since all horses in our study were between 7-35 years. Age positively correlated to cartilage arthroscopy/gross scores, IL-1β, and IL-10 for all horses and to arthroscopic synovial scores in case horses. This suggests that as horses get older, there are more OA changes present in the synovial membrane and articular cartilage with variable cytokine responses. There were no direct correlations between age and direct biomarkers suggesting, as others have [73], that this interaction is likely more complex.

In our study, 4 case horses had communication of DIT and TMT joints with only 1 horse having communication from distal joints to PIT and TT joints. The number of horses with communication may be underestimated because we did not try to achieve maximal pressure as described by others using a similar medium [109]. Nonetheless, our results are similar to other study populations [109; 110]. We thought that horses with communication from distal joints might make TT joint OA worse, but based on our results, it is uncertain what role joint communication has on biomarker concentrations and OA status. The one horse (horse 6) that had communication between all joints examined had much higher indirect biomarker concentrations compared to the other horses (Figure 2.5; Table 2.7), but relatively average direct biomarker concentrations (Figure 2.4; Table 2.6) with mild distal joint radiographic scores (Table 2.3). Based on

lack of radiographic disease, it is unknown if elevated indirect biomarker concentrations are from joint communication or individual horse variation. Additional studies comparing biomarker concentrations between joints with direct communication are needed to determine what effect communication has on biomarker concentrations across different stages of OA.

It has been stated that arthroscopic surgery is the gold standard for OA diagnosis in TT joints [45; 46; 108]. Our results agree since the vast majority of lesions could be identified via arthroscopy, with gross examination only adding a median score of 3 per horse to the total pathology score. However, it is important to note that while arthroscopy provided the most information on OA severity in TT joints, additional lesions may be present in locations that cannot be examined via arthroscopy such as the distal tibia. It has been shown that lesions can be present on the distal tibia that can cause a substantial lameness [57; 58; 111]. These reports required the use CT and/or MRI to diagnose subchondral cystic lesions, which demonstrates the difficulty in identifying distal tibia lesions. We identified distal tibia cartilage lesions in 7 horses in our study. Four of these horses had apparent cartilage degeneration at the periphery of the synovial fossa (Figure 2.3), with 3 of these 4 horses having additional focal cartilage lesions or score lines elsewhere on the distal tibia. We could not identify any pattern of clinical, radiographic, arthroscopic, or biomarker changes that clearly helped identify distal tibia pathology.

Synovial fossae (normal cartilage-free depression) in TT joints have been reported to be present on the distal tibia, and in the intertrochlear groove, and medial trochlear ridge of the talus in horses [108; 112]. In our study, we found a wide array of

appearances of these synovial fossae; further study of these areas may be warranted, as it was difficult at times to determine if pathology was present surrounding them. We considered them normal unless there appeared to be extension from what has been described. On the distal tibia, we gave a score of 1 if there appeared to be a partial ring of thinning cartilage around the fossa periphery. In addition, we also gave a score of 1 if the proximal and distal synovial fossa on the intertrochlear groove connected. These areas may not be true lesions; since we did not perform histology we cannot say definitively one way or another. Nonetheless, by giving low scores to those fossae that appeared different, we did not over-represent these findings. On top of that, even if those scores were removed, the mild and moderate groupings of the horses would not change.

There were several limitations in this study. Only a small number of horses with wide ranging degrees of TT joint OA were enrolled. Further studies are needed with larger numbers of horses, and our data can help researchers develop them. In addition, it is difficult to obtain disease-free controls from an older population of horses. Therefore, we had to use horses from a different study that did not have all the clinical, radiographic, arthroscopic, and biomarker end points as the current study. This may mean that we classified some of these horses as controls, even though more pathology could have been identified if all modalities had been utilized. Finally, we did not have the luxury of enrolling only those horses that had lameness localized to the tarsus. This would be ideal for future studies since that would help determine if certain clinical signs represent the presence and/or severity of OA better than others.

In conclusion, to the authors' knowledge, this is the first study to examine biomarkers in TT joints of older horses, and hope that it provides the template for future

studies. Our results suggest that C2C and IL-6 are the best biomarkers, of those examined, at distinguishing the presence of TT joint OA. In addition, there was more pathology present in TT joints than could be seen on radiographs, with no correlation to PIT, DIT, and TMT disease; suggesting that arthroscopic surgery is still the best method to evaluate TT joint OA. Based on our study, biomarkers could provide a valuable source of information about the OA disease process in TT joints that is currently only attainable with arthroscopic surgery, however further research is needed.

CHAPTER 3:
THE USE OF BIOMARKERS TO DETERMINE THE SEVERITY OF OSTEOARTHRITIS IN THE DISTAL INTERTARSAL AND TARSOMETATARSAL JOINTS OF OLDER HORSES

Osteoarthritis (OA) commonly develops in the distal intertarsal (DIT) and tarsometatarsal (TMT) joints of performance horses (collectively termed "the distal tarsal joints"). Arguably, these joints are some of the most frequently medicated across disciplines since OA in the tarsal joints is one of the most common causes of pelvic limb lameness that limits performance in horses [44].

Currently, the most common methods of identifying OA in the distal tarsal joints are physical, lameness, and radiographic examinations [47-49]. Physical examination includes conformation assessment and the response to the Churchill hock test which consists of application of pressure on first and second tarsal bones, and head of medial splint bone [47]. Lameness examination of horses with active distal tarsal OA often reveals a bilateral hind limb lameness and positive response to full limb flexion tests. Intra-articular anesthesia of the distal joints can also be used to localize the lameness. Radiography is the most common imaging modality used to assess OA in the distal tarsal joints, but many horses may have pain localized to these joints with minimal radiographic changes [48-54]. Radiographic evidence of OA (narrowed joint space, sclerotic bone, osteophytes, and bony bridging) takes time to develop, which is likely why radiographic findings are highly variable compared to clinical findings and the degree of lameness. For example, up to a 50% decrease in bone density can occur before changes are visualized on radiographs [55]. This points out that there is an important gap in our knowledge about the onset and progression of OA within these joints.

The main reason for this gap in knowledge is because accurate diagnosis of OA in the distal tarsal joints is difficult. Readily attainable and reliable diagnostics are not available. Compared to most other joints in the distal limb of the horse, additional diagnostics in the distal tarsal joints are limited. Arthroscopy is considered to be the "gold standard" for diagnosing cartilage lesions in most joints such as the tibiotarsal joint [108] (Chapter 2), but the small size of the distal tarsal joints prevents the use of this valuable diagnostic. Since direct visualization of the distal tarsal joints is not possible, Magnetic Resonance Imaging (MRI) is considered the gold standard for evaluating them. MRI is a superior imaging modality in identifying specific anatomic lesions affecting cartilage, subchondral bone, synovium and surrounding soft tissues when compared to radiography, ultrasonography, and nuclear scintigraphy [61-64]. Unfortunately, MR imaging of the tarsus in a live horse in a high field magnet is often unattainable due to financial limitations as well as anatomical restraints of fitting the leg into some magnets with small bores. The lack of a clear diagnostic tool to not only determine the presence of OA in the distal hock joints but to also track disease progression and response to treatment, often leads to over-treatment of these joints. In fact, it is common clinical practice to inject the distal tarsal joints of horses with intra-articular corticosteroids up to every 6 months throughout horses' careers even though there is plenty of suggestion in the literature that corticosteroid treatment into a joint is not benign [66-68]. Therefore, there is an urgent need for minimally invasive, reliable, quantitative diagnostic tests in horses that can detect early OA in these joints; ideally one that can then monitor the progression of OA such that the timing of treatment can be appropriately controlled.

A novel way to detect early OA changes in the joint is through the measurement

of biomarkers from the synovial fluid (SF) from the joint of interest [35]. To the authors' knowledge, there is only one biomarker study within the tarsus that has focused on OA in distal hock joints (specific to TMT joint) and found that certain biomarker assays may be useful in detecting early OA [105]. No study has examined biomarkers from the distal tarsal joints in a population of older horses and compared the biomarker changes to high field MRI.

There were two objectives of this research. The first objective was to compare the degree of OA found on radiographs to MRI in the distal tarsal joints. The second objective was to measure SF concentrations of specific biomarkers from the distal tarsal joints to determine if they correlated to the following: (1) the degree of DIT and TMT OA identified on radiographs, (2) the degree of DIT and TMT OA identified on MRI and, (3) biomarker concentrations between the DIT and TMT joints. We hypothesized that (1) the degree of OA found on the radiographs would correlate to MRI but underestimate amount of OA present compared to MRI findings; (2) the biomarkers of the distal tarsal joints would correlate to the degree of OA identified on MRI more so than radiographs; (3) the biomarkers would be able to distinguish distal joints with moderate/severe OA from distal joints with mild OA, and (4) biomarkers would correlate to each other within each specific DIT and TMT joint.

Materials and methods

Horses were enrolled from a population of animals donated to the University (IACUC Approval #1411-32001A) for educational purposes (Chapter 2; Table 2.1). The inclusion criterion was radiographic evidence of tarsal OA in the distal tarsal joints that ranged from mild to severe using the following criteria: joint space narrowing, soft tissue swelling, subchondral bone sclerosis/lucency, and presence of osteophytes/enthesophytes. Both tarsi of each horse were radiographed using standard views (lateromedial, dorsoplantar, and dorsolateral to plantaromedial and dorsomedial to plantarolateral obliques). The tarsus with the most overall radiographic changes was chosen as the limb of interest (Table 2.2), and radiographic scores were individually determined for each DIT and TMT joint (Table 3.1) using a modified scoring system [13] for a total possible score of 18 (Chapter 2; Table 2.3). A blinded surgeon and radiologist scored the radiographs. Mean scores were recorded.

Horses were then euthanized and DIT and TMT joint SF were obtained within 30 minutes. Samples from the DIT joint were collected on the medial aspect of the limb, just distal to the cunean tendon, at the junction of the central, third tarsal bone, and second tarsal bones. The TMT joint SF samples were collected on the plantarolateral aspect of the limb, just proximal to the head of the fourth metatarsal bone. SF samples were centrifuged, aliquoted, and stored at -80C° until further analyses.

Contrast radiography was performed on the limb of interest to determine presence of direct communication between tarsal joints. Three mL of iohexol (Omnipaque 240[e]) were injected into the TMT joints, with needle placement verified by radiographs. A

lateromedial radiograph was obtained to assess and record information. This was repeated for the DIT joint.

DIT and TMT joint SF was analyzed for several direct biomarkers using commercially available ELISAs. Carboxy-terminal propeptide of type II collagen (CPII[a]) was measured to represent type II collagen synthesis. Neoepitopes generated by collagenase cleavage of type II collagen (C2C[a]) as well as C-terminal crosslinked telopeptides of type II collagen (CTXII[b]) were measured for cartilage degradation. Aggrecan turnover was assessed using the chondroitin sulfate epitope 846 (CS846[a]) and early osteoblastic turnover was assessed using bone alkaline phosphatase (BAP[c]). Biomarkers were analyzed according to manufacturer instructions at appropriate dilutions.

