Autologous conditioned serum and subsequent synovial fluid concentrations of cytokines, matrix metalloproteinases, and tissue inhibitors of metalloproteinases after injection into equine osteoarthritic distal interphalangeal joints

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Dedication

I would like to dedicate this thesis to all family and friends in Canada and U.S.A. for their endless encouragement and support. I would also like to thank the many mentors through veterinary school, internship, and residency who have taught me the ways of veterinary medicine.

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ABSTRACT

INTRODUCTION: Autologous conditioned serum (ACS) is used for treatment of osteoarthritis in horses. However, there is a lack of critical knowledge of the contents of ACS and any biological effects after injection into osteoarthritic joints.

OBJECTIVE: The specific aims of this study were to (1) evaluate content of cytokine, matrix metalloproteinases (MMP), and tissue inhibitors of matrix metalloproteinases (TIMP) in ACS, (2) validate/refute that ACS increases anti-inflammatory or pro-inflammatory biomarkers in synovial fluid from naturally occurring osteoarthritic joints, (3) demonstrate whether freeze/thaw effects IL-1ra concentrations in ACS, and (4) evaluate for clinical improvement in lameness following ACS therapy.

METHODS: Eleven horses with naturally occurring osteoarthritis in a forelimb distal inter-phalangeal joint were given 3 consecutive intra-articular doses of ACS separated by 7-day intervals. Synovial fluid was collected before administration of each dose at day 0, 7, 14, and 21. Concentrations of cytokines (IL-1ra, IL-1β, TNFα, IL-4, IL-6, IL-8, IL-10), MMPs (-1, -3, -9, -13), and TIMPs (-1, -2, -3, -4) were quantified using ELISA and multiplex assays. Serum was compared to unincubated and incubated controls and synovial fluid was compared to baseline (day 0). Horses were videotaped trotting (at day 0, 7, 14, and 21) and graded blindly for degree of lameness.

RESULTS: Concentrations of IL-1ra, as well as MMP-1 to TIMP (-1, -2, -3, -4) and MMP-9 to TIMP (-1, -2, -4) ratios were increased in ACS (vs. unincubated control), and IL-4, IL-6, and IL-8 were decreased in ACS (vs. incubated control). Freeze/thaw did not affect biomarker concentrations in ACS. Although ACS contained high concentrations of IL-1ra, no changes in IL-1ra concentrations were observed in synovial fluid 7 days after each injection. No change in median lameness grade was noted throughout study duration (median grade of 1/5 at all time points).

Table of Contents

List of Tables

List of Figures

CHAPTER 1:
PRELIMINARY LITERATURE REVIEW

PART A - STRUCTURE, FUNCTION, AND HOMEOSTASIS OF A NORMAL EQUINE JOINT

Synovial joints are present throughout the mammalian body, and act as an organ that facilitates pain-free movement of the limb. Joints are composed of a variety of tissues (including articular cartilage, bone, synovial membrane, joint capsule, synovial fluid, and ligaments) that work together to provide a functional articulation over the ends of two or more bones. Articular cartilage covers the subchondral bone and is surrounded by synovial fluid that is produced in the synovial membrane. Synovial fluid facilitates ease of movement, while also acting as a nutritional reservoir for cartilage. Depending on the joint, extra-capsular, and/or intra-capsular ligaments are present to provide further support (Frisbie 2012a).

The synovial membrane is composed of two main tissue layers, called the intimal and subintimal layers. The intimal layer interacts directly with the joint cavity, and lines the underlying subintimal layer. Unique to the intimal layer are the lack of basement membranes and tight junctions. This allows synovial fluid, which originates as an ultrafiltrate of plasma from capillaries within the subintimal layer, to secrete into the joint cavity. The intimal layer itself can be further subdivided into two main categories of cells: type A (macrophage-like) synoviocytes and type B (fibroblast-like) synoviocytes. The type A synoviocytes mainly engage in phagocytosis, a function that helps remove foreign material from the joint and contributes to the natural turnover of synovial fluid. Alternatively, type B synoviocytes predominately act to secrete proteins that are important constituents for normal synovial fluid and cartilage health. Such proteins include: hyaluronic acid, lubricin, fibronectin, laminin, and proteoglycans (amongst others). Cytokines and matrix metalloproteinases (MMPs) from synoviocytes also play an important role in influencing anabolic and catabolic processes within the joint (Todhunter

1996, Frisbie 2012a). The subintimal layer merges with the true joint capsule, contributing to synovial membrane structural support. Together, the intimal and subintimal layers become arranged grossly as synovial villi, which act to increase the effective surface area of synoviocytes within the joint.

Articular (hyaline) cartilage is a specialized tissue that contributes to smooth movement during flexion and extension of the joint. Articular cartilage is composed of a low population of cells (chondrocytes), which produce the more prevalent surrounding extracellular matrix. Extracellular matrix is composed predominately of water, collagen, and proteoglycan, and to a lesser extent, glycoproteins and minerals. In hyaline cartilage, collagen is predominately composed of the type II subtype which acts to provide a scaffold for the extracellular matrix and helps withstand tensile forces. Proteoglycans are composed of a protein core with glycosaminoglycan side chains. Aggrecan is the most common proteoglycan, and is predominately responsible for withstanding compressive forces due to the capability of the glycosaminoglycan side chains to attract and retain water. Articular cartilage is a unique tissue, in that it lacks vasculature, lymphatics, and nerves. Therefore, nutrition for cartilage heath is resultant from diffusion from synovial fluid (Todhunter 1996, Frisbie 2012a).

Within the normal joint environment, a highly regulated balance exists between the synthesis (anabolism) and degradation (catabolism) of the extracellular matrix in articular cartilage. This balance allows for the natural turnover and repair of injured tissue, which maintains the health of the joint in response to the distribution of forces constantly applied through the limb (Mueller 2011). To an extent, cyclical and dynamic compression of cartilage stimulates an increase in extracellular matrix deposition (Guilak 2004). Conversely, static compression or excessive dynamic compression will deplete the extracellular matrix and hinder further synthesis (Guilak 2004). This normal balance of

synthesis and degradation is constantly ongoing, helping to maintain the health and function of a joint.

PART B - PATHOPHYSIOLOGY OF OSTEOARTHRITIS

Osteoarthritis (OA) is a complex, irreversible disease that affects both animals and humans. In humans, OA has been described as the most frequent cause of pain and discomfort, with widespread implications for economic loss in the workplace due to disability and loss of function (Goldring 2007). Post trauma, the risk of developing secondary osteoarthritis ranges from 20 to 50% (Weigel 2002, Dirschl 2004). With age (over 65), the majority of human patients develop osteoarthritis in a wide variety of joints, and overall, over 27 million adults suffer from clinical OA (Goldring 2007, Lawerence 2008). Similar limitations are seen in the equine athlete. Todhunter et. al. reported that joint disease was the underlying cause of lameness in 28 to 42% of cases (Todhunter 1990). In 1998, horse owners, veterinarians, and trainers were surveyed regarding equine health. This study noted lameness (of which osteoarthritis is a major contributor) as the most important concern (78% response) (Kane 2000). When evaluating annual incidence, cost, and days of lost use, lameness again was found to be the main health limitation in the equine industry (Kane 2000). The total cost was estimated at $678 million to $1 billion (Kane 2000).

Globally, OA is defined as deterioration of articular cartilage, with concurrent damage to the synovium and bone. By definition, the Orthopedic Research Society International (OARSI) currently defines OA as “a disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic

derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function) that can culminate in illness" (Kraus 2015).

The word osteoarthritis can be considered a misnomer, as it does not represent a traditional inflammatory process due to the lack of neutrophils and cardinal signs of inflammation (heat, pain, hyperemia, swelling and loss of function) (Goldring 2007). The pathophysiology of OA degeneration follows a similar progression, regardless of the initiating cause. Injury to the cartilage can occur from direct trauma or from chronic, repetitive stress. Direct damage can cause chondrocyte cell death and structural damage to the extracellular matrix. Alternatively, indirect cytokine and chemokine activity can increase the concentration of proteolytic enzymes produced by synoviocytes and chondrocytes (specifically, MMPs and aggrecanases) within the joint. Central to mediating this inflammation are the pro-inflammatory cytokines IL-1 and TNF-α, as well as chemokines, prostaglandins and nitric oxide (Goldring 2007). Aggrecanase and MMPs are enzymes that degrade the extracellular matrix of cartilage (ie, proteoglycan and type II collagen). Normally, these enzymes help maintain normal homeostasis. During osteoarthritis, however, abnormal production and activation of proteolytic enzymes result in loss of cartilage structure and function. With partial-thickness defects in the superficial zone of cartilage, some repair occurs from increased type II collagen and glycosaminoglycan synthesis. In mature defects, a healing response with normal hyaline cartilage is difficult to attain (Goldring 2007). This is partially due to the low metabolic activity for chondrocytes present in the intermediate or deep zones (Goldring 2007). Instead, full thickness defects preferentially repair by in-growth of subchondral fibrous tissue (composed of type I collagen), which then mature into a fibrocartilage patch (Goldring 2007).

Primary synovitis can also lead to secondary changes in articular cartilage, and may actually be an early mediator of OA (Benito 2005). McIlwraith, et. al. demonstrated this potential in the horse, using intra-articular injection of filipin (a polyene antibiotic, produced by *Streptomyces* organisms) in the intercarpal joint (McIlwraith 1981). Following a 12-week course of filipin injections, the histopathological appearance and loss of glycosaminoglycan content was similar to that in naturally occurring OA cases. Based on these results, it was proposed that primary synovitis may be capable of producing pro-inflammatory cytokines that up-regulate catabolic enzymes within the joint environment, leading to secondary degeneration of articular cartilage (McIlwraith 1981).

The subchondral bone also contributes to OA development and progression. In progressively deteriorated joints, common pathological features include increased bone deposition (osteophyte formation), increased subchondral bone thickness, and with chronicity, development of osseous bone cysts (Buckland-Wright 2004). An increase in subchondral bone thickness has been observed in the tarsus of horses with increasing age, leading to the proposition that subchondral bone thickening precedes OA formation (Murray 2009).

OA is a multifactorial disease that can cause variable amounts of damage to the joint. One major determinant for degree of injury is the underlying cause. Acute trauma causes joint instability through damage to various soft tissue structures (ie, ligaments, joint capsule, menisci, or peri-articular soft tissue) (Frisbie 2012a). Alternatively, fracture(s) of the osteochondral surface can displace and heal with a maligned joint surface. This leads to irregular cartilage surface congruency and chronic secondary release of inflammatory mediators (Frisbie 2012a). Developmentally, failure of normal endochondral ossification (ie, abnormal cartilage and subchondral bone) can lead to an irregular joint surface, detachment of cartilage and subchondral bone, and in select cases,

involution and subchondral cyst formation (van Weeren 2012). Depending on the anatomical location, normal forces applied to osteochondrosis lesions may result in OA (van Weeren 2012). The diseased cartilage and subchondral bone can also up-regulate anabolic, anti-catabolic and catabolic cytokines, as well as produce and activate MMPs, affecting the entire joint (Trumble 2001). Together, both the irregular cartilage surface and secretion of degradative proteins will sustain inflammation within the joint. Lastly, and most commonly in the horse, chronic and cyclical absorption of forces distributed through the appendicular skeleton can lead to both inflammation of the synovium (synovitis) and degeneration of the weight-bearing surface (breakdown of cartilage and remodeling of subchondral bone) such that OA is established (Frisbie 2012a).

Pain, manifested as lameness, is a common clinical presentation of OA. Inflammatory debris, resultant from OA, increases effusion (total synovial fluid volume) within the joint environment. This leads to overall distension and pressure exerted on the joint capsule, where many afferent nociception fibers are present (Frisbie 2012a). During the inflammatory cascade, proteins involved in pain transmission (such as substance P) are also released (Platt 1996). Distortion of normal joint anatomy (ie, irregular cartilage surface, joint capsule fibrosis, and new peri-articular bone formation) can also create a functional impairment of locomotion by decreasing the range of motion (Frisbie 2012a). During the early stages of OA, horses can compensate for low-grade pain and mild loss of function (Frisbie 2012a). However, if unmanaged, both effusion and lameness symptoms progressively worsen. In horses, this is paralleled by a notable decrease in the athletic performance (Frisbie 2012a). It is at this point that many owners seek veterinary intervention for localization of the lameness and prescription of disease- and symptom-modifying therapeutics.

OA in the horse must always remain a differential diagnosis whenever poor performance and/or lameness are localized to a joint(s). Clinical signs of joint injury

include decreased range of motion, increased synovial effusion, palpable fibrosis of the joint capsule, or enlargement of the adjacent peri-articular bone (Frisbie 2012a). Inducing stress by flexing the affected joint can help localize pain by a worsening of the lameness ("flexion test"). Once a joint(s) is isolated as symptomatic, radiographic assessment commonly follows. It is important to note that radiographs are best for visualization of mineralized tissue, with poor visualization of soft tissue structures. As such, cartilage defects are not apparent. However, secondary changes in the subchondral bone can be seen, such as osteophyte formation, increased bone density ("sclerosis"), decrease(s) in joint space, and/or osseous cystic lesions (McIlwraith 2012). With the lack of visualization of the cartilage and soft tissue structures, earlier cases of OA are difficult to identify. In other words, the lack of radiographic signs of OA does not preclude its existence. In fact, the development of radiographic abnormalities in the distal interphalangeal joint of the horse is often considered delayed during the early stages of OA (Butler 2008). In humans, early cartilage defects can be visualized using magnetic resonance imaging (MRI) (Goldring 2007). However, due to limitations in the size of horses, technical difficulties, and increased cost of diagnosis, the use of any novel imaging (ie, MRI or computed tomography) is less commonly applied.

Other methods of identifying OA during the early stages of disease progression thus remain valuable for diagnosis. With arthroscopy, cartilage lesions can be visualized, as well as any pathology of the synovium or internal soft tissue structures (ie, menisci, cruciate ligaments) (McIlwraith 2005). Common characteristics of cartilage lesions include partial or complete erosion, fibrillation, and/or wear lines (McIlwraith 2005). In fact, arthroscopy remains more sensitive than magnetic resonance imaging for detection of articular defects, and is considered the 'gold standard' ante mortem diagnostic test (VanBeek, 2014). Depending on the amount and extent of injuries visualized, prognosis for the joint can be inferred during arthroscopy and therapeutic debridement of abnormal cartilage can be attempted (McIlwraith 2005, Cohen 2009). Historically, the most

important limitation of diagnostic arthroscopy was the necessity of general anesthesia, with the risks of traumatic recovery. However, standing arthroscopic techniques have been developed for various equine joints. These techniques have the distinct advantage of avoiding the inherent risks of anesthetizing horses (Elce 2002, Gasiorowski 2014, Frisbie 2014).

Alternate to imaging, measurement of various molecules in body fluids (such as synovial fluid) is an active area of research as a means for identifying OA. The molecules assessed in samples are generally called 'biomarkers', and can either measure molecules that directly result from extracellular matrix synthesis/degradation, or those that indirectly play a role in OA pathogenesis such as cytokines, growth factors, MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs). Historically, cytokines have been measured in equine synovial fluid by using enzyme linked immunoabsorbance assay (ELISA) or reverse transcriptase polymerase chain reaction (rtPCR) to detect mRNA expression (Trumble 2001, Kamm 2010). Alternatively, gelatin zymography has been used to quantify MMP concentrations (Clegg 1997, Trumble 2001) and reverse zymography for TIMP concentrations (Clegg 1998). More recently multiplex assay has been used to quantify multiple indirect biomarkers simultaneously from the same serum or synovial fluid sample. Multiplex assays utilize a bead based, flow cytometer system that can evaluate up to 100 different analytes. Each bead is internally color-coded with varying ratios of 2 fluorescent dyes. This allows an analyte of interest to be measured through covalent coupling to specific capture antibodies. One laser identifies the specific bead set, and another laser quantifies the florescent reaction to the antibody. Then, the florescent reaction can be compared against a standard curve (similar to an ELISA), to also provide quantitative results. The obvious advantage of this technology compared to the previously mentioned techniques is the relative efficiency in measuring multiple analytes from small fluid volumes. Additionally, it has been suggested that the multiplex technology may be more sensitive than traditional ELISAs (van Gageldonk 2008). Using

multiplex technology for investigating OA has been successfully performed in human medicine, and has similar potential for equine research (Cattano 2011, Beekhuizen 2013, Heard 2013, Tsuchida 2014, Monibi 2015).

