Inflammatory Response in Equine Joints

Studies on Proinflammatory Cytokines in Diseased Joints and Chondrocyte Cultures

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Inflammatory Response in Equine Joints. Studies on Proinflammatory Cytokines in Diseased Joints and Chondrocyte Cultures

Abstract

Proinflammatory cytokines mediate inflammatory responses as well as regulate tissue metabolism. Thus, they may provide a link between inflammation and other pathologic findings seen in equine joint disease. The aims of this thesis were to gain a deeper understanding of the development of chondral pathology in equine osteoarthritis (OA) by obtaining increased knowledge of inflammatory processes in the joint, and to investigate proinflammatory cytokines as markers of joint pathology.

Measurements of interleukin (IL)-6 and tumour necrosis factor (TNF) activities in synovial fluid from clinical cases of carpal joint disease revealed increased concentrations of bioactive IL-6 in joints with osteochondral fragments (OCF). Immunohistochemical staining for IL-6 and high mobility group box protein (HMGB)-1 in tissues from diseased carpal and fetlock joints identified both OCF and synovial membranes as cellular sources of IL-6 and extracellular/cytoplasmic HMBG-1. Histological assessment of synovial membrane biopsies from immunostained joints showed increased grade of synovitis in diseased compared to healthy joints. The effects of IL-6, IL-1β and HMGB-1 on chondrocyte metabolism was studied *in vitro*, and focused on gene expression analyses. A catabolic response to IL-1β was detected, whereas the response to IL-6 and HMGB-1 was varied but indicated promotion of cartilage formation. Analyses of the ultrastructural immunolocalisation of HMGB-1 in IL-1β and IL-6 treated pellets, showed a trend for decreased nuclear/cytoplasmic ratio, and increased extracellular matrix density of HMGB-1 after IL-6 treatment.

Theses studies gained insight to presence of TNF, IL-6 and HMGB-1 in equine joints, and indicated an increased inflammatory response in joints with OCF. The difference in metabolic response of chondrocytes to IL-1β versus IL-6 or HMGB-1 *in vitro*, and differences in HMGB-1 translocation after IL-1β and IL-6 treatment suggest that the outcome of cartilage pathology in equine joint disease may relate to the individual contributions of cytokine activity and cytokine interactions.

Keywords: cytokines, HMGB-1, horse, IL-1, IL-6, osteoarthritis, osteochondral fragmentation, synovitis, TNF

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Dedication

To my husband Charles, and my children Timothy, Kristoffer and Edward

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Ley, C., Ekman, S., Elmén, A., Nilsson, G. and Eloranta, ML. (2007). Interleukin-6 and tumour necrosis factor in synovial fluid from horses with carpal joint pathology. *Journal of Veterinary Medicine. A, Physiology, Pathology, Clinical Medicine* 54, 346-351.

II Ley, C., Ekman, S., Ronéus, B. and Eloranta, ML. (2009). Interleukin-6 and high mobility group box protein-1 in synovial membranes and osteochondral fragments in equine osteoarthritis. *Research in Veterinary Science* 86, 490-497.

III Ley, C., Svala, E., Nilton, A., Lindahl, A., Eloranta, ML., Ekman, S. and Skiöldebrand, E. Effects of high mobility group box protein-1, interleukin-1β and -6 on cartilage matrix metabolism in three dimensional equine chondrocyte cultures (manuscript).

IV Ley, C., Svala, E., Skiöldebrand, E., Blomén, E., Hultenby, K., Eloranta, ML. and Ekman, S. Gene expression and immunolocalisation of high mobility group box protein (HMGB)-1 in interleukin (IL)-1β and -6 stimulated three dimensional chondrocyte cultures (manuscript).

Abbreviations

ADAMTS	a disintegrin and matrix metalloproteinase with thrombospondin motifs
C3	third carpal bone
cDNA	complementary deoxyribonucleic acid
COMP	cartilage oligomeric matrix protein
CT	cycle treshold
DNA	deoxyribonucleic acid
DRF	dorsal radial facet
ECM	extracellular matrix
GAG	glycosaminoglycan
GUSB	beta glucoronidase
HMGB	high mobility group box protein
IL	interleukin
IL-1R	interleukin-1 receptor
IL-1Ra	interleukin-1 receptor antagonist
IL-6Rα	interleukin-6 alpha receptor
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
N/C	nuclear/cytoplasmic
OA	osteoarthritis
OCF	osteochondral fragment
PC	palmar condyle
PGE2	prostaglandin E2
qRT-PCR	quantitative real time reverse transcriptase polymerase chain reaction
RNA	ribonucleic acid
sIL-6Rα	soluble interleukin-6 alpha receptor
Sox9	sex determining region on the Y chromosome-related high

	mobility group box 9
TIMP	tissue inhibitor of metalloproteinase
TNF	tumour necrosis factor

1 Introduction

1.1 General background

Traumatically induced arthritis is a common cause of joint disease in athletic horses and is associated with numerous conditions, including traumatic synovitis and capsulitis, ligamentous or meniscal injuries, subchondral bone disease, osteochondral fractures and osteoarthritis (McIlwraith, 2001). A recent study showed that 11 and 13% of ailments in two and three year old Thoroughbred horses, respectively, related to joint problems (Wilsher *et al.*, 2006). In athletic horses, the carpal and fetlock joints are often involved, and accounted (in equal parts) for 28% of cases with lameness resulting in lost days of training in Thoroughbred racehorses (Rossdale *et al.*, 1985). Furthermore, middle carpal lameness has been reported to develop in 30% of Standardbred horses during their first 12-18 months of training (Hopper *et al.*, 2004), and was the most common cause for extended rest (Steel *et al.*, 2006). However, also in non-racing horses joint problems are common. A Swedish study on cause-specific morbidity, based upon insurance company data on non-racing horses between 1997-2000, showed that the joints were the most commonly affected part of the body, and that fetlock arthritis was the most common diagnosis (Penell *et al.*, 2005).

Joint lesions involving the articular cartilage pose special concerns as the adult articular cartilage shows little capacity for intrinsic healing, and satisfactory repair of the tissue is difficult to achieve (Nixon & Fortier, 2001; Buckwalter *et al.*, 1990; Convery *et al.*, 1972). In addition, subtle or early changes of cartilage disease are difficult to diagnose with routinely available clinical aids. When cartilage lesions of training and racing horses are diagnosed concurrent bone and soft tissue changes are commonly present (Pool & Meagher, 1990), making it difficult to determine the sequence of

events in the diseased joint. As any traumatic injury will initiate an inflammatory response, studies of inflammatory processes in joint tissues may increase the understanding of the mechanisms contributing to cartilage damage and increase the possibilities to detect early pathologic changes in the cartilage, thereby preventing chronic and irreversible joint disease.

1.2 The carpal and fetlock joints

Joints are formed by unions of bone or cartilage by other tissue. The carpal and fetlock joints are diarthrodial joints, i.e. movable joints that demonstrate a joint cavity (containing synovial fluid), a lining of synovial membrane, and articular cartilage (Sisson, 1953). However, the subchondral bone and other structural elements, such as muscles, tendons, ligaments, bursae and menisci are also required to provide appropriate joint function (Sledge *et al.*, 2001).

The carpal joint consists of three major compartments and two rows of carpal bones. The proximal row include the radial, intermediate, ulnar and accessory carpal bones, and the distal row the first (sometimes missing) second, third, fourth and fifth (often missing) carpal bones (Butler *et al.*, 2000). In addition to several peri- and intra-articular ligaments, the proximal and distal rows of the carpal bones are connected via the medial and lateral palmar intercarpal ligaments. The medial palmar intercarpal ligament attaches proximally to the radial carpal bone and distally to the second and third carpal bones (C3) (Whitton *et al.*, 1997b; Phillips & Wright, 1994). The lateral palmar intercarpal ligament, attaches proximally to the ulnar and intermediate carpal bones and distally to C3 and the fourth carpal bone (Whitton *et al.*, 1997b; Phillips & Wright, 1994) (Fig. 1). The palmar intercarpal ligaments have been proposed to absorb loads created by medial to lateral displacement of the carpal bones during axial weight bearing (Bramlage *et al.*, 1988), but they also prevent dorsal displacement of the proximal row of carpal bones (Whitton & Rose, 1997).

The large amount of traumatic injuries seen in the dorsal parts of the radiocarpal and middle (inter-) carpal joint may partly relate to altered contact and pressure patterns seen with increased mechanical loading, as described for C3 (Palmer *et al.*, 1994). In addition, fractures arise as strenuous exercise exposes the dorsal margins of the bones of the intercarpal joint to harmful forces when they come together with sudden focal high impact, or the dorsal margins of the bones of the radiocarpal joint are brought together when the joint is overrotated at high racing speed (Bramlage, 1983).

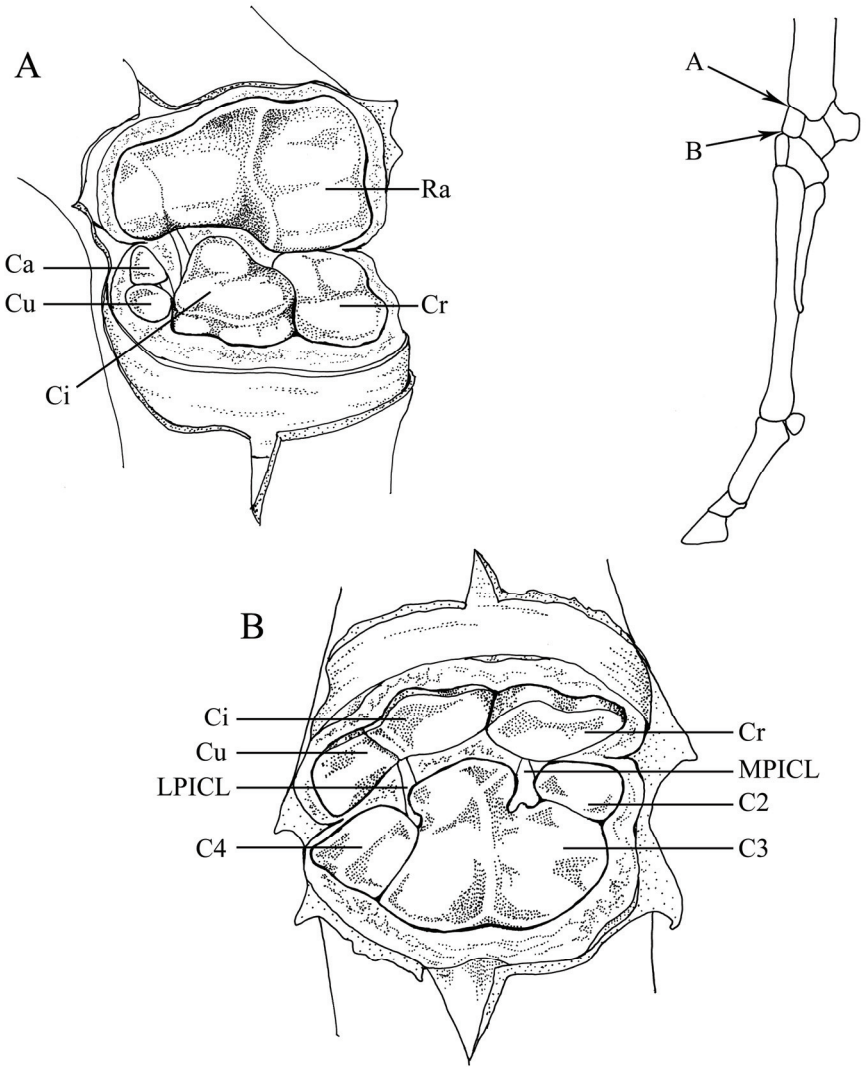

Figure 1. Line drawings of the articular surfaces of the upper (radiocarpal, A) and middle (intercarpal, B) compartments of the equine carpus. (A) Ca = accessory carpal bone; Ci = intermediate carpal bone; Rc = radial carpal bone; Ra = radius. (B) C2 = second carpal bone, C3 = third carpal bone; C4 = fourth carpal bone; LPICL = lateral palmar intercarpal ligament; MPICL = medial palmar intercarpal ligament. (Drawings by Karolina Larsson.)

The fetlock joints (metacarpo-/metatarsophalangeal joints) are formed by the distal third metacarpal/metatarsal bone, the proximal part of the proximal phalanx, and the two proximal sesamoid bones, embedded in the intersesamoidean ligament (Fig. 2). The joint has a thick joint capsule, several external ligaments, including the suspensory ligament (Sisson, 1953).

It has been proposed that the joint is particularly susceptible to traumatic injury due to its small surface area, its great range of motion, and its role in weight transmission during stride (Pool & Meagher, 1990). The contact area between the third metacarpal bone and the proximal phalanx is increased with loading, and the highest pressures are created at the dorsal articular margins (Brama *et al.*, 2001), which is a common location for osteochondral pathology (Pool & Meagher, 1990).

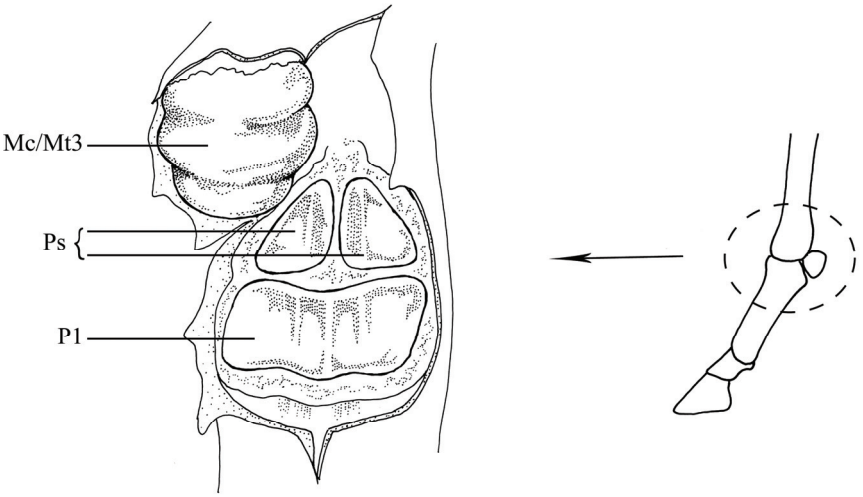

Figure 2. Line drawing of the articular surfaces of the equine metacarpo-/metatarsophalangeal joint. Mc/Mt3 = third metacarpal/-tarsal bone; Ps = proximal sesamoid bones; P1 = proximal phalanx. (Drawings by Karolina Larsson.)

1.3 Joint structures

The joint can only properly operate as an organ when the articular tissues are in metabolic balance, and provide appropriate interaction with each other. In this section, breif descriptions of components (Fig. 3A-C) and functions of the tissues of the synovial joints are given.

1.3.1 Osteochondral tissues

Articular cartilage provides low friction movements between bones, and absorbs and distributes mechanical loads applied to the joint. It is composed of extracellular matrix (ECM), matrix water, and a small proportion of chondrocytes, which are responsible for matrix synthesis and metabolic homeostasis. The main structural components of the ECM are collagens

(approximately 60% of the dry weight of the cartilage), proteoglycans (25-30% of the dry weight) and non-collagenous proteins and glycoproteins (15-20% of the dry weight) (Buckwalter & Hunziker, 1999).

The collagen framework provides the cartilage with tensile strength. Collagen type II, the principal collagen in articular cartilage, is arranged in crosslinked triple helices and form a fibrillar network together with smaller amounts of other types of collagens, such as type IX, XI, III and V (Sandell *et al.*, 2007). In addition, collagen type VI is found in the pericellular matrix and may bridge the chondrocyte to surrounding ECM (Poole *et al.*, 1992).

Proteoglycans provide the cartilage with compressive properties, due to their negatively charged glycosaminoglycan (GAG) side chains, which attract water by creating a large osmotic swelling pressure (Kiani *et al.*, 2002). The chondroitin sulphate rich proteoglycan aggrecan is the dominating proteoglycan in articular cartilage, and it binds to hyaluronan to form large aggregates (Kiani *et al.*, 2002).

In addition, the ECM contains a large number of proteins, several belonging to the family of leucine- rich repeat proteins, which may bind to collagens and membrane molecules (integrins), and the family of thrombospondins, including the collagen binding glycoprotein cartilage oligomeric matrix protein (COMP) (Sandell *et al.*, 2007).

The adult articular cartilage is avascular, alymphatic and aneural, and relies upon nourishment from the synovial fluid and the vasculature of the subchondral bone (Goldring & Goldring, 2005). Morphologically articular cartilage is divided into four zones: superficial, intermediate, deep and calcified (Fig. 3B). The superficial zone shows chondrocytes with a flattened cellular shape, low proteoglycan content and abundant collagen fibrils, arranged parallel to the cartilage surface. In the intermediate zone cells become more spheroid, the collagen fibrils thinner and more dispersed and proteoglycan content increased. In the deep zone chondrocytes are arranged perpendicular to the joint surface, thick collagen fibrils run parallel to chondrocyte orientation and proteoglycan content is high (Goldring & Goldring, 2005). Influences on the biochemical content of cartilage from site and/or exercise has been demonstrated in several equine studies of carpal cartilage (Murray *et al.*, 2001a; Murray *et al.*, 2001b; Murray *et al.*, 2000; Palmer *et al.*, 1995a), as well as in cartilage from the proximal phalanx (Brama *et al.*, 2009; Brama *et al.*, 2002; Brama *et al.*, 2000a; Brama *et al.*, 2000b; Brama *et al.*, 2000c; Brama *et al.*, 1999) and the third metacarpal bone (Brama *et al.*, 2000c).

The so called tidemark provides a demarcation between non-calcified and calcified cartilage (Fig. 3B). The tidemark can be distinguished due to its

enhanced metachromatic staining pattern, and has been suggested to be the result of the accumulation of nuclear debris (Simkin, 2008) and non-specific molecules caused by discontinuous mineralisation (Oegema *et al.*, 1997). The zone of calcified cartilage is located between the tidemark and the subchondral bone. Its boundaries are determined by the moving of the tidemark into the non-calcified cartilage and by replacement of calcified cartilage by subchondral bone. Increased thickness of the calcified cartilage has been reported in carpal cartilage from horses subjected to high intensity exercise compared to walking exercise (Murray *et al.*, 1999). The zone of calcified cartilage is involved in force transmission and dissipation at the bone-cartilage interface and constitutes a diffusion barrier between the bone and non-calcified articular cartilage (Oegema *et al.*, 1997). As indicated from ultrastructural studies, chondrocytes of the calcified cartilage have little metabolic activity (Buckwalter & Hunziker, 1999).

The subchondral bone absorbs mechanically induced stresses to provide cushioning for the joint, and ensure maintenance of an incongruent joint surface (Kawcak *et al.*, 2001). The subchondral bone is composed of a cortical bone plate (compact bone) and of trabecular (cancellous) bone. Bone is continuously transformed in response to loading conditions, and its strength depends on material properties as well as structural arrangement (Burr, 2004). Influence from loading on bone characteristics in horses has been demonstrated in the C3 and the radial carpal bone, where increased bone formation, remodeling and collagen synthesis was found in racing horses compared to non-racing horses (Tidswell *et al.*, 2008). In studies of the distal equine third metacarpal bone, a sagittal direction of main trabeculae of the subchondral cancellous bone and less significant connections mediolaterally was demonstrated, suggesting maximum stiffness in the direction of the greatest peak loads (Boyde *et al.*, 1999). Bone formation is carried out by osteoblasts, which synthesize osteoid that subsequently is mineralised, whereas bone resorption involves the breakdown of mineralised tissue by osteoclasts (Schenk *et al.*, 2002).

1.3.2 Joint capsule and ligaments

The outer parts of the joint capsule and the ligaments have joint stabilizing function. Ligaments share macroscopic similarities with tendons but the biochemical and structural composition differ (Rumian *et al.*, 2007; Amiel *et al.*, 1984), so that ligaments can withstand high tensile forces applied in multiaxial patterns. Bundles of collagen (predominanty collagen type I) are arranged in a wavy "crimp" pattern, with a relatively higher collagen type III conent in ligaments compared to tendons (Amiel *et al.*, 1984).

The internal part of the joint capsule, i.e. the synovial membrane or synovium, produces components of the synovial fluid and provides nutrients for the articular cartilage. It may form villous projections into the joint cavity. Cells of the inner cellular lining layer (synovial intima) consist of macrophage-derived type A synoviocytes (Fig. 3C) with phagocytic and antigen presenting abilities, fibroblast-derived secretory type B synoviocytes, and transitional forms thereof (type C cells) (Goldring & Goldring, 2005; Iwanaga *et al.*, 2000). In the sublining tissue (synovial stroma or subintima) cells are predominantly fibroblasts. In the horse, three types of synovial membrane are recognized depending on the structure of the underlying stroma; areolar (characterized by loose connective tissue), adipose and fibrous (Johansson & Rejno, 1976), and four types of villi (bell, tongue, leaf and sword type) have been identified, distinguished by their shape and length (Izumisawa *et al.*, 1996). A predominance of type A synoviocytes has been reported in synovial villi from fetlock joints (Izumisawa et al., 1996), although another study showed a predominance of type B synoviocytes in healthy fetlock, hock and stifle joints, with a relative increase in type A cells in diseased joints (Johansson & Rejno, 1976). In carpal joints, type A synoviocytes were mainly localised at the tip of the villi and type B synoviocytes showed a more even distribution (Shikichi et al., 1999).

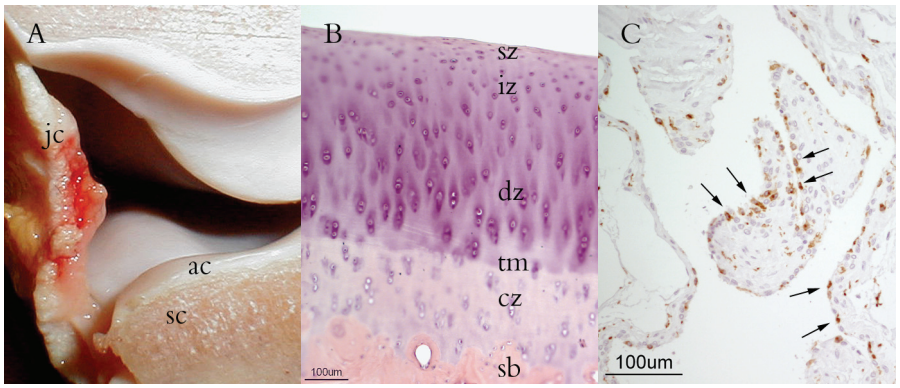

Figure 3. (A) Sagittal section of a synovial joint (radiocarpal joint), showing joint capsule (jc), articular cartilage (ac) and subchondral bone (sc). (B) Photomicrograph of articular cartilage, showing the cartilage zones (superficial = sz; intermediate = iz; deep = dz; calcified = cz). (C) Photomicrograph of synovial membrane immunostained for the macrophage marker CD163, showing presence of numerous type A synoviocytes in the lining layer (arrows).