MR imaging was performed on the DIT and TMT joints. All imaging was done on a GE Signa HD x 3.0 Tesla magnet. Prior to imaging the limbs of interest, the feasibility of MRI of the tarsus was evaluated in a pilot study using two cadaver legs; one leg with and one leg without clinical and radiographic evidence of OA in the distal tarsal joints (Figure 3.1; images A-E). For both the pilot and limbs of interest in the study population, the same protocol developed by a boarded radiologist, was used. T1-weighted images were acquired in the sagittal plane. Proton density and T1-weighted images were used to evaluate morphological features associated with OA (joint effusion, synovial proliferation, subchondral bone sclerosis, number and size of osteophytes). T2- weighted short tau inversion recovery (STIR) images were acquired in the sagittal plane to remove the signal from the subchondral bone fat and allow evaluation of subchondral bone hyperintensity. Thin slices 3-Dimensional Fast Spoiled Gradient Recall (3D FSPGR)

were acquired in the sagittal plane, allowing high-resolution multiplanar reconstruction in any plane. A multi-echo T2-relaxation mapping sequence was obtained in the sagittal plane. The 3D-FSPGR and T2-relaxation mapping images were used to evaluate cartilage. Each joint from the limb of interest was graded according to several criteria, modified from previous grading scales [113]. Details of the grading scheme are available in Table 3.2, with the maximum possible MRI score being 21. A boarded radiologist graded all MRIs.

Gaussian distribution could not be assumed and no transformation was appropriate for all data. Non-parametric Spearman's rho tests were used to determine correlations amongst all horses for: age, DIT and TMT radiographic scores, total MRI scores, subcategory MRI scores, and biomarker concentrations. Based on distal joint MRI scores, a clear line was evident to separate horses into subgroups of mild (score <7; DIT n= 5; TMT n=6) and moderate/severe (score ≥7; DIT n=6, TMT n=5) OA severity. Full data sets were not available for all biomarkers due to limitations in SF collection amount, and the number of horses in each group are indicated in the results section. The mild and moderate/severe OA groups were tested for normality using a Shapiro- Wilk normality test. Data that passed the Shapiro-Wilk normality test were analyzed using a parametric Unpaired T test with Welch's correction to compare ranks of biomarker concentrations between mild and moderate/severe OA groups. This included the following: BAP, CPII, C2C, and CTX II in the DIT joint, and C2C and CS846 in the TMT joint. Data that did not pass the Shapiro-Wilk normality test were analyzed using unpaired Mann-Whitney tests to compare ranks of biomarker concentrations between mild and moderate/severe OA severity groups. This included the following: CS846 in the

DIT joint; BAP, CPII, and CTX II in the TMT joint. MRI subgroups were compared between mild and moderate/severe OA groups in both the DIT and TMT joints using unpaired Mann-Whitney tests to compare ranks of grading categories. ROC analysis and Fisher's exact test were performed to discriminate between mild and moderate/severe groups in the DIT and TMT joints using sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio, and odds ratio. P values of ≤ 0.05 were considered significant using statistical software[f,g] when applicable.

Results

Eleven horses met inclusion criteria for case horses, ranging in age from 9-35 years old (mean=21 years). All eleven horses described in Chapter 2 had the DIT and TMT joints of the limb of interest analyzed. The horse data is summarized in Tables 2.1 and 2.2.

Radiographic scores for the DIT and TMT joints are listed in Table 3.1. In the DIT joint, radiographic scores ranged from 1-14.5 with a mean of 6.3, while the TMT joint radiographic scores ranged from 0.5 to 12.0 with a mean of 5.8. The DIT and TMT radiographic scores were positively correlated ($R=0.740$, $P= 0.009$).

MRI scores for the DIT and TMT joints are listed in Table 3.2. See Figures 3.2 and 3.3 for examples of MR images obtained from study horses. MRI scores for the distal joints were evaluated using the entire group (n=11) as well as by separating the OA severity in joints based on MRI scores into mild (<7) and moderate/severe (≥7). In the DIT joint, 5 horses had mild OA and 6 horses had moderate/severe OA. In the TMT joint, 6 horses had mild OA and 5 horses had moderate/severe OA.

In the DIT joint, MRI scores ranged from 3-15 with a mean score of 7.6 points. The DIT joint radiographic and MRI scores were positively correlated (R=0.840, P=0.001). However, further analysis of DIT MRI (Table 3.3) demonstrated that MRI images provided more detail about the OA changes present than radiographs. MRI demonstrated cartilage destruction in 9 horses (grades: 6 mild, 2 moderate, 1 severe). Mild joint effusion was present in 2 horses and moderate joint effusion was present in 1 horse. Synovial proliferation was not seen in any of the horses. Subchondral bone sclerosis was noted in all DIT joints (grades: 3 mild, 7 moderate, 1 severe) and when compared to radiographs, MRI identified greater severity of subchondral bone sclerosis in 4 horses. However, there was no significant difference in the severity of subchondral bone sclerosis between the horses in the mild versus moderate/severe OA groups. Subchondral bone hyperintensity was identified in 6 horses (grades: 4 mild, 2 severe). Results of DIT joint MRI categories mild OA (<7) and moderate/severe OA (≥7) groups are available in Table 3.3. Significant differences between groups were present in subchondral bone hyperintensity (P=0.022). Within MRI subcategories, subchondral bone hyperintensity was correlated to the number of osteophytes (R= 0.748, P= 0.012) and to the amount of cartilage destruction (R=0.630, P= 0.030); subchondral bone sclerosis was correlated to the number of osteophytes present (R=0.600, P= 0.050).

In the TMT joint, MRI scores ranged from 3-14 points with a mean of 7.2. The TMT joint radiographic and MRI scores were positively correlated (R=0.870, P= 0.001). However, further analysis of TMT MRI (Table 3.4) demonstrated that MRI provided more detail about the OA changes present than radiographs. MRI demonstrated 10 horses with cartilage destruction (grades: 6 mild, 3 moderate, 1 severe). Mild joint

effusion was identified in 2 horses, with no synovial proliferation seen in any of the horses. Subchondral bone sclerosis was noted in all horses (grades: 4 mild, 6 moderate, 1 severe) and when compared to radiographs, MRI identified greater subchondral bone sclerosis severity in 4 horses, and decreased severity in 1 horse. Subchondral bone hyperintensity was identified in 4 horses (grades: 2 mild, 1 moderate, 1 severe). Osteophytes were identified in all TMT joints on MRI and when compared to radiographs, additional osteophytes in number and size were only identified in 1 horse (horse 5). Results of TMT joint MRI subcategories mild OA (<7) and moderate/severe OA (≥7) groups are available in Table 3.4. Significant differences between groups were present in cartilage destruction severity (P= 0.013), SCB hyperintensity severity (P= 0.015), and the size of the largest osteophyte (P= 0.015). Within MRI categories, subchondral bone hyperintensity was correlated to the number of osteophytes (R= 0.876, P= 0.004) and the size of the largest osteophyte present (R= 0.712, P= 0.014). TMT joint cartilage destruction severity was also correlated to the size of the largest osteophyte (R= 0.754, P= 0.014).

When comparing the DIT and TMT joint MRI results, the DIT joint had mildly increased severity of OA but overall disease present was similar with mean total MRI scores of 7.6 and 7.2, and the scores correlated to each other (R=0.662, P= 0.030). Subchondral bone sclerosis was found in all DIT and TMT joints and their severity was correlated (R=0.699, P= 0.021). Nine horses had cartilage destruction in both joints concurrently but severity was not correlated. The number and size of osteophytes were similar between the joints but were not correlated.

The amount of SF collected from the DIT joints ranged from 0-2.0 mL with an average collection volume of 0.45 mL. The amount of SF collected from the TMT joints ranged from 0-1.0 mL with an average collection volume of 0.4 mL. Biomarker dilutions used and concentrations obtained for the distal joints are available in Tables 3.5 and 3.6. Correlations between biomarker concentrations are available in Table 3.7. The separation of DIT and TMT OA groups based on MRI scores into mild (<7) and moderate/severe (≥7) are represented graphically in Figures 3.4 and 3.5. In the DIT joint, 5 horses had mild OA and 6 horses had moderate/severe OA. In the TMT joint, 6 horses had mild OA and 5 horses had moderate/severe OA.

BAP concentrations from the DIT joint were available for 9 horses. As an entire group, DIT joint BAP concentrations were strongly correlated to total MRI scores (R= 0.932, P=0.001). The DIT joint BAP concentrations were also correlated to cartilage destruction (R= 0.772, P= 0.016), subchondral bone hyperintensity (R= 0.767, P= 0.025), number of osteophytes (R=0.757, P= 0.032), and the size of the largest osteophyte (R= 0.730, P= 0.048). When examining BAP concentrations after horses were split into DIT joint MRI scores of mild (n=4) and moderate/severe (n=5), moderate/severe horses had significantly higher BAP concentrations (mean of 33.2 U/L) than mild (mean of 13.57 U/L) horses (P= 0.039; Figure 3.4). Based on ROC analysis, a BAP concentration of 25 U/L was used to determine predictive value (0.048) for discriminating moderate/severe OA in the DIT joint (MRI score ≥ 7) yielding: sensitivity=80%, specificity=100%, PPV=100%, NPV=80%, a positive likelihood ratio of 8, and an odds ratio of 27. BAP concentrations in the TMT joint were higher in the moderate/severe OA group (mean of

24.84U/L) compared to the mild OA group (mean of 17.66 U/L) but were not statistically different (P= 0.904; Figure 3.5).

CPII concentrations from the DIT joint were available for 8 horses. As an entire group, DIT joint CPII concentrations were strongly correlated to DIT joint subchondral bone hyperintensity (R=0.809, P= 0.029) and to total DIT joint MRI scores (R=0.743, P=0.042). When examining CPII concentrations after horses were split into DIT joint MRI scores of mild (n=4) and moderate/severe (n=4), moderate/severe horses had significantly higher CPII concentrations (mean of 2387 ng/mL) than mild (mean of 1094 ng/mL) horses (P= 0.034; Figure 3.4). Based on ROC analysis, a CPII concentration of 1405 ng/mL was used to determine a predictive value (P= 0.028) for discriminating moderate/severe OA in the DIT joint (MRI score ≥7) yielding sensitivity=100%, specificity=100%, PPV = 100%, NVP= 100%. CPII concentrations from the TMT joint were available for 8 horses. As an entire group, the CPII concentrations correlated to total TMT joint MRI scores (R=0.634, P= 0.047). When examining CPII concentrations after horses were splint into TMT joint MRI scores of mild (n=6) and moderate/severe (n=2), moderate/severe horses had higher CPII concentrations (mean of 2880.5 ng/mL) than mild horses (mean of 1697.7 ng/mL), but were not significantly different (P=0.071; Figure 3.5).