As previously mentioned, OA is an irreversible disease. As such, management strategies for therapy are ultimately aimed at minimizing the inflammatory and degradative damage of OA, thereby decreasing pain and minimizing ongoing damage. Surgically, this can be accomplished via removal of incongruent osteochondral fragments and/or abnormal cartilage, such that fibrocartilage ingrowth can occur. Therapeutics are directed towards providing a net anti-inflammatory change. Common medications include systemic non-steroidal anti-inflammatories (ie, phenylbutazone, firocoxib) and joint supplements (ie, glucosamine, chondroitin sulfate); and/or intra-articular corticosteroids (ie, methylprednisolone, triamcinolone) and hyaluronic acid (Frisbie 2012b). Novel biological therapies, such as autologous conditioned serum, platelet-rich plasma, or stem cells have also become popular recently (Frisbie 2007, Textor 2011, Brehm 2014).

In the equine athlete, OA can occur in any joint. Depending on the type of sport a horse participates in, certain joints develop OA more often. Commonly, the metacarpal (tarsal)-phalangeal, radiocarpal, and middle carpal joints are affected in Thoroughbred racehorses (Frisbie 2012b). In fact, 33% of all 2- and 3- year old racing Thoroughbreds had cartilage lesions and pathological signs of osteoarthritis in the metacarpophalangeal joint in a retrospective study of 50 racehorses (Neundorf 2010). Alternatively, in Western and English performance horses, injury to the femorotibial, distal intertarsal, and tarsometatarsal joints is more prevalent (Ferris 2011). The distal interphalangeal joint (DIP) was commonly affected in dressage and hunter/jumpers in another retrospective study (Kristiansen 2007). Interestingly, the presence of DIP joint effusion alone does not

always correlate with clinical signs of lameness. Thus, physical exam alone can make the diagnosis of DIP joint OA difficult and potentially over-represented (Olive 2014).

In horses, the incidence of OA increases with age. As OA is a degenerative process, manifestations of OA worsen over time. However, there are other structural changes known to develop within the joint as the result of age, and not exclusively attributable to OA. In humans, increasing stiffness and decreasing tensile strength of extracellular matrix occurs because of changes in type II collagen and proteoglycan (Aigner 2007). This is thought to result from decreased anabolic capabilities of cells (Aigner 2007). Limited specific studies of microscopic structural changes in cartilage with respect to horse age have revealed elevated concentrations of prostaglandin-E2 in cartilage samples of older horses (>20 years) compared to young (<10 years) following exposure to lipopolysaccharide (Briston 2010). In another study, cartilage in tarsometatarsal joints showed decreasing hyaline cartilage and increasing calcified cartilage quantity with age (Murray 2009). With this knowledge, one can hypothesize that as horses become older, articular cartilage may become less able to withstand normal cyclical forces, increasing the risk of OA.

In general, OA is a complex disease resulting in significant debility for the equine patient and notable economic strains in the horse industry. Osteoarthritis can develop from a wide variety of inciting causes. Cartilage, synovium, and subchondral bone all play a role in OA pathophysiology. Proper management of OA is highly dependent on early detection. However, no ideal diagnostic tool yet exists. Depending on the severity of OA, a variety of therapeutics exists, but none can cure established OA. Furthermore, the study of OA in horses not only will benefit that species, but also provides a good translational model for human OA.

PART C - CYTOKINES

Cytokines are a diverse group of immunological proteins that are ubiquitous in all organs and tissues. Cytokines play a key role in synovitis and osteoarthritic disease (Goldring 2004). Cytokines have an important role in cell signaling and influence the inflammatory environment either positively or negatively. This is accomplished predominately by autocrine (internal signaling within the same cell) or paracrine (external signaling between two cells within the same organ) function (Goldring 2004). A third, unique mechanism exists where cytokines remain associated with the surface of their origin cell, but interact with receptors on other cells (juxtacrine signaling) (Goldring 2004). Cytokines can be either a part of the cell surface or soluble within the joint fluid, and are produced by both chondrocytes and synoviocytes (Goldring 2004). During osteoarthritic progression, it has been proposed that cytokines increase in concentration and diffuse through synovial fluid, thereby influencing articular cartilage locally (Martel-Pelletier 1999).

Within the joint environment, cytokines influence the progression of osteoarthritis through a variety of mechanisms, which can be subdivided into four broad categories (Goldring 2004). Cytokines can bind to a target receptor, which subsequently up-regulate other proteolytic factors within the joint (catabolism). Opposing this, certain cytokines act to directly or indirectly antagonize the catabolic cytokines (anti-catabolism). Other cytokines can directly signal and influence the synthesis of chondrocytes and synoviocytes (anabolic). Lastly, some cytokines lack an obvious beneficial or detrimental effect, yet have the ability to modulate the effects of other cytokines (modulatory). Classification of the various types of cytokines present can be broadly simplified into separate groups by these outcomes (Table 1.1).

Function in Joint	Cytokine
Catabolic	IL-1, TNF-α, IL-2, IL-8, IL-17, IL-18,
Anti-Catabolic	IL-1ra, IL-4, IL-10, IL-13
Anabolic	IGF-1, TGF-β, VEGF
Modulatory	IL-6, IL-11

TABLE 1.1 – Categorization of cytokines based on functional effect on cartilage.

Of the catabolic cytokines, interleukin-1 (IL-1) is central in promoting inflammation. Interleukin-1 has two structurally different, but functionally similar molecular subtypes, denoted as alpha (α) and beta (β). Previous human studies have demonstrated the importance of IL-1 in osteoarthritis pathogenesis (Dodge 1989, Smith 1989, Lefebvre 1990). The important role of IL-1 has also been demonstrated in equine studies (Alwan 1991, Trumble 2001). In vitro, when cartilage is exposed to IL-1, up-regulation of proteolytic enzymes (including the wide array of MMPs) occurs (McGuire-Goldring 1984). In vivo, when recombinant IL-1 is injected directly into the joint environment, proteoglycan loss occurs (Pettipher 1986). This method has also been used as a model of arthritis in horses (Pearson 2009, Ross 2012).

The source of IL-1 is predominately from synoviocytes or chondrocytes, but can also come from inflammatory cells that infiltrate the joint (Goldring 2004). IL-1 predominately signals through binding of specific IL-1 receptors, which initiates cellular signaling and influences gene transcription of other cytokines and proteolytic enzymes (Richardson 2000, Sutton 2009). Beyond up-regulation and activation of proteolytic enzymes, other notable cellular effects of IL-1 include inhibition of proteoglycan synthesis, indirect up-regulation of additional catabolic cytokines (ie, TNFα), release of angiogenic factors, chondrocyte apoptosis, inhibition of TIMPs, and nitric oxide release (Sutton 2009).

One previous investigation examined IL-1 mRNA expression from chondrocyte and synovial membrane tissue in normal, osteochondrosis, and osteoarthritis affected horses (Trumble 2001). No significant difference in IL-1α expression was noted between normal and osteoarthritic joints. As well, no effect of age was reported (Trumble 2001). In contrast, a significant difference in expression of IL-1β was noted between osteoarthritic and normal samples of synovial tissue and an age related effect was also observed, with younger horses demonstrating increased expression. In articular cartilage, IL-1β showed no significant difference between healthy and osteoarthritic joints. However, IL-1β expression in articular cartilage was correlated with both the white blood cell count and MMP-3 concentrations (Trumble 2001). A correlation also existed between IL-1α and IL-1β expression (Trumble 2001). Conversely, in osteoarthritic equine radiocarpal joints, IL-1 was found to be highly elevated within articular cartilage, but not synovial tissue (Kamm 2010). In another study, IL-1β showed a high variability in concentration within normal joints. As such, using measurements of IL-1β concentrations yields low specificity for inferring underlying osteoarthritic disease (Bertone 2001).

Another important catabolic cytokine involved in equine osteoarthritis is tumor necrosis factor alpha (TNFα). TNFα induces an almost identical list of physiological effects within the joint as previously described for IL-1 (Goldring 2004). By comparison, IL-1 is 100 to 1000 times more potent than TNFα (van den Berg 2001). There is a strong synergistic relationship between IL-1 and TNFα, with each causing up-regulation of production of the other (Henderson 1989). In osteoarthritic equine radiocarpal joints, TNFα was found to be significantly increased in both synovial and chondrocyte tissue (Kamm 2010). In equine chondrocyte tissue, osteoarthritic joints had higher TNFα expression compared to normal joints (Trumble 2001). TNFα may be more active during the early stages of osteoarthritis (Billinghurst 1995, Trumble 2001). When evaluating

concentrations of TNFα in normal and osteoarthritic joints, it was concluded that horses with TNFα concentrations in the top 75th quartile were 5.7 times more likely to have acute, severe joint disease (Bertone 2001). It was concluded that elevated TNFα (50th percentile or higher) is a very good indicator of osteoarthritis, but not progression. However, concentrations of TNFα do not appear to correlate well with the degree of joint pathology (Ley 2007, Jouglin 2000). TNFα concentrations in osteoarthritic joints do not seem to be influenced by a previous history of intra-articular corticosteroid injections (Ley 2007).

The third main catabolic cytokine is IL-8. This protein acts to promote inflammation in the joint, predominately through recruitment of neutrophils and induction of phagocytosis (David 2007). IL-8 is also referred to as a chemokine due to this function (David 2007). Other cellular responses to IL-8 are promotion of angiogenesis (Koch 1992) and release of MMPs (Borzi 2000). In an in vitro equine chondrocyte culture, IL-8 was significantly up-regulated following exposure to IL-1 (David 2007). IL-2 also has catabolic function in the joint through effects on T-lymphocyte recruitment and proliferation (Martel-Pelletier 1999). Relatively little is known about IL-2 concentrations in equine arthropathies.

Anti-catabolic cytokines, as the name implies, act to diminish the effect of catabolic proteins (ie, IL-1 and TNFα). One of the most fundamental anti-catabolic cytokines is interleukin-1 receptor antagonist protein (IL-1ra) (Goldberg 2004). Normally, the relationship between IL-1ra and IL-1/TNFα helps promote chondrocyte metabolism and turnover during normal joint homeostasis (Goldberg 2004). IL-1ra antagonistically binds to IL-1 receptors on the chondrocyte cell surface (Hannum 1990, Goldring 2004) to prevent IL-1 from binding to that site, thereby preventing cellular signaling in response to IL-1 (Hannum 1990). If an ample concentration of IL-1ra exists in the joint, then IL-1 signal is suppressed, and less MMP up-regulation will occur. This

concept was confirmed in horses using gene therapy to up-regulate intrinsic IL-1ra in the joint (Frisbie 2002). A significant improvement in osteoarthritis scores was seen when higher concentrations of circulating IL-1ra were present (Frisbie 2002).

The other major anti-catabolic cytokines are IL-4, IL-10, and IL-13. These 3 cytokines prevent and suppress cartilage degradation in a variety of ways. Interleukin-4, -10, and -13 can directly suppress proteolytic-induced cartilage degradation (Goldring 2004). Indirectly, IL-4, -10, and -13 can decrease the production of various pro-inflammatory cytokines (such as IL-1 and TNFα) from synoviocytes and chondrocytes (Alaaeddine 1999). In an in vitro study, IL -4, -10, and -13 have been shown to increase the concentration of IL-1ra (Fernandes 2002). IL-4 has been shown to inhibit osteoclasts, which are involved in bone remodeling during osteoarthritis. (Shioi 1991). IL-4 and IL-13, but not IL-10, have been shown to inhibit synoviocyte apoptosis (Relic 2001) which can perpetuate further synovial membrane hyperplasia in chronic, osteoarthritic joints. This effect illustrates one potentially negative effect of the aforementioned cytokines. In an in vitro study of the effects of IL-1 on various other cytokine families, equine chondrocyte cells were exposed to recombinant IL-1 and no up-regulation of IL-4 occurred; therefore IL-4 activity may not be linked to the catabolic cytokines (David 2007).

Anabolic cytokines directly promote synthesis by chondrocytes, synoviocytes, and osteoblasts. The two most important cytokines in this family include insulin-like growth factor -1 (IGF-1) and transforming growth factor – beta (TGF-β). IGF-1 promotes chondrocyte proliferation and synthesis of the extracellular matrix (Maor 1993, Davenport-Goodall 2004). When IGF-1 is co-cultured with osteogenic protein, both normal and osteoarthritic tissue cultures increased proteoglycan quantity (Loeser 2003). Additionally, IGF-1 influences aggrecan, decorin, and type II collagen production (Chubinskaya 2007). When equine synoviocytes and chondrocytes are exposed to IGF-1

in-vitro, an increase in production of type II collagen and proteoglycan is identified. (Haupt 2005). Indirectly, IGF-1 antagonizes IL-1 production, such that less proteolytic enzymes are present (Im 2003). Using gene therapy for IGF-1 or IGF-1 combined with IL-1ra, an improvement in proteoglycan content and type II collagen was noted in cartilage samples (Morisset 2007).

A second important anabolic cytokine is TGF-β. Similar to IGF-1, TGF-β appears to increase proteoglycan concentrations in the joint. Also similar to IGF-1, TGF-β appears to reduce production of IL-1β by both decreased expression of IL-1 itself, as well as decreased expression of IL-1 receptors (Harvey 1991). Through this path, TGF-β indirectly minimizes cartilage degradation (Harvey 1991). Interestingly, for both IGF-1 and TGF-β, the physiologic changes induced in the joint are not always beneficial. Both cytokines may contribute to osteophyte formation during the later stages of osteoarthritis (Horner 1998, Okazaki 1999). In one study, TGF-β increased concentrations of MMP-9 (but not MMP-2) in normal equine chondrocytes (Thompson 2001). Lastly, vascular endothelial growth factor (VEGF) is an anabolic cytokine produced by chondrocytes that can promote angiogenesis within synovium and other inflamed tissue (Honorati 2004).

The last group of cytokines is classified as modulatory. Cytokines that are a part of this group include IL-6 and IL-11. Modulatory is a term used to describe the ability of these cytokines to regulate the inflammatory environment, either in a pro- or anti-inflammatory manner, depending on the circumstance. IL-11 has apparently not been investigated in horses. IL-6 can induce anti-inflammatory and chondroprotective effects, through induction of IL-1ra and TIMPs (Goldring 2004). Indirectly, IL-6 blocks IL-1 ability to inhibit proteoglycan synthesis. Yet, counter-intuitively, IL-1 stimulates IL-6 production in the joint (Neitfeld 1994). In some instances, IL-6 directly inhibits proteoglycan and chondrocyte synthesis (Goldring 2004). IL-6 also up-regulates MMP and aggrecanase activity within the joint (Goldring 2004). In one study, IL-6 was

significantly elevated in joints with osteochondral fragment(s) and secondary osteoarthritic changes, compared to healthy joints (Ley 2007). In this study, the grade of osteoarthritis present correlated with IL-6 concentrations in the synovial fluid (Ley 2007). This same study also found that IL-6 was usually higher in those joints that had no previous intra-articular corticosteroid injection (Ley 2007). Using joint fluid from normal (n=50) and diseased (n=69) horses, elevated concentrations of IL-6 were highly sensitive and specific for ruling in/out osteoarthritic disease (Bertone 2001).

A wide variety of cytokines exist, and within the joint environment, the cellular effects from cytokine activity (ie, pro- or anti-inflammatory action) are highly variable. Many complex relationships exist between the multitude of cytokines present, with many having a positive or negative influence towards each other. Measuring an individual cytokine concentration alone does not provide clarity regarding the health of a joint. Comparatively, profiling groups of cytokines may provide a better understanding of the entire joint environment. Assessment of cytokines from each of the four sub-families (catabolic, anti-catabolic, anabolic, modulatory) would be ideal. A cross-sectional sampling of cytokines will help understanding of the function and interactions of each cytokine, as well as their relationship to the onset and progression of joint disease. Secondarily, a more objective assessment of the efficacy of joint therapeutics can be attained.

PART D – MATRIX METALLOPROTEINASES AND TISSUE INHIBITORS OF METALLOPROTEINASES

Matrix metalloproteinases (MMPs) can be found in a variety of organ systems within the body, including the joint. They are zinc dependent enzymes that are active in the normal remodeling of articular cartilage. MMPs are secreted as inactive zymogens, requiring enzymatic cleavage to become functional (Murphy 1990). In the joint

environment, MMPs cause degradation of the extracellular matrix (Clutterbuck 2010). For normal growth and regeneration to occur, MMP-induced degradation is an important initial step (Clutterbuck 2010). Degradation of the extracellular matrix by MMPs also imparts indirect, secondary effects, such as release of various growth factors, such as fibroblast growth factor (Whitelock 1996). However, MMP degradation of the extracellular matrix is closely controlled by an opposing family of endogenous proteins, aptly called tissue inhibitors of MMPs (TIMPs). TIMPs are proteins that directly antagonize the effects of MMPs (Clutterbuck 2010). In normal joint physiology, a controlled balance exists between the activated MMPs and TIMPs, which allows for the continued health of articular cartilage (Clutterbuck 2010).

