Biomarker Assessment for Detection of Joint Pathology in Horses and Evaluation of the Nutritional Supplement Steadfast Equine as a Therapeutic

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By

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

Biomarker Assessment for Detection of Joint Pathology in Horses and Evaluation of the Nutritional Supplement Steadfast Equine as a Therapeutic

Presented by Emily Vija Fyfe

A candidate for the degree of Masters of Science

and hereby certify that in their opinion it is worthy of acceptance

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Dr. Trista Strauch

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Dr. Kevin Fritsche

In dedication to my father, who amazed me with his knowledge and inspires me with his curiosity about the world around him, to my mother, who has allowed me to venture into the unknown knowing that I will always have a safe place to return to, and to Molly, Colin, Stephen and Shamrock for their unconditional support and companionship.

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ABBREVIATIONS

AA	Affected Area
AAEP	American Association of Equine Practitioners
A-G	Accelerometer-Gyroscope
C2C	¾ Length Helical Domain of Type II Collagen
CTX-II	Carboxyl-Terminal Crosslinking Telopeptide of Type II Collagen
DJD	Degenerative Joint Disease
ECM	Extra Cellular Matrix
GAG	Glycosaminoglycan
HA	Hyaluronan
IGF	Insulin-like Growth Factor
IL	Interleukin
JSN	Joint Space Narrowing
LS	Lameness Score
MMP	Matrix Metalloproteinase
MRI	Magnetic Resonance Imaging
NO	Nitric Oxide
NSAID	Non-Steroidal Anti-Inflammatory Drug
PGE2	Prostaglandin E2
PIIANP	N-Terminal Procollagen Propeptide of Type II Collagen
TNF	Tumor Necrosis Factor

CHAPTER I

REVIEW OF LITERATURE

Introduction

Improvements in veterinary care, husbandry, and nutrition have led to increased longevity for domestic animals.[1-3] The resulting geriatric population has increased the incidence of age-related illnesses seen in veterinary medicine.[3-5] Degenerative joint disease (**DJD**), also known as osteoarthritis, is a common disease in geriatric populations and has been found ubiquitously across medium and large mammalian species.[6] Like many diseases of aging, it is believed that chronic inflammation is fundamental both to the onset and progression of DJD.[7,8]

Degenerative joint disease is characterized by cartilage degradation and loss leading to joint space narrowing (**JSN**), hypertrophy and hyperplasia of the synovial capsule, loss of synovial fluid and eventually calcification of the articular cartilage and osteophyte formation.[9,10] Degenerative joint disease can take years to clinically manifest in affected individuals.[11] Radiographs and magnetic resonance imaging (**MRI**) remain the most prevalent methods of diagnosing DJD, but, significant and irreversible damage has often occurred in the joint by the point that it can be detected using these techniques.[11,12]

Currently there is no cure for DJD and most interventions target alleviating pain and retarding disease progression.[13,14] Common interventions range from weight loss and physical therapy to palliative pharmaceuticals such as non-steroidal anti-inflammatory drugs (**NSAIDS**) and corticosteroids.[15,16] Nutritional therapies may offer a safe and effective treatment alternative that could be used for extended periods of time without adverse health effects. However, nutrition is more effective when used as a preventative measure or in slowing disease development, highlighting the need for practical and reliable detection techniques for DJD onset and progression. Serum biomarkers that indicate rates of tissue turnover in the joint have shown promise for the diagnosis and prognosis of joint ailments in both human and veterinary medicine.[17-20] With specific regard to horses, the animal model for this study, DJD is one of the most common musculoskeletal disorders, with 60% of equine lameness caused by or related to DJD.[21]

This literature review provides background information on articular joints and joint tissues, the etiology of DJD, the use of biomarkers in the diagnosis of DJD, equine lameness as it relates to DJD, and the joint support supplement, Equine® Steadfast.

Articular Joints and Joint Tissues

Articular joints are specifically designed for movement. Also known as synovial joints, or diarthroses, they are characterized by a synovial capsule: a dense, fibrous connective tissue lined by a metabolically active synovial membrane. The membrane secretes lubricating synovial fluid into the space confined between two connecting bones.

The ends of these bones are capped with articular cartilage, a class of hyaline cartilage. Outside the capsule, the bones are stabilized by ligaments and attached to muscle by tendons, which allow for movement.[22] Articular cartilage distributes mechanical weight, allowing bone to withstand compressive force. Without a protective layer of cartilage, the spongy bone collapses easily.[23,24]

Bone is composed primarily of thick type I collagen fibers connected by crosslinking pryridinoline and deoxypyridinoline molecules.[22] The synovium is constructed of a synovial membrane, or intima, and a fibrous outer-layer. The intima is metabolically active and highly vascular.[22] The primary collagen fibers in the synovium are collagen III and VI, although these collagens are not specific to the synovium.[25] The synovial membrane is permeable to water, electrolytes, glucose, amino acids and non-protein associated drugs, but, not to larger hyaluronanand lubricin molecules, the polymers which give synovial fluid its viscous quality.[22] The synovial membrane is composed of three primary types of synoviocytes: macrophages (type A), fibroblasts (type B), and dendritic cells. Type A synoviocytes have a primarily phagocytic function whereas type B synoviocytes produce fibronectin, collagen and hyaluronic acid for release into the synovial fluid.[26]

Articular cartilage is the primary weight bearing surface in the synovial joint.[27] Cartilage cells, known as chondrocytes, secrete and are embedded in a large extra cellular matrix (**ECM**). Similar to other connective tissues, approximately 90% of cartilage is ECM. The main components of the ECM are collagen fibers, HA's, proteoglycans and glycoproteins. The most abundant collagen fiber found in articular cartilage is type II

collagen, which is cartilage specific, and makes up approximately 60% of the dry weight of the ECM.[22,28] Collagen fibers and proteoglycans are arranged to withstand tensile and shear stress in the surface layers and compressive stress in the deeper layers. Collagen tends to orient parallel to the surface in the superficial layers, but, becomes gradually more aslant in the deeper radial layers until it is perpendicular near the tidemark (i.e. the junction between articular cartilage and calcified cartilage).[27] Proteoglycans are held-in place by the collagen matrix. It is primarily these classes of molecules that provide cartilage its unique qualities as an elastic, weight bearing tissue. Cartilage undergoes a constant turnover throughout the life of chondrocytes and the ECM, although this metabolic rate is low in healthy adult animals.[29]

Type II collagen is composed of three homogeneous polypeptide chains that are bound in a triple-helix by crosslinking telopeptides.[30] It is synthesized from procollagen monomers, secreted from chondrocytes. There are two forms of type II collagen due to alternate RNA splicing. Form IIA has a 69-amino-acid, cysteine-rich globular domain encoded by exon II in the N-Terminal Procollagen Propeptide of Type II Collagen (**PIINP**).[31] This domain is not present in the IIB form. Form IIB is expressed by mature chondrocytes, while form IIA is expressed only by chondrogenic precursor cells and during chondrocyte hypertrophy.[31,32] As procollagen molecules polymerize into collagen fibrils, the non-helicoid domains at the C and N terminus of the molecules[22] are cleaved, released into the synovial fluid and cleared into the blood. Type II collagen fibrils are degraded by proteolytic enzymes released from chondrocytes and synoviocytes. The main class of proteolytic enzymes in the joint is matrix metalloproteinase's (**MMP**s). The primary MMP's involved in cartilage degradation are MMP-1, -8, -13 and -14. These

enzymes cleave collagen fibers within the crosslinking telopeptide domain, resulting in a ¾ length helical fragment,**C2C**, and a ¼ length fragment, the C-terminal cross-linking telopepetide of collagen type II (**CTX-II).**[33]

Degenerative Joint Disease

Arthritis is generally divided into degenerative or inflammatory categories. However, this division is somewhat misleading because it is now known that inflammation is a major catalyst in the propagation of both disease types.[9] While DJD is caused by changes in tissue metabolism and/or altered biomechanics, inflammatory arthritis is typically caused by an infection of the joint (septic arthritis) or a non-antigen related hyper-immunologic reaction in the joint (rheumatoid arthritis).[9,34] Many of the physical symptoms are common to both etiologies including localized pain due to manipulation of and/or pressure on the joint, decreased range of movement, thickening of the joint capsule, osteophyte formation, loss of articular cartilage, and production of heat.[35] Age is the primary risk factor for DJD, although the chances of onset are increased by conditions including obesity, genetic predisposition, mechanical stress, nutritional deficiencies and previous joint injuries.[36]

The hallmark of DJD is loss of articular cartilage,[9,22] however, as the understanding of cellular pathology in DJD increases, it appears that multiple tissue types are involved. Bone, synovium and cartilage have all been shown to be involved in the onset and progression of DJD.[22] In a diseased state all three tissues undergo pathological

biochemical and biomechanical changes simultaneously, affecting the other tissue types, through anatomical remodeling and release of molecular by-products.[23] Disease progression is a self-perpetuating cycle of the cross-talk between all three tissue types.[23]

Inflammation of the synovium, or synovitis, appears to be one of the most important features of early DJD and correlates well with joint pain and function.[37] All early stages of arthritis are described by some degree of synovitis, characterized by the infiltration of neutrophils, T-lymphocytes and monocytes, along with increased hyperplasia and vascularization of the synovium.[38] The inflamed synovial membrane up-regulates expression of cytokines including interleukin (**IL**)-1β and tumor necrosis factor (**TNF**)-α. Increased cytokine concentration in the joint causes chondrocytes and synoviocytes to increase production and release of MMP's, which degrade the collagen matrix, disrupting stability.[26] Focal cartilage loss in areas adjacent to the inflamed synovium results in the release of molecular fragments from ECM molecules.[23] These molecular fragments are phagocytosed by the cells of the synovial membrane, causing a further pattern of inflammation.[9,38]

Release of cytokines also stimulates chondrocytes to up-regulate production of other biologically active molecules such as prostaglandin-E2 and nitric oxide (**NO**).[38] Long-term oxidative stress has been implicated as the major cause of many degenerative diseases of aging, including arthritis.[39] IL-1 and TNF-α potentiate the production of nitrite in bone, causing the release of NO derivatives that lead to bone loss.[9] Reactive oxygen species such as NO have been shown *in vitro* to adversely affect collagen structure and integrity and may be responsible for the depolymerizarion of hyaluronate,

increasing friction and causing damage to superficial cartilage layers.[40] NO, in particular, is a complex, multifunctional signaling molecule that may propagate the progression of DJD through tissue destruction and up-regulation of other inflammatory signaling molecules.[41]

The integrity of articular joint tissues is ultimately determined by the balance between cytokine-driven anabolic and catabolic pathways which, when uncoupled, can contribute to the onset and prognosis of arthritic disease.[42] The overall metabolic rate in chondrocytes affected by DJD is higher than that of healthy chondrocytes.[33] This could be due to increased production by chondrocytes and synoviocytes of growth factors, such as transforming growth factor-β, bone morphogenic proteins and insulin-like growth factor (**IGF**)-1.[33] Additionally, programmed cell death is a governing event in cartilage loss during the progression of DJD. As the number of chondrocytes decreases, the ECM is less efficiently produced, decreasing the ability of cartilage to repair itself.[33] In summary, DJD leads to a higher rate of cartilage degradation than synthesis due to excessive catabolic activity, loss of the structural integrity of the ECM and a high rate of apoptosis, causing a decrease in chondrocyte concentration which, in turn, reduces the ability of cartilage to facilitate repair of the ECM. Fissures in the surface, and eventually in the deep, cartilage layers increase friction in the joint, inducing inflammation, causing pain and impairing joint function.

Biomarkers of Degenerative Joint Disease

Observed lameness, radiography and MRI are the standard diagnostics for arthritic conditions.[22] However, by the time these tools can confirm a diagnosis, significant and possibly irreversible damage to the joint has already occurred.[43] Radiographs are used to measure JSN, however, because measurable changes are relatively small compared to the precision error of radiographs, at least one, and, preferably two, years are needed before the progression of joint damage can be accurately assessed.[22,31,44] Using MRI to arthroscopically evaluate the joint provides a direct image of the cartilage surface.[37] However, arthroscopy is an invasive technique and impractical for the type of repeated measures that would be needed for routine checks, to track disease progression or to monitor efficacy of treatment.[31]

Measuring concentrations of biomarkers associated with the synthesis and degradation of joint tissues has shown promise for the diagnosis and prognosis of joint ailments.[17,19,45] Biomarkers for joint condition are molecules, or fragments of molecules, which make up connective tissue matrices and are released into biologic fluids during tissue turnover. Relative concentrations of biomarkers can yield information about disease onset, expected rate of progression and effects of therapy.[31] As the most abundant protein component of cartilage, type II collagen molecules have been extensively investigated as biomarkers of joint condition.[46] A critical review to assess the usefulness of different type II collagen biomarkers found that urinary CTX-II concentration was beneficial for the purpose of diagnosing DJD, evaluating the burden of disease,

determining a prognosis and quantifying treatment effects.[47] No other type II collagen biomarker was found to be as ubiquitously useful.[47] Other studies have now shown that high concentrations of urinary CTX-II correspond to an increased risk of DJD and faster progression of JSN as assessed by radiography, and cartilage loss as assessed by MRI.[48-51]

The N-terminal procollagen propeptide of type II collagen (**PIIANP**) has also shown diagnostic merit as a biomarker in DJD. It is not expressed in healthy adult chondrocytes, but, is expressed in chondrocytes from human patients with DJD.[32] It has been suggested that chondrocytes under inflammatory stress reexpress PIIANP.[32] Because PIIANP is also expressed by chondrogenic precursors, it would be expected that concentrations of PIIANP would be greatest during the growth or degradation of cartilage.[52] A five-year longitudinal study in humans that tracked JSN found that people with the fastest rates of disease progression were those with high concentrations of both CTX-II and PIIANP.[53] Other studies have foundfound contradictory results suggesting that the most precise predictor of both disease onset and progression was a low concentration of PIIANP and a high CTX-II concentration, indicating uncoupling of cartilage synthesis and degradation.[31,54]

Another biomarker that has received considerable attention in the field of DJD research is NO. Nitrite (NO^{2-}) concentration, a metabolite of NO, is often used as a measure of NO concentration and, thus, oxidative stress. Excess NO may trigger chondrocyte apoptosis and matrix destruction.[55] It has been determined that serum NO concentration may increase the catabolic activity in arthropathies that lead to cartilage

destruction.[56] Several human studies have shown that nitrite concentrations, the form to which NO is converted in the blood, are significantly higher in the serum, synovial fluid and/or plasma of patients affected by DJD than control patients.[41,57-59] In addition, *in vitro* chondrocyte cultures have been shown to up-regulate NO production in situations meant to emulate DJD (i.e. incubation with IL-1).[60,61] Further investigations need to be conducted before biomarker concentrations can be interpreted with confidence in relation to DJD, however, such research is merited as joint disease biomarkers have potential as diagnostics for the early detection and prognosis of DJD, as well as offering a means to evaluate therapeutics.

Lameness in Horses

Lameness is the most prevalent performance limiting medical condition affecting horses, and the second most frequently diagnosed disorder in geriatric horses.[2,21] Equine lameness resulted in an estimated $600 million to $1 billion annual loss to horse-owners in 1998, a figure that has no doubt continued to grow.[21] In addition, lameness due to joint injury or disease is the single greatest cause of diminished athletic ability and wastage in race horses.[62] Degenerative joint disease has been identified as the most common cause of tarsal lameness and may involve the centrodistal, tarsometatarsal, and less frequently the talocalcaneal-centroquartal and talocalcaneal joints.[63] Even in the absence of DJD, or in the subclinical stages of the disease, there are age-related changes in equine joints including decreased thickness of the articular cartilage, increased thickness of calcified cartilage and more variation in the orientation of collagen fibers, decreasing the ability of

the tissue to withstand shear and compressive stress.[64] With a growing population of geriatric horses[2], it can be surmised that lameness will continue to be a prevalent issue in equine veterinary medicine. In response, reliable diagnostics and novel therapies are needed.

Before a diagnosis can be made, the locale and degree of lameness must be determined. Currently the American Association of Equine Practitioners has defined a scale from one to five to quantify lameness. However, there are often large discrepancies in assigning lameness scores due to the subjective opinions of individual practitioners.[21,65-67] Objective quantification of lameness is now possible using a wireless accelerometer-gyroscope (**A-G**) system that evaluates the biomechanics of normal and pathological equine movement.[21]

One motion detector is placed on the front right fetlock to measure gait. A stride is defined as the period from which the right front leg is lifted from the ground until it is lifted again. Two other motion detectors are placed-at the poll and on the croup, centered on the horse's mid-line. During a single stride a horse will move its head and hind quarters up and down twice. In a sound horse, this would result in a sinusoidal movement curve. If a horse is lame, weight is often shifted to alleviate stress on that limb. This will cause a repeating irregularity in the sinusoidal curve as the horse shifts its head or hind quarters during each stride in response to the shifted weight loading.[67] Often a primary lameness can cause compensatory weight shifting to either of the contralateral limbs.[21] Thus, when evaluating lameness scores, it is important to observe whether a horse is truly lame in multiple limbs or whether a second area of detected lameness is an artifact from a

primary lameness. Although uncommon, another potential area for error is a horse that is equally lame in either both fore-limbs or both hind limbs.[21] In this case the horse may be evaluated as sound. Therefore, the wireless A-G system does not replace the evaluation of an experienced equine practitioner, but, may assist in standardizing lameness scoring, a particularly important attribute in research trials.[21]

While such a system is useful for quantifying the degree and area of lameness, it can only do so after lameness is clinically present, affirming the need for early diagnostic measures. In horses, lameness is often attributable to some degree of DJD.[68] Even if DJD is not the initial cause of lameness, chronic, uneven loading on the joints due to other forms of injury increases the risk of developing DJD.[69] The structure and protein composition of human and equine cartilage has been found to be comparable.[62] Therefore, type II collagen biomarkers may have potential as indicators of the onset and severity of DJD in horses as has been demonstrated in humans. Additionally, serum CTX-II concentration has been found to be increased in horses with carpal injury.[70] The high incidence of naturally occurring DJD, the growing population of geriatric horses, the significant cost to horse owners and, the causal effects of other forms of lameness on the induction of DJD, warrants investigation into the use of joint-tissue biomarker in horses.

Steadfast® Equine

Treatment for DJD in horses has generally been symptomatic, with little objective research into the long-term disease modifying effects.[68] Currently the most common

interventions for equine DJD are non-steroidal anti-inflammatory drugs (**NSAID**'s), including phenylbutazone, ketoprofen, naproxen or carprofen, intraarticular steroid injections, viscosupplementation to reduce the effects of decreased synovial fluid concentrations and chondroprotectives, such as glycosaminoglycans (**GAG**'s).[62] However, both NSAID's and steroids have side effects which may actually impair chondrocyte function in addition to adding stress on the liver and suppressing immune function.[71,72] Nutraceutical interventions, defined here as foods or nutritional supplements that provide medical and/or health benefits, including the prevention and treatment of disease,[73] could provide a safe alternative to replace or reduce the use of conventional therapies.

Steadfast® Equine (Novus International, Inc., St. Charles, MO, USA) is a comprehensive dietary supplement designed to maintain the health and growth of joints, hooves, tendons and ligaments as well as support immune response in mature horses. It is composed of biotin and methionine; eggshell membrane (Natural Eggshell Membrane, NEM®, Carthage, Mo, USA) containing type I collagen, glucosamine, chondroitin sulfate and hyaluronic acid[74]; calcium, phosphate, ascorbic acid, vitamin D; and a micronutrient mix, TelaFIRM™, which includes organic, chelated zinc, copper, manganese and selenium.[75] It is intended to prevent or slow the progression of joint disease through reduction of oxidative stress and inflammation, which in turn affects the mechanisms of tissue turnover.

Both biotin and methionine have been implicated in the growth and strength of keratinized tissues. Biotin is crucial for the differentiation of epidermal layers and

required for the production of keratin, the structural molecule that gives strength to hoof horn tissue.[76] A human study found that women who consumed less biotin were more likely to develop DJD.[77] In a study on the long-term effects of biotin supplementation in cattle, it was found that supplemented animals had better locomotion scores than control animals.[78] In horses, biotin supplementation is often used to improve hoof health.[79,80] Poor hoof-health can lead to uneven loading of the joints and potentiate the onset of DJD.[69] In a porcine study it was found that pigs given a methionine supplement had a lower risk for developing lameness due to osteochondrosis than pigs fed a control corn-soybean meal diet or pigs supplemented with fish oil.[81] If the methionine and biotin in Steadfast® Equine produces similar effects it may reduce lameness in horses through improved hoof-health, lower incidence of osteochondrosis, and improved strength of soft-tissues.

GAG's have shown promise for cartilage protection and regeneration *in vitro,* though not all of these affects have been repeatable when evaluated *in vivo*.[82] Glucosamine and chondroitin sulfate have both been shown, independently and/or in combination, to decrease pro-inflammatory molecules, decrease MMP synthesis, and increase proteoglycans and GAG synthesis.[83-87] The biological availability of glucosamine hydrochloride in the horse is estimated to be between 2.5% to 5.9%.[88] Glucosamine sulfate, the form of glucosamine found in NEM®, is only half as bioavailable as glucosamine hydrochloride and, thus, in theory, would require twice the amount to achieve the same effects.[89] Apart from incorporation into joint tissues, glucosamine has also been shown to decrease cyclo-oxygenase-2 gene expression, inhibiting PGE2 concentration, and reducing inflammation in the joint.[90] Orally

administered chondroitin sulfate is also biologically available to horses, however, source and molecular weight have been shown to directly affect absorption across the gastrointestinal tract. Molecular weight is inversely proportional to bioavailability, with a 32% bioavailability for 8-kd chondroitin sulfate and 22% bioavailability for 16-kd chondroitin sulfate.[88,91] Certain equine studies have found up-regulation of collagen, and inhibition of inflammatory mediators *in vivo*,[92] however, these findings remain controversial.[93] NEM® is believed to be both highly bioavailable and in a ratio to optimally stimulate cartilage repair.[74]

Ascorbic acid is required for normal collagen synthesis.[13,94] Additionally, ascorbic acid acts as an anti-oxidant, scavenging free-radicals.[95] Reduced plasma ascorbic acid concentrations may contribute to cartilage loss, disease progression and joint pain in horses with degenerative joint disease.[96] Ascorbic acid supplementation may be particularly important for horses as plasma concentrations in unsupplemented horses are lower than those found in other herbivorous species.[97] In chondrocyte cultures, vitamin D has been shown to increase synthesis of proteoglycans.[98] In a human study, patients in the middle or lowest tertiles of serum vitamin D metabolite, 25-hydroxyvitamin D, were found to be three times more likely to have significant joint disease progression as defined by narrowing of the joint space and osteophyte formation due to improper structural remodeling of the bone.[96]

The absorption level for certain minerals is thought to be affected by age, meaning that lower trace mineral concentrations may cause or perpetuate some of the biochemical changes in DJD, increasing the need for supplementation.[99] Zinc has a protective role in preserving bone mass by stimulating bone formation and inhibiting

bone resorption.[100,101] Zinc, manganese, and selenium have anti-oxidant properties, and manganese is also a cofactor for the synthesis of GAG's.[102] On the basis of its nutrient composition, Steadfast® Equine, has therapeutic potential for the amelioration of equine lameness, however, to date there is no objective evidence to confirm this.

Conclusions

As the lifespan of domestic animals continues to increase, DJD is likely to become more present in veterinary medicine. In response, there is a new focus on preventative strategies, including life style changes and nutraceutical interventions. In order for early intervention to become a possibility, there must be reliable diagnostics for DJD in the preclinical stages. Collagen biomarkers and biomarkers for oxidative stress may have the potential to aid in determining a diagnosis and prognosis for joint disease. In addition, nutraceuticals may offer a means to reduce the use of conventional therapies with adverse side-effects. These studies focus on creating a profile of type II collagen and oxidative stress biomarker concentrations in an equine population, assessing if they relate to patterns of equine lameness and evaluating Steadfast® Equine as a therapeutic for the improvement of lameness in horses.

