Studies of articular cartilage macromolecules in the equine middle carpal joint, in joint pathology and training

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"Always look at the bright side of life"

Monty Python

Abstract

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Lameness caused by osteoarthritis (OA) is the most common reason for failure to race among horses. Degenerative changes to the articular cartilage are commonly seen often with subchondral bone sclerosis. Cartilage is a connective tissue with tensile strength and resilience, consisting of cells and abundant extracellular matrix. The cells are adapting the matrix to environmental conditions by synthesis and degradation of the matrix structural elements. A change in the concentration of macromolecules in synovial fluid and serum can indicate early biochemical changes in the joint. This provides a tool for research into and monitoring pathogenic mechanisms of OA.

The content of COMP, aggrecan and collagen type II was measured in serum and synovial fluid (sf) from trotters and riding horses with either normal joints or joints with different stages of cartilage pathology. The trotters with a training background and cartilage degeneration had low concentrations of COMP and aggrecan in synovial fluid and serum as well as sf-collagen (paper I). The effect of long-term training on the concentration of sf-COMP, -aggrecan and -collagen type II from young trotters was measured (paper II). The amount of training and age influenced sf-COMP, where lower concentrations were related to more training and higher age. The concentration of collagen type II degradation products increased with total days of training. *In vitro* dynamic compression of cartilage explants from trained horses showed a down-regulation of COMP synthesis compared to untrained horses (paper II). COMP in cartilage matrix was presented in an ultrastructural study (paper III), where COMP concentration, was much less abundant in loaded and unloaded matrix of articular cartilage from a strenuously trained horse compared to untrained horses. The untrained horses often displayed a higher immunolabeling in loaded areas compared to unloaded areas, indicating that dynamic load can promote COMP synthesis and /or retention, while excessive load have opposite effect.

Galloping horses with osteochondral fractures had higher concentration of sf-COMP compared to normal or osteoarthtritic joints from trotters or galloping horses (paper IV). In conclusion the present work shows that strenuously training of racehorses alter the expression of macromolecules in the articular cartilage of the third carpal bone.

Keywords: COMP, aggrecan, collagen type II, osteoarthritis, synovial fluid, training, dynamic compression, osteochondral fractures, middle carpal joint, horses.

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Contents

List of papers

Papers I-IV

This thesis is based on the following papers which are referred to in the text by the Roman numerals as set below:

I. 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*, 394-402.

II. Skiöldebrand, E., Heinegård, D., Wong, M., Siegrist, M., Önnerfjord, P., Olofsson, B., Rucklidge, G.J., Roneus, N. & Ekman, S. Altered homeostasis of extracellular matrix proteins in articular cartilage of intensely training Standardbred trotters. *Manuscript submitted for publication.*

III. Skiöldebrand, E., Ekman, S., Idsund, E., Heinegård, D. & Hultenby, K. Ultrastructural immunolocalization of cartilage oligomeric matrix protein (COMP) in loaded and unloaded areas of the articular cartilage from the equine middle carpal joint. *In manuscript.*

IV. Skiöldebrand, E., Heinegård, D., Eloranta, M-J., Nilsson, G., Dudhia, J., Sandgren, B. & Ekman, S. (2004) Enhanced concentration of cartilage oligomeric matrix protein (COMP) in osteochondral fractures from racing Thoroughbreds.
Journal of Orthopaedic Research. In press.

Abbreviations

ADAM/-TS-	A disintegrin and metalloprotease with thrombospondin repeat
COMP	Cartilage oligomeric matrix protein
ELISA	Enzyme-linked immunosorbant assay
GAG	Glycosaminoglycan
Galloping horse	Thoroughbred horse
IL	Interleukin
KS	Keratan sulphate
MMP	Matrix metalloprotease
MT-MMP	Membrane-type MMP
OA	Osteoarthritis
PRELP	Proline-arginine-rich end and leucine-rich repeat protein
PsoA	Psoriatic arthritis
RA	Rheumatoid arthritis
Riding horse	Swedish Warmblood horse
Sf-aggrecan	Concentration of aggrecan in synovial fluid
Sf-collagen	Concentration of collagen type II in synovial fluid
Sf-COMP	Concentration of COMP in synovial fluid
TIMP	Tissue inhibitor of metalloproteases
TNF	Tumour necrosis factor
Trotter	Standardbred trotter

Background

The horse racing industry suffers significant economic losses due to a high prevalence of lameness in racehorses (Rossdale *et al*., 1985).

Osteoarthritis (OA) of the middle carpal joint is a common cause of lameness in racehorses and the medial aspect of the radial facet in the third carpal bone is the most common site of degenerative lesions (Fig. 1). These lesions include cartilage fraying and erosions, subchondral bone sclerosis and incomplete bone fractures (McIlwraith, 1982; Palmer, 1986; Pool & Meagher, 1990; Norrdin *et al*., 1998).

Early stages of tissue changes in equine OA are impossible to detect clinically. An early diagnosis, with subsequent appropriate treatment and accurate convalescence program are of great value to prevent irreversible joint destruction. A lameness evaluation of the horse includes a clinical examination and palpation of the joints to identify and localise signs of heat, pain and swelling. Grading of the lameness before and after flexion tests, together with intra-articular anaesthesia can accurately localise the exact joint responsible for the lameness. The tissue destruction can be characterised by the aid of radiology, magnetic resonance imaging, ultrasonography, computed tomography, scintigraphy and diagnostic arthroscopy. However, clinical identification of early biochemical as well as microscopically catabolic processes of the cartilage and bone is not possible with techniques available today. Hence, molecular markers in synovial fluid and/or serum correlated to catabolic and anabolic processes in the joint tissues would be of great value.

Most joint lesions are induced by acute trauma, repetitive load, or overload (Pool & Meagher, 1990). OA is defined as a joint disorder characterised by degeneration of the articular cartilage and sclerosis and /or microfractures of underlying bone with an inflammatory reaction in the synovial membrane and capsule (Sandell & Aigner, 2001). The pathophysiological mechanisms involved in OA are not fully understood. Findings implicate an alteration in the dynamic equilibrium between the biosynthetic phase (in which the chondrocytes synthesise and restore extracellular matrix) and the degradative phase (in which proteolytic enzymes are activated) (Sandell & Aigner, 2001). Release of different macromolecules and their fragments into synovial fluid and serum follow these anabolic and catabolic processes in the cartilage (Saxne & Heinegård, 1992). Hence, these macromolecules can be used as markers of cartilage homeostasis.

This literature review covers Getty, 1975; Stryer, 1981; Poole, 1986; Mankin, 1989; Mayne, 1989; Pool & Meagher, 1990; Buckwalter & Mow, 1992; Kuettner 1992; Levick, 1992; Poole *et al*., 1995; McIlwraith, 1996; Pool, 1996; Grodzinsky *et al*., 1998; Heinegård *et al*., 1998; Lohmander & Felson, 1998; McCarthy & Frassia, 1998; Neame *et al*., 1999; Grodzinsky *et al*., 2000; Kawacak *et al*., 2001; Sandell & Aigner, 2001; Heinegård *et al*., 2002; Kielty & Grant, 2002; Martin & Buckwalter, 2002; McIlwraith, 2002; Morris *et al*., 2002; Murphy & Reynolds, 2002; Stashak & Hill, Garrick & Requa, 2003, and includes several original references. References to review papers are identified by “rev” before the reference.

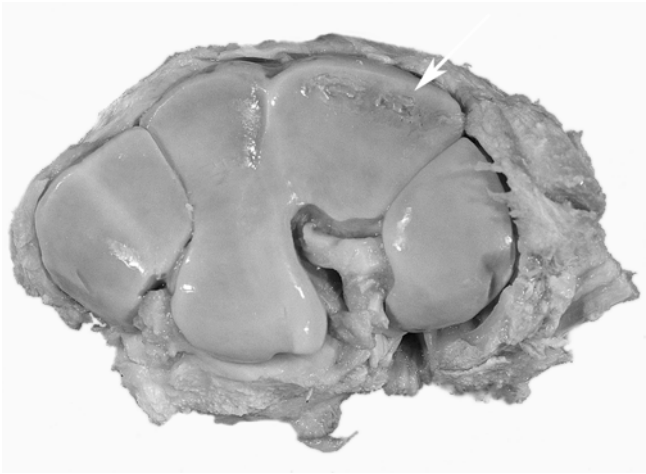

Figure 1. *Macroscopic section of the proximal surfaces of the fourth, third and second carpal bones in the middle carpal joint of a four-year-old trotter. Moderate articular fraying and erosions of the third carpal bone are present at the radial facet (arrow).*

Literature review

The carpal joint

The carpal joint is a diarthrodial joint comprised of bony parts covered by hyaline cartilage. Its major functions are to absorb and distribute load and to enable low-friction *motion*. The cartilage surfaces are separated by the joint cavity containing synovial fluid enclosed by a synovial membrane. The carpal joint is composed of three main joints with collateral and intercarpal ligaments including distal radius, carpal bones, and proximal metacarpal bones. The carpal bones consist of seven to eight bones arranged in a proximal and distal row (rev Getty, 1975) (Fig. 2). The proximal row contains radial-, intermediate-, ulnar- and accessory carpal bones and the distal row contains first (sometimes absent), second, third and fourth bones.

The radial, intermediate and third carpal bones are axial weight bearing subjected to most of the load (Colahan *et al*., 1988). These three carpal bones and the distal radius are the most frequently injured parts of the joint (Bramlage *et al*., 1988). The ulnar, and fourth and second carpal bones are subjected to minor load and the accessory carpal bone is non-weight bearing but interposed in flexor tendons (Colahan, *et al*.,1988; rev Getty, 1975). During the stride, the radiocarpal and middle carpal joints have a great range of movement compared to the more distal carpometacarpal joint. During the non-weightbearing phase, the different bones of the carpal joints do not fit perfectly, but when the hoof hits the ground, the bones slide into a locked position, absorbing large axial forces (Bramlage *et al*., 1988).