If the balance between activated MMPs and TIMPs becomes disrupted, damage to the joint can occur. Either an excessive increase in MMP concentrations or decrease in TIMP concentrations can contribute to a degradative state (Yoshihara 2000). Joint disease is a relevant example of normal and abnormal MMP/TIMP balance in physiology (Clutterbuck 2010). Approximately 5% of the total MMP concentration is present in an active form within the healthy joint (Murphy 1990). The remaining 95% of inactive MMPs are present latently within the joint, or are bound with respective TIMPs (Brama 2000). The ratio of active to inactive MMPs increases with the establishment of osteoarthritis (Murphy 1990). Logically, the higher the concentration of active MMPs present, the more degradation that occurs within the joint. The pro-inflammatory cytokine IL-1 up-regulates various MMPs (Clutterbuck 2010). Interestingly, MMPs can, in-turn, degrade IL-1. This implies that a negative feedback loop exists, further exemplifying the close inter-relationship between the many various proteins within the joint (Clutterbuck 2010). Beyond IL-1, up-regulation of MMPs can occur from inherent extracellular matrix components, various growth factors, and certain exogenous pathogens (Khokha 2013).

Aside from the joint, the pathologic effect of excessive MMPs has been widely documented in a variety of other clinical diseases in the horse, such as laminitis, hepatitis, nephritis, recurrent airway obstruction, colic, epidermal wounds, ocular ulcerative keratitis, and neoplasia (Iredale 2006, Arosalo 2007, Clutterbuck 2010). In a study of equine osteoarthritis in the metacarpo- (metatarso-) phalangeal joint, general MMP activity (as measured by a fluorometric assay technique) was significantly increased (van den Boom 2005). This MMP activity was positively correlated with the degree of cartilage erosion, as well as the concentration of hydroxyproline, an amino-acid sequence in type II collagen, shed during degradation (van den Boom 2005).

A variety of subtypes exist within the MMP family, and are either classified numerically (ie, MMP-1) or based on the enzymatic name, which infers the substrate that is most actively degraded by a specific MMP (ie, collagenases breaks down collagen). Most MMPs may degrade multiple substrates, but generally prefer the substrate found within the enzymatic name (Clutterbuck 2010). An example of this multi-functional activity is MMP-1, which is predominately active in degrading collagen, but will also degrade glycoprotein, gelatin, and proteoglycan. An overview of the more common and active MMPs in joint disease, correlated to their general enzymatic substrate name, is provided (Clutterbuck 2010) (Table 1.2). It is important to note that the complete list of identified MMPs in mammals is much more comprehensive than those described below.

PREDOMINANT SUBSTRATE SPECIFICITY	**MATRIX METALLOPROTEINASE**
Collagenases	MMP -1, -8, -13,
Gelatinases	MMP -2, -9
Stromelysins	MMP -3, -10,

TABLE 1.2 - Categorization of the common families of matrix metalloproteinases (MMPs) in joint disease.

Collagenases (MMP-1, -8, -13) have a central role in osteoarthritis, as articular cartilage has a high component of type II collagen. Collagenases denature collagen by cleaving all 3 alpha chains of the collagen triple helix 3/4 of the length from the amino terminus (Billinghurst 1997). They have minor roles in breaking down glycoproteins, gelatin, and proteoglycans (Clutterbuck 2010). MMP-13 is more efficient at type II collagen degradation than the other collagenases, being 10 times faster than MMP-1 (Mitchell 1996). In general, MMP-13 is not considered a major contributor to joint homeostasis due to the low concentrations present in normal joints when compared to diseased joints (Reboul 1996, Trumble 2001). Alternatively, MMP-13 is thought to be active in the remodeling process of osteoarthritis, and therefore more likely to be elevated in diseased joint environments (Tardif 1999, Trumble 2001). The significance of MMP-1 in osteoarthritis is conflicting. MMP-1 concentrations were found to be higher than MMP-13 in human osteoarthritic joints (Shlopov 1997). mRNA expression of both MMP -1 and -13 were elevated in equine chondrocyte cultures that were stimulated with recombinant IL-1β (Tung 2002). In synovial tissue samples collected from horses with osteoarthritis (n=59), only 5% of cases identified mRNA expression of MMP-1, whereas 8% of cases identified mRNA expression of MMP-13 (Trumble 2001). No chondrocytes expressed MMP-1, whereas 22% of chondrocytes had MMP-13 expression (Trumble 2001). When evaluating MMP-1 concentrations in fetal, juvenile, and adult horses with normal joints, the concentration of MMP-1 progressively declined with age (Brama 2004). In horses with metacarpophalangeal joint osteoarthritis, concentration of MMP-1 was significantly elevated compared to normal age-matched horses (Brama 2004).

The function of the gelatinase family of MMPs (-2 and -9) is to degrade gelatin and unwind collagen (Murphy 1990). These MMPs also display activity against proteoglycan, collagen, laminin, elastin, and glycoproteins (Clutterbuck 2010). Gelatinases play an important role in equine osteoarthritis (Clegg 1999, Jouglin 2000). A

2 to 3 fold elevation of MMP-2 concentration has been identified in osteoarthritic joints, compared to normal horse joints (Clegg 1997). Increased MMP activity was found in metacarpo- (metatarso-) phalangeal osteoarthritis, which was predominately attributed to MMP-2 and -9 (van den Boom 2005). Gelatinase activity was noted in 76 joint samples from horses with naturally occurring osteoarthritis. The concentration of inactivated MMP-2 was elevated in 100% of OA joints. Activated MMP-2, however, was only detected in 3% of the study population (Trumble 2001). Inactivated MMP-2 may be an important component of normal joint homeostasis, and only becomes activated in response to disease (Clegg 1999). Unique to MMP-2, the inactivated form becomes activated by exposure to cell surface membranes, which is in contrast to other MMPs that require proteolytic enzyme cleavage (Clegg 1999). Elevated MMP-2 concentrations were positively correlated with older horses with acute, traumatic osteoarthritis, and negatively correlated with older horses with chronic, traumatic osteoarthritis (Trumble 2001).

Inactivated MMP-9 was found in only 43% of joints, and activated concentrations in 17% (Trumble 2001). MMP-9 does not appear to be a major contributor to normal joint homeostasis, however does become increasingly active during osteoarthritis secondary to joint sepsis (Trumble 2001). MMP-9 is highly abundant in the tertiary granules of neutrophils, and is released upon stimulation by IL-8 or TNFα (Khokha 2013). Interestingly, monitoring the concentration of MMP -2 and -9 allows for the prediction of survivability of horses with septic arthritis (Kidd 2007).

Similar to other MMP families, the production and activation of gelatinases during osteoarthritis occurs mainly by exposure to IL-1. This effect was demonstrated with MMP-2 using in vitro equine articular chondrocyte and synovial fibroblast cell culture (Clegg 1999). In this same study, IL-1 also influenced MMP-9 in synovial tissue, however it did not affect MMP-9 in articular cartilage itself (Clegg 1999). In human osteoarthritis studies, MMP-9 is also up regulated directly by exposure to MMP-2, and

other MMP subtypes, such as MMP -1, -3, -7 (Fridman 1995, Woessner 1991). In horses, pro-MMP-2 may increase MMP-9 (Trumble 2001).

The stromelysin family of MMP enzymes (-3, -10) functions in multiple pathways within the joint. Stromelysins contribute primarily to proteoglycan degradation, but also effect collagen (IX and XI), laminin, glycoproteins, elastin, and gelatin (Brama 2000, Clutterbuck 2010). Stromelysins can also activate MMP-1, -9, and -13 (Brama 2000). Of the stromelysins, MMP-3 has been implicated as the most active in osteoarthritis (Brama 2000). In human osteoarthritic joints, MMP-3 was found at high concentrations in synovium and extracellular matrix, whereas MMP-10 was not observed (Hembry 1995). In samples from horses with osteoarthritis, 86% of synovial samples (n=59) and 80% of chondrocyte (n=45) samples had elevated MMP-3 expression (Trumble 2001). In normal metacarpophalangeal and tibiotarsal joints, MMP-3 concentrations were found to be significantly elevated during fetal and juvenile growth (Brama 2000). MMP-3 concentrations decreased as horses aged (Brama 2000). However, osteoarthritic metacarpophalangeal joints had MMP-3 concentrations that were 4-fold higher than age matched controls (Brama 2000), highlighting the importance of MMP-3 in osteoarthritis. Chondrocytes and synoviocytes can up-regulate MMP-3 production in the joint, and this up-regulation is initiated mainly by IL-1 (Hasty 1990).

A variety of factors beyond osteoarthritis influence changes in MMP concentrations within the joint. Younger horses have increased joint metabolism, leading to increased cartilage turnover. As such, juvenile horses (those less than 4 years old) have increased MMP concentrations compared to mature horses in which cartilage metabolism slows down (Brama 1998). MMPs play an important role in cartilage development and maturation. Arthrocentesis likely incites local inflammation within the synovium, and increases MMP concentrations (van den Boom 2004). After 2.5 and 7 days, MMP concentrations were abnormally elevated due to arthrocentesis (van den Boom 2004). In

another study, MMP-1 concentrations were found to be elevated at 12 hours, but returned to baseline by 60 hours (Brama 2004). When correlating different joints and MMP concentrations following arthrocentesis, the radiocarpal and metacarpo- (metatarso-) phalangeal joints showed higher elevations (relative to baseline) compared to the tibiotarsal joint (van den Boom 2004). It was postulated that the larger volume of the tibiotarsal joint resulted in more dilution of MMPs, accounting for the difference (van den Boom 2004). Moderate exercise did not change MMP concentrations in normal metacarpophalangeal joints (van den Boom 2004). However, excessive exercise or joint disease may give a different response (van den Boom 2004, Pap 1998, Roos 1995).

Tissue inhibitors of matrix metalloproteinases (TIMPs) are endogenous proteins that oppose MMP function. Four TIMPs are involved in MMP antagonism (TIMP -1, -2, -3, and -4). TIMPs bind directly to the active site for MMP proteins and render its degradative function inert (Khokha 2013). TIMPs bind only active MMP proteins; they exert no interaction with the inactive MMP state (Khokha 2013). Each of the four subtypes of TIMPs exert an effect against multiple MMPs (Khokha 2013). In addition to the primary role of antagonizing MMPs and aggrecanases, TIMPs contribute to extracellular matrix remodeling by influencing cell growth and apoptosis (Chirco 2006). IL-1 stimulates production of various MMPs and actively suppresses TIMP production, augmenting the pro-inflammatory environment in osteoarthritis (Martel-Pelletier 1994). In horses, TIMP-1 and -2 are produced by chondrocytes and fibroblastic synoviocytes, with TIMP-1 also from monocytes in blood (Clegg 1998). Further equine-specific studies evaluating TIMP-3 and -4 are not available; however, inferring from human literature, TIMP-3 and -4 also originate from chondrocytes, synoviocytes and monocytes (Khokha 2013).

MMPs and TIMPs are vital participants in the development, maturation, and maintenance of the normal equine joint and are also important in synovitis and

osteoarthritis. Detrimental effects of MMPs occur through a wide variety of degradative functions. To improve understanding of the development and efficacy of various joint therapies, it will be important to define the role of MMP and TIMP activity in the joint.

PART E – AUTOLOGOUS CONDITIONED SERUM

Autologous conditioned serum (ACS) is a novel therapeutic that has been used in human and veterinary medicine for a variety of disorders. The word 'autologous' defines this therapeutic as being derived from the same individual that would be treated. ACS is an acellular composition of multiple blood-derived immunological proteins. Serum is specifically altered to up-regulate IL-1ra, which is a pure, specific antagonist to IL-1 (Meijer 2003).

The first report of IL-1ra came from a case series of 3 patients affected with monocytic leukemia, in which a protein was present in high concentrations in the patients' urine that inhibited the pro-inflammatory effects of IL-1 (Balavoine 1986). This unknown, new protein, which later became classified as IL-1ra, was further explored in a cultured fibroblast model and found to reduce up-regulation of prostaglandin E2 and collagenase (MMP-1) through antagonism of IL-1 (Balavoine 1986). Due to the underlying cancer lineage in these patients, it was postulated that IL-1ra is predominately produced by monocytes (Balavoine 1986). Further investigation showed that IL-1ra binds to IL-1 receptors present on a variety of cell surfaces throughout the body (Hannum 1990). Binding affinity of IL-1ra for the IL-1 receptor was found to be nearly equal to that of IL-1 (Arend 1990). These receptors, when bound with IL-1ra (instead of IL-1) showed no agonist response. As such, IL-1ra was identified as a pure, receptor antagonist that facilitates an indirect anti-inflammatory response through the direct blockage of IL-1 activity (Hannum 1990).

The beneficial effect of the IL-1ra in joints has previously been demonstrated in various animal models of OA. In one study, canine stifle joints were medicated with human-recombinant IL-1ra, following transection of the cranial cruciate ligament. Dogs received 2 or 4 mg of human-recombinant IL-1ra (vs. saline control) intra-articularly twice weekly, for 4 weeks duration. This study found that human-recombinant IL-1ra exerted a dose dependent effect, noted by a reduced osteophyte size and number, as well as decreased size and severity of cartilage lesions (Caron 1996). Additionally, expression of mRNA of matrix metalloproteinase-1 (MMP-1) concentrations decreased following therapy (Caron 1996). Another study utilized a homologous gene sequence carried by an adenoviral vector to induce up-regulation of IL-1ra within the joint. Following induction of osteoarthritis using an osteochondral model of OA in the equine carpus, intra-articular injections of adenovirus IL-1ra vector were performed (Frisbie 2002). Following IL-1ra gene therapy, a subjective improvement in lameness scores, decrease in synovial effusion, and decrease in proteoglycan loss (based on Safranin-O and Fast Green staining) was identified in treated joints (Frisbie 2002). The intra-articular concentration of IL-1ra was also up regulated at day 7, 14, and 21 following introduction of the vector (Frisbie 2002).

With continued research, up-regulation of IL-1ra from the monocyte could be externally stimulated by exposure to a variety of factors, such as immunoglobulin G, lipopolysaccharide, and most notably, various glass surfaces (Arend 1990, Meijer 2003). This allowed for the development of medical devices that could be used bedside relatively easily, and has been utilized for an assortment of medical disorders in humans. Even though ACS is a generic term, these medical devices have generally been referred to as ACS. In humans, ACS has mostly been used to treat osteoarthritis (Yang 2008, Baltzer 2009, Baltzer 2013), however it has also been administered with positive outcomes to patients with lumbar radicular compression and muscle injury (Wright-Carpenter 2004, Becker 2007, Chevalier 2009). ACS has been utilized extensively in

veterinary medicine predominately in the horse for the management of lameness due to synovitis and osteoarthritis. Thomas Weinberger was one of the first veterinarians to use ACS and he reported his clinical experience and success using ACS in Germany in 262 horses treated since 2001 (Weinberger 2008). In a 2007 study, it was estimated that over 3000 equine patients had received ACS in clinical practice (Frisbie 2007). During a review of joint therapy utilized by veterinarians, 54.1% of 791 practitioners stated they had experience using autologous conditioned serum as an intra-articular therapy for osteoarthritis (Ferris 2011). Of those practitioners who used ACS clinically, 21.9% stated frequent use and 32.2% stated occasional use (Ferris 2011). Most commonly, veterinarians prescribed ACS when previous attempts to alleviate inflammation in the joint with intra-articular corticosteroids were unsuccessful (36.2% of the time) (Ferris 2011). Secondarily, ACS was also used when the expense of medication was not a limitation for the owner (24.1% of the time) (Ferris 2011). Clinically, most veterinarians felt that metacarpo- (metatarso-) phalangeal joint displayed the best response to ACS injection (37.3%), however the femorotibial joint (21.6%) and distal interphalangeal joint (20.3%) also were highly implicated as showing improvement (Ferris 2011). Finally, the study also found that sport horse veterinarians (those that predominately treat horses in dressage, jumping, eventing and polo disciplines) were more likely to use ACS than veterinarians focused on other disciplines (Ferris 2011). In addition to treatment of osteoarthritis, equine surgeons have also used ACS in a prophylactic manner following arthroscopic surgery, to provide anti-inflammatory benefit to the joint during the post-operative rehabilitation period (Textor 2011).