CHAPTER II

ASSESSMENT OF JOINT CONDITION BIOMARKERS IN AN EQUINE POPULATION

Abstract

The objective of this trial was to establish inter- and intra-individual variance over time of selected joint condition biomarkers in an equine population. Concentrations of biomarkers were then evaluated for correspondence to joint pathology. Thirty-six horses of various breeds and gender were used in this study. Horses ranged in age from 5- to 23-years. Serum samples were analyzed for concentrations of CTX-II, PIIANP and NO. Horses were evaluated for lameness using a wireless accelerometer-gyroscope based lameness system, and were assigned a lameness score of 0 to 3 (0=sound, 1=mildly lame, 2=moderately lame, and 3=markedly lame). The area affected by lameness was also scored from 0 to 3 (0=no lameness, 1=primary forelimb lameness, 2=primary hind limb lameness, and 3=mixed fore- and hind limb lameness). Population means ± standard error, population ranges and individual repeatability were determined for the serum concentration of each biomarker. The effects of lameness score, affected area, age and gender on biomarker concentrations were evaluated. Within the population, the mean CTX-II concentration was 42.8±3.8 pg/mL; the mean PIIANP concentration was 837.44±68.43 ng/mL; and the mean NO concentration was 12.8±1.8 μM. The individual repeatability for CTX-II, PIIANP and NO was 85.5%, 88.7 % and 3.4%, respectively.

CTX-II concentration decreased with increasing lameness score ($P<0.0001$). There was no overall effect of lameness score on PIIANP concentration ($P>0.21$), however there was decreased PIIANP concentration in lame horses, regardless of score, compared to sound horses ($P<0.05$). Age had an effect on both CTX-II ($P<0.0002$) and PIIANP ($P<0.0001$). Gender had an effect on mean concentrations of CTX-II ($P<0.0001$), however, there was no effect of gender on PIIANP concentration. There was no effect of lameness score, affected area, age or gender on NO concentration. Establishing an initial horse population profile was a first step to subsequent trials evaluating lameness and biomarker concentrations in horses.

Abbreviations

A-G	Accelerometer-Gyroscope
CTX-II	Carboxyl-Terminal Crosslinking Telopeptide of Type II Collagen
DJD	Degenerative Joint Disease
NO	Nitric Oxide
NSAID	Non-Steroidal Anti-Inflammatory Drug
MMP	Matrix Metalloproteinase
PIIANP	N-Terminal Procollagen Propeptide of Type II Collagen

Introduction

Degenerative joint disease (**DJD**) is both a common cause and complication in equine lameness. An estimated 60% of equine lameness cases are related to DJD.[103] Physical examination, the standard preliminary procedure for assessing equine lameness, can usually localize pathology to a particular joint.[62] However, physical examination alone is not sufficient to assess the severity and duration of joint disease and there is a great deal of variability in assessment from one veterinarian to the next.[66] Radiographs that measure joint space narrowing are the primary diagnostic for DJD, however a significant change in joint structure can require one to two years to detect.[33] Even with more sensitive techniques, such as magnetic resonance imaging, by the time a definitive diagnosis can be established, there is often already significant joint damage.[22,104] Additionally, current imaging technologies are not adequately sensitive to monitor the efficacy of treatments aimed at preventing or slowing DJD.[22] Alternatively, biomarkers that reflect the rate of turnover of cartilage, bone or the synovial membrane, or biomarkers that provide information about the level of oxidative stress or inflammation, may be useful for the early detection and monitoring of DJD.[19,105,106]

The etiology of DJD is not fully understood, however, it is a progressive, self-perpetuating disease that results from abnormalities in mechanical, biological, biochemical, immunoregulatory and enzymatic feedback loops.[30] The disease is characterized by uncoupling of the catabolic and anabolic processes that control cartilage turnover, leading to excessive cartilage degradation and loss.[22] Type II collagen is the

major protein component of articular cartilage. Degradation of type II collagen is mediated by matrix metalloproteinases (**MMP)**-1, -8, -13 and -14, which cleave the collagen helix in the crosslinking telopeptide domain, resulting in ¾ and ¼ length fragments.[33,107] The ¼ length fragment is the C-terminus of the telopeptide of type II collagen (**CTX-II**), and is a well-documented correlate of cartilage degradation in both human and veterinary medicine. [51,108-110] The rate of cartilage synthesis can be quantified by measuring the concentration of type IIA collagen N-propeptide (**PIIANP**) a molecular fragment that is cleaved and released into the synovial cavity as procollagen monomers polymerize to form fibrils. Unlike collagen IIB, which is synthesized by adult chondrocytes, collagen IIA is only synthesized in chondrocyte progenitor cells and chondrocytes affected by DJD.[33] Nitric oxide (**NO**) plays an important role in arthropathies by increasing the catabolic processes that lead to cartilage destruction.[56] The release of interleukin-1β up-regulates inducible nitric oxide synthase production by chondrocytes, in turn inducing the release of NO. Nitric oxide promotes cartilage degradation by increasing cytokine production, suppressing matrix synthesis, elevating catabolic enzyme concentrations (i.e. MMP's) and reducing the activity of tissue inhibitors of metalloproteinases.[83] Several studies have shown that nitrite concentrations, the form to which nitric oxide is converted in the blood, are significantly higher in the synovial fluid and serum of human patients affected by DJD compared with control patients.[58,59]

Concentrations of joint condition biomarkers in synovial fluid have been predictive of joint pathology in horses.[12,82,111] However, arthroscopy to collect synovial fluid is invasive and introduces the risks of infection into the joint. Therefore, it would

not be practical as a routine screening procedure to detect or monitor DJD. Serum biomarkers offer an alternative source of information about the joint, as blood sampling is minimally invasive and presents very little risk to the patient. Each of the biomarkers evaluated in this trial has shown promise for the detection of joint disease from serum concentrations.[70,112,113]

There is no published data on average serum concentrations for CTX-II or PIIANP in horses. Therefore, the primary objectives for this trial were to create an equine population profile of mean values and standard deviations of serum CTX-II, PIIANP and NO and to examine average individual variance. The third objective was to determine whether patterns existed between serum biomarker concentrations and lameness in horses, as assessed with a wireless accelerometer-gyroscope (**A-G**) based system.

Materials and Methods

Horses- The research protocol was reviewed and approved by the University of Missouri Animal Care and Use Committee. Thirty-six horses from Stephen's College Equestrian Program in Columbia, Missouri were utilized in this study. Horses were of various breeds [American Saddlebred (n=14); Thoroughbred (n=6); Quarter Horse (n=14); Arabian (n=1); American Paint Horse (n=1)], and ranged in age from 5- to 23-years. The population consisted of 26 geldings and 10 mares. The horses were housed at the Stephen's College Equestrian Stables and were ridden and/or turned out daily in accordance with the Stephen's College Equestrian Program, receiving a moderate to

heavy exercise load daily. Fourteen days prior to trial commencement, administration of nutraceuticals, pharmaceuticals and/or other supplements, including joint supplements, non-steroidal anti-inflammatory drugs (**NSAID**'s), and systemic or intra-articular steroids or glycosaminoglycans, was discontinued. Horses were fed individual diets consisting of grass or alfalfa hay, supplemented with grains or pellets, and given *ad libitum* access to water.

Serum Collection and Processing- Whole blood samples (20 mL) were collected from each horse using vacutainer tubes (BD Vacutainer®, Franklin Lakes, NJ, USA) and 20-gauge needles via jugular veinipuncture. Blood samples were obtained on days 0, 14 and 28 of the trial. (**Figure 2.1**) Blood was drawn between 0630 and 0830 hours and stored at 2-8ºC until processed. Whole blood was spun at 3000 rpm for 20 minutes in a centrifuge with a 19.2 cm rotating radius. Serum was harvested thereafter and stored, within eight hours of collection, at -20ºC until analyzed (no more than 120 days). CTX-II serum concentration was measured using a commercial ELISA kit (Serum Pre-Clinical CrossLaps® ELISA, Immunodiagnostics Int., Fountain Hills, AZ, USA) and recorded in picograms per milliliter (pg/mL). CTX-II samples below the detection limit of the standard curve were entered for statistical analysis as an average between the lowest point on the standard curve, 6.30 ng/mL and 0, in this case 3.15 ng/mL. PIIANP concentration was also measured by use of a commercial competitive ELISA kit (Human PIIANP ELISA, Millipore™, Billerica, MA, USA) and recorded in nanograms per milliliter (ng/mL). Nitric oxide concentration was determined using the Griess method. Nitrite (NO^{2-}) a stable, oxidized form of nitric oxide was quantified using a colorimetric assay kit (Nitrate/Nitrite Colorimeteric Assay Kit® ,LDH Method, Caymen Chemical,

Ann Arbor, MI) and recorded in micromoles (μM). All serum samples for nitrite analysis were micro filtered before being assayed (10,000 Nominal Molecular Weight Unit, Microcon®, Millipore™, Billerica, MA, USA). Intra and inter-assay coefficients of variance were 11.89% and 7.87% for CTX-II; 10.63% and 17.77% for PIIANP; and 6.02% and 2.87% for NO, respectively.

Wireless Accelerometer-Gyroscope Lameness Evaluation- On days 0 and 28, each horse was evaluated for lameness using the wireless A-G based system Equinosis™ (Equinosis LLC, Columbia, MO). (**Figure 2.1)** An accelerometer was attached to the right front fetlock to measure gait, while two gyroscopes were placed- one at the poll and one on the croup (at the midline)- to assess head or hind-end motion patterns. Abnormal patterns are indicative of weight shifting due to lameness.[21,114] Horses were hand-led and trotted on a compacted, in-door arena surface for approximately 100 meters. Each assessment was performed in duplicate. Based on the severity of lameness, horses were assigned a lameness score ranging from 0 to 3 (0=sound, 1=mild lameness, 2=moderate lameness and 3=marked lameness). The affected area was also scored from the A-G system data along with the assessment of the equine veterinarian running the evaluations to discern between primary and compensatory affected areas. The same veterinarian ran all evaluations and assigned lameness and affected area scores. Each horse was given a score from 0 to 3 (0=no lameness, 1=forelimb, 2=hind limb lameness, 3=mixed fore- and hind limb lameness). **(Table 2.1)**

Statistical Analysis- All statistical analysis was performed on SAS (v.9.1, SAS Institute Inc., Cary, NC, USA). Population means and ranges were determined for serum

concentrations of CTX-II, PIIANP, and NO. To estimate the individual variance of CTX-II, PIIANP and NO the Nested data procedure was used. The effect of lameness score over time was examined using the Mixed data procedure, with time (Day0, D14, and Day 28) as the independent variable, and concentrations of CTX-II, PIIANP and NO as the dependent variables.

Effects of lameness score, affected area, age and gender on concentrations of CTX-II, PIIANP and NO were analyzed by the GLM (general linear model) procedure, with horse as the experimental unit. The statistical model included lameness score, affected area, age and gender as main effects, and biomarker concentrations as the dependent variable(s). Horses were blocked into four groups for assessment of age effect: (Group A=5-6 years, n=4; Group B=7-13 years, n=14; Group C=14-19 years, n=14; and Group D=20-23 years, n=4). These assignments corresponded to the 0-10th percentile, the 10th-50th percentile, the 50th-90th percentile and the 90st-100th percentile. The least squares means (LSMEANS) function of SAS with PDIFF and STDERR options was used to calculate group means, establish differences between groups, and calculate the standard error associated with each mean. Results are reported as the mean ± standard error for all variables. The effect of lameness score on biomarker concentrations in sound versus lame horses (0 v. 1, 2, 3), mildly-lame versus markedly-lame horses (1 v. 3), and moderately-lame horses versus markedly-lame horses (2 v. 3) were assessed using a *post hoc* test of orthogonal contrasts. Contrasts for affected area were for sound versus lame horses (0 v. 1, 2, 3), forelimb lameness versus hind limb lameness (1 v. 2), and single versus mixed area lameness (1, 2 v. 3). Significance was defined as values of $P<0.05$ for all calculations; values approaching significance were defined as $0.05<P<0.10$.

Results

Population Profile- The population mean, range and the individual repeatability for each biomarker was calculated from all measurements (n=36 horses x 3 samples=108). The mean ± standard error was 42.8±3.8 pg/mL for CTX-II, 837.4±68.4 ng/mL for PIIANP, and 12.8±1.8 μM for NO. Concentrations ranged from 3.2 to 188.5 pg/mL for CTX-II; from 162.2 to 1694.0 ng/mL for PIIANP; and 0.90-103.3 μM for NO. Individual repeatability was 85.5% for CTX-II, 88.9% for PIIANP and 3.4% for NO. (**Table 2.2**)

There was no effect of time on CTX-II concentration (P>0.32). There was, however, an effect of time on PIIANP (P<0.02) and NO concentrations (P<0.0003). The first collection date had a lower population mean for PIIANP concentration (749.0±127.0ng/mL) compared to the second (880.4±127.1 ng/mL; P<0.03) or third (907.7±127.1 ng/mL; P<0.008) collection dates. There was no difference in PIIANP concentration between the second and third collection dates (P>0.64). The population mean for NO was increased at the first collection date (22.7±3.1 μM) compared to the second (8.9±3.1 μM; P<0.001) and third (6.8±3.1 μM; P<0.002) collection dates. There was no difference between the second and third collection dates (P>0.61) for serum concentration of NO. **(Table 2.3; Figure 2.2)**

Effects of Lameness Score – Horses were given a single lameness score for the evaluations at day 0 and 28. Seven horses had a lameness score of 0, eleven a score of 1, twelve a score of 2, and six a score of 3.**(Table 2.1; Table 2.4)** There was an effect of lameness score on CTX-II concentration (P<0.0001) with a pattern of decreasing CTX-II

concentration corresponding to an increase in lameness score. Mean CTX-II concentration in sound horses (69.8±7.9 pg/mL) was greater than the concentration in horses with moderate- (28.9±6.0 pg/mL; P<0.0001) and marked-lameness (23.2±8.6 pg/mL; P<0.0001). The difference between sound horses and horses with mild-lameness (51.4±6.3 pg/mL) approached significance (P<.08). There was no difference in CTX-II concentration between moderately- and markedly-lame horses (P>0.59). There was a greater CTX-II concentration in sound horses than horses with any degree of lameness (P<0.0001) and in mildly-lame compared to markedly-lame horses (P<0.01). **(Table 2.4; Figure 2.3(A))**

There was not an effect of lameness score on PIIANP concentration (P>0.21), however, PIIANP concentration in sound horses (1112.3±154.0 ng/mL) was numerically higher in horses with moderate- (759.6±117.7 ng/mL) compared to marked lameness (674.8±166.4 ng/mL). PIIANP concentration was greater in sound horse than horses with any level of lameness (P<0.05). There was no effect of lameness score on NO serum concentration (P>0.99), and no difference between sound and lame horses (P>0.93). **(Table 2.4; Figure 2.3(B))**

Effects of Affected Area- There were seven horses with an affected area score of 0, ten with a score of 1, seven with a score of 2 and twelve with a score of 3. Affected area had an overall effect on CTX-II serum concentration (P<0.0001). CTX-II concentration was lower in horses with fore-limb lameness (22.3±6.5 pg/mL; P<0.0001) and horses with hind limb lameness (28.1±7.8; P<0.003) and tended to be lower in horses with mixed fore-limb and hind limb lameness (52.6±5.9pg/mL; P<0.08) compared to

horses with no lameness (69.8±7.9 pg/mL). CTX-II concentration was higher in sound versus horses with any area affected with lameness (P<0.0001), and lower in horses with a single area of lameness compared to horses with mixed fore- and hind limb lameness (P<0.0007). There was no difference in CTX-II concentration between horses with forelimb compared to hind limb lameness (P>0.6). (**Table 2.5; Figure 2.4(A)**)

Affected area did not have an effect on PIIANP concentration (P>0.21), however, sound horses (1112.3±154.1 ng/mL) had a numerically higher concentration of PIIANP compared to horses with hind limb lameness (670.4±154.1 ng/mL) and numerically lower concentration than horses with forelimb lameness (779.2±128.9 ng/mL). PIIANP concentrations were higher in sound horses versus horses with any area of affected lameness (P<0.04). There was no difference in concentration between horses with single area versus mixed fore- and hind limb lameness (P>0.52) or between horses with forelimb compared to horses with hind limb lameness (P>0.52). NO concentration showed no effect by affected area (P>0.63) and no differences between any affected area score group. (**Table 2.5; Figure 2.4(B)**)

Effects of Age – CTX-II serum concentration was affected by age (P<0.0002). The youngest 10th percentile (Group A; 71.4±8.6 pg/mL; n=4) had a higher concentration of CTX-II than group B (23.9±6.1 pg/mL; P<0.0001; n=14), group C (49.5±6.7 pg/mL; P<0.05; n=14) and group D (41.2±8.6 pg/mL; P<0.001; n=4). Group B had a CTX-II concentration that approached a significant decrease from the two oldest groups, group C (P<0.06) and group D (P<0.08). PIIANP concentration was also affected by age

(P<0.0001) with a higher concentration of PIIANP in group D (1625.0±117.7 ng/mL) than group A (482.5±135.9 ng/mL);, group B (648.6±96.1 ng/mL) and group C (646.9±105.3; P<0.0001). PIIANP concentration was not different between any other age groups. There was no effect of age on NO concentration (P>0.83). **(Table 2.6; Figure 2.5)**

Effects of Gender- There was an effect of gender on CTX-II concentration (P<0.0001) with a higher concentration of CTX-II in mares (73.7±6.4 pg/mL) than in geldings (30.9±3.9). There was no effect of gender on PIIANP concentration (P>0.40). NO concentration in geldings (7.8±3.4μM) approached a significant decrease (P<0.09) from the concentration in mares (14.7±2.1μM). (**Table 2.7; Figure 2.5)**

Discussion

An investigation of joint biomarkers in an equine population has provided a preliminary profile of expected mean concentrations and variation for CTX-II, PIIANP and NO. This information may be helpful in identifying appropriate biomarkers for the diagnosis of DJD and the evaluation of therapeutics.

The repeatability over a 28 day period with a single horse for type two collagen biomarkers was high (>85%) and time did not show an effect on CTX-II. On the other hand, NO had an individual repeatability of less than 5%. These data provide evidence that, in the absence of treatment, individual values of CTX-II and PIIANP remain relatively constant within an individual, suggesting that non-lameness related variables,

such as exercise and diet, had a minimal effect on the concentration of these biomarkers over a 28 day period. It should be noted that horses were involved in a summer-riding program for the duration of this trial, thus, the level of exercise they received over the course of the 28 days was fairly consistent. On the other hand, because NO varied greatly in the absence of treatment, there appears to be confounding effects of non-lameness related variables on the systemic concentration of NO.

A number of human studies have found that both synovial fluid and serum concentrations of NO are increased in patients with DJD compared to control patients.[56,57,115] Cartilage explant studies have confirmed that chondrocytes, both human and equine, produce ample quantities of NO when under inflammatory duress.[60,61] On the other hand, the equine synovial membrane has a uniquely low capacity for NO synthesis.[116] It has also been reported in horses that joints affected with moderate DJD may have a higher concentration of inflammatory mediators, including NO, than joints affected with severe DJD.[117] Another study reported a greater variance in NO concentration for horses with DJD compared to healthy controls, but, no difference in mean plasma or synovial fluid concentrations.[112] Similarly, in this study there were no differences in NO based on lameness score, affected area, age or gender, but, there was a large amount of variance within and across individuals.

There was a higher mean population concentration of NO on day 0 than on days 14 or 28. Although the reason for the increased concentration is unknown, horses had gone from a rest period to the beginning of a summer riding camp the week before collections started. The high NO concentration may account for the decreased PIIANP

concentration on day 0 as compared to days 14 and 28. NO suppresses matrix synthesis, repressing type II collagen in the joint and thus could account for a decrease in PIIANP concentration.[56,57,83] There was no effect of time on CTX-II despite the fact that NO is also believed to up-regulate matrix metalloproteinase activity.

Contrary to what was expected based upon the results of human studies[51,53,118,119], CTX-II decreased as lameness score increased. However, an important difference is that many of these studies used high CTX-II concentrations to predict the onset of DJD, whereas lameness score detects the current state of disability. The lower CTX-II concentration may be in response to the loss of articular cartilage that occurs in late-stage DJD.[64] A short-coming of this trial is that horses were not prescreened for the presence of DJD, therefore, lameness score as measured by the wireless A-G system is not limited to lameness caused by DJD. Although there is a clear pattern of decreasing CTX-II with increasing lameness score, this is not the same thing as a decrease in CTX-II that corresponds to increased severity of DJD.

The lower CTX-II concentrations in higher lameness categories, along with the trend toward lower PIIANP concentration in lameness categories 2 and 3, provides evidence that cartilage metabolism is impaired in moderately- and markedly-lame horses as compared to mildly-lame and sound horses. Programmed cell death of chondrocytes has been identified in late stage DJD, impairing cartilage metabolism,[120] and may account for the decreased turnover of the cartilage matrix observed in this trial in moderately- and markedly-lame horses.

Despite evidence for different rates of type II collagen degradation for different joints in horses when experimentally induced with DJD[103] there was no difference in CTX-II or PIIANP concentrations between horses with primary forelimb lameness and horses with primary hind limb lameness. Horses with mixed fore- and hind limb lameness had higher concentrations of CTX-II than horses with a single area of lameness, suggesting that serum concentration of CTX-II is a cumulative pool of all diseased joints. In a human study, a positive association was found between the number of joints affected by DJD (knee, hip, hand, and cervical and lumbar facet discs and joints) and the urinary concentration of CTX-II.[108]

CTX-II was higher in the lowest 10th percentile of horses (5-6 years) than in any other age group, indicating a higher level of cartilage metabolism in immature horses. A higher rate of cartilage metabolism in growing horses has been similarly observed in other studies.[121,122] CTX-II levels decline in 7-13 year olds, and then increase again in 14-23 year olds. Both human and equine studies have found that increasing age positively correlates to CTX-II concentration.[123,124] This pattern reflects the propensity of geriatric individuals to develop diseases caused by chronic, low-level inflammation.[125]

The concentration of PIIANP was highest in the 90th -100th percentile of horses as compared to any other age groups. A longitudinal, 5-year human study found that patients with high concentrations of both CTX-II and PIIANP were more likely to have more severe disease progression.[53] It has also been shown that in healthy adult chondrocytes, no mRNA for PIIANP was found, however, PIIANP was reexpressed in chondrocytes affected by DJD.[32] Because age is the primary risk factor for the

development of DJD,[124,126] individuals in the top 10th percentile for age are in a high risk-group for the development of DJD, a fact which may be further confirmed by an increased expression of PIIANP.

Mares had a higher concentration of CTX-II than geldings. It has been shown that estrogen deprivation in post-menopausal women increases CTX-II concentration, but has no effect on other type II collagen biomarkers, including PIIANP.[127] In congruence with those findings, there was no effect of gender on PIIANP. It is believed that both estrogens and androgens have a chondroprotective effect.[128] However, mares would have been in a reproductive cycling season during this trial, so increased CTX-II is not likely due to estrogen deprivation. The reason for gender differences in CTX-II concentration may be due to the smaller number of mares (n=11) involved in the study compared to geldings (n=26). In order to evaluate whether there is a true effect of gender on CTX-II concentration a study would need to be conducted that involved an equal ratio of mares to geldings.

CTX-II and PIIANP may be useful candidate biomarkers for the detection of DJD, determining the degree of pathology, and evaluating the effects of therapy. They are highly repeatable within an individual over a 28 day period and are independently different between sound and lame horses. However, it should be taken into account that in the population of horses used in this trial there were effects of both age and gender on the serum concentrations of CTX-II and PIIANP. Further investigation involving imaging of affected joints is needed to determine if a higher lameness score and/or lower

concentration of CTX-II is correlated to decreased articular cartilage and more progressed DJD.