1.3.3 Synovial fluid

Synovial fluid provides nutrients and lubrication for the articular cartilage. It is formed by an ultrafiltrate of plasma and locally produced substances. Hyaluronan, synthezised by type B synoviocytes, provides the viscoelastic properties of synovial fluid, and is involved in cartilage lubrication, restriction of plasma proteins from the synovial fluid, and retention of synovial fluid water (Ghosh & Guidolin, 2002). In addition, lubrication is provided by lubricin (Swann *et al.*, 1985; Swann *et al.*, 1981), which is synthesized by synovial fibroblasts (Jay *et al.*, 2000), and the highly homologous proteoglycan superficial zone protein (Neu *et al.*, 2007; Jay *et al.*, 2001), produced by chondrocytes of the superficial zone of the articular cartilage (Schumacher *et al.*, 1994).

Whereas red blood cells are not normally present in equine synovial fluid, the leukocyte count varies between joints (Persson, 1971). However, more recent literature on equine synovial fluid gives a general reference of 50–500 leukocytes/μl, with less than 10% neutrophils (Caron, 2003). Concentrations of proteins in synovial fluid are dependent of several factors; plasma concentration, synovial blood flow, microvascular permeability, lymphatic drainage, local protein production and degradation (Goldring & Goldring, 2005). However, total protein concentrations in equine synovial fluid normally do not exceed 2.5 g/dl (Caron, 2003; McIlwraith, 1980).

1.4 Definition and aetiology of osteoarthritis (OA)

In humans, OA has been defined as a group of diseases sharing similar biological, morphological and clinical outcomes (Flores & Hochberg, 2003). It has been classified as either idiopathic (primary) or secondary, with the former further categorized as localised or generalised (Flores & Hochberg, 2003). However, the existence of primary OA is debated, and it has been suggested that OA should be viewed as a process representing the pathophysiological response of the joint to mechanical insults (Brandt *et al.*, 2008). The term OA points out involvement of subchondral bone, and the suffix -itis implies an inflammatory component of the disease.

Equine OA has been described as the common end stage of a group of disorders characterized by progressive and permanent deterioration of the articular cartilage, accompanied by changes in the subchondral bone and articular soft tissues (McIlwraith, 2002; McIlwraith, 1996). Further classification of equine OA into three types was proposed by McIlwraith (1996); type I, associated with synovitis and capsulitis, and commonly seen in carpal, fetlock, distal tarsal and distal interphalangeal joints; type 2,

associated with (and usually secondary to) other identified injuries or problems; type 3, incidental or non-progressive cartilage erosion. Other terms commonly used for OA include degenerative joint disease and osteoarthrosis.

Repeated microtrauma caused by mechanical forces has been suggested to be the major aetiology of equine OA (Caron, 2003). Whether the articular cartilage is primary affected, or cartilage lesions arise secondary to changes in other tissues (subchondral bone or synovial membrane) has not been clarified. Increased bone stiffness and reduced load absorbing capability, seen as a result of suboptimal healing and remodeling of mechanically induced subchondral microfractures, may expose the cartilage to harmful stresses when normal deformation of the bone-cartilage zone fails (Caron, 2003). Furthermore, microcracks in the calcified cartilage and subchondral bone are thought to trigger reactivation of secondary centers of ossification, resulting in advancement and reduplication of the tidemark and thinning of the articular cartilage, with increases in shear stresses at the base of the cartilage and loss of cartilage as result (Burr & Radin, 2003).

In addition, synovitis is usually present in equine OA (McIlwraith, 1996), and may precede cartilage degeneration in carpal and fetlock joints of racing horses (McIlwraith, 1982). Several theories on how soft tissue injuries may contribute to OA exist, and include induction of joint instability, impairment of synovial capillary blood flow and reperfusion injury, and production of inflammatory mediators and degradative enzymes in mechanically damaged tissues (McIlwraith, 1996).

1.5 Inflammation and OA

Traditionally regarded inflammation is a reaction of vascularised tissues to infection or injury. Optimally, the inflammatory processes provide protection of the individual, and contribute to processes promoting healing.

As part of the early (acute) inflammatory response there is vascular adaptation to increase blood flow, vascular permeability and recruitment of leukocytes, processes that are regulated by a large number of the soluble mediators, including vasoactive amines (eg histamine and serotonin), arachidonic acid metabolites (prostaglandins, leukotrienes and lipoxins), platelet-activating factor, reactive oxygen species, nitric oxide, cytokines and chemokines (Kumar *et al.*, 2010). Cytokines act as signalling molecules inducing a cellular response after binding to their appropriate receptors. The fine tuning of their biological actions are promoted by cytokine networks, intricately regulated by receptor antagonists, soluble receptors, cytokines

with opposing effects and decoy receptors (Balkwill, 2001). Chronic inflammation, morphologically characterized by neovascularisation, involvement of mononuclear inflammatory cells and fibrosis may occur with extensive tissue destruction, involvement of tissues incapable of regeneration, and failure of the early inflammatory response to resolve (Kumar *et al.*, 2010).

Studies of inflammation in OA provide special concerns, as the articular cartilage is not a vascularised tissue. Morphologically, inflammation may be present in the synovial membrane, and synovitis is not an uncommon finding in either equine (McIlwraith, 1996) or human OA (Haywood *et al.*, 2003; Smith *et al.*, 1997; Goldenberg *et al.*, 1982). Increased mononuclear infiltrates, blood vessel formation, and numbers of synovial lining layers have been reported in early (duration < 1 year) human OA compared to late stages (Benito *et al.*, 2005). Another human study reported the most pronounced inflammatory changes in synovial membrane from patients with severe OA; however, overlap of morphological findings in the synovial membrane was seen between different grades of OA, and between normal and OA joints (Smith *et al.*, 1997). In naturally occurring equine joint disease, it was reported that synovitis and articular cartilage lesions often were of comparable grades (Ronéus *et al.*, 1997), however synovial membrane biopsies have also been found to be non-specific diagnostically (Trotter & McIlwraith, 1996). Short (84 days post trauma) and long (180 days post trauma) term studies of experimental impact-induced OA in the horse revealed few changes in synovial membranes, with minor infiltration of inflammatory cells (Bolam *et al.*, 2006). No difference in synovitis was found between OA and control joints in an instability-induced equine OA model (Simmons *et al.*, 1999). Similar findings are described in mechanically induced experimental OA in rabbits, where only synovial hyperplasia (without other morphologic signs of inflammation) was noted to precede cartilage destruction (Walker *et al.*, 1991).

However, inflammation on a molecular level may be present in OA joints. Initial stages of OA are characterized by increased metabolic activity of chondrocytes, including cell proliferation, increased synthesis of growth factors and matrix proteins, but also by production of proinflammatory cytokines and other inflammatory mediators (Goldring & Goldring, 2007), and with disease progression catabolic processes, which may be induced by inflammatory mediators, become dominating (schematically outlined in Fig. 4).

The proinflammatory cytokines interleukin (IL)-1 beta (β) and tumour necrosis factor (TNF) alpha (α) have been suggested to be of major

importance in the development of human OA (Pelletier *et al.*, 2001). They are thought to influence the disease by inducing production of other inflammatory mediators, including prostaglandin E2 (PGE2), nitric oxide, IL-6, IL-8, IL-17, IL-18, leukemia inhibitory factor, and matrix degrading enzymes, and suppressing genes associated with differentiated chondrocytes (Goldring & Goldring, 2007). IL-1 (α and/or β) and TNFα induce similar activities on articular tissues, including leukocyte infiltration (Henderson & Pettipher, 1989), inhibition of proteoglycan synthesis (Wilbrink *et al.*, 1991), and induction of collagenase and PGE2 production in synovial cells (Hauptmann *et al.*, 1991; Lotz & Guerne, 1991). However, IL-1 has been shown to be considerably more potent than TNFα, and together they can show synergistic effects (Wilbrink *et al.*, 1991; Henderson & Pettipher, 1989). Apoptotic effects of IL-1 and TNFα on chondrocytes *in vitro* have been described (Kim *et al.*, 2010; Schuerwegh *et al.*, 2003), although conflicting results have been reported by others (Relić *et al.*, 2002; Kühn *et al.*, 2000; Blanco *et al.*, 1995). Several other proinflammatory cytokines, such as IL-6 and IL-17, have also been discussed to be involved in the process of cartilage destruction in OA (Malemud, 2004).

Matrix metalloproteinases (MMP)s, which traditionally can be subgrouped according to their substrates into collagenases, gelatinases and stromelysins, appear to be important matrix degrading agents contributing to cartilage loss in OA (Poole *et al.*, 2007). In particular, MMP-13 may be important in cleaving collagenase type II (Knäuper *et al.*, 1996), however also MMP-1, -3 and -8 are found in OA cartilage (Tetlow *et al.*, 2001). In addition, the aggrecanases ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs)-4 and -5, have been shown to cleave aggrecan in several sites, and are proposed to be important in early stages of OA (Huang & Wu, 2008).

Several equine studies have investigated presence of MMP activities in naturally occurring joint disease. In such studies, OA has sometimes been grouped together with other types of aseptic joint disease, leaving levels in OA joints not further specified. Increased synovial fluid MMP activity (Brama *et al.*, 1998), and activities of MMP-1 (collagenase I) and -3 (stromelysin I) have been detected in osteoarthritic equine joints (Brama *et al.*, 2004; Brama *et al.*, 2000d). In addition, increased synovial fluid activities of MMP-2 (gelatinase A) and -9 (gelatinase B) in aseptic arthritic joints (including OA, articular fractures, osteochondrosis dissecans and traumatic arthritis) have been found (Clegg *et al.*, 1997). Although in another study, presence of MMP-2 and -9 in synovial fluid in aseptic joint disease (including acute and chronic traumatic arthritis and osteochondrosis

dissecans) was mainly related to the detection of pro-forms and less often to active enzymes (Trumble *et al.*, 2001). Furthermore, increased synovial fluid activity of MMP-9 was found in equine joints with severe cartilage lesions related to OA, osteochondrosis or enlarged synovial fossae (Jouglin *et al.*, 2000). Activity of tissue inhibitor of MMPs (TIMP)s has been demonstrated in normal and arthritic equine joints, with increased activity in aseptic joint disease (Clegg *et al.*, 1998).

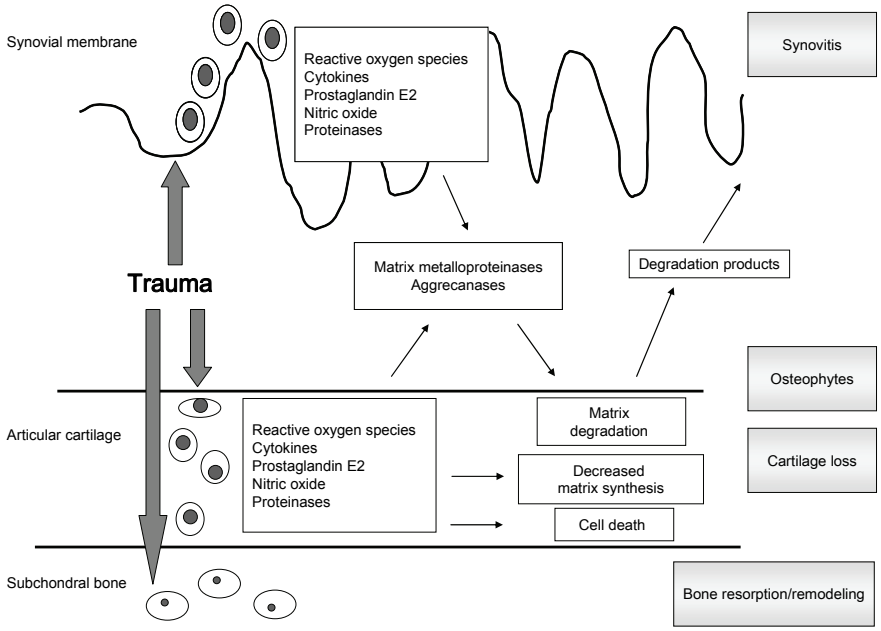

Figure 4. Simplified schematic description of metabolic processes in traumatically induced osteoarthritis.

The work included in this thesis focused on the following proinflammatory cytokines: TNF, IL-6, IL-1 and high mobility group box protein (HMGB)-1. Thus, these inflammatory mediators will be discussed in further detail.

1.6 Interleukin-1

IL-1 belongs to the interleukin-1 cytokine family, which currently consists of 11 members (Dinarello, 2009). IL-1β, as well as IL-1α and IL-1 receptor antagonist (IL-1Ra) binds to the IL-1 receptors (IL-1R) I and II. IL-1RI is

found on most cells, and signalling is induced by IL-1α and IL-1β, but not by IL-1Ra (Arend, 2002). IL-1RII is found mainly on neutrophils, monocytes/macrophages and B-lymphocytes, and functions as a decoy-receptor (Colotta et al., 1993). IL-1 activity is further regulated by the soluble forms of IL-1RI and IL-1RII, and soluble IL-1R accessory protein (Jacques *et al.*, 2006). Precursor IL-1β is processed by caspase-1 (also known as IL-1β converting enzyme) and other non-caspase-1 mechanisms to form a biologically active protein (Dinarello, 2009; Dinarello, 1998), which in horses is proposed to have a molecular mass of 17,300 Da (Howard *et al.*, 1998) and show 66.4-66.7% amino acid homology with human IL-1β (Howard *et al.*, 1998; Kato *et al.*, 1995).

Increased bioactivity of IL-1 has been detected in various equine joint conditions including OA (Alwan *et al.*, 1991b; Morris *et al.*, 1990), and IL-1β has been demonstrated in articular cartilage from OA affected equine joints (Weaver et al., 2006). Messenger ribonucleic acid (mRNA) expression for IL-1α and β has been detected in synovial membrane and articular cartilage from joints with acute and chronic traumatic disease, as well as from normal joints (Trumble *et al.*, 2001), and in experimentally induced synovitis immunostaining for IL-1β was increased in cartilage and synovial membrane (Todhunter *et al.*, 1996). Early *in vitro* studies on IL-1 in equine joint research identified this cytokine as an inducer of PGE2 production (May *et al.*, 1992c; May *et al.*, 1992a; May *et al.*, 1989) in chondrocytes and synoviocytes, and of protease activity (Spiers *et al.*, 1994) in synoviocytes. Since then, IL-1β dependent up-regulation of PGE2 production in chondrocytes has been shown to be associated with up-regulation of cyclooxygenase-2 (Farley *et al.*, 2005; Tung *et al.*, 2002b) and microsomal prostaglandin E synthase-1 expression (Farley *et al.*, 2005). In addition, IL-1β stimulation of equine chondrocytes results in nitric oxide production (Frean *et al.*, 1997). Several studies on equine chondrocytes have demonstrated induction of gene expressions of matrix degrading enzymes in response to IL-1 (Little *et al.*, 2005; Takafuji *et al.*, 2005; Tung *et al.*, 2002a; Richardson & Dodge, 2000; Caron *et al.*, 1996). In addition, inhibition of proteoglycan synthesis (Takafuji *et al.*, 2002; Bird *et al.*, 1997; Morris & Treadwell, 1994; Platt & Bayliss, 1994; MacDonald *et al.*, 1992), and induction of GAG release after IL-1 exposure have been shown in equine cartilage explants (Garvican *et al.*, 2008; Gregg *et al.*, 2006; Little *et al.*, 2005; Takafuji *et al.*, 2002; Frean *et al.*, 2000; Bird *et al.*, 1997; MacDonald *et al.*, 1992). These data suggest that IL-1 plays a role in the disease process of equine OA.

Synovial fluid from normal equine joints and joints with early and chronic joint disease showed an inhibitory effect on synoviocyte PGE2 production induced by human recombinant IL-1α *in vitro* (May *et al.*, 1992b). However, when the synoviocyte cultures were stimulated with human IL-1β the inhibitory effect was only demonstrated in synovial fluid from normal joints and joints with early disease, and absent in synovial fluid from chronically diseased joints (May *et al.*, 1992b). Therapy aimed at reducing the biological action of IL-1 has been investigated in experimentally induced equine OA, by the use of as autologous conditioned serum (Frisbie *et al.*, 2007), and by gene therapy using equine IL-1Ra and an adenoviral vector (Frisbie *et al.*, 2002). In both studies, clinical improvement was noted with specific treatment. In addition, the former study showed decreased synovial membrane hyperplasia with treatment, but no improvement in articular cartilage morphology, and the latter study showed improved macroscopic cartilage erosions, preservation of articular cartilage proteoglycan content, decreased synovial membrane vascularity, but increased synovial membrane lymphocytic infiltrates. Combined transduction of equine synoviocytes with IL-1Ra and the anabolic growth factor insulin-like growth factor-1 using adenoviral vectors, prevented IL-1β induced proteoglycan loss in co-culures with cartilage explants (Haupt *et al.*, 2005). Furthermore, intra-articular injection with equine IL-1Ra/insulin-like growth factor-1 adenoviral preparations in joints with microfractured chondral defects increased the defect content of proteoglycans (although this was also seen in placebo treated joints) and collagen type II, but macroscopic and histomorphometric analyses indicated formation of similar repair tissue between treated (including placebo) and control joints (Morisset *et al.*, 2007).

1.7 Tumour necrosis factor α

TNFα belongs to the TNF superfamily, which consists of 19 ligands and 30 receptors (Croft, 2009). The mature protein is formed by cleavage of precursor TNFα by TNFα converting enzyme (Black *et al.*, 1997). Trimerized TNFα and the homologous cytokine TNFβ are ligands to the TNF receptors 1 and 2, the former expressed on most cell types, and suggested to be the receptor for soluble TNF, and the latter present mainly on immune and endothelial cells, and suggested to be the receptor for membrane bound TNF (Aggarwal, 2003; Grell *et al.*, 1995). Depending on receptor ligation and activation of different intracellular signalling pathways, TNFα is known to either induce apoptosis or transcription factors involved

in the induction of genes of the inflammatory response (Aggarwal, 2003). The nucleotide sequence of the equine TNFα gene was demonstrated by Su *et al.* (Su *et al.*, 1991), who also generated recombinant products that could be cleaved into a 17,000 Da protein (Su *et al.*, 1992), consistent with the size of human mature TNFα (Aggarwal *et al.*, 1985). The amino acid sequence homology between human and equine TNFα was reported to be 87% (Su *et al.*, 1992).

As for IL-1, TNFα has been shown to induce matrix degradation and increased gene expression of matrix degrading enzymes in equine chondrocytes (Little *et al.*, 2005; Richardson & Dodge, 2000). In experimentally induced synovitis, increased immunostaining for TNFα and its two receptors have been shown in cells of the synovial membrane and in cartilage (Todhunter *et al.*, 1996). Whereas, articular cartilage in acute traumatic joint disease showed increased mRNA expression for TNFα, cartilage from joints with chronic traumatic disease did not (Trumble *et al.*, 2001). Increased bioactivity of TNF was found in synovial fluid from joints with major intra-articular fractures, but not from joints with synovitis (Billinghurst *et al.*, 1995). In a study focusing on cartilage pathology no correlation was found between degree of joint damage and TNF activity, although a tendency for increased TNF activity was seen with numerous or severe changes, and no differences in TNF activity was seen in regard to aetiology of lesions (Jouglin et al., 2000).

Also in normal synovial membrane and articular cartilage TNFα mRNA expression has been demonstrated (Trumble *et al.*, 2001). Bioactivity of TNF has also been detected in synovial fluid from normal joints (Billinghurst *et al.*, 1995) and after exercise (van den Boom *et al.*, 2004).

1.8 Interleukin-6

IL-6 belongs to the IL-6 subfamily of cytokines, which all share the signal transducing receptor gp130 (Rose-John *et al.*, 2006). By the binding of IL-6 and the ligand-binding IL-6 α receptor (IL-6Rα) to membrane bound gp130, a signalling complex is formed (Boulanger *et al.*, 2003). Soluble forms of IL-6Rα (sIL-6Rα) may mediate the effects of IL-6 on cells that naturally lack expression of IL-6Rα, a phenomenon referred to as trans-signalling (Rose-John *et al.*, 2006). In addition, gp130 also exists in soluble forms and can bind to IL-6/sIL-6Rα and inhibit induction of biological effects (Narazaki *et al.*, 1993).

Equine IL-6 was originally sequenced and cloned as an 180 amino acid mature protein with a predicted molecular mass of 20,471 Da and 77.6%

amino acid homology with human IL-6 (Swiderski *et al.*, 2000). Studies on IL-6 in equine joint disease are few, although IL-6 is known to be produced in the inflammatory response in experimental lipopolysaccaride induced arthritis (Hawkins *et al.*, 1995; Hawkins *et al.*, 1993) and in synovectomy induced synovitis (Theoret *et al.*, 1998). Also fibrous tissue of subchondral bone cysts show an up-regulation of IL-6 mRNA (von Rechenberg *et al.*, 2001). In addition, IL-6 mRNA expression is increased in equine chondrocytes by IL-1β stimulation (David *et al.*, 2007).

The exact role of IL-6 in OA and on cartilage metabolism is not clear. IL-6 has been found in synovial fluid from human osteoarthritic joints (Doß *et al.*, 2007; Kaneko *et al.*, 2000; Remick *et al.*, 1992), and increased IL-6 activity has been reported in canine OA secondary to cranial cruciate ligament rupture (Hay *et al.*, 1997). IL-6 appears to have a chondroprotective effect (including reduced cartilage loss) in murine models of arthritis (de Hooge *et al.*, 2005), and IL-6 reduces IL-1 induced PGE2 production (Hauptmann *et al.*, 1991) and stimulates TIMP production (Shingu *et al.*, 1995; Shingu *et al.*, 1993; Lotz & Guerne, 1991) in human chondrocytes and synoviocytes. On the hand, other studies have shown IL-6 induced inhibition of proteoglycan synthesis and potentiation of IL-1 induced proteoglycan catabolism (Jikko *et al.*, 1998; Nietfeld *et al.*, 1990). Together with sIL-6Rα, IL-6 induced inhibition of proteoglycan synthesis was potentiated (Guerne *et al.*, 1999), and IL-1 induced proteoglycan and collagen degradation enhanced (Rowan *et al.*, 2001; Flannery *et al.*, 2000), suggesting that the effects from IL-6 can be related to the presence of sIL-6Rα.