The early introduction of ACS in North America resulted from a German company (Orthogen) marketing a human ACS preparation kit called Orthokine IRAP[a] for use in equine veterinary medicine, following the popularity of ACS in Europe (Weinberger 2008). A few years later, a North American company (Arthrex) developed an equine specific ACS preparation kit labeled IRAP II[b]. Both products have been

previously studied in the horse, and shown to increase production of IL-1ra in equine serum samples (Frisbie 2007, Hraha 2011). According to a 2009 survey of equine practitioners, approximately 54% of respondents had experience using the Orthokine IRAP[a] kit, which was the only ACS product available at the time of survey (Ferris 2011). The Orthokine IRAP product has been used for an in vivo investigation (Frisbie 2007), whereas, the Arthrex IRAP II product has been studied in an in vitro chondrocyte culture (Carlson 2013). One study performed a direct comparison of the two kits and determined that both the Orthokine IRAP[a] and Arthrex IRAP II[b] systems increased IL-1ra compared to 1-hour control serum (93-fold increase for IRAP[a] and 119-fold increase for IRAP II[b]) (Hraha 2011). In recent years it can be argued that use of the Arthrex IRAP II[b] product has become more common amongst equine veterinarians in North America. This change may be a reflection of the modestly improved cytokine profile identified using the Arthrex IRAP II[b] kit, as reported (Hraha 2011). The Arthrex IRAP II[b] kit contains a pre-made blood collection syringe, butterfly catheter, and a second (closed system) syringe that contains beads of borosilicate origin. Borosilicate glass has previously been recognized as one of the surfaces that can induce IL-1ra up-regulation from monocytes (Meijer 2003). Specifically, the Arthrex IRAP II kit contains 327 borosilicate beads, with each bead 3.0 mm in diameter and 92.4 mm^2 in surface area (Bare 2010). In comparison, approximately 200 borosilicate beads are present within the Orthokine IRAP syringe, with each bead 2.5 mm in diameter and 21 mm^2 in surface area (Meijer 2003).

Specific for the Arthrex IRAP II kit[b], the process begins by collecting 50 ml of whole blood aseptically from the individual equine patient to be treated. The whole blood is transferred into a second polypropylene syringe containing the beads, by connecting the two syringes by a Leur-lock mechanism. The current recommendation by the manufacturer is to hold the syringe at a 60-degree angle and inject the blood slowly, such that the blood runs down the wall of the bead-syringe. The bead-syringe is then gently inverted a minimum of 10 times to mix the whole blood and beads, and the syringe is

placed in a horizontal plane within an Arthrex specific incubator[b]. The incubator is set at a temperature of 37°C, and the syringe is left untouched for a recommended 16 to 24 hours (with special care not to exceed 24 hours). Following incubation, the serum is spun in an Arthrex specific centrifuge[b] for 10 minutes at 4000 rpm (which, when taking into account the radius of the rotor, equates to approximately 1,800g). Following bead-based surface activation, incubation and centrifugation, processed serum is considered 'conditioned' and as such meets the definition of ACS.

The recommendation for incubation time for the Arthrex IRAP II[b] system is likely derived from the original description of ACS. Elevation in IL-1ra concentration began at 30 minutes, and continued to increase for 24 hours. However, after 24 hours of incubation, the concentrations then steadily decreased (Meijer 2003). Interestingly, one human study utilized an incubation time of only 6 hours, which successfully elevated IL-1ra when combined with glass beads (Rutgers 2010). Recently, it has been questioned whether exposure to glass beads are necessary. This originates from several studies wherein IL-1ra concentrations increase in the non-conditioned, yet incubated, control serum group (Hraha 2011, Magalon 2014, Fjordbakk 2015). Hraha et. al. postulated that exposure to the glass wall of the non-heparinized blood tube, combined with incubation, may be the underlying mechanism for the observed increase of cytokine concentrations (Hraha 2011). In their study, when concentrations of IL-1ra were compared to 24-hour incubated control, the Orthokine IRAP[a] product (but not Arthrex IRAP II[b]) was not statistically different. However, both Magalon et. al. and Fjordbakk et. al. reported a similar IL-1ra increase in incubated control serum when using plastic (not glass) syringes. In fact, both authors proposed that exposure to glass beads may not be necessary for preparation of cytokine-rich serum. Since it appears that incubation alone influences IL-1ra elevation, further research is required to determine whether glass beads may impact other anti-catabolic, catabolic and anabolic proteins, thereby providing further therapeutic efficacy via alternate mechanisms.

Under aseptic conditions, the resultant ACS is withdrawn from the IRAP syringe using a sterile spinal needle, and aliquoted into individual syringes. During transfer of ACS into individual syringes, it is common for ACS to be passed through a 0.2 μm micro-filter to help improve the purity of the product during in-house processing. Transfer through a micro-filter is also commonly performed a second time, immediately prior to intra-articular injection. Aliquots of ACS are generally frozen in a conventional freezer at -20°C for future use. To the author's knowledge, no studies have been performed evaluating the effect of freeze-thaw cycles on the composition of ACS in the horse, which is important to know since freeze-thaw cycles can induce cell membrane rupture and/or protein denaturation (Guo 2013).

It is important to note that the manufacturer recommends dividing the ACS to attain 4 ml aliquots per syringe, but in a clinical setting the volume stored per aliquot is predominately dictated by the practitioner. This preference is influenced by the volume needed per intra-articular injection, as well as the total volume of ACS attained following processing. Although no standard dose for ACS is universally accepted, Weinberger reported a treatment protocol based on the type of joint medicated based on his personal experience (Table 1.3) (Weinberger 2008).

ACS TREATMENT PROTOCOL (ADAPTED FROM WEINBERGER 2008)	
JOINT	**TREATMENT PROTOCOL**
Coffin Joint	Dose: 4 – 6 ml; Number: 2 - 3 times; Interval: 8 - 14 days
Pastern Joint	Dose: 2 - 4 ml; Number: 2 - 3 times; Interval: 8 - 14 days
Fetlock Joint	Dose: 4 - 6 ml; Number: 2 - 3 times; Interval: 8 - 14 days
Radiocarpal / Intercarpal Joint	Dose: 4 - 6 ml; Number: 2 - 3 times; Interval: 8 - 14 days
Elbow Joint	Dose: 4 – 6 ml; Number: 2 - 3 times; Interval: 8 - 14 days
Shoulder Joint	Dose: 4 - 8 ml; Number: 2 - 3 times; Interval: 8 - 14 days
Tarsometatarsal and Distal Intertarsal Joint	Dose: 1 - 2 ml; Number: 2 - 3 times; Interval: 8 - 14 days
Tibiotarsal Joint	Dose: 6 – 8 ml; Number: 2 - 3 times; Interval: 8 - 14 days
Femorotibial / Femoropatellar Joint	Dose: 4 - 8 ml; Number: 2 - 3 times; Interval: 8 - 14 days
Coxofemoral Joint	Dose: 4 – 8 ml; Number: 2 - 3 times; Interval: 12 - 21 days

TABLE 1.3 – ACS treatment protocol developed by Thomas Weinberger for use of IRAP for treatment of joint disease in horses (Adapted from Weinberger 2008).

The ideal volume of ACS required to impart the maximal amount of therapeutic benefit remains unclear. In normal joints, type B synoviocytes have thousands of IL-1 receptors present per cell (Larrick 1989). In osteoarthritis, IL-1 receptors are further up-regulated in the cartilage and synovium (Fernandes 2002). The abundance of IL-1 receptors increases the likelihood of IL-1 binding, which initiates pro-inflammatory change in the joint. Additionally, very minimal IL-1β binding overall is required to initiate pro-inflammatory cellular signaling (Fernandes 2002). As such, the quantity of IL-1ra that is needed to successfully compete against IL-1 is disproportionally high. The disparity in the ratio of IL-1ra to IL-1β has been supported in vitro. During an early investigation of IL-1ra, it was found that a 50% reduction in IL-1α could be achieved with a 30-fold greater concentration of IL-1ra, while a 50% reduction in IL-1β induced prostaglandin E2 could be achieved with a 10-fold greater concentration of IL-1ra compared to IL-1 (Arend 1990). Furthermore, this same study also showed that a 100-fold greater concentration of IL-1ra was required for 50% reduction of IL-1α in hyaline cartilage (Arend 1990). With ACS therapy, the ratio of IL-1ra to IL-1 can effectively represent therapeutic potential (Meijer 2003). In human medicine, a proposed ratio of 10 to 1 (IL-1ra to IL-1) has been derived as an ideal threshold for therapeutic efficacy (Granowitz 1991). However, to date, no calculations of the ideal IL-1ra to IL-1 ratios have been investigated for veterinary species. When comparing the IL-1ra to IL-1β ratio of Orthokine IRAP[a] and Arthrex IRAP II[b] kits, only the IRAP II[a] kit (with a ratio of 6:1) had a statistically higher ratio than that of 1-hour serum control (Hraha 2011). Hraha et al. recognized that the equine IL-1ra to IL-1 ratio they found was low compared to human-derived ACS (Hraha 2011).

Another unique consideration of ACS joint therapy is the inability to standardize the consistency of the product, such as is accomplished with traditional pharmaceutical drugs. This unpredictability is most apparent when appreciating the high standard of error of reported IL-1ra concentrations in prior equine ACS studies (Frisbie 2007, Hraha 2011,

Fjordbakk 2015). Since ACS is derived from individual horses, inherent variability exists between horses and within the same horse after exposure to external factors, such as stress (Fjordbakk 2015). Fjordbakk et. al. studied the impact of surgery, as determined by serum amyloid A (SAA) concentration, on resultant cytokine production in equine ACS. Horses classified with 'marked' stress (SAA > 200 mg/L) had significantly lower concentrations of IL-1ra, TGF-β, and IGF-1 than those horses classified with 'moderate' or 'mild' stress (SAA < 200mg/L) (Fjordbakk 2015). Potentially, an influence of systemic OA severity could also influence IL-1ra production in ACS. However, to the author's knowledge, no studies have explored this concept.

Within the ACS product, IL-1ra is marketed as the major anti-inflammatory factor present. However, other biologically active proteins change in concentration. Increases in anti-catabolic and anabolic proteins such as IL-10 (Meijer 2003, Rutgers 2010, Magalon 2014), IL-4 (Meijer 2003), and TGF-β (Rutgers 2010) have been reported in human ACS. Hraha et. al. evaluated both Orthokine IRAP[a] and Arthrex IRAP II[b] systems with equine blood, and found increased concentrations of IL-10, TGF-β, and IGF-1 in both kits (compared to unincubated serum). In conflict with these findings, Fjordbakk et. al. found no difference in IL-10, TGF-β, and IGF-1 in equine ACS. However, this study also utilized a human and a proprietary, unmarketed ACS kit, rather than the prevalent Orthokine IRAP[a] and Arthrex IRAP II[b] systems.

Also of importance is whether conditioning of serum may increase pro-inflammatory cytokines. In the original description of the Orthokine IRAP[a] kit using human blood, no increase in the pro-inflammatory cytokines IL-1β or TNF-α were reported (Meijer 2003). Contrary to these findings, both Rutgers et. al. and Magalon et. al. have identified significant increases in IL-1β, TNF-α, and the immuno-modulatory cytokine IL-6 in human ACS (Rutgers 2010, Magalon 2014). Using equine blood, both IL-1β and TNF-α were elevated in the Orthokine IRAP[a] and Arthrex IRAP II[b] systems

compared to unincubated control (Hraha 2011). Yet, Fjordbakk et. al. could only identify an increase in IL-1β, but not TNF-α, in their study (Fjordbakk 2015). Understanding of the anti- and pro-inflammatory biological spectrum of ACS remains conflicted. This disagreement may be a cumulated result of variability between sample populations, differences in species, differences in ACS kits utilized, or varying laboratory methods. Further research to define the actual composition of ACS is needed, to help better understand how it works.

Assessment of clinical efficacy from ACS treatment for osteoarthritis has conflicting opinions in the human medical field. In a prospective, non-blinded, clinical trial treating patients with hip osteoarthritis (n = 46 patients) with ACS, therapy was more effective when administered alone in reducing pain scores when compared to ACS/ corticosteroid combination and ACS/corticosteroid/recombinant IL-1ra combination (Baltzer 2013). Another prospective, randomized, patient/observer blinded, placebo-control clinical trial was performed on patients with knee osteoarthritis (n = 376 patients) (Baltzer 2009). In this study, ACS was found to reduce clinical symptoms and pain scores for up to 6 months, when compared to hyaluronic acid (HA) and saline controls (Baltzer 2009). However, a major flaw in the study design was that ACS patients received 6 intra-articular injections total, whereas HA and saline patients only received 3 (Baltzer 2009). A separate group performed another prospective, randomized, patient/observer blinded clinical trial that also used knee osteoarthritis (n = 167 patients) (Yang 2008). In this study, outcome was based on a variety of established pain scales. Although ACS treated patients trended towards more comfort and improved joint function, no parameters measured were statistically significant (Yang 2008).

In horses, a limited amount of peer-reviewed, scientific research is available to help understand ACS therapy. One in vitro study collected cartilage from cadavers, which were in turn transformed into chondrocyte pellets and then exposed to either IL-1β and

ACS, or IL-1β and unconditioned serum (Carlson 2013). In this experiment, no significant changes were identified when evaluating glycosaminoglycan release, total glycosaminoglycan concentration, MMP-3 concentration, total DNA content, or mRNA expression of cyclo-oxygenase-2, aggrecan and type II collagen (Carlson 2013). The concentration of IGF-1 was identified as significantly higher in the ACS-treated pellets, which was interpreted as a beneficial effect. The authors concluded that very minimal difference with ACS exposure occurred (Carlson 2013).

Only one in vivo ACS study has been performed using horses, thus far. As such, this individual clinical investigation has become a noteworthy scientific basis to justify ACS use in equine veterinary practice. In 2007, Frisbie et al. used an osteochondral fragment model for induction of osteoarthritis in the middle carpal joint (Frisbie 2007). Eight horses had one forelimb induced with an osteochondral fragment and the contralateral joint sham operated (treatment group). For therapy intervention, 6 ml of ACS (Orthokine IRAP[a] kit) was administered into the osteochondral fragment joint while saline was injected into the sham-operated joint. A second group of eight placebo horses also underwent surgery (one limb for osteochondral fragmentation, other only sham operated) and were subsequently treated with placebo. Injections were started on day 14 of the study, and repeated once per week for a total of 4 weeks (Frisbie 2007). Several outcome measures were evaluated at various time points, including lameness grading (including flexion stress tests), radiographic evaluation, synovial fluid analysis (gross characteristics, white blood cell count/differential, total protein concentration, mucin content, glycosaminoglycan [GAG] concentration, prostaglandin-E2 [PGE2], IL-1ra concentration), synovial membrane gross pathology/histology, cartilage gross pathology/histology, and cartilage matrix metabolism analysis (Frisbie 2007).

Clinically, a statistically significant improvement in the degree of lameness was subjectively noted 35 days following the last intra-articular ACS injection. However, this

improvement was only identified at one single time point. Also of interest, the improvement in lameness was less than one full grade (based on American Association of Equine Practitioner scale [Anonymous 1991]) in magnitude (Frisbie 2007). It is unclear how the OA and sham operated limbs were evaluated independently. A more objective lameness analysis (ie, force plate) may have improved the strength of lameness observation. Other subjective assessments of clinical improvement, such as joint effusion and joint flexion response, were unchanged (Frisbie 2007). Radiographic changes of osteoarthritis became apparent using the carpal osteochondral fragment model; however, no improvements in radiographic signs were seen in the ACS-treated group when compared to controls (Frisbie 2007). Additionally, when examining the synovial fluid for IL-1ra concentrations, the investigators combined the IL-1ra concentrations from the OA and sham operated joints, and demonstrated a significant elevation in IL-1ra compared to the placebo horse group when measured 35 days after the last ACS injection (Frisbie 2007). It remains unclear why both the OA and sham operated joints were evaluated together, and not assessed individually. After combining the two joints, the authors then inferred that administration of exogenous IL-1ra (via ACS) may potentially stimulate endogenous, systemic IL-1ra production (Frisbie 2007).

In this study, there was no significant difference in neutrophil percentage of the total white blood cell population, total protein concentration, GAG concentration, or PGE2 concentration found between groups (Frisbie 2007). Scores for gross cartilage erosion and synovial membrane hemorrhage were assigned, and although ACS-treated joints displayed a trend for improved (lower) scores, this outcome was not statistically significant (Frisbie 2007). Histologically, there was a significant decrease in intimal hyperplasia in the ACS-treated joints; however, grades of subintimal fibrosis, synovial vascularity, and cartilage erosion/fibrillation showed no difference between groups (Frisbie 2007). Finally, no significant difference in cartilage GAG content from metabolism was present between groups (Frisbie 2007). Although many parameters

showed no statistical difference, based on the improved lameness the authors concluded that IL-1ra showed promise as an intra-articular therapy (Frisbie 2007).