C2C concentrations from the DIT joint were available for 10 horses. As an entire group, C2C concentrations correlated to DIT joint subchondral bone hyperintensity identified on MRI (R= 0.804, P= 0.010) and total DIT joint MRI score (R=0.640, P=0.046). When examining C2C concentrations after horses were split into DIT joint MRI scores of mild (n=5) and moderate/severe (n=5), moderate/severe horses (mean of

292.4 pmol/mL) had significantly higher C2C concentrations than mild (mean of 200.9 pmol/mL) horses (P= 0.008; Figure 3.4). Based on ROC analysis, a C2C concentration of 240 pmol/mL was used to determine a predictive value (P=0.038) for discriminating moderate/severe OA in the DIT joint (MRI score ≥ 7) yielding: 100% sensitivity, 100% specificity, PPV = 100%, NPV= 100%. In the TMT joint, C2C concentrations were higher in the mild OA group (mean of 224.3 pmol/mL) than the moderate/severe OA group (mean of 214.8 pmol/mL) but were not statistically different (P=0.762).

CTX II concentrations from the DIT joint were available for 9 horses. As an entire group, DIT joint CTX II concentrations were correlated to subchondral bone sclerosis severity identified on MRI (R= 0.689, P= 0.044). When examining CTX II concentrations after horses were split into mild (n=4) and moderate/severe (n=5) groups, moderate/severe horses had higher CTX II concentrations (mean of 25.29 pg/mL) than mild horses (mean of 17.11 pg/mL) but were not significantly different (P= 0.286; Figure 3.4). However, it should be noted that one horse in the mild group (horse 7) had a much higher value (48.57 pg/mL) than the rest of the mild horses (mean value of 6.63 pg/mL), potentially skewing the data. In the TMT joint, results were available for 7 horses. When examining CTX II concentrations after horses were divided into mild OA (n= 5), and moderate/severe OA (n=2) groups, the mild OA group had higher CTX II values (mean of 15.58 pg/mL) than the moderate/severe OA group (mean of 13.7 pg/mL), but were not statistically different (P=0.857; Figure 3.5).

CS846 concentrations from the DIT joint were available for 9 horses. When examining CS846 concentrations after horses were splint into DIT joint MRI scores of mild (n=4) and moderate/severe (n=5), mild OA horses had higher CS846 concentrations

(mean of 7104 ng/mL) than moderate/severe OA horses (mean value of 5720 ng/mL), but were not significantly different (P=0.114; Figure 3.4). In the TMT joint, CS846 concentrations were available for 9 horses. As an entire group, CS846 concentrations correlated to the amount of cartilage damage identified on MRI in the TMT joint (R= 0.697, P=0.032). When examining CS846 concentrations after horses were splint into TMT joint MRI scores of mild (n=6) and moderate/severe (n=3), mild OA horses had higher CS846 concentrations (mean of 6953 ng/mL) than moderate/severe OA horses (mean of 4356 ng/mL), but were not significantly different (P=0.547; Figure 3.5).

Contrast radiography identified direct communication between TMT and DIT joints in 4 horses (36%), with one (9%) of these also communicating with PIT and TT joints (Table 2.3). Two horses had mild DIT and TMT OA, and 2 horses had moderate DIT and TMT OA based on MRI scores. The presence of distal joint communication was not correlated with imaging or biomarker results. When looking at concentrations between the joints that communicated there were no similarities in concentrations between the joints for any of the biomarkers examined with the exception of C2C in 2 horses that had mild OA in both the DIT and TMT joints (horse 5 and 6).

Discussion

To the authors' knowledge, this is the first study that examined the correlation of OA disease severity in the distal tarsal joints of older horses with radiographs, MRI, and biomarkers of OA. We hope this provides a template for future studies of biomarkers in the equine tarsus. This study demonstrated that MRI provides superior detail compared to radiographs and could be useful in identifying early tarsal OA. Of the biomarkers examined BAP, CPII, and C2C correlated to DIT joint OA severity and could distinguish

moderate/severe from mild disease. In the TMT, none of the biomarkers analyzed could be used to determine moderate/severe OA from mild OA.

Radiographs of the DIT and TMT joints correlated to the MRI scores. The DIT joint had slightly more pathology than the TMT joint on both radiographs and MRI, similar to previous studies [51; 54; 114]. The variance in disease severity may be due to the difference in the bones that make up the DIT and TMT joints. The DIT joint is encompassed by small tarsal bones on both the proximal and distal articular surfaces. As the joint is loaded, forces are spread across the proximal and distal row of tarsal bones that have a small area to disperse such forces. The TMT joint has a proximal surface of small tarsal bones, but the distal surface is composed of the larger metatarsal bones, with the third metatarsal bone bearing most of the weight. The larger bone has more area to disperse forces placed upon it, protecting the TMT joint. Comparing radiographs to MRI, radiographs were a good indicator of OA for the distal tarsal joints in this population. 10 of 11 horses were put in the same mild or moderate/severe groupings for the DIT joint and 9 of 11 horses for the TMT joint based on the grading scales used. However, radiographs did underestimate the degree SCB hypointensity (sclerosis), number of osteophytes, and size of osteophytes in many of the cases.

The advantage to using MRI for the distal tarsal joints is to identify boney and cartilaginous abnormalities with greater sensitivity compared to radiographs, and to find OA changes unidentifiable on radiographs to allow early detection of OA. An area where MRI was more sensitive than radiographs was SCB sclerosis severity. Knowing the presence of SCB sclerosis could help clinically localize lameness to the tarsus. A previous study showed that when subchondral bone sclerosis is present in the tarsus,

intra-articular anesthesia does not always block out pain, altering the interpretation of the block [63]. In this study, SCB sclerosis was found in all DIT and TMT joints on MRI. On radiographs, SCB sclerosis was identified in most of the joints (10 of 11 DIT and TMT joints), however, the severity of SCB sclerosis found on MRI compared to radiographs was greater in 4 DIT joints and 3 TMT joints. There is evidence to support that SCB sclerosis precedes articular damage in OA [32; 40-42]. The mechanism of action for the initial subchondral bone changes likely originates with the joint receiving abnormal forces from either traumatic injury or chronic irregular weight bearing (such as poor conformation). The subchondral bone is damaged and thickens in the process of remodeling. The incongruities of areas of thick and normal subchondral bone create abnormal shear forces on the overlying cartilage that lead to cartilage damage and fibrillation [32]. In fact, in this population, SCB sclerosis severity had a higher grade than cartilage destruction in 9 of 11 DIT and TMT joints suggesting these joints have more SCB damage than articular damage. Therefore, early identification is valuable and could allow treatment or exercise changes before cartilage damage occurs.

Of the MRI subcategories evaluated, SCB hyperintensity separated the moderate/severe from mild OA in the DIT and TMT joints the best. Similar to previous studies, SCB hyperintensity had the greatest visualization on the T2-weighted STIR images [63; 115]. SCB hyperintensity can occur from any combination of bone marrow necrosis or fibrosis, and bone edema [63]. In humans, it has been noted that SCB hyperintensity viewed on T2 STIR images could be indicative of reactive sclerosis with newly formed fibrovascular tissue, versus mature sclerosis that often appears hypointense [65]. If horses act in a similar manner, identification of SCB hyperintensity could be a

good indicator of early and active disease since it has been stated that SCB hyperintensity of the distal tarsal joints may correlate with clinical lameness [63]. In the study population, SCB hyperintensity was found in 100% of the moderate/severe OA DIT joints and was not present in any of the mild DIT joints. In the TMT joint, SCB hyperintensity was found in 4 of 5 (80%) of the moderate/severe OA joints and was not present in any of the mild joints. These findings suggest that the presence of SCB hyperintensity is indicative of moderate/severe OA. Identifying SCB hyperintensity is a great advantage of MRI since it is not visualized on radiographs. Although our results clearly demonstrate the value of MRI when evaluating the distal tarsal joints, it is important to remember that the availability of the MRI is limited due to financial constraints. In addition, to get comparable images to those examined in this study, there is a need for anesthesia and the ability to get the distal tarsal joint far enough into magnets with smaller bores. It is unknown as to whether these changes could be identified using standing MRI units, but it is worth further investigation. The discrepancy in information gathered on MRI versus radiographs is an area where biomarkers have a potential valuable role, specifically in terms of SCB hyperintensity and cartilage damage.

Unlike previous studies, age did not correlate to any biomarker concentrations, [73; 116]. This discrepancy is most likely because this population consisted of all mature horses while many previous studies looked at younger horses still in the process of maturing musculoskeletally, either through skeletal growth or exercise-related development. One problem with examining an older population of horses, however, was that finding normal control horses is difficult since many horses in this age range have

distal tarsal joint OA. In addition, it is difficult to identify subtle OA changes in the distal tarsal joints since gross examination is unreliable due to the difficulty in disarticulating these joints. This is why mild and moderate/severe OA groups were identified in this study based on total MRI scores, even though the degree of OA could have been underestimated on MRI and radiographs, especially with regards to articular cartilage. While it would be ideal to have normal controls for comparison, it was felt that by dividing the horses into two groups based on MRI severity, it would be possible to see if the biomarkers could distinguish those with greater disease severity from milder changes.

In general, the biomarkers examined were able to distinguish the severity of OA in the DIT joint more than the TMT joint. There was less separation in the TMT joint than in the DIT joint when mild and moderate/severe OA groups were formed, lowering the statistical power and ability to distinguish a difference between groups. Additionally, less data was available for biomarkers of the TMT joint moderate/severe OA group for most of those examined compared to the DIT joint..

Of the biomarkers examined, those that represent synthesis of the matrices appeared to be the best indicators of OA in this population. In the DIT joint, BAP and CPII were the best at distinguishing OA severity, and had the highest correlation to total MRI DIT scores. In the TMT joint, CPII had the highest correlation to total TMT joint MRI scores. The up-regulation of synthetic biomarkers is indicative of early OA, while end stage OA results in an increase in degradation with a decreased production of bone and cartilage [35]. Therefore, despite MRI scores ranging from mild to severe, the global

degree of OA in these joints was not yet end stage, as they were still able to regenerate bone and cartilage.

BAP, a marker of early osteoblastic activity is increased in environments of bone turnover [82]. A value of 25 U/L was able to distinguish all but one horse with moderate/severe disease compared to those with mild OA. More importantly, BAP also correlated to several MRI subcategories such as SCB hyperintensity, a finding that cannot be identified via radiographs. Of the 6 horses with SCB hyperintensity, BAP values were available for 5 horses. Of the 5 horses, 4 of them had BAP values over 25 U/L. Therefore, BAP concentrations have the potential to provide important information about the severity of disease in the DIT joints of horses in which MRI is not an option. It is interesting to note that BAP concentrations reported in this study in the mild and moderate/severe DIT joint groups were similar to concentrations reported in previous studies of normal and diseased carpal and fetlock joints, respectively [59; 83]. Within the TMT joint, similar trends occurred but were not significant. While BAP from the TMT joint SF could also potentially be useful, only three samples were available for moderate/severe OA horses.