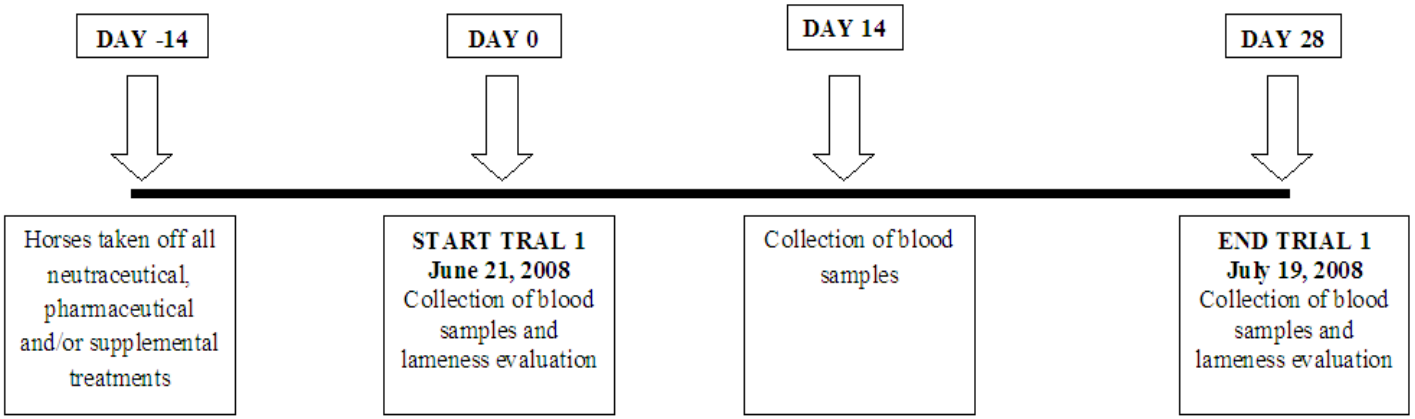

Figure 2.1- Timeline for Trial 1

Table 2.1 Classification of Lameness Score and Affected Area Score

Lameness Score	Severity of Lameness
0	Sound (none)
1	Mild
2	Moderate
3	Marked

Affected Area Score	Area Affected by Lameness
0	None
1	Forelimb
2	Hindlimb
3	Mixed Forelimb and Hindlimb

Table 2.2 Population Mean± Standard Error, Range and Individual Repeatability of Serum Biomarker Concentration

Biomarker	Population Mean (n=36)	Population Range (n=36)	Individual Repeatability (n=36 horses x 3 samples=108)
CTX-II (pg/mL)	42.8±3.8	3.2-188.5	85.5%
PIIANP (ng/mL)	837.4±68.4	164.2-1694.0	88.9%
NO (µM)	12.8±1.8	0.9-103.3	3.4%

Table 2.3 Effect of Time on Biomarker Concentration (n=108). Mean ± SE for each biomarker over all collection dates

Biomarker	Day 0	Day 14	Day 28	**SE**	P<[1]
CTX-II (ng/mL)	45.4	42.8	41.7	6.5	NS
PIIANP (pg/mL)	749^{A}	880^{B}	907^{B}	127	0.02
NO (µM)	22.7^{A}	8.9^{B}	6.8^{B}	3.1	0.0005

[1]Significance (P<0.05)

NS=Not Significant

Upper-case letters indicate significant differences between means (P<0.05)

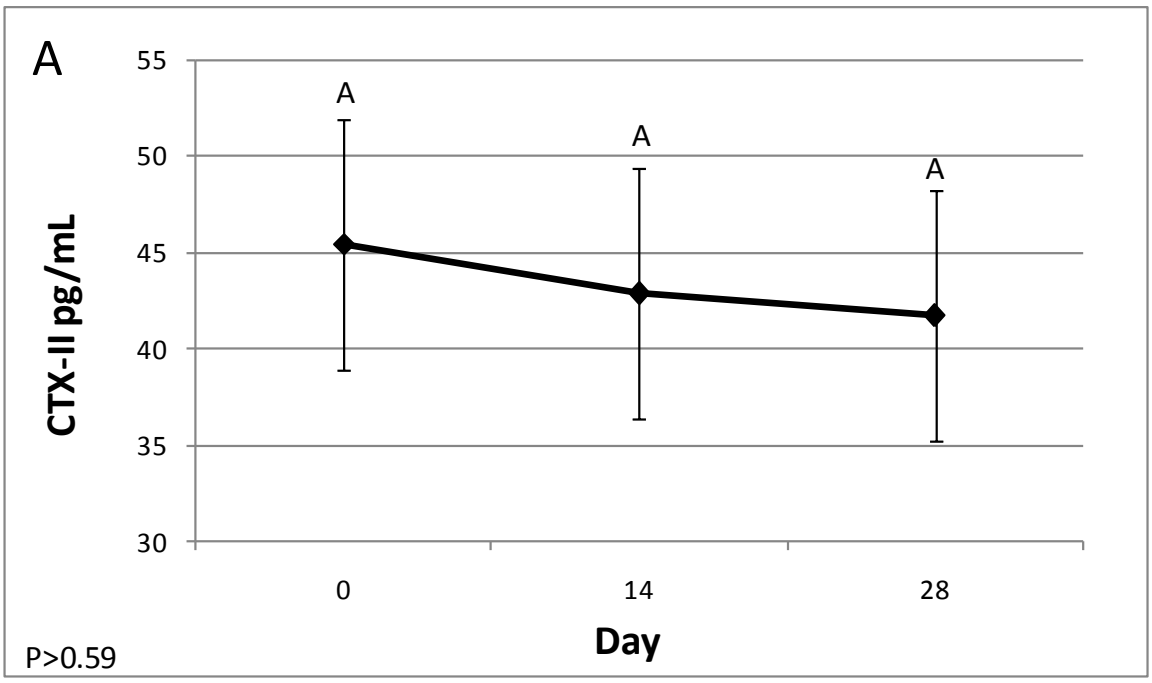

Figure 2.2(A) CTX-II Concentration over Time in an Equine Population- Mean ± SE CTX-II concentrations (n=36) at day 0 (45.4±6.5), day 14 (42.8±6.5) and day 28 (41.7±6.5). Capital letters indicate a difference in means (P<0.05).

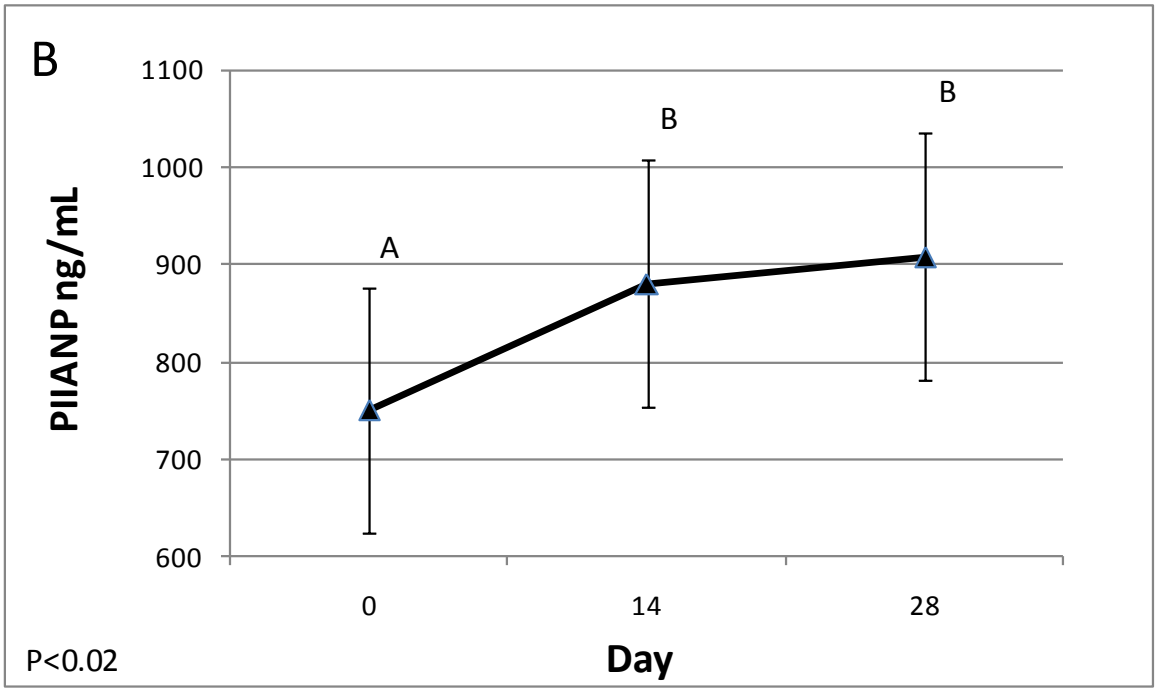

Figure 2.2(B) PIIANP Concentration over Time in an Equine Population- Mean ± SE PIIANP concentrations (n=36) at day 0 (749±127), day 14 (880±127) and day 28 (907±127). Capital letters indicate a difference in means (P<0.05).

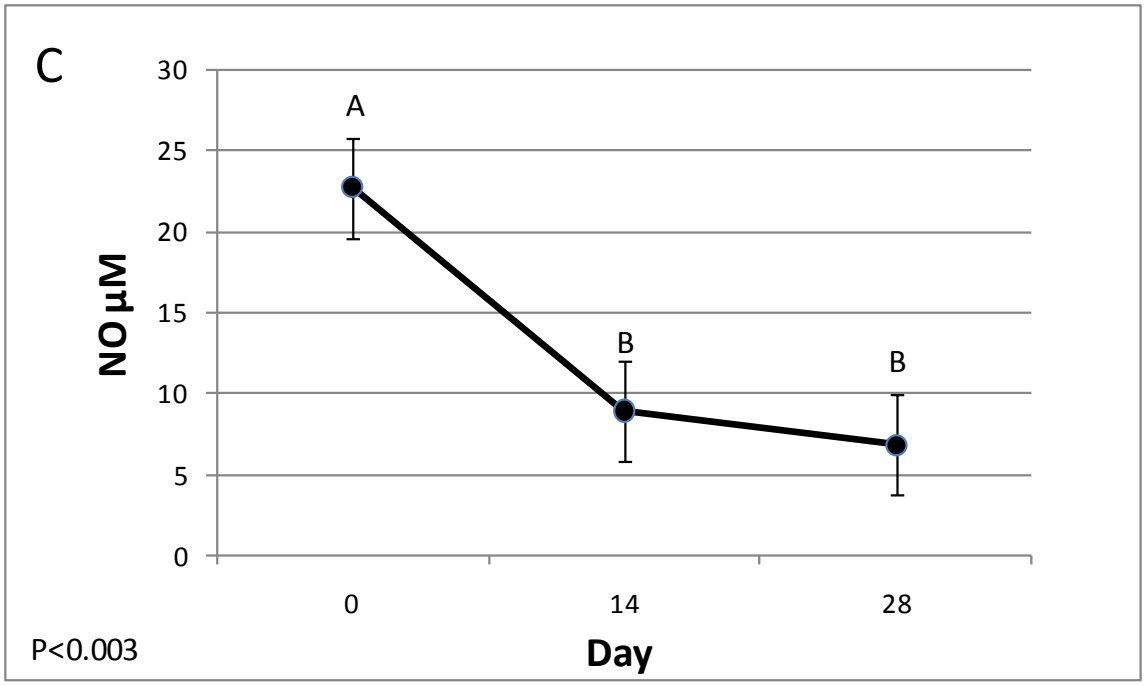

Figure 2.2(C) NO Concentration over Time in an Equine Population-
Mean ± SE NO concentrations (n=36) at day 0 (22.7±3.1), day 14 (8.9±3.1), and day 28 (6.8±3.1). Capital letters indicate a difference in means (P<0.05).

Table 2.4 Effect of Lameness Score on Biomarker Concentration (n=36). Mean ± SE biomarker concentrations for horses assigned to lameness score groups 0 to 3.

	Lameness Score				
Biomarker	**0 (sound) (n= 7)**	**1 (mild) (n=11)**	**2 (moderate) (n=12)**	**3 (marked) (n=6)**	**P<[1]**
CTX-II (pg/mL)	69.8±7.9^{A}	51.4±6.3^{a}	28.9±6.0^{B}	23.2±8.6^{B}	0.0001
PIIANP (ng/mL)	1112±154	836±123	759±118	674±166	NS
NO (µM)	12.5±4.2	13.1±3.3	12.6±3.2	12.9±4.5	NS

[1]Significance (P<0.05)

NS=Not Significant

Upper-case letters indicate significant differences between means (P<0.05)

Lower-case letters indicate a means that approach significance as compared to means with the corresponding upper-case letter (P<0.10)

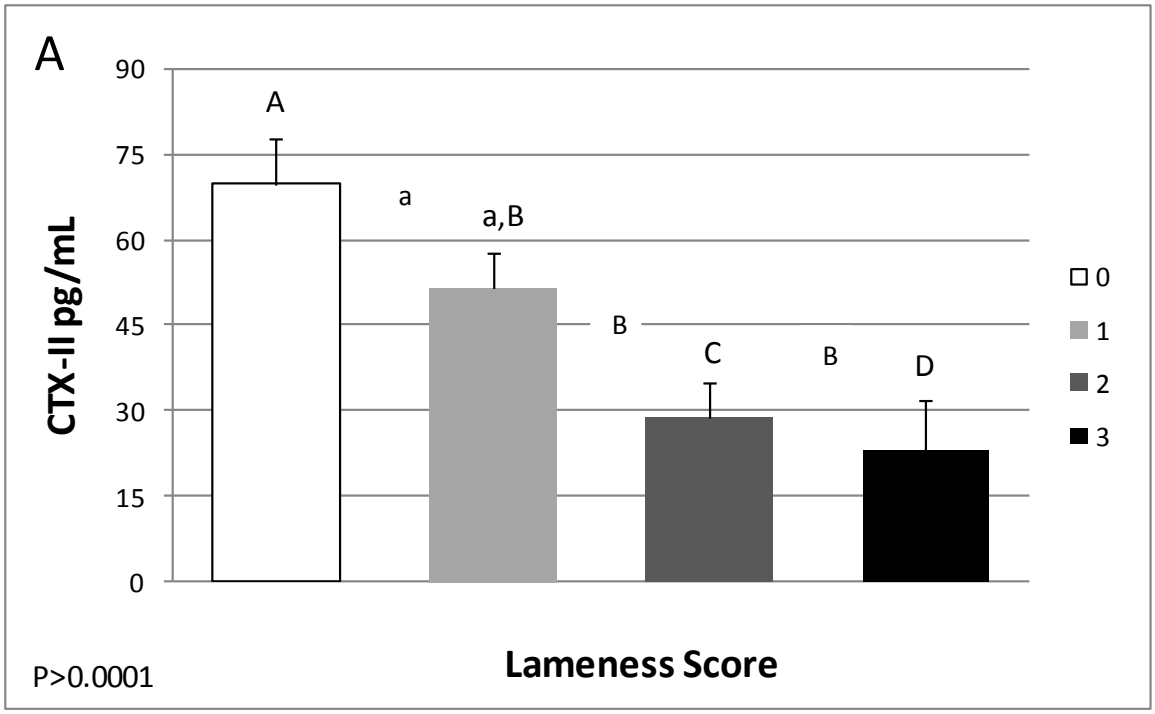

Figure 2.3(A) Effect of Lameness Score on CTX-II Concentration in an Equine Population- Mean ± SE CTX-II concentrations (n=36) at LS=0 (69.8±7.9), LS=1 (51.4±6.3), LS=2 (28.9±6.1) and LS=3 (23.2±8.6) where 0=sound, 1=mild lameness, 2=moderate lameness, and 3=marked lameness. Capital letters indicate a difference in means ($P<0.05$). Lower-case letters indicate a difference in means approaching significance as corresponding to the upper-case letter ($P<0.10$).

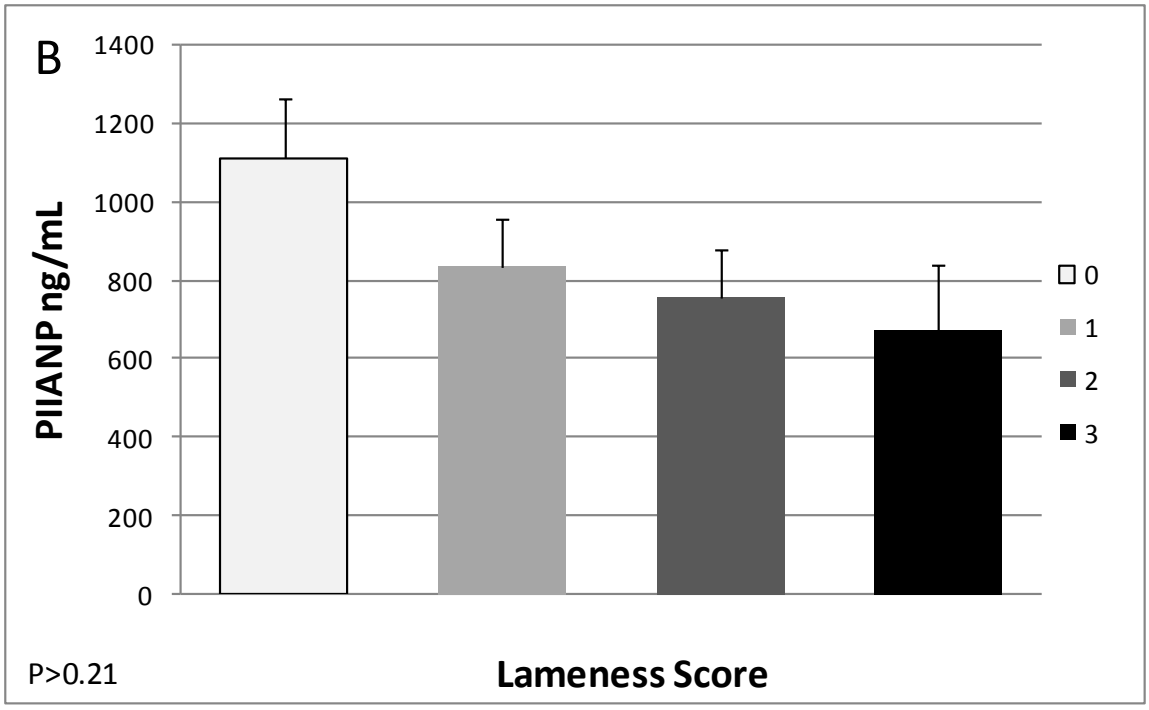

Figure 2.3(B) Effect of Lameness Score on PIIANP Concentration in an Equine Population - Mean ± SE PIIANP concentration (n=36) at LS=0 (1112.3±154.0), LS=1 (836.1±122.9), LS=2 (759.6±117.7) and LS=3 (674.8±166.4) where 0=sound, 1=mild lameness, 2=moderate lameness, and 3=marked lameness. Capital letters indicate a difference in means ($P<0.05$). Lower-case letters indicate a difference in means approaching significance ($P<0.10$).

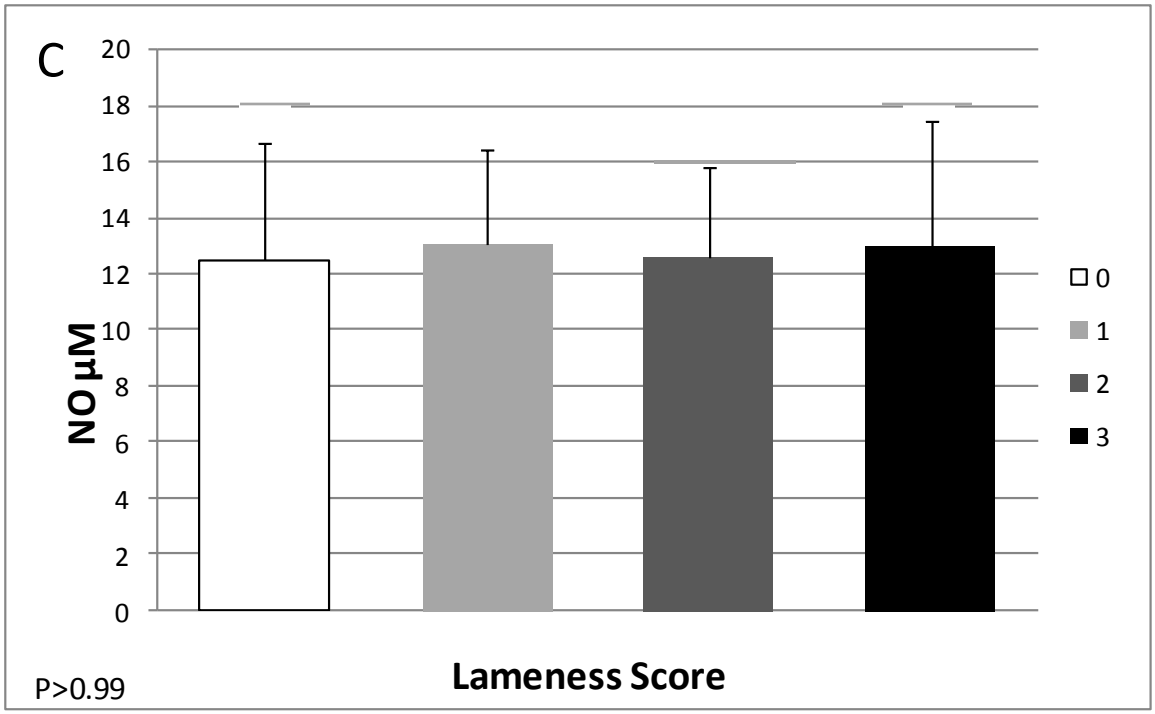

Figure 2.3(C) Effect of Lameness Score on NO Concentration in an Equine Population- Mean ± SE NO concentration (n=36) at LS=0 (12.5±4.2), LS=1 (13.1±3.3), LS=2 (12.6±3.2) and LS=3 (12.9±4.5) where 0=sound, 1=mild lameness, 2=moderate lameness, and 3=marked lameness. Capital letters indicate a difference in means ($P<0.05$).

Table 2.5 Effect of Affected Area on Biomarker Concentration (n=36). Mean ± SE biomarker concentrations for horses assigned affected area score groups 0 to 3.

Affected Area					
Biomarker	**0 (no lameness) (n=7)**	**1 (forelimb) (n=10)**	**2 (hind limb) (n=7)**	**3 (mixed fore- & hind limb) (n=12)**	**P<[1]**
CTX-II (pg/mL)	69.8±7.9[A]	22.3±6.5[B]	28.1±7.8[B]	52.6±5.9[C]	0.0001
PIIANP (ng/mL)	1112±154	779±128	670±154	823±117	NS
NO (µM)	12.4±4.1	15.8±3.5	8.7±4.1	12.9±3.2	NS

[1]Significance (P<0.05)

NS= Not Significant

Upper-case letters indicate significant differences between means (P<0.05)

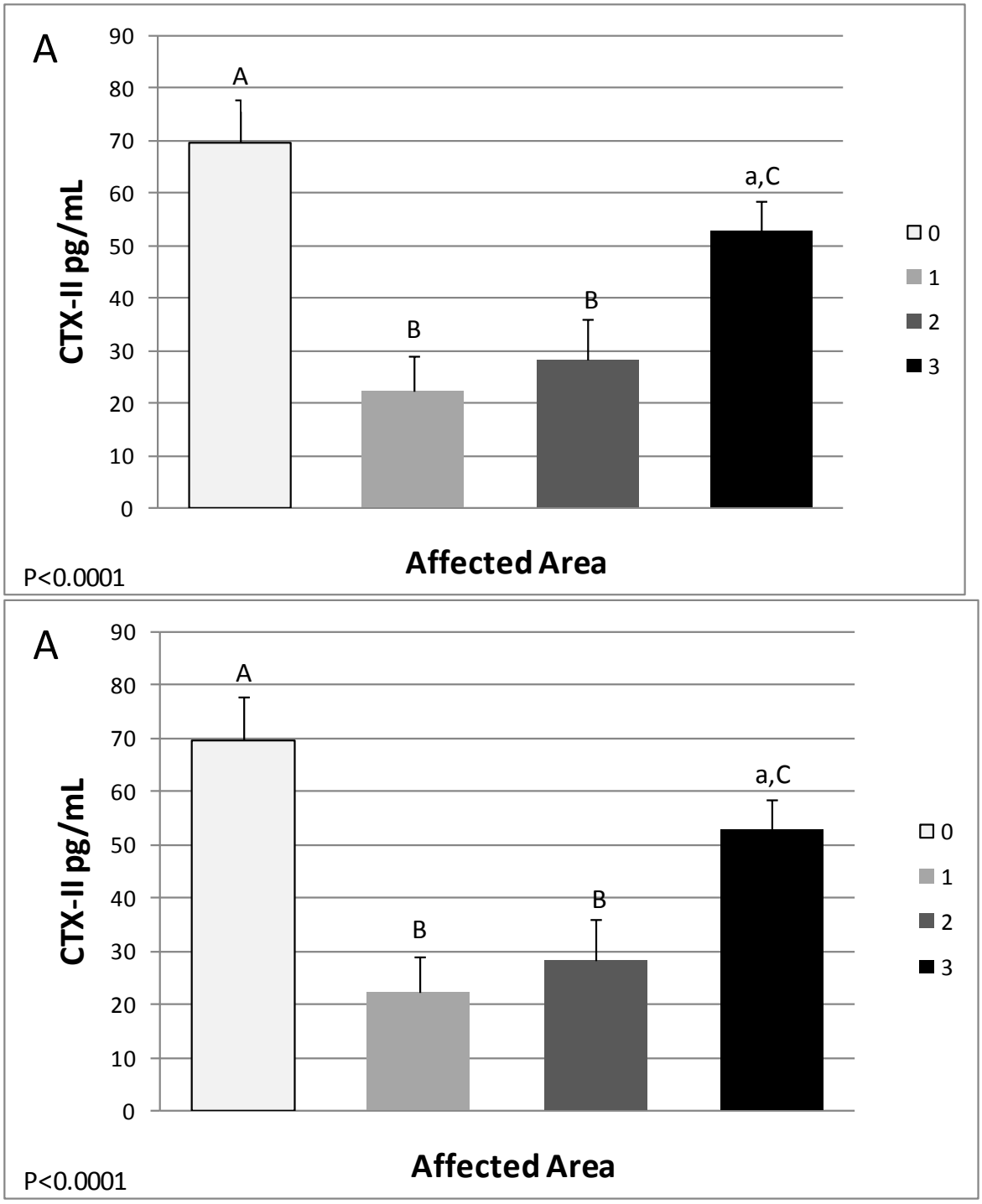

Figure 2.4(A) Effect of Affected Area on CTX-II Concentration in an Equine Population - Mean ± SE CTX-II concentration (n=36) at AA=0 (69.8±7.9), AA=1 (22.3±6.5), AA=2 (28.1±7.8) and AA=3 (52.7±5.9) where 0=no lameness 1=forelimb lameness, 2=hind limb lameness, and 3=mixed fore- and hind limb lameness. Capital letters indicate a difference in means (P<0.05). Lower-case letters indicate a difference in means approaching significance as corresponding to the upper-case letter (P<0.10).