It is unknown how long exogenous IL-1ra remains in the joint following injection. Repeated doses of ACS are commonly administered when treating OA. The justification for serial doses is unclear, however may originate from treatment protocols that use recombinant IL-1ra, wherein several doses are required for therapeutic efficacy (Gouze 2003). Baltzer et. al. administered 2 doses of recombinant IL-1ra per week for 3 weeks (6 total doses) (Baltzer 2009). Rapid intra-articular clearance of IL-1ra occurs within 3 days following intra-articular ACS injections, which may justify repeated dosing (Rutgers 2010). Veterinarians commonly administer 3 to 5 doses, with each dose separated by 7 to 14 day intervals (Weinberger 2008, Textor 2011). In the Frisbie et. al. study, the authors developed their treatment protocol (4 doses, 7 days apart) based on common use of the medication in Europe (Frisbie 2007). The availability of large volumes of ACS from one individual ACS kit readily allows veterinarians to repeat intra-articular injections.

ACS is marketed as a novel therapy that is more biologic than traditional pharmaceuticals. Due to it autologous and biological composition, there is the added bonus that ACS cannot be detected by conventional drug testing. This becomes an important advantage for many owner/trainer and veterinarian teams that are actively managing OA in athletic horses that compete in regulated disciplines. By definition, ACS therapy is categorized as a medical device. Although medical devices can provide alternative means for therapy, there is no requirement for safety or efficacy evaluation. In fact, the American Association of Equine Practitioner (AAEP) guidelines maintain that if an approved pharmaceutical product exists for a specific disease, the pharmaceutical should be used instead. Additionally, AAEP states that it is unethical to promote a medical device as equivalent to an approved pharmaceutical, and clients must be informed of the difference between the two products prior to therapy (Anonymous 2010).

The economic implication of offering ACS therapy is favorable for veterinarians. The average wholesale cost of each individual ACS kit is around $250. When combined with the professional costs for intra-articular injection and repeated dosage, the total cost of ACS often totals $1000 to $1200 (Textor 2011). In comparison, intra-articular corticosteroids may be a third to a quarter of this price.

In spite of increased ACS usage in horses, no prospective or retrospective evaluations of short and long term clinical use has been published to date (Ferris 2011, Textor 2011). Anecdotally, many practitioners regard the medication as safe, with the same risk for joint inflammation (flare) or infection as other intra-articular medications. Practitioners apparently do not commonly mix antibiotics with ACS when injecting the serum into a joint. The effects of adding antibiotics on ACS composition are unknown. Maintenance of ACS product sterility is almost solely dependent on those handling it. To the author's knowledge, no adverse systemic effects (such as those seen with non-steroidal anti-inflammatories or corticosteroids) have been associated with ACS.

The clinical benefits of IL-1ra have been previously demonstrated by administering recombinant IL-1ra intra-articularly or using gene therapy as a method of increasing concentrations (Caron 1996, Frisbie 2002). To date, rationale for ACS use in horses is limited to one in vivo study, which was funded by the manufacturer (Frisbie 2007). Anecdotal evidence from practitioners suggests that the therapy may be effective in reducing OA pain. Detailed knowledge of the composition of ACS and its effect on arthritic joints need to be defined. A better understanding of the variability of the product from horse to horse, the ratio of IL-1ra to IL-1β required for efficacy, influence of glass beads and incubation on conditioning of serum, and validation of repeated doses would provide better scientific rationale for treatment. Well-designed clinical trials are necessary to validate therapeutic use of ACS.

CHAPTER 2:
HYPOTHESIS, SPECIFIC AIMS, AND RATIONALE

PART A - HYPOTHESIS

Our central hypothesis is that autologous conditioned serum (ACS) will be associated with anti-inflammatory effects when injected into osteoarthritic distal interphalangeal joints. The main objectives of this study are: (1) to evaluate the cytokine, MMP, and TIMP content of ACS produced by the Arthrex® IRAP II system[b] and (2) to measure cytokine, MMP, and TIMP content of osteoarthritic distal interphalangeal joint synovial fluid 7 days following three repeated intra-articular ACS injections.

Aliquots of ACS are usually frozen for use at a later date. However, the effect of freeze-thaw cycle on ACS is unknown. It is unknown whether repeated doses are more effective than a single dose. Clinical improvement of osteoarthritis following intra-articular ACS therapy has not been documented under controlled conditions.

Our working hypothesis is that ACS will have increased anti-inflammatory cytokines and tissue inhibitors of metalloproteinases, with smaller increases in pro-inflammatory cytokines and matrix metalloproteinases, when compared to unconditioned 1-hour serum. We also hypothesize that injection of ACS into osteoarthritic distal interphalangeal joints will be associated with increased synovial fluid concentrations of anti-inflammatory cytokines and tissue inhibitors of metalloproteinases, in greater proportion to changes seen in pro-inflammatory cytokines and MMPs, when compared to synovial fluid prior to ACS injection. We hypothesize that high variability in ACS composition will exist between individual horses, that repeated dosing will result in greater increases in anti-inflammatory biomarkers compared to that of a single dose, and that all biomarker concentrations in ACS will decrease following an additional freeze-thaw sample. We hypothesize that clinical improvement in lameness will be present following ACS therapy. Knowledge gained from this study may lead to future studies on

the efficacy of ACS for treatment of osteoarthritis in horses.

PART B - SPECIFIC AIMS

1. Define the cytokine, matrix metalloproteinase, and tissue inhibitor of metalloproteinase content of ACS produce from the commercially available Arthrex® IRAP II[b] system.

2. Evaluate variability in cytokine, matrix metalloproteinase, and tissue inhibitor of metalloproteinase concentrations in ACS between individual horses.

3. Evaluate the effect of freeze-thaw cycle on ACS composition.

4. Evaluate whether repeated (serial) doses of ACS will further increase anti-inflammatory biomarker concentrations in synovial fluid, compared to concentrations after a single dose of ACS.

5. Evaluate improvement of lameness grade following intra-articular ACS therapy in cases of naturally occurring osteoarthritis.

6. Validate/refute that intra-articular injection of ACS into an osteoarthritic joint will result in increased synovial fluid concentrations of anti-inflammatory cytokines and tissue inhibitors of metalloproteinases, with minimal concurrent changes in matrix metalloproteinase or pro-inflammatory cytokine concentrations.

PART C - RATIONALE

The rationale for this study is that in spite of the common clinical usage of ACS, little is known about the actual composition of the product injected into the horse, how much ACS varies from horse to horse, or the effects of freeze-thaw cycles on ACS composition. Additionally, knowledge regarding any change to the composition of cytokines, MMPs and TIMPs in synovial fluid after treatment, or whether any clinical improvement occurs after intra-articular ACS therapy, also remains unknown. Given the popularity of ACS in equine veterinary medicine, there is clearly a need for more scientific information regarding the clinical use of ACS in horses.

CHAPTER 3:
MATERIALS AND METHODS

PART A - POWER CALCULATIONS

To determine the number of horses required for the study, power calculations were based on changes demonstrated in our pilot data (Table 3.1 and 3.2). Eight analytes listed in Tables 3.1 and 3.2 that were either significantly different between groups (normal vs. osteoarthritic synovial fluid or serum) or approached significance ($P<0.3$) were used to determine power. Unpaired means were compared between two groups (normal vs. osteoarthritis) for each analyte. Differences between the means were assumed to be the true difference. Standard deviations between two groups (normal vs. osteoarthritis) of each analyte were averaged. Then, sample size required to detect a difference for each analyte was calculated, with power set at 80%. Finally, averaging the previously calculated sample sizes across the 8 analytes set the total number of horses required for the study. Therefore, for this investigation, 12 horses were required to achieve 80% power at an alpha of $P<0.05$.

SF	*IL-1β*	*IL-1ra*	*TNFα*	*IL-4*	*IL-6*	*IL-8*	*IL-10*
Norm N=4	**0.10 * ±0.14**	33.4 ±26.6	**0.00 † ±0.01**	1.47 ±1.47	0.20 ±0.28	**17.1 † ±21.4**	**0.09 † ±0.05**
OA N=7	**0.62 ±0.46**	60.4± 73	**0.56 ±0.69**	1.23 ±1.41	0.22 ±0.53	**2.1 ±4.3**	**0.05 ±0.04**
Serum							
Norm N=3	0.55 ±0.95	**47.2 † ± 45**	0 ± 0	0 ± 0	0 ± 0	2730 ±884	0 ± 0
OA N=4	1.03 ±1.03	**113.2 ±80**	0.25 ±0.29	0 ± 0	0.64 ±1.29	1628 ±208	0.11 ±0.23

TABLE 3.1 - Pilot multiplex assay data for cytokines and growth factors using commercially available human kits (R&D Systems[c]). Mean (±SD) concentrations from

synovial fluid (carpi) and serum from normal (Norm) horses and those with naturally occurring OA associated with osteochondral injury (OA). Note: Pilot assays used polystyrene beads, whereas magnetic beads were used for the subsequent ACS investigation. Significant differences and trends are bolded. * P<0.05, † P<0.3.

SF	*MMP3*	*MMP9*	*MMP13*	*TIMP1*	*TIMP3*	*TIMP4*
Norm n=4	709 ±252	296 ±332	**0 ± 0***	207 ±9.4	**1499 † ±439**	**15.2 * ±1.17**
OA n=7	923 ±385	259 ±425	**39.6 ±56.9**	212 ±7.6	**1062 ±484**	**19.8 ±2.7**
Serum						
Norm N=3	**192 † ±102**	186949 ±106405	0 ± 0	-	-	-
OA N=4	**95 ±45.1**	95269 ±47410	0 ± 0	-	-	-

TABLE 3.2 - Pilot multiplex assay data for MMPs and tissue inhibitors of metalloproteinases (TIMPs) using commercially available human kits (R&D Systems[c]). Mean (±SD) concentrations from synovial fluid (carpi) and serum from normal (Norm) horses and those with naturally-occurring OA associated with osteochondral injury (OA). Note: Pilot assays used polystyrene beads, whereas magnetic beads were used for the subsequent ACS investigation. Significant differences and trends are bolded. * P<0.05, † P<0.3

PART B - STUDY DESIGN

Client-owned horses with unilateral forelimb distal interphalangeal (DIP) joint osteoarthritis (OA) were identified. For inclusion, horses needed to satisfy at least 3 of the following criteria: palpable DIP joint effusion, positive response to distal limb flexion, positive response to DIP joint intra-articular analgesia, radiographic evidence of

OA in the DIP joint, and/or previous positive response to intra-articular corticosteroid injection. Three blinded investigators (T.N.T., N.S.E., M.C.M.) graded radiographs for severity of DIP OA and changes to the navicular bone (Manfredi 2012), using a modified scheme (Table 3.3 and 3.4). Exclusion criteria included horses with moderate to severe radiographic signs of navicular bone abnormalities (≥ grade 3 on Table 4) or history of previous corticosteroid injection in the affected joint within 6 months.

0	Normal appearance of distal interphalangeal joint
1	Small osteophyte present in either dorsal or palmar aspect of joint
2	Small osteophytes present in both dorsal and palmar aspect of joint
3	Moderate or large sized osteophytes present, presence of subchondral lucencies
4	Joint collapse, cyst formation in articular surface of P2 or P3

TABLE 3.3 – Radiographic grading scale for distal interphalangeal osteoarthritis. The average measure intraclass correlation coefficient for agreement of the observers' distal interphalangeal joint radiographic scores in this study was 0.89 (≥ 0.80 is considered acceptable).

0	Normal appearance of navicular bone
1	Multiple distal border lucencies of variable shapes
2	Sclerosis mildly obscuring corticomedullary junction
3	Poor corticomedullary junction, thick cortices, large enthesophytes, distal border fragments
4	Large cysts, productive bone on flexor surface

TABLE 3.4 – Radiographic grading scale for navicular bone pathology. The average measure intraclass correlation coefficient for agreement of the observers' navicular bone radiographic scores was 0.95 (≥ 0.80 is considered acceptable).

On the day prior to first intra-articular ACS injection, 50 ml of blood was collected aseptically via jugular venipuncture into an ACS syringe containing borosilicate beads[b], following manufacturer directions. The syringe was incubated[b] at 37°C for 19 hours (range: 18-20 hours), then centrifuged[b] for 10 minutes at 1,800 x g. This conditioned serum (ACS) was aseptically aspirated from the syringe, and passed through a 0.2 μm filter[d] into three separate 5 ml aliquots that were used for weekly intra-articular treatments as described below. Any remaining ACS (range: 3-10 ml) was further aliquoted and frozen at -80°C for future analysis. Day 0 aliquot was injected immediately, but day 7 and 14 doses were stored frozen (-20°C) and thawed prior to administration. As a control, an additional 20 ml of blood was simultaneously collected in non-heparinized vacuum tubes. Ten ml were immediately centrifuged[e] for 10 minutes at 1,800 x g, and serum decanted within 1 hour following venipuncture (baseline control – 1-hr serum). The remaining 10 ml was incubated[b] at 37°C for 18-20 hours, and similarly centrifuged[e]. Serum was decanted within 19 hours of venipuncture, similar to ACS, and was used as an incubated, unconditioned control (19-hr serum). Control serum was aliquoted and frozen at -80°C for future analysis.

For each horse, arthrocentesis of the OA-affected DIP joint was performed on day 0, 7, 14, and 21, with day 0 serving as pre-treatment OA baseline. Horses were sedated with detomidine hydrochloride[f] (0.004 – 0.02 mg/kg IV), and a lateral and medial abaxial sesamoid nerve block was performed with 2 ml of lidocaine[g] per palmar digital nerve. The DIP joint was graded for effusion (mild, moderate, or severe), and then aseptically prepared for dorsal and lateral approaches (Moyer 2011). To obtain synovial fluid, a 20-gauge, 1.5-inch needle was inserted into the DIP joint using a dorsal approach initially, followed by a lateral approach if necessary. Arthrocentesis occurred during late morning to early afternoon for all horses over all sampling periods. Number of attempts required

to obtain fluid, as well as presence/absence of blood contamination was documented for each collection. Synovial fluid was centrifuged[e] for 10 minutes at 2,500 x g and decanted if blood contamination was present; aliquots were frozen at -80°C for future analysis.

On days 0, 7, and 14, immediately following arthrocentesis, a 5 ml aliquot of fresh ACS (day 0) or thawed ACS (days 7 and 14) was again passed through a 0.2 μm filter[d], and 4 ml of ACS injected into the DIP joint. For days 7 and 14, the remaining 1 ml of ACS was refrozen a second time (at -80°C) for future laboratory analysis. On day 21, no ACS was injected; only arthrocentesis was performed to assess previous ACS treatments. Throughout the study, horses were maintained on strict stall rest with no exercise allowed. No anti-inflammatory medications or joint supplements were given.

At each time point (day 0, 7, 14, and 21), prior to arthrocentesis, horses were videotaped walking and trotting in a straight line, on a hard surface. Additionally, bilateral distal forelimb flexions were performed. At study completion, three blinded investigators (T.N.T., N.S.E., M.C.M.) graded the lameness videos based on the American Association of Equine Practitioners scale of lameness (Anonymous 1991). Median scores were used for analysis.

PART C - ELISA AND MULTIPLEX METHODOLOGY

Serum and synovial fluid samples were evaluated using an equine specific ELISA[c] (IL-1ra) or human multiplex assays[c] using Luminex® xMAP® technology[h] to evaluate cytokine (IL-1β, -4, -6, -8, -10 and TNFα), matrix metalloproteinase (MMP-1, -3, -9, and -13) and tissue inhibitor of MMP (TIMP-1, -2, -3, and -4) concentrations. For human multiplex assays, magnetic beads (rather than polystyrene beads) were chosen. All samples were run in duplicate or triplicate at pre-determined dilutions based on pilot studies (Table 3.1 and 3.2) where partial validation was performed with equine serum and

synovial fluid to demonstrate that each analyte could be recognized in each equine fluid (unpublished data). Equine and human sequences have the highest percent homology overall (>60% homology required for potential cross-reactivity with cytokines [Scheerlinck 1999]), making human commercial kits the best option for identifying cytokines in equine fluids (Table 3.5). Both the equine specific ELISA[c] and the human multiplex assays[c] were run according to manufacturer recommendations, with no alterations in laboratory technique.

The ELISA used is sandwich type, as it utilizes a capture antibody immobilized within the plate wells to bind the analyte of interest (ie, IL-1ra). Binding of detection antibody to the analyte of interest then provides fluorescence that can be measured as mean absorbance by a plate reader. When compared against a standard curve, quantitative results are available. The Luminex platform technology is a bead based, flow cytometer system that can evaluate up to 100 different analytes attained from a single sample of fluid. Each bead is internally color-coded with varying ratios of 2 fluorescent dyes. This allows an analyte of interest to be measured through covalent coupling to specific capture antibodies. One laser identifies the specific bead set, and another laser quantifies the florescent reaction to the antibody. Then, the florescent reaction can be compared against a standard curve (similar to an ELISA), to also provide quantitative results.