Over-extension of the carpal joint (Fig. 3) during the weight bearing phase of the stride occurs due to the joint's rotating action (Bramlage *et al*., 1983; Johnston & Roepstorff, 1995) and maximum overextension of the carpus is correlated with high trotting and galloping speeds (Johnston *et al*., 1995; rev Stashak & Hill, 2002). During this overextension phase, the dorsal parts of the joints receive an excessive amount of vertical load causing high intrarticular pressure between the radial and third carpal bones (Johnston & Roepstorff, 1995). The dorsal part of the radial facet of the third carpal bone is also most prone to develop cartilage lesions; such as fraying, erosions, ulceration and subchondral bone sclerosis with incomplete fractures (Palmer, 1986; rev Pool & Meagher, 1990; Norrdin *et al*., 1998).

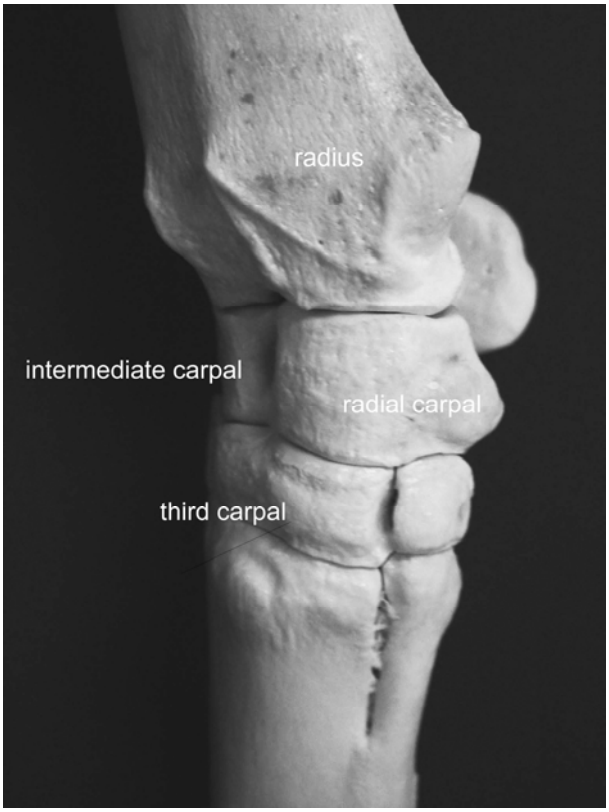

Figure 2. *Macerated specimen of the equine carpal joint viewed dorsomedial. The radius, radial carpal, intermediate carpal and third carpal bone are indicated.*
(Photo: Cecilia Ley)

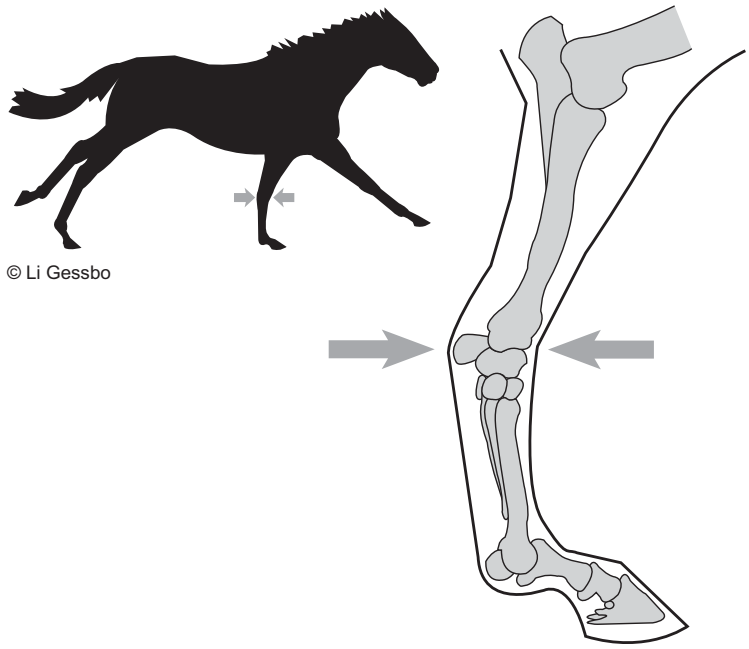

Figure 3. *Schematic drawing showing overextension of the carpal joint at gallop.*

Articular cartilage

The articular cartilage is a connective tissue covering joint surfaces with specialised load-bearing properties, and the ability to withstand compressive, tensile, and shear forces due to the composition and structural integrity of its extracellular matrix (rev Grodzinsky *et al*., 1998). Adult articular cartilage is aneural, avascular, alymphatic and has a high matrix to cell ratio (rev Morris *et al*., 2002; rev McIlwraith, 2002). The major constituents of this matrix are fibril-forming collagen type II (giving the cartilage its tensile strength) and large aggregating proteoglycans (essential for its load-bearing properties). Collagen fibers and aggregating proteoglycans form a network together with non-collagenous proteins responsible for the functional properties of the tissue (rev Heinegård *et al*., 2002).

Morphology

Adult articular cartilage is structurally arranged in four zones—superficial, intermediate, deep and calcified (Fig. 4) (Palmer & Bertone, 1994). The content of collagen is highest in the superficial zone with fibers orientated parallel to the surface. Collagen content decreases with increasing distance from the surface, while proteoglycan content shows a trend in the opposite direction (Poole *et al.*, 1982; rev McIlwraith, 2002). Extracellular water is more concentrated in the superficial layer, contributing to lubrication during load through fluid exudation into the synovial space. Chondrocytes in the superficial layer are oval and oriented parallel to the articular surface. The intermediate layer contains larger

chondrocytes, single or paired in lacunes (chondrones), and randomly oriented. Proteoglycan content in the intermediate layer is higher than the superficial layer and has a network of obliquely oriented collagen fibrils (rev Morris *et al*., 2002). The deep layer is characterised by rounded chondrocytes often arranged in vertical columns separated by radially aligned collagen fibrils, with the highest proteoglycan content. The most basal layer is mineralised, separated from the non-mineralised articular cartilage by a distinct thin layer of enhanced calcification—known as the tidemark (rev Morris *et al*., 2002). Each layer has a different inherent biomechanical capacity due to different biochemical composition and collagen fiber arrangements.

The fine structural organisation of the extracellular matrix can be divided into different compartments—the pericellular, territorial and interterritorial. The pericellular matrix, close to each cell (the lacunae), contains molecules interacting with the cell surface membrane providing feedback from the matrix. The territorial matrix (the capsular) surrounds the cell or groups of cells, with molecules regulating early stages of matrix assembly. The interterritorial matrix is the most abundant compartment occupying the intervening spaces at a distance from the cell. The main role of the extracellular matrix is to provide the unique biomechanical capacity of the articular cartilage (rev Heinegård *et al*., 2002).

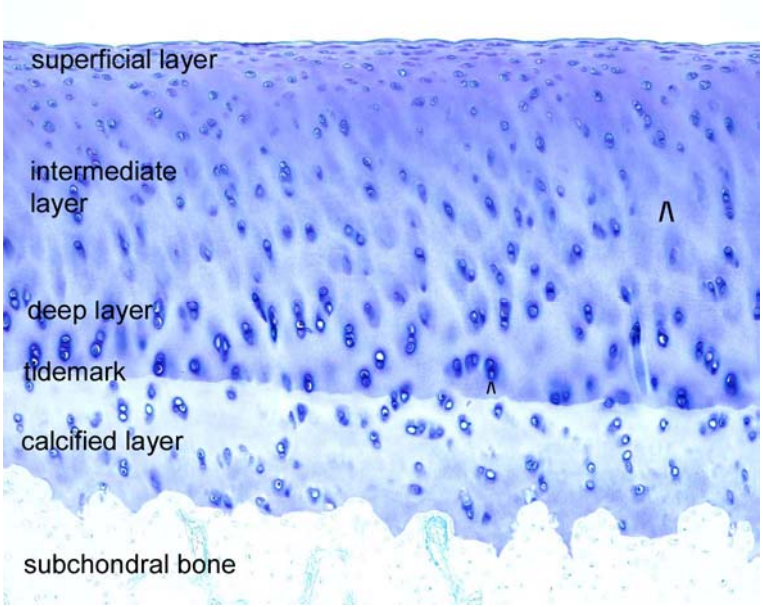

Figure 4. *Micrographs of Tolouidine blue stained section of equine articular cartilage from the third carpal bone of a four-year-old trotter.*
Interterritorial matrix (large arrowhead) and territorial matrix (small arrowhead).

Molecular organisation

The molecular organisation in articular cartilage is complex. This review focuses on macromolecules studied in this thesis (COMP, aggrecan and collagen type II) and other macromolecules of interest in adult articular cartilage with OA (Fig. 5).

Articular cartilage is composed of a highly hydrated matrix in which the chondrocytes are embedded. The chondrocytes lack cell to cell contact and communication between cells occurs via the matrix (rev Kuettner, 1992). To adapt to changes within the matrix, the chondrocytes respond to signals (mechanical, electrical and physiochemical) in the pericellular microenvironment. This occurs through cell/matrix interactions responsible for cell proliferation, differentiation, and metabolism (Huber *et al*., 2000; Giannoni *et al*., 2003).

Chondrocytes are responsible for synthesis, assembly and degradation of extracellular matrix constituents and cytokines, growth factors and degrading enzymes regulate the turnover of matrix constituents. Cartilage is a dynamic tissue that changes with many factors including age, mechanical load and inflammation with a continuous turnover of matrix constituents in order to meet changed functional needs. During homeostasis, the cartilage continuously undergoes remodelling and repair in which macromolecules of the extracellular matrix are continuously removed and replaced (Setton *et al*., 1999).

In pathological processes such as OA, the balance between anabolic and catabolic processes is disturbed causing increased or decreased synthesis or degradation (rev Sandell & Aigner, 2001).