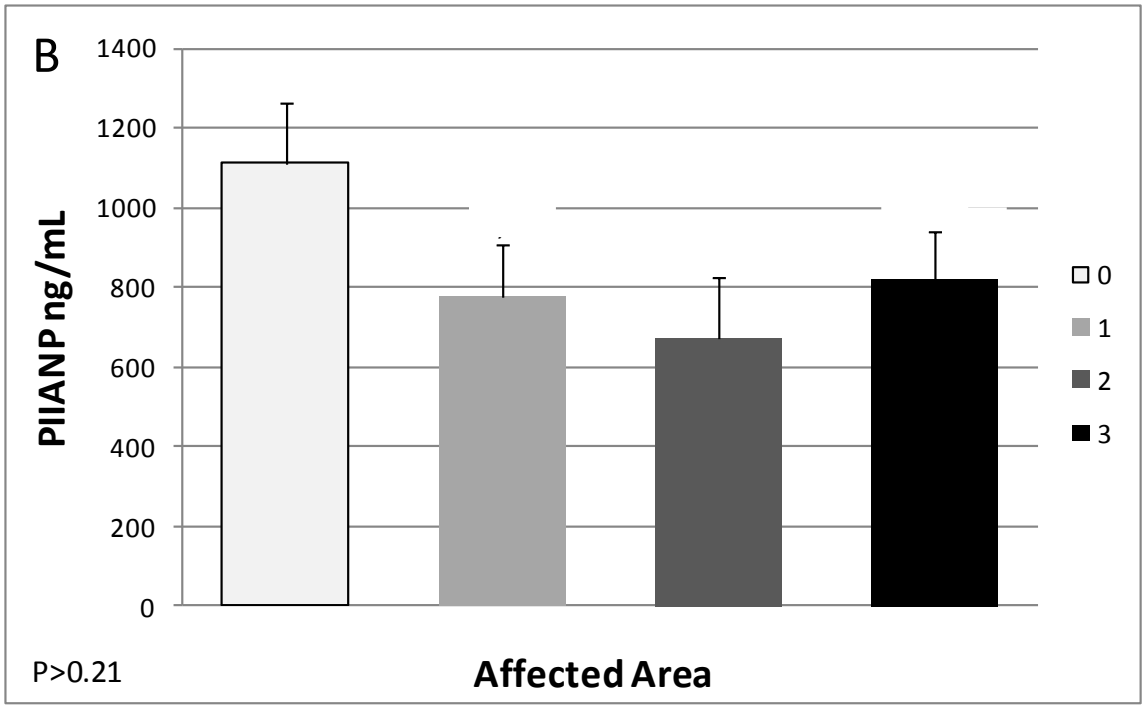

Figure 2.4(B) Effect of Affected Area on PIIANP Concentration in an Equine Population- Mean ± SE PIIANP concentration (n=36) at AA=0 (1112±154), AA=1 (779±128), AA=2 (670±154) and AA=3 (823±117) where 0=no lameness 1=forelimb lameness, 2=hind limb lameness, and 3=mixed fore- and hind limb lameness. Capital letters indicate a difference in means ($P<0.05$). Lower-case letters indicate a difference in means approaching significance ($P<0.10$).

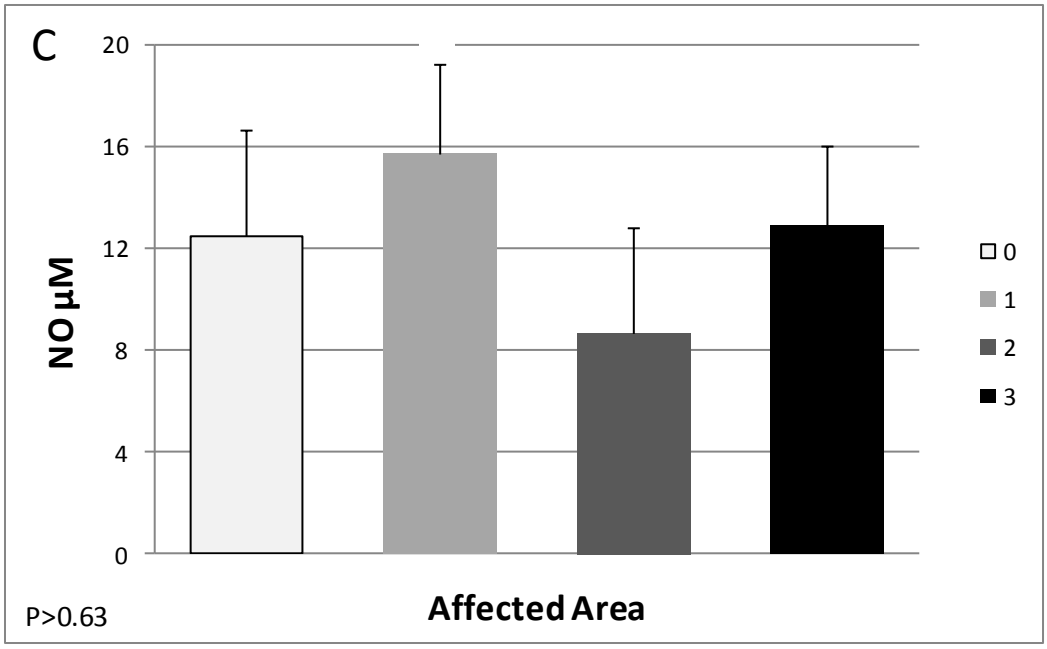

Figure 2.4(C) Effect of Affected Area on NO Concentration in an Equine Population- Mean ± SE NO concentration (n=36) at AA=0 (12.5±4.1), AA=1 (15.8±3.5), AA=2 (8.7±4.1) and AA=3 (12.9±3.2) where 0=no lameness 1=forelimb lameness, 2=hind limb lameness, and 3=mixed fore- and hind limb lameness. Capital letters indicate a difference in means ($P<0.05$).

Table 2.6 Effect of Age on Biomarker Concentration (n=36). Mean ± SE biomarker concentrations for age groups. The 0-10th percentile was designated group A, the 11th-50th percentile group B, the 51st to 90th percentile group C, and the 91th to 100th percentile group D.

	Age				
Biomarker	**Group A 5-6 years (n=4)**	**Group B 7-13 years (n=14)**	**Group C 14-19 years (n=14)**	**Group D 20-23 years (n=4)**	**P<[1]**
CTX-II (pg/mL)	71.4±8.6^{A}	23.9±6.1^{B,C}	49.5±6.7^{B,c}	41.2±8.6^{B,c}	0.0002
PIIANP (ng/mL)	483±136^{A}	649±96^{A}	647±105^{A}	1625±118^{B}	0.0001
NO (µM)	10.3±4.5	12.4±3.2	12.4±3.5	15.7±3.9	NS

[1]Significance (P<0.05)

NS= Not Significant

Upper-case letters indicate significant differences between means (P<0.05)

Lower-case letters indicate a means that approach significance as compared to means with the corresponding upper-case letter (P<0.10)

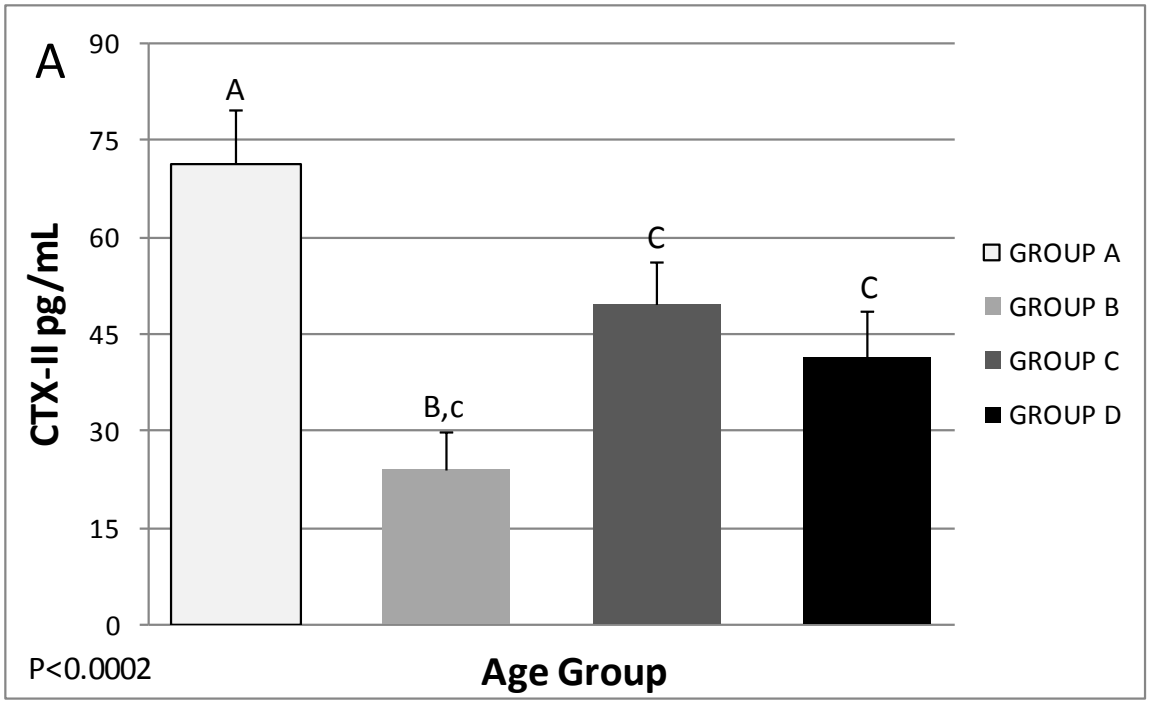

Figure 2.5(A) Effect of Age on CTX-II Concentration in an Equine Population- Mean ± SE CTX-II concentration for group A (71.4±8.6), group B (23.9±6.1), group C (49.5±6.7), and group D (41.2±8.6) where group A consists of 5-6 year-olds (n=4), group B of 7-13 year-olds (n=14), group C of 14-19 year-olds (n=14), and group D of 20-23 year-olds (n=4). Capital letters indicate a difference in means ($P<0.05$). Lower-case letters indicate a difference in means approaching significance ($P<0.10$) compared to upper-case letters.

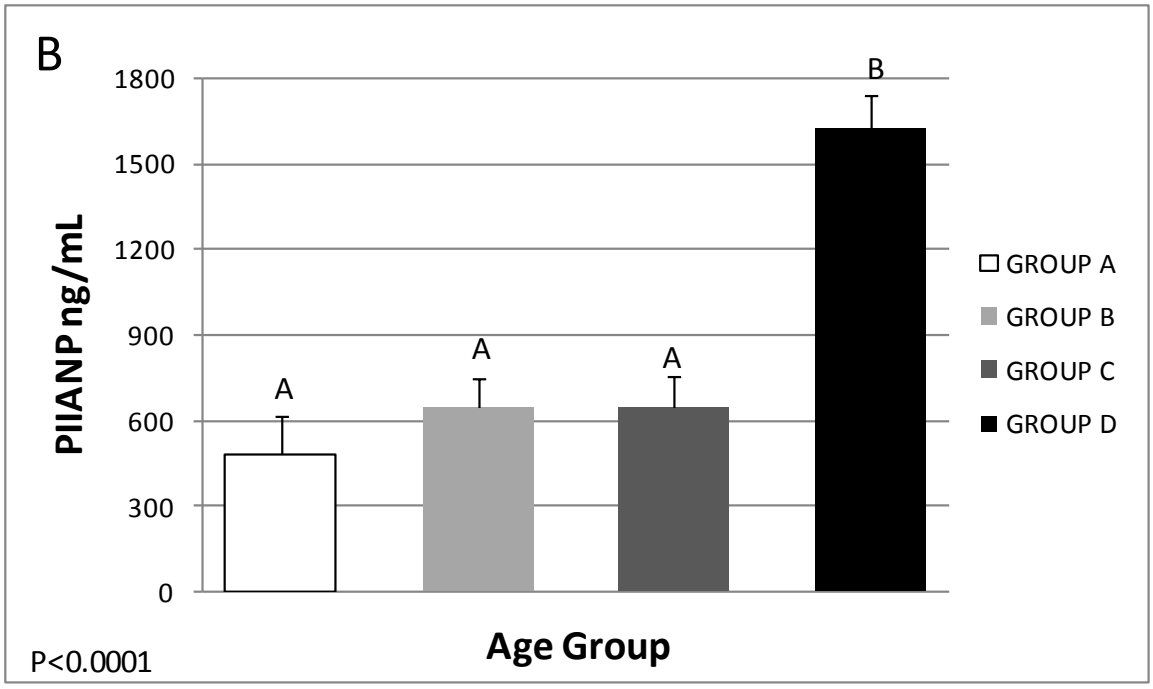

Figure 2.5(B) Effect of Age on PIIANP Concentration in an Equine Population- Mean ± SE PIIANP concentration for group A (482±135), group B (648±96), group C (646±5), and group D (1625±117) where group A consists of 5-6 year-olds (n=4), group B of 7-13 year-olds (n=14), group C or 14-19 year-olds (n=14), and group D of 20-23 year-olds (n=4). Capital letters indicate a difference in means (P<0.05).

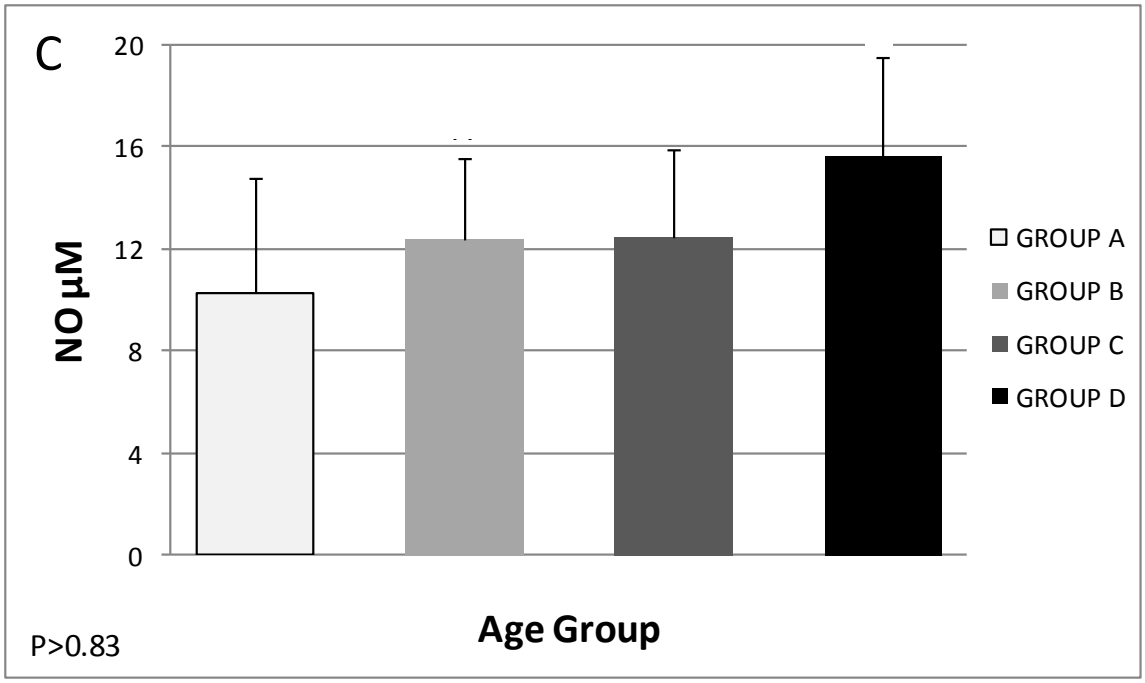

Figure 2.5(C) Effect of Age on NO Concentration in an Equine Population- Mean ± SE NO concentration for group A (10.3±4.5), group B (12.4±3.2), group C (12.4±3.5), and group D (15.7±3.9) where group A consists of 5-6 year-olds (n=4), group B of 7-13 year-olds (n=14), group C or 14-19 year-olds (n=14), and group D of 20-23 year-olds (n=4). Capital letters indicate a difference in means ($P<0.05$).

Table 2.7- Effect of Gender on Biomarker Concentration (n=36). Mean ± SE biomarker concentrations for mares and geldings.

Biomarker	Mares (n=11)	Geldings (n=25)	P<[1]
CTX-II (pg/mL)	73.7±6.4[A]	30.9±3.9[B]	0.0001
PIIANP (ng/mL)	743±129	873±80	NS
NO (µM)	14.7±2.1	7.8±3.4	NS

[1]Significance (P<0.05)

NS= Not Significant

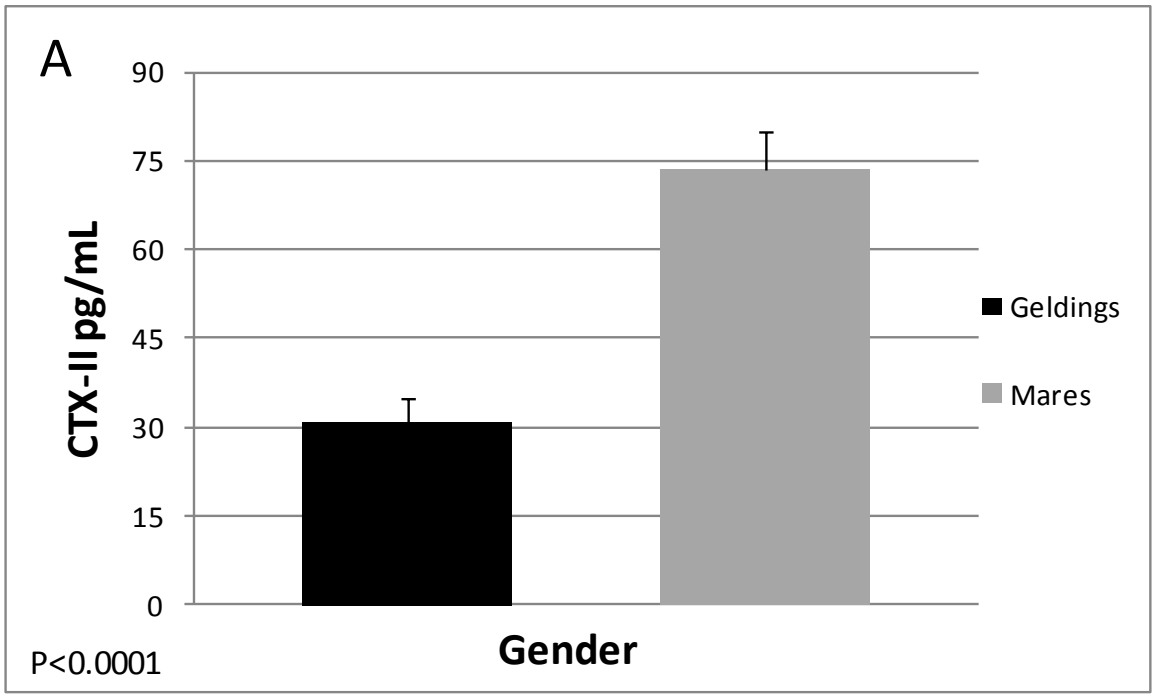

Figure 2.6(A) Effect of Gender on CTX-II Concentration in an Equine Population- Mean ± SE CTX-II concentration for mares (73.7±6.4; n=11) and geldings (30.9±3.9; n=26).

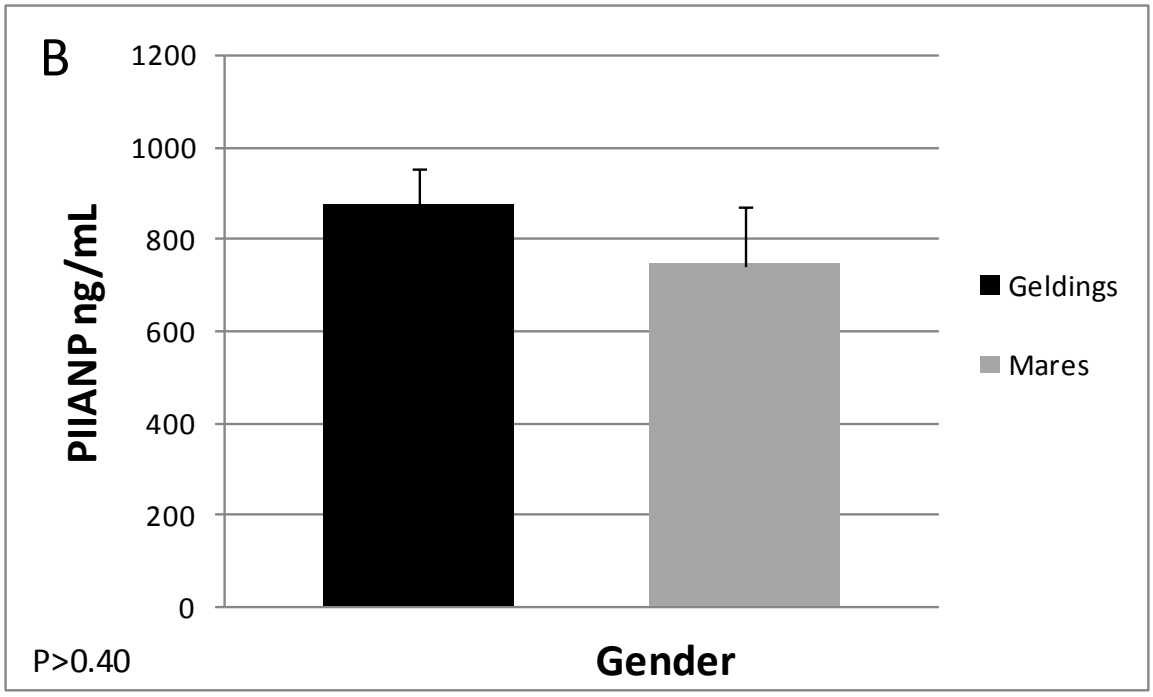

Figure 2.6(B) Effect of Gender on PIIANP Concentration in an Equine Population- Mean ± SE PIIANP concentration for mares (743.5±129.9; n=11) and geldings (873.6±80.6; n=26).