Because values in some samples were below the lower limit of quantification (LLOQ - lowest standard curve point) for multiple analytes (Table 3.6), half of the lower limit of detection for each individual analyte was used as the representative concentration to allow statistical analyses (Croghan 2003). Mean intra-assay and inter-assay coefficient of variation (CV) for IL-1ra was 9% and 8%, respectively, whereas the remaining analytes measured by multiplex assay averaged 8% and 12%, respectively.

Protein	Equine NCBI Accession #	% Human protein homology	Human accession #	% Mouse protein homology	Mouse accession #	% Rat protein homology	Rat accession #
IL-1 β	AAC39256	65	EAW73607	62	NP_032387	60	NP_113700
IL-1 Ra	NP_001075994	77	NP_776213 isof 2	76	NP_112444 isof 1	75	NP_071530
IL-4	NP_001075988	55	CAP72493	40	BAD83771	43	NP_958427
IL-6	NP_001075965	61	NP_000591	43	AAA68814	44	NP_036721
IL-8	NP_001077420	72	CAA77745				
IL-10	NP_001075959	84	CAG46825	75	NP_034678	76	NP_036986
TNF α	NP_001075288	85	NP_000585	77	NP_038721	75	CAA47146
MMP-1	NP_001075316	86	AAV38949	59	NP_114395	59	NP_001128 002
MMP-2	CAB46656	95	BAG35588	94	NP_032636	94	CAA50583
MMP-3	AAB05774	81	NP_002413	73	NP_034939	71	EDL78534
MMP-9	ABU80399	84	P14780	78	BAA02208	77	EDL96479
MMP-13	NP_001075273	90	AAH67523	86	NP_032633	86	P23097
TIMP-1	NP_001075984	82	BAG52016	66	NP_0010378 49	71	AAB08483
TIMP-2	ABL11344	98	AAC50729	100	AAA40446	100	AAA21553
TIMP-3	CAB51854	99	NP_000353	96	NP_035725	95	NP_037018
TIMP-4	XP_001492627 Predicted	90	AAB40391	84	NP_542370	84	NP_001102 863

TABLE 3.5 - Homology of equine cytokine, MMP, and TIMP protein sequences compared to human, mouse, and rat (determined using NCBI BLASTp software). Note that equine and human sequences have the highest percent homology overall (>60% homology required for potential cross-reactivity with cytokines [Scheerlinck 1999]), making human commercial kits the best option for identifying cytokines in equine fluids.

Please note that homology alone does not indicate whether equine proteins will interact with human-based assays, but provided the starting point as to which species to use. Pilot data with pooled serum and synovial fluid from horses was performed by the investigators (unpublished data) to partially validate use of human assays with equine matrices (precision, linearity of dilution, parallelism, percent recovery, and stability). Note that MMP-2 did not reliably react with either equine fluid and as such was not examined in this study.

BIOMARKER	1 HOUR	19 HOUR	ACS	D0 SF	D7 SF	D14 SF	D21 SF
IL-1ra	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11
IL-1β	2 / 11	0 / 11	2 / 11	7 / 11	7 / 11	6 / 11	7 / 11
TNFα	3 / 11	1 / 11	4 / 11	7 / 11	7 / 11	9 / 11	7 / 11
IL-4	3 / 11	1 / 11	9 / 11	9 / 11	10 / 11	10 / 11	11 / 11
IL-6	4 / 11	2 / 11	8 / 11	8 / 11	11 / 11	9 / 11	10 / 11
IL-8	0 / 11	0 / 11	0 / 11	0 / 11	1 / 11	1 / 11	0 / 11
IL-10	6 / 11	3 / 11	8 / 11	7 / 11	9 / 11	9 / 11	7 / 11
MMP-1	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11
MMP-3	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11
MMP-9	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11
MMP-13	11 / 11	11 / 11	11 / 11	4 / 11	3 / 11	7 / 11	4 / 11
TIMP-1	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11
TIMP-2	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11
TIMP-3	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11
TIMP-4	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11	0 / 11

TABLE 3.6 - Description of the number of samples that were measured below the lower limit of quantification for all analytes in serum and synovial fluid. (1 HOUR = unincubated serum control; 19 HOUR = incubated serum control; ACS = autologous conditioned serum; D0 SF = Day 0 synovial fluid sample; D7 SF = Day 7 synovial fluid sample; D14 SF = Day 14 synovial fluid sample; DAY 21 SF = Day 21 synovial fluid sample).

PART D - STATISTICAL ANALYSIS

Serum and synovial fluid concentrations of each analyte were assessed using Shapiro-Wilk normality test to determine appropriate analysis for each individual analyte. In serum, parametric data was compared using one-way ANOVA, with Tukey's post-hoc multiple comparisons for IL-1ra, IL-8, MMP-1 and -9, TIMP -1, -2, -3, and -4; non-parametric data was compared using Kruskal-Wallis with Dunn's post-hoc multiple comparisons for IL-1β, -4, -6, and -10, TNFα, MMP-3 and -13. MMP:TIMP ratios in serum were assessed similarly, with normality of individual ratios detailed in Figure 4.3. All analytes in synovial fluid were non-parametric and were assessed using the Friedman test, with Wilcoxon signed-rank test for post-hoc multiple comparisons. Percent change calculations (ie, $[[x_2 - x_1]/[x_1]*100]$) was used to facilitate identifying trends of increase or decrease in synovial fluid analytes.

Coefficient of variation was calculated to determine variability in biomarker concentrations in ACS between horses, as well as determine precision of assays. Average measures intra-class correlation coefficient was used to evaluate reliability of lameness and radiographic grades among clinicians. Spearman rank tests were used to assess correlations between arthrocentesis attempts, blood contamination, joint effusion, lameness grades, radiographic scores, and all analytes to determine any confounding variables and/or relationships with disease severity on clinical response to ACS. Significance was set at $P<0.05$ for all analyses.

CHAPTER 4:
RESULTS

PART A - POPULATION AND INCLUSION

Eleven horses met inclusion criteria and were recruited for the study. Study population included mean (±SD) age of 16.9 years (±4.4), weight of 540 kg (±55), and height of 159.3 cm (±6.6). Breeds included Thoroughbred (4), Quarter Horse (3), Warmblood (2), and American Paint Horse (2), with 6 mares and 5 geldings. The affected limbs included 10 right and 1 left fore.

All horses (11/11) had palpable DIP joint effusion and responded to distal limb flexion. Radiographic signs of DIP joint OA (as based on blinded review – Table 3.3) were present in 8/11 horses. Of these eight horses, six (6/8) were determined as grade 1, one (1/8) as grade 2, and one (1/8) as grade 3. Of the three horses without radiographic DIP OA, two (2/3) responded to intra-articular analgesia and one (1/3) had previously responded favorably to a corticosteroid injection. When assessing for changes to the navicular bone (as based on blinded review – Table 3.4), six horses (6/11) were deemed normal, four horses (4/11) classified as grade 1, and one horse as grade 2 (1/11). An intra-articular DIP joint block improved the lameness by >50% within 10 minutes in 6/11 horses, whereas 5/11 horses had a previous improvement in lameness noted after intra-articular corticosteroid administration.

PART B - SERUM

ACS and 19-hr control serum were incubated for a mean (±SD) of 19 hours (±51 min). Fifty ml of whole blood resulted in 20.2 (±2.3) ml of total ACS product. Median number of arthrocentesis attempts was 1, with 39 of 44 synovial fluid samples having

blood contamination (Table 4.1). At sampling times (n=44), effusion was graded as absent (2), mild (35), and moderate (7) (Table 4.2).

SAMPLING	Day 0		Day 7		Day 14		Day 21	
Mean Arthrocentesis Attempts	1.6		1.2		1.4		1.4	
Blood Contamination	YES	9	YES	10	YES	9	YES	11
	NO	2	NO	1	NO	2	NO	0

TABLE 4.1 – Representation of the mean arthrocentesis attempts for arthrocentesis and the presence of blood contamination within synovial fluid samples on days 0, 7, 14, and 21.

EFFUSION	Day 0	Day 7	Day 14	Day 21
Absent	0	0	1	1
Mild	7	8	10	10
Moderate	4	3	0	0
Severe	0	0	0	0

TABLE 4.2 – Representation of the degree of effusion, subjectively graded as absent, mild, moderate, or severe within DIP joints on days 0, 7, 14, and 21.

Median IL-1ra concentration in ACS was 36 times higher ($P<0.0001$) than 1-hr control serum, but was not significantly different from 19-hr control serum (Figure 4.1). Median IL-1ra to IL-1β ratio in ACS was significantly higher compared to 1-hr ($p=0.011$), but not 19-hr control serum (Figure 4.1). ACS contained significantly less IL-4 and IL-8 compared to 1-hr and 19-hr serum controls, whereas IL-6 was significantly lower in ACS compared to 19-hr serum control (Figure 4.1). MMP-1 and MMP-9 were significantly elevated in ACS compared to 1-hr control serum, but not 19-hr control

serum (Figure 4.2). No significant differences in IL-1β, TNFα, IL-10, MMP-3, MMP-13 or TIMP-1, -2, -3, and -4 were present when comparing ACS to serum controls (Figures 4.1 and 4.2). Within serum, the only MMP:TIMP ratios that demonstrated a significant increase in ACS compared to control were MMP-1 to TIMP-1, -2, -3, -4 and MMP-9 to TIMP-1, -2, -4 (Figure 4.3). Marked variability was present between horses in IL-1ra concentration in ACS (42% CV). Thirteen of the 14 additional analytes measured in ACS had a CV >15%, exceeding the inter-assay CV of 12% (Table 4.3).

Concentration of IL-1ra decreased by approximately 10% when comparing thawed ACS at day 7 or 14 to fresh ACS at day 0; however, no significant change in IL-1ra or other analytes could be identified as a result of freeze/thaw (Figures 4.1 and 4.2).

Biomarker	Mean Concentration (with range) of Biomarkers in ACS [pg/ml]	Standard Deviation	Coefficient of Variation	Number of Values Below LLOQ
IL-1ra	6585 (3600 – 13299)	2750	41.76%	0/11
IL-1β	0.3860 (0.043 – 0.91)	0.2926	75.81%	2/11
TNFα	2.481 (0.191 – 12.24)	3.502	141.15%	4/11
IL-4	4.513 (3.56 – 12.92)	2.809	62.24%	9/11
IL-6	0.2387 (0.052 – 0.97)	0.3366	141.01%	8/11
IL-8	261.2 (18.4 – 686.23)	292.2	111.89%	0/11
IL-10	0.1198 (0.056 – 0.44)	0.1238	103.32%	8/11
MMP-1	1441 (817.57 – 2187.52)	466.2	32.36%	0/11
MMP-3	76.91 (33.71 – 194.04)	50.19	65.25%	0/11
MMP-9	62720 (32545 – 103756)	21658	34.53%	0/11
MMP-13	47.16	0.0	0.00%	11/11
TIMP-1	378.9 (248.71 – 465.57)	60.85	16.06%	0/11
TIMP-2	166343 (92980 – 577762)	138033	82.98%	0/11
TIMP-3	46858 (17666 – 93908)	25716	54.88%	0/11
TIMP-4	73.69 (51.39 – 101.97)	16.00	21.72%	0/11

TABLE 4.3 - Overview of cytokine, matrix metalloproteinase, and tissue inhibitors of matrix metalloproteinase profile within autologous conditioned serum. Number of values below LLOQ (LLOQ = lower limit of quantification) outlines number of analytes that measured below lowest standard curve point. For these values, half of the lower limit of detection [lowest value statistically different from a blank at 99% confidence] for each individual analyte was used as the concentration.

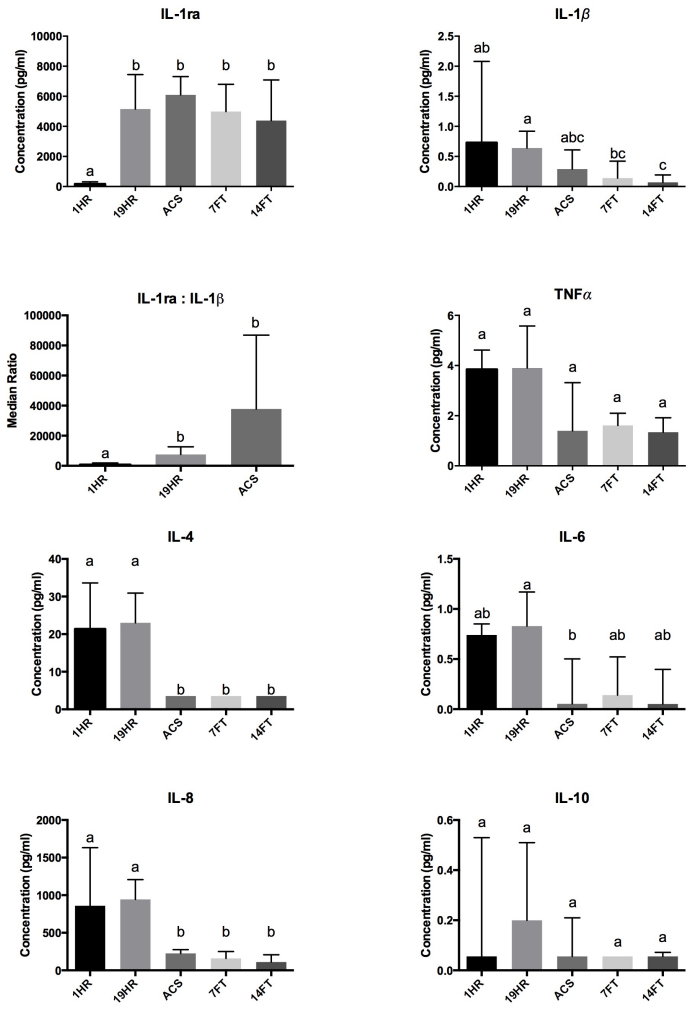

FIGURE 4.1 - Median serum concentrations (with interquartile range) of cytokines IL-1ra, IL-1β, IL-1ra to IL-1β ratio, TNFα, IL-4, IL-6, IL-8, IL-10. Groups included 1-hour unconditioned, unincubated control serum (1HR), 19-hour unconditioned, incubated control serum (19HR), fresh autologous conditioned serum (ACS), day 7 freeze-thaw ACS (7FT) and day 14 freeze-thaw ACS (14FT). Parametric analyses were performed on IL-1ra and IL-8, whereas non-parametric analyses were performed on IL-1β, IL-1ra:IL-1β ratio, IL-4, IL-6, IL-10, and TNFα. Different letters indicate significant differences between groups ($P<0.05$).

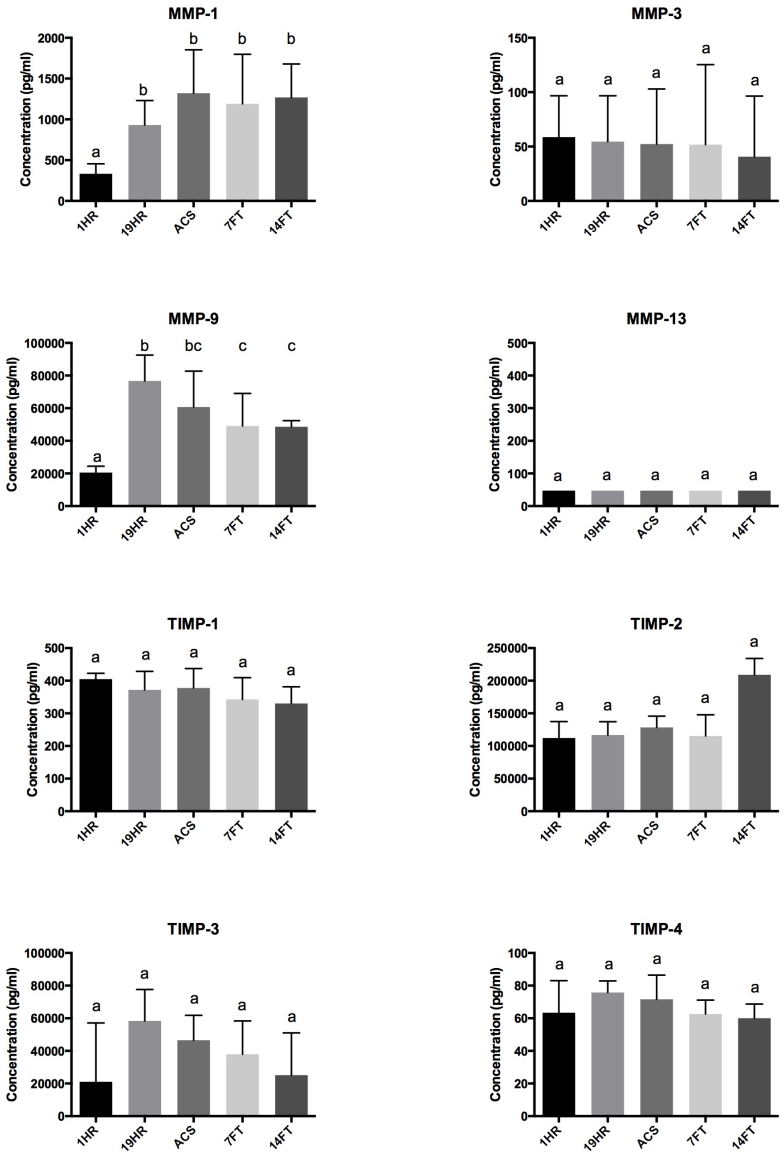

FIGURE 4.2 – Median serum concentrations (with interquartile range) of matrix metalloproteinases (MMP) -1, -3, -9, and -13, and tissue inhibitors of matrix metalloproteinases (TIMP) -1, -2, -3, and -4. See Figure 4.2 legend for group definitions. Parametric analyses were performed on MMP-1, MMP-9, TIMP-1, TIMP-2, TIMP-3, and TIMP-4, whereas non-parametric analyses were performed on MMP-3 and MMP-13. Different letters indicate significant differences between groups ($P<0.05$). Note: all values for MMP-13 represent 50% of the lower limit of detection.