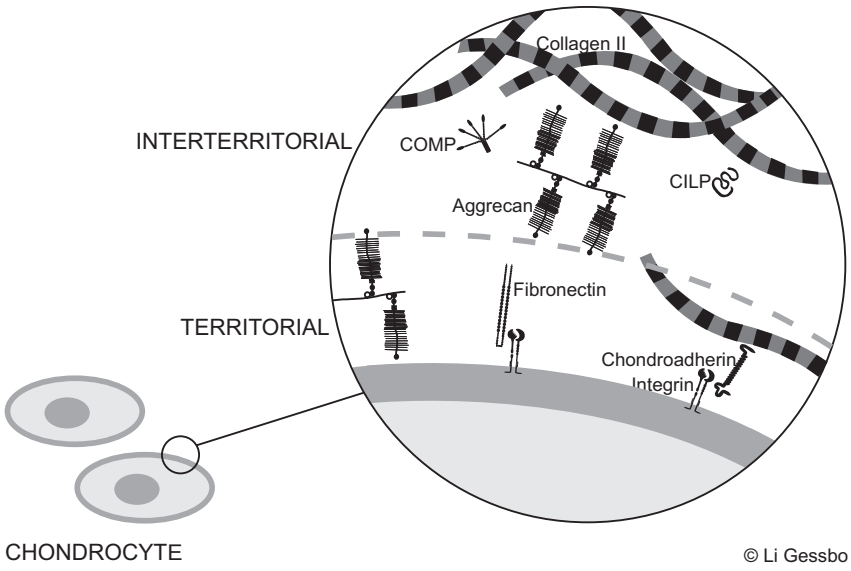

Figure 5. *Schematic drawing of chondrocytes and their extracellular matrix, illustrating a limited number of macromolecules relevant for this Thesis and suggested future research. For complete matrix review see Heinegård et al., 2002.*

Aggrecan

A major constituent of cartilage is aggrecan, a large macromolecule having a central core protein with many negatively charged glycosamnioglycan side chains covalently bound to it (rev Heinegård *et al*., 2002). The closely packed negatively charged groups attract and bind water and repel each other, leading to a large osmotic swelling pressure that contributes to the compressive stiffness of the articular cartilage (Heinegård & Oldberg, 1989; rev Heinegård *et al*., 1998). Several functional domains can be identified in the core protein.

The first globular (G1) domain, in the amino-terminal part, allows a specific interaction with hyaluronan, stabilised by the link-protein enabling a large number of aggrecan molecules to bind to one molecule of hyaluronan (Hardingham & Muir, 1972). Aggrecan also consists of a second globular (G2) domain, with an unknown function, that is separated from the G1 domain by a short stretch of amino acids, the interglobular domain (IGD). A third globular (G3) domain, which appears to play a role in matrix assembly, is found at the carboxyl terminal part of the proteoglycan (Olin *et al*., 2001; rev Heinegård *et al*., 2002). Interposed between the G2 and G3 domains are the keratan sulphate (KS) and chondroitin sulphate (CS) attachment regions. The KS-region, with approximately 30 keratan sulphate chains, and the CS region with 100 some chondroitin sulphate chains bound to the core protein of a single proteoglycan monomer. KS and CS glycosaminoglycans consist of anionic polysaccharides with repeating disaccharide units each containing an amino sugar, (KS- glucosamine; CS- and galactosamine) and a hexose (KS) or hexuronic acid (CS). Each chain consists on average of 40 to 50 disaccharide units each with two negatively charged groups. The hexosamine carries a sulphate ester group as occasionally does the other sugar. Proteoglycan turnover primarily results in the release of large GAG substituted fragments cleaved between G1 and G2 domains (Ratcliffe *et al*., 1986), however further degradation results in the release of the G1 domain and link protein (Witter *et al*., 1987; Ratcliff *et al*., 1992). The enzymatic degradation is mediated by metalloproteases (stromelysin) and aggrecanases (ADAM-TS -4, -5, -11) (Poole *et al*., 1995; Abbaszade, I. *et al*., 1999; Tortorella *et al*., 2000).

Collagens

The extracellular matrix in the articular cartilage is composed of a three-dimensional crosslinked network of collagen type II (together with smaller amounts of other minor collagens such as collagen type IX, and XI) essential for the tensile strength and stiffness of the tissue (rev Mayne, 1989; Huber *et al*., 2000, rev Kielty & Grant, 2002). Collagen type II represents 50% of the dry weight and 95% of the total collagen content in articular cartilage (rev Kuettner 1992). The collagen type II molecule is fibril forming and composed of three identical polypeptide chains, $[\alpha_1(II)]_3$, wound around each other forming a right-handed triple helix.

Chondrocytes synthesise and secrete collagen type II. Many modifications unique to collagen occur during biosynthesis. Collagen contains proline and lysine, and during synthesis hydroxylation of the residues results in the formation of hydroxyproline and hydroxylysine (rev Stryer, 1981) almost unique to collagens. The molecule, secreted as a procollagen, contains nonhelical propeptide

extensions at their amino- and carboxy terminal ends. The extensions are enzymatically removed, allowing the molecules to form fibrils. These are later stabilised by covalent, intermolecular crosslinks following modification of certain lysin residues by lysoloxidase, further increasing tensile strength (Eyre *et al*, 1984).

Other collagens in articular cartilage are also classified as *fibril forming*: collagen type III and XI, *short-chain*: collagen type VI and X, *fibril-associated collagen with interrupted triple helices (FACIT)*: collagen type IX, XII, XIV. Collagen type XI, located within the interior of collagen type II fibrils probably organise type II molecules and determine the size of the final collagen fibril (rev Kuettner, 1992; Blaschke *et al*., 2000). Collagen type IX is covalently linked to the surface of collagen type II fibrils, which is believed to mediate fibril-fibril and fibril-proteoglycan interactions (Eyre, *et al*., 2004). Collagen type X, involved in calcification of matrix, is present in the growth plate around hypertrophic chondrocytes and in the calcified zone of articular cartilage (Grant *et al*., 1985). Collagen type VI, located in the pericellular compartment is proposed to have a bridging function between chondrocyte and matrix. (Horikawa *et al*., 2004). Collagen type III fibrils are rarely found in normal articular cartilage (Young *et al*., 2000).

Collagenases (MMP-1, -2 –8, -13 and –14) cleave the collagen molecule, with ensuing unwinding the helical structure with subsequent susceptibility to degradation by other proteases (Murphy *et al*., 1987; rev Murphy &Reynolds, 2002).

Non-collagenous proteins

Articular cartilage also consists of non-collagenous matrix proteins (rev Heinegård *et al*., 2002) which are important for the interaction and assembly of the various macromolecules. These molecules have different functions and interact specifically with other matrix molecules, either contributing to the structural network or interacting directly with the chondrocytes by modulating the phenotype (rev Neame *et al*., 1999).

Leucine rich repeat proteins

A major non-collagenous protein family of the extracellular matrix is the small leucine-rich repeat proteins (sLRP), some of which contain glycosaminoglycan. These proteins have a central leucine rich repeat (LRR) domain and represent an important superfamily known to participate in protein-protein interactions (Hocking *et al*., 1998). They are sub-grouped according to their amino acid sequences and gene organisation. Several sLRPs are found in cartilage including decorin, biglycan, asporin, fibromodulin, lumican, PRELP (proline-arginine-rich end leucine-rich repeat protein), ephiphycan and chondroadherin.

Decorin and biglycan, interact with collagens via the LRR domain (Oldberg *et al*., 1989), and asporin (which is structurally related to biglycan and decorin but without glycosaminoglycan chains) is upregulated in articular cartilage in early OA (Lorenzo *et al*., 2001). PRELP, a heparin binding protein, binds to a basement membrane proteoglycan—perlecan via its heparin-binding domain. Additional, PRELP binds to collagen type I and II via the LRR domain. The putative role for

this protein is to anchor the basement membranes to the underlying tissue (Bengtsson *et al*., 2002).

Chondroadherin interacts with chondrocytes and fibroblasts via a surface receptor (integrin α2β1) (Camper *et al*., 1997). Chondroadherin also binds to two sites on collagen type II (Månsson *et al*., 2001a), and collagen type VI (Wiberg *et al*., 2002) with probable functional importance in regulating collagen fibril assembly and anchor the cell to matrix. Chondroadherin is localised in the territorial matrix of late proliferative cells in the growth plate and in articular cartilage of the maturing femoral head of the rat. The protein may have an important role in the regulation of chondrocyte proliferation and growth (Shen *et al*., 1998). Biglycan, decorin and chondroadherin have the ability to interact with collagen type VI and biglycan or decorin with GAG–chains can organise the molecule into extensive hexagonal-like networks (Wiberg *et al*., 2002).

Fibronectin

Fibronectin is found in most tissues and body fluids, binding to cell surfaces via several different integrin receptors and integrin subunits α5β1 to chondrocytes (Lucchinetti *et al*., 2004). Mechanical load up-regulates the synthesis of fibronectin in bovine explants and in osteoarthritic joints (Xie *et al*., 1992; Wong *et al*., 1999). Additional fragments of fibronectin can initiate degradation and release of COMP and chondroadherin, inducing production of nitric oxide from equine cartilage explants *in vitro* (Johnson *et al*., 2004).

Increased concentration of fibronection has also been found in cartilage explants (Burton-Wurster *et al*., 1999) and synovial fluid (Lust *et al*., 1997) from OA joints in dogs.

Cartilage intermediate layer protein (CILP)

CILP, present in the middle/deep layer of articular cartilage increases in concentration with age. The function of the protein is not known, though its restricted distribution in the articular cartilage suggests a specific function (Lorenzo *et al*., 1998).

Cartilage Oligomeric Matrix Protein (COMP)

COMP is a glycoprotein belonging to the thromospondin family also named thrombospondin 5. COMP is made up of five identical subunits (pentamer) linked together at their N-terminal part via a coiled coil domain stabilised by disulphide bonds. Each subunit continues with a flexible arm that contains four epidermal growth factor (EGF) domains and seven calcium-binding domains (Zaia *et al*., 1997). The chains end with a C-terminal globular domain creating a bouquet that is tulip-like appearance (Mörgelin *et al*., 1992). The intact pentamer is 435 kDA in size, and is dissociated by reduction of disulphide bonds resulting in 5 subunits on 86,949 kDa (Zaia *et al*., 1997).