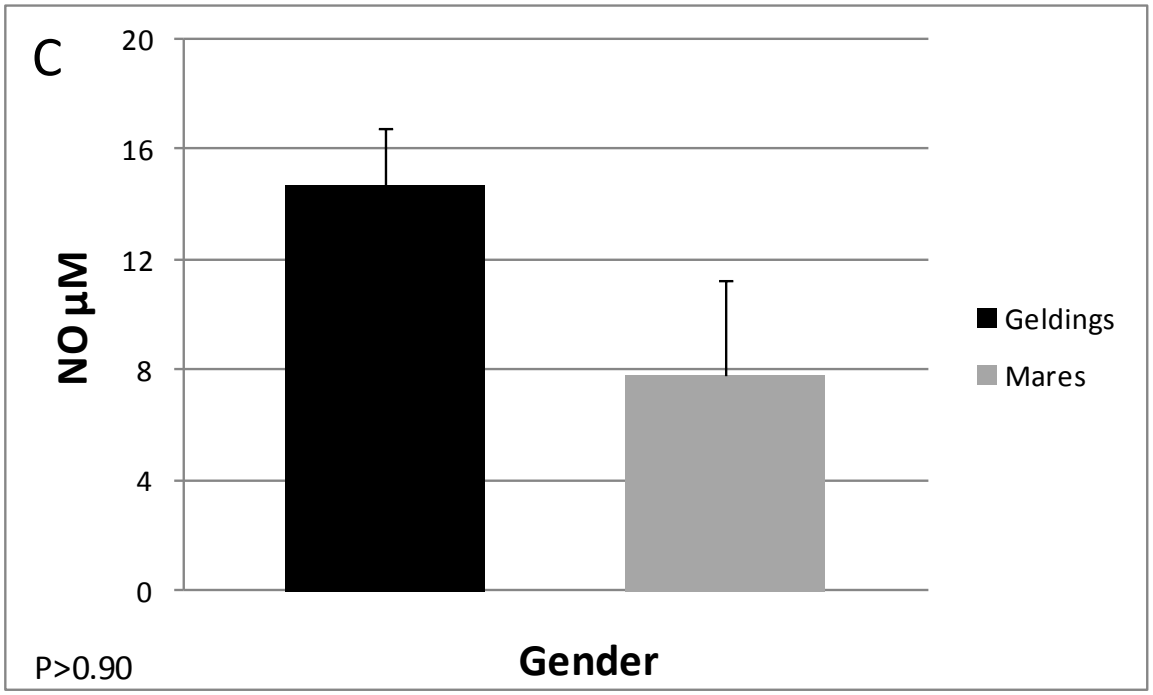

Figure 2.6(C) Effect of Gender on NO Concentration in an Equine Population- Mean ± SE NO concentration for geldings (14.7±2.1; n=26) and mares (7.8±3.4; n=11).

CHAPTER III

EVALUATION OF THE NUTRITIONAL SUPPLEMENT STEADFAST® EQUINE FOR TREATMENT OF HORSES WITH NATURALLY ACQUIRED LAMENESS

Abstract

The objective of this trial was to evaluate the efficacy of Steadfast® Equine for use in horses with naturally acquired lameness. Thirty-three horses of various breeds, ranging in age from 4 to 23 years-old were utilized. Horses were blocked into two groups that were equivalent for mean age, initial lameness score, and initial affected area score and given either Steadfast® Equine or a placebo (alfalfa-molasses base-product). Lameness was evaluated on days 0, 28, 56, 84 and 112 using a wireless accelerometer-gyroscope system. Based on lameness data, horses were given a score of 0 to 3 at each time point (0=sound, 1=mild lameness, 2=moderate lameness, 3=marked lameness). Based on lameness data, horses were also assigned a primary affected area score from 0 to 3 for each lameness evaluation (0=none, 1=forelimb, 2=hind limb, 3=mixed fore- and hind limb). Blood was collected on days 0, 7, 14, 28, 42, 56, 70, 84, 91, 98 and 112. Serum from collection days 0, 28 and 84 was analyzed for concentrations of type II collagen biomarkers CTX-II and PIIANP. There was no effect of supplementation with Steadfast® Equine on lameness score (P=0.64), affected area (P=0.86), CTX-II concentration (P=0.58) or PIIANP concentration (P=0.71) compared to the placebo

group. There was an effect of time on lameness score (P=0.004), PIIANP concentration (P=0.003) and an increase in CTX-II concentration between day 0 and day 84 (P<0.05). indicating a possible confounding effect of season and/or exercise load. No effects of Steadfast® Equine on equine lameness were observed under the conditions of this trial.

Abbreviations

A-G	Accelerometer-Gyroscope
CTX-II	C-Terminal Telopeptide of Type II Collagen
DJD	Degenerative Joint Disease
GAG	Glycosaminoglycan
IGF	Insulin-Like Growth Factor
NEM®	Natural Egg Membrane
NSAID	Non-Steroidal Anti-Inflammatory Drug
PIIANP	N-Terminal Procollagen Propeptide of Type II Collagen
Prostaglandin	PG

Introduction

Lameness is one of the most common and debilitating issues in equine medicine as it results in decreased work ability and early retirement for horses, and significant economic loss for horse owners.[21,62,129] In horses, 60% of lameness cases are associated with some degree of degenerative joint disease (**DJD)**.[129] Conventional treatments for DJD in horses are largely palliative, including non-steroidal anti-inflammatory drugs (**NSAID**'s) and intra-articular injections with corticosteroids or polysulfated-glycosaminoglycans (**GAG's**).[103,130] However, DJD is a chronic disorder, necessitating long-term treatment and there has been evidence that conventional therapies may have deleterious effects with persistent usage. Long-term use of NSAIDS may increase the risk of ulcers,[14,131,132] and both NSAIDS and corticosteroids have been shown to alter the metabolism of articular cartilage.[14,133,134]

Possibly due to the current limitations of conventional therapies, DJD is the most common human and veterinary medical condition for which people seek alternative therapies.[135] Nutraceuticals, nutritional supplements intended to alter disease progression, comprise a newer class of therapies that are widely used, but, whose efficacies in the treatment of DJD are largely untested.[130] The claimed effects are often based in anecdotal evidence or clinical trials not subject to peer review.[136] Currently there are more than 60 name-brand equine nutraceuticals for the treatment of joint pathology, resulting in a $20 to $50 million industry.[137]

Steadfast® Equine (Novus International, Inc.; St. Charles, Mo, USA) is a comprehensive nutraceutical designed to maintain the health and growth of joints,

hooves, tendons and ligaments as well as support immune response in mature horses. It is composed of biotin, methionine, Natural Eggshell Membrane **(NEM®**, Carthage, Mo, USA), calcium, phosphate, ascorbic acid, vitamin D, and a micronutrient mix, TelaFIRM®, containing organic chelated zinc, copper, manganese and selenium.[75] Eggshell membrane, the only ingredient in NEM®, is composed of fibrous proteins, including type I collagen (<25% dry weight), glucosamine sulfate (<1% dry-weight), chondroitin sulfate (<2% dry weight) and hyaluronic acid (<2% dry weight).[74] Only the percent composition of the "active ingredients" was given in the label; the nutrient composition of the other 70% was not specified.

In the field of joint disease, nutraceuticals containing glucosamine and/or chondroitin sulfate have received particular attention due to *in vitro* evidence that these compounds have caused up-regulation of collagen and GAG synthesis and inhibition of inflammatory mediators.[82] Glucosamine is commercially available in three forms: glucosamine hydrochloride, glucosamine sulfate and N-acetyl-D-glucosamine. Of these three, glucosamine hydrochloride and glucosamine sulfate are more efficacious than N-acetyl-D-glucosamine at inhibiting cartilage degradation *in vitro*.[85,86,138] Serum and synovial fluid sulfate concentrations increased in *in vivo* studies after oral administration of glucosamine sulfate.[139] On the other hand, large oral doses of glucosamine alone have failed to increase serum glucosamine concentrations *in vivo*.[140] Therefore, it has been proposed that sulfate may be a necessary component for the incorporation of glucosamine in to cartilage.[139] Glucosamine sulfate, the form of glucosamine found in NEM®, is only half as bioavailable as glucosamine hydrochloride; thus, in theory, would require twice the amount to achieve the same effects.[89] It has been shown that cartilage chondroitin

sulfate concentration is higher than plasma concentration for an extended period after administration, suggesting that chondroitin sulfate has a tropism for cartilage.[141] Similar to glucosamine, chondroitin sulfate appears to mediate chondroproductive and chondroprotective effects by decreasing inflammatory mediators, such as nitric oxide and prostaglandin-E2, and increasing production of GAG's.[84,142] The realization of the effects of glucosamine and/or chondroitin sulfate *in vivo*, remains contentious.[83,92,93,143] One problem is that concentrations of glucosamine and chondroitin are often much higher in trials using cartilage explants than what joint tissues are likely to be exposed to *in vivo*.[68] trials evaluating the effects of orally administered hyaluronic acid on equine joint tissues have also rendered mixed results and merit further research.[143,144] One important consideration is that the high doses of both glucosamine and chondroitin sulfate are associated with a good safety profile with long term use.[145,146]

Other ingredients in Steadfast® Equine also have the potential to slow the onset and progression of equine DJD. Biotin and methionine are necessary for the healthy growth of keratinized tissues and supplementation may decrease lameness in cattle and swine.[78,81] Biotin is regularly used as a supplement for horses to improve hoof-health.[79]

In addition to being required for normal collagen and aggrecan synthesis, ascorbic acid acts as an antioxidant, scavenging free radical and reducing oxidative stress.[13,94,95] Vitamin D increases the synthesis of proteoglycans by chondrocytes in cartilage explants.[98] Manganese is a cofactor in the biosynthesis of GAG's[147] and zinc may have a protective role in preserving bone mass and inhibiting bone resorption.[100,101] In addition, selenium, zinc, copper and manganese all have the potential to act as

antioxidants.[147] The nutrient components of Steadfast® Equine indicate that it may have potential as a nutraceutical for use in equine lameness, particularly for the restoration of the extra cellular matrix by means of increasing production of matrix components and reducing inflammatory signals which up-regulate catabolic pathways. However, at the time of this study there was no objective data to confirm this. To assess lameness, the wireless accelerometer-gyroscope (**A-G**) system (Equinosis™, Columbia, MO, USA) was utilized. Additionally serum concentrations of the type-II collagen biomarkers, the C-terminus telopeptide of type II collagen (**CTX-II**) and the N-propeptide of type II A procollagen (**PIIANP**), were used as indicators of cartilage metabolism in the joint.[47] The purpose of this trial was to assess Steadfast® Equine as a therapeutic for use in equine lameness.

Materials and Methods

Horses-Following approval of the University of Missouri Animal Care and Use Committee, thirty-eight horses from Stephen College Equestrian Program (Columbia, Mo, USA) were utilized for this trial. Horses ranged in age from 4- to 23- years old and were of various breeds [American Saddlebred (n=15), Quarter Horse (n=10). Thoroughbred (n=5), American Paint (n=1), Arabian (n=1), Miniature American Hackney(1), and National Show Horse (n=1)] and mixed gender [geldings (n=23) and mares (n=10)]. Horses were stabled at the Stephen's College Equestrian Stables and exercised daily in accordance with their riding program. Although exercise regime varied over the duration of the trial, it varied consistently between all horses. The most defined

changes in daily exercise load occurred over winter break (December 18, 2009 through January 14, 2009) and the spring break (March 21, 2009 through March 29, 2009). During these periods horses were subjected to a lower level of daily exercise (turn-out and lunging only) compared to the daily-riding schedule when the program was in session. Horses were fed individually designed diets consisting of grass and/or alfalfa hay, and supplemented with grains or pellets. Horses had ad libitum access to water.

Steadfast® Equine- Horses were assigned to groups such that pretrial lameness score, affected area and age were balanced between the two groups. Horses were appointed either to a treatment group (Steadfast® Equine supplementation, n=18) or a placebo group (alfalfa-molasses base-product, n=18). Both the treatment and placebo supplements were prepackaged in individual 25 gram packets. Individual packets ensured consistent dosing and helped protect the product from damage to moisture or oxidation. This is the same packaging used for the commercially available product. Beginning on day 0 each horse was given one packet of supplement (Steadfast® Equine or placebo) twice daily, during morning and evening feeding, by means of top-coating grains or pellets. Supplement administration for both groups was stopped on day 84. All packets were color-coded and researchers were blind to treatment.

Exclusion Criteria- Fourteen-days before the commencement of supplement-administration, horses ceased taking all other nutraceuticals, pharmaceuticals and/or other nutritional supplements including NSAIDS, corticosteroids, and oral or intraacticular GAG's. If horses required pharmaceuticals within the duration of the trial, an equine veterinarian assessed whether the timing and duration of treatment merited removal from

the trial. All removal assessments were made by the same veterinarian. Horse 30, was removed from the trial due to long-term administration of NSAIDs following a non-study related leg injury. In addition, horses 1 and 20 were removed due to retirement from the Stephen's Equestrian Program. Horse 41, the miniature American hackney, was removed due to differences in supplement dosing by weight and trotting pattern. Finally, horse 44 was removed because his temperament and aversion to the equipment for lameness evaluation made it too difficult to collect reliable lameness data. This resulted in n=16 horses in the Steadfast group and n=17 horses in the placebo group.

Serum Collection and Processing- Whole blood samples (20 mL) were collected on days 0, 7, 14, 28, 42, 56, 70, 84, 91, 98, and 112. Only samples from days 0, 28 and 84 were assayed for serum concentrations of biomarkers. **(Figure 3.1)** Samples were collected via jugular veinipuncture using Vacutainer tubes (BD Vacutainer®, Franklin Lakes, NJ, USA) and 20-gauge needles. Horses were bled between 0700 and 0900 hours and samples were stored at 2-8˚C for no longer than five-hours before being centrifuged at 3000 rpm for 20 minutes (rotating-radius of 19.2 cm). Serum was harvested and stored at -80˚C until analyzed in duplicate for biomarker concentrations. Serum was stored for no longer than 140 days before being analyzed for CTX-II and PIIANP concentrations. Commercially available ELISA kits were used to measure both CTX-II (Serum Pre-Clinical CrossLaps® ELISA, Immunodiagnostic Systems Inc., Fountain Hills, AZ, USA) and PIIANP (Human PIIANP® ELISA, Millipore, Billerica, MA, USA). CTX-II was measured in picograms per milliliter (pg/mL) and PIIANP in nanograms per milliliter (ng/mL). CTX-II samples below the detection limit of the standard curve were entered for statistical analysis as an average between the lowest point on the standard curve, 5.0

pg/mL and 0, in this case 2.5 pg/mL. Intra- and inter-assay coefficients of variance (CV) were 7.75% and 5.07% for CTX-II and 5.90% and 13.15% for PIIANP, respectively.

Wireless Accelerometer-Gyroscope Lameness Evaluation- Horses were evaluated for lameness using the wireless A-G system (Equinosis™, Columbia, Mo, USA). Lameness evaluations were conducted on days 0, 28, 56, 84 and 112. Lameness evaluations from all time points were considered in evaluation of the effects of treatment. **(Figure 3.1)** After an accelerometer had been fastened to the right, front fetlock and gyroscopes placed at the poll and on the mid-line of the croup, horses were trotted on a compacted earth surface in an in-door arena for approximately 100 meters. All evaluations were performed in duplicate. The affected area was also scored from the A-G system data along with the assessment of the equine veterinarian running the evaluations to discern between primary and compensatory affected areas. The same veterinarian ran all evaluations and assigned lameness and affected area scores. Each horse was given a score from 0 to 3 (0=no lameness, 1=forelimb, 2=hind limb lameness, 3=mixed fore- and hind limb lameness). **(Table 2.1)**

Statistical Analysis- Statistical analysis was conducted on SAS (v.9.1, SAS Institute Inc., Cary, NC, USA). Horse was the experimental unit. Population means and ranges were determined for serum concentrations of CTX-II and PIIANP. The individual repeatability for each biomarker was determined using horse as the class-statement variable and CTX-II and PIIANP concentrations as the dependent variables. The effects of age and/or gender were assessed using the GLM (general linear model) procedure, with age and gender as main effects and biomarker concentration as the dependent

variable. Horses were blocked by age into four groups corresponding to the 0-10th percentile (group A; 4-5 year-olds; n=7), the 10th-50th percentile (group B; 6-10 year-olds; n=10), the 50th-90th percentile (group C; 11-18 year-olds; n=12), and the 90th-100th percentile (group D;19-23 year-olds; n=4).

The effects of treatment and treatment over time on lameness score, affected area and biomarker concentrations were evaluated using the Mixed procedure. The statistical model included treatment, time and treatment by time as main effects and lameness score, affected area, CTX-II and PIIANP as dependent variables. The Mixed procedure was also used to determine if an effect existed of lameness score and/or affected area over time on serum concentrations of CTX-II and PIIANP. The least squares mean (LSMEANS) function was used to calculate means, standard error and differences between groups. All means are reported as the mean ± standard error. For analyses where lameness score was a class variable, a *post hoc* test of orthogonal contrasts was run to compare sound versus lame horses (0 v. 1, 2, 3), mildly-lame versus markedly-lame horses (1 v. 3), and moderately-lame horses versus markedly-lame horses (2 v. 3). Orthogonal contrasts for affected area were sound versus lame horses (0 v. 1, 2, 3), forelimb lameness versus hind limb lameness (1 v. 2), and single versus mixed area lameness (1, 2 v. 3). Significance was defined as $P<0.05$ and approaching significance as $P<0.10$.

Results

Population Profile- The mean concentration for the population as a whole across days 0, 28, and 84 was 25.2±2.13 pg/mL for CTX-II and 1417±136 ng/mL for PIIANP. The range for CTX-II was from 2.5 to 144.5 pg/mL and for PIIANP from 151 to 6849

ng/mL. Individual repeatability across these same time points was 25.2% for CTX-II and 63.2% for PIIANP. **(Table 3.1)** Over all five evaluations, lameness score had an individual repeatability of 35.8% and affected area of 20.7%.

There was no effect (P>0.43) of gender on either CTX-II concentration or PIIANP concentration. Mares had a mean CTX-II concentration of 24.0±3.9 pg/mL and gelding's 27.6±2.6 pg/mL. PIIANP concentration was 1581±247 ng/mL in mares and 1346±163 ng/mL in geldings. **(Table 3.2; Figure 3.2)** The effect of age on CTX-II concentration approached significance (P<0.09). CTX-II was numerically higher in group A (33.7±4.5pg/mL) than in group B (22.0±3.5 pg/mL) and group C (23.8±3.8 pg/mL). There was no difference between group A and group D (34.4±6.0 pg/mL; P>0.92) and no difference between group B and group C (P>0.73). PIIANP concentration was also affected by age (P<0.0001). Group D had a higher PIIANP concentration (3516±318 ng/mL) than groups A (904±241 ng/mL; P<0.0001), B (1011±201 ng/mL; P<0.0001) and C (1355±184 ng/mL; P<0.0001). There were no other differences in PIIANP concentration between age groups. **(Table 3.3; Figure 3.3)**

Effect of Steadfast® Equine on Lameness Score, Affected Area and Biomarker Concentrations- There was no effect of treatment (P>0.6) or treatment over time (P>0.98) on lameness score. Similarly, there was no effect of treatment (P>0.9) or treatment over time (P>0.4)) on affected area. **(Table 3.5; Figure 3.5)** There was no effect of treatment (P>0.6) or treatment over time (P>0.4) on CTX-II concentration, **(Table 3.6; Figure 3.6)** and no effect of treatment (P>0.7) or treatment over time (P>0.3) on PIIANP concentration. **(Table 3.7; Figure 3.7)**

Effect of Time on Lameness Score, Affected Area and Biomarker Concentration- Time had an effect on lameness score ($P<0.004$). The average lameness score for each trial date was 1.6 at day 0, 1.5 at day 28, 1.8 at day 56, 1.7 at day 84 and 2.2 at day 112 (SE=0.2). Day 112 had a higher mean lameness score than day 0 ($P<0.003$), day 28 ($P<0.0003$), and day 84 (P.0.02) and tended to be higher than day 56 ($P<0.09$). **(Table 3.4; Figure 3.4)** Both treatment groups follow a similar pattern for lameness score [6]over time. **(Figure 3.4)** There was no effect of time on affected area ($P>0.9$) or CTX-II concentration ($P>0.1$). **(Table 3.5; Figure 3.5 and Table 3.6; Figure 3.6)** However, the population concentration of CTX-II was numerically higher at day 84 (30.1±4.1 pg/mL) than at day 0 (23.9±4.5 pg/mL). There was an effect of time on PIIANP concentration ($P<0.003$) with a concentration at day 84 (1857±250 ng/mL) that was higher than at day 0 (946±269 ng/mL; $P<0.0006$), and tended to be higher than day 28 (1471±269; $P<0.10$). **(Table 3.7; Figure 3.7)**

Effect of Lameness Score and Affected Area on Biomarker Concentration- There was an effect of lameness score on CTX-II concentration ($P<0.05$), with a lower CTX-II concentration in sound horses (16.3± 6.5 pg/mL) and markedly-lame horses (18.4±4.8 pg/mL) than in mildly-lame horses (29.8±4.0; $P<0.05$) and moderately-lame horses (33.0±5.3; $P<0.04$). **(Table 3.8; Figure 3.8)** There was no effect of lameness score on PIIANP concentration ($P>0.3$). **(Table 3.9; Figure 3.9**) Affected area did not have an effect on either CTX-II concentration ($P>0.42$) or PIIANP concentration ($P>0.7$).

Discussion

In horses with naturally acquired lameness, Equine® Steadfast administered over 84 days, failed to produce quantifiable effects on lameness score, affected area, or concentrations of type-II collagen biomarkers CTX-II and PIIANP. The relatively low repeatability of lameness score within an individual horse compared to trial 1 and the increase in both CTX-II and PIIANP concentrations over time, in spite of a lack of treatment effects, suggests confounding effects from variables such as exercise load and season.

Among the horses in this trial, supplementation with Steadfast® Equine did not decrease mean lameness scores compared to the placebo group. There was a significant increase in lameness scores from day 84 to day 112, twenty-eight days after stopping supplement administration; however, this same pattern was observed in both groups. Across all evaluation points the changes in mean lameness scores of the Steadfast® Equine supplemented group parallels the placebo group, without significant difference between treatments at any time point. Furthermore, mean lameness score fluctuates, rather than increasing or decreasing steadily for either group. These two patterns may imply that differential exercise loads are affecting lameness evaluation. It should be noted that although exercise load changed over the course of the study based on the demands of the equestrian program in which the horses were involved, exercise load and patterns of change were not different between treatment groups. A study conducted on horses without DJD found that prolonged immobilization induced lameness, that was somewhat ameliorated by excersise.[148] On the other hand, the systemic concentration of pro-inflammatory signaling molecules increases following excersise,[149] and periods of high-intensity lead to muscle soreness due to the build-up of lactic acid.[150] Thus, there is

evidence that both periods of low exercise load and periods of high exercise load can increase lameness. Exercise logs were kept for all horses throughout the duration of this trial; however, there was not a consistent pattern between exercise load and the fluctuations in lameness scores. Lameness scores decreased from day 0 to day 28, a period that began with a normal riding schedule and went into the winter break. A contradictory pattern was seen with an increase in lameness scores after the spring break. **(Figure 3.4)** The complex pattern between exercise load and lameness may account for the lack of convergence between exercise load and lameness scores. In a sheep trial, looking at the effects of forced exercise on lameness, it was postulated that changes in exercise regime had a greater effect on lameness than maintaining either a consistently high or low exercise load.[151]

There was also no difference between treatment groups in CTX-II or PIIANP serum concentrations. When accounting for bioavailability, concentrations of glucosamine sulfate and chondroitin sulfate are much lower in Steadfast® Equine (and NEM®) than the concentration used in *in vitro* studies that have found an effect of these compounds on cartilage matrix synthesis.[74,84,89] Furthermore, the product label does not provide minimum percent composition of collagen type I, glucosamine sulfate, chondroitin sulfate and hyaluronic acid, so concentrations may be even lower than the claimed percentage dry weight amounts. Steadfast® Equine may contain insufficient concentrations of glucosamine sulfate and/or chondroitin sulfate to induce a difference in cartilage metabolism. On the other hand, glucosamine sulfate and chondroitin sulfate, in some studies, have shown little direct effect on type II collagen synthesis.[152-154] In this case, effects of Steadfast® Equine (and NEM®) may not have been reflected in the

measurement of type II collagen biomarkers and measuring GAG synthesis of synovial fluid concentrations or PG-E2, may provide improved information.