1.9 High mobility group box protein-1

HMGB-1 is a chromosomal protein belonging to the HMGB superfamily (Bustin, 2001). It is evolutionary conserved, and its nuclear functions include nucleosome stabilisation and regulation of gene transcription (Bianchi & Agresti, 2005). Structurally, HMGB-1 is a 215 amino acid protein composed of two deoxyribonucleic acid (DNA)-binding domains (the A and B boxes) and a carboxyterminal acidic tail (Dumitriu *et al.*, 2005a; Bustin, 1999). Whereas the inflammatory action of HMGB-1 resides in the B-box (Li *et al.*, 2003), the A-box shows antagonistic action to HMGB-1 mediated effects (Yang *et al.*, 2004; Kokkola *et al.*, 2003).

HMGB-1 is classified as an alarmin, an endogenous molecule signalling cell or tissue damage (Bianchi, 2007). Extracellular HMGB-1 may arise through leakage from damaged or dying cells (Scaffidi *et al.*, 2002; Degryse

et al., 2001). Furthermore, it appears that HMGB-1 release from necrotic cells stimulate an inflammatory response (El Mezayen *et al.*, 2007; Scaffidi *et al.*, 2002). In addition, immune cells, such macrophages (Wang *et al.*, 1999a), monocytes (Gardella *et al.*, 2002) and dendritic cells (Dumitriu *et al.*, 2005b) secrete HMGB-1 upon activation by proinflammatory cytokines (TNFα and IL-1β) or bacterial endotoxin, a process that in activated monocytes have been shown to involve exocytosis via vesicle mediated pathways (Gardella *et al.*, 2002). However, also other types of cells may release HMGB-1 in response to inflammatory stimuli (Liu *et al.*, 2006; Wang *et al.*, 1999b). Apoptotic cells were shown to firmly bind HMGB-1, preventing extracellular release and induction of an inflammatory response (Scaffidi *et al.*, 2002). However, subsequently HMGB-1 release has been detected also from apoptotic cells (Jiang *et al.*, 2007; Bell *et al.*, 2006). It has been shown that the danger signal of HMGB-1 is quenched by caspase-triggererd formation of reactive oxygen species and oxidization of HMGB-1, providing a possible explanation for apoptosis associated immunologic tolerance (Kazama *et al.*, 2008).

The effects from HMGB-1 may be mediated after binding to the receptor for advanced glycation end products (Tian *et al.*, 2007; Dumitriu *et al.*, 2005b; Loeser *et al.*, 2005; Hori *et al.*, 1995), Toll-like receptor 2, 4 (Park *et al.*, 2006; Park *et al.*, 2004) and 9 (Ivanov *et al.*, 2007), and triggering receptor expressed on myeloid cells-1 (El Mezayen *et al.*, 2007). Although HMGB-1 has been found to induce production of proinflammatory mediators in monocytes (Andersson *et al.*, 2000) and up-regulation of IL-1β, IL-8 and TNFα mRNA in neutrophils (Park *et al.*, 2003), it has lately been proposed that the proinflammatory action of HMGB-1 is coupled to complexes formed between HMGB-1 and other molecules (such as lipopolysaccarides, IL-1β, single stranded DNA and nucleosomes), and that HMGB-1 on its own has mainly mitogenic and chemoattractive properties (Bianchi, 2009).

Investigations on HMGB-1 in equine joint disease are few, and little is known about its effects on cartilage. Recently, synovial fluid concentrations of HMGB-1 were shown to be increased in Thoroughbred racehorses with carpal and fetlock dorsal osteochondral injury compared to normal joints (Brown *et al.*, 2009). Furthermore, intra-articular injection with HMGB-1 induces synovitis in mice (Pullerits *et al.*, 2003), and anti-HMGB-1 treatment reduces the severity of disease in collagen-induced arthritis in mice and rats (Kokkola *et al.*, 2003). Extracellular deposits and/or cytoplasmic HMGB-1 have been observed in synovial membrane of human chronic inflammatory arthritides, including rheumatoid arthritis (af Klint *et*

al., 2005; Taniguchi *et al.*, 2003; Kokkola *et al.*, 2002), and synovial fluid concentrations of HMGB-1 have been reported to be higher in human rheumatoid arthritis than OA (Taniguchi *et al.*, 2003).

In osteoarthritic human cartilage marginal increases in HMGB-1 mRNA, and HMGB-1 induced increases in PGE2, nitric oxide, IL-6 and IL-8 production have briefly been reported (Attur *et al.*, 2003). In addition, HMGB-1 induced increased activities of mitogen activated protein kinase and nuclear factor-kappa B, and MMP-13 production in human chondrocyte cultures (Loeser *et al.*, 2005).

1.10 Joint changes associated with equine OA

1.10.1 Osteochondral tissues

Osteoarthritic articular cartilage is typically dull, yellowish discoloured, and, depending on severity, shows varying degrees of fibrillation, ulceration, and eburnation (Pool & Meagher, 1990) (Fig. 5A). Wear lines, represented by fine, parallel and sagitally orientated lines in wide-range hinge joints, have been suggested to represent a self-limiting degenerative change in its mild forms that under certain circumstances become extensive and contribute to generalised cartilage degeneration (Pool & Meagher, 1990). Their presence has been positively correlated with exercise, and negatively correlated with hydroxylysylpyridinoline crosslinking of collagen (Brama *et al.*, 2000a). In fetlock joints, the cartilage degeneration index (CDI) technique (Brommer *et al.*, 2003) has been used to assess distribution of cartilage degeneration on the proximal surface of the proximal phalanx, and identified the dorsal aspect as the initial location for cartilage degeneration (Brommer *et al.*, 2004). Furthermore, loss of cartilage stiffness has been related to degenerative changes in cartilage of the proximal phalanx (Brommer *et al.*, 2005).

Microscopically, OA cartilage shows loss of proteoglycans, fragmentation of collagen fibrils, splitting and loss of extracellular matrix, chondrocyte death and proliferation of viable chondrocytes in adjacent cartilage (Pool & Meagher, 1990) (Fig. 5B). A positive correlation between the microscopic severity of cartilage changes and extent of apoptotic chondrocytes has been demonstrated (Thomas *et al.*, 2007; Kim *et al.*, 2003).

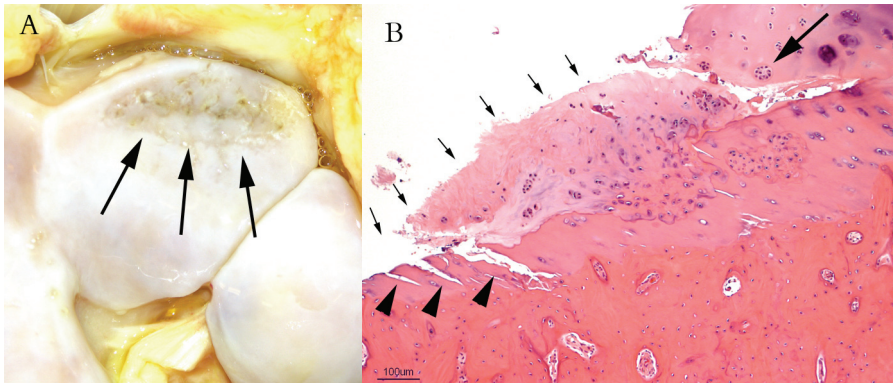

Figure 5. (A) Discoloured and fissured cartilage of the dorsal radial facet (arrows) of the third carpal bone (photo S. Ekman). (B) Photomicrograph of osteoarthritic cartilage of the radial facet showing eroded cartilage and cartilage loss (small arrows), chondrocyte proliferation (large arrow), and cracks in the calcified cartilage (arrow heads).

Increases in thickness of calcified carpal cartilage have been demonstrated at sites predisposed to osteochondral injury (Murray *et al.*, 1999). In addition, sclerosis of the subchondral bone is often present in areas of cartilage degeneration (Anastasiou *et al.*, 2003; Cantley *et al.*, 1999; Pool & Meagher, 1990). Bone density and stiffness have been shown to be higher in pathologic C3 (cartilage degeneration or slab fractures) compared to that of C3 in trained and untrained horses (Young *et al.*, 1991), and a heterogenenous pattern of focal resorptive lesions and sclerosis of subchondral bone has been demonstrated in osteoarthritic third metacarpal condyles (Young *et al.*, 2007). Microcracks and microfractures in the subchondral bone may be seen in association with severe sclerosis and cartilage lesions (Norrdin & Stover, 2006), and a co-localisation of microcracks in the calcified cartilage and ingrowth of blood vessels has been described in the distal third metacarpal/-tarsal bone (Muir *et al.*, 2006).

Reactive changes in the joint tissue margins may be seen as periarticular osteophytes or as enthesiophytes (Pool & Meagher, 1990). Osteochondral fragments (OCF) of bones in the dorsal part of the carpal joints, and on the dorsal aspect of the first phalanx, so called chip-fractures (Fig. 6A and B), are in most cases thought to arise either from fragmentation of periarticular osteophytes at the joint margin, or fragmentation of original joint margins, where sclerotic subchondral bone has become necrotic and replaced by granulation tissue (Pool & Meagher, 1990). In addition, so called central subchondral osteophytes, formed by focal projections of dense bone into the calcified cartilage and the deep zone of articular cartilage, have been

described in equine metacarpophalangeal joints with cartilage pathology (Olive *et al.*, 2009).

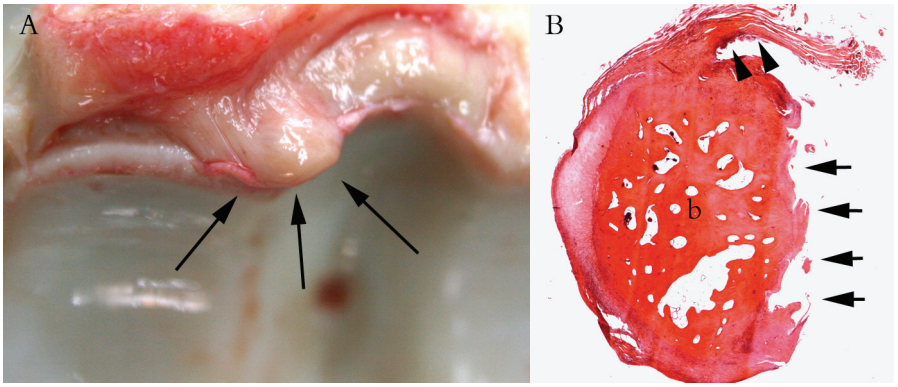

Figure 6. (A) Dorsal osteochondral fragment (OCF) of the proximal phalanx attached to the joint capsule. (B) Photomicrograph of OCF showing articular cartilage degeneration (arrows), a central core of bone (b), and attachment to the joint capsule (arrow heads).

1.10.2 Joint capsule and ligaments

The joint capsule may macroscopically show oedema, congestion, discolouration (due to hemosiderosis) and thickening (due to fibrosis) with hyperplasia and hypertrophy of synovial villi (Trotter & McIlwraith, 1996; Pool & Meagher, 1990). The microscopic appearance of the synovial membrane is that of a non-specific inflammatory reaction, and may show vascular congestion, hemorrhage, oedema, fibrosis, villi formation, and, less commonly, granulocytic and mononuclear/lymphoplasmacytic stromal infiltrates (Ronéus *et al.*, 1997; Trotter & McIlwraith, 1996) (Fig. 7A and B). In long-standing cases synovial chondromas may form (Pool & Meagher, 1990).

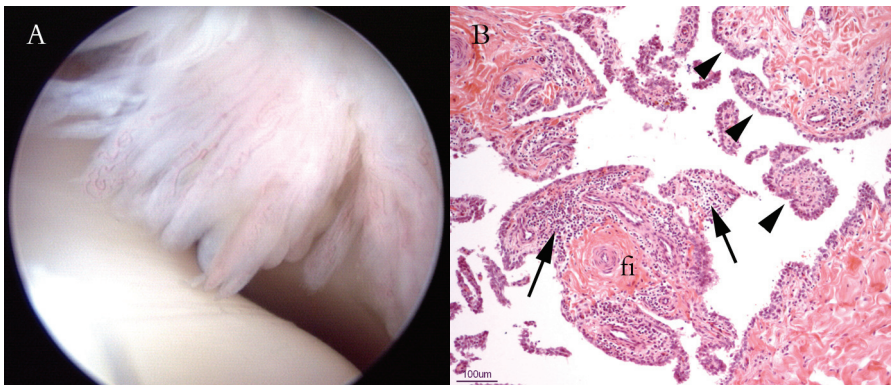

Figure 7. (A) Arthroscopic photograph of fetlock synovial villi with mild to moderate chronic hypertrophic synovitis. Multiple superficial wear lines are seen in the articular cartilage (photo B. Ronéus). (B) Photomicrograph of synovial membrane in a fetlock joint affected by osteoarthritis secondary to fracture of the third metacarpal bone, showing infiltration of mononuclear inflammatory cells (arrows), hypercellular lining layer (arrow heads) and fibrosis (fi).

The relation between ligamentous and osteochondral injury in equine carpal OA is unclear. However, damage to the medial palmar intercarpal ligament is a common finding in horses undergoing carpal arthroscopy (Whitton *et al.*, 1997a; Phillips & Wright, 1994; Kannegieter & Colgan, 1993). Whereas increased degree of ligament tearing was seen in joints with more severe osteochondral damage (Kannegieter & Colgan, 1993), other studies failed to detect such a relation (Whitton *et al.*, 1997a; McIlwraith, 1992).

1.10.3 Synovial fluid

Currently, there are no routinely used paramenters measured in synovial fluid diagnostic for OA. Increases in leukocyte counts and total protein concentrations are often minor, and the search for useful biomarkers has been extensive (McIlwraith, 2005).

Although increased concentrations of GAG may be detected in OA (Alwan *et al.*, 1991a; Little *et al.*, 1990), other types of injuries may also affect GAG concentrations (Palmer *et al.*, 1995b; Alwan *et al.*, 1991a). Neither GAG nor aggrecan concentrations have been found to correlate with grade of cartilage lesions (Fuller *et al.*, 2001; Skiöldebrand *et al.*, 2001; Little *et al.*, 1990). Whereas concentrations of keratan sulphate in chronic (but not acute) joint disease were increased compared to normal joints (Palmer *et al.*, 1995b), clinically active carpal and fetlock joints (of which the majority showed cartilage pathology) showed decreased keratan sulphate concentrations and decreased keratan sulphate/GAG ratios compared to the

contralateral joint (Fuller *et al.*, 2001). Furthermore, a negative correlation was reported for concentration of keratan sulphate and keratan sulphate/GAG ratio and grade of cartilage damage (Fuller *et al.*, 2001).

Decreased hyaluronan concentrations were also found in clinically active joints (Fuller *et al.*, 2001), and in joints affected by traumatic arthritis/OA (Tulamo *et al.*, 1996; Hilbert *et al.*, 1984). However, in another study concentrations of hyaluronan did not differ between OA and normal joints (Little *et al.*, 1990), and both presence (Tulamo *et al.*, 1996) and absence (Fuller *et al.*, 2001) of correlations between hyaluronan concentrations and degree of cartilage damage has been reported.

Also, increased COMP concentrations have been reported from osteoarthritic equine joints (Arai *et al.*, 2005). Presence of moderate cartilage lesions in the middle carpal joint were associated with decreased COMP concentrations in Standardbred trotters, but increased concentrations in riding horses (Skiöldebrand *et al.*, 2001). Neither concentrations of COMP nor collagen type II correlated with grade of cartilage lesions (Skiöldebrand *et al.*, 2001), although detection of the collagen type II degradation neoepitope C2C was correlated to the severity of joint injury in carpal and fetlock joints with OCF (Trumble *et al.*, 2009). Furthermore, in carpal joints with osteochondral injury both increased COMP (Skiöldebrand *et al.*, 2005), CS 846, total protein concentrations (Frisbie *et al.*, 1999) and bone alkaline phosphatase (Trumble *et al.*, 2008) have been found, as well as increased chondroitin sulphate chain length and decreased hyaluronan chain length (Brown *et al.*, 2007).

A positive correlation between bone alkaline phosphatase and grade of cartilage lesions has also been found (Fuller *et al.*, 2001), suggesting that bone derived markers are of value to evalualte in OA.

When OA is induced experimentally (osteochondral defect created surgically followed by treadmill exercise) an inflammatory response, increased bone formation and metabolism of GAG and collagen is induced, reflected by increased concentrations of PGE2, osteocalcin, CS 846, and markers of collagen synthesis and degradation (Frisbie *et al.*, 2008). Investigations of presence of inflammatory mediators, proteolytic enzymes, and enzyme inhibitors in diseased equine joints were partly described in sections 1.5-1.7 and 1.9. In addition, increased concentrations of PGE2 (Kirker-Head *et al.*, 2000; Gibson *et al.*, 1996) and substance P (Kirker-Head *et al.*, 2000; Caron *et al.*, 1992) have been found in synovial fluid from OA affected equine joints.

2 Aims of thesis

The general aims of this thesis were to gain a deeper understanding of the development of chondral pathology in equine osteoarthritis (OA) by obtaining increased knowledge of inflammatory processes in the joint, and to investigate proinflammatory cytokines as markers of joint pathology.

The specific aims were:

- Determine synovial fluid concentrations of IL-6 and TNF in diseased carpal joints, and investigate possible associations between cytokine concentrations and categories of joint lesions (paper I).
- Determine cellular sources of IL-6 and HMGB-1 in tissues of diseased carpal and fetlock joints, and investigate their expressions in the synovial membrane in regard to presence of chondral/ostochondral pathology (paper II).
- Characterize morphologic changes in synovial membranes of diseased carpal and fetlock joints, to investigate possible relations between grade of synovitis and presence of chondral/ostochondral pathology (paper II).
- Investigate metabolic changes in cultured chondrocytes from healthy joints after exposure to IL-1β, IL-6 and HMGB-1, and to investigate if the responses to cytokine treatment differ between cells harvested from differently *in vivo* loaded sites (paper III).
- Investigate the effects of IL-1β and IL-6 on HMGB-1 gene expression and localisation in cultured chondrocytes (paper IV).

3 Hypotheses

The following hypotheses were raised for the studies performed on diseased joints:

- Concentrations of IL-6 and TNF bioactivities in synovial fluid reflect intra-articular pathology (paper I).
- Dorsal carpal and fetlock osteochondral fragments (OCF) contribute to an increased inflammatory response in diseased equine joints (paper II).

The following hypotheses were raised for the studies performed on cultured chondrocytes:

- Chondrocyte metabolism is affected by exposure to IL-1β, IL-6 and HMGB-1 (paper III).
- A different metabolic response to IL-1β, IL-6 or HMGB-1 can be demonstrated in chondrocytes from anatomical sites which show different *in vivo* loading and susceptibility to develop cartilage lesions in equine OA (paper III).
- HMGB-1 is translocated and released from chondrocytes when exposed to IL-1β or IL-6 (paper IV).

4 Material and methods

This section summarises the material and methods used in the studies performed for this thesis. Complementary descriptions of the procedures are presented in the individual papers.

4.1 Animals and sample collection (papers I-IV)

Specimens from diseased joints, were obtained from horses presented for arthroscopic examination and/or surgery, and consisted of synovial fluid (Paper I), synovial membranes and OCF (paper II), collected under general anaesthesia. Synovial fluid from healthy joints were additionally obtained from young untrained Standardbred trotters and used as control samples in paper I. Represenatitive control material (osteochondral slabs and synovial membrane biopsies, paper II) and articular cartilage biopsies (paper III-IV) were collected from macroscopically healthy joints from horses presented for post mortem examination. The cartilage biopsies were aseptically collected as shavings of the dorsal radial facet (DRF) (papers III and IV) and palmar condyle (PC) (paper III) of C3 (see Fig. 9A). In several of these joints, synovial membrane biopsies and osteochondral slabs were also collected for microscopic evaluation from adjacent areas of cartilage biopsies. Demographic data for sampled animals and joints are presented in Table 1.

Table 1. Demographic data for sampled horses and joints

Paper	Category	Breed	Horses (n=)	Joint studied (n=)	Age (years; mean (range))
I	Diseased	STB	40	39 MC, 1 RC	3.6 (2-8)
		TB	9	3 MC, 6 RC	3.4 (3-5)
		SWH	2	2 MC	8
	Healthy	STB	6	6 MC	1
II	Diseased	STB	14	14 MC	3.9 (2-6)
		SWH	6	2 RC, 3 MCP, 1 MTP	7.5 (2-12)
		Other	1	1 MCP	10
	Healthy	SWH	2	1 MC, 1 MCP	6 (2-10)
		TB	1	1 MC	3
		STB	1	1 MC	1
III-IV	Healthy	SWH	2	3 MC	2.5 (2-3)
		Other	2*	4 MC	4.2 (0.5-8)
		STB	1	2 MC	1

STB = Standardbred trotter; SWH = Swedish warmblood riding horse; TB= Thoroughbred racehorse; MC = middle carpal; RC = radiocarpal; MCP = metacarpophalangeal; MCT = metatarsophalangeal

*Paper III did not include the ½ year old horse, and paper IV did not include the 8 year old horse.

4.2 Fixation of tissue specimens (papers II-IV)

Specimens intended for light microscopic evaluation were fixed in 10% buffered formalin or 4% phosphate buffered paraformaldehyde. Osteochondral material was decalcified in either in 3.4% (w/v) sodium formiate and 15.1% (v/v) formic acid, 20% (v/v) formic acid (formalin fixed samples), or in 7% (w/v) EDTA with 0.5% (w/v) paraformaldehyde (paraformaldehyde fixed samples). Material intended for electron microscopy was fixed in 3% phosphate buffered paraformaldehyde.

4.3 Evaluation of tissues

4.3.1 Macroscopic assessment (papers I and II)

To ensure consistency in examinations and tissue handling only one surgeon performed the arthroscopic examinations for each paper. The material used

in paper I originated from joints that were macroscopically assessed in regard to pathologic findings in a previous study (Skiöldebrand *et al.*, 2001). The criteria of grading are presented in Table 2.