CPII measures an epitope of protein fragments produced when type II collagen is synthesized [70]. In early OA, cartilage production increases secondarily to cartilage degradation [117], which could be why CPII concentrations are increased. It has been shown in humans that as cartilage damage continues during OA, eventually synthesis cannot keep up with degradation and CPII concentrations are lower in end stage OA [71]. In this population, CPII was the only biomarker that correlated to OA severity in both of the distal tarsal joints; CPII concentrations from the DIT and TMT were correlated to

both total MRI and radiographic scores. This demonstrated that even as the disease severity increases, the CPII concentration continued to increase, showing the ability for these joints to still synthesize cartilage, indicating some capacity to heal. A CPII concentration of 1405 ng/mL in the DIT joint separated moderate/severe from mild OA joints in all horses. The DIT joint CPII concentrations in horses with mild OA were similar to values from previous studies in normal adult and young horse fetlock joints; moderate/severe OA DIT joint concentrations were similar to young horses with OC affected fetlock joints [73]. Both the DIT and TMT joint concentrations were higher than those found in young horses with normal and OCD affected TT joints [74], and lower than young horses with carpal bone fractures [72]. In humans, CPII was able to predict successive radiographic changes in early knee OA [17]. In this study, we found CPII was correlated to both radiographic and MRI scores of the distal joints, so in future studies it would be interesting to follow horses out for longer periods of time starting earlier in the OA disease process to see if there are similar trends.

C2C was the best biomarker of degradation examined as it correlated to total DIT joint MRI scores and DIT joint subchondral bone hyperintensity. C2C measures the degradation of type II collagen by identifying the neoepitope created after collagenase cleavage of the collagen triple helix at the ¾ fragment of the carboxy terminus region [16]. The cleavage of type II collagen is an abnormal finding in a joint and increases when the cartilage matrix is disturbed, as occurs in OA [80]. In this study population, a DIT joint C2C concentration of 240 pmol/mL was able to distinguish moderate/severe horse from all mild OA horses. C2C concentrations in the DIT joint were also strongly correlated to SCB hyperintensity, making it a potentially useful biomarker clinically.

Within the DIT joint, C2C concentrations correlated to CPII concentrations (Table 3.7), demonstrating the simultaneous cartilage degeneration and regeneration occurring in the joint. C2C concentrations of the mild OA DIT joints were similar to C2C values found in synovial fluid from normal adult horse (median age 4 years) metacarpophalangeal joints [73]. C2C concentrations of the moderate/severe OA DIT joints were similar to metacarpophalangeal joints with osteochondral injury [73], and to young horses (median age 2) with carpal bone fractures [72]. Interestingly, C2C did not distinguish OA severity in the TMT joint, despite similar amounts of cartilage damage determined on MRI. However, as mentioned previously, the amount of cartilage damage may have been underestimated on MRI. It is possible that there could have been more pathology in the DIT joint compared to the TMT joint than determined in the study. Further research is warranted.

The two biomarkers that did not correlate to total MRI scores or separate moderate/severe from mild OA in the distal tarsal joints were CTX II and CS846. CTX II is a catabolic biomarker that is a measurement of type II collagen degradation [69]. It should be noted that with the exception of one value (horse 7, CTX II = 48.57 pg/mL), mild OA horses had lower CTX II concentrations (< 10 pg/mL) than the moderate/severe groups (>20 pg/mL). While significant differences were not noted between groups, CTX II is a biomarker that would be worth studying further. The CTXII concentrations in the DIT and TMT joints in both mild and moderate/severe OA categories were lower compared to a previous studies evaluating CTX II in the middle carpal and metacarpophalangeal joints in young horses with osteochondral injury [78], but were similar to values in another study in horses that developed traumatic OA of the

metacarpophalangeal joint from time of injury to two years after [79]. The lack of success of CTX II to distinguish OA severity in this study could be from the low number of horses enrolled or because the joints are still predominately in the anabolic stage of OA. Future studies with long-term follow up would be beneficial to evaluate if CTX II increases as OA progresses. CS846 is a biomarker that measures fetal forms of aggrecan that is normal in young animals, but only seen in adult animals when repairing cartilage [80]. CS846 concentrations in this study did not correlate to MRI scores and were not different among mild and moderate/severe DIT or TMT joints in this study, again, possibly due to underestimating OA severity on MRI. The concentrations of the groups were similar to those of polo ponies that developed arthritis in the metacarpophalangeal joints within 24 months compared to horses that did not develop OA [81] and to 2 year old horses with induced OA in the middle carpal joints after about 50 days of treadmill exercise [76]. The concentrations in the mentioned studies showed elevations of CS846 overtime. The older population in this study may have already had initial CS846 concentration increases and are now in a chronic steady state. Further research is warranted to follow older horses out for a longer period of time to see if CS846 concentrations would elevate further, or decline as the disease progresses.

In joints with direct anatomic communication, biomarker concentrations between the joints were not similar. We demonstrated similar communication patterns as those reported in previous studies between the DIT and TMT (36%), as well as up to the PIT and TT Joints (9%) [109; 110]. In the study population, it appears that each joint has its own microenvironment and the biomarker concentrations reflected pathology specific to each individual DIT or TMT joints. The number of horses with communication in this

study was low, so further research is needed to determine the impact of anatomical joint communication on biomarker concentrations.

A potential clinical challenge of using biomarkers in the distal tarsal joints could come in the ability to collect enough synovial fluid. The following amounts of SF are needed to run the following biomarkers of interest, in duplicates: 0.05 mL for C2C, 0.032 mL for CPII, and 0.04 mL for BAP; totaling 0.122 mL. A mean SF sample of 0.4 mL was collected in the DIT and TMT joints in this study, but SF sample collection was performed after euthanasia, allowing larger needles to be used for collection than may be possible in the live horse. With that said, it is still possible to get fluid back when performing synoviocentesis or joint injections on the distal tarsal joints in some clinical cases. However, a recent study showed that accuracy of getting a needle into the TMT joint is high (96%), while much lower in the DIT joint (42%) [118], so radiographic guidance may help with accurate needle placement for SF collection. In this study, the amount of SF collected from the distal joints limited the number of biomarkers that could be evaluated. Future studies could look at additional direct and indirect biomarkers. Another limitation of this study was that only a small number of horses were enrolled. Further research is warranted with a larger number of horses. In addition, it is difficult to obtain disease-free controls, as many older horses have a degree of OA in the distal tarsal joints, so further studies may have to divide the population into mild and moderate/severe OA as done here. Finally, the focus of this study was on the amount of OA present on MRI compared to biomarker concentrations, not clinical lameness. Future research with horses with lameness localized to the tarsus would be advantageous.

Conclusions: Radiographs of the DIT and TMT joints correlated to the corresponding MRIs in this study but underestimated the degree of SCB bone sclerosis, and number and size of osteophytes in many of the cases. MRI was more sensitive in identifying these abnormalities and provided information about cartilage damage and SCB hyperintensity. The severity of SCB sclerosis and presence of SCB hyperintensity was a good indicator for separating moderate/severe from mild OA. Of the biomarkers evaluated, synthetic biomarkers were the best in identifying OA and separating moderate/severe from mild disease. The biomarkers of synthesis with the highest correlation to disease in the DIT joint were BAP and CPII and in the TMT was CPII. Of the catabolic biomarkers, C2C correlated to total MRI score and to CPII in the DIT joint. In the DIT joint, BAP, CPII, and C2C also correlated to SCB hyperintensity. This demonstrates the potential use of biomarkers to give information relevant to detecting early OA that is not visible on radiographs. Early identification of OA could help guide treatment and training regimens to better shape the career and longevity of the equine athlete.

Chapter 4:
The use of biomarkers from the tibiotarsal joint to determine osteoarthritis in the distal intertarsal and tarsometatarsal joints in horses

The tarsus is comprised of 5 joints: the tibiotarsal (TT), proximal intertarsal (PIT), distal intertarsal (DIT), tarsometatarsal (TMT), and talocalcaneal (TC) joints. The DIT and TMT joints (collectively termed "distal tarsal joints") are prone to developing osteoarthritis (OA). Biomarkers of OA can be collected from the synovial fluid from the joint of interest and measured (Chapters 2 and 3). The main challenge in using biomarkers to measure OA in the distal tarsal joints is the ability to collect synovial fluid due to their small size. However, the tibiotarsal (TT) joint is much larger and repeated synoviocentesis is easily achievable. The TT joint and proximal intertarsal (PIT) joint always communicate anatomically. The distal tarsal joints communicate up to 36% and direct communication between the TT joint and distal hock joints is uncommon, ranging from 1-4% [109; 110]. However, functional communication may be increased between the proximal and distal tarsal joints. It has been shown in a previous study that when smaller molecules like steroids are examined versus using traditional latex or Iohexol to determine joint communication, functional communication in the distal tarsal joints was found to be up to 100% [119]. Therefore, it may be possible for small biomarkers of OA to diffuse between joints in a similar manner. Clinically, it has been noted that after distal joints are treated with corticosteroids in some horses, effusion in the tibiotarsal joint decreases. If synovial fluid from the tibiotarsal joint can provide information about disease in the distal tarsal joints, it could serve as a quantitative method of evaluating OA in the DIT and TMT joints and provide a means for veterinarians to have an objective method to diagnose, stage, and determine response to treatment of OA. The first

objective of this research was to see if SF from the TT joint could be used to assess OA in the distal joints by comparing specific SF biomarker concentrations in the TT/PIT joints from older horses with varying degrees of OA and determine if they correlate to the following: (1) the degree of PIT, DIT, or TMT joint OA identified on radiographs, (2) the degree of PIT, DIT, or TMT OA identified on MRI, (3) specific SF biomarker concentrations from the DIT and TMT joints.

Materials and methods

Refer to Chapters 2 and 3 for detailed materials and methods. The PIT joints from the legs of interest were also imaged with MRI and graded under the same protocol as the DIT and TMT joints. Biomarkers examined in the TT/PIT joints are listed in Tables 2.6 and 2.7 and the biomarkers examined in the distal joints are listed in Tables 3.5 and 3.6.

Gaussian distribution could not be assumed and no transformation was appropriate for all data. Non-parametric Spearman's rho tests were used to determine correlations between the proximal (TT and PIT) joints and the distal (DIT and TMT) joints for: radiographic scores (TT, PIT, DIT, TMT joints), MRI scores (PIT, DIT, TMT joints), and biomarker concentrations (TT/PIT, DIT, TMT joints). Non-parametric Mann Whitney tests were used to compare mild (MRI score <7) and moderate/severe (MRI score ≥7) DIT and TMT joints (Chapter 3) using TT SF values.

Results

Radiographic scores for the TT, PIT, DIT, and TMT joints are listed in Table 2.3. Radiographic scores from the proximal joints did not correlate to radiographic scores of the distal joints. Radiographic scores in the proximal joints were lower compared to the distal joints and were highest in the DIT joint. The mean radiograph scores for the joints of interest were: 0.5 in the TT joint, 2.6 in the PIT joint, 6.3 in the DIT joint, and 5.8 in the TMT joint.