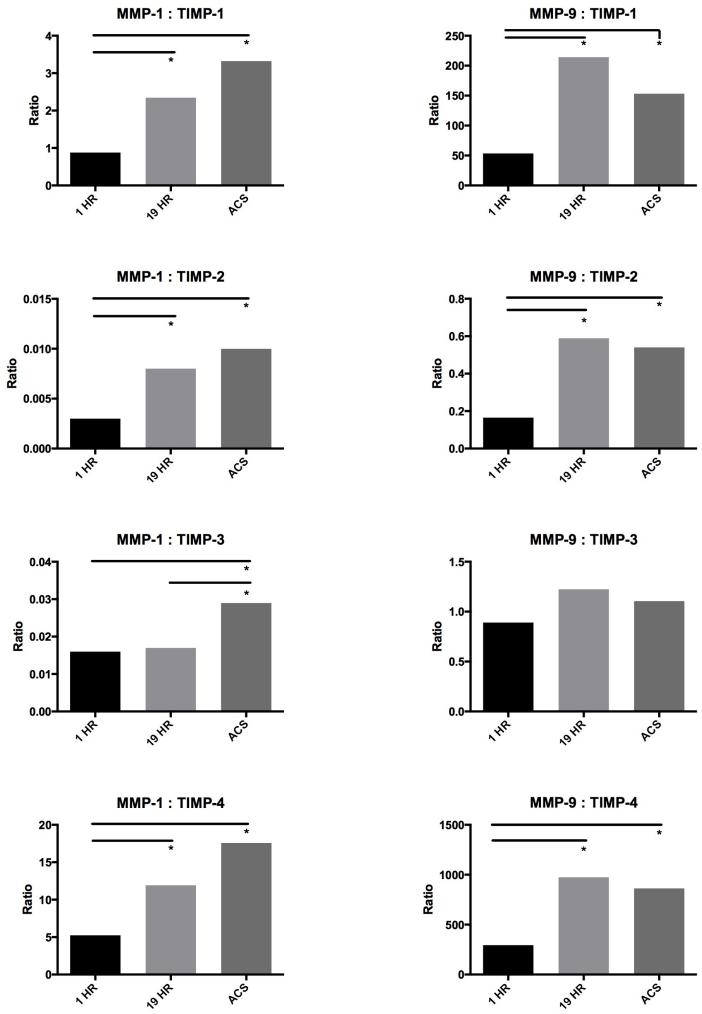

FIGURE 4.3 - Median serum MMP to TIMP ratios in 1-hour unconditioned, unincubated control serum (1 HR), 19-hour unconditioned, incubated control serum (19 HR), and autologous conditioned serum (ACS) groups. Asterisks denote significant difference between 1 HR, 19 HR or ACS groups. Significance was set at $P<0.05$. Parametric ratios include MMP1:TIMP1, MMP1:TIMP3, MMP1:TIMP4, MMP3:TIMP3, MMP9:TIMP1, MMP9:TIMP3, MMP9:TIMP4, MMP13:TIMP3, and MMP13:TIMP4. Non-parametric ratios include MMP1:TIMP2, MMP3:TIMP1, MMP3:TIMP2, MMP3:TIMP4, MMP9:TIMP2, MMP13:TIMP1, and MMP13:TIMP2.

PART C - SYNOVIAL FLUID

Synovial fluid collected 7 days after intra-articular ACS injection only had 1% of the IL-1ra concentration that was present within the injected ACS product. This finding was consistent after all 3 injections (Figure 4.4). There was no significant change in concentrations of any cytokine, MMP, TIMP or MMP:TIMP ratio when comparing post-ACS treatment synovial fluid to baseline.

When evaluating synovial fluid for changes in IL-1ra concentrations based on percent change from baseline, 5/11 horses displayed a consistent decrease in concentration at day 7, 14, 21, whereas 2/11 horses displayed a consistent increase in concentration. The remaining 4 horses showed a mixed response (both decrease/increase noted). For other analyte changes, trends were grouped relative to whether IL-1ra increased, decreased or were mixed in response. For these trends, it is imperative to note that no statistics could be performed due to the small sizes present per group. In horses that demonstrated decreased IL-1ra concentrations over time, a concurrent decrease in IL-8, IL-10 and TIMP-3 also simultaneously occurred. In horses where IL-1ra increased over time, concentrations of IL-1β, TNFα (day 7, 14 only), and MMP-9 increased (Figure 4.5). Overall, higher concentrations of MMP -1, -9, and -13 and TIMP -1, -2, -3, and -4 were measured when IL-1ra consistently increased compared to the IL-1ra decrease group (Table 4.4). No trends in other analytes were discernable when IL-1ra response was mixed over time.

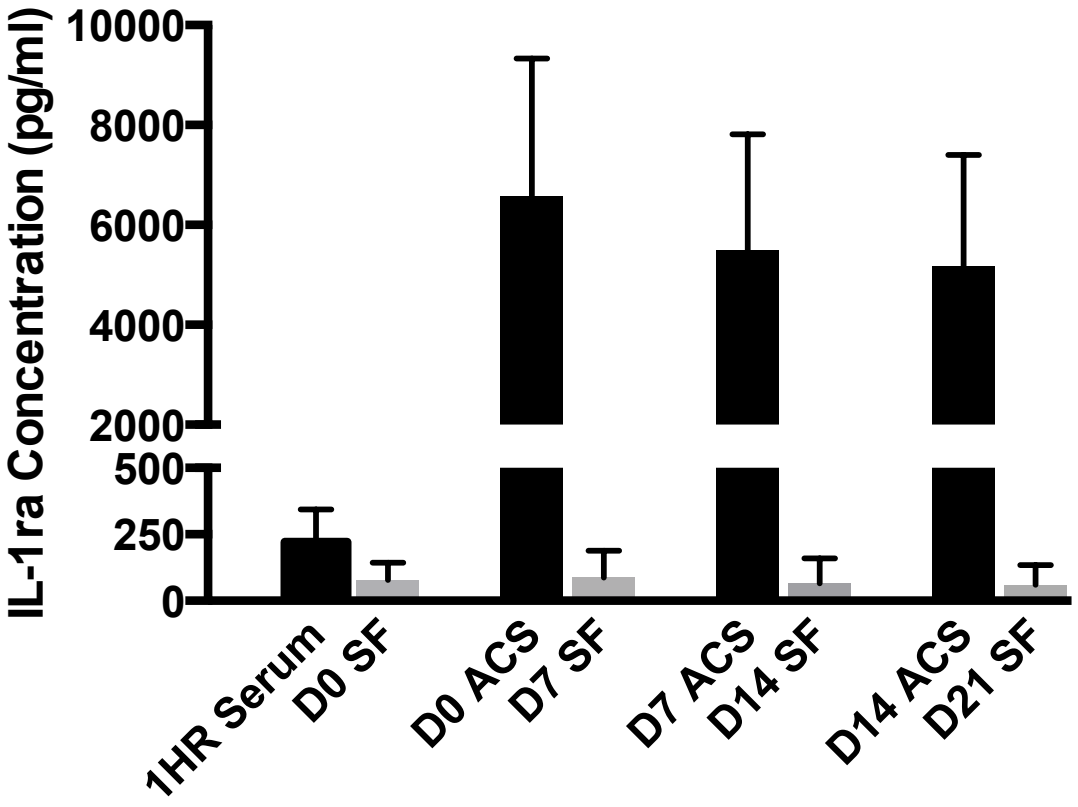

FIGURE 4.4 – Median concentrations (with interquartile range) of IL-1ra from autologous conditioned serum (ACS-black bars) administered intra-articularly (IA) compared to subsequent synovial fluid (SF-grey bars) samples collected after 7-day intervals. [1HR Serum=Serum IL-1ra concentration prior to conditioning (control); D0 SF=Synovial fluid IL-1ra concentration in OA joints at baseline; D0 ACS=ACS IL-1ra concentration on day 0; D7 SF=SF IL-1ra concentration 7 days after IA administration of D0 ACS; D7 ACS=ACS IL-1ra concentration after freeze/thaw on Day 7; D14 SF=SF IL-1ra concentration 7 days after IA administration of D7 ACS; D14 ACS=ACS IL-1ra concentration after freeze/thaw on Day 14; D21 SF=SF IL-1ra concentration 7 days after IA administration of D14 ACS].

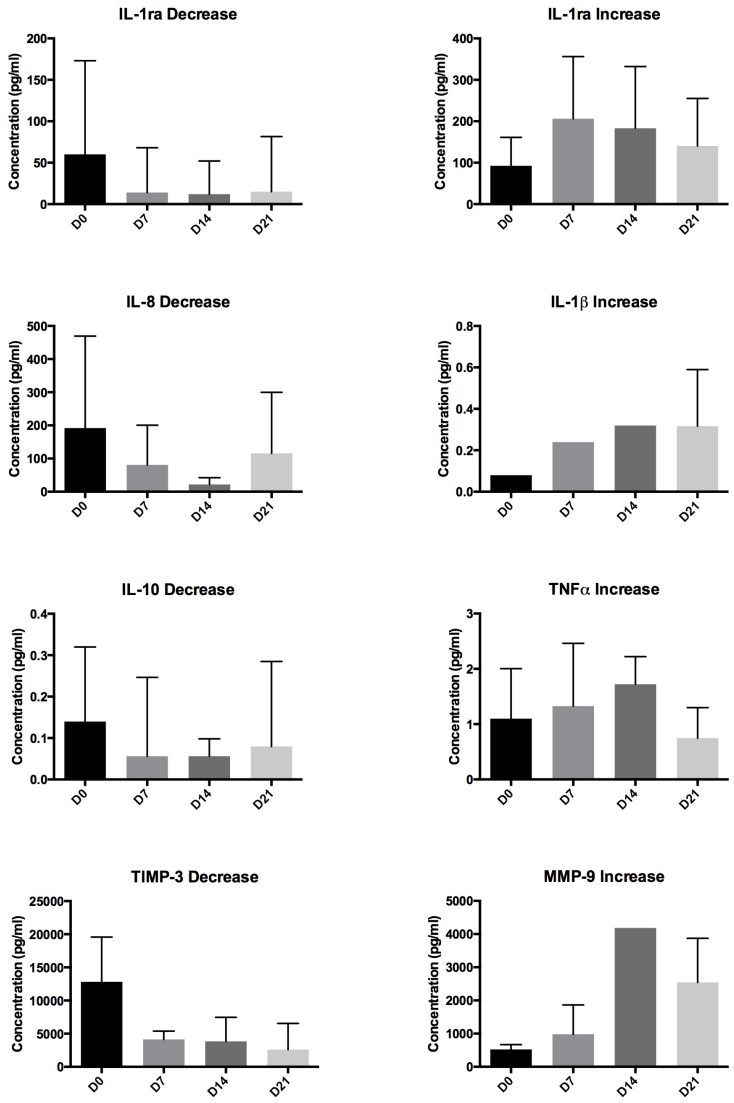

FIGURE 4.5 – Median concentrations (with interquartile range) of analytes in synovial fluid, following further grouping of horses based on whether IL-1ra consistently increased or decreased compared to baseline. Note: These changes were not analyzed statistically due to the small numbers per group. (A) Left Column: In horses (n = 5) where concentration of IL-1ra consistently decreased on day 7, 14, 21 (compared to day 0), there were also concurrent decreases in IL-8, IL-10, and TIMP-3. (B) Right Column: In horses (n = 2) where concentration of IL-1ra consistently increased on day 7, 14, 21 (compared to day 0), there were also concurrent increases in IL-1β, TNFα (day 7, 14 only), and MMP-9.

ANALYTE	IL-1ra Increase Group (pg/ml)	IL-1ra Decrease Group (pg/ml)
MMP-1	261.1	127.8
MMP-9	1215	139.6
MMP-13	193.3	89.27
TIMP-1	158.8	134.3
TIMP-2	365121	122824
TIMP-3	7860	5050
TIMP-4	71.62	21.81

TABLE 4.4 - Higher median concentrations (pg/ml) of MMP -1, -9, and -13 and TIMP -1, -2, -3, and -4 were measured for horses where IL-1ra concentration consistently increased (n=2) compared to horses where the IL-1ra concentration consistently decreased (n=5), when averaged across study duration.

PART D - LAMENESS

Two horses switched lameness to the contralateral forelimb between inclusion exam and day 0. Assessment of horses for inclusion occurred on average 45 days prior to the start of the study (range 7 to 97 days). Because we were interested in the response of the treated limb only, these 2 horses were removed from further lameness analyses. Median lameness grade for the remaining 9 horses before and after ACS administration was 1/5 for all time points (days 0, 7, 14, and 21). The average measure intraclass correlation coefficient for agreement of observers' lameness grades was 0.946 ($\geq$ 0.80 is acceptable). There was no significant difference noted within the study population when evaluating flexion test response following ACS therapy.

PART E - CORRELATIONS

IL-1ra was positively correlated with IL-1β and TNFα in ACS, and TNFα, IL-8, MMP -9 and -13, as well as TIMP -1, -2, -3, and -4 in synovial fluid (Table 4.5). No significant correlations were present between arthrocentesis attempts, blood contamination, and joint effusion. Number of arthrocentesis attempts required was positively correlated to MMP-13 ($p=0.04$, $r=0.323$) and TIMP-1 ($p=0.007$, $r=0.415$) synovial fluid concentrations. The presence of blood contamination in synovial fluid was positively correlated to IL-8 ($p=0.003$, $r=0.447$), MMP-1 ($p=0.0008$, $r=0.491$), and MMP-9 ($p=0.024$, $r=0.345$) concentrations. When radiographic grade of DIP joint OA and navicular disease were summed with joint effusion and lameness and then compared to IL-1ra concentration in ACS, a negative correlation was present ($P=0.036$, $R= -0.690$).

AUTOLOGOUS CONDITIONED SERUM		
	P	**R**
IL-1ra to IL-1 β	0.009	0.758
IL-1ra to TNFα	0.032	0.637
SYNOVIAL FLUID		
	P	**R**
IL-1ra to TNFα	0.004	0.435
IL-1ra to IL-8	0.005	0.417
IL-1ra to MMP-9	0.04	0.432
IL-1ra to MMP-13	0.027	0.345
IL-1ra to TIMP-1	<0.001	0.680
IL-1ra to TIMP-2	<0.001	0.541
IL-1ra to TIMP-3	0.01	0.398
IL-1ra to TIMP 4	0.001	0.514

TABLE 4.5 – Significant ($P<0.05$) correlations between IL-1ra and various biomarkers in ACS and synovial fluid from all sampling time points, determined using Spearman rank correlation.