In paper II focus was placed on the presence of OCF, and although cartilage lesions were present in all joints without OCF, grading of cartilage lesions was not performed as part of the study. Since synovial membrane biospsies were collected for further immunohistochemical investigation of cytokine production, the synovial membrane reaction was graded histologically instead of macroscopically.

Table 2. Grading of cartilage lesions (paper I)

Joint tissue	Grade of lesion	Macroscopic finding
Articular cartilage	Normal	No abnormalities detected
	Mild	Superficial fraying in a small area
	Moderate	Fraying over a larger area, erosions with focal small areas with denuded bone
	Severe	Fraying, erosions, cartilage loss with denuded bone in a large area
	Chip-fracture	OCF arising in the dorsal parts of distal radius, distal RCB, distal IC or proximal C3
Synovial membrane	Normal	No abnormalities detected
	Mild	Hyperemia, mild thickening of villi
	Moderate	Hyperemia, mild thickening of villi, focal formation of new villi
	Severe	Hyperemia, mild thickening of villi, focal formation of new villi, adhesions between villi
Intercarpal ligaments	Intact	No abnormalities detected
	Partial rupture	Fiber fraying in either or both ligaments

OCF = osteochondral fragment; RCB = radial carpal bone; IC = intermediate carpal bone; C3 = third carpal bone

4.3.2 Light microscopy (papers II-IV)

Haematoxylin and eosin stained sections were used to assess morphology of synovial membrane biopsies, OCF, osteochondral slabs, and pellets of three dimensional (3D) chondrocyte cultures.

Grading of inflammatory changes in synovial membranes followed a protocol modified from previously described (Krenn *et al.*, 2006), and

assessed lining layer hyperplasia, density of subintimal stromal cells and inflammatory infiltrates (paper II).

Safranin-O and Masson's trichrome staining were used to assess proteoglycan and collagen content in pellets, respectively (paper III).

4.4 Detection of cytokines in diseased joints

4.4.1 Bioassays (paper I)

To detect the bioactivities of IL-6 and TNF in synovial fluid, proliferation of IL-6 dependent murine B9 hybridoma cells subclone B13.29 (Aarden *et al.*, 1987) (Fig. 8), and the cytotoxic effect of TNF on the murine fibroblast cell line L929 NCTC clone 929 in the presence of actinomycin D (Wang *et al.*, 1985) were determined. Cytokine bioactivities were measured by analysing the capability of synovial fluid exposed cells to reduce tetrazolium salts into formazan derivates, and comparing the results to standard curves generated from known concentrations of recombinant human IL-6 and TNFα.

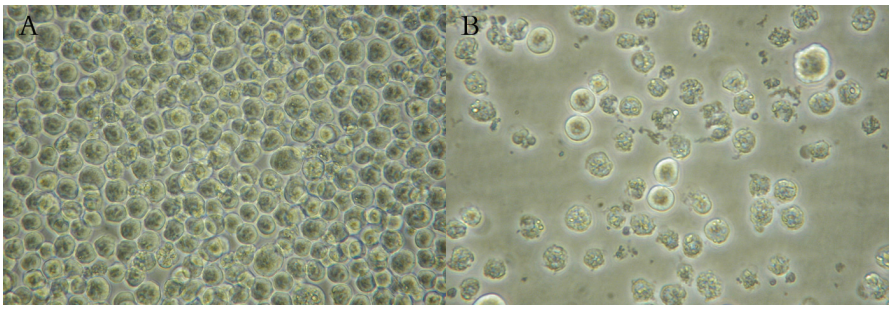

Figure 8. B9 cells cultured in the presence (A) and absence (B) of IL-6 (photos M-L. Eloranta).

4.4.2 Immunohistochemistry (paper II)

Presence and localisation of cytokines in tissue specimens was demonstrated with immunohistochemistry. When different fixation and decalcification techniques were used for material in the same study, additional material was studied to compare levels of antigen detection for the relevant antibodies (IL-6 and HMGB-1), and no differences were found. Primary antibodies and used concentrations are listed in Table 3. Negative controls were always treated with isotype-matched irrelevant antibodies. Light microscopic detection of antigen was made by visualization of avidin-biotin-peroxidase

complexes using Sigma Fast™ 3,3' diaminobenzidine tablet sets (Sigma-Aldrich Chemical Co., St. Louis, MO, USA).

Table 3. Primary antibodies used for immunohistochemistry

Paper	Primary antibody	Dilution
II	Goat anti-equine IL-6[a]	1:100
II	Rabbit anti-human HMG-1[b]	1:500

[a]R&D Systems, Abingdon, UK

[b]BD Bioscience Pharmingen, San Jose, CA, USA

4.5 Cytokine stimulation of chondrocyte cultures (papers III and IV)

The procedure of chondrocyte culture is described in detail in papers III and IV. Following mechanical mincing of cartilage shavings from DRF and/or PC, ECM was digested using collagenase. Cells were expanded in monolayer culture (Fig. 9B), then cultured in 3D cell cultures (pellets) using protocols modified from previously described (Stenhamre *et al.*, 2008; Penick *et al.*, 2005; Tallheden *et al.*, 2004). After 14 days of 3D culture (Fig. 9C), pellets were treated with proinflammatory cytokines or further cultured untreated. For paper III cells were stimulated for a total of 48 h, whereas for paper IV cells were stimulated for 48 or 96 h, or 7 days (Table 4). At the end of stimulation, cell culture supernatants and/or pellets were collected for further analyses.

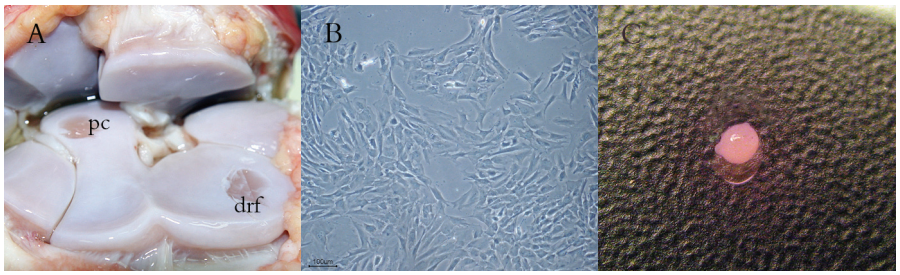

Figure 9. (A) Sampling sites on the third carpal bone (drf = dorsal radial facet; pc = palmar condyle). (B) Chondrocytes cultured in monolayer (photo E. Svala). (C) Chondrocyte pellet after 14 days in three dimensional culture.

Table 4. Protocol for cytokine stimulation

Cytokine	Concentration	Duration of stimulation
Recombinant equine IL-1β[a]	5 ng/ml	48 h, 96 h, 7 days
Recombinant equine IL-6[a]	5 ng/ml	48 h, 96 h, 7 days
Recombinant human HMGB-1[a,b]	1 μg/ml	48 h

[a]R&D Systems, Abingdon, UK

[b]Sigma-Aldrich, St. Louis, MO, USA

4.6 Gene expression analyses (papers III and IV)

4.6.1 Isolation of ribonucleic acid (RNA)

RNA isolation was performed on 3-4 pooled pellets from each individual and sampling site. Mechanical homogenization (bead-milling) of pooled pellets was performed using a TissueLyser (Qiagen, Hilden, Germany) on samples repeateadly frozen in liquid nitrogen with intervals of 4 minutes. Once homogenized, RNA was extracted using QIAzol Lysis Reagent (Qiagen) and chloroform. RNA was further purified using RNeasy mini kit (Qiagen). Genomic DNA was removed by DNase I (Qiagen). The purity of sample RNA was determined by measuring concentrations of RNA (NanoDrop® ND-1000 spectrophotometer, Thermo Scientific, Wilmington, DE, USA) at 260 and 280 nm. We considered ratios (260/280 nm) between 1.8-2.1 acceptable (Martin *et al.*, 2001).

4.6.2 Synthesis of complementary deoxyribonucleic acid (cDNA)

Total RNA (100 ng/sample) in 20 μl reaction mixtures was used to produce cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Stockholm, Sweden). cDNA synthesis was performed according to the manufacturers' instructions and by using a 2720 Thermal Cycler (Applied Biosystems).

4.6.3 Quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR)

The qRT-PCR procedure used in the TaqMan® Gene Expression Assay (Applied Biosystems) relies upon the 5′-3′exonuclease activity of *Thermus aquaticus* (*Taq*) DNA polymerase (Holland *et al.*, 1991). In this system, a reporter probe, which is designed to anneal to a specific DNA sequence between the forward and reverse primer sites for the target gene, is labelled with a fluorescent dye and a quencher. During each amplification cycle, the exonuclease activity separates the fluorescent-labelled part of the probe from

the quencher and fluorescence is emitted. The number of cycles required for a critical level of fluorescence to be reached (the cycle threshold, CT value) is thus dependent on the amount of mRNA for the investigated gene present in the sample, as emitted fluorescence is proportional to the produced amount of amplicon. The higher the gene expression of a specific gene is, the sooner the CT value is reached.

Human primers and probes were used in the analyses of sex determining region on the Y chromosome-related high mobility group box 9 (Sox9), HMGB-1 and collagen type II (Applied Biosystems, Sweden). Equine primers and reporter sequences were used for versican, collagen type I, aggrecan, COMP, MMP-9, MMP-13, ADAMTS-5, TIMP-1 and beta glucoronidase (GUSB), the latter used as endogenous control (Applied Biosystems) (Table 5). GUSB was selected as housekeeping gene after comparing human DNA sequences for housekeeping genes presented in a human endogenous control panel (TATAA Biocentre) with equine sequences, found in the NCBI (National Centre for Biotechnology Information) data base (www.ncbi.nlm.nih.gov), and searching the literature for the most commonly used endogenous controls for equine material. The efficiencies of all equine specific primers and probes were verified by performing 1:2 dilution series of cDNA template and a standard curve. Satisfactory stability of GUSB was found throughout a series of experiments of pellets treated with hyaluronan, IL-1β and IL-6.

Table 5. *Equine primer and reporter sequences*

Gene	Primer/probe	Sequence (5′-3′)
ADAMTS-5[b]	Forward primer	TGGCTCACGAAATTGGACATC
	Reverse primer	AGGTCTAGCAAACAGTTACCATGACC
	Reporter	TGGTCCAAATGCACCTC
Aggrecan[a]	Forward primer	GCGAGGCCACCCTAGAG
	Reverse primer	CGTGGAGATGGCTCTGTAATGG
	Reporter	AACACGATGCCTTTCACC
Coll I[a]	Forward primer	GAGGCCGTCCTGTATGCA
	Reverse primer	CAAGAGGAGGGCCAAGAAGAAG
	Reporter	CTGCTGGGATGTCTTC
COMP[a]	Forward primer	GGGCAACGGCTCATACTGT
	Reverse primer	GCGAGGGAAGCAGGGATT
	Reporter	CAACGAGTGCAACGCC
GUSB[a]	Forward primer	GTGACCAACTCCAACTATGAAGCA
	Reverse primer	AGGAGTAGTAACTATTCACACAGATGACA
	Reporter	CATATGGCGCCCCTAGGTC
MMP-9[b]	Forward primer	TTGGACATGCACGACGTCTT
	Reverse primer	GAAGCTCACGTAGCCCACTTG
	Reporter	TACCGAGAGAAAGCTTACTT
MMP-13[a]	Forward primer	GAGCATCCTTCCAAAGACCTTATCT
	Reverse primer	GGATAACCTTCCAGAATGTCATAACCA
	Reporter	TTTAGAGGCCGAAAATT
TIMP-1[a]	Forward primer	CCGCAGCGAGGAGTTTCT
	Reverse primer	GTGATGTACAGCTTCTCGTCCAA
	Reporter	CATCGCCGGACAACTA
Versican[a]	Forward primer	CCTACGTGTGCACCTGTGT
	Reverse primer	GCACGTGGCTCCATTGC
	Reporter	CAGTGTGAGCTTGATTTT

ADAMTS-5 = a disintegrin and metalloproteinase with thrombospondin motifs 5; Coll I = collagen type I; COMP = cartilage oligomeric matrix protein; GUSB = beta glucoronidase; MMP-13 = matrix metalloproteinase 13; MMP-9 = matrix metalloproteinase 9; TIMP-1 = tissue inhibitor of metalloproteinase 1

[a]Custom TaqMan® Gene Expression Assays/Assay by design, Applied Biosystems, Sweden

[b]Oligos, Applied Biosystems, Sweden

qRT-PCR was performed on duplicate samples of 2.5 ng cDNA/reaction using ABI 7900HT (Applied Biosystems) and TaqMan Gene Expression master mix (Applied Biosystems). Samples were analysed in ABI Prism® 96-well Opitcal Reaction plates (Applied Biosystems). Activation of polymerase

was performed at 95°C for 10 minutes, followed by 40 PCR cycles with annealing at 60°C, extension at 72°C, and denaturation at 95°C. Data were analysed using Sequence Detection System (SDS) 2.2 (Applied Biosystems).

4.6.4 Evaluation of gene expression

Relative gene expressions were calculated by using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001), which is based upon comparisons of CT values between target and reference (housekeeping) genes (ΔCT) in the evaluated sample and a reference sample (calibrator).

To investigate gene expression profiles in untreated pellets, and to determine the overall effect of cytokine treatments a calibrator made of a mix of pellets exposed to different teatments was used (paper III). However, to evaluate the response to cytokine treatment in individual sites untreated control pellets were used as calibrator (papers III and IV).

4.7 Detection of HMGB-1 in chondrocyte cultures (paper IV)

4.7.1 Immunohistochemistry

Immunolocalisation of HMGB-1 in pellets was demonstrated using mouse monoclonal 2G7 anti-rat HMGB-1 antibody (Critical Therapeutics Inc., Boston, MA, USA; kind gift from Helena Erlandsson Harrris, Karolinska Institutet, Stockholm) at a dilution of 1:5000. Treatment of negative controls and antigen detection were performed as described in 4.4.2. Presence of nuclear versus cytoplasmic and/or extracellular immunostaining for HMGB-1 was evaluated.

4.7.2 Immunelectron microscopy

Ultrathin sections from one quarter of each pellet were obtained from specimens infiltrated with 2.3 M sucrose and frozen at -95°C. Sections were placed on carbon-treated formvar coated nickel grids, used as a supporting film, and thawed. As primary antibody, rabbit anti-rat HMGB-1 antibody (Serotec, Oxford, UK) diluted 1:200 was used. Isotype matched irrelevant antibody was applied to a single sample. Quantitative detection of HMGB-1 was made with gold coated (10 nm) protein A (GE Healtcare, Amersham, UK). The densities of HMGB-1 in the different compartments were assessed in digitally obtained electron microscopy images by counting numbers of gold particles/squared micrometer ($Au/\mu m^2$). One image each from a total of 15 cells and 10 pericellular areas were evaluated, which according to a

pilot study using cumulative plot (Weibel, 1979) were appropriate sampling sizes.

4.8 Detection of other chondrocyte derived substances (paper III)

4.8.1 Immunoassays

To complement results from gene expression analyses of aggrecan and MMP-13, concentration of the chondroitin sulphate epitope CS 846 (IBEX Diagnostics, Montreal, Quebec, Canada) and activated MMP-13 (Fluorokine E, R&D Systems) were measured in duplicate samples according to the manufacturers' instructions.

4.8.2 Immunohistochemistry

To further evaluate effects from cytokine stimulation on COMP metabolism in pellets, immunolabelling for COMP was performed using a rabbit anti-bovine COMP antibody (Hedbom *et al.*, 1992), diluted 1:2000. Treatment of negative controls and antigen detection were performed as described in 4.4.2.

4.9 Data analyses

In general, statistic significance was considered when P values were <0.05.

In paper I, comparisons of cytokine concentrations in synovial fluid between categories of joint lesions were analysed using Kruskal-Wallis analysis of variance (ANOVA) on ranks or Mann-Whitney rank sum test. Dunn's method with Bonferroni correction was used to isolate groups that differed when significance was found by ANOVA on ranks, and in these analyses P-values <0.0001 were considered significant. Associations between IL-6, TNF and total leukocyte counts and total protein concentrations were estimated by Spearman rank order correlation. Analyses were performed using SigmaStat 3.0 (SPSS Science Software GmbH, Erkrath, Germany) and StatView 5.0.1 (SAS Institute Inc. Copyright© 1992-1998, Cary, USA) software.

In paper II, differences in immunostaining for IL-6 and HMGB-1 in synovial membranes from OA and OCF joints were analysed using the Fisher exact test, and differences in synovitis score between OA joints, OCF joints, and healthy joints were analysed with Mann Whitney rank sum test (SigmaStat 3.0; SPSS Science Software GmbH, Erkrath, Germany).

In paper III, differences in pellet gene expressions and concentrations of MMP-13 and CS 846 in pellet supernatants were analysed by ANOVA (PROC GLM) in the SAS Software version 9 (SAS Institute Inc., Cary, NC, USA). The statistical model included the fixed effects of animal, site, treatment, and interactions between site and treatment. Pairwise comparisons of least square means between cytokine treated and untreated pellets, as well as between sites were tested for significance.

In paper IV, mean numbers of gold particles per squared micrometer ($Au/\mu m^2$) in the nucleus, cytoplasm, and pericellular ECM were calculated, and nuclear/cytoplasmic (N/C) ratios determined. Due to the low number of individuals included in the study statistical tests were not performed.

5 Results

5.1 Cytokines in synovial fluid (paper I)

Concentrations of bioactive IL-6 and TNF were measured in synovial fluid from a population (n = 51) of athletic horses with carpal pathology. When macroscopic OA-like lesions were present, these joints were classified as suffering from OA (type I or II, according to the classification by McIlwraith, 1996). Several horses did not show cartilage lesions and were classified as having traumatic arthritis.

Increased concentrations of IL-6 activity were detected in joints with OCF (so called chip-fractures) compared to diseased joints without OCF ($P < 0.001$), and compared to joints with normal, mild or moderate cartilage lesions ($P < 0.0001$). Due to the low number of joints with severe cartilage lesions (n= 2) statistical comparisons with this group was not performed, however IL-6 was not detected in synovial fluid from these two joints. Although highly increased IL-6 activity indicated presence of chip-fracture, low or undetectable levels did not exclude their presence. All joints with chip-fractures showed some degree of synovitis, however, there was no significant difference in IL-6 values between catergories of synovitis. No significant correlation was detected between concentrations of IL-6 and synovial leukocyte counts, although significance was detected between IL-6 and total protein concentrations. Activity of IL-6 was not detected in synovial fluid from non-lame horses.

Concentrations of active TNF were low and did not differ between categories of joint lesions. Its activity was detected in one synovial fluid sample from the non-lame horses.

5.2 Cytokines in joint tissues (paper II)

Immunohistochemistry was used to investigate the immunolocalisation of IL-6 and HMGB-1 in synovial membrane biopsies and osteochondral tissues from carpal and metacarpo-/metatarsophalangeal joints with macroscopic lesions associated with equine OA (n = 21), and normal joints (n = 4). Depending on presence of OCF in diseased joints, a subdivision into OCF (n = 10) and OA (n= 11) joints was done, where the former was represented by joints with OCF (with or without concurrent cartilage lesions) and the latter by joints with cartilage lesions only (absence of OCF). Light microscopy was used to morphologically characterize changes in sampled tissues.

Immunostaining for IL-6 was demonstrated in cells of synovial membranes from all normal joints, and inconsistently demonstrated in cells of the synovial membranes from diseased joints. When IL-6 positive cells were found, these were present in large numbers and found in the lining layer as well as the subintimal stroma and endothelium. Cells with a rounded nucleus and abundant cytoplasm, commonly seen in hypercellular lining layers were most prominently immunostained. There was no statistical difference in frequency of numbers of biopsies with deteced IL-6 between OCF and OA joints.

Whereas nuclear staining for HMGB-1 was the dominating pattern of immunostaining in all normal synovial membranes, cytoplasmic and widespread extracellular HMGB-1 was detected in 5/10 of synovial membrane biopsies from joints with OCF, and in one biopsy from the OA joints, however this difference was not statistically significant.

Immunostaining for IL-6 and HMGB-1 was increased in cartilage of OCF compared to normal cartilage. In all examined OCF (n = 8), IL-6 positive cells were identified, and chondrocytes in the middle and deeper zones were often the most intensely immunostained cells. Presence of cytoplasmic and/or extracellular HMGB-1 was observed along reduplicated tidemarks, and occasionally in areas adjacent to cartilage loss and in fibrous tissue.

Increased grade of synovitis was detected in OA and OCF joints compared to normal joints ($P = 0.042$ and $P = 0.013$, respectively). However, morphologic signs of synovitis in diseased joints was sometimes absent (n = 4), most commonly low grade (n = 15), and seldom high grade (n = 2). Most often synovitis was related to increases in subintimal stromal cells and hypercellular lining layers, and not to increased inflammatory infiltrates, which were only seen in two biopsies.

5.3 Effects of cytokines on cultured chondrocytes (paper III and IV)

5.3.1 Effects of IL-1β, IL-6 and HMGB-1 on cartilage matrix metabolism (paper III)

The effects of IL-1β, IL-6 and HMGB-1 on chondrocyte metabolism were evaluated in 3D chondocyte cultures (pellets), established with cells harvested from the *in vivo* highly loaded DRF (n=4) and the less loaded PC (n=4). The effects were evaluated after 48 h of cytokine treatment by gene expression analyses, in part complemented by protein analyses in pellet supernatants and of pellet content.

Compared to untreated controls, pellets stimulated with IL-1β showed increased gene expression of versican ($P<0.0001$), MMP-9 ($P = 0.0137$) and TIMP -1 ($P = 0.0059$), and decreased gene expression of aggrecan ($P < 0.0001$), collagen type I ($P = 0.0077$) and II ($P = 0.0082$), and COMP (not statistically significant, $P = 0.0543$). In addition, IL-1β treatment induced markedly increased gene expression of MMP-13 and ADAMTS-5, as well as concentrations of activated MMP-13 in culture supernatant (significance of statistical tests not considered due to absence of normally distributed residuals), increased COMP immunostaining but decreased safranin-O staining of pellets.

The effects of IL-6 and HMGB-1 treatment on investigated gene expressions were variable, although up-regulation of Sox9 was often present and statistically increased in HMGB-1 treated pellets ($P = 0.012$). No other significant effects on gene expressions were detected in IL-6 and HMGB-1 treated pellets compared to controls, and the appearances of pellet safranin-O staining and immmunostaining for COMP were in most IL-6 and HMGB-1 treated pellets similar to that of controls.