MRI scores were available for the PIT, DIT, and TMT joints. Specific MRI findings for the distal joints are available in the Chapter 3 results section. PIT joint radiographic and MRI scores are available in Table 4.1. No correlations existed between the distal joints and the PIT joint MRI scores. Similar to the distal joints, additional pathology was identified in the PIT joint on MRI compared to radiographs. In the PIT joint, MRI scores ranged from 1-8 with a mean of 4.2 out of a possible 21 points. Joint effusion was noted in 5 horses (4 mild, 1 moderate). Mild cartilage destruction was identified in 3 horses. Synovial proliferation was identified in 3 horses (2 mild, 1 severe). Subchondral bone sclerosis was noted in all PIT joints and when compared to radiographic findings, the severity was greater in 5 horses. Subchondral bone hyperintensity was not identified in any PIT joints. The number of osteophytes found on MRI compared to radiographs was decreased, with more found on radiographs in 3 horses. The size of the largest osteophyte in the same 3 horses in which additional osteophytes were found was also graded larger on radiographs compared to MRI.

Comparing PIT joint MRI results to the distal joints, globally less disease was present. Overall mean MRI scores were lowest in the PIT joint. Cartilage damage was less severe compared to the distal joints in 7 horses. In contrast to the distal joints, no subchondral bone hyperintensity was found around any PIT joints. Subchondral bone sclerosis in the PIT joint was similar to the distal joints. A decreased number of osteophytes were found in 8 of the PIT joints.

OA in the TT joint was assessed by arthroscopy and gross examination (Table 2.4). TT joint arthroscopy scores negatively correlated to DIT radiographic scores (R= -0.620; P=0.042) and did not correlate to PIT or TMT joint radiographic scores. Arthroscopy scores did not correlate to PIT, DIT, or TMT joint MRI scores.

Correlations between proximal and distal tarsal joint biomarker concentrations are available in Table 4.2. In summary, all correlations identified were negative. When TT joint biomarkers were assessed based on the severity groupings of the distal joints that were determined by MRI (i.e. mild or moderate/severe), TT concentrations for all biomarkers were unable to distinguish any difference between the groups (Figures 4.1-4.4).

Discussion

Biomarkers have a potential role in identifying early OA in the distal tarsal joints of horses (Chapter 3) but a clinical limitation is the amount of synovial fluid that can be collected from the small DIT and TMT joints. In contrast, synoviocentesis is easy in the TT joint. It would be ideal if there were a biomarker that could be measured from TT SF that correlated to distal tarsal joint OA. However, the results of this study did not identify such a biomarker. Of the biomarkers examined from the TT SF, none of them correlated

to disease in the distal joints. Biomarkers of the TT SF only had negative correlations to biomarkers from the distal tarsal joints (Table 4.2).

When evaluating the TT joint biomarker concentrations based on the DIT and TMT OA severity (mild <7 MRI score, moderate/severe ≥ 7 MRI score) groups, there were no differences observed between groups (Figures 4.1-4.4). In addition, TT joint biomarker concentrations did not correlate to distal tarsal OA severity on MRI, and imaging of the TT joint (radiographs, arthroscopy, gross pathology) did not correlate to distal joint imaging (radiographs, MRI).

In the study population, 4 horses had communication between joints, 3 between the DIT and TMT joints, and 1 with communication from the distal joints all the way to the TT joint. Similar to findings in Chapter 2 and 3, joints with anatomic communication determined by contrast radiology did not have similar biomarker concentrations among them. Further research is warranted to determine the role of joint communication and OA.

Limitations: Only a small number of horses were enrolled in the study. In addition, it is difficult to obtain disease-free controls, as many older horses have a degree of OA in the distal tarsal joints, so further studies may have to divide the population into mild and moderate/severe OA as done here. Finally, the focus of this study was on amount OA present on MRI compared to biomarker values, not clinical lameness. Future research with horses with lameness localized to the tarsus would be advantageous. A larger sample size with a broader range of OA would be ideal to evaluate biomarker value trends.

In conclusion, the results of this study suggest that SF from the TT joint cannot be used to identify OA in the distal tarsal joints. It appears that information about OA in the

distal joints must be collected from the DIT and TMT joints themselves even if anatomical communication is present.

Chapter 5:

Future Directions

The research of this thesis focused on both imaging and biomarkers of OA in the equine tarsus - an anatomic location in the horse that is prone to the development of OA and subsequent lameness that limits careers [99]. The focus of this research was twofold: 1) Imaging of the tarsus with radiography and MRI and 2) Correlating biomarkers of OA to disease found by radiographs, arthroscopy (TT joint), gross examination (TT joint), and MRI (PIT, DIT, TMT joints).

In the TT Joint, the amount of OA identified on radiographs was compared to the amount of OA identified in arthroscopy and gross examination. In the PIT, DIT, and TMT joints, radiographs were compared to pathology found on MRI. In the TT joint, radiographs were a poor indicator of disease. The TT joint radiographs were all normal or showed subtle changes, but arthroscopy and gross examination scores ranged from mild to severe. Arthroscopy remains the gold standard to assess the TT joint, allowing direct visualization of the cartilage. Arthroscopy requires general anesthesia that is a risk factor for older horses [60] and can be cost prohibitive. In this study, even with the use of arthroscopy, additional lesions were found after joint disarticulation. Additional lesions were found in 7 of 11 horses: 4 in the proximity of the synovial fossa of the distal tibia and 3 elsewhere in the joint not visible on arthroscopy. Future studies should also look at MRI in the tibiotarsal joint to see the degree of accuracy it provides in identifying cartilaginous lesions. Further research is warranted to determine the impact cartilage lesions have on lameness, if they can be identified on MRI, and if there is a biomarker that correlates to presence of such lesions. High-field strength MRI magnets should be

used initially to provide the best chance at identifying subtle lesions, and if they are identifiable, standing low-field MRI should be evaluated to determine its efficacy. If a standing low-field MRI is a valid imaging modality for this purpose, horses could avoid having to undergo general anesthesia. Additionally, histopathology should be conducted on lesions present at the synovial fossae to determine if they represent pathology or are variations of normal.

In the distal tarsal joints, radiographs were significantly better at identifying disease than in the TT joint. Radiographic scores correlated to MRI scores in both the DIT and TMT joints. However in this population of horses, factors that contributed to the identification of early OA were: the presence of subchondral bone hypointensity (sclerosis) and subchondral bone hyperintensity. Subchondral bone sclerosis was identified on both radiographs and MRI, but the latter allowed identification with higher sensitivity. Subchondral bone hyperintensity is only visualized on MRI, and is not seen on radiographs. 100% of the DIT joints and 80% of the TMT joints in the moderate/severe OA groups had hyperintensity on MRI, while none of mild OA horses had hyperintensity in either distal tarsal joints. This finding could be crucial in identifying moderate/severe OA from early OA. More research is needed looking at OA and the presence/absence of subchondral bone hyperintensity. Similar to the TT joint mentioned above, the use of standing MRI could be very beneficial in this population of horses and should also be researched to see if the lower field strength magnets can identify subchondral bone hyperintensity. Therefore, while radiographs had the horses in similar categories of overall mild versus moderate/severe OA, MRI was able to pick up

specific abnormalities that could allow the clinician to detect OA earlier in the disease process, and prescribe treatment and exercise regimes appropriately.

OA in the distal tarsal joints may have been underestimated on MRI as well. It would be ideal to disarticulate the distal tarsal joints to allow for direct visualization of the articular surfaces, and sample for histopathology. This is extremely difficult in horses with OA as bridging osteophytes are present and disarticulation results in iatrogenic damage.

In terms of biomarkers, more research is needed with a larger number of horses. In the TT joint, C2C and IL-6 were the biomarkers that correlated to disease severity. In the DIT joint, BAP, CPII, and C2C were the best, with potential for CTX II, and in the TMT joint, CPII was the best biomarker to identify OA severity. However, only 11 horses were included in the study. Further breakdown of groups was made within the 11 horses to compare mild to moderate/severe disease, further decreasing the statistical power. Future research could use this study design as a template to contribute more biomarker results. Additionally, enrolling horses with a wider range of disease severity would be beneficial. Biomarkers of synthesis were the best in these joints, meaning they were still in stages of anabolism. In end stage OA, catabolism predominates, and biomarkers would be expected to change. It would be nice to follow horses from early/no OA to severe/end stage OA and to track biomarkers during disease progression as long as possible.

The volume of SF collected limited the number of biomarkers evaluated in the distal tarsal joints. Future studies could examine the same biomarkers as in this study, as well as more direct biomarkers and include indirect biomarkers. SF was easily collected

from the TT joint, so more biomarkers were examined. Biomarkers that were examined in the TT joint but not the distal joints were: direct biomarker C12C, and indirect biomarkers IL-1β, IL-6, Il-8, IL-10, and TNFα. For all the joints, additional biomarkers examined in other studies should be considered including matrix metalloproteinases (MMPs), and hyaluronic acid (HA) [76; 79; 83; 84; 100-102; 105; 116; 120-124].

A large limitation of this research was the lack of true controls. It is hard to get older horses without OA in the tarsus, which is why groups were broken down into mild and moderate/severe disease. It would be ideal to have controls in future studies, but similar breakdowns might be necessary as studies using younger horses showed that age influenced biomarker values [73; 100]. Also, we did not have the luxury of enrolling horses that had lameness localized to the tarsus, so focus was placed on disease severity determined by imaging, not lameness. In future studies, localizing the lameness to the tarsus with diagnostic local analgesia would be beneficial to correlate clinical findings to imaging and biomarkers, because ultimately, the ideal purpose of sampling biomarkers would be for interpretation of disease status in clinical cases.

A substantial amount of research is still required to understand the role of biomarkers and their potential value clinically in the equine tarsus. To the authors' knowledge, this is the first study examining biomarkers of OA in the TT/PIT, DIT, and TMT joints in an older horse population. We hope this study provides a template for future studies.

CHAPTER 6:

FOOTNOTES

a IBEX Technologies, Inc. Montréal, Québec.

b Serum Pre-Clinical CartiLaps IDS-Nordic. Helve, Denmark.

c Microfuge BAP. Quidel Corporation. San Diego, CA.

d Genorise Scientific, INC. Glen Mills, PA.

e Omnipaque 240 (Iohexol 518mg/mL) from GE Healthcare, Princeton, NJ.

f IBM SPSS Statistics Software. Armonk, NY.

g GraphPad Prism 7.0b. GraphPad Software, Inc. La Jolla, CA.

CHAPTER 7:

REFERENCES

[1] Goldring, S.R. and Goldring, M.B. (2006) Clinical aspects, pathology and pathophysiology of osteoarthritis. *Journal of musculoskeletal & neuronal interactions* **6**, 376-378.

[2] Frisbie, D.D. (2012) Chapter 78: Synovial Joint Biology and Pathobiology In: *Equine Surgery*, 4th edn., Ed: J.A. Auer, Stick, J.A. Elsevier (Saunders), St. Louis, Missouri. pp 1096-1114.

[3] Lee, D.W., Banquy, X. and Israelachvili, J.N. (2013) Stick-slip friction and wear of articular joints. *Proceedings of the National Academy of Sciences of the United States of America* **110**, E567-574.