The COMP molecule consists of 737 amino acids. The distribution in different parts of cartilage as well as between cartilages differs. The protein is present in the territorial matrix of the growth plate (Ekman *et al*., 1997) and immature cartilage (Shen *et al*., 1995) and the interterritorial matrix of the adult articular cartilage

(Shen *et al*., 1995). Its function is not fully understood but it interacts with collagen type I, II (Rosenberg *et al*., 1998) and IX molecules (Thur *et al*., 2001; Holden *et al*., 2001) through the C-terminal domain and catalyses the assembly of collagen type I and II into fibrils *in vitro* (Rosenberg, PhD Thesis 2001). COMP also interacts with fibronectin found in the extracellular matrix of cartilage (Di Cesare *et al*., 2002) and is found in tendon (Di Cesare *et al*., 1994; Smith *et al*., 1997), and in trace amounts in synovial membrane (DiCeasare *et al*., 1997). However, the concentration of COMP in intra-articular ligaments and synovial membrane of the equine middle carpal joint is a thousand and hundred-fold lower, respectively, than in synovial fluid (Skiöldebrand *et al*., 2001).

COMP plays an important role in cartilage development and mutations in the gene give rise to multiple epiphyseal dysplasia (Hecht *et al*., 1998) and pseudoachondroplasia (Briggs *et al*., 1995) characterised by limb dwarfism and cartilage abnormalities. Suprisingly, COMP-deficient mice have normal skeletal development suggesting that mutations in the gene result in defects of the protein or abnormalities in matrix assembly, rather than reduced amounts of COMP (Svensson *et al*., 2002).

The enzymes responsible for COMP degradation *in vivo* have yet to be identified. Bovine explants stimulated with Il-1 release aggrecan prior to COMP, which in turn was released prior to collagen (Dickinson *et al*., 2003). COMP was cleaved by ADAMTS-4 (a disintegrin and metalloprotease with thrombospondin repeat) creating fragments with an apparent molecular mass of approximately 110 kDa similar to fragments of COMP released in OA synovial fluid.

Age-related changes in articular cartilage

Age changes in cartilage matrix are mostly related to a decline in the ability of the cells to synthesise and assemble matrix macromolecules (rev Martin & Buckwalter 2002). Macromolecular homeostasis in ageing healthy joints must be considered when interpreting pathological processes occurring in OA. Age-related articular cartilage degeneration such as fibrillation is the most obvious structural change, not always associated with joint pain or dysfunction in humans (Byers *et al*., 1970; Koepp *et al*., 1999). There is a strong relationship between increased levels of pentosidine crosslinks and degeneration in articular cartilage in man, resulting in a stiffer collagen network more susceptible to mechanical load (Verzijl *et al*., 2000). Additional, increased pentosidine crosslink levels are present in articular cartilage from older horses (Brama *et al*., 1999). Articular cartilage degeneration was found to increase with age, suggesting that OA also may be a natural occurring age-related process in the horse (Brommer *et al*., 2003a).

Age-related articular cartilage fibrillation does not necessarily lead to progressive articular cartilage degeneration present in OA (rev Martin & Buckwalter, 2002). Low metabolic activity in chondrocytes with a decreased responsiveness to growth factors have been found in articular cartilage from old horses compared to young horses (Iqbal *et al*., 2000; Morries & Treadwell, 1994). Also, an age-related decrease in the ratio of cell (DNA) to matrix (dry-weight) has been described in equine articular cartilage from weight bearing areas of the metacarpophalangeal joint (Platt *et al*., 1998; Brama *et al*., 2002). This is in agreement with studies of human articular cartilage showing decreased cellularity

correlated to higher age (Vignon *et al*., 1976; rev Martin & Buchwalter, 2002). It is suggested ”that the functional adaptation of articular cartilage, as reflected in the function of biochemical heterogeneity in the horse, occurs for the most part during the first 5 month post-partum” (Brama *et al*., 2002).

In synovial fluid of neonates, high concentrations of hydroxyproline , GAG and MMP were found (van den Boom *et al*., 2004), and during maturation the concentrations decreased. However, in horses more than 4 years of age the collagen content and hydroxyproline and GAG concentrations did not change with age in adult horses (Brama, *et al*., 1999; van den Boom, *et al*., 2004). This is in agreement with GAG in synovial fluid from different equine joints (8-30 years) (Fuller *et al*., 1996). The total proteoglycan content (mainly composed of aggrecan) remains constant with age in equine articular cartilage, however a heterogeneity in size of the proteoglycans, with an increased level of free hyaluronan binding region suggesting a retained functional capacity to aggregate is present (Platt *et al*., 1998). Also, there is an age-related change in sulphation of newly synthesised and endogenous proteoglycans, in human (Hardingham & Bayliss, 1990; Bayliss *et al*., 1995) and equine (Platt *et al*., 1998) articular cartilage. The decrease in sulphation at the 6 loci compared to the 4 loci of CS in equine and the increase in the 6-sulphation and decrease in 4-sulphation of CS in human (Bayliss *et al*., 1995) suggest a species related difference in the sulphotransferas enzyme. The content of hyaluronan in equine and human articular cartilage increases with age, presenting more binding sites for protoglycan monomers (Hardingham & Bayliss, 1990; Platt *et al*., 1998). The structures of decorin and link protein in equine articular cartilage of metacarpophalangeal joints are similar in size and change with age (Platt *et al*., 1998). No significant correlation is found between KS, total GAGs, and age in synovial fluid from different equine joints (Fuller *et al*., 1996).

The biochemical characteristics of the collagen network such as water and collagen content, lysylhydroxylation, and crosslinking of hydroxylysylpyridinoline in equine articular cartilage of the proximal first phalanx does not change with age (4 to 30 years) (Brama *et al*., 1999). No significant correlation between age and concentration of COMP in serum was found but the concentration of KS in serum from young horses was higher compared to the older horses (Misumi *et al*., 2002). Collagenase (MMP-1) and stromelysin (MMP-3) activity in synovial fluid declined with maturation of the joint after high activity in foetal joints, suggesting a rapid tissue turnover necessary for maturation of the tissue (Brama *et al*., 2000; Brama *et al*., 2004).

Load-related changes in articular cartilage

It is well known that immobilisation of joints or lack of exercise negatively affects the metabolism of articular cartilage and that exercise is necessary to maintain normal cartilage homeostasis (Richardson & Clark, 1993; van den Hoogen *et al*., 1999). Also lack of exercise during the first 5 months of life delay the functional adaptation of the collagen network in equine articular cartilage (Brama *et al*., 2002).

In vivo, axial, shear, and tensile strains are produced in cartilage when loaded (rev Grodzinsky *et al*., 2000). In general, static compression of cartilage, *in vitro*,

decreases synthesis of proteoglycans, most likely due to reduction in cell and nucleus structures (Buschmann *et al*., 1996). However, low-amplitude dynamic compression *in vitro* with different frequencies stimulates chondrocyte metabolism (Sah *et al*., 1989). Injurious dynamic compression of articular cartilage, *in vitro*, results in cell necrosis and apoptosis (Chen *et al*., 2001; Kurz *et al*., 2001).

The exact level of exercise for an adequate dynamic load, beneficial for physiologic turnover of matrix constituents is not known. Mechanical load classified as overload or repetitive load is not a static process and the transformation from physiologic load to overload with a subsequent dominance of catabolic processes is not well characterised. The cellular responses in *in vitro* compression studies are difficult to compare since different loading and labeling times are used, and normal turnover of many proteins is not known.

Physical and structural alterations occur in articular cartilage during dynamic compression, including cell and nucleus deformation, extracellular matrix changes in fluid flow, streaming potentials and currents, altered water content, and fixed charge density. All these parameters have been found to modulate matrix metabolism after compression *in vitro* (rev Grodzinsky *et al*., 1998).

The modulation of metabolic activity in tissue caused by mechanical load is orchestrated by the chondrocytes (Wu & Chen, 2000; Buschmann *et al*., 1996), which can respond through stretch-activated ion channels and integrin signalling. Mechanical load has been found to increase the number of integrin subunit α5 (Lucchinetti *et al*., 2004) and subunit β-1 in articular cartilage (Giannoni *et al*., 2003). These subunits form the receptor for fibronectin, a matrix protein involved in mechanotransduction (Millward-Sadler *et al*., 2000; Enomoto *et al*., 1993) and regulation of cytokine production (Arner & Tortorella, 1995).

The equine third carpal bone in the middle carpal joint is characterised by different load-bearing regions of the proximal articular surface (Palmer *et al*., 1994). Contact area and pressure distribution changes during load and the dorsal part of the radial facet is subjected to a high load compared to the palmar aspect of the intermediate facet, characterised as an unloaded non-contact area (Palmer *et al*., 1994). The articular cartilage from moderately and strenuously trained horses has a similar total DNA content in loaded and unloaded areas (Little *et al*., 1997) indicating no cell loss. A significant reduction in aggrecan and increase in decorin synthesis is present in the dorsal radial facet of articular cartilage from long-term strenuously trained racehorses. The abnormalities in proteoglycan biosynthesis are retained four months after termination of the training (Little *et al*., 1997). No change in biglycan synthesis or ability of aggrecan to aggregate was found.

Short-term, high-speed training in trotters will increase KS concentration in synovial fluid (Yovich *et al*., 1993), as is also found in serum of human athletes after running for 1-1.5 hours (Roos *et al*., 1995). After long-term strenuous training of 2-year-old galloping horses, a decrease in hydroxylysylpyridinoline (HP) crosslinking in the proximal first phalanx (Brama *et al*., 2000a) and a difference of collagen content and crosslinking between sites in the equine articular cartilage of this bone is reported (Brama *et al*., 1999). The areas subjected to constant load show a lower collagen content, HP and pentosidine crosslinks than the dorsal areas subjected to intermittent peaks of load.

The collagen content was also significantly lower in the dorsal area of the third carpal bone of the middle carpal joint in galloping horses exposed to high-

intensity training compared to those in low-intensity training (Murray *et al*., 2001a). These findings suggest that the collagen network is influenced and damaged in strenuous training. Also, lower serum concentrations of CPII were found in five-month old foals exposed to training compared to rested foals and foals in pasture—suggesting a negative effect on the collagen turnover in relation to forced exercise early in life (Billinghurst *et al*., 2003).

COMP expression in articular cartilage is also correlated to load. COMP and fibronectin is up-regulated in bovine adult cartilage explants subjected to cyclic unconfined compression *in vitro* (Wong *et al*., 1999). The up-regulation may be an attempt by the cartilage to stabilise the extracellular matrix in response to the compression. Cell/receptor/matrix interactions are important in the transduction of mechanical signals. The up-regulation of COMP could be blocked with antibodies against the integrin subunits β-1, suggesting activation of a matrix/receptor complex (Giannoni *et al*., 2003).