Across treatment, expression of versican was higher in DRF compared to PC pellets ($P = 0.0020$), and the opposite was found for aggrecan ($P = 0.0017$). Overall, similar responses to cytokine treatment were found between sites. Significant interaction between treatment and site was found for aggrecan, where decreased expression was found in IL-1β treated PC pellets ($P < 0.0001$) but not DRF pellets.

The relative gene expressions of untreated DRF and PC pellets were compared, and in the three younger horses higher expression of versican and collagen type I was noted in DRF compared to PC pellets, whereas higher Sox9, collagen type II and aggrecan expression was noted in PC compared to DRF pellets.

5.3.2 Effects of IL-1β and IL-6 on HMGB-1 immunolocalisation and gene expression (paper IV)

The effects of IL-1β, IL-6 on HMGB-1 immunolocalisation and gene expression were evaluated in 3D cultured chondrocytes originating from the DRF (n=4 or n=3) at three different times (48 h, 96 h and 7 days of treatment). Immunolocalisation of HMGB-1 was evaluated by immunohistochemistry and immunolelectron microscopy, and gene expression was determined using qRT-PCR. A quantitative assessment of HMGB-1 translocation and extracellular presence was performed by comparing N/C ratios and ECM densities between cytokine treated and untreated pellets.

It was not possible to detect a difference in HMGB-1 immunolocalisation between treated and untreated pellets by immunohistochemistry, and presence of nuclear as well as cytoplasmic HMGB-1 was detected in treated as well as untreated pellets. Presence of extracellular HMGB-1 was sometimes suspected but the exact location was made uncertain due to indistinct cytoplasmic borders and/or undiscernable nuclei.

However, by using immunoelectron microscopy increased ECM density for HMGB-1, compared to untreated controls, was detected in 6/10 IL-6 treated pellets and decreased N/C ratios were found in 7/10 IL-6 treated pellets. In IL-1β treated pellets, there was an increase in ECM density of HMGB-1 in 2/10 pellets and decreased N/C ratios in 5/10 pellets. Increased ECM density of HMGB-1 was detected after IL-6 treatment in pellets from all horses at some time point, but only in one horse after IL-1β treatment. In addition, a trend toward decreased N/C ratios was detected with time, particularly in the untreated pellets.

Changes in HMGB-1 relative gene expression were often small. A clear increase (ratio treated/untreated >2) in HMGB-1 gene expression was only detected in one IL-1β and one IL-6 treated pellet, although in an additional two IL-1β treated samples the ratio (treated/untreated) exceeded 1.7. A clear decrease (ratio treated/untreated <0.5) was detected in one IL-1β treated pellet. When the gene expression ratios were evaluated together with the ratios (treated/untreated) for HMGB-1 immunolocalisation and time, increasing nuclear, but not cytoplasmic or ECM, immunolabelling was preceded by or concurrent with increased HMGB-1 relative gene expression.

6 General discussion

In order to identify proinflammatory cytokines with possible involvement in equine OA, the first two studies of the thesis investigated if IL-6, TNF or HMGB-1 could be demonstrated in joints suffering from OA associated pathology, and if they were related to a type or grade of joint lesion (i.e. could be regarded as a marker of the pathologic finding). By using *in vitro* studies, findings from the initial studies were followed up to investigate effects from detected cytokines on cartilage metabolism (paper III), and possible causes of extracellular HMGB-1 in cartilage (paper IV). The results from the *in vitro* studies need to be cautiously interpreted in regard to possible *in vivo* effects of investigated cytokines. *In vitro*-induced phenotype alterations of the cultured chondrocytes, and lack of natural biomechanical stimuli, interactions between the joint tissues, synovial fluid components and inflammatory cells were excluded from the culture system, but may influence the outcome of cytokine actions *in vivo*.

6.1 Cytokines in diseased joints

Increased concentrations of IL-6 activity were identified in joints with chip-fractures, whereas concentrations of TNF activity did not indicate presence of a specific category of joint lesion. The increase in IL-6 activity in chip-fracture joints suggests that IL-6 may be important in the disease process of osteochondral fragmentation. Carpal chip-fractures are often removed by arthroscopic surgery in order to relieve pain and to prevent progression of OA (McIlwraith & Bramlage, 1996), and it is possible that IL-6 is a mediator of chip-fracture-induced advancement of OA. IL-6 is known to induce bone resorption (Ishimi *et al.*, 1990), and has previously been associated with pathologic bone resorption in equine subchondral cystic lesions, indicated by

an upregulation of IL-6 mRNA in fibrous tissue in the cyst centres (von Rechenberg *et al.*, 2001).

In experimentally induced synovitis, synovial fluid concentrations of TNF and IL-6 activities increase in the early inflammatory response (Cornelissen *et al.*, 1998; Hawkins *et al.*, 1995; Hawkins *et al.*, 1993). In these studies, peak TNF activity was detected prior to peak synovial fluid leukocyte counts (Cornelissen *et al.*, 1998; Hawkins *et al.*, 1995; Hawkins *et al.*, 1993), whereas peak IL-6 activity was detected with peak leukocyte counts (Hawkins *et al.*, 1995; Hawkins *et al.*, 1993). These findings suggest that resident cells (in case of TNF production) as well as infiltrating inflammatory cells (in case of IL-6 production) may be important in cytokine production. Interestingly, *in vitro* treatment of equine chondrocytes and synoviocyte with lipopolysaccaride did not induce TNF activity, but increased concentrations of IL-6 activity in culture media from both cell types (Armstrong & Lees, 2002). Thus, both IL-6 and TNF may be involved in the inflammatory response of the joint, but clearly, depending on experimental conditions, the cytokine response may vary.

In the present study of naturally diseased joints, macroscopic signs of synovitis were often found despite no detection of IL-6 and TNF activities in the synovial fluid. All joints with chip-fractures showed synovitis macroscopically. However, there were no differences in concentrations of IL-6 between categories of synovitis, or correlation between IL-6 and synovial fluid leukocyte counts, suggesting that IL-6 activity did not primarily reflect grade of synovitis, and that other cells than infiltrating inflammatory cells may be important in the production of IL-6. Studies on human osteoarthritic tissues have demonstrated IL-6 production in several cell types including synoviocytes (Field *et al.*, 1991; Guerne *et al.*, 1989), plasma cells in the synovial lining layer (Doß *et al.*, 2007), chondrocytes (Moos *et al.*, 1999; Guerne *et al.*, 1990), and osteoblasts from subchondral bone (Sakao *et al.*, 2008; Massicotte *et al.*, 2002) and osteophytes (Sakao *et al.*, 2009). Thus, several joint tissues and cell types could have been responsible for IL-6 production in the present study, and this was further investigated in paper II.

The results on TNF are in agreement with other studies on naturally occurring equine joint disease, where no significant correlation between TNF activity and grades of cartilage lesions was found (Jouglin *et al.*, 2000), and no increase in TNF activity with chip-fractures or synovitis was seen

(Billinghurst *et al.*, 1995). Neither TNF nor IL-6 bioactivities in synovial fluid are strictly associated with clinical signs of joint disease (van den Boom *et al.*, 2004; Billinghurst *et al.*, 1995; Hawkins *et al.*, 1995), and TNF activity detected in synovial fluid from one of the non-lame horses might have been triggered by non-disease-associated mechanisms, such as athletic activity (van den Boom *et al.*, 2004).

Only low bioactivity of TNF was detected in the diseased joints. Hence, we did not find TNF useful as marker of joint pathology. Since concentrations of IL-6 were found in most joints with chip-fractures, IL-6 could theoretically be regarded as a marker of such lesions. Synovial fluid concentrations of TNFα and IL-6 in naturally diseased equine joints have previously been determined by using human immunoassays, and it was suggested that concentrations of TNFα well predicted, and of IL-6 excellently predicted joint disease (Bertone *et al.*, 2001). However, the amino acid sequence homology between human and equine cytokines, reported to be 77.6% for IL-6 (Swiderski *et al.*, 2000) and 87% for TNFα (Su *et al.*, 1992), may not be high enough to allow confidence in results based upon human immmunoassays. Other researchers have reported difficulties in interpreting results based on human immunoassays due to low cross-reactivity (Theoret *et al.*, 1998), and claimed that human immunoassays do not accurately detect equine cytokines (Armstrong & Lees, 2002). In the present work, a commersial immunoassay for equine TNFα (Endogen, Rockford, IL, USA) was used to detect TNFα in the synovial fluid samples previously analyzed for TNF activity. In samples diluted 1:2 or 1:4 TNFα could not be detected (unpublished results), supporting that presence of TNFα was indeed low in analysed samlpes.

In paper II, cells in the synovial membrane were identified as producers of IL-6 in healthy as well as in OA and OCF joints. Absence of immunostaining for IL-6 in synovial membrane from several diseased joints is in accordance with the results in paper I, where IL-6 activity was detected in less than half (47%) of the synovial fluid samples. Absence of a difference in frequency of IL-6 positive synovial membrane biopsies between OA and OCF joints suggested that other joint tissues than the synovial membrane may be involved in IL-6 production. This was confirmed by presence of immunostaining for IL-6 in cells of all examined OCF. This further suggests that the OCF itself is likely to take active part in the inflammatory response of diseased joints.

The finding of IL-6 positive cells in normal synovial membranes was somewhat unexpected since IL-6 was not detected in synovial fluid from normal joints (paper I). However, as discussed above, activity of IL-6 has been detected by others in synovial fluid from normal equine joints (Hawkins *et al.*, 1995; Hawkins *et al.*, 1993), and it is possible that the production is related to maintenance of metabolic homeostasis in healthy joint. Since the biopsies from normal joints were obtained after death, it cannot be excluded that IL-6 production in the synovial membranes could be related to post mortem-associated changes, such as tissue hypoxia (Ahn *et al.*, 2008; Yan *et al.*, 1995). Compared to chondrocytes, synvoviocytes are more sensitive to hypoxia (Schneider *et al.*, 2005), which may explain why many synoviocytes but few chondrocytes in normal tissue showed IL-6 immunostaining.

A cytoplasmic/extracellular pattern of HMGB-1 in the synovial membrane was mainly seen in joints with OCF, although it was also present in one OA joint. Thus, it may be speculated that presence of OCF contributed to release of HMGB-1 in the synovial membrane. Release of HMGB-1 in the synovial membrane has been associated with inflammatory arthritides such as rheumatoid arthritis (Kokkola *et al.*, 2002). The present study showed for the first time that extracellular HMGB-1 may arise from cells in the synovial membrane also in osteoarthritic equine joints. A later study have further confirmed presence of extracellular HMGB-1 in carpal and feltlock joints with OCF by demonstrating significant increased synovial fluid concentrations of HMGB-1 in injured compared to normal joints (Brown *et al.*, 2009). In synovial fluid from the joints of paper II, HMGB-1 was detected using an immunoassay (HMGB-1 ELISA kit II, IBL International GmbH, Hamburg, Germany) in 7/10 chip fracture joints, and in 3/10 (one missing sample) OA joints (unpublished results).

Presence of extracellular HMGB-1 in the OCF further supports a role for HMGB-1 in equine OA. Chondrocytes stimulated with HMGB-1 have been found to produce MMP-13 (Loeser *et al.*, 2005), which is highly efficient in degrading collagen type II (Knäuper *et al.*, 1996), although this was not confirmed in the *in vitro* studies of the present work (paper III). Furthermore, HMGB-1 deposits at reduplicated tidemarks may suggest involvement in the advancement of subchondral bone, since HMGB-1 has been found to have a regulatory role in enchondral ossification (Taniguchi *et al.*, 2007).

Based upon a small number of joints, increased grade of synovitis was detected microscopically in OA and OCF joints compared to normal joints. However, in general the synovial reaction in diseased joints was mild. The minor infiltration of inflammatory infiltrates is similar to previous descriptions in joints with traumatic arthritis and naturally occurring OA (Ronéus *et al.*, 1997; Trotter & McIlwraith, 1996).

The material used for papers I and II was obtained from cases of naturally occurring and clinically relevant equine traumatic arthritis/OA. The results of cytokine detection may be confounded by natural fluctuations in disease activity, and presence of joint lesions may have related both to previous or ongoing disease activity. Athletic horses suffering from joint disease are often treated medically before presented for arthroscopy. This means that the majority of joints had received intra-articular medication at some stage before arthroscopy. However, in cases of chip-fractures intra-articular medication had less often been given. It cannot be excluded that cytokine levels in the medicated joints were influenced by treatment. However, little is known about how the commonly used antiiflammatory treatments affect cytokine production in equine joints *in vivo*. Recurrence of clinical signs despite treatment indicated that previous medication had not managed to control the disease process.

6.2 Effects of cytokines on cultured chondrocytes

An *in vitro* system using 3D chondrocyte cultures was established to investigate the effects of the cytokines identified in diseased joints (IL-6 and HMGB-1) on cartilage metabolism. In addition, IL-1β was used in the *in vitro* experiments. Whereas the effects of IL-6 and HMGB-1 on equine chondrocytes previously have not been reported, cells were also stimulated with IL-1β, which is known to induce a catabolic response in regard to proteoglycan metabolism in equine cartilage explants (Garvican *et al.*, 2008; Gregg *et al.*, 2006; Little *et al.*, 2005; Frean *et al.*, 2000; Bird *et al.*, 1997; Morris & Treadwell, 1994; Platt & Bayliss, 1994; MacDonald *et al.*, 1992), and upregulate matrix degrading enzymes (Little *et al.*, 2005; Takafuji *et al.*, 2005; Tung *et al.*, 2002a; Richardson & Dodge, 2000; Caron *et al.*, 1996) and TIMP-1 (Tung *et al.*, 2002a) in cultured equine chondrocytes.

There are differences in loading patterns between the DRF and PC of C3 (Palmer *et al.*, 1994), and these two sites were chosen to study possible differences in chondrocyte phenotypes and cytokine-induced responses in

matrix metabolism as an explanation for site-associated cartilage pathology of C3. However, in the studies of HMGB-1 immunolocalisation and gene expression only DRF derived chondrocytes were studied, as the DRF represents an area commonly affected by chondral and osteochondral pathology in equine OA (Pool & Meagher, 1990). Only macroscopically healthy joints were studied in order to ensure that the phenotypes of sampled chondrocytes were not influenced by presence of concurrent intra-articular disease.

An overall catabolic response on proteoglycan metabolism was demonstrated in IL-1β treated pellets, indicated by the reduced safranin-O staining compared to untreated control pellets. The effect could partly be related to an anti-chondrogenic or dedifferentiating effect induced by IL-1β treatment, as the gene expression for Sox9 was decreased and versican increased in the majority of IL-1β treated pellets. However, reduced matrix proteoglycan content could also relate to increased production of matrix degrading enzymes, as increased MMP-9, MMP-13 and ADAMTS-5 gene expression was detected in IL-1β treated pellets. Although protein detection was not performed for MMP-9 and ADAMTS-5, increased concentrations of activated MMP-13 was detected in pellet supernatants from IL-1β treated pellets.

Absence of reduced safranin-O staining, and minor changes to gene expression for MMP-13 and ADAMTS-5 suggest that IL-6 and HMGB-1 treatment did not induce substantial matrix degradation. Instead, in IL-6 and HMGB-1 treated pellets Sox9 expression was often increased, which indicated that IL-6 and HMGB-1 treatment promoted chondrocyte differentiation and cartilage formation. This effect was most noticable in the two youngest horses, where untreated pellets showed the most fibrocartilage-like phenotype (i.e. DRF pellets).

The difference in gene expression profiles in untreated DRF and PC pellets of the younger horses, suggested a more hyalin cartilage phenotype in PC compared to DRF. This might be associated with different loading patterns *in vivo*, and could indicate a predisposition for the DRF to *in vivo* produce cartilage matrix with inadequate biomechanical properties. The DRF is a biomechanically more intensely loaded area than the PC (Palmer *et al.*, 1994), and previous studies have shown lowered aggrecan and increased decorin synthesis in the DRF compared to PC in strenously, but not moderately, trained three to five year old horses (Little *et al.*, 1997). Both

explant and alginate bead cultures from highly loaded ovine cartilage show lower aggrecan and higher decorin synthesis compared to cultures from less loaded areas, suggesting that loading is of importance in modulating the chondrocyte phenotype (Little & Ghosh, 1997).

The notable differences in gene expressions after cytokine treatment in the younger horses, may reflect increased responsiveness of young cartilage to cytokines, which is in accordance with previous findings on IL-1α (Morris & Treadwell, 1994; MacDonald *et al.*, 1992) and TGF-β (Iqbal *et al.*, 2000) in equine cartilage explants. Thus, it can be speculated that increased cytokine production *in vivo* will have greater effect on chondrocyte metabolism in younger than older individuals.

The similar response to cytokine stimulation in cells from DRF and PC does not support site-associated differences to cytokine exposure *in vivo*, and is in accordance with previously reported results based upon stimulation of equine cartilage explants with human recombinant IL-1β and TNFα (Little *et al.*, 2005).

The 3D cultured chondrocytes appeared relatively insensitive to IL-1β and IL-6 treatment in regard to translocation and release of HMGB-1 and changes in HMGB-1 gene expression. However, there was a trend for increased ECM density of HMGB-1 and decreased HMGB-1 N/C ratios in IL-6 treated pellets, which suggests that HMGB-1 was translocated and released in response to IL-6 treatment. In contrast, no trend for increased ECM density of HMGB-1 was detected in IL-1β treated samples, although at 48 h all IL-1β treated pellets showed a decreased N/C ratio.

It can not be excluded that HMGB-1 was released in response to cytokine treatment at earlier time points than those investigated. It has been shown that monocytes stimulated with lipopolysaccaride redistribute nuclear HMGB-1 to cytoplasmic vesicles already within 1 h of exposure (Bonaldi *et al.*, 2003), and pituicytes release HMGB-1 after 3-4 h of IL-1 stimulation (Wang *et al.*, 1999b). However, in order to simulate the *in vivo* exposure to IL-6 of chondrocytes in chip-fracture joints, where increased bioactivity of IL-6 is suspected to remain high for prolonged periods (paper I), the pellet cultures were evaluated after prolonged cytokine treatment. Increased relative HMGB-1 gene expression preceding or concurrent with increasing nuclear HMGB-1 density may indicate that early translocation and release of HMGB-1 triggered increased HMGB-1 gene expression to restore nuclear

HMGB-1 concentrations. However, in a previous 18 day study where pelleted murine rib chondrocytes of growth cartilage (but not chondrocytes from human articular cartilage) released HMGB-1 for up to day 3 of pellet culture, increased mRNA levels were not demonstrated (Taniguchi *et al.*, 2007). In the present study, the finding of increased gene expression and increasing nuclear densities was based upon ratios between treated and untreated controls, and the clear decrease in N/C ratios in controls needs to be taken into account when evaluating the relation between gene expression and nuclear densities, as well as cytoplasmic densities.

The reason for decreased N/C ratios with time is unclear. It is possible that decreased N/C ratios are associated with increased grade of differentiation of the pelleted cells. 3D cultured chondrocytes are known to gradually redifferentiate from the fibroblastic phenotype seen during expansion in monolayer culture with time (Tallheden *et al.*, 2005; Tallheden *et al.*, 2004). In embryonic murine growth cartilage HMGB-1 was not demonstrated in resting and proliferating chondrocytes, showed a nuclear localisation in prehypertrophic chondrocytes, was translocated to the cytoplasm of hypertrophic chondrocytes, and released extracellularly from terminal hypertrophic chondrocytes (Taniguchi *et al.*, 2007). Although in the present study, growth cartilage was only detected in the deep layer of the cartilage in samples from one horse (six months old), all horses were young and it is possible that the harvested chondrocytes had retained some properties of chondrocytes of growth cartilage. In addition, it can not be ruled out that other culture-induced factors also could have influenced the N/C ratios.

Based upon the trends for increased ECM density of HMGB-1 and decreased HMGB-1 N/C ratios in IL-6 treated pellets, it is possible that HMGB-1 is relased in equine articular cartilage as a consequence of IL-6 stimulation. If so, this could provide a link between high levels of bioactive IL-6 in synovial fluid of joints with OCF (paper I) and presence of extracellular HMGB-1 in the fragment (paper II).

7 Conclusions

- Concentrations of bioactive IL-6 were often greatly increased in synovial fluid from carpal joints with OCF (chip-fractures). This suggests a role for IL-6 in the disease development of affected joints and a potential use for IL-6 as a marker of carpal osteochondral fragmentation. In contrast, synovial fluid concentrations of TNF bioactivity were not associated with any type of joint lesion.
- Synovial membrane and OCF may both serve as sources of IL-6 and extracellular HMGB-1. Production of IL-6 in OCF indicates that the fragment is actively involved in the inflammatory response. This is also supported by presence of extracellular HMGB-1 in cartilage and fibrous tissue of OCF, and possibly by the relatively more common finding of extracellular HMGB-1 in synovial membranes from chip-fracture joints.
- Increased synovitis was morphologically detected in OA and OCF joints compared to healthy joints, but no difference in grade of synovitis was found between OA and OCF joints.
- IL-1β induced a catabolic matrix response (matrix degradation) in chondrocyte pellets, whereas both IL-6 and HMGB-1 appeared to promote chondrocyte differentiation and cartilage formation. These results propose that the proinflammatory cytokines have different roles in cartilage biology, and suggest that the inflammatory response of the joint can contribute to cartilage deterioration as well as induce processes promoting healing. There was minimal support for topographic variation in response to cytokine treatment between chondrocytes from different anatomical sites.
- Trends for increased ECM density of HMGB-1 and lowered HMGB-1 N/C ratios in IL-6 treated chondrocyte pellets support that HMGB-1 is involved in the inflammatory response of

chondrocytes, and that extracellular HMGB-1 in OCF may be a consequence of IL-6 stimulation.

8 Future research

Studies on the inflammatory response and presence of cytokines in equine OA is hampered by few species-specific comercially available reagents, such as antibodies and immunoassays. However, such reagents would be of great help in further clarifying the role of proinflammatory cytokines in the development of disease.