[4] Jay, G.D. and Waller, K.A. (2014) The biology of lubricin: near frictionless joint motion. *Matrix biology: Journal of the International Society for Matrix Biology* **39**, 17-24

[5] Gleghorn, J.P., Jones, A.R., Flannery, C.R. and Bonassar, L.J. (2009) Boundary mode lubrication of articular cartilage by recombinant human lubricin. *Journal of orthopaedic research: official publication of the Orthopaedic Research Society* **27**, 771-777.

[6] Flannery, C.R., Hughes, C.E., Schumacher, B.L., Tudor, D., Aydelotte, M.B., Kuettner, K.E. and Caterson, B. (1999) Articular cartilage superficial zone protein (SZP) is homologous to megakaryocyte stimulating factor precursor and Is a multifunctional proteoglycan with potential growth-promoting, cytoprotective, and lubricating properties in cartilage metabolism. *Biochemical and biophysical research communications* **254**, 535-541.

[7] Schmidt, T.A., Schumacher, B.L., Klein, T.J., Voegtline, M.S. and Sah, R.L. (2004) Synthesis of proteoglycan 4 by chondrocyte subpopulations in cartilage explants, monolayer cultures, and resurfaced cartilage cultures. *Arthritis Rheum* **50**, 2849-2857.

[8] Jahn, S., Seror, J. and Klein, J. (2016) Lubrication of Articular Cartilage. *Annual review of biomedical engineering* **18**, 235-258.

[9] Houard, X., Goldring, M.B. and Berenbaum, F. (2013) Homeostatic mechanisms in articular cartilage and role of inflammation in osteoarthritis. *Current rheumatology reports* **15**, 375.

[10] Sophia Fox, A.J., Bedi, A. and Rodeo, S.A. (2009) The basic science of articular cartilage: structure, composition, and function. *Sports health* **1**, 461-468.

[11] O'Hara, B.P., Urban, J.P. and Maroudas, A. (1990) Influence of cyclic loading on the nutrition of articular cartilage. *Ann Rheum Dis* **49**, 536-539.

[12] Alford, J.W. and Cole, B.J. (2005) Cartilage restoration, part 1: basic science, historical perspective, patient evaluation, and treatment options. *The American journal of sports medicine* **33**, 295-306.

[13] Buckwalter, J.A. and Mankin, H.J. (1998) Articular cartilage: tissue design and chondrocyte-matrix interactions. *Instructional course lectures* **47**, 477-486.

[14] Farnum, C.E. and Wilsman, N.J. (2011) Orientation of primary cilia of articular chondrocytes in three-dimensional space. *Anatomical record (Hoboken, N.J. : 2007)* **294**, 533-549.

[15] Mayne, R. (1989) Cartilage collagens. What is their function, and are they involved in articular disease. *Arthritis Rheum* **32**, 241-246.

[16] Billinghurst, R.C., Dahlberg, L., Ionescu, M., Reiner, A., Bourne, R., Rorabeck, C., Mitchell, P., Hambor, J., Diekmann, O., Tschesche, H., Chen, J., Van Wart, H. and Poole, A.R. (1997) Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *The Journal of clinical investigation* **99**, 1534-1545.

[17] Sugiyama, S., Itokazu, M., Suzuki, Y. and Shimizu, K. (2003) Procollagen II C propeptide level in the synovial fluid as a predictor of radiographic progression in early knee osteoarthritis. *Annals of Rheumatic Diseases* **62**, 27-32.

[18] Teshima, R., Otsuka, T., Takasu, N., Yamagata, N. and Yamamoto, K. (1995) Structure of the most superficial layer of articular cartilage. *The Journal of bone and joint surgery. British volume* **77**, 460-464.

[19] Oegema, T.R., Jr., Carpenter, R.J., Hofmeister, F. and Thompson, R.C., Jr. (1997) The interaction of the zone of calcified cartilage and subchondral bone in osteoarthritis. *Microscopy research and technique* **37**, 324-332.

[20] Lane, L.B., Villacin, A. and Bullough, P.G. (1977) The vascularity and remodelling of subchondrial bone and calcified cartilage in adult human femoral and humeral heads. An age- and stress-related phenomenon. *The Journal of bone and joint surgery. British volume* **59**, 272-278.

[21] Burnstein, A.H., Wright, T.M. (1994). In: *Fundamentals of Orthopaedic Biomechanics*, Williams and Wilkins, The University of Michigan. pp 73-86.

[22] Goldring, S., Goldring M.B. (2017) Chapter 1: Biology of the Normal Joint. In: *Kelly and Firestein's Textbook of Rheumatology* 10th edn., Ed: G.S. Firestein, Budd, R.C., Gabriel, S.E., McInnes, I.B., O'Dell, J.R., Elsevier, Inc., Philadelphia, PA. pp 1-17.

[23] Li, G., Yin, J., Gao, J., Cheng, T.S., Pavlos, N.J., Zhang, C. and Zheng, M.H. (2013) Subchondral bone in osteoarthritis: insight into risk factors and microstructural changes. *Arthritis research & therapy* **15**, 223.

[24] Milz, S. and Putz, R. (1994) Quantitative morphology of the subchondral plate of the tibial plateau. *Journal of anatomy* **185 (Pt 1)**, 103-110.

[25] Caron, J.P. (2010) Osteoarthritis. In: *Diagnosis and Management of Lameness in the Horse*, 2nd edn., Ed: M.W. Ross, Dyson, S.J., Saunders, an imprint of Elsevier, Inc., St. Louis, MO. pp 655-668.

[26] Knowlton, R.G., Katzenstein, P.L., Moskowitz, R.W., Weaver, E.J., Malemud, C.J., Pathria, M.N., Jimenez, S.A. and Prockop, D.J. (1990) Genetic linkage of a polymorphism in the type II procollagen gene (COL2A1) to primary osteoarthritis associated with mild chondrodysplasia. *The New England journal of medicine* **322**, 526-530.

[27] Goldenberg, D.L., Egan, M.S. and Cohen, A.S. (1982) Inflammatory synovitis in degenerative joint disease. *The Journal of rheumatology* **9**, 204-209.

[28] Kohlhof, H., Gravius, S., Kohl, S., Ahmad, S.S., Randau, T., Schmolders, J., Rommelspacher, Y., Friedrich, M. and Kaminski, T.P. (2016) Single Molecule Microscopy Reveals an Increased Hyaluronan Diffusion Rate in Synovial Fluid from Knees Affected by Osteoarthritis. *Scientific reports* **6**, 21616.

[29] Scanzello, C.R. and Goldring, S.R. (2012) The role of synovitis in osteoarthritis pathogenesis. *Bone* **51**, 249-257.

[30] Schmidt, T.A., Gastelum, N.S., Nguyen, Q.T., Schumacher, B.L. and Sah, R.L. (2007) Boundary lubrication of articular cartilage: role of synovial fluid constituents. *Arthritis and Rheumatology* **56**, 882-891.

[31] Svala, E., Jin, C., Ruetschi, U., Ekman, S., Lindahl, A., Karlsson, N.G. and Skioldebrand, E. (2017) Characterisation of lubricin in synovial fluid from horses with osteoarthritis. *Equine Veteinary Journal* **49**, 116-123.

[32] Radin, E.L. and Rose, R.M. (1986) Role of subchondral bone in the initiation and progression of cartilage damage. *Clinical orthopaedics and related research*, 34-40.

[33] Suri, S., Gill, S.E., Massena de Camin, S., Wilson, D., McWilliams, D.F. and Walsh, D.A. (2007) Neurovascular invasion at the osteochondral junction and in osteophytes in osteoarthritis. *Annals of Rheumatic Diseases* **66**, 1423-1428.

[34] Fernandes, J.C., Martel-Pelletier, J. and Pelletier, J.P. (2002) The role of cytokines in osteoarthritis pathophysiology. *Biorheology* **39**, 237-246.

[35] McIlwraith, C.W. (2010) Use of synovial fluid and serum biomarkers in equine bone and joint disease: a review. *Equine Veterinary Journal* **37**, 473-482.

[36] Goldring, S.R. and Goldring, M.B. (2004) The role of cytokines in cartilage matrix degeneration in osteoarthritis. *Clinical orthopaedics and related research*, S27-36.

[37] Goldring, M.B. (2001) Anticytokine therapy for osteoarthritis. *Expert opinion on biological therapy* **1**, 817-829.

[38] Cuellar, J.M., Scuderi, G.J., Cuellar, V.G., Golish, S.R. and Yeomans, D.C. (2009) Diagnostic utility of cytokine biomarkers in the evaluation of acute knee pain. *The Journal of bone and joint surgery. American volume* **91**, 2313-2320.

[39] Bobacz, K., Sunk, I.G., Hofstaetter, J.G., Amoyo, L., Toma, C.D., Akira, S., Weichhart, T., Saemann, M. and Smolen, J.S. (2007) Toll-like receptors and chondrocytes: the lipopolysaccharide-induced decrease in cartilage matrix synthesis is dependent on the presence of toll-like receptor 4 and antagonized by bone morphogenetic protein 7. *Arthritis and Rheumatology* **56**, 1880-1893.

[40] Carlson, C.S., Loeser, R.F., Jayo, M.J., Weaver, D.S., Adams, M.R. and Jerome, C.P. (1994) Osteoarthritis in cynomolgus macaques: a primate model of naturally occurring disease. *Journal of orthopaedic research: official publication of the Orthopaedic Research Society* **12**, 331-339.

[41] Dieppe, P., Cushnaghan, J., Young, P. and Kirwan, J. (1993) Prediction of the progression of joint space narrowing in osteoarthritis of the knee by bone scintigraphy. *Annals of Rheumatic Diseases* **52**, 557-563.

[42] Bailey, A.J. and Mansell, J.P. (1997) Do subchondral bone changes exacerbate or precede articular cartilage destruction in osteoarthritis of the elderly? *Gerontology* **43**, 296-304.

[43] Gondim Teixeira, P.A., Balaj, C., Marie, B., Lecocq, S., Louis, M., Braun, M. and Blum, A. (2014) Linear signal hyperintensity adjacent to the subchondral bone plate at the knee on T2-weighted fat-saturated sequences: imaging

aspects and association with structural lesions. *Skeletal radiology* **43**, 1589-1598.

[44] Zubrod, C.J., Schneider, R.K., Hague, B.A., Ragle, C.A., Gavin, P.R. and Kawcak, C.E. (2005) Comparison of three methods for arthrodesis of the distal intertarsal and tarsometatarsal joints in horses. *Veterinary surgery : VS* **34**, 372-382.

[45] Kawcak, C.E. (2016) Chapter 24: *Tarsus*. In: *Joint Disease in the Horse,* 2nd edn., Ed: McIlwraith, W., Frisbie, D.D., Kawcak, C.K., van Weeren, R. Elsevier, St. Louis, Missouri. pp 340-353.

[46] Dabareiner, R.M., Carter, G.K. and Dyson, S.J. (2003) The tarsus. In: *Diagnosis and Management of Lameness in the Horse*, 1st edn., Eds: M.W. Ross and S.J. Dyson, Saunders, St. Louis, MO. pp 440-449.