Distribution of COMP, assessed by light microscopic immunohistochemistry, is more prominent in the interterritorial compartments of articular cartilage from horses undergoing a 19-week high-intensity training program (Murray *et al*., 2001b) compared to non-exercised horses. In the latter, a generalised distribution in territorial and interterritorial compartments is found, suggesting exercise related enhancement of the structural maturity of the articular cartilage. This reorganisation of COMP is also present in articular cartilage from older individuals (King and Heinegård, unpublished data).

The content of COMP also differs in loaded and unloaded equine articular cartilage with lower concentration of COMP in the dorsal areas of radial facet compared to the palmar areas of the intermediate facet of the third carpal bone in exercised horses (Murray *et al*., 2001b). A correlation between COMP and load is also present in other connective tissues. The concentration of COMP in equine tendons varies with age and tendon type with low levels in the neonatal tendon and an increase in concentration up to the age of three years (Smith *et al*., 1997). The superficial digital flexor tendon, which experiences a high load, has a high concentration of COMP compare to the deep digital flexor tendon and extensor tendon.

Another non-collagenous protein in cartilage, fibronectin, is also affected by load. Increased fibronectin content, assessed by light microscopic immuno-chemistry, is present in articular cartilage from loaded area of the third carpal bone in two-year-old galloping horses that were strenuously trained for 19 weeks, compared to non-trained horses (Murray *et al*., 2000).

Biomechanical properties such as the aggregate modulus and permeability constant of the articular cartilage from the third carpal bone were assessed by creep indentation testing (Palmer *et al*., 1995). Exercise significantly increased the permeability constant, suggesting an increased fluid movement through the articular cartilage, resulting in thinner cartilage. Similar results were found in young galloping horses strenuously trained for 19 weeks. The articular cartilage of the middle carpal joint, assessed by creep indentation, showed less permeability, and was thinner in the loaded area of the third carpal bone as compared to untrained horses (Murray *et al*., 1999).

Joint diseases

Joint diseases in horses have been categorised into three main groups, according to etiologic factors: traumatic, developmental, and infectious (rev McIlwraith, 1996).

In man, more than 100 specific diagnostic entities of joint diseases exist, with the most common being, degenerative, inflammatory and metabolic (rev McCarthy & Frassia, 1998). Degenerative joint disease is mainly exemplified by OA; the inflammatory by rheumatoid arthritis (RA), reactive arthritis and psoriatic arthritis (PsoA); and the metabolic by gout and calcium pyrophosphate deposition disease. Other major disease categories in humans include traumatic and infectious disease that can lead to OA when no successful healing is achieved.

OA comprises a heterogeneous group of disorders in the joints with pain and loss of joint function. OA in humans and equine has been defined in various ways—degenerative joint disease, osteoarthrosis, primary and secondary OA—which has led to confusion when comparing the aetiopathogensis. Osteoarthritis and osteoarthrosis are often used interchangeably, but it is useful to remember the difference between them. The latter being joint failure from primarily degenerative changes whereas osteoarthritis are primarily an inflammatory process with secondary degenerative changes (Radin, 1995). Differentiating between the two is often impossible since the difference is found only in the acute event. Both osteoarthritis and osteoarthrosis result in a mixture of inflammatory and degenerative processes within the joint. There appears to always be a component of inflammation in OA. Hence, the term osteoarthritis (OA) will be used throughout this thesis.

The aetiopathogenesis of OA is multifactorial and differs between man (Dieppe, 1995) and horse (rev McIlwraith, 1996). OA in man often has an age-related factor (rev Mankin, 1989) not as predominant in equine OA (Brommer *et al*., 2003a). Excessive, rapid and repetitive load has been suggested to induce joint damage and is an important factor in clinical OA of racehorses (Bramlage *et al*., 1988; rev Pool & Meagher, 1990) and athletes (rev, Garrick & Requa, 2003).

OA in the horse is characterised by articular cartilage degeneration, subchondral bone sclerosis, bone necrosis, marginal osteophytes, and inflammation of the synovial membrane (synovitis), capsule (capsulitis) and ligaments (rev McIlwraith, 1996). Synovitis is almost always present and the reaction is reported to range from mild synovitis to more severe degenerative joint disease (McIlwraith, 1982). Joints differ in form and function, and joints of the limbs develop unique patterns of changes—where the carpal, fetlock, distal intertarsal and tarsometatarsal joints are most commonly affected (rev Pool, 1996).

Subchondral bone sclerosis is often found in OA of the carpal joints of racehorses (Norrdin *et al*., 1998), and it has been suggested that bone sclerosis precedes cartilage fibrillation (Hayami *et al*., 2004). A sclerotic bone is less elastic and may have a reduced ability to dissipate and absorb load (Radin & Rose, 1986). In an assessment of cartilage and bone lesions (by magnetic resonance imaging—MRI), in carpal joints of racehorses, the different cartilage lesions were related to the degree of sclerosis in the underlying bone (Anastasiou *et al*., 2003). However, the sequence of events in the interactions between articular cartilage and subchondral bone is not fully understood (rev Kawcak *et al*., 2001).

Articular cartilage changes in osteoarthritis

The normal articular cartilage is a metabolically active tissue with a balance between anabolic and catabolic processes (rev Heinegård *et al*., 2002). In OA, an imbalance occurs between synthesis and the degradation rate of cartilage extracellular matrix components, inflammatory mediators released from the synovium as well as changes in the subchondral bone (Fig. 6). Joint surface osteoarthritis is grossly characterised by a roughening and fibrillation of the superficial cartilage and in late stages, erosions with denuded bone and the formation of osteophytes. Histologically, superficial fibrillation together with chondrocyte necrosis and proliferation (chondrocyte clustering) to vertical clefts and full thickness loss of articular cartilage are seen (rev McIlwraith, 1996; rev Sandell & Aigner, 2001).

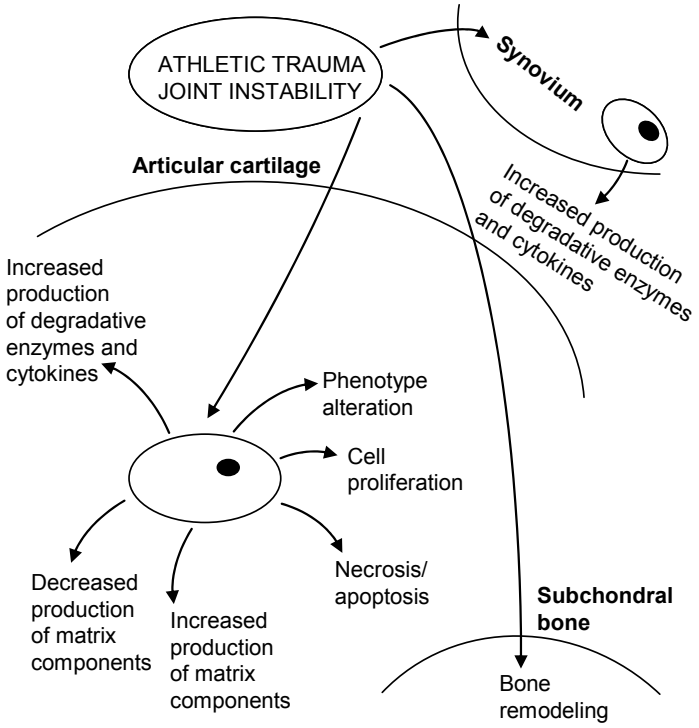

Figure 6. *Suggested cellular responses in equine osteoarthritis.*

Biochemical reactions in osteoarthritic cartilage are complex with degenerative and repair processes present simultaneously. Early cartilage degeneration induced by proinflammatory enzymes is characterised by a net loss of proteoglycans and increased tissue volume (Mankin *et al*., 1971; Maroudas, 1976). KS concentration is significantly increased in serum prior to the onset of macroscopic cartilage damage in dogs with ligament transection of the knee (Manicourt *et al*., 1991). When proteoglycan loss has reached a certain level, degradation of the collagen

network follows with disruption of the network leading to tissue swelling and loss of tensile properties (Maroudas, 1976). The synthesis of collagen has a slow turnover rate, so damage to collagen type II fibrillar network is a critical event in disease progression (Maroudas, 1976; Verzijl *et al*., 2000). Phenotype alteration of the chondrocytes in ostearthritic cartilage with ensuing synthesis of proteoglycans of heterogeneous size and collagen type I (fibrocartilage), results in matrix being less resistance to mechanical load (rev Poole, 1986; Pfander *et al*., 1999).

Although the chondrocytes increases its anabolic activity as an attempt to repair, the tissue is characterised by increased enzymatic degradation. Proinflammatory cytokines such as IL-1, -6, -17, -18 and tumour necrosis factor alpha (TNFα) stimulates chondrocytes and synovial cells to synthesise proteases and nitric oxide with subsequent matrix degradation (rev Sandell & Aigner, 2001; Min *et al*., 2001). Proteases, especially metalloproteases (MMP), are important enzymes in cartilage degradation. MMPs are classified into collagenases, gelatinases, stromelysins, matrilysins, ADAMs and MT-MMP (membrane-type MMPs). Collagenases (MMP -1, -2, -8, -13 and 14), cleave and degrade fibrillar collagen at a single bond, three-quarters of the way along the collagen molecule from the N-terminus, thereby destabilising the conformation (Gadher *et al*., 1988). Stromelysins (MMP -3, -10 and -11) degrade proteoglycans, and collagens such as collagen types II, IX, XI. Gelatinases (MMP -2 and -9) degrade gelatin and unwound collagen. ADAMS-TS -4 and -5 (aggrecanase) cleave the aggrecan molecules at specific sites (Tortorella *et al*., 2000). The inactive form of the MMPs can be activated by stromelysin or MT- MMPs (Woessner, 1991; Murphy *et al*., 1990).