To follow up the work performed as part of this thesis, it would be of interest to:

- Investigate presence of IL-1β and IL-1Ra in diseased equine joints. Although thought to be of major importance in equine OA, no equine studies have examined synovial fluid concentrations of IL-1β (or IL-1Ra) in regard to cartilage pathology in naturally occurring joint disease, and protein detection of IL-1β in joint tissues have relied upon the use of non-species specific antibodies.
- Investigate presence of membrane bound IL-6Rα and gp130 on chondrocytes in culture and in cartilage, and of sIL-6Rα and gp130 in synovial fluid. Both IL-6 receptors may modulate the effects of IL-6, but their presences in equine joints have not been studied.
- Study chondrocyte phenotypes in articular cartilage from the DRF and PC in untrained and trained young and old athletic horses, with the aim to establish if DRF chondrocytes are predisposed to *in vivo* synthesis of extracellular matrix with inferior biomechanical properties compared to cells from PC.
- Characterize the early HMGB-1 response in chondrocytes exposed to IL-1β and IL-6, by evaluating HMGB-1 production

and translocation in pellet cultures prior to 48 hours of stimulation.

9 References

Aarden, L.A., De Groot, E.R., Schaap, O.L. & Lansdorp, P.M. (1987). Production of hybridoma growth factor by human monocytes. *Eur J Immunol* 17(10), 1411-6.

af Klint, E., Grundtman, C., Engström, M., Catrina, A.I., Makrygiannakis, D., Klareskog, L., Andersson, U. & Ulfgren, A.K. (2005). Intraarticular glucocorticoid treatment reduces inflammation in synovial cell infiltrations more efficiently than in synovial blood vessels. *Arthritis Rheum* 52(12), 3880-9.

Aggarwal, B.B. (2003). Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 3(9), 745-56.

Aggarwal, B.B., Kohr, W.J., Hass, P.E., Moffat, B., Spencer, S.A., Henzel, W.J., Bringman, T.S., Nedwin, G.E., Goeddel, D.V. & Harkins, R.N. (1985). Human tumor necrosis factor. Production, purification, and characterization. *J Biol Chem* 260(4), 2345-54.

Ahn, J.K., Koh, E.M., Cha, H.S., Lee, Y.S., Kim, J., Bae, E.K. & Ahn, K.S. (2008). Role of hypoxia-inducible factor-1α in hypoxia-induced expressions of IL-8, MMP-1 and MMP-3 in rheumatoid fibroblast-like synoviocytes. *Rheumatology (Oxford)* 47(6), 834-9.

Alwan, W.H., Carter, S.D., Bennett, D. & Edwards, G.B. (1991a). Glycosaminoglycans in horses with osteoarthritis. *Equine Vet J* 23(1), 44-7.

Alwan, W.H., Carter, S.D., Dixon, J.B., Bennett, D., May, S.A. & Edwards, G.B. (1991b). Interleukin-1-like activity in synovial fluids and sera of horses with arthritis. *Res Vet Sci* 51(1), 72-7.

Amiel, D., Frank, C., Harwood, F., Fronek, J. & Akeson, W. (1984). Tendons and ligaments: a morphological and biochemical comparison. *J Orthop Res* 1(3), 257-65.

Anastasiou, A., Skiöldebrand, E., Ekman, S. & Hall, L.D. (2003). Ex vivo magnetic resonance imaging of the distal row of equine carpal bones: assessment of bone sclerosis and cartilage damage. *Vet Radiol Ultrasound* 44(5), 501-12.

Andersson, U., Wang, H., Palmblad, K., Aveberger, A.C., Bloom, O., Erlandsson-Harris, H., Janson, A., Kokkola, R., Zhang, M., Yang, H. & Tracey, K.J. (2000). High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. *J Exp Med* 192(4), 565-70.

Arai, K., Misumi, K., Carter, S.D., Shinbara, S., Fujiki, M. & Sakamoto, H. (2005). Analysis of cartilage oligomeric matrix protein (COMP) degradation and synthesis in equine joint disease. *Equine Vet J* 37(1), 31-6.

Arend, W.P. (2002). The balance between IL-1 and IL-1Ra in disease. *Cytokine Growth Factor Rev* 13(4-5), 323-40.

Armstrong, S. & Lees, P. (2002). Effects of carprofen (R and S enantiomers and racemate) on the production of IL-1, IL-6 and TNF-α by equine chondrocytes and synoviocytes. *J Vet Pharmacol Ther* 25(2), 145-53.

Attur, M., Dave, M., Akamatsu, M., Nakagawa, N., Miki, J., Yang, H., Katoh, M., Wisniewski, J., Tracey, K.J. & Amin, A. (2003). Differential expression of high mobility group protein in human normal and arthritic cartilage; functional genomic analysis. In: *Proceedings of 49th Annual Meeting of the Orthopaedic Research Society* pp. 18.

Balkwill, F. (2001). Cytokines and cytokine receptors. In: Roitt, I.*, et al.* (Eds.) *Immunology*. Edinburgh: Mosby. pp. 119-129.

Bell, C.W., Jiang, W., Reich, C.F., 3rd & Pisetsky, D.S. (2006). The extracellular release of HMGB1 during apoptotic cell death. *Am J Physiol Cell Physiol* 291(6), C1318-25.

Benito, M.J., Veale, D.J., FitzGerald, O., van den Berg, W.B. & Bresnihan, B. (2005). Synovial tissue inflammation in early and late osteoarthritis. *Ann Rheum Dis* 64(9), 1263-7.

Bertone, A.L., Palmer, J.L. & Jones, J. (2001). Synovial fluid cytokines and eicosanoids as markers of joint disease in horses. *Vet Surg* 30(6), 528-38.

Bianchi, M.E. (2007). DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol* 81(1), 1-5.

Bianchi, M.E. (2009). HMGB1 loves company. *J Leukoc Biol* 86(3), 573-6.

Bianchi, M.E. & Agresti, A. (2005). HMG proteins: dynamic players in gene regulation and differentiation. *Curr Opin Genet Dev* 15(5), 496-506.

Billinghurst, R.C., Fretz, P.B. & Gordon, J.R. (1995). Induction of intra-articular tumour necrosis factor during acute inflammatory responses in equine arthritis. *Equine Vet J* 27(3), 208-16.

Bird, J.L.E., Wells, T., Platt, D. & Bayliss, M.T. (1997). IL-1β induces the degradation of equine articular cartilage by a mechanism that is not mediated by nitric oxide. *Biochem Biophys Res Commun* 238(1), 81-5.

Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K.A., Gerhart, M., Davis, R., Fitzner, J.N., Johnson, R.S., Paxton, R.J., March, C.J. & Cerretti, D.P. (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-α from cells. *Nature* 385(6618), 729-33.

Blanco, F.J., Ochs, R.L., Schwarz, H. & Lotz, M. (1995). Chondrocyte apoptosis induced by nitric oxide. *Am J Pathol* 146(1), 75-85.

Bolam, C.J., Hurtig, M.B., Cruz, A. & McEwen, B.J. (2006). Characterization of experimentally induced post-traumatic osteoarthritis in the medial femorotibial joint of horses. *Am J Vet Res* 67(3), 433-47.

Bonaldi, T., Talamo, F., Scaffidi, P., Ferrera, D., Porto, A., Bachi, A., Rubartelli, A., Agresti, A. & Bianchi, M.E. (2003). Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *Embo J* 22(20), 5551-60.

Boulanger, M.J., Chow, D.C., Brevnova, E.E. & Garcia, K.C. (2003). Hexameric structure and assembly of the interleukin-6/IL-6 α-receptor/gp130 complex. *Science* 300(5628), 2101-4.

Boyde, A., Haroon, Y., Jones, S.J. & Riggs, C.M. (1999). Three dimensional structure of the distal condyles of the third metacarpal bone of the horse. *Equine Vet J* 31(2), 122-9.

Brama, P.A.J., Holopainen, J., van Weeren, P.R., Firth, E.C., Helminen, H.J. & Hyttinen, M.M. (2009). Influence of exercise and joint topography on depth-related spatial distribution of proteoglycan and collagen content in immature equine articular cartilage. *Equine Vet J* 41(6), 557-63.

Brama, P.A.J., Karssenberg, D., Barneveld, A. & van Weeren, P.R. (2001). Contact areas and pressure distribution on the proximal articular surface of the proximal phalanx under sagittal plane loading. *Equine Vet J* 33(1), 26-32.

Brama, P.A.J., TeKoppele, J.M., Bank, R.A., Barneveld, A., Firth, E.C. & van Weeren, P.R. (2000a). The influence of strenuous exercise on collagen characteristics of articular cartilage in Thoroughbreds age 2 years. *Equine Vet J* 32(6), 551-4.

Brama, P.A.J., TeKoppele, J.M., Bank, R.A., Barneveld, A. & van Weeren, P.R. (2000b). Functional adaptation of equine articular cartilage: the formation of regional biochemical characteristics up to age one year. *Equine Vet J* 32(3), 217-21.

Brama, P.A.J., TeKoppele, J.M., Bank, R.A., Barneveld, A. & van Weeren, P.R. (2002). Development of biochemical heterogeneity of articular cartilage: influences of age and exercise. *Equine Vet J* 34(3), 265-9.

Brama, P.A.J., TeKoppele, J.M., Bank, R.A., Karssenberg, D., Barneveld, A. & van Weeren, P.R. (2000c). Topographical mapping of

biochemical properties of articular cartilage in the equine fetlock joint. *Equine Vet J* 32(1), 19-26.

Brama, P.A.J., TeKoppele, J.M., Bank, R.A., van Weeren, P.R. & Barneveld, A. (1999). Influence of different exercise levels and age on the biochemical characteristics of immature equine articular cartilage. *Equine Vet J Suppl*(31), 55-61.

Brama, P.A.J., TeKoppele, J.M., Beekman, B., van El, B., Barneveld, A. & van Weeren, P.R. (2000d). Influence of development and joint pathology on stromelysin enzyme activity in equine synovial fluid. *Ann Rheum Dis* 59(2), 155-7.

Brama, P.A.J., TeKoppele, J.M., Beekman, B., van Weeren, P.R. & Barneveld, A. (1998). Matrix metalloproteinase activity in equine synovial fluid: influence of age, osteoarthritis, and osteochondrosis. *Ann Rheum Dis* 57(11), 697-9.

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

Bramlage, L.R. (1983). Surgical diseases of the carpus. *Vet Clin North Am Large Anim Pract* 5(2), 261-74.

Bramlage, L.R., Schneider, R.K. & Gabel, A.A. (1988). A clinical perspective on lameness originating in the carpus. *Equine Vet J Suppl*(6), 12-8.

Brandt, K.D., Dieppe, P. & Radin, E.L. (2008). Etiopathogenesis of osteoarthritis. *Rheum Dis Clin North Am* 34(3), 531-59.

Brommer, H., Brama, P.A.J., Barneveld, A. & van Weeren, P.R. (2004). Differences in the topographical distribution of articular cartilage degeneration between equine metacarpo- and metatarsophalangeal joints. *Equine Vet J* 36(6), 506-10.

Brommer, H., Laasanen, M.S., Brama, P.A.J., van Weeren, P.R., Helminen, H.J. & Jurvelin, J.S. (2005). Functional consequences of cartilage degeneration in the equine metacarpophalangeal joint: quantitative assessment of cartilage stiffness. *Equine Vet J* 37(5), 462-7.

Brommer, H., van Weeren, P.R. & Brama, P.A.J. (2003). New approach for quantitative assessment of articular cartilage degeneration in horses with osteoarthritis. *Am J Vet Res* 64(1), 83-7.

Brown, M.P., Trumble, T.N. & Merritt, K.A. (2009). High-mobility group box chromosomal protein 1 as a potential inflammatory biomarker of joint injury in Thoroughbreds. *Am J Vet Res* 70(10), 1230-5.

Brown, M.P., Trumble, T.N., Plaas, A.H., Sandy, J.D., Romano, M., Hernandez, J. & Merritt, K.A. (2007). Exercise and injury increase chondroitin sulfate chain length and decrease hyaluronan chain length in synovial fluid. *Osteoarthritis Cartilage* 15(11), 1318-25.

Buckwalter, J.A. & Hunziker, E.B. (1999). Articular cartilage morhology and biology. In: Archer, C.W., *et al.* (Eds.) *Biology of the synovial joints*. Singapore: Harwood Academic Publishers. pp. 75-100.

Buckwalter, J.A., Rosenberg, L.C. & Hunziker, E.B. (1990). Articular cartilage: composition, structure, response to injury, and methods of facilitating repair. In: Ewing, J.W. (Ed.) *Articular cartilage and knee joint function: basic science and arthroscopy*. New York: Raven Press Ltd. pp. 19-56.

Burr, D.B. (2004). Anatomy and physiology of the mineralized tissues: role in the pathogenesis of osteoarthrosis. *Osteoarthritis Cartilage* 12 Suppl A, S20-30.

Burr, D.B. & Radin, E.L. (2003). Microfractures and microcracks in subchondral bone: are they relevant to osteoarthrosis? *Rheum Dis Clin North Am* 29(4), 675-85.

Bustin, M. (1999). Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins. *Mol Cell Biol* 19(8), 5237-46.

Bustin, M. (2001). Revised nomenclature for high mobility group (HMG) chromosomal proteins. *Trends Biochem Sci* 26(3), 152-3.

Butler, J.A., Colles, C.M., Dyson, S.J., Kold, S.E. & Poulos, P.W., Jr. (2000). The carpus. In: *Clinical radiology of the horse*. Oxford: Blackwell Science Ltd. pp. 171-204.

Cantley, C.E., Firth, E.C., Delahunt, J.W., Pfeiffer, D.U. & Thompson, K.G. (1999). Naturally occurring osteoarthritis in the metacarpophalangeal joints of wild horses. *Equine Vet J* 31(1), 73-81.

Caron, J.P. (2003). Osteoarthritis. In: Ross, M.W., *et al.* (Eds.) *Diagnosis and management of lameness in the horse*. St. Louis: Saunders. pp. 572-591.

Caron, J.P., Bowker, R.M., Abhold, R.H., Toppin, D.S., Sonea, I.M. & Vex, K.B. (1992). Substance P in the synovial membrane and fluid of the equine middle carpal joint. *Equine Vet J* 24(5), 364-6.

Caron, J.P., Tardif, G., Martel-Pelletier, J., DiBattista, J.A., Geng, C. & Pelletier, J.P. (1996). Modulation of matrix metalloprotease 13 (collagenase 3) gene expression in equine chondrocytes by interleukin 1 and corticosteroids. *Am J Vet Res* 57(11), 1631-4.

Clegg, P.D., Coughlan, A.R. & Carter, S.D. (1998). Equine TIMP-1 and TIMP-2: identification, activity and cellular sources. *Equine Vet J* 30(5), 416-23.

Clegg, P.D., Coughlan, A.R., Riggs, C.M. & Carter, S.D. (1997). Matrix metalloproteinases 2 and 9 in equine synovial fluids. *Equine Vet J* 29(5), 343-8.

Colotta, F., Re, F., Muzio, M., Bertini, R., Polentarutti, N., Sironi, M., Giri, J.G., Dower, S.K., Sims, J.E. & Mantovani, A. (1993). Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science* 261(5120), 472-5.

Convery, F.R., Akeson, W.H. & Keown, G.H. (1972). The repair of large osteochondral defects. An experimental study in horses. *Clin Orthop Relat Res* 82, 253-62.

Cornelissen, B.P., Rijkenhuizen, A.B., van den Hoogen, B.M., Rutten, V.P. & Barneveld, A. (1998). Experimental model of synovitis/capsulitis in the equine metacarpophalangeal joint. *Am J Vet Res* 59(8), 978-85.

Croft, M. (2009). The role of TNF superfamily members in T-cell function and diseases. *Nat Rev Immunol* 9(4), 271-85.

David, F., Farley, J., Huang, H., Lavoie, J.P. & Laverty, S. (2007). Cytokine and chemokine gene expression of IL-1β stimulated equine articular chondrocytes. *Vet Surg* 36(3), 221-7.

de Hooge, A.S.K., van de Loo, F.A.J., Bennink, M.B., Arntz, O.J., de Hooge, P. & van den Berg, W.B. (2005). Male IL-6 gene knock out mice developed more advanced osteoarthritis upon aging. *Osteoarthritis Cartilage* 13(1), 66-73.

Degryse, B., Bonaldi, T., Scaffidi, P., Müller, S., Resnati, M., Sanvito, F., Arrigoni, G. & Bianchi, M.E. (2001). The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. *J Cell Biol* 152(6), 1197-206.

Dinarello, C.A. (1998). Interleukin-1β, interleukin-18, and the interleukin-1β converting enzyme. *Ann N Y Acad Sci* 856, 1-11.

Dinarello, C.A. (2009). Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* 27, 519-50.

Doß, F., Menard, J., Hauschild, M., Kreutzer, H.J., Mittlmeier, T., Müller-Steinhardt, M. & Müller, B. (2007). Elevated IL-6 levels in the synovial fluid of osteoarthritis patients stem from plasma cells. *Scand J Rheumatol* 36(2), 136-9.

Dumitriu, I.E., Baruah, P., Manfredi, A.A., Bianchi, M.E. & Rovere-Querini, P. (2005a). HMGB1: guiding immunity from within. *Trends Immunol* 26(7), 381-7.

Dumitriu, I.E., Baruah, P., Valentinis, B., Voll, R.E., Herrmann, M., Nawroth, P.P., Arnold, B., Bianchi, M.E., Manfredi, A.A. & Rovere-Querini, P. (2005b). Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products. *J Immunol* 174(12), 7506-15.

El Mezayen, R., El Gazzar, M., Seeds, M.C., McCall, C.E., Dreskin, S.C. & Nicolls, M.R. (2007). Endogenous signals released from necrotic cells augment inflammatory responses to bacterial endotoxin. *Immunol Lett* 111(1), 36-44.

Farley, J., Sirois, J., MacFarlane, P.H., Kombé, A. & Laverty, S. (2005). Evaluation of coexpression of microsomal prostaglandin E synthase-1 and cyclooxygenase-2 in interleukin-1-stimulated equine articular chondrocytes. *Am J Vet Res* 66(11), 1985-91.

Field, M., Chu, C., Feldmann, M. & Maini, R.N. (1991). Interleukin-6 localisation in the synovial membrane in rheumatoid arthritis. *Rheumatol Int* 11(2), 45-50.

Flannery, C.R., Little, C.B., Hughes, C.E., Curtis, C.L., Caterson, B. & Jones, S.A. (2000). IL-6 and its soluble receptor augment aggrecanase-mediated proteoglycan catabolism in articular cartilage. *Matrix Biol* 19(6), 549-53.

Flores, R.H. & Hochberg, M.C. (2003). Definition and classification of osteoarthritis. In: Brandt, K.D., *et al.* (Eds.) *Osteoarthritis*. Oxford: University Press. pp. 1-16.

Frean, S.P., Bryant, C.E., Fröling, I.L., Elliott, J. & Lees, P. (1997). Nitric oxide production by equine articular cells in vitro. *Equine Vet J* 29(2), 98-102.

Frean, S.P., Gettinby, G., May, S.A. & Lees, P. (2000). Influence of interleukin-1β and hyaluronan on proteoglycan release from equine navicular hyaline cartilage and fibrocartilage. *J Vet Pharmacol Ther* 23(2), 67-72.

Frisbie, D.D., Al-Sobayil, F., Billinghurst, R.C., Kawcak, C.E. & McIlwraith, C.W. (2008). Changes in synovial fluid and serum biomarkers with exercise and early osteoarthritis in horses. *Osteoarthritis Cartilage* 16(10), 1196-204.

Frisbie, D.D., Ghivizzani, S.C., Robbins, P.D., Evans, C.H. & McIlwraith, C.W. (2002). Treatment of experimental equine osteoarthritis by in vivo delivery of the equine interleukin-1 receptor antagonist gene. *Gene Ther* 9(1), 12-20.

Frisbie, D.D., Kawcak, C.E., Werpy, N.M., Park, R.D. & McIlwraith, C.W. (2007). Clinical, biochemical, and histologic effects of intra-articular administration of autologous conditioned serum in horses with experimentally induced osteoarthritis. *Am J Vet Res* 68(3), 290-6.

Frisbie, D.D., Ray, C.S., Ionescu, M., Poole, A.R., Chapman, P.L. & McIlwraith, C.W. (1999). Measurement of synovial fluid and serum concentrations of the 846 epitope of chondroitin sulfate and of carboxy propeptides of type II procollagen for diagnosis of osteochondral fragmentation in horses. *Am J Vet Res* 60(3), 306-9.

Fuller, C.J., Barr, A.R., Sharif, M. & Dieppe, P.A. (2001). Cross-sectional comparison of synovial fluid biochemical markers in equine osteoarthritis and the correlation of these markers with articular cartilage damage. *Osteoarthritis Cartilage* 9(1), 49-55.

Gardella, S., Andrei, C., Ferrera, D., Lotti, L.V., Torrisi, M.R., Bianchi, M.E. & Rubartelli, A. (2002). The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO Rep* 3(10), 995-1001.

Garvican, E.R., Vaughan-Thomas, A., Redmond, C. & Clegg, P.D. (2008). MT3-MMP (MMP-16) is downregulated by in vitro cytokine

stimulation of cartilage, but unaltered in naturally occurring equine osteoarthritis and osteochondrosis. *Connect Tissue Res* 49(2), 62-7.
Ghosh, P. & Guidolin, D. (2002). Potential mechanism of action of intra-articular hyaluronan therapy in osteoarthritis: are the effects molecular weight dependent? *Semin Arthritis Rheum* 32(1), 10-37.
Gibson, K.T., Hodge, H. & Whittem, T. (1996). Inflammatory mediators in equine synovial fluid. *Aust Vet J* 73(4), 148-51.
Goldenberg, D.L., Egan, M.S. & Cohen, A.S. (1982). Inflammatory synovitis in degenerative joint disease. *J Rheumatol* 9(2), 204-9.
Goldring, M.B. & Goldring, S.R. (2007). Osteoarthritis. *J Cell Physiol* 213(3), 626-34.
Goldring, S.R. & Goldring, M.B. (2005). Biology of the normal joint. In: Harris, J.H.E., *et al.* (Eds.) *Kelley's textbook of rheumatology.* Philadelphia: Elsevier Saunders.1). pp. 1-34.
Gregg, A.J., Fortier, L.A., Mohammed, H.O., Mayr, K.G., Miller, B.J. & Haupt, J.L. (2006). Assessment of the catabolic effects of interleukin-1β on proteoglycan metabolism in equine cartilage cocultured with synoviocytes. *Am J Vet Res* 67(6), 957-62.
Grell, M., Douni, E., Wajant, H., Löhden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K. & Scheurich, P. (1995). The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell* 83(5), 793-802.
Guerne, P.A., Carson, D.A. & Lotz, M. (1990). IL-6 production by human articular chondrocytes. Modulation of its synthesis by cytokines, growth factors, and hormones in vitro. *J Immunol* 144(2), 499-505.
Guerne, P.A., Desgeorges, A., Jaspar, J.M., Relic, B., Peter, R., Hoffmeyer, P. & Dayer, J.M. (1999). Effects of IL-6 and its soluble receptor on proteoglycan synthesis and NO release by human articular chondrocytes: comparison with IL-1. Modulation by dexamethasone. *Matrix Biol* 18(3), 253-60.
Guerne, P.A., Zuraw, B.L., Vaughan, J.H., Carson, D.A. & Lotz, M. (1989). Synovium as a source of interleukin 6 in vitro. Contribution to local and systemic manifestations of arthritis. *J Clin Invest* 83(2), 585-92.
Haupt, J.L., Frisbie, D.D., McIlwraith, C.W., Robbins, P.D., Ghivizzani, S., Evans, C.H. & Nixon, A.J. (2005). Dual transduction of insulin-like growth factor-I and interleukin-1 receptor antagonist protein controls cartilage degradation in an osteoarthritic culture model. *J Orthop Res* 23(1), 118-26.
Hauptmann, B., Van Damme, J. & Dayer, J.M. (1991). Modulation of IL-1 inflammatory and immunomodulatory properties by IL-6. *Eur Cytokine Netw* 2(1), 39-46.
Hawkins, D.L., Cargile, J.L., MacKay, R.J., Broome, T.A. & Skelley, L.A. (1995). Effect of tumor necrosis factor antibody on synovial fluid

cytokine activities in equine antebrachiocarpal joints injected with endotoxin. *Am J Vet Res* 56(10), 1292-9.