Despite the lack of treatment effects, there was a significant increase in PIIANP concentration over time and an increase from day 0 and day 84 in CTX-II concentration. A study comparing CTX-II synovial fluid and serum concentrations of rested horses, exercised horses (same horses after four to seven months of race-training), and horses with osteochondral fragments, found there was no difference between the serum CTX-II concentrations of rested and exercised horse, but, that both had lower concentrations than horses with osteochondral fragments.[110] These results would suggest that differential exercise loading is not the cause of changing CTX-II concentration. Rather, increased PIIANP and CTX-II concentrations could be a reflection of seasonally-induced increased cartilage metabolism, although seasonal changes in cartilage metabolism have not yet been characterized. In horses, a seasonal effect in insulin like growth factor-1 IGF-1, has been established with increasing concentrations through the spring and summer months.[155] IGF-1 increases type II collagen synthesis in human articular cartilage, [156] and was found to increase collagen content in equine cartilage explants.[157] Seasonal increases in the concentration of IGF-1 could account for the up-regulation of cartilage metabolism seen in this trial, as evidenced by increased concentrations of both CTX-II and PIIANP from December 5th (day 0) to February 27th (day 84). In order to provide evidence for a seasonal effect more sample dates would need to be assayed.

In contrast to trial 1 (Chapter 2), there was no difference due to gender in the concentration of CTX-II. Concentrations of CTX-II have been linked sex-steroids which

have a chondroprotective effect.[127,128] Mares would not have been in estrus through-out the majority of this trial; in contrast the majority would have been cycling during trial 1. Although, mean estrus concentrations would likely have been lower, there would have been less fluctuation. The effects of sex steroids on type II collagen biomarkers is a very new area of research and there is not yet data to support a conjecture as to whether absolute concentrations or fluctuations in sex steroid concentrations have a greater effect on CTX-II concentration. Age showed a pattern of increased PIIANP in older horses in both this trial and trial 1. There was also a parallel between the two trials concerning the effects of age on CTX-II concentration, with the oldest and youngest age groups having the highest concentration of CTX-II. In trial 1 there was no difference between groups C and D, whereas, in trial 2, there was a significant increase in CTX-II concentration in group D compared to group C. The age blocks were based on percentiles of the population and, thus, divided-up differently between the trials. In general, trial 2, tended to have younger age cut-offs, which could account for the different patterns. Despite these differences, the important similarities across trials are that both the lowest 10^{th} percentile and the upper 90^{th} percentile had increased CTX-II concentrations and the upper 90^{th} percentile had an increased PIIANP concentration. High CTX-II in the youngest age group for both studies may be a reflection of the increased metabolism in growing horses. [121] Increased CTX-II in the highest age group across both studies is in convergence with other studies that have found a similar pattern in both horses and humans.[64,124] Both increased age and high PIIANP concentrations are risk factors for the development of DJD,[29,53] which may explain the correlation between the two seen in both trials.

In this trial there was an effect of lameness score on CTX-II concentration, however, the pattern differed from that seen in trial 1. Sound horses and markedly lame horses had the lowest concentrations of CTX-II, with increased concentration in mildly- and moderately-lame horses. It is postulated that low CTX-II values may be due to decreased catabolism in sound horses and decreased remaining articular cartilage in markedly lame horses. The important similarity across both trials is that CTX-II is decreased in markedly-lame horses. In conclusion, supplementation with Equine® Steadfast over an 84 day period did ameliorate equine lameness, as assessed using a wireless A-G system and/or alter type II collagen biomarker concentrations, over an 84 day period, as compared to supplementation with a placebo. However, exercise load and seasonal effect may have been significant confounding effects. Additionally, type II collagen biomarkers may not be the most appropriate choice to measure the effects of glucosamine sulfate and chondroitin sulfate. Data from this study provided evidence of a possible, although complex, relationship between exercise and lameness, a seasonal effect on type II collagen metabolism, and a correlation between lameness and CTX-II concentration.

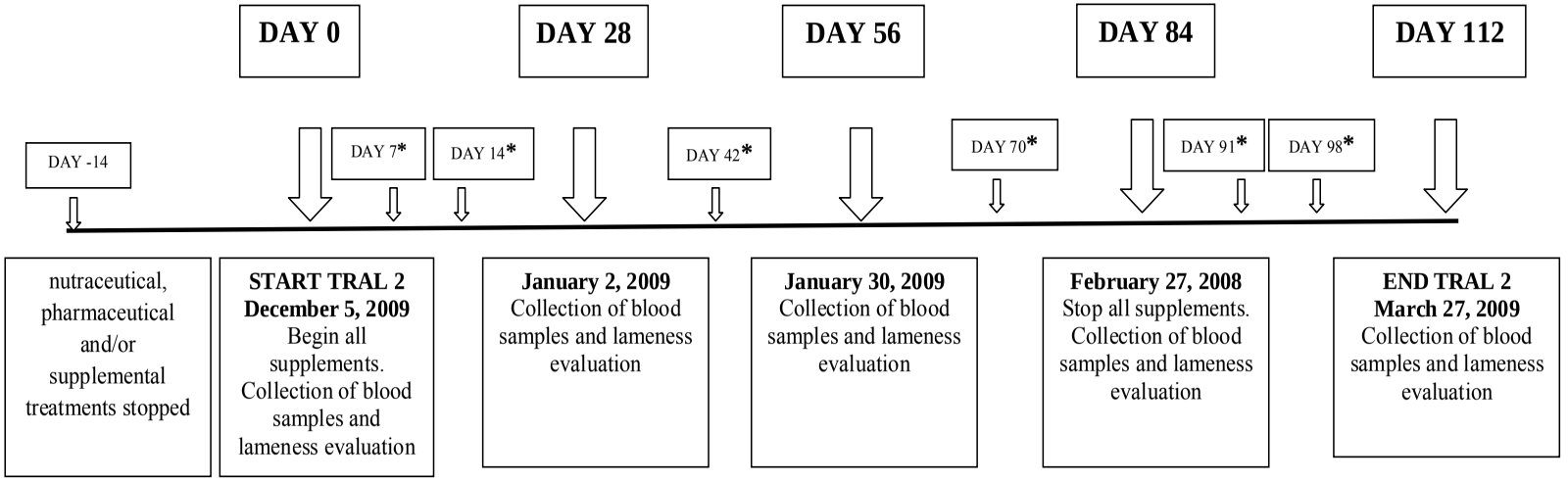

Figure 3.1- Time-Line for Trial 2

*Additionally blood was collected on days 7, 14, 42, 70, 91 and 98.

** Only samples from days 0, 28 and 84 were assayed for CTX-II, PIIANP and NO concentrations.

Table 3.1 Population Mean ± Standard Error, Range and Individual Repeatability of Serum Biomarkers in an Equine Population

Biomarker	Mean ± SE (n=33)	Range (n=33)	Individual Repeatability (n=33 horses x 3 samples=99)
CTX-II (pg/mL)	25.2±2.1	2.5-144.5	25.2%
PIIANP (ng/mL)	1417±136	151-6849	63.2%

Table 3.2 Effect of Gender on Biomarker Concentration in an Equine Population (n=33)- Mean ± SE biomarker concentrations for mares and geldings.

Biomarker	Mares (n=10)	Geldings (n=23)	P
CTX-II (pg/mL)	24.0±3.9	27.6±2.6	NS
PIIANP (ng/mL)	158±247	1346±163	NS

Significance (P<0.05)

NS=Not Significant

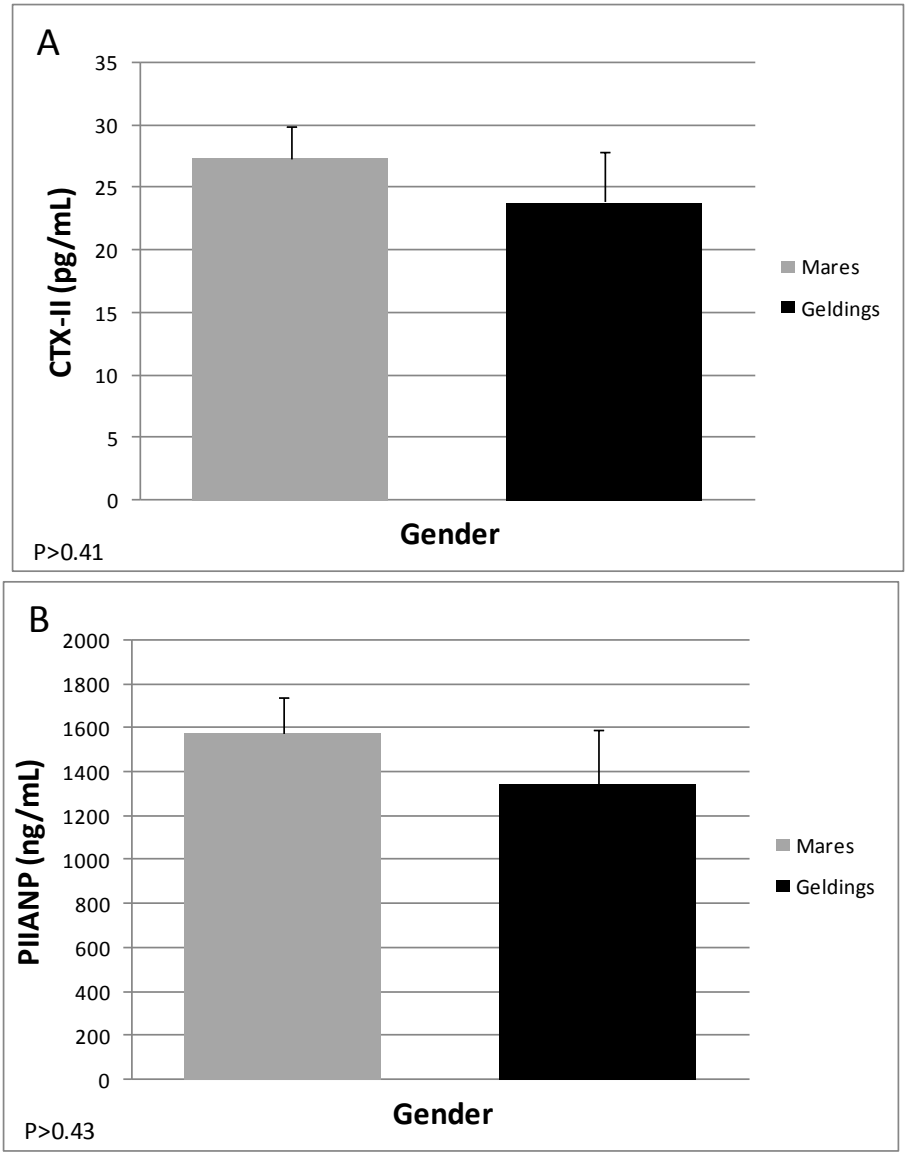

Figure 3.2 Effect of Gender on Biomarker Concentration in an Equine Population- Mean ± SE for CTX-II (A) and PIIANP (B).

Table 3.3 Effect of Age on Biomarker Concentration in an Equine Population (n=33)-Mean ± SE biomarker concentrations for age groups. The 0-10th percentile was designated group A, the 10th-50th percentile group B, the 50th to 90th percentile group C and the 90th to 100th percentile group D.

	Age				
Biomarker	**Group A 4-5 years (n=7)**	**Group B 6-10 years (n=10)**	**Group C 11-18 years (n=12)**	**Group D 19-23 years (n=4)**	**P**
CTX-II (pg/mL)	33.7±4.54	23.8±3.8	22.0±3.5	34.4±6.0	0.09
PIIANP (ng/mL)	904±241^{A}	1011±201.14^{A}	1355±184^{A}	3516±318^{B}	0.0001

Significance (P<0.05)

NS=Not Significant

All superscripts apply across rows. Upper-case letters indicate significant differences between means.

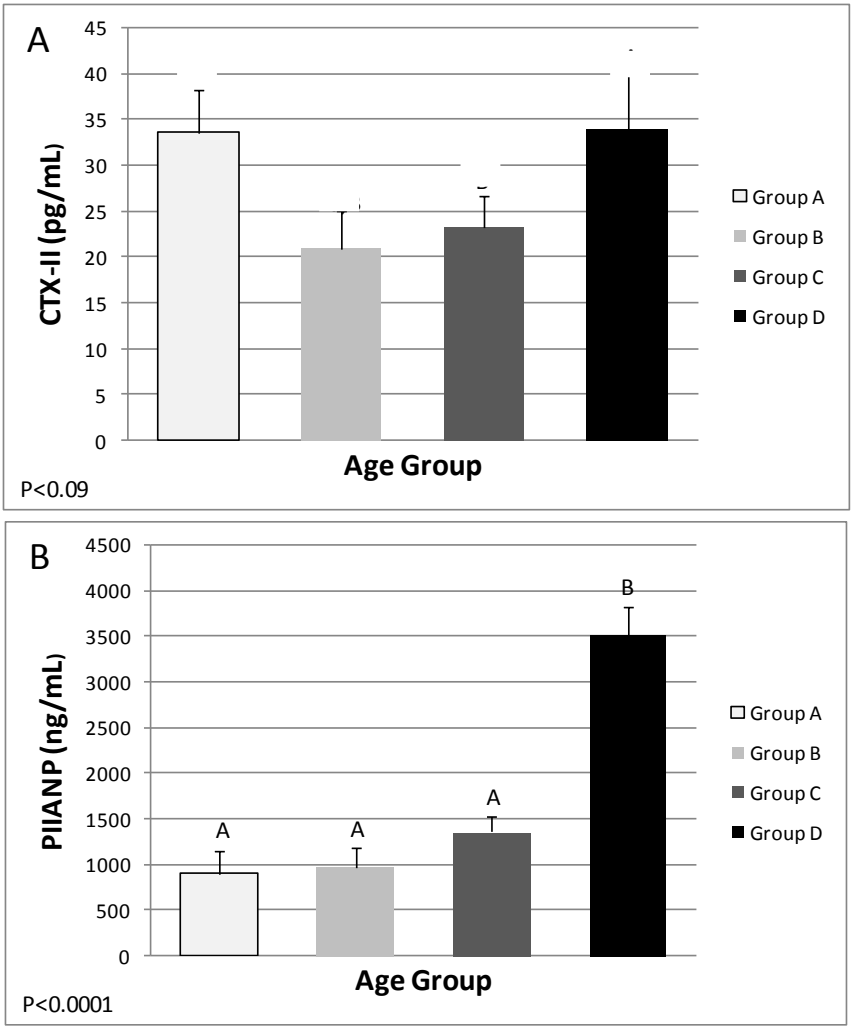

Figure 3.3 Effect of Age on Biomarker Concentration in an Equine Population- Mean ± SE for CTX-II (A) and PIIANP (B). Only samples from day 0. 24 and 84 were assayed. CTX-II concentration was 33.7±4.5 for group A (n=7), 23.8±3.8 for group B (n=10), 22.0±3.5 for group C (n=12), and 34.4±6.00 for group D (n=4). PIIANP concentration was 904±241 for group A, 1011±201 for group B, 1355±184 for group C and 3316±318 for group D. Lower-case letters indicate a difference in means approaching significance (P<0.10) compared to upper-case letters.

Table 3.4 Effect of Treatment on Lameness Score over Time in an Equine Population (n= 33 horses x 5 evaluation dates=164)-Mean lameness score for Steadfast and placebo treatment groups, and for the population as a whole (All) on days 0, 28, and 84.

Treatment	Day 0	Day 28	Day 56	Day 84	Day 112	Standard Error	P[1]	P[2]	P[3]
Steadfast (n=16)	1.5	1.4	1.8	1.7	2.2	0.3	NS	0.004	NS
Placebo (n=17)	1.7	1.5	1.9	1.8	2.2	0.3			
All (n=33)	1.6	1.5	1.9	1.7	2.2	0.2			

[1]Treatment
[2]Time
[3]Treatment*Time

Significance (P<0.05)

NS=Not Significant

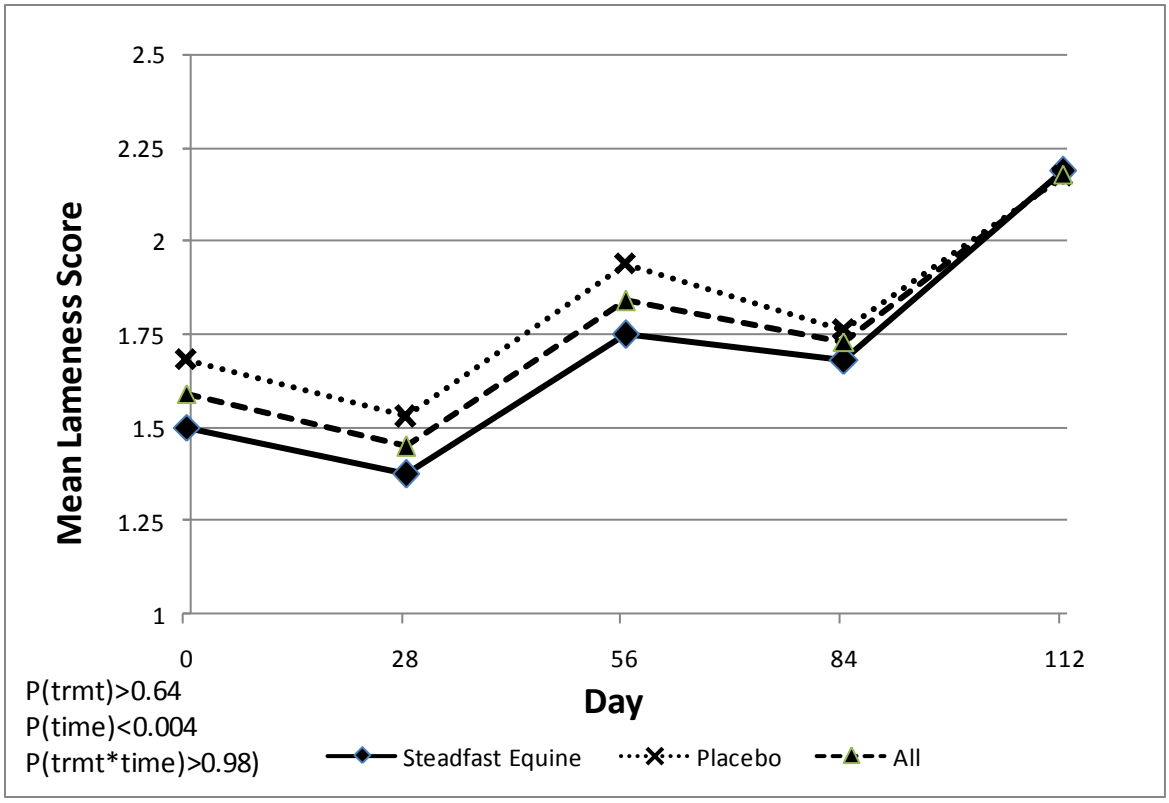

Figure 3.4 Effect of Treatment over Time on Lameness Score in an Equine Population (n= 33 horses x 5 evaluation dates=164)

Table 3.5 Effect of Treatment on Affected Area over Time in an Equine Population (n=33 horses x 5 evaluation dates=164)-Mean lameness score for Steadfast and placebo treatment groups, and for the population as a whole (All) on days 0, 28 and 84.

Treatment	Day 0	Day 28	Day 56	Day 84	Day 112	Standard Error	P[1]	P[2]	P[3]
Steadfast (n=16)	1.6	1.3	1.7	1.5	1.4	0.2	NS	NS	NS
Placebo (n=17)	1.4	1.5	1.4	1.4	1.7	0.2			
All (n=33)	1.5	1.4	1.5	1.4	1.5	0.1			

[1]Treatment
[2]Time
[3]Treatment*Time

Significance (P<0.05)

NS=Not Significant

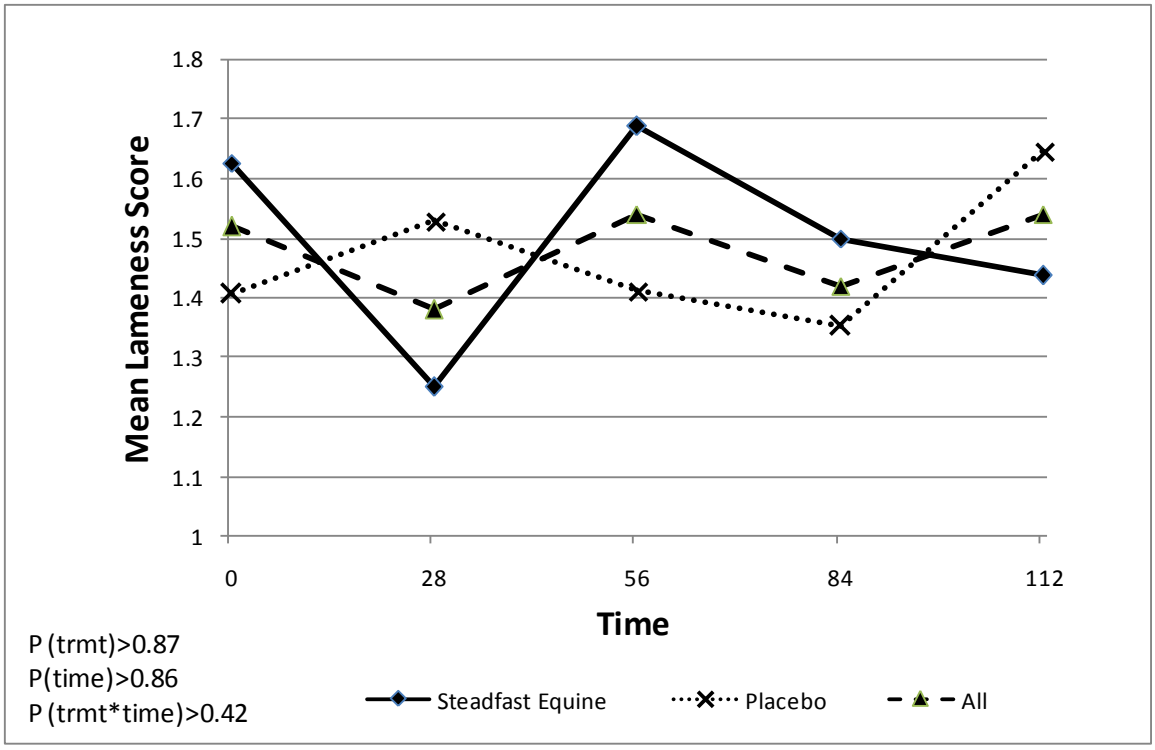

Figure 3.5 Effects of Treatment over Time on Affected Area in an Equine Population (n=33 horses x 5 evaluation dates=164)

Table 3.6 Effect of Treatment on CTX-II Concentration over Time in an Equine Population (n= 33 horses x 3 sample dates=99)-Mean ± SE CTX-II concentration for Steadfast and placebo treatment groups, and for the population as a whole (All) on days 0, 28 and 84.