Production of MMPs by equine chondrocytes and subsequent cartilage degradation has been induced by TNFα and Il-1 ß (Alwan *et al*., 1991b; Billinghurst *et al*., 1995; Clegg & Carter, 1999; Trumble *et al*., 2001). However, the activity of TNFα in synovial fluid does not correlate to the degree of joint destruction in horses as does the activity of MMP -9, where high levels are found in joints with severe cartilage lesions (Joughlin *et al*., 2000). High concentrations of MMP -2 and -9 are present in synovial fluid from equine aseptic arthritic joints (Clegg *et al*., 1997; Trumble *et al*., 2001). Also, high MMP activity as well as high concentrations of MMP -3 and MMP -1 are found in synovial fluid from OA joints (Brama *et al*., 1998; 2000; 2004). Regulation of MMPs is achieved by controlling production and activation of the pro-enzymes together with the presence of inhibitors, such as tissue inhibitors of metalloproteases (TIMP) (Clegg *et al*., 1998).

Molecular markers in joint disease

The early stages of cartilage and bone changes in OA are impossible to define and monitor, hence there is a search for biochemical and immunological markers that indicate the disease process. In order to prevent the onset of tissue destruction and progression, it is crucial to find molecular markers that can be used to monitor inflammatory and catabolic as well as anabolic processes in cartilage and bone (Heinegård & Saxne, 1991; Lohmander *et al*., 1995).

A set of synovial fluid and serum markers, indicating events in different compartments of the joint tissues would be valuable in monitoring tissue

destruction and repair over time. These markers could also be used to diagnose, prognosticate, and evaluate therapeutic intervention. The effects of load, inflammation and age on the degradation, remodelling and synthesis of matrix molecules in connective tissues could be evaluated using such markers.

Molecular markers are classified as inflammation and skeletal markers (Otterness *et al*., 2000). Inflammation markers, comprise MMPs (Clegg *et al*., 1997; Brama *et al*., 1998; 2000b; 2004), cytokines (Morris EA *et al*., 1990, Bertone *et al*., 2001, Billinghurst *et al*., 1995) acute phase proteins (Hultén *et al*., 2002; Otterness, 1994) and hyaluronan (Tulamo *et al*., 1996). However, this review concentrates on skeletal markers and in particular, markers of cartilage metabolism.

Skeletal markers comprise constituents of cartilage and bone extracellular matrix. Specified concentrations of these tissue derived markers in body fluid compartments (synovial fluid, serum and urine) may serve as a window of ongoing pathological processes in the joint (Saxne *et al*., 1993; Lohmander *et al*., 1998; Petersson *et al*., 1997, 1998). Skeletal markers are related to anabolic and catabolic processes of cartilage and bone matrix (Otterness *et al*., 2000; rev Lohmander & Felson, 1998). The correlation between metabolic processes in the joint and the concentration of markers in synovial fluid is influenced by such parameters as: degradation/synthesis rate of the matrix constituents; amount of tissue, cell viability and intensity of inflammation—as the rate of elimination from the joint fluid compartment increases with inflammation (rev Levick, 1992; Myers *et al*., 1996). Concentrations of molecular markers in serum and urine are further dependent on the rate of elimination by lymph nodes, liver and kidney (Fig. 7).

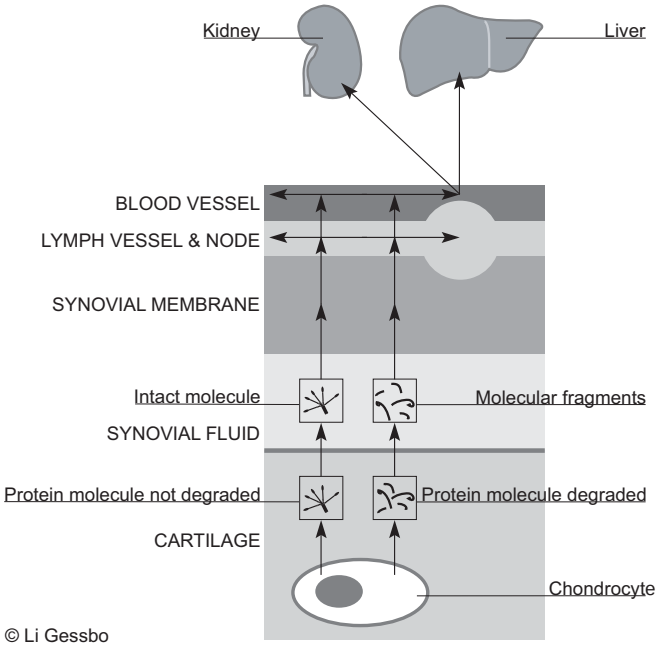

Figure 7. *Schematic drawing illustrating elimination of cartilage matrix constituents.*

The concentration and fragmentation of a certain molecular marker in synovial fluid can reflect the tissue metabolism of the specific joint (Heinegård *et al*., 1985; Heinegård & Saxne, 1991; Saxne & Heinegård, 1992). Serum, however, contains intact or degraded macromolecules released from many joints, reflecting systemic changes (Simkin *et al*., 1995). Also, human joint cartilage represents less than 10% of the total body hyaline cartilage, hence, serum concentrations of a cartilage derived macromolecule will reflect pathological changes in other organs than joints (rev Lohmander & Felson, 1998).

Cartilage matrix molecules originate from newly synthesised molecules never incorporated in the matrix or resident molecules released as degraded or intact molecules. In addition, the released molecules originate from different compartments in the matrix (pericellular, territorial and interterritorial) and/or from different morphological layers (superficial, intermediate and deep) of the articular cartilage (rev Lohmander & Felson, 1998).

The results of the many studies of skeletal markers in OA can be difficult to compare since different methods are often used. It is important to consider the type of joint, the pathological changes, the age, and the athletic activity of the horse when comparing results.

KS concentration in serum, (as measured by an inhibition ELISA using monoclonal antibodies against a sulphated epitope on KS (5D4)), was significantly higher in horses with OA joints compared to normal, however no difference in synovial fluid from normal and OA joints was noted. (Okumura *et al*., 1998). Significantly increased levels of KS in synovial fluid and serum, assessed by an identical immunoassay and glycosaminoglycans measured by a specific dye

binding assay (DMB) were found in fetlock and carpal joints of horses with OA as compared to normal joints (Alwan *et al*., 1990). However, using an identical immunoassay, KS in synovial fluid from fetlock and carpal joints of galloping horses, subjected to arthroscopy after a short period of lameness, decreased in correlation to the severity of pathological changes (Fuller *et al*., 2001). But, this comparison was made between the clinically active joint (lame) and the contralateral joint, not clearly defined as normal. Also, a low KS concentration in synovial fluid from osteoarthritic carpal joints has been reported (Todhunter *et al*., 1997). The differences presented in these studies can be due to different athletic profiles of the horses, different definition of OA and/or different activity of the inflammation in the osteoarthritic joints.

High levels of GAGs (DMB-assay) were found in synovial fluids, serum, and urine from horses with OA and in each case, the level was higher in the synovial fluid than in the serum or urine from the same horse (Alwan *et al*., 1991a). High levels were also found in horses with osteochondritis dissecans and traumatic arthritis, but not in normal or infected joints. A change in glycosaminoglycan structure depicted by the 846 epitope on chondroitin sulphate is an indicator of altered proteoglycan metabolism. High levels of this epitope were found in synovial fluid and serum from horses with osteochondral fractures of the carpal joints as compared to healthy joints. As well, an elevated concentration of CPII (pro-peptide of collagen type II)—an indicator of collagen type II production in serum from horses with fractured joints was found (Frisbie *et al*., 1999). Further, a significant increase in CPII and a decrease of 846 and KS epitopes were present in young horses with osteochondrosis (Laverty *et al*., 2000). Collagen type II fragments [COL2-3/4C], biomarkers of collagen degradation (collagenase-generated neoepitopes of type-II collagen fragments) in serum, are indicators of the severity of osteochondrosis in foals, aged 5 months (Billinghurst *et al*., 2004). However, osteochondrosis in foals, aged 11 months, showed lower concentrations of [COL2-3/4C] and higher of CPII in serum, indicating reparation. In another study (van den Boom *et al*. 2004), no increase in concentrations of hydroxyproline (high-performance liquid chromatography) and GAG (DMB-assay) in synovial fluid from horses with OA compared to unaffected joints was found. However, hydroxyproline was positively correlated with cartilage degeneration index and MMP activity.

COMP has been an extensively studied candidate in the search for molecular markers of OA in man. COMP concentrations in synovial fluid and serum increase in RA, reactive arthritis, and in early stages of osteoarthtritis (Forslind *et al*., 1992; Saxne *et al*., 1993; Lohmander *et al*., 1994; Di Cesare *et al*., 1996), and the high concentration remains for many years. However, in advanced OA with cartilage erosions and loss of cartilage with denuded bone, no high COMP concentrations are observed (Lohmander *et al*., 1994). In a one-year prospective study of patients with hip OA, COMP concentrations in serum were higher in patients with progressive disease (Conrozier *et al*., 1998).

COMP in synovial fluid and serum has also been tested as a marker candidate of OA in horses. The concentrations of COMP in synovial fluid from joints with aseptic and septic arthritis were significantly lower compared to normal joints, with no difference between aseptic and septic joints (Misumi *et al*., 2001). A higher proportion of fragmented COMP was also found in these joints.

Additionally, low concentrations of COMP and KS in serum were found in horses (unknown breeds) with synovitis, osteochondral chip fractures and osteochondritis dissecans. The concentration of COMP in synovial fluid also varies between different joints (Viitanen *et al*., 2000). Higher concentrations of COMP, total protein, and GAGs were found in synovial fluid from the coffin joint as compared to the fetlock joint, possibly reflecting differences in load.

Studies of experimental induced arthritis in rats showed high serum levels of COMP reflecting cartilage destruction, and fibrinogen and hyaluronan reflecting inflammation (Larsson *et al*., 2002). Inflammation and tissue destruction appears to be uncoupled processes in human RA and in animal models of RA (Joosten *et al*., 1999a; Joosten *et al*., 1999b; Roux-Lombard *et al*., 2001). An increased concentration of COMP can be an indicator of anabolic and /or catabolic processes in the cartilage. Inflammatory joint diseases, such as RA and PsoA include both synovial inflammation and tissue destruction and PsoA patients had a high concentration of sf-COMP and a low concentration of sf-aggrecan compared to patients with destructive and non-destructive RA (Månsson *et al*., 2001b). The low concentration of sf-aggrecan and high concentration of sf-COMP in PsoA may reflect a repair process where the high concentration of COMP primarily represents release of newly synthesised molecules.