Hawkins, D.L., MacKay, R.J., Gum, G.G., Colahan, P.T. & Meyer, J.C. (1993). Effects of intra-articularly administered endotoxin on clinical signs of disease and synovial fluid tumor necrosis factor, interleukin 6, and prostaglandin E2 values in horses. *Am J Vet Res* 54(3), 379-86.

Hay, C.W., Chu, Q., Budsberg, S.C., Clayton, M.K. & Johnson, K.A. (1997). Synovial fluid interleukin 6, tumor necrosis factor, and nitric oxide values in dogs with osteoarthritis secondary to cranial cruciate ligament rupture. *Am J Vet Res* 58(9), 1027-32.

Haywood, L., McWilliams, D.F., Pearson, C.I., Gill, S.E., Ganesan, A., Wilson, D. & Walsh, D.A. (2003). Inflammation and angiogenesis in osteoarthritis. *Arthritis Rheum* 48(8), 2173-7.

Hedbom, E., Antonsson, P., Hjerpe, A., Aeschlimann, D., Paulsson, M., Rosa-Pimentel, E., Sommarin, Y., Wendel, M., Oldberg, A. & Heinegård, D. (1992). Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage. *J Biol Chem* 267(9), 6132-6.

Henderson, B. & Pettipher, E.R. (1989). Arthritogenic actions of recombinant IL-1 and tumour necrosis factor α in the rabbit: evidence for synergistic interactions between cytokines in vivo. *Clin Exp Immunol* 75(2), 306-10.

Hilbert, B.J., Rowley, G. & Antonas, K.N. (1984). Hyaluronic acid concentration in synovial fluid from normal and arthritic joints of horses. *Aust Vet J* 61(1), 22-4.

Holland, P.M., Abramson, R.D., Watson, R. & Gelfand, D.H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc Natl Acad Sci U S A* 88(16), 7276-80.

Hopper, B.J., Steel, C., Richardson, J.L., Alexander, G.R. & Robertson, I.D. (2004). Radiographic evaluation of sclerosis of the third carpal bone associated with exercise and the development of lameness in Standardbred racehorses. *Equine Vet J* 36(5), 441-6.

Hori, O., Brett, J., Slattery, T., Cao, R., Zhang, J., Chen, J.X., Nagashima, M., Lundh, E.R., Vijay, S., Nitecki, D. & et al. (1995). The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *J Biol Chem* 270(43), 25752-61.

Howard, R.D., McIlwraith, C.W., Trotter, G.W. & Nyborg, J.K. (1998). Cloning of equine interleukin 1α and equine interleukin 1β and determination of their full-length cDNA sequences. *Am J Vet Res* 59(6), 704-11.

Huang, K. & Wu, L.D. (2008). Aggrecanase and aggrecan degradation in osteoarthritis: a review. *J Int Med Res* 36(6), 1149-60.

Iqbal, J., Dudhia, J., Bird, J.L. & Bayliss, M.T. (2000). Age-related effects of TGF-β on proteoglycan synthesis in equine articular cartilage. *Biochem Biophys Res Commun* 274(2), 467-71.

Ishimi, Y., Miyaura, C., Jin, C.H., Akatsu, T., Abe, E., Nakamura, Y., Yamaguchi, A., Yoshiki, S., Matsuda, T., Hirano, T. & et al. (1990). IL-6 is produced by osteoblasts and induces bone resorption. *J Immunol* 145(10), 3297-303.

Iwanaga, T., Shikichi, M., Kitamura, H., Yanase, H. & Nozawa-Inoue, K. (2000). Morphology and functional roles of synoviocytes in the joint. *Arch Histol Cytol* 63(1), 17-31.

Ivanov, S., Dragoi, A.M., Wang, X., Dallacosta, C., Louten, J., Musco, G., Sitia, G., Yap, G.S., Wan, Y., Biron, C.A., Bianchi, M.E., Wang, H. & Chu, W.M. (2007). A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. *Blood* 110(6), 1970-81.

Izumisawa, Y., Yamaguchi, M., Bertone, A.L., Tangkawattana, P., Masty, J., Yamashita, K. & Kotani, T. (1996). Equine synovial villi: distinctive structural organization of vasculature and novel nerve endings. *J Vet Med Sci* 58(12), 1193-204.

Jacques, C., Gosset, M., Berenbaum, F. & Gabay, C. (2006). The role of IL-1 and IL-1Ra in joint inflammation and cartilage degradation. *Vitam Horm* 74, 371-403.

Jay, G.D., Britt, D.E. & Cha, C.J. (2000). Lubricin is a product of megakaryocyte stimulating factor gene expression by human synovial fibroblasts. *J Rheumatol* 27(3), 594-600.

Jay, G.D., Tantravahi, U., Britt, D.E., Barrach, H.J. & Cha, C.J. (2001). Homology of lubricin and superficial zone protein (SZP): products of megakaryocyte stimulating factor (MSF) gene expression by human synovial fibroblasts and articular chondrocytes localized to chromosome 1q25. *J Orthop Res* 19(4), 677-87.

Jiang, W., Bell, C.W. & Pisetsky, D.S. (2007). The relationship between apoptosis and high-mobility group protein 1 release from murine macrophages stimulated with lipopolysaccharide or polyinosinic-polycytidylic acid. *J Immunol* 178(10), 6495-503.

Jikko, A., Wakisaka, T., Iwamoto, M., Hiranuma, H., Kato, Y., Maeda, T., Fujishita, M. & Fuchihata, H. (1998). Effects of interleukin-6 on proliferation and proteoglycan metabolism in articular chondrocyte cultures. *Cell Biol Int* 22(9-10), 615-21.

Johansson, H.E. & Rejno, S. (1976). Light and electron microscopic investigation of equine synovial membrane. A comparison between healthy joints and joints with intraarticular fractures and osteochondrosis dissecans. *Acta Vet Scand* 17(2), 153-68.

Jouglin, M., Robert, C., Valette, J.P., Gavard, F., Quintin-Colonna, F. & Denoix, J.M. (2000). Metalloproteinases and tumor necrosis factor-alpha activities in synovial fluids of horses: correlation with articular cartilage alterations. *Vet Res* 31(5), 507-15.

Kaneko, S., Satoh, T., Chiba, J., Ju, C., Inoue, K. & Kagawa, J. (2000). Interleukin-6 and interleukin-8 levels in serum and synovial fluid of patients with osteoarthritis. *Cytokines Cell Mol Ther* 6(2), 71-9.

Kannegieter, N.J. & Colgan, S.A. (1993). The incidence and severity of intercarpal ligament damage in the equine carpus. *Aust Vet J* 70(3), 89-91.

Kato, H., Ohashi, T., Nakamura, N., Nishimura, Y., Watari, T., Goitsuka, R., Tsujimoto, H. & Hasegawa, A. (1995). Molecular cloning of equine interleukin-1α and -β cDNAs. *Vet Immunol Immunopathol* 48(3-4), 221-31.

Kawcak, C.E., McIlwraith, C.W., Norrdin, R.W., Park, R.D. & James, S.P. (2001). The role of subchondral bone in joint disease: a review. *Equine Vet J* 33(2), 120-6.

Kazama, H., Ricci, J.E., Herndon, J.M., Hoppe, G., Green, D.R. & Ferguson, T.A. (2008). Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity* 29(1), 21-32.

Kiani, C., Chen, L., Wu, Y.J., Yee, A.J. & Yang, B.B. (2002). Structure and function of aggrecan. *Cell Res* 12(1), 19-32.

Kim, D.Y., Taylor, H.W., Moore, R.M., Paulsen, D.B. & Cho, D.Y. (2003). Articular chondrocyte apoptosis in equine osteoarthritis. *Vet J* 166(1), 52-7.

Kim, J., Xu, M., Xo, R., Mates, A., Wilson, G.L., Pearsall, A.W.,4th & Grishko, V. (2010). Mitochondrial DNA damage is involved in apoptosis caused by pro-inflammatory cytokines in human OA chondrocytes. *Osteoarthritis Cartilage* 18(3), 424-32.

Kirker-Head, C.A., Chandna, V.K., Agarwal, R.K., Morris, E.A., Tidwell, A., O'Callaghan, M.W., Rand, W. & Kumar, M.S. (2000). Concentrations of substance P and prostaglandin E2 in synovial fluid of normal and abnormal joints of horses. *Am J Vet Res* 61(6), 714-8.

Knäuper, V., López-Otin, C., Smith, B., Knight, G. & Murphy, G. (1996). Biochemical characterization of human collagenase-3. *J Biol Chem* 271(3), 1544-50.

Kokkola, R., Li, J., Sundberg, E., Aveberger, A.C., Palmblad, K., Yang, H., Tracey, K.J., Andersson, U. & Erlansson Harris, H. (2003). Successful treatment of collagen-induced arthritis in mice and rats by targeting extracellular high mobility group box chromosomal protein 1 activity. *Arthritis Rheum* 48(7), 2052-8.

Kokkola, R., Sundberg, E., Ulfgren, A.K., Palmblad, K., Li, J., Wang, H., Ulloa, L., Yang, H., Yan, X.J., Furie, R., Chiorazzi, N., Tracey, K.J., Andersson, U. & Erlandsson Harris, H. (2002). High mobility

group box chromosomal protein 1: a novel proinflammatory mediator in synovitis. *Arthritis Rheum* 46(10), 2598-603.

Krenn, V., Morawietz, L., Burmester, G.R., Kinne, R.W., Mueller-Ladner, U., Muller, B. & Haupl, T. (2006). Synovitis score: discrimination between chronic low-grade and high-grade synovitis. *Histopathology* 49(4), 358-64.

Kühn, K., Hashimoto, S. & Lotz, M. (2000). IL-1β protects human chondrocytes from CD95-induced apoptosis. *J Immunol* 164(4), 2233-9.

Kumar, V., Abbas, A.K., Fausto, N. & Aster, J.C. (2010). Acute and chronic inflammation. In: *Pathologic basis of disease*. Philadelphia: Saunders Elsevier. pp. 43-77.

Li, J., Kokkola, R., Tabibzadeh, S., Yang, R., Ochani, M., Qiang, X., Harris, H.E., Czura, C.J., Wang, H., Ulloa, L., Wang, H., Warren, H.S., Moldawer, L.L., Fink, M.P., Andersson, U., Tracey, K.J. & Yang, H. (2003). Structural basis for the proinflammatory cytokine activity of high mobility group box 1. *Mol Med* 9(1-2), 37-45.

Little, C.B., Flannery, C.R., Hughes, C.E., Goodship, A. & Caterson, B. (2005). Cytokine induced metalloproteinase expression and activity does not correlate with focal susceptibility of articular cartilage to degeneration. *Osteoarthritis Cartilage* 13(2), 162-70.

Little, C.B. & Ghosh, P. (1997). Variation in proteoglycan metabolism by articular chondrocytes in different joint regions is determined by post-natal mechanical loading. *Osteoarthritis Cartilage* 5(1), 49-62.

Little, C.B., Ghosh, P. & Rose, R. (1997). The effect of strenuous versus moderate exercise on the metabolism of proteoglycans in articular cartilage from different weight-bearing regions of the equine third carpal bone. *Osteoarthritis Cartilage* 5(3), 161-72.

Little, C.B., Hilbert, B.J., Wickstrom, S. & Hedlund, B.E. (1990). Quantitative microanalysis of equine synovial fluid glycosaminoglycan concentration. *Am J Vet Res* 51(10), 1534-9.

Liu, S., Stolz, D.B., Sappington, P.L., Macias, C.A., Killeen, M.E., Tenhunen, J.J., Delude, R.L. & Fink, M.P. (2006). HMGB1 is secreted by immunostimulated enterocytes and contributes to cytomix-induced hyperpermeability of Caco-2 monolayers. *Am J Physiol Cell Physiol* 290(4), C990-9.

Livak, K.J. & Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25(4), 402-8.

Loeser, R.F., Yammani, R.R., Carlson, C.S., Chen, H., Cole, A., Im, H.J., Bursch, L.S. & Yan, S.D. (2005). Articular chondrocytes express the receptor for advanced glycation end products: Potential role in osteoarthritis. *Arthritis Rheum* 52(8), 2376-85.

Lotz, M. & Guerne, P.A. (1991). Interleukin-6 induces the synthesis of tissue inhibitor of metalloproteinases-1/erythroid potentiating activity (TIMP-1/EPA). *J Biol Chem* 266(4), 2017-20.
MacDonald, M.H., Stover, S.M., Willits, N.H. & Benton, H.P. (1992). Regulation of matrix metabolism in equine cartilage explant cultures by interleukin 1. *Am J Vet Res* 53(12), 2278-85.
Malemud, C.J. (2004). Cytokines as therapeutic targets for osteoarthritis. *BioDrugs* 18(1), 23-35.
Martin, I., Jakob, M., Schäfer, D., Dick, W., Spagnoli, G. & Heberer, M. (2001). Quantitative analysis of gene expression in human articular cartilage from normal and osteoarthritic joints. *Osteoarthritis Cartilage* 9(2), 112-8.
Massicotte, F., Lajeunesse, D., Benderdour, M., Pelletier, J.P., Hilal, G., Duval, N. & Martel-Pelletier, J. (2002). Can altered production of interleukin-1β, interleukin-6, transforming growth factor-β and prostaglandin E_2 by isolated human subchondral osteoblasts identify two subgroups of osteoarthritic patients. *Osteoarthritis Cartilage* 10(6), 491-500.
May, S.A., Hooke, R.E. & Lees, P. (1989). Identity of the E-series prostaglandin produced by equine chondrocytes and synovial cells in response to a variety of stimuli. *Res Vet Sci* 46(1), 54-7.
May, S.A., Hooke, R.E. & Lees, P. (1992a). Equine chondrocyte activation by a variety of stimuli. *Br Vet J* 148(5), 389-97.
May, S.A., Hooke, R.E. & Lees, P. (1992b). Inhibition of interleukin-1 activity by equine synovial fluid. *Equine Vet J* 24(2), 99-102.
May, S.A., Hooke, R.E. & Lees, P. (1992c). Interleukin-1 stimulation of equine articular cells. *Res Vet Sci* 52(3), 342-8.
McIlwraith, C.W. (1980). Synovial fluid analysis in the diagnosis of equine joint disease. *Equine Practice* 2(2), 44-48.
McIlwraith, C.W. (1982). Current concepts in equine degenerative joint disease. *J Am Vet Med Assoc* 180(3), 239-50.
McIlwraith, C.W. (1992). Tearing of the medial palmar intercarpal ligament in the equine midcarpal joint. *Equine Vet J* 24(5), 367-71.
McIlwraith, C.W. (1996). General pathobiology of the joint and response to injury. In: McIlwraith, C.W. & Trotter, G.W. (Eds.) *Joint disease in the horse*. Philadelphia: W. B. Saunders Company.1). pp. 40-70.
McIlwraith, C.W. (2001). Disease processes of synovial membrane, fibrous capsule, ligaments, and articular cartilage. In: *Proceedings of 47th Annual Convention of the American Association of Equine Pratitioners*, San Diego, CA, USA pp. 142-156.
McIlwraith, C.W. (2002). Diseases of joints, tendons, ligaments, and related structures. In: Troy, D. (Ed.) *Adams' lameness in horses*. Philadelphia: Lippincott Williams & Wilkins. pp. 459-644.
McIlwraith, C.W. (2005). Use of synovial fluid and serum biomarkers in equine bone and joint disease: a review. *Equine Vet J* 37(5), 473-82.

McIlwraith, C.W. & Bramlage, L.R. (1996). Surgical treatment of joint injury. In: McIlwraith, C.W. & Trotter, G.W. (Eds.) *Joint disease in the horse*. Philadelphia: W.B. Saunders Company. pp. 292-317.

Moos, V., Fickert, S., Müller, B., Weber, U. & Sieper, J. (1999). Immunohistological analysis of cytokine expression in human osteoarthritic and healthy cartilage. *J Rheumatol* 26(4), 870-9.

Morisset, S., Frisbie, D.D., Robbins, P.D., Nixon, A.J. & McIlwraith, C.W. (2007). IL-1ra/IGF-1 gene therapy modulates repair of microfractured chondral defects. *Clin Orthop Relat Res* 462, 221-8.

Morris, E.A., McDonald, B.S., Webb, A.C. & Rosenwasser, L.J. (1990). Identification of interleukin-1 in equine osteoarthritic joint effusions. *Am J Vet Res* 51(1), 59-64.

Morris, E.A. & Treadwell, B.V. (1994). Effect of interleukin 1 on articular cartilage from young and aged horses and comparison with metabolism of osteoarthritic cartilage. *Am J Vet Res* 55(1), 138-46.

Muir, P., McCarthy, J., Radtke, C.L., Markel, M.D., Santschi, E.M., Scollay, M.C. & Kalscheur, V.L. (2006). Role of endochondral ossification of articular cartilage and functional adaptation of the subchondral plate in the development of fatigue microcracking of joints. *Bone* 38(3), 342-9.

Murray, R.C., Birch, H.L., Lakhani, K. & Goodship, A.E. (2001a). Biochemical composition of equine carpal articular cartilage is influenced by short-term exercise in a site-specific manner. *Osteoarthritis Cartilage* 9(7), 625-32.

Murray, R.C., Janicke, H.C., Henson, F.M.D. & Goodship, A. (2000). Equine carpal articular cartilage fibronectin distribution associated with training, joint location and cartilage deterioration. *Equine Vet J* 32(1), 47-51.

Murray, R.C., Smith, R.K., Henson, F.M.D. & Goodship, A. (2001b). The distribution of cartilage oligomeric matrix protein (COMP) in equine carpal articular cartilage and its variation with exercise and cartilage deterioration. *Vet J* 162(2), 121-8.

Murray, R.C., Whitton, R.C., Vedi, S., Goodship, A.E. & Lekeux, P. (1999). The effect of training on the calcified zone of equine middle carpal articular cartilage. *Equine Vet J Suppl* 30, 274-8.

Narazaki, M., Yasukawa, K., Saito, T., Ohsugi, Y., Fukui, H., Koishihara, Y., Yancopoulos, G.D., Taga, T. & Kishimoto, T. (1993). Soluble forms of the interleukin-6 signal-transducing receptor component gp130 in human serum possessing a potential to inhibit signals through membrane-anchored gp130. *Blood* 82(4), 1120-6.

Neu, C.P., Khalafi, A., Komvopoulos, K., Schmid, T.M. & Reddi, A.H. (2007). Mechanotransduction of bovine articular cartilage superficial zone protein by transforming growth factor β signaling. *Arthritis Rheum* 56(11), 3706-14.

Nietfeld, J.J., Wilbrink, B., Helle, M., van Roy, J.L.A.M., den Otter, W., Swaak, A.J.G. & Huber-Bruning, O. (1990). Interleukin-1-induced interleukin-6 is required for the inhibition of proteoglycan synthesis by interleukin-1 in human articular cartilage. *Arthritis Rheum* 33(11), 1695-701.

Nixon, A.J. & Fortier, L.A. (2001). New horizons in articular cartilage repair. In: *Proceedings of 47th Annual Convention of the American Association of Equine Practitioners*, San Diego, CA, USA pp. 217-226.

Norrdin, R.W. & Stover, S.M. (2006). Subchondral bone failure in overload arthrosis: a scanning electron microscopic study in horses. *J Musculoskelet Neuronal Interact* 6(3), 251-7.

Oegema, T.R., Jr., Carpenter, R.J., Hofmeister, F. & Thompson, R.C., Jr. (1997). The interaction of the zone of calcified cartilage and subchondral bone in osteoarthritis. *Microsc Res Tech* 37(4), 324-32.

Olive, J., D'Anjou, M.A., Girard, C., Laverty, S. & Theoret, C.L. (2009). Imaging and histological features of central subchondral osteophytes in racehorses with metacarpophalangeal joint osteoarthritis. *Equine Vet J* 41(9), 859-864.

Palmer, J.L., Bertone, A.L. & Litsky, A.S. (1994). Contact area and pressure distribution changes of the equine third carpal bone during loading. *Equine Vet J* 26(3), 197-202.

Palmer, J.L., Bertone, A.L., Malemud, C.J., Carter, B.G., Papay, R.S. & Mansour, J. (1995a). Site-specific proteoglycan characteristics of third carpal articular cartilage in exercised and nonexercised horses. *Am J Vet Res* 56(12), 1570-6.

Palmer, J.L., Bertone, A.L. & McClain, H. (1995b). Assessment of glycosaminoglycan concentration in equine synovial fluid as a marker of joint disease. *Can J Vet Res* 59(3), 205-12.

Park, J.S., Arcaroli, J., Yum, H.K., Yang, H., Wang, H., Yang, K.Y., Choe, K.H., Strassheim, D., Pitts, T.M., Tracey, K.J. & Abraham, E. (2003). Activation of gene expression in human neutrophils by high mobility group box 1 protein. *Am J Physiol Cell Physiol* 284(4), C870-9.