CHAPTER 5:
DISCUSSION

PART A - SERUM

IL-1ra has been advocated as one of the main contributors to the anti-inflammatory effect of ACS. Our study confirms previous reports that IL-1ra concentrations are substantially elevated in equine-derived ACS compared to 1-hr control serum [Frisbie 2007, Hraha 2011, Fjordbakk 2015]. Our results also show that IL-1ra concentrations in ACS were not significantly different compared to 19-hr control serum, which is in contrast to those reported by Hraha et al. where the IRAP II[b] kit demonstrated higher IL-1ra concentration in ACS compared to both unincubated and incubated control serum [Hraha 2011]. The reason for this disparity is not clear, but could be related to our shorter incubation time (19 vs. 24 hours) since it has been shown that IL-1ra concentration continues to steadily increase up to 24 hours [Meijer 2003]. In our study, aiming for 18-20 hours of incubation remained within manufacturer recommendations (16-24 hours)[b] and was more convenient when used with client owned-horses. Our results do, however, concur with other human and veterinary studies that used other commercial ACS kits [Hraha 2011, Fjordbakk 2015, Magalon 2014]. This lack of difference in IL-1ra concentrations between ACS and incubated serum has led some to propose that conditioning of serum with glass beads may be unnecessary [Hraha 2011, Fjordbakk 2015, Magalon 2014]. This conclusion was based on the fact that control serum incubated in plastic (non-glass) syringes [Fjordbakk 2015, Magalon 2014] or glass blood-collection tubes [Hraha 2011] demonstrated elevated IL-1ra concentrations that were no different than ACS. Nonetheless, it is important to consider that there are other components in ACS besides IL-1ra that may contribute to its action in vivo.

Our study identified lower concentrations of IL-4, -6, and -8 compared to 19-hr control. Physiologically, these cytokines have an anti-inflammatory (IL-4), immuno-

modulatory (IL-6), and pro-inflammatory (IL-8) effect within the joint [Platt 1996] making it difficult to interpret the clinical relevance of their suppression. Similar differences between ACS and incubated controls (24-hr) have been found in previous equine studies, where IGF-1 concentrations in ACS were elevated compared to incubated controls [Hraha 2011]. Combined, these results suggest that ACS may be different in composition compared to incubated control serum (19- or 24-hr), such that one cannot assume that incubating serum without glass beads will result in the same product. To our knowledge, this has not been previously reported in equine-derived ACS.

ACS is considered anti-inflammatory and concurrent increases in catabolic cytokines or degradative enzymes would be undesirable. We noted no change in IL-1β and TNFα concentrations, which is different than previous equine-derived ACS studies [Hraha 2011, Fjordbakk 2015]. Those studies both demonstrated increased IL-1β in ACS compared to unincubated controls, whereas TNFα was increased in ACS in only one study [Hraha 2011]. We could, however, demonstrate a significant positive correlation between IL-1ra and both IL-1β and TNFα in ACS, suggesting that there is at least a modulatory response between all 3 cytokines. Our results also illustrated a significant elevation in MMP-1 and MMP-9 activity in ACS when compared to 1-hr control serum. This may be important because the increase in MMPs was disproportionate to the TIMPs as demonstrated by their ratios. To our knowledge, no previous studies have investigated MMP or TIMP profile in equine-derived ACS. MMPs are actively involved in catabolism of articular cartilage; MMP-1 functions to denature type II collagen, whereas MMP-9 degrades gelatin and unwind denatured collagen [McIlwraith 1996]. Although the increase in MMPs within ACS is clearly undesirable, a concurrent increase in MMP concentration or MMP:TIMP ratios was not evident in synovial fluid 7 days after any of the intra-articular injections of ACS. This might be because the TIMPs in synovial fluid positively correlated to IL-1ra concentration, leading to a protective effect even though

TIMP concentrations were not different in synovial fluid. Further research is required to better understand why MMPs increase in ACS.

In equine practice, it is common for ACS aliquots to be frozen after processing and stored for subsequent treatments at weekly or bi-weekly intervals. As such, ACS undergoes an additional freeze-thaw cycle and passage through a filter. To our knowledge, the effect of this has not been previously examined in equine ACS. Concentrations did decrease by approximately 10%, but there was no significant loss of the original ACS composition following one freeze-thaw cycle. We did not investigate prolonged storage, so it is unknown whether results would be the same for storage >2 weeks.

Similar to other equine ACS investigations, concentration of IL-1ra in our study showed wide variability between individual horses [Frisbie 2007, Hraha 2011, Fjordbakk 2015]. Variability was not isolated to IL-1ra alone, as most other cytokine, MMP, and TIMP analytes had high CVs. Therefore, each dose of ACS must be considered unique. The effect of this variability on clinical response is unclear.

In osteoarthritis, IL-1 receptors within cartilage and synovium increase, leading to further potentiation of IL-1 induced inflammation [Fernandes 2002]. In addition, IL-1β itself is potent, such that small increases in IL-1β binding result in marked inflammation. As such, a disproportionally high amount of IL-1ra is required to antagonize IL-1β-induced inflammation [Fernandes 2002]. The ratio of IL-1ra to IL-1β has been postulated as a means of estimating therapeutic efficacy of an ACS product [Meijer 2003], with 10:1 (IL-1ra:IL-1β) being the minimum needed [Granowitz 1991]. When the overall clinical score of disease increased in our study, IL-1ra concentration in ACS decreased, meaning horses with more disease ultimately produced less IL-1ra in ACS. Nonetheless, our IL-1ra:IL-1β ratios were much greater than 10:1, so IL-1ra concentrations achieved within

the ACS product was presumably adequate to inhibit IL-1β binding regardless of disease status.

PART B - SYNOVIAL FLUID

In our study, IL-1ra concentrations were high in ACS, and ACS was serially administered intra-articularly. As such, we expected that synovial fluid IL-1ra concentrations would increase over time as the number of available IL-1 receptors decreased due to increased IL-1ra binding. However, similar to another equine study [Frisbie 2007], serial intra-articular administration at 7-day intervals did not result in increased IL-1ra concentrations in synovial fluid through day 21. Combined, this seems to suggest that IL-1ra becomes summarily bound to IL-1 receptors, with less free IL-1ra in synovial fluid. It is possible that if we followed these horses out longer, we may have seen a change in synovial fluid concentrations since horses with experimental OA demonstrated increased IL-1ra in synovial fluid 35 days after the 4^{th} serial dose of ACS (Frisbie 2007). However, it is not clear why it took that long for concentrations to increase. It is important to recall that IL-1ra concentrations were combined from both the OA and sham operated joints within the osteochondral fragment study (Frisbie 2007). The reason why concentrations were combined, rather than being assessed individually, remains unclear. However, combining the concentrations may have misled the interpretation of change within IL-1ra.

It may be more likely that some IL-1ra will bind to IL-1 receptors in the joint, while most is either degraded or transported out of the joint within 7 days following ACS injection. One human osteoarthritis study concluded that ACS has minimal influence within joints due to rapid intra-articular clearance of IL-1ra [Rutgers 2010]; they saw no change in synovial fluid IL-1ra concentration when measured at 3-day intervals following 6 ACS injections into human knees. The authors theorized that IL-1ra is not the central

protein responsible for the reported efficacy of ACS therapy, as is commonly assumed [Rutgers 2010]. Nonetheless, when combined with our study, it is clear that further research into the disposition of IL-1ra following intra-articular injection is needed to determine its effect in the joint. Additionally, due to the minimal change in IL-1ra concentration in synovial fluid, it was impossible to determine any influence on the necessity of serial dosing.

For many of the remaining analytes, concentrations in ACS were not different compared to 1-hr serum and no change would be expected following ACS therapy. For MMP-1 and MMP-9 (also significantly increased in ACS versus 1-hr serum), the lack of change in synovial fluid could have similarly been a direct result of rapid intra-articular clearance or protein degradation within the joint. Although not significant, IL-1β, TNFα, and MMP-9 illustrated a trend of concurrently increasing along with IL-1ra in synovial fluid. As previously mentioned, these three analytes were also significantly correlated in serum. Together, the modulatory relationship between IL-1ra, IL-1β, and TNFα may reflect response of the joint to increased IL-1ra. In other words, as IL-1ra increases due to ACS therapy, a natural increase in catabolic proteins occurs as well to maintain joint homeostasis. In the five horses that had consistently decreased IL-1ra, a trend of concurrently decreased IL-8, IL-10, and TIMP-3 was also noted. The mixed catabolic (IL-8) and anti-catabolic (IL-10 and TIMP-3) and low sample size (n=5) makes the interpretation of these trends difficult.

Of note, several biomarkers measured in this study were below the lower limit of detection, namely IL-1β, TNF-α, IL-4, IL-6, IL-10 and MMP-13 (Table 3.6). Another study using similar multiplex assay methodology compared normal vs. OA synovial fluid in humans, and found a high prevalence of samples below the lower limit of detection when evaluating IL-1β, IL-6 and IL-10 (Beekhuizen 2013). In other studies, (one comparing healthy vs. OA cartilage and another comparing normal vs. mild to moderate

OA) there was no significant change in IL-1ra, IL-1β, IL-4, IL-6, IL-8 and IL-10 concentrations identified between groups (Heard 2013, Tsuchida 2014). These similar results within various multiplex studies may suggest that the aforementioned biomarkers are not highly increased in osteoarthritis, in both humans and horses.

PART C - LAMENESS

Lameness improvement following ACS therapy was not evident in our study. Comparatively, when treating the carpal joint, significant improvement in lameness was reported 35 days after the last of 4 ACS doses [Frisbie 2007]. However, closer examination of that study reveals that the improvement in lameness was less than one full grade (based on American Association of Equine Practitioner scale) in magnitude. Potentially, examining more horses, providing more than 3 ACS doses, or monitoring for change in lameness beyond day 21 may have shown improved lameness scores in our study. Although three separate investigators graded gait videos blindly, video evaluation of lameness can be limited and not provide a complete representation of a horse's movement. Additionally, visual lameness evaluation is inherently subjective. The addition of force-plate or kinematic gait analysis technology could better clarify the clinical effect of ACS therapy on lameness symptoms.

PART D - STUDY LIMITATIONS

There are important limitations to our study. First, an inherent variability exists when using naturally occurring cases of OA, which may negatively affect the power of the study. However, naturally occurring osteoarthritic cases provide the most accurate in vivo representation of clinical practice. Therefore, each joint served as its own control by

comparing synovial fluid concentrations before and after ACS therapy to minimize this variability. Inclusion criteria for study horses with DIP osteoarthritis required exclusion of advanced navicular disease. Performing magnetic resonance imaging prior to study inclusion would provide better information compared to radiographs, however this was not financially feasible for this study.

Another limitation, especially when using client-owned horses, is the potential inability to recruit adequate numbers of osteoarthritic cases for study inclusion. In this study, 11 horses were enrolled and treated within the confines of the study timeframe. Based on power calculations, recruitment of 12 horses was proposed to achieve 80% power at an alpha of $P<0.05$. As such, the slightly lower sample size present may have contributed to overall lower power.

Other variables, such as arthrocentesis attempts and blood contamination, were found to correlate with certain analytes in synovial fluid. However, since no changes in the aforementioned biomarkers were seen over time in synovial fluid, the clinical impact of these correlations are unknown. Interestingly, blood contamination correlated with both IL-8 and MMP-9 concentrations. Both of these analytes are related to neutrophils, which may imply that a relevant correlation existed. Inclusion of a saline-injected osteoarthritic control group could provide better clarity regarding any influence of needle-induced inflammation.

The equine IL-1ra ELISA has been used successfully in previous investigations [Hraha 2011, Fjordbakk 2015]. However, this investigation is one of the first equine studies to use a human-based multiplex assay to quantitate multiple analytes. Human multiplex technology has been partially validated by the authors prior to onset of investigation (unpublished data). Human bead-based kits were utilized over rat or mice due to the high homology between amino acid sequences present between horses and

humans (Table 3.5). Potentially, using equine specific cytokine ELISAs or zymographic techniques for MMPs and TIMPs could yield more accurate results.

PART E - CONCLUSION

In conclusion, although commercially available ACS kits appear to increase the concentration of IL-1ra in serum from horses, the lack of IL-1ra concentration change within synovial fluid is concerning. Quantifying the retention time of ACS-derived IL-1ra within the joint is an important question that remains. Although gait evaluation was a secondary focus of this study, the lack of improvement warrants future research regarding the ability of ACS to provide lameness improvement in naturally occurring cases of OA. Lastly, multiplex assay using human cytokine, MMP and TIMP kits are effective for measurement of equine biomarker concentrations.

CHAPTER 6:
FUTURE DIRECTIONS

This investigation provides further clarity on the biomarker composition of equine-derived ACS, however not all potentially relevant biomarkers were assessed. Unfortunately, certain analytes of interest provided poor protein homology between human and equine amino acid sequences, had poor validation when using human-based kits and Luminex® xMAP® technology during preliminary studies, or were not financially feasible to include due to budget restrictions. Various other cytokines (IL-2, IL-5), growth factors [platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), transforming growth factor beta (TGF-β)], or matrix metalloproteinases (MMP-2) could change in both ACS and subsequent synovial fluid following ACS therapy. In fact, others have documented change in biomarkers unmeasured in our study, such as TGF-β (Rutgers 2010, Hraha 2011) and IGF-1 (Hraha 2011).

To further characterize the role of individual biomarkers within ACS, and learn about their individual influence on joint inflammation, further in vitro studies could be performed utilizing harvested chondrocyte samples that are exposed to IL-1β. Potentially, cartilage samples could be individually exposed to biomarkers, with subsequent measurements on cartilage metabolism. This study design has been previously utilized by Carlson et. al., however the study was directed towards exposure to ACS as a whole rather than individual biomarkers (Carlson 2013). Secondary to in vitro data, translation to an in vivo study using naturally occurring osteoarthritis could be attempted using recombinant versions of those proteins found to be relevant.

Horses enrolled in this study had low grades of radiographic OA (9 out of 11 horses as either grade 0 or 1) and low grades of lameness (median grade 1 out of 5). As

such, the study population was characterized as mild. Potentially, enrollment of horses with more severe OA may result in ACS imparting a more noticeable clinical effect. That said, in this study a negative correlation was identified between IL-1ra concentrations in synovial fluid and worsening OA (identified by summation of various clinical parameters). Alternatively, creating two or more groups of horses with experimental OA (ie, osteochondral fragments) that are of varying severity may allow for interpretation of whether ACS is best suited for early/mild or advanced/severe OA.

This study also only provides insight on horses affected with naturally occurring osteoarthritis in the distal interphalangeal (DIP) joint. However, it remains unknown whether ACS acts similarly when utilized in other joints. Potentially, further in vivo research evaluating both biomarker change and clinical improvement when administering ACS into other joints of the horse could provide better indications for which joints ACS is best suited.

Potentially examining horses and synovial fluid samples beyond 21 days could identify different changes within various biomarker concentrations that were unidentified in our current study. Because Frisbie et. al. reported improvement in lameness scores following 4 serial ACS injections, performing a study using naturally occurring OA cases and >4 serial injections may provide further insight on ACS efficacy (Frisbie 2007).

We did not observe changes in biomarker concentrations within synovial fluid when samples were attained at 7 days intervals post-ACS administration. Potentially, decreasing the sampling interval such that samples are attained every 12 to 48 hours could provide further detail on the duration of exogenous IL-1ra (or other biomarkers) within the joint environment. However, with additional arthrocentesis attempts, more iatrogenic inflammation can result which can make interpretation of biomarker concentrations difficult (Brama 2004).

This study was designed as a prospective clinical trial, wherein each treated joint was compared to baseline synovial fluid values collected at day 0. However, a stronger level of evidence-based medicine could be attained through developing a randomized, blinded, controlled clinical trial. With this study design, a true placebo-injected control group would exist. This would allow better separation of change in biomarker concentrations due to arthrocentesis or fluid volume change within the joint. Alternatively, enrolling more horses into the current study would help further strengthen the study.

Lameness is the ultimate clinical symptom that drives client concern. Although ACS may provide anti-inflammatory benefit to the joint, if lameness symptoms (which are driven by pain within the joint environment) persist following ACS therapy, then the effectiveness of ACS as a practical therapy should be questioned. Strengthening the assessment of clinical outcome using force-plate or kinematic motion analysis software would strengthen the acceptance/rejection of ACS as a successful therapeutic for management of lameness due to the limitations of subjective gait assessment.

CHAPTER 7:
FOOTNOTES

(a) Orthokine® Vet IRAP 10, Dechra Veterinary Products, Overland Park, Kansas, USA.

(b) IRAP^{TM} II System, Arthrex Vet Systems, Naples, Florida, USA.

(c) R & D Systems, Minneapolis, Minnesota, USA.

(d) 0.2µm Whatman Syringe Filter, GE Healthcare, Barrington, Illinois, USA

(e) Centrifuge Model #5702, Eppendorf North America, Hauppauge, New York, USA.

(f) Dormosedan® (10 mg/ml), Zoetis Animal Health, Florham Park, New Jersey, USA.

(g) Lidocaine hydrochloride 2%®, Zoetis Animal Health, Florham Park, New Jersey, USA.

(h) Luminex® Liquichip Workstation IS200, BioRad Labratories, Hercules, California, USA.

CHAPTER 8:

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