Treatment	Day 0	Day 28	Day 84	P[1]	P[2]	P[3]
Steadfast (n=16)	23.0±5.8	21.2±7.1	33.7±5.8	NS	NS	NS
Placebo (n=17)	15.3±7.1	26.7±5.5	26.4±5.7			
All (n=33)	19.2±4.6	23.9±4.5	30.1±4.1			

[1]Treatment
[2]Time
[3]Treatment*Time

Significance (P<0.05)

NS=Not Significant

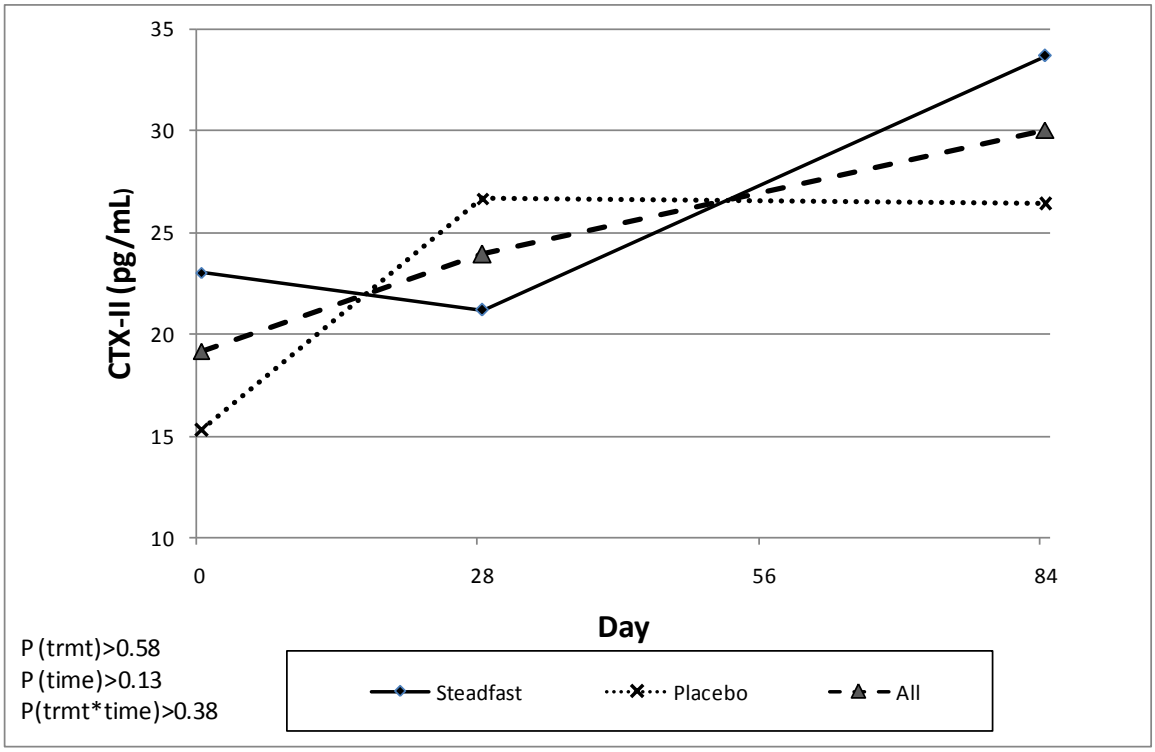

Figure 3.6 CTX-II Concentration by Treatment over Time in an Equine Population (n= 33 horses x 3 sample dates=99)

Table 3.7 Effect of Treatment on PIIANP Concentration over Time in an Equine Population (N=33 horses x 3 sample dates=99)-Mean ± SE PIIANP concentration for Steadfast and placebo treatment groups, and for the population as a whole (All) on days 0, 28 and 84.

Treatment	Day 0	Day 28	Day 84	P[1]	P[2]	P[3]
Steadfast (n=16)	1181±354	1537±408	1803±353	NS	0.003	NS
Placebo (n=17)	711±405	1406.31±348	1910±354			
All (n=33)	946±269	1471±268	1857±250			

[1] Treatment
[2] Time
[3] Treatment*Time

Significance (P<0.05)

NS=Not Significant

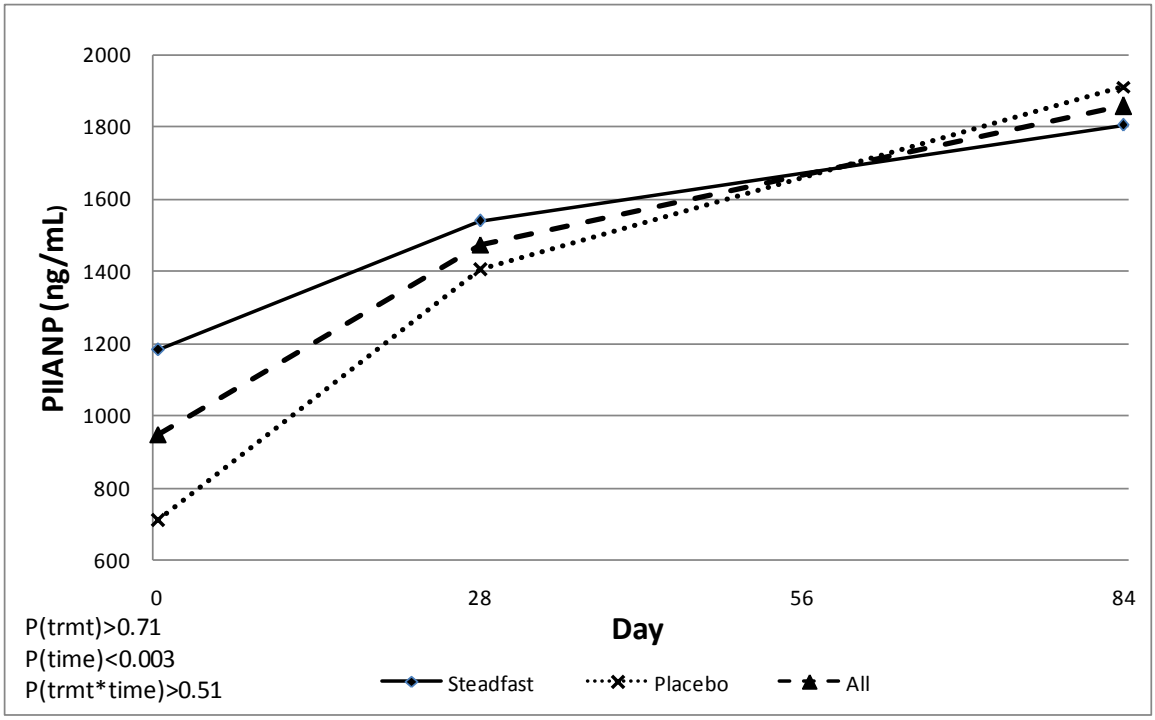

Figure 3.7 PIIANP Concentration by Treatment over Time (n=33 horses x 3 sample dates=99)

Table 3.8 Effect of Lameness Score on CTX-II Concentration in an Equine Population (n= 33 horses x 5 evaluation dates=164)- Mean ± SE CTX-II concentration for Steadfast and placebo treatment groups, and the population as a whole (All), for horses assigned to lameness score groups 0 to 3. (0=sound, 1=mild lameness, 2=moderate lameness and 3= marked lameness).

Lameness Score					
Treatment	**0 (n=17)**	**1 (n=58)**	**2 (n=36)**	**3 (n=53)**	**P**
Steadfast (n=16)	$19.2\pm7.9^{A,b}$	29.1 ± 5.1^{B}	35.2 ± 8.9^{B}	$20.3\pm6.0^{A,b}$	0.05
Placebo (n=17)	13.3 ± 9.3^{A}	30.5 ± 6.2^{B}	30.9 ± 6.0^{B}	16.6 ± 7.7^{A}	
All (n=33)	16.3 ± 6.5^{A}	29.8 ± 4.0^{B}	33.0 ± 5.3^{B}	18.4 ± 4.9^{A}	

Significance (P<0.05)

NS=Not Significant

All superscripts apply across rows. Upper-case letters indicate significant differences between means; lower-case letters indicate a means that approach significance as compared to means with the corresponding upper-case letter

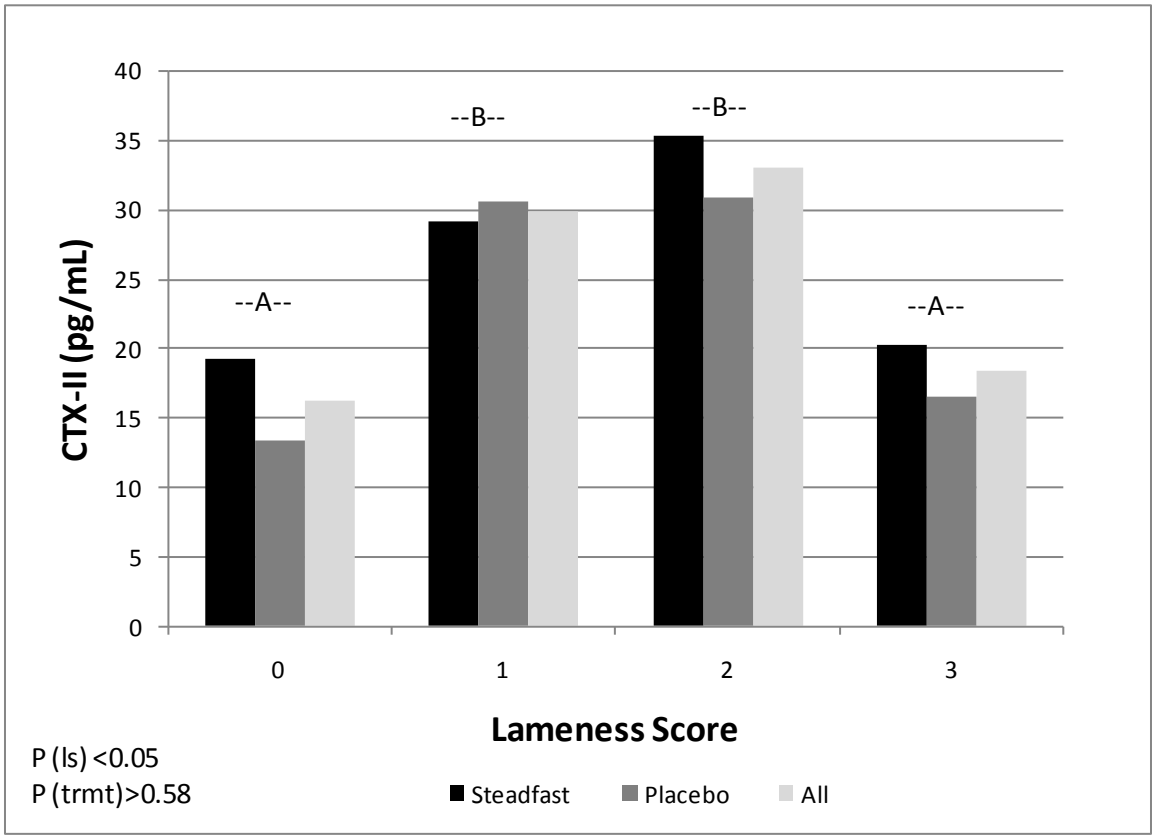

Figure 3.8 Effect of Lameness Score on CTX-II Concentration by Treatment (n= 33 horses x 3 sample dates=99)- Upper-case letters indicate significant differences between means; lower-case letters indicate means that approach significance as compared to means with the corresponding upper-case letter.

Table 3.9 Effect of Lameness Score on PIIANP Concentration in an Equine Population (n= 33 horses x 5 evaluation dates=164)- Mean ± SE PIIANP concentration for Steadfast and placebo treatment groups, and the population as a whole (All), for horses assigned to lameness score groups 0 to 3. (0=sound, 1=mild lameness, 2=moderate lameness and 3= marked lameness).

Lameness Score					
Treatment	**0 (n=17)**	**1 (n=58)**	**2 (n=36)**	**3 (n=53)**	**P**
Steadfast (n=16)	1799±517	1455±335	1559±474	1215±378	NS
Placebo (n=17)	1327±506	818±379	1773±375	1450±439	
All (n=33)	1563±361	1136±253	1666±302	1333±289	

Significance (P<0.05)

NS=Not Significant

All superscripts apply across rows. Upper-case letters indicate significant differences between means; lower-case letters indicate a means that approach significance as compared to means with the corresponding upper-case letter

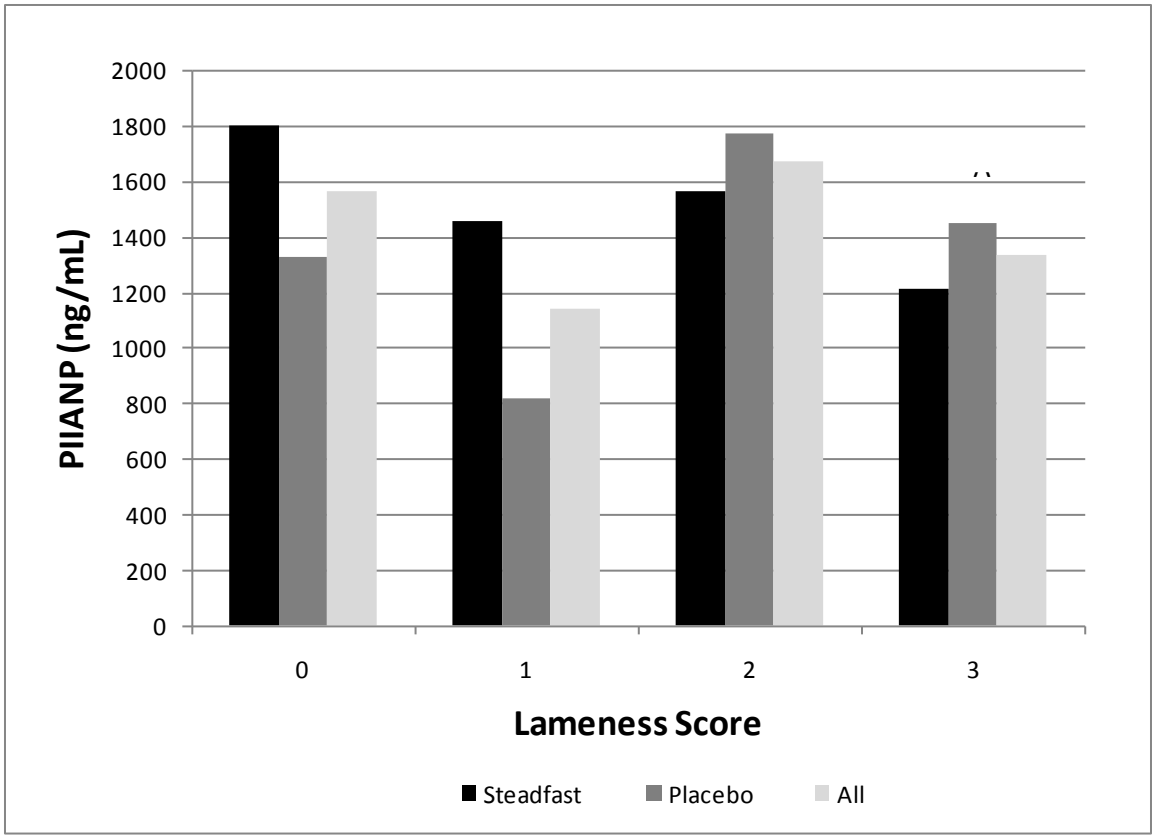

Figure 3.9 Effect of Lameness Score on PIIANP Concentration by Treatment (n= 33 horses x 3 sample dates=99)-

CHAPTER IV

SUMMARY: MAJOR FINDINGS AND WHERE TO GO NEXT

The design of two trials involved in this thesis endeavored to address the issues of detecting and monitoring degenerative joint disease in horses and assessing the use of nutraceutical supplementation to reverse said degeneration via collagen catabolites shed into the serum. Major findings of the work completed here include establishing baseline values for PIIANP and CTX-II serum concentrations, finding significantly decreased CTX-II concentrations in horses with marked lameness, and positively correlating age with an increase in both CTX-II and PIIANP.

There are discrepancies in the patterns of biomarker concentration across the two trials indicating that further investigation is needed in order to understand the cause and effect of cartilage metabolism and/or that the experimental design needs to be altered. While CTX-II and PIIANP concentrations were relatively stable over a 28 day period they showed an increasing trend in the concentration of both biomarkers over a longer time period, suggesting a possible seasonal effect. In order to investigate the hypothesis that cartilage biomarker concentrations change in a seasonal pattern a trial would need to monitor a horse population with a consistent exercise regime over a minimum period of 12 months.

Another finding that differed between the two trials is the effect of gender on CTX-II concentration. In the first trial mares had a higher mean concentration of CTX-II, however, CTX-II concentration did not differ between mares and geldings in the second trial. Again a follow-up study of longer duration may help clarify whether at certain times

of the year there is an effect of gender due to hormonal fluctuations such as increased androgens during the breeding season which have a chondroprotetive effect.[127,128]

This thesis used lameness as measurement of DJD. While DJD is the most common cause of non-traumatic lameness in horses this assumption inherently contains a certain amount of error. Further investigation in this area would benefit from more stringent screening of the involved equine population such that only horses with a definitive diagnosis of DJD are included in the trial. Furthermore, imaging studies at baseline and then throughout the course of the trial (radiographs, MRI and/or arthroscopy) would be needed in order to definitively tie biomarker concentrations to progressive joint damage. Such studies were not possible for this thesis due to financial limitations and conflicting interests of the equine population involved.

The lack of difference in CTX-II and PIIANP concentrations between placebo and supplemented trial groups may indicate that Steadfast® Equine has no effect on collagen catabolism, that the selected biomarkers were not adequately sensitive to measure the change in catabolism and/or that 84 days was not long enough to have serum biomarker concentrations reflect the changes in the rate of catabolism. The higher degree of fluctuations in lameness score within individual horses compared to trial 1 suggests confounding effects. The suggested use of nutraceuticals in the management of DJD has also been largely aimed at prevention rather than treatment, a parameter which would be much more difficult to measure. It must also be considered that while collagen type II is the major structural molecule in articular cartilage, its catabolism is not a direct

measurement of cartilage destruction and there may be other biomarkers that are more sensitive to the measure the potential benefits of nutraceutical supplementation.

At this early stage in understanding of the connection between equine DGD and the mechanism and rate of cartilage catabolism, it is equally important to establish both the potential uses and limitations of type II collagen biomarkers as tools in making a diagnosis, monitoring the progression, and measuring the therapeutic interventions for DJD. The culmination of the data of these two trials demonstrates the potential utility of serum CTX-II concentration in order to non-invasively evaluate DJD and also several confounding variables and limitations, such as season, gender and a precise standard against which to measure joint damage. It is my hope that the data collected over the course of these trials, as well as both the flaws and successes in experimental design, should provide beneficial information to help to guide future research in this area.

REFERENCES

1. Lawler DF, Larson BT, Ballam JM, et al. Diet restriction and ageing in the dog: major observations over two decades. *British Journal of Nutrition* 2008;99:793-805.

2. Brosnahan MM, Paradis MR. Demographic and clinical characteristics of geriatric horses: 467 cases (1989-1999). *J Am Vet Med Assoc* 2003;223:93-98.

3. D. Gunn-Moore KM, L.-A. Christie, E. Head,. Cognitive dysfunction and the neurobiology of ageing in cats. *Journal of Small Animal Practice* 2007;48:546-553.

4. Swanson KS, Schook LB. Canine Nutritional Model: Influence of Age, Diet, and Genetics on Health and Well-Being. *Current Nutrition & Food Science* 2006;2:115-126.

5. S. E. GLATT KEF, J. L. SCHEELS,. A survey of current dental problems and treatments of zoo animals. *International Zoo Yearbook* 2008;42:206-213.

6. Greer M, Greer, J.K., Gillingham, J. Osteoarthritis in selected wild animals. *Proceedings of the Oklahoma Academy of Sciences* 1977;57:39-43.

7. Ehrlich GE. Osteoarthritis beginning with inflammation. Definitions and correlations. 1975. *Bull World Health Organ* 2003;81:691-693.

8. Hrycaj PZ. Systemic inflammation in osteoarthritis. *Ann Rheum Dis* 2004;63:750-751; author reply 751.

9. Pelletier JP, Martel-Pelletier J, Abramson SB. Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. *Arthritis Rheum* 2001;44:1237-1247.

10. Zhang Y, Jordan JM. Epidemiology of osteoarthritis. *Rheum Dis Clin North Am* 2008;34:515-529.

11. Matyas JR, Atley L, Ionescu M, et al. Analysis of cartilage biomarkers in the early phases of canine experimental osteoarthritis. *Arthritis Rheum* 2004;50:543-552.

12. Bertone AL, Palmer JL, Jones J. Synovial fluid cytokines and eicosanoids as markers of joint disease in horses. *Vet Surg* 2001;30:528-538.

13. Gaby AR. Natural treatments for osteoarthritis. *Altern Med Rev* 1999;4:330-341.

14. Moskowitz RW. The appropriate use of NSAIDs in arthritic conditions. *Am J Orthop* 1996;25:4-6.

15. Bijlsma JW. Diagnosis and nonsurgical management of osteoarthritis. *Ann Rheum Dis* 2001;60:6.

16. Manek NJ, Lane NE. Osteoarthritis: current concepts in diagnosis and management. *Am Fam Physician* 2000;61:1795-1804.

17. Felson DT, Lohmander LS. Whither osteoarthritis biomarkers? *Osteoarthritis Cartilage* 2009;17:419-422.

18. Frisbie DD, Al-Sobayil F, Billinghurst RC, et al. Changes in synovial fluid and serum biomarkers with exercise and early osteoarthritis in horses. *Osteoarthritis Cartilage* 2008;16:1196-1204.

19. Williams FM, Spector TD. Biomarkers in osteoarthritis. *Arthritis Res Ther* 2008;10:101.

20. Garnero P. Use of biochemical markers to study and follow patients with osteoarthritis. *Curr Rheumatol Rep* 2006;8:37-44.

21. Keegan KG. Evidence-based lameness detection and quantification. *Vet Clin North Am Equine Pract* 2007;23:403-423.

22. Garnero P, Rousseau JC, Delmas PD. Molecular basis and clinical use of biochemical markers of bone, cartilage, and synovium in joint diseases. *Arthritis Rheum* 2000;43:953-968.

23. Samuels J, Krasnokutsky S, Abramson SB. Osteoarthritis: a tale of three tissues. *Bull NYU Hosp Jt Dis* 2008;66:244-250.

24. Wright V, Dowson D, Kerr J. The structure of joints. *Int Rev Connect Tissue Res* 1973;6:105-125.

25. Gineyts E, Garnero P, Delmas PD. Urinary excretion of glucosyl-galactosyl pyridinoline: a specific biochemical marker of synovium degradation. *Rheumatology (Oxford)* 2001;40:315-323.

26. Benito MJ, Veale DJ, FitzGerald O, et al. Synovial tissue inflammation in early and late osteoarthritis. *Ann Rheum Dis* 2005;64:1263-1267.

27. Burr DB. Anatomy and physiology of the mineralized tissues: role in the pathogenesis of osteoarthrosis. *Osteoarthritis Cartilage* 2004;12 Suppl A:S20-30.

28. Jung M, Christgau S, Lukoschek M, et al. Increased urinary concentration of collagen type II C-telopeptide fragments in patients with osteoarthritis. *Pathobiology* 2004;71:70-76.

29. Loeser RF, Jr. Aging and the etiopathogenesis and treatment of osteoarthritis. *Rheum Dis Clin North Am* 2000;26:547-567.

30. Martel-Pelletier J, Boileau C, Pelletier JP, et al. Cartilage in normal and osteoarthritis conditions. *Best Pract Res Clin Rheumatol* 2008;22:351-384.

31. Garnero P, Ayral X, Rousseau JC, et al. Uncoupling of type II collagen synthesis and degradation predicts progression of joint damage in patients with knee osteoarthritis. *Arthritis Rheum* 2002;46:2613-2624.

32. Aigner T, Zhu Y, Chansky H, et al. Reexpression of Type IIA Procollegen by Adult Articular Chondrocytes in Osteoarthritic Cartilage. *Arthritis and Rheumatism* 1999;42:1443-1450.

33. Sandell LJ, Aigner T. Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res* 2001;3:107-113.

34. Loeser R. The biology of Osteoarthritis. American College of Veterinary Pathologists 2005.

35. Read R. Diagnosis and Management of Osteoarthritis. WSAVA 2002.

36. Loeser RF. The biology of osteoarthritis *56th Annual Meeting of the American College of Veterinary Pathobiologists (ACVP) and 4oth Annual Meeting of the American Society for Veterinary Clinical Pathology (ASVCP)* 2005.

37. Ayral X, Dougados M, Listrat V, et al. Arthroscopic evaluation of chondropathy in osteoarthritis of the knee. *J Rheumatol* 1996;23:698-706.

38. Sutton S, Clutterbuck A, Harris P, et al. The contribution of the synovium, synovial derived inflammatory cytokines and neuropeptides to the pathogenesis of osteoarthritis. *Vet J* 2007.

39. McAlindon TE, Jacques P, Zhang Y, et al. Do antioxidant micronutrients protect against the development and progression of knee osteoarthritis? *Arthritis Rheum* 1996;39:648-656.

40. Greenwald RA, Moy WW. Inhibition of collagen gelation by action of the superoxide radical. *Arthritis Rheum* 1979;22:251-259.

41. Sakurai H, Kohsaka H, Liu MF, et al. Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. *J Clin Invest* 1995;96:2357-2363.