Aims of thesis

The overall aim was to study the metabolism of equine articular cartilage in relation to training and injury using the pattern of release of extracellular matrix proteins into the synovial fluid as a biologic window. Detection of subclinical biochemical cartilage destruction may provide knowledge into the pathogenesis of OA.

The specific objectives were:

* To define the baseline values for COMP, aggrecan, and collagen type II degradation products in synovial fluid and serum in normal middle carpal joints from two horse breeds with different athletic background. Additionally, to identify the relationship, if any, between the concentration of macromolecules released into the synovial fluid and serum and the extent of articular cartilage lesions.

* To measure changes in the concentration of COMP, aggrecan, and collagen type II degradation products released into synovial fluid in the middle carpal joints from racehorses during a long term training program (24 month) and to investigate the synthesis of COMP in dynamic compressed cartilage explants from horses with different athletic backgrounds.

* To delineate the presence and distribution of COMP in relation to collagen fibril thickness in different matrix compartments and zones from loaded and unloaded areas of proximal articular cartilage of the third carpal bone.

* To determine the concentration of COMP and aggrecan released into synovial fluid of carpal joints in racehorses with acute lameness and to identify any relationship between the macromolecular concentration and the type of joint lesion present.

Present investigation

Three common horse breeds in Sweden are the Swedish Warmblood horse (riding horse), Thoroughbred horse (galloping horse) and Standardbred trotter (trotter). The trotter and galloping horse are the two most popular racehorses and the different gait at which the horses perform creates different loading conditions within the carpal joints—leading to differences in the location of lesions and type of fractures to the third carpal bone (Palmer, 1986; Schneider *et al*., 1988). Greater force is generated in the carpal joints during the gallop as compared to trot, which is a more balanced gait. However, the third carpal bone is subjected to more frequent repetitive cyclic load during trotting, though with lower magnitude forces than in the gallop (Schneider *et al*., 1988). Racehorses start to train at an early age (1.5 years) and to qualify for youth races at 2.5 years. Riding horses used for competition and pleasure riding start their training at 2 to 3 years of age, with a slower progression of intensity during the first years compared to the racehorses. The riding horse will qualify for advanced competition at the age of 8-10 years.

This thesis focuses on these three horse breeds, with different athletic backgrounds. Synovial fluids are sampled from the left middle carpal joints (paper I, II, IV) and radiocarpal joints (paper IV), which are high-motion joints subjected to high load with frequent micro-trauma and subsequent cartilage destruction and subchondral bone sclerosis. The studies concentrate on the left carpal joint often associated with lameness in the racehorse (Bramlage, 1988). Also, trotters in Sweden race counter clock-wise with subsequent lameness more frequently originating in the left carpal joint than the right carpal joint (Magnusson, Thesis 1985). Only one joint is evaluated, since different joints normally express varying concentrations of synovial fluid constituents (Viitanen *et al*., 2000) and the cartilage metabolism of macromolecules in one joint can influence the contralateral joint (Dahlberg *et al*., 1994).

Molecular markers of cartilage metabolism would be powerful tools in investigations into the pathogenic mechanisms of articular cartilage destruction in the athletic horse. Much research has been done to develop biochemical markers that identify and quantify cartilage breakdown. To be able to detect and monitor the early stages of osteoarthritic changes, when the balance between anabolic and catabolic processes is disturbed, is crucial for an understanding of the pathogenesis of OA.

The biochemical and morphological methods used are thoroughly described in four papers included in the Thesis. The following antibodies have been used: a) rabbit polyclonal antibody against purified porcine collagen type II, denatured thermally (paper I, II), not cross reacting with any of the other collagen types, but with collagen type II from rat and horse. After immunoadsorption against native collagen type II by ELISA it did not react with native collagen type II from any species; b) rabbit polyclonal antibody against the CS-region of the core protein of aggrecan (paper I, II, IV); and c) rabbit polyclonal antibody against bovine COMP reacting with intact protein as well as fragments of the pentamer ín the ELISA (paper I, II, IV) and immunolocalisation (paper II, III).

Summary of papers

Concentration of collagen, aggrecan and cartilage oligomeric matrix protein (COMP) in synovial fluid from equine middle carpal joints (Paper I)

Hypothesis: The concentration of cartilage derived molecular markers in synovial fluid and serum correlates to tissue destruction of cartilage in the middle carpal joint, with higher values in osteoarthritic joints with cartilage degeneration.

To test this, we evaluated metabolic activity in the cartilage of the third carpal bone by measuring the release of COMP, aggrecan, and collagen type II molecules into the synovial fluid and serum and correlating the values to different cartilage lesions and metabolically labelled cartilage explants.

Seventy-three horses of two different breeds, trotters (52) and riding horses (21) aged 1 to 19 years, with different athletic backgrounds having articular cartilage with normal appearance or mild, moderate, or severe macroscopic lesions were included. A significantly lower concentration of sf-COMP and sf-aggrecan were found in trotters with moderate joint lesions as compared to trotters with normal articular cartilage. This lower sf-COMP was not present in riding horses, instead, a slight increase in concentration was found in joints with moderate lesions compared to normal joints and the sf-aggrecan concentration was similar in joints with normal articular cartilage and lesions. The COMP content in serum was also significantly lower in the trotter with moderate joint lesions and highest in the riding horse with normal joints. Also, a correlation between age and lower sf-COMP was found in the trotters. Additional metabolic labeling confirmed a lower synthesis of COMP in cartilage with moderate lesions compared to cartilage with normal cartilage or mild lesions. The total content of COMP in the articular cartilage was lowest in the trotter with moderate lesions. The content of COMP in the cartilage extracts was expressed as mg/wet weight and in relation to µg hydroxyprolin, with similar results.

The level of collagen type II degradation products in synovial fluid was higher in the riding horses compared to the trotters (indicating a difference in release of molecules in horses with different activity), but no correlation between joint lesions and collagen concentrations were found.

The hypothesis, put forward, could not be proven and the results contradict the higher COMP release found in humans with OA.

Interestingly a correlation between age and lower sf-COMP was found in the trotters but not in the riding horses. These results suggest that the trotter, subjected to earlier and more intense training than the riding horse, has an altered cartilage metabolism. This difference between breeds can reflect a difference in release of macromolecules in loaded normal and/or osteoarthritic cartilage. Hence the effect of load on articular cartilage achieved during training needed to be further evaluated. To shed some light on these questions, the second study was conducted.

Altered metabolism of extracellular matrix proteins in articular cartilage of intensely training Standardbred trotters (Paper II)

Hypothesis: Training will increase the turnover of macromolecules in normal articular cartilage with a subsequent increase of molecules or fragments into the synovial fluid.

To test this hypothesis, we followed 28 trotters during a two-year training program from start of training at the age of 1.5 years until high speed racing at 3.5 years. To minimise effects from variables other than training and age, the horses were trained by the same trainer at the same campus during the same time of year, and synovial fluid was collected every third month (before 2 days of rest). The concentration of COMP, aggrecan and collagen type II degradation products was correlated to total amount of training, age, rest and lameness. At each visit, the horse was classified as lame or not lame and the values of the macromolecules were recorded as coming from a lame or non-lame joint.

The statistical evaluation was constructed to find possible relationships, and the magnitude of any relationship, between the concentration of macromolecules in the synovial fluid and each of the parameters measured (age, days of training, days of rest, lame/non-lame).

The concentration of collagen type II degradation products increased with the amount of training, indicating degeneration of the collagen network. This was found in both lame and non-lame joints. Sf-aggrecan was not significantly influenced by the parameters studied. However, a somewhat lower concentration of aggrecan was found in the raced horses compared to the non-raced at visit 6. Total days of training and the age of the horse influenced COMP concentration in both lame and non-lame joints. However, age and total days of training correlated strongly to each other, hence the result indicated that both age and load could influence COMP metabolism.

To obtain information on possible mechanisms responsible for the lower COMP concentration, an *in vitro* compression study was undertaken. Cartilage from age-matched, trained and untrained horses was collected and subjected to 48-h cyclic unconfined dynamic load. In the untrained horses, an up-regulation of COMP synthesis was seen, and in the cartilage from trained horses a down-regulation of the protein was found, though this was not influenced by age. The up-regulation in the untrained horses is in agreement with previous results from compression studies of bovine cartilage from untrained animals (Wong *et al*., 1999).

Our working hypothesis was not supported by the results, instead we concluded that long-term strenuous training results in alterations of cartilage metabolism and an inhibited ability of the cell to respond with increased synthesis of matrix proteins. The down-regulation of COMP synthesis may be one part of the early development of cartilage degeneration in osteoarthritic joints.

Ultrastructural immunolocalization of cartilage oligomeric matrix protein (COMP) in loaded and unloaded areas of the articular cartilage from the equine third carpal bone (Paper III)

Hypothesis: Mechanical load creates a structural difference in the articular cartilage matrix with changes in collagen fibrils and amount of COMP.

To test this hypothesis, an ultrastructural semi-quantification of COMP was undertaken—to compare the delineation of COMP in cartilage matrix from a high weight-bearing area in the dorsal radial facet (loaded) and a low weight-bearing area in the palmar intermediate facet (unloaded) of the third carpal bone from trained and untrained horses.

The untrained horses often displayed a higher COMP concentration, assessed by density of gold markers, in the loaded areas compared to unloaded. Also, immunolabeling was higher in the untrained horses compared to the trained 3-year-old horse, which showed the lowest levels of all horses. The untrained 3-year-old horse displayed the highest density of gold markers in the loaded area of all horses. This marked difference between the age-matched horses clearly indicates that the high-speed training of the horse has a drastic effect on cartilage metabolism. The collagen fibril diameter was also measured and the young untrained horses had a similar diameter distribution in unloaded and loaded areas. The 3-year-old horses, also showed a similar distribution of fibril diameter, but the trained 3-year-old presented a higher proportion of thin fibrils in loaded areas compared to unloaded. This was not found in the three-year-old untrained horse, where a tendency to a somewhat higher proportion of thicker fibrils could be seen in loaded areas.