[47] Baxter, G.M., Southwood, L. L., Dechant, J. E. (2003) Diagnosis of distal tarsal osteoarthritis in horses. *Compendium on Continuing Education for the Practicing Veterinarian* **2**, 138-147.

[48] Butler, J.A., Colles, C. M., Dyson, S. J., Kold, S. E., Poulos, P. W. (2000) Chapter 6: The tarsus. In: *Clinical radiology of the horse*, 2nd edn., Blackwell Science Oxford. pp 247-284.

[49] Labens, R., Innocent, G.T. and Voute, L.C. (2007) Reliability of a quantitative rating scale for assessment of horses with distal tarsal osteoarthritis. *Veterinary radiology & ultrasound* **48**, 204-211.

[50] Verschooten, F., Schramme, M. (1994) Radiological examination of the tarsus. *Equine Veterinary Education* **6**, 323-332.

[51] Byam-Cook, K.L. and Singer, E.R. (2009) Is there a relationship between clinical presentation, diagnostic and radiographic findings and outcome in horses with osteoarthritis of the small tarsal joints? *Equine Veterinary Journal* **41**, 118-123.

[52] Hartung, K., Munzer, B., Keller, H. (1983) Radiologic evaluation of spavin in young trotters. *Veterinary radiology & ultrasound: the official journal of the American College of Veterinary Radiology and the International Veterinary Radiology Association* **24**, 153-155.

[53] van Hoogmoed, L.M., Snyder, J.R., Thomas, H.L. and Harmon, F.A. (2003) Retrospective evaluation of equine prepurchase examinations performed 1991-2000. *Equine Veterinary Journal* **35**, 375-381.

[54] Fairburn, A., Dyson, S. and Murray, R. (2010) Clinical significance of osseous spurs on the dorsoproximal aspect of the third metatarsal bone. *Equine Veterinary Journal* **42**, 591-599.

[55] Vanderperren, K., Raes, E., Bree, H.V. and Saunders, J.H. (2009) Diagnostic imaging of the equine tarsal region using radiography and ultrasonography. Part 2: bony disorders. *Veterinary journal (London, England : 1997)* **179**, 188-196.

[56] Sullins, K.E. (2011) Chapter 5: Lameness in the extremities- the Tarsus and Tibia. In: *Adams and Stashak's Lameness in Horses*, 6th edn., Ed: G.M. Baxter, Wiley-Blackwell, Ames, IA. pp 725-782.

[57] Garcia-Lopez, J.M. and Kirker-Head, C.A. (2004) Occult subchondral osseous cyst-like lesions of the equine tarsocrural joint. *Veterinary surgery : VS* **33**, 557-564.

[58] Hanson, J.A., Seeherman, H.J., Kirker-Head, C.A. and O'Callaghan, M.W. (1996) The role of computed tomography in evaluation of subchondral osseous lesions in seven horses with chronic synovitis. *Equine Veterinary Journal* **28**, 480-488.

[59] Trumble, T.N., Brown, M.R., Merritt, K.A. and Billinghurst, R.C. (2008) Joint dependent concentrations of bone alkaline phosphatase in serum and synovial fluids of horses with osteochondral injury: an analytical and clinical validation. *Osteoarthritis and Cartilage* **16**, 779-786.

[60] Muir, W.W. and E., H.J.A. (2009) Chapter 22: Anesthetic-associated complications. In: *Equine Anesthesia: Monitoring and Emergency Therapy*, 2 edn., Eds: W.W. Muir and J.A.E. Hubbell, Elsevier Inc., St. Louis, MO. pp 397-417.

[61] Blaik, M.A., Hanson, R.R., Kincaid, S.A., Hathcock, J.T., Hudson, J.A. and Baird, D.K. (2000) Low-field magnetic resonance imaging of the equine tarsus: normal anatomy. *Veterinary radiology & ultrasound* **41**, 131-141.

[62] Latorre, R., Arencibia, A., Gil, F., Rivero, M., Henry, R.W., Ramirez, G. and Vaquez, J.M. (2006) Correlation of magnetic resonance images with anatomic features of the equine tarsus. *American Journal of Veterinary Research* **67**, 756-761.

[63] Daniel, A.J., Judy, C.E., Rick, M.C., Saveraid, T.C. and Herthel, D.J. (2012) Comparison of radiography, nuclear scintigraphy, and magnetic resonance imaging for detection of specific conditions of the distal tarsal bones of horses: 20 cases (2006-2010). *Journal of the American Veterinary Medical Association* **240**, 1109-1114.

[64] Choi, J.A. and Gold, G.E. (2011) MR imaging of articular cartilage physiology. *Magnetic resonance imaging clinics of North America* **19**, 249-282.

[65] Kanberoglu, K., Kantarci, F. and Yilmaz, M.H. (2005) Reactive sclerosis: hyperintense appearance on T2-weighted magnetic resonance imaging. *Actaradiologica (Stockholm, Sweden : 1987)* **46**, 708-715.

[66] Shoemaker, R.S., Bertone, A.L., Martin, G.S., McIlwraith, C.W., Roberts, E.D., Pechman, R. and Kearney, M.T. (1992) Effects of intra-articular administration of methylprednisolone acetate on normal articular cartilage and on healing of experimentally induced osteochondral defects in horses. *American Journal of Veterinary Research* **53**, 1446-1453.

[67] Frisbie, D.D., Kawcak, C.E., Trotter, G.W., Powers, B.E., Walton, R.M. and McIlwraith, C.W. (1997) Effects of triamcinolone acetonide on an in vivo equine osteochondral fragment exercise model. *Equine Veterinary Journal* **29**, 349-359.

[68] Dechant, J.E., Baxter, G.M., Frisbie, D.D., Trotter, G.W. and McIlwraith, C.W. (2003) Effects of dosage titration of methylprednisolone acetate and triamcinolone acetonide on interleukin-1-conditioned equine articular cartilage explants in vitro. *Equine Veterinary Journal* **35**, 444-450.

[69] Garnero, P. and Delmas, P.D. (2003) Biomarkers in osteoarthritis. *Current Opinion in Rheumatology* **15**, 641-646.

[70] Nelson, F., Dahlberg, L., Laverty, S., Reiner, A., Pidoux, I., Ionescu, M., Fraser, G.L., Brooks, E., Tanzer, M., Rosenberg, L.C., Dieppe, P. and Robin Poole, A. (1998) Evidence for altered synthesis of type II collagen in patients with osteoarthritis. *The Journal of clinical investigation* **102**, 2115-2125.

[71] Rousseau, J. and Garnero, P. (2012) Biological markers in osteoarthritis. *Bone* **51**, 265-277.

[72] Lettry, V., Sumie, Y., Mitsuda, K., Tagami, M., Hosoya, K., Takagi, S. and Okumura, M. (2010) Divergent diagnosis from arthroscopic findings and identification of CPII and C2C for detection of cartilage degradation in horses. *The Japanese journal of veterinary research* **57**, 197-206.

[73] Nicholson, A.M., Trumble, T.N., Merritt, K.A. and Brown, M.P. (2010) Associations of horse age, joint type, and osteochondral injury with serum and synovial fluid concentrations of type II collagen biomarkers in Thoroughbreds. *American Journal of Veterinary Research* **71**, 741-749.

[74] de Grauw, J.C., Brama, P.A., Wiemer, P., Brommer, H., van de Lest, C.H. and van Weeren, P.R. (2006) Cartilage-derived biomarkers and lipid mediators of inflammation in horses with osteochondritis dissecans of the distal intermediate ridge of the tibia. *American Journal of Veterinary Research* **67**, 1156-1162.

[75] Billinghurst, R.C., Buxton, E.M., Edwards, M.G., McGraw, M.S. and McIlwraith, C.W. (2001) Use of an antineoepitope antibody for identification of type-II collagen degradation in equine articular cartilage. *American Journal of Veterinary Research* **62**, 1031-1039.

[76] Frisbie, D.D., Al-Sobayil, F., Billinghurst, R.C., Kawcak, C.E. and McLlwraith, C.W. (2008) Changes in synovial fluid and serum biomarkers with exercise and early osteoarthritis in horses. *Osteoarthritis and Cartilage* **16**, 1196-1204.

[77] Trumble, T.N., Scarbrough, A.B. and Brown, M.P. (2009) Osteochondral injury increases type II collagen degradation products (C2C) in synovial fluid of Thoroughbred racehorses. *Osteoarthritis and Cartilage* **17**, 371-374.

[78] Cleary, O.B., Trumble, T.N., Merritt, K.A. and Brown, M.P. (2010) Effect of exercise and osteochondral injury on synovial fluid and serum concentrations of carboxy-terminal telopeptide fragments of type II collagen in racehorses. *American Journal of Veterinary Research* **71**, 33-40.

[79] Bertuglia, A., Pagliara, E., Grego, E., Ricci, A. and Brkljaca-Bottegaro, N. (2016) Pro-inflammatory cytokines and structural biomarkers are effective to categorize osteoarthritis phenotype and progression in Standardbred racehorses over five years of racing career. *BMC veterinary research* **12**, 246.

[80] Poole, A.R. (1993) Cartilage in health and disease. In: *Arthritis and Allied Conditions: A Textbook of Rheumatology*, Ed: D.J.M.a.W.J. Koopman, Lea and Febiger, Philadelphia, PA. pp 279-333.

[81] Baccarin, R.Y., Rasera, L., Machado, T.S. and Michelacci, Y.M. (2014) Relevance of synovial fluid chondroitin sulphate as a biomarker to monitor polo pony joints. *Canadian journal of veterinary research* **78**, 50-60.

[82] Golub, E.E., Harrison, G., Taylor, A.G., Camper, S. and Shapiro, I.M. (1992) The role of alkaline phosphatase in cartilage mineralization. *Bone and mineral* **17**, 273-278.

[83] Fuller, C.J., Barr, A.R., Sharif, M. and Dieppe, P.A. (2001) Cross-sectional comparison of synovial fluid biochemical markers in equine osteoarthritis and the correlation of these markers with articular cartilage damage. *Osteoarthritis Cartilage* **9**, 49-55.

[84] Bertone, A.L., Palmer, J.L. and Jones, J. (2001) Synovial fluid cytokines and eicosanoids as markers of joint disease in horses. *Veterinary Surgery* **30**, 528-538.

[85] Trumble, T.N., Trotter, G.W., Oxford, J.R., McIlwraith, C.W., Cammarata, S., Goodnight, J.L., Billinghurst, R.C. and Frisbie, D.D. (2001) Synovial fluid gelatinase concentrations and matrix metalloproteinase and cytokine expression in naturally occurring joint disease in horses. *American Journal of Veterinary Research* **62**, 1467-1477.

[86] Henderson, B. and Pettipher, E.R. (1989) Arthritogenic actions of recombinant IL-1 and tumour necrosis factor alpha in the rabbit: evidence for synergistic interactions between cytokines in vivo. *Clinical and experimental immunology* **75**, 306-310.

[87] Kamm, J.L., Nixon, A.J. and Witte, T.H. (2010) Cytokine and catabolic enzyme expression in synovium, synovial fluid and articular cartilage of naturally osteoarthritic equine carpi. *Equine Veterinary Journal* **42**, 693-699.