Park, J.S., Gamboni-Robertson, F., He, Q., Svetkauskaite, D., Kim, J.Y., Strassheim, D., Sohn, J.W., Yamada, S., Maruyama, I., Banerjee, A., Ishizaka, A. & Abraham, E. (2006). High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am J Physiol Cell Physiol* 290(3), C917-24.

Park, J.S., Svetkauskaite, D., He, Q., Kim, J.Y., Strassheim, D., Ishizaka, A. & Abraham, E. (2004). Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J Biol Chem* 279(9), 7370-7.

Pelletier, J.P., Martel-Pelletier, J. & Abramson, S.B. (2001). Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. *Arthritis Rheum* 44(6), 1237-47.

Penell, J.C., Egenvall, A., Bonnett, B.N., Olson, P. & Pringle, J. (2005). Specific causes of morbidity among Swedish horses insured for veterinary care between 1997 and 2000. *Vet Rec* 157(16), 470-7.

Penick, K.J., Solchaga, L.A. & Welter, J.F. (2005). High-throughput aggregate culture system to assess the chondrogenic potential of mesenchymal stem cells. *Biotechniques* 39(5), 687-91.

Persson, L. (1971). *On the synovia in horses. A clinical and experimental study.* Diss. Royal Veterinary College. Stockholm.

Phillips, T.J. & Wright, I.M. (1994). Observations on the anatomy and pathology of the palmar intercarpal ligaments in the middle carpal joints of thoroughbred racehorses. *Equine Vet J* 26(6), 486-91.

Platt, D. & Bayliss, M.T. (1994). An investigation of the proteoglycan metabolism of mature equine articular cartilage and its regulation by interleukin-1. *Equine Vet J* 26(4), 297-303.

Pool, R.R. & Meagher, D.M. (1990). Pathologic findings and pathogenesis of racetrack injuries. In: Turner, A.S.*, et al.* (Eds.) *Veterinary Clinics of North America Equine Practice.* Philadelphia: W. B. Saunders Company. pp. 1-30.

Poole, A.R., Guilak, F. & Abramson, S.B. (2007). Etiopathogenesis of osteoarthritis. In: Moskowitz, R.W.*, et al.* (Eds.) *Osteoarthritis.* Philadelphia: Lippincott Williams & Wilkins. pp. 27-49.

Poole, C.A., Ayad, S. & Gilbert, R.T. (1992). Chondrons from articular cartilage. V. Immunohistochemical evaluation of type VI collagen organisation in isolated chondrons by light, confocal and electron microscopy. *J Cell Sci* 103, 1101-10.

Pullerits, R., Jonsson, I.M., Verdrengh, M., Bokarewa, M., Andersson, U., Erlandsson-Harris, H. & Tarkowski, A. (2003). High mobility group box chromosomal protein 1, a DNA binding cytokine, induces arthritis. *Arthritis Rheum* 48(6), 1693-700.

Relić, B., Bentires-Alj, M., Ribbens, C., Franchimont, N., Guerne, P.A., Benoît, V., Merville, M.P., Bours, V. & Malaise, M.G. (2002). TNF-α protects human primary articular chondrocytes from nitric oxide-induced apoptosis via nuclear factor-κB. *Lab Invest* 82(12), 1661-72.

Remick, D.G., DeForge, L.E., Sullivan, J.F. & Showell, H.J. (1992). Profile of cytokines in synovial fluid specimens from patients with arthritis. Interleukin 8 (IL-8) and IL-6 correlate with inflammatory arthritides. *Immunol Invest* 21(4), 321-7.

Richardson, D.W. & Dodge, G.R. (2000). Effects of interleukin-1β and tumor necrosis factor-α on expression of matrix-related genes by cultured equine articular chondrocytes. *Am J Vet Res* 61(6), 624-30.

Ronéus, B., Andersson, A.M. & Ekman, S. (1997). Racing performance in standardbred trotters with chronic synovitis after partial arthroscopic synovectomy in the metacarpophalangeal, metatarsophalangeal and intercarpal (midcarpal) joints. *Acta Vet Scand* 38(1), 87-95.

Rose-John, S., Scheller, J., Elson, G. & Jones, S.A. (2006). Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. *J Leukoc Biol* 80(2), 227-36.

Rossdale, P.D., Hopes, R., Wingfield Digby, N.J. & Offord, K. (1985). Epidemiological study of wastage among racehorses 1982 and 1983. *Vet Rec* 116(3), 66-9.

Rowan, A.D., Koshy, P.J.T., Shingleton, W.D., Degnan, B.A., Heath, J.K., Vernallis, A.B., Spaull, J.R., Life, P.F., Hudson, K. & Cawston, T.E. (2001). Synergistic effects of glycoprotein 130 binding cytokines in combination with interleukin-1 on cartilage collagen breakdown. *Arthritis Rheum* 44(7), 1620-32.

Rumian, A.P., Wallace, A.L. & Birch, H.L. (2007). Tendons and ligaments are anatomically distinct but overlap in molecular and morphological features--a comparative study in an ovine model. *J Orthop Res* 25(4), 458-64.

Sakao, K., Takahashi, K.A., Arai, Y., Saito, M., Honjo, K., Hiraoka, N., Asada, H., Shin-Ya, M., Imanishi, J., Mazda, O. & Kubo, T. (2009). Osteoblasts derived from osteophytes produce interleukin-6, interleukin-8, and matrix metalloproteinase-13 in osteoarthritis. *J Bone Miner Metab* 27(4), 412-23.

Sakao, K., Takahashi, K.A., Mazda, O., Arai, Y., Tonomura, H., Inoue, A., Saito, M., Fujioka, M., Takamiya, H., Imanishi, J. & Kubo, T. (2008). Enhanced expression of interleukin-6, matrix metalloproteinase-13, and receptor activator of NF-κB ligand in cells derived from osteoarthritic subchondral bone. *J Orthop Sci* 13(3), 202-10.

Sandell, L.J., Heinegård, D. & Hering, T.M. (2007). Cell biology, biochemistry, and molecular biology of articular cartilage in osteoarthritis. In: Moskowitz, R.W., *et al.* (Eds.) *Osteoarthritis*. Philadelphia: Lippincott Williams & Wilkins. pp. 73-106.

Scaffidi, P., Misteli, T. & Bianchi, M.E. (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 418(6894), 191-5.

Schenk, R.K., Hofstetter, W. & Felix, R. (2002). Morphology and chemical composition of connective tissue: Bone. In: Royce, P.M. & Steinmann, B. (Eds.) *Connective tissue and its heritable disorder: Molecular, genetic, and medical aspects*. New York: Wiley-Liss Inc. pp. 67-120.

Schneider, N., Mouithys-Mickalad, A.L., Lejeune, J.P., Deby-Dupont, G.P., Hoebeke, M. & Serteyn, D.A. (2005). Synoviocytes, not chondrocytes, release free radicals after cycles of anoxia/re-oxygenation. *Biochem Biophys Res Commun* 334(2), 669-73.

Schuerwegh, A.J., Dombrecht, E.J., Stevens, W.J., Van Offel, J.F., Bridts, C.H. & De Clerck, L.S. (2003). Influence of pro-inflammatory (IL-

1α, IL-6, TNF-α, IFN-γ) and anti-inflammatory (IL-4) cytokines on chondrocyte function. *Osteoarthritis Cartilage* 11(9), 681-7.
Schumacher, B.L., Block, J.A., Schmid, T.M., Aydelotte, M.B. & Kuettner, K.E. (1994). A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage. *Arch Biochem Biophys* 311(1), 144-52.
Shikichi, M., Kitamura, H.P., Yanase, H., Konno, A., Takahashi-Iwanaga, H. & Iwanaga, T. (1999). Three-dimensional ultrastructure of synoviocytes in the horse joint as revealed by the scanning electron microscope. *Arch Histol Cytol* 62(3), 219-29.
Shingu, M., Miyauchi, S., Nagai, Y., Yasutake, C. & Horie, K. (1995). The role of IL-4 and IL-6 in IL-1-dependent cartilage matrix degradation. *Br J Rheumatol* 34(2), 101-6.
Shingu, M., Nagai, Y., Isayama, T., Naono, T., Nobunaga, M. & Nagai, Y. (1993). The effects of cytokines on metalloproteinase inhibitors (TIMP) and collagenase production by human chondrocytes and TIMP production by synovial cells and endothelial cells. *Clin Exp Immunol* 94(1), 145-9.
Simkin, P.A. (2008). A biography of the chondrocyte. *Ann Rheum Dis* 67(8), 1064-8.
Simmons, E.J., Bertone, A.L. & Weisbrode, S.E. (1999). Instability-induced osteoarthritis in the metacarpophalangeal joint of horses. *Am J Vet Res* 60(1), 7-13.
Sisson, S. (1953). Arthrology. In: Grossman, J.D. (Ed.) *Anatomy of the domestic animals*. Phildelphia: W. B. Saunders Company. pp. 209-253.
Skiöldebrand, E., Heinegård, D., Eloranta, M.L., Nilsson, G., Dudhia, J., Sandgren, B. & Ekman, S. (2005). Enhanced concentration of COMP (cartilage oligomeric matrix protein) in osteochondral fractures from racing Thoroughbreds. *J Orthop Res* 23(1), 156-63.
Skiöldebrand, E., Lorenzo, P., Zunino, L., Rucklidge, G.J., Sandgren, B., Carlsten, J. & Ekman, S. (2001). Concentration of collagen, aggrecan and cartilage oligomeric matrix protein (COMP) in synovial fluid from equine middle carpal joints. *Equine Vet J* 33(4), 394-402.
Sledge, C.B., Reddi, A.H., Walsh, D.A. & Blake, D.R. (2001). Biology of the normal joint. In: Ruddy, S*., et al*. (Eds.) *Kelley's textbook of rheumatology*. Philadelphia: W. B. Saunders Company.1). pp. 1-26.
Smith, M.D., Triantafillou, S., Parker, A., Youssef, P.P. & Coleman, M. (1997). Synovial membrane inflammation and cytokine production in patients with early osteoarthritis. *J Rheumatol* 24(2), 365-71.
Spiers, S., May, S.A., Bennett, D. & Edwards, G.B. (1994). Cellular sources of proteolytic enzymes in equine joints. *Equine Vet J* 26(1), 43-7.
Steel, C.M., Hopper, B.J., Richardson, J.L., Alexander, G.R. & Robertson, I.D. (2006). Clinical findings, diagnosis, prevalence and predisposing

factors for lameness localised to the middle carpal joint in young Standardbred racehorses. *Equine Vet J* 38(2), 152-7.

Stenhamre, H., Slynarski, K., Petrén, C., Tallheden, T. & Lindahl, A. (2008). Topographic variation in redifferentiation capacity of chondrocytes in the adult human knee joint. *Osteoarthritis Cartilage* 16(11), 1356-62.

Su, X., Morris, D.D., Crowe, N.A., Moore, J.N., Fischer, K.J. & McGraw, R.A. (1992). Equine tumor necrosis factor alpha: cloning and expression in *Escherichia coli*, generation of monoclonal antibodies, and development of a sensitive enzyme-linked immunosorbent assay. *Hybridoma* 11(6), 715-27.

Su, X., Morris, D.D. & McGraw, R.A. (1991). Cloning and characterization of gene TNFα encoding equine tumor necrosis factor alpha. *Gene* 107(2), 319-21.

Swann, D.A., Silver, F.H., Slayter, H.S., Stafford, W. & Shore, E. (1985). The molecular structure and lubricating activity of lubricin isolated from bovine and human synovial fluids. *Biochem J* 225(1), 195-201.

Swann, D.A., Slayter, H.S. & Silver, F.H. (1981). The molecular structure of lubricating glycoprotein-I, the boundary lubricant for articular cartilage. *J Biol Chem* 256(11), 5921-5.

Swiderski, C.E., Sobol, G., Lunn, D.P. & Horohov, D.W. (2000). Molecular cloning, sequencing, and expression of equine interleukin-6. *Vet Immunol Immunopathol* 77(3-4), 213-20.

Takafuji, V.A., Howard, R.D., Ward, D.L., Sharova, L.V. & Crisman, M.V. (2005). Modulation of equine articular chondrocyte messenger RNA levels following brief exposures to recombinant equine interleukin-1β. *Vet Immunol Immunopathol* 106(1-2), 23-38.

Takafuji, V.A., McIlwraith, C.W. & Howard, R.D. (2002). Effects of equine recombinant interleukin-1α and interleukin-1β on proteoglycan metabolism and prostaglandin E2 synthesis in equine articular cartilage explants. *Am J Vet Res* 63(4), 551-8.

Tallheden, T., Bengtsson, C., Brantsing, C., Sjögren-Jansson, E., Carlsson, L., Peterson, L., Brittberg, M. & Lindahl, A. (2005). Proliferation and differentiation potential of chondrocytes from osteoarthritic patients. *Arthritis Res Ther* 7(3), R560-8.

Tallheden, T., Karlsson, C., Brunner, A., van der Lee, J., Hagg, R., Tommasini, R. & Lindahl, A. (2004). Gene expression during redifferentiation of human articular chondrocytes. *Osteoarthritis Cartilage* 12(7), 525-35.

Taniguchi, N., Kawahara, K., Yone, K., Hashiguchi, T., Yamakuchi, M., Goto, M., Inoue, K., Yamada, S., Ijiri, K., Matsunaga, S., Nakajima, T., Komiya, S. & Maruyama, I. (2003). High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. *Arthritis Rheum* 48(4), 971-81.

Taniguchi, N., Yoshida, K., Ito, T., Tsuda, M., Mishima, Y., Furumatsu, T., Ronfani, L., Abeyama, K., Kawahara, K., Komiya, S., Maruyama, I., Lotz, M., Bianchi, M.E. & Asahara, H. (2007). Stage-specific secretion of HMGB1 in cartilage regulates endochondral ossification. *Mol Cell Biol* 27(16), 5650-63.

Tetlow, L.C., Adlam, D.J. & Woolley, D.E. (2001). Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. *Arthritis Rheum* 44(3), 585-94.

Theoret, C.L., Barber, S.M. & Gordon, J.R. (1998). The expression of IL-1, IL-6, and TGF-β in the synovial fluid of horses with surgically-induced transient synovitis. *Vet Comp Orthop Traumatol* 11, 141-145.

Thomas, C.M., Fuller, C.J., Whittles, C.E. & Sharif, M. (2007). Chondrocyte death by apoptosis is associated with cartilage matrix degradation. *Osteoarthritis Cartilage* 15(1), 27-34.

Tian, J., Avalos, A.M., Mao, S.Y., Chen, B., Senthil, K., Wu, H., Parroche, P., Drabic, S., Golenbock, D., Sirois, C., Hua, J., An, L.L., Audoly, L., La Rosa, G., Bierhaus, A., Naworth, P., Marshak-Rothstein, A., Crow, M.K., Fitzgerald, K.A., Latz, E., Kiener, P.A. & Coyle, A.J. (2007). Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nat Immunol* 8(5), 487-96.

Tidswell, H.K., Innes, J.F., Avery, N.C., Clegg, P.D., Barr, A.R., Vaughan-Thomas, A., Wakley, G. & Tarlton, J.F. (2008). High-intensity exercise induces structural, compositional and metabolic changes in cuboidal bones - findings from an equine athlete model. *Bone* 43(4), 724-33.

Todhunter, P.G., Kincaid, S.A., Todhunter, R.J., Kammermann, J.R., Johnstone, B., Baird, A.N., Hanson, R.R., Wright, J.M., Lin, H.-C. & Purohit, R.C. (1996). Immuhistochemichal analysis of an equine model of synovitis-induced arthritis. *Am J Vet Res* 57(7), 1080-1093.

Trotter, G.W. & McIlwraith, C.W. (1996). Clinical features and diagnosis of equine joint disease. In: McIlwraith, C.W. & Trotter, G.W. (Eds.) *Joint disease in the horse.* Philadelphia: W.B. Saunders Company. pp. 120-145.

Trumble, T.N., Brown, M.P., Merritt, K.A. & Billinghurst, R.C. (2008). Joint dependent concentrations of bone alkaline phosphatase in serum and synovial fluids of horses with osteochondral injury: an analytical and clinical validation. *Osteoarthritis Cartilage* 16(7), 779-86.

Trumble, T.N., Scarbrough, A.B. & Brown, M.P. (2009). Osteochondral injury increases type II collagen degradation products (C2C) in

synovial fluid of Thoroughbred racehorses. *Osteoarthritis Cartilage* 17(3), 371-4.

Trumble, T.N., Trotter, G.W., Oxford, J.R., McIlwraith, C.W., Cammarata, S., Goodnight, J.L., Billinghurst, R.C. & Frisbie, D.D. (2001). Synovial fluid gelatinase concentrations and matrix metalloproteinase and cytokine expression in naturally occurring joint disease in horses. *Am J Vet Res* 62(9), 1467-77.

Tulamo, R.M., Houttu, J., Tupamäki, A. & Salonen, M. (1996). Hyaluronate and large molecular weight proteoglycans in synovial fluid from horses with various arthritides. *Am J Vet Res* 57(6), 932-7.

Tung, J.T., Fenton, J.I., Arnold, C., Alexander, L., Yuzbasiyan-Gurkan, V., Venta, P.J., Peters, T.L., Orth, M.W., Richardson, D.W. & Caron, J.P. (2002a). Recombinant equine interleukin-1β induces putative mediators of articular cartilage degradation in equine chondrocytes. *Can J Vet Res* 66(1), 19-25.

Tung, J.T., Venta, P.J., Eberhart, S.W., Yuzbasiyan-Gurkan, V., Alexander, L. & Caron, J.P. (2002b). Effects of anti-arthritis preparations on gene expression and enzyme activity of cyclooxygenase-2 in cultured equine chondrocytes. *Am J Vet Res* 63(8), 1134-9.

Walker, E.R., Boyd, R.D., Wu, D.D., Lukoschek, M., Burr, D.B. & Radin, E.L. (1991). Morphologic and morphometric changes in synovial membrane associated with mechanically induced osteoarthrosis. *Arthritis and Rheumatism* 34(5), 515-524.

van den Boom, R., Brama, P.A., Kiers, G.H., DeGroot, J., Barneveld, A. & van Weeren, R.R. (2004). The influence of repeated arthrocentesis and exercise on matrix metalloproteinase and tumour necrosis factor α activities in normal equine joints. *Equine Vet J* 36(2), 155-9.

Wang, A.M., Creasey, A.A., Ladner, M.B., Lin, L.S., Strickler, J., Van Arsdell, J.N., Yamamoto, R. & Mark, D.F. (1985). Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science* 228(4696), 149-54.

Wang, H., Bloom, O., Zhang, M., Vishnubhakat, J.M., Ombrellino, M., Che, J., Frazier, A., Yang, H., Ivanova, S., Borovikova, L., Manogue, K.R., Faist, E., Abraham, E., Andersson, J., Andersson, U., Molina, P.E., Abumrad, N.N., Sama, A. & Tracey, K.J. (1999a). HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 285(5425), 248-51.

Wang, H., Vishnubhakat, J.M., Bloom, O., Zhang, M., Ombrellino, M., Sama, A. & Tracey, K.J. (1999b). Proinflammatory cytokines (tumor necrosis factor and interleukin 1) stimulate release of high mobility group protein-1 by pituicytes. *Surgery* 126(2), 389-92.

Weaver, R.E., Sharif, M., Livingston, L.A., Andrews, K.L. & Fuller, C.J. (2006). Microscopic change in macroscopically normal equine cartilage from osteoarthritic joints. *Connect Tissue Res* 47(2), 92-101.

Weibel, E.R. (1979). *Stereological methods. Practical methods for biological morphometry*. London: Academic Press Ltd.

Whitton, R.C., Kannegieter, N.J. & Rose, R.J. (1997a). The intercarpal ligaments of the equine midcarpal joint, Part 3: Clinical observations in 32 racing horses with midcarpal joint disease. *Vet Surg* 26(5), 374-81.

Whitton, R.C., McCarthy, P.H. & Rose, R.J. (1997b). The intercarpal ligaments of the equine midcarpal joint, Part 1: The anatomy of the palmar and dorsomedial intercarpal ligaments of the midcarpal joint. *Vet Surg* 26(5), 359-66.

Whitton, R.C. & Rose, R.J. (1997). The intercarpal ligaments of the equine midcarpal joint, Part 2: The role of the palmar intercarpal ligaments in the restraint of dorsal displacement of the proximal row of carpal bones. *Vet Surg* 26(5), 367-73.

Wilbrink, B., Nietfeld, J.J., den Otter, W., van Roy, J.L., Bijlsma, J.W. & Huber-Bruning, O. (1991). Role of TNFα, in relation to IL-1 and IL-6 in the proteoglycan turnover of human articular cartilage. *Br J Rheumatol* 30(4), 265-71.

Wilsher, S., Allen, W.R. & Wood, J.L.N. (2006). Factors associated with failure of thoroughbred horses to train and race. *Equine Vet J* 38(2), 113-8.

von Rechenberg, B., Leutenegger, C., Zlinsky, K., McIlwraith, C.W., Akens, M.K. & Auer, J.A. (2001). Upregulation of mRNA of interleukin-1 and -6 in subchondral cystic lesions of four horses. *Equine Vet J* 33(2), 143-9.

Yan, S.F., Tritto, I., Pinsky, D., Liao, H., Huang, J., Fuller, G., Brett, J., May, L. & Stern, D. (1995). Induction of interleukin 6 (IL-6) by hypoxia in vascular cells. Central role of the binding site for nuclear factor-IL-6. *J Biol Chem* 270(19), 11463-71.

Yang, H., Ochani, M., Li, J., Qiang, X., Tanovic, M., Harris, H.E., Susarla, S.M., Ulloa, L., Wang, H., DiRaimo, R., Czura, C.J., Wang, H., Roth, J., Warren, H.S., Fink, M.P., Fenton, M.J., Andersson, U. & Tracey, K.J. (2004). Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proc Natl Acad Sci U S A* 101(1), 296-301.

Young, B.D., Samii, V.F., Mattoon, J.S., Weisbrode, S.E. & Bertone, A.L. (2007). Subchondral bone density and cartilage degeneration patterns in osteoarthritic metacarpal condyles of horses. *Am J Vet Res* 68(8), 841-9.

Young, D.R., Richardson, D.W., Markel, M.D. & Nunamaker, D.M. (1991). Mechanical and morphometric analysis of the third carpal bone of Thoroughbreds. *Am J Vet Res* 52(3), 402-9.

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