42. Martel-Pelletier J. Pathophysiology of osteoarthritis. *Osteoarthritis Cartilage* 2004;12 Suppl A:S31-33.

43. Thonar EJ, Shinmei M, Lohmander LS. Body fluid markers of cartilage changes in osteoarthritis. *Rheum Dis Clin North Am* 1993;19:635-657.

44. Ravaud P, Giraudeau B, Auleley GR, et al. Variability in knee radiographing: implication for definition of radiological progression in medial knee osteoarthritis. *Ann Rheum Dis* 1998;57:624-629.

45. Garnero P, Delmas PD. Biomarkers in osteoarthritis. *Curr Opin Rheumatol* 2003;15:641-646.

46. Henrotin Y, Addison S, Kraus V, et al. Type II collagen markers in osteoarthritis: what do they indicate? *Curr Opin Rheumatol* 2007;19:444-450.

47. Henrotin Y, Addison S, Kraus V, et al. Type II collagen markers in osteoarthritis: what do they indicate? *Current Opinion in Rheumatology* 2007;19:444-450 410.1097/BOR.1090b1013e32829fb32823b32825.

48. Vosse D, Landewe R, Garnero P, et al. Association of markers of bone- and cartilage-degradation with radiological changes at baseline and after 2 years follow-up in patients with ankylosing spondylitis. *Rheumatology (Oxford)* 2008;47:1219-1222.

49. Dam EB, Byrjalsen I, Karsdal MA, et al. Increased urinary excretion of C-telopeptides of type II collagen (CTX-II) predicts cartilage loss over 21 months by MRI. *Osteoarthritis Cartilage* 2008.

50. Oestergaard S, Chouinard L, Doyle N, et al. The utility of measuring C-terminal telopeptides of collagen type II (CTX-II) in serum and synovial fluid samples for estimation of articular cartilage status in experimental models of destructive joint diseases. *Osteoarthritis Cartilage* 2006;14:670-679.

51. Jordan KM, Syddall HE, Garnero P, et al. Urinary CTX-II and glucosyl-galactosyl-pyridinoline are associated with the presence and severity of radiographic knee osteoarthritis in men. *Ann Rheum Dis* 2006;65:871-877.

52. Rousseau JC, Sandell LJ, Delmas PD, et al. Development and clinical application in arthritis of a new immunoassay for serum type IIA procollagen NH2 propeptide. *Methods Mol Med* 2004;101:25-37.

53. Sharif M, Kirwan J, Charni N, et al. A 5-yr longitudinal study of type IIA collagen synthesis and total type II collagen degradation in patients with knee osteoarthritis--association with disease progression. *Rheumatology (Oxford)* 2007;46:938-943.

54. Henrotin YE, Deberg MA, Crielaard JM, et al. Avocado/soybean unsaponifiables prevent the inhibitory effect of osteoarthritic subchondral osteoblasts on aggrecan and type II collagen synthesis by chondrocytes. *J Rheumatol* 2006;33:1668-1678.

55. Feelisch M. The chemical biology of nitric oxide--an outsider's reflections about its role in osteoarthritis. *Osteoarthritis Cartilage* 2008;16 Suppl 2:S3-S13.

56. Karan A, Karan MA, Vural P, et al. Synovial fluid nitric oxide levels in patients with knee osteoarthritis. *Clin Rheumatol* 2003;22:397-399.

57. Abramson SB. Nitric oxide in inflammation and pain associated with osteoarthritis. *Arthritis Res Ther* 2008;10 Suppl 2:S2.

58. Ersoy Y, Ozerol E, Baysal O, et al. Serum nitrate and nitrite levels in patients with rheumatoid arthritis, ankylosing spondylitis, and osteoarthritis. *Ann Rheum Dis* 2002;61:76-78.

59. Borysewicz K, Sewern E, Szmyrka-Kaczmarek M, et al. Serum and synovial fluid nitrite levels in patents with rheumatoid arthritis and osteoarthritis *Advanced Clinical Experimantal Medicine* 2008;17:395-398.

60. Frean SP, Bryant CE, Froling IL, et al. Nitric oxide production by equine articular cells *in vitro*. *Equine Vet J* 1997;29.

61. Hauselmann HJ, Opplinger L, Michel BA, et al. Nitric oxide and proteoglycan biosyntheisis bu human articular chondrocytes in alginate culture. *FEBS Lett* 1994;352:361-364.

62. Goodrich LR, Nixon AJ. Medical treatment of osteoarthritis in the horse - a review. *Vet J* 2006;171:51-69.

63. Vanderperren K, Raes E, Hoegaerts M, et al. Diagnostic imaging of the equine tarsal region using radiography and ultrasonography. Part 1: The soft tissues. *The Veterinary Journal* 2009;179:179-187.

64. Murray RC, Blunden TS, Branch MV, et al. Evaluation of age-related changes in the structure of the equine tarsometatarsal osteochondral unit. *American Journal of Veterinary Research* 2009;70:30-36.

65. Keegan KG, Yonezawa Y, Pai PF, et al. Evaluation of a sensor-based system of motion analysis for detection and quantification of forelimb and hind limb lameness in horses. *Am J Vet Res* 2004;65:665-670.

66. Keegan KG, Wilson DA, Wilson DJ, et al. Evaluation of mild lameness in horses trotting on a treadmill by clinicians and interns or residents and correlation of their assessments with kinematic gait analysis. *Am J Vet Res* 1998;59:1370-1377.

67. Keegan KG, Yonezawa Y, Pai PF, et al. Accelerometer-based system for the detection of lameness in horses. *Biomed Sci Instrum* 2002;38:107-112.

68. Trumble TN. The use of nutraceuticals for osteoarthritis in horses. *Vet Clin North Am Equine Pract* 2005;21:575-597, v-vi.

69. Moger CJ, Arkill KP, Barrett R, et al. Cartilage collagen matrix reorientation and displacement in response to surface loading. *J Biomech Eng* 2009;131:031008.

70. Mack SJ, Jackson BF, Dyson PK, et al. Evaluation of the C-Terminal Cross-Linked Telopeptide of Type II Collagen (CTX-II) as a Potential Bioamrker for use in Horses *British Equine Veterinary Association Congress* Hertfordshire, UK: The Royal Veterinary College, 2007.

71. Jaffre B, Watrin A, Loeuille D, et al. Effects of antiinflammatory drugs on arthritic cartilage: a high-frequency quantitative ultrasound study in rats. *Arthritis Rheum* 2003;48:1594-1601.

72. Gineyts E, Mo JA, Ko A, et al. Effects of ibuprofen on molecular markers of cartilage and synovium turnover in patients with knee osteoarthritis. *Ann Rheum Dis* 2004;63:857-861.

73. Ameye LG, Chee WS. Osteoarthritis and nutrition. From nutraceuticals to functional foods: a systematic review of the scientific evidence. *Arthritis Res Ther* 2006;8:R127.

74. Ruff KJ, DeVore DP, Leu MD, et al. Eggshell Membrane:A possible new theraputic for joints and connective tissue disorders. Results from two open-lable human studues. *Clinical Interventions in Ageing* 2009;4:235-240.

75. Equine Steadfast: A unique formulation for total structural health. 2007.

76. Fitzgerald T, Norton B, Elliott R, et al. The influence of long-term supplementation with biotin on the prevention of lameness in dairy cows. *The Journal of Dairy Science* 2000;83:338-344.

77. Povorozniuk V, Grigorieva N. On the role of nutrition in the developement of knee osteoarthritis. *Gerontologia* 2007;8:26-30.

78. Fitzgerald T, Norton BW, Elliott R, et al. The influence of long-term supplementation with biotin on the prevention of lameness in pasture fed dairy cows. *J Dairy Sci* 2000;83:338-344.

79. Geyer H, Schultz J. The long term influence of Biotin supplemtation on hoof horn quality in horses. *Schweitz Arch Tierheilk* 1994;Supp. 4:137-149.

80. Geyer H, Schulze J. The long-term influence of biotin supplementation on hoof horn quality in horses. *Schweiz Arch Tierheilkd* 1994;136:137-149.

81. Frantz N, Andrews G, Tokach M, et al. Effects if dietary nutrients on osteochondrosis lesions and cartilage properties in pigs. *American Journal of Veterinary Research* 2008;69:617-624.

82. Frisbie DD, Kawcak C, McIlwraith CW, et al. Evaluation of polysulfated glycosaminoglycan or sodium hyaluronan adminestered intra-articularly for treatment of horses

with experimentally induced osteoarthritis. *American Journal of Veterinary Research* 2009;70:203-209.

83. Chan PS, Caron JP, Rosa JM, et al. Glucosamine and chondroitin sulfate gene expression and synthesis of nitric oxide and prostaglandin E_2 in articular cartilage explants. *Osteoarthritis and Cartilage* 2005;13.

84. Dechant JE, Baxter GM, Frisbie DD, et al. Effects of glucosamine hydrochloride and chondroitin sulphate, alone and in combination, on normal and interleukin-1 conditioned equine articular cartilage explant metabolism. *Equine Vet J* 2005;37:227-231.

85. Fenton JI, Chlebek-Brown KA, Peters TL, et al. The effects of glucosamine derivatives on equine articular cartilage degradation in explant culture. *Osteoarthritis Cartilage* 2000;8:444-451.

86. Fenton JI, Chlebek-Brown KA, Peters TL, et al. Glucosamine HCl reduces equine articular cartilage degradation in explant culture. *Osteoarthritis Cartilage* 2000;8:258-265.

87. Orth MW, Peters TL, Hawkins JN. Inhibition of articular cartilage degradation by glucosamine-HCl and chondroitin sulphate. *Equine Vet J Suppl* 2002:224-229.

88. Du J, White N, Eddington ND. The bioavailability and pharmacokinetics of glucosamine hydrochloride and chondroitin sulfate after oral and intravenous single dose administration in the horse. *Biopharm Drug Dispos* 2004;25:109-116.

89. Deal CL, Moskowitz RW. Nutraceuticals as therapeutic agents in osteoarthritis. The role of glucosamine, chondroitin sulfate, and collagen hydrolysate. *Rheum Dis Clin North Am* 1999;25:379-395.

90. Chan PS, Caron JP, Rosa GJ, et al. Glucosamine and chondroitin sulfate regulate gene expression and synthesis of nitric oxide and prostaglandin E(2) in articular cartilage explants. *Osteoarthritis Cartilage* 2005;13:387-394.

91. Lequesne M. [Symptomatic slow-action anti-arthritic agents: a new therapeutic concept?]. *Rev Rhum Ed Fr* 1994;61:75-79.

92. Glade MJ. Polysulfated glycosaminoglycans accelerates net synthesis of collagen and glycosaminoglycans by arthritic equine cartilage tissue and chondrocytes *American Journal of Veterinary Research* 1990;51:779-785.

93. Caron JP, Toppin DS, Block JA. Effects of polysulfated glycosaminoglycans on osteoarthritic equine cartilage in explant culture. *American Journal of Veterinary Research* 1993;54:1116-1121.

94. Clark AG, Rohrbaugh AL, Otterness I, et al. The effects of ascorbic acid on cartilage metabolism in guinea pig articular cartilage explants. *Matrix Biol* 2002;21:175-184.

95. Machlin LJ, Bendich A. Free radical tissue damage: protective role of antioxidant nutrients. *FASEB J* 1987;1:441-445.

96. McAlindon T, Felson DT. Nutrition: risk factors for osteoarthritis. *Ann Rheum Dis* 1997;56:397-400.

97. Snow DH, Harris RC. The use of conventional and unconventional supplements in the Thoroughbred horse. *Proc Nutr Soc* 1989;48:135-139.

98. Gerstenfeld LC, Kelly CM, Von Deck M, et al. Effect of 1,25-dihydroxyvitamin D3 on induction of chondrocyte maturation in culture: extracellular matrix gene expression and morphology. *Endocrinology* 1990;126:1599-1609.

99. Coudray C, Feillet-Coudray C, Rambeau M, et al. The effect of aging on intestinal absorption and status of calcium, magnesium, zinc, and copper in rats: a stable isotope study. *J Trace Elem Med Biol* 2006;20:73-81.

100. Yamaguchi M, Fukagawa M. Role of zinc in regulation of protein tyrosine phosphatase activity in osteoblastic MC3T3-E1 cells: zinc modulation of insulin-like growth factor-I's effect. *Calcif Tissue Int* 2005;76:32-38.

101. Yamaguchi M, Uchiyama S. Receptor activator of NF-kappaB ligand-stimulated osteoclastogenesis in mouse marrow culture is suppressed by zinc in vitro. *Int J Mol Med* 2004;14:81-85.

102. Leach RM, Jr., Muenster AM, Wien EM. Studies on the role of manganese in bone formation. II. Effect upon chondroitin sulfate synthesis in chick epiphyseal cartilage. *Arch Biochem Biophys* 1969;133:22-28.

103. Kawcak CE, Frisbie DD, McIlwraith CW, et al. Evaluation of avocado and soybean unsaponifiable extracts for treatment of horses with experimentally induced osteoarthritis. *Am J Vet Res* 2007;68:598-604.

104. Garnero P, Gineyts E, Christgau S, et al. Association of baseline levels of urinary glucosyl-galactosyl-pyridinoline and type II collagen C-telopeptide with progression of joint destruction in patients with early rheumatoid arthritis. *Arthritis Rheum* 2002;46:21-30.

105. Jansen NW, Roosendaal G, Lundin B, et al. The combination of the biomarkers urinary C-terminal telopeptide of type II collagen, serum cartilage oligomeric matrix protein, and serum chondroitin sulfate 846 reflects cartilage damage in hemophilic arthropathy. *Arthritis Rheum* 2009;60:290-298.

106. de Grauw JC, Brama PA, Wiemer P, et al. Cartilage-derived biomarkers and lipid mediators of inflammation in horses with osteochondritis dissecans of the distal intermediate ridge of the tibia. *Am J Vet Res* 2006;67:1156-1162.

107. Poole AR, Kobayashi M, Yasuda T, et al. Type II collagen degradation and itd regulation in articular cartilage in osteoarthritis. *Ann Rheum Dis* 2002;61(Suppl 2):ii78-81.

108. Meulenbelt I, Kloppenburg M, Kroon HM, et al. Urinary CTX-II levels are associated with radiographic subtypes of osteoarthritis in hip, knee, hand, and facet joints in subject with familial osteoarthritis at multiple sites: the GARP study. *Ann Rheum Dis* 2006;65:360-365.

109. Oestergaard S, Chouinard L, Doyle N, et al. The utility of measuring C-terminal telopeptides of collagen type II (CTX-II) in serum and synovial fluid samples for estimation of articular cartilage status in experimental models of destructive joint diseases. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2006;14:670-679.

110. Cleary OB, Trumble TN, Brown MP, et al. 110 Effects of exercise and osteochondral injury on CTX II concentrations in equine synovial fluid and serum.. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society* 2007;15:C69-C70.

111. Trumble TN, Scarbrough BS, Brown MP. Osteochondral injury increases type II colllagen degradation products (C2C) in synovial fluid of thouroughbred racehorses. *Osteoarthritis and Cartilage* 2009;17.

112. de la Calle J, Burba DJ, Ramaswamy CM, et al. Plasma and synovial fluid endothelin-1 and nitric oxide concentration in horses with and without joint disease. *American Journal of Veterinary Research* 2002;63:1648-1654.

113. Sandell LJSL, MO, US), Garnero, Patrick (Villeurbanne, FR). Uncoupled collagen synthesis and degradation assays. United States: Barnes-Jewish Hospital, 2004.

114. Keegan KG, Satterley JM, Skubic M, et al. Use of gyroscopic sensors for objective evaluation of trimming and shoeing to alter time between heel and toe lift-off at end of the stance phase in horses walking and trotting on a treadmill. *Am J Vet Res* 2005;66:2046-2054.

115. Afonso V, Champy R, Mitrovic D, et al. Reactive oxygen species and superoxide dismutases: role in joint diseases. *Joint Bone Spine* 2007;74:324-329.

116. Simmons EJ, Bertone AL, Hardy J, et al. Nitric oxide synthase activity in healthy and and interleukin 1beta-exposed equine synovial membrane *American Journal of Veterinary Research* 1999;60.

117. Von Rechenberg B, Guenther H, McIlwraith CW. Fibrous tissue of subchondral cystic lesions in horses produce local mediators and neutral metalloproteinases and cause bone resorption in vitro. *Vet Surg* 2000;29.

118. Cibere J, Zhang H, Garnero P, et al. Association of biomarkers with pre-radiographically defined and radiographically defined knee osteoarthritis in a population-based study. *Arthritis Rheum* 2009;60:1372-1380.

119. Garnero P, Conrozier T, Christgau S, et al. Urinary type II collagen C-telopeptide levels are increased in patients with rapidly destructive hip osteoarthritis. *Ann Rheum Dis* 2003;62:939-943.

120. Thomas CM, Fuller CJ, Whittles CE, et al. Chondrocyte death by apoptosis is associated with cartilage matrix degradation. *Osteoarthritis and Cartilage* 2007;15:27-34.

121. Jeffcott LB, Henson FDM. Studies on growth cartilage in the horse and their application to aetiopathogenisis of dyschondroplasia (Osteochondrosis). *The Veterinary Journal* 1998;156.

122. Fradette ME, Celeste C, Richard H, et al. Effects of continuous oral administration of phenylbutazone on biomarkers of cartilage and bone metabolism in horses. *Am J Vet Res* 2007;68:128-133.

123. Murray RC, Blunden TS, Branch MV, et al. Evaluation of age-related changes in the structure of the equine tarsometatarsal osteochondral unit. *Am J Vet Res* 2009;70:30-36.

124. Loeser RF. Aging and osteoarthritis: the role of chondrocyte senescence and aging changes in the cartilage matrix. *Osteoarthritis Cartilage* 2009.

125. Brinkley TE, Leng X, Miller ME, et al. Chronic inflammation is associated with low physical function in older adults across multiple comorbidities. *J Gerontol A Biol Sci Med Sci* 2009;64:455-461.

126. Loeser RF, Jr. Aging cartilage and osteoarthritis--what's the link? *Sci Aging Knowledge Environ* 2004;2004:pe31.

127. Bay-Jensen AC, Tabassi NC, Sondergaard LV, et al. The response to estrogen deprivation on cartilage collagen degradation markers; CTX-II is unique compared to other markers of collagen turnover. *Arthritis Res Ther* 2009;11:R9.

128. Ganesan K, Tiwari M, Balachandran C, et al. Estrogen and testosterone attenuate extracellular matrix loss in collagen-induced arthritis in rats. *Calcif Tissue Int* 2008;83:354-364.

129. Ross RW, Dyson SJ. *Diagnosis and management of lameness in the horse*. Philadelphia, PA: Saunders Edu., 2003.

130. Troy NT. The Use of Nutraceuticals for Osteoarthritis in Horses. *The Veterinary clinics of North America Equine practice* 2005;21:575-597.

131. Snow DH, Douglas TA, Thompson H, et al. Phenylbutazone toxicosis in equidae: a biochemical and pathophysiological study. *Am J Vet Res* 1981;42:1754-1759.

132. Traub-Dargatz JL, Bertone JJ, Gould DH, et al. Chronic flunixin meglumine therapy in foals. *Am J Vet Res* 1988;49:7-12.

133. Brandt KD. Effects of nonsteroidal anti-inflammatory drugs on chondrocyte metabolism in vitro and in vivo. *Am J Med* 1987;83:29-34.

134. Frisbie DD, Kawcak CE, Baxter GM, et al. Effects of 6alpha-methylprednisolone acetate on an equine osteochondral fragment exercise model. *Am J Vet Res* 1998;59:1619-1628.

135. Resch KL, Hill S, Ernst E. Use of complementary therapies by individuals with 'arthritis'. *Clin Rheumatol* 1997;16:391-395.

136. McIlwraith CW. Liscensed medications, "generic" medication, compounding, and nutraceuticals-what has been scientifically validated, where do we encounter scientific mistruth, and where are we legally? . Proceedings of the 50th Annual Convention of the American Association of Equine Practitioners 2004;459-475.

137. Boothe DM. Balancing fact and fiction of novel ingredients: definitions, regulations and evaluation. *Vet Clin North Am Small Anim Pract* 2004;34:7-38.

138. Shikhman AR, Kuhn K, Alaaeddine N, et al. N-acetylglucosamine prevents IL-1 beta-mediated activation of human chondrocytes. *J Immunol* 2001;166:5155-5160.

139. Hoffer LJ, Kaplan LN, Hamadeh MJ, et al. Sulfate could mediate the therapeutic effect of glucosamine sulfate. *Metabolism* 2001;50:767-770.

140. Setnikar I, Giacchetti C, Zanolo G. Pharmacokinetics of glucosamine in the dog and in man. *Arzneimittelforschung* 1986;36:729-735.

141. Conte A, Volpi N, Palmieri L, et al. Biochemical and pharmacokinetic aspects of oral treatment with chondroitin sulfate. *Arzneimittelforschung* 1995;45:918-925.

142. Uebelhart D, Thonar EJ, Zhang J, et al. Protective effect of exogenous chondroitin 4,6-sulfate in the acute degradation of articular cartilage in the rabbit. *Osteoarthritis Cartilage* 1998;6 Suppl A:6-13.

143. Frean SP, Lees P. Effects of polysulfated glycosaminoglycan and hyaluronan on prostaglandin E2 production by cultured equine synoviocytes. *Am J Vet Res* 2000;61:499-505.

144. Lynch TM, Caron JP, Arnoczky SP, et al. Influence of exogenous hyaluronan on synthesis of hyaluronan and collagenase by equine synoviocytes. *Am J Vet Res* 1998;59:888-892.

145. Kirker-Head C, Kirker-Head R. Safety of an oral chondroprotective agent in horses. *Vet Ther* 2001;2:345-353.

146. Pearson W, Orth MW, Lindinger MI. Evaluation of inflammatory responses induced via intra-articular injection of interleukin-1 in horses receiving a dietary nutraceutical and assessment of the clinical effects of long-term nutraceutical administration. *Am J Vet Res* 2009;70:848-861.

147. Malone ED. Managing chronic arthritis. *Vet Clin North Am Equine Pract* 2002;18:411-437.

148. Harreveld PDv, Lillich JD, Kawcak CE, et al. Clinical evaluation of the effects of immobilization followed by remobilization and exercise on the metacarpophalangeal joint in horses. *American Journal of Veterinary Research* 2002;63:282-288.

149. Donovan DC, Jackson CA, Colahan PT, et al. Exercise-induced alterations in pro-inflammatory cytokines and prostaglandin F2alpha in horses. *Vet Immunol Immunopathol* 2007;118:263-269.

150. Frape DL. Diet and exercise performance in the horse. *Proceedings of the Nutrition Society* 1994;53:189-206.

151. Vernonâˆ— KL, Riggs L, Coverdale J, et al. Forced Exercise Induces Histological Evidence of Osteoarthritis on the Distal Metacarpus of Sheep. 2009;29:305-307.

152. Oegema TR, Jr., Deloria LB, Sandy JD, et al. Effect of oral glucosamine on cartilage and meniscus in normal and chymopapain-injected knees of young rabbits. *Arthritis Rheum* 2002;46:2495-2503.

153. Bassleer C, Rovati L, Franchimont P. Stimulation of proteoglycan production by glucosamine sulfate in chondrocytes isolated from human osteoarthritic articular cartilage in vitro. *Osteoarthritis Cartilage* 1998;6:427-434.

154. Bassleer CT, Combal JP, Bougaret S, et al. Effects of chondroitin sulfate and interleukin-1 beta on human articular chondrocytes cultivated in clusters. *Osteoarthritis Cartilage* 1998;6:196-204.

155. W. B. Staniar, D. S. Kronfeld, R. M. Akers, et al. Insulin-like growth factor I in growing thoroughbreds. *Journal of Animal Physiology and Animal Nutrition* 2007;91:390-399.

156. Hui W, Rowan AD, Cawston T. Modulation of the expression of matrix metalloproteinase and tissue inhibitors of metalloproteinases by TGF-beta1 and IGF-1 in primary human articular and bovine nasal chondrocytes stimulated with TNF-alpha. *Cytokine* 2001;16:31-35.

157. Frisbie DD, Nixon AJ. Insulin-like growth factor 1 and corticosteroid modulation of chondrocyte metabolic and mitogenic activities in interleukin 1-conditioned equine cartilage. *Am J Vet Res* 1997;58:524-530.