[88] Ley, C., Ekman, S., Elmén, A., Nilsson, G. and Eloranta, M.L. (2007) Interleukin-6 and Tumour Necrosis Factor in Synovial Fluid from Horses with Carpal Joint Pathology. *Journal of Veterinary Medicine Series A* **54**, 346-351.

[89] Jouglin, M., Robert, C., Valette, J.P., Gavard, F., Quintin-Colonna, F. and Denoix, J.M. (2000) Metalloproteinases and tumor necrosis factor-alpha activities in synovial fluids of horses: correlation with articular cartilage alterations. *Veterinary research* **31**, 507-515.

[90] Billinghurst, R.C., Fretz, P.B. and Gordon, J.R. (1995) Induction of intra-articular tumour necrosis factor during acute inflammatory responses in equine arthritis. *Equine Veterinary Journal* **27**, 208-216.

[91] Goldring, M.B. (2000) The role of the chondrocyte in osteoarthritis. *Arthritis and Rheumatology* **43**, 1916-1926.

[92] Hardy, J., Bertone, A.L., Weisbrode, S.E., Muir, W.W., O'Dorisio, T.M. and Masty, J. (1998) Cell trafficking, mediator release, and articular metabolism in acute inflammation of innervated or denervated isolated equine joints. *American Journal of Veterinary Research* **59**, 88-100.

[93] Hawkins, D.L., MacKay, R.J., Gum, G.G., Colahan, P.T. and Meyer, J.C. (1993) Effects of intra-articularly administered endotoxin on clinical signs of disease and synovial fluid tumor necrosis factor, interleukin 6, and prostaglandin E2 values in horses. *American Journal of Veterinary Research* **54**, 379-386.

[94] Lotz, M.K. (2001) Cytokines and their receptors. In: *Arthritis and Allied Conditions - A textbook of Rheumatology*, 14th edn., Williams and Wilkins, Philadelphia, PA. pp 436-477.

[95] Borzi, R.M., Mazzetti, I., Marcu, K.B. and Facchini, A. (2004) Chemokines in cartilage degradation. *Clinical orthopaedics and related research*, S53-61.

[96] Koch, A.E., Polverini, P.J., Kunkel, S.L., Harlow, L.A., DiPietro, L.A., Elner, V.M., Elner, S.G. and Strieter, R.M. (1992) Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science (New York, N.Y.)* **258**, 1798-1801.

[97] David, F., Farley, J., Huang, H., Lavoie, J.P. and Laverty, S. (2007) Cytokine and chemokine gene expression of IL-1beta stimulated equine articular chondrocytes. *Veterinary surgery : VS* **36**, 221-227.

[98] Pigott, J.H., Ishihara, A., Wellman, M.L., Russell, D.S. and Bertone, A.L. (2013) Investigation of the immune response to autologous, allogeneic, and xenogeneic mesenchymal stem cells after intra-articular injection in horses. *Veterinary immunology and immunopathology* **156**, 99-106.

[99] McIlwraith, C.W. (2015) Traumatic arthritis and posttraumatic osteoarthritis in the horse. In: *Joint Disease in the Horse*, 2nd edn., Eds: C.W. McIlwraith, D.D. Frisbie, C.E. Kawcak and R. van Weeren, Elsevier - Health Sciences Division, China. pp 33-48.

[100] Brama, P.A., TeKoppele, J.M., Beekman, B., van Weeren, P.R. and Barneveld, A. (1998) Matrix metalloproteinase activity in equine synovial fluid: influence of age, osteoarthritis, and osteochondrosis. *Annals of Rheumatic Diseases* **57**, 697-699.

[101] Brink, P., Smith, R.K.W., Tverdal, A. and Dolvik, N.I. (2015) Changes in synovial fluid biomarker concentrations following arthroscopic surgery in horses with osteochondritis dissecans of the distal intermediate ridge of the tibia. *American Journal of Veterinary Research* **76**, 599-607.

[102] Verwilghen, D.R., Martens, A., Busschers, E., Franck, T., Deberg, M., Henrotin, Y., Vanderheyden, L. and Serteyn, D. (2011) Coll2-1, Coll2-1NO2 and myeloperoxidase concentrations in the synovial fluid of equine tarsocrural joints affected with osteochondrosis. *Veterinary research communications* **35**, 401-408.

[103] Laverty, S., Ionescu, M., Marcoux, M., Boure, L., Doize, B. and Poole, A.R. (2000) Alterations in cartilage type-II procollagen and aggrecan contents in synovial fluid in equine osteochondrosis. *Journal of orthopaedic research* **18**, 399-405.

[104] Verwilghen, D.R., Enzerink, E., Martens, A., Franck, T., Balligand, M., Henrotin, Y., Detilleux, J. and Serteyn, D. (2011) Relationship between arthroscopic joint evaluation and the levels of Coll2-1, Coll2-1NO(2), and myeloperoxidase in the blood and synovial fluid of horses affected with osteochondrosis of the tarsocrural joint. *Osteoarthritis Cartilage* **19**, 1323-1329.

[105] Taylor, S.E., Weaver, M.P., Pitsillides, A.A., Wheeler, B.T., Wheeler-Jones, C.P.D., Shaw, D.J. and Smith, R.K.W. (2006) Cartilage oligomeric matrix protein and hyaluronan levels in synovial fluid from horses with osteoarthritis of the tarsometatarsal joint compared to a control population. *Equine Veterinary Journal* **38**, 502-507.

[106] Fradette, M.E., Celeste, C., Richard, H., Beauchamp, G. and Laverty, S. (2007) Effects of continuous oral administration of phenylbutazone on biomarkers of cartilage and bone metabolism in horses. *American Journal of Veterinary Research* **68**, 128-133.

[107] AAEP, P. (1991) Definition and classification of lameness. In: Guide for Veterinary Services and Equestrian Events. In: *American Association of Equine Practitioners* Lexington, KY.

[108] McIlwraith, C.W., Wright, I. and Nixon, A.J. (2015) Chapter 7: Diagnostic and surgical arthroscopy of the tarsocrual (tibiotarsal) joint. In: *Diagnostic and Surgical Arthroscopy in the Horse* 4th edn., Eds: C.W. McIlwraith, I. Wright and A.J. Nixon, Elsevier Health Sciences, China. pp 243-272.

[109] Kraus-Hansen, A.E., Jann, H.W., Kerr, D.V. and Fackelman, G.E. (1992) Arthrographic analysis of communication between the tarsometatarsal and distal intertarsal joints of the horse. *Veterinary surgery : VS* **21**, 139-144.

[110] Bell, B.T., Baker, G.J., Foreman, J.H. and Abbott, L.C. (1993) In vivo investigation of communication between the distal intertarsal and tarsometatarsal joints in horses and ponies. *Veterinary surgery : VS* **22**, 289-292.

[111] Raes, E.V., Bergman, E.H., van der Veen, H., Vanderperren, K., Van der Vekens, E. and Saunders, J.H. (2011) Comparison of cross-sectional anatomy and computed tomography of the tarsus in horses. *American Journal of Veterinary Research* **72**, 1209-1221.

[112] Sisson, S. and Grossman, J.D. (1975). In: *Sisson and Grossman's The Anatomy of the Domestic Animals*, Ed: R. Getty, Saunders, Philadelphia. pp 117-120.

[113] Ley, C.J., Ekman, S., Dahlberg, L.E., Bjornsdottir, S. and Hansson, K. (2013) Evaluation of osteochondral sample collection guided by computed tomography and magnetic resonance imaging for early detection of

osteoarthritis in centrodistal joints of young Icelandic horses. *American Journal of Veterinary Research* **74**, 874-887.

[114] Eksell, P., Axelsson, M., Brostrom, H., Roneus, B., Haggstrom, J. and Carlsten, J. (1998) Prevalence and risk factors of bone spavin in Icelandic horses in Sweden: a radiographic field study. *Acta veterinaria Scandinavica* **39**, 339-348.

[115] Winegardner, K.R., Scrivani, P.V., Krotscheck, U. and Todhunter, R.J. (2007) Magnetic resonance imaging of subarticular bone marrow lesions in dogs with stifle lameness. *Veterinary radiology & ultrasound* **48**, 312-317.

[116] Brama, P.A., van den Boom, R., DeGroott, J., Kiers, G.H. and van Weeren, P.R. (2004) Collagenase-1 (MMP-1) activity in equine synovial fluid: influence of age, joint pathology, exercise and repeated arthrocentesis. *Equine Veterinary Journal* **36**, 34-40.

[117] Sandell, L.J. and Aigner, T. (2001) Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis research* **3**, 107-113.

[118] Seabaugh, K.A., Selberg, K.T., Mueller, P.O.E., Eggleston, R.B., Peroni, J.F., Claunch, K.M., Markwell, H.J. and Baxter, G.M. (2017) Clinical study evaluating the accuracy of injecting the distal tarsal joints in the horse. *Equine Veterinary Journal* **49**, 668-672.

[119] Serena, A., Schumacher, J., Schramme, M.C., et al (2004) Concentration of methylprednisolone in the distal intertarsal joint after administration of methylprednisolone acetate into the tarsometatarsal joints In: *50th Annual American Association of Equine Practitioners Convention*. pp 296-298.

[120] Billinghurst, R.C., Brama, P.A.J., van Weeren, P.R., Knowlton, M.S. and McIlwraith, W. (2004) Evaluation of serum concentrations of biomarkers of skeletal metabolism and results of radiography as indicators of severity of osteochondrosis in foals. *American Journal of Veterinary Research* **65**, 143-150.

[121] Brama, P.A.J., TeKoppele, J.M., Beekman, B., van Weeren, P.R. and Barneveld, A. (1998) Matrix metalloproteinase activity in equine synovial fluid: influence of age, osteoarthritis, and osteochondrosis. *Annals of the Rheumatic Diseases* **57**, 697-699.

[122] de Grauw, J.C., van de Lest, C.H.A., Brama, P.A.J., Rambags, B.P.B. and van Weeren, P.R. (2009) In vivo effects of meloxicam on inflammatory mediators, MMP activity and cartilage biomarkers in equine joints with acute synovitis. *Equine Veterinary Journal* **41**, 693-699.

[123] Tatarniuk, D.M., Groschen, D.M., Merritt, K.A., Maher, M.C., Ernst, N.S., Brown, M.P. and Trumble, T.N. (2015)Concentrations of cytokines, matrix metalloproteinases, and tissue inhibitors of matrix metalloproteinases in autologous conditioned serum from horses with distal interphalangeal joint osteoarthritis. *Osteoarthritis and Cartilage* **23**, A399-A400.

[124] van den Boom, R., van der Harst, M.R., Brommer, H., Brama, P.A., Barneveld, A., van Weeren, P.R. and DeGroot, J. (2005) Relationship between synovial fluid levels of glycosaminoglycans, hydroxyproline and general MMP activity and the presence and severity of articular cartilage change on the proximal articular surface of P1. *Equine Veterinary Journal* **37**, 19-25.