In conclusion, an altered immunolabeling of COMP with low concentration in all compartments of articular cartilage matrix from an intensely trained horse is presented— supporting our hypothesis. An indication of altered collagen fibril diameter together with low COMP content indicates the need for further studies into the effect of strenuous training with high load on the equine articular cartilage.

Enhanced concentration of COMP (cartilage oligomeric matrix protein) in osteochondral fractures from racing Thoroughbreds (Paper IV)

Hypothesis: A correlation between COMP and aggrecan concentration in synovial fluid and macroscopic lesions of articular cartilage, osteochondral fractures and ligament tears in acutely lame horses exists.

Sixty-three lame horses (49 trotters and 14 galloping horses: 2.5-8.3 years old) in conventional training/racing with acute lameness underwent arthroscopy to the intercarpal or middle carpal joints and synovial fluid samples were collected. The joint pathology was characterised macroscopically and correlated to the sf-COMP and sf-aggrecan. No correlation between sf-COMP and age was present in the galloping horses, however, sf-COMP decreased with age in the trotters.

The concentration of COMP was higher in galloping horses with osteochondral fractures than in trotters and galloping horses with osteoarthritic lesions. The concentration also increased in correlation to days after injury. The chondrocytes in middle and deep zones of the articular cartilage in the osteochondral fragments from two horses expressed COMP mRNA, in contrast to the articular cartilage of the third carpal bone on the opposite side of the fracture with no expression detected. In the synovial fluid from a joint with osteochondral fracture only intact COMP was present, whereas, fragmented COMP was more prominent in synovial fluid from a joint with osteoarthritic lesions. The concentration of aggrecan did not differ between the two breeds, or between different joint pathology.

The increased concentration of COMP in joints with osteochondral fractures and the dominance of intact COMP together with expression of COMP mRNA in the fragments, suggests that the protein is up-regulated in this type of pathology where compressive load of the osteochondral fragment is low.

Our hypothesis was partly supported by the findings of significant elevation of sf-COMP in the joints from galloping horses with osteochondral fractures. The elevated COMP concentration in the joints of the galloping horses can be a useful clinical marker for carpal joint osteochondral fragments.

Conclusions

● The concentration of COMP, aggrecan and collagen type II degradation products in synovial fluid from trotters with moderate articular cartilage lesions is lower than in riding horses with similar lesions. The COMP synthesis and total amount of COMP in explants from trained trotters are lower compared to untrained horses. This indicates that chondrocytes from cartilage subjected to high and repeated load have a low capacity to synthesise COMP. The baseline values differed between the two horse breeds.

● The concentration of COMP in synovial fluid from young trotters in training decreases with the amount of training and higher age.

● Cartilage explants—collected from trained horses—subjected to dynamic unconfined compression, *in vitro*, showed inhibition of COMP synthesis compared to cartilage from age-matched untrained horses.

● COMP concentration, assessed by immunolabeling is higher in loaded areas compared to unloaded areas of articular cartilage in untrained horses. Low COMP levels are seen in both loaded and unloaded areas of a trained horse.

● A high concentration of COMP in synovial fluid is seen in galloping horses with osteochondral fractures compared to trotters with normal articular cartilage and articular cartilage with moderate lesions or osteochondral fractures. The articular cartilage of the osteochondral fragments expressed COMP mRNA and the synovial fluid contained intact COMP molecules, indicating COMP synthesis with increased release or primary increased release of COMP from the matrix.

General discussion

A progressive destruction of articular cartilage in combination with subchondral bone changes, leading to pain, lameness and subsequent loss of joint function is seen in OA. The exact aethiopathogenesis behind the cartilage degeneration in OA of equine athletes is poorly understood. A high and/or repetitive load is believed to play a significant role in the destruction of the articular cartilage of the dorsal radial facet on the third carpal bone in the middle carpal joint of racehorses (Bramlage *et al*., 1988; Palmer *et al*., 1994).

The work in this thesis focused on three cartilage matrix macromolecules; COMP, aggrecan, and collagen type II—each reflecting different functional units of the cartilage. The present studies concentrated on the left middle carpal joint focusing on the third carpal bone in order to minimise variations of biomechanics and patterns of joint disease. Also, metabolic changes in one joint can influence the contralateral joint (Dahlberg *et al*., 1994).

The overall aim was to detect subclinical biochemical articular cartilage destruction in the horse with the aid of cartilage-derived macromolecules in the synovial fluid. The metabolic reactions of articular cartilage to high repetitive load of the dorsal radial facet in the middle carpal joint and its role in pathogenesis of OA were also of great interest.

In paper I, we measured the concentrations of COMP, aggrecan or fragments of these molecules and collagen type II degradation products released to synovial fluid and serum from trotters and riding horses with articular cartilage degeneration or normal joints. Sf-COMP and sf-aggrecan decreased in the joints with moderate cartilage lesions from the trotters compared to the normal joints. This decrease was not found in the joints from riding horses. A marked difference between normal and osteoarthritic cartilage was seen in COMP synthesis (identified by metabolic labeling), where the lowest amount was found in articular cartilage with moderate lesions from the trotter. Also, no increase of sf- GAG from the metacarpophalangeal joints (van den Boom *et al*., 2004) and sf-COMP (unknown joints) (Misumi *et al*., 2001) of horses (unknown breed and athletic background) with OA is seen.

In contrast, an increase in COMP and aggrecan concentration is found in joints with rheumatoid arthritis, OA, reactive arthritis, and juvenile arthritis in man (Saxne & Heinegård, 1992). A distinct difference between horse and man is that the equine athletes will continue to expose the articular cartilage to a high load, while the human patient will not. A lower concentration of collagen type II was also found in the trotter compared to the riding horse; this was not correlated to the cartilage lesions or age. Lower hydroxyproline concentration in synovial fluid is correlated to cartilage degeneration index (CDI) in the metacarpophalangeal joints (van den Boom *et al*., 2004) and age. The difference in results can be explained by different joints and different classification and activity of cartilage lesions. Using the more precise CDI classification (Brommer *et al*., 2003b) may have showed a correlation with cartilage lesions and the lower collagen type II concentration of the trotters. The reported age difference is most significant between the neonates and 5-month-old foals. At the age of 1-4 years, the levels are stable throughout the

lifespan of the horses (van den Boom *et al*., 2004), which explains the lack of age correlation in our study.

In conclusion paper I showed that there is a difference between two breeds (with different activity profiles) in the articular cartilage metabolic activity of the middle carpal joint. The lower metabolic activity can be related to intensive high load training experienced by trotters. To test the hypothesis that strenuous training and lameness alter the articular cartilage metabolic activity and influence the release of COMP, aggrecan, and collagen type II in synovial fluid, a longitudinal study with young trotters in training was set up in paper II.

Young trotters were sampled every third month during a long-term training program (24 months). Due to economic limitations it was not possible to sample an age-matched non-exercising group. However, the correlations between the amount of training and lameness remains valid and important observations were made. The concentration of COMP decreased with increasing age (19.5 to 40 months) and total days of training. Also, the concentration of collagen type II degradation products increased with total days of training. Additionally, *in vitro* dynamic compression of cartilage explants from trained and untrained, age-matched horses showed a decrease of COMP synthesis in the compressed explants compared to the free swelling explants from trained horses. An increase of COMP synthesis was found in the explants from the untrained horses. Stimulation of COMP synthesis is always found when untrained bovine cartilage is compressed *in vitro* (Wong *et al*., 1999). Hence, it was concluded that the strenuous training with high load to the articular cartilage would alter the cartilage metabolism with a subsequent lower COMP synthesis.

To further evaluate the effect of load on the presence of COMP, an ultrastructural semi-quantitative delineation of the protein was done in loaded and unloaded areas of trained and untrained horses. The samples from the trained trotter displayed a very low amount of COMP, assessed by gold-markers, in both territorial and interterritorial matrix compartments from two different depths of the cartilage. The COMP concentration was much higher in the cartilage from the untrained horses, where often the loaded areas showed higher levels compared to unloaded areas, indicating a stimulatory effect of load. Riding horses were compared to trotters, since their activity profiles are different and the trotters are subjected to an earlier and more intensive training, aimed to qualify for fast speed racing at the age of 2 years. The galloping horse also is trained and raised at this early age. However, the difference in motion results in different mechanical load of the carpal joints with different types of fractures. The trotter experiences mostly large and deep fractures, while galloping horses develop small superficial fractures in the carpal joints (Schneider *et al*., 1988).

In paper IV, COMP and aggrecan concentrations in synovial fluids from acutely lame trotters and galloping horses, subjected to arthroscopy, were evaluated and a higher concentration of sf-COMP in galloping horses with osteochondral fractures compared with the trotters and galloping horses with OA was found. The articular cartilage of the unloaded osteochondral fragments expressed COMP mRNA, in contrast to the articular cartilage on the opposite side of the fracture, still subjected to load. Intact COMP was present in the joint with osteochondral fracture, which suggests an up-regulation of this protein where compressive loading of the fragment is low. Dynamic load can stimulate the chondrocyte to active synthesis.

The cells may respond to changes in cell shape (deformation), sensed by cytoskeletal proteins or stretch-activated ion-channels, or to changes in integrin- (Wu & Chen, 2000) and IL-4- signalling (Salter *et al*., 2002). A non-physiological high load may lead to cell necrosis or apoptosis and/or production of MMPs and cytokines. An increased expression of fibronectin fragments is present in OA (Zie *et al*., 1992; Homandberg *et al*., 1999) and these fragments induce the production of MMPs and cytokines (Homandberg *et al*., 1997). This can result in higher levels of protein fragments in the synovial fluid; though in a long-term period of high repetitive load, the cell may react with altered integrin composition and fatigue of the cell where the mechanical signal transducers are blocked. The decreased levels of proteins found in the present studies can be due to cell fatigue.

The results from this thesis clearly indicate that dynamic compression at high load and high frequency, as a result from strenuous training, has drastic effects on the metabolism of articular cartilage from the dorsal radial facet of the equine third carpal bones.

Development of new assays, quantifying fragments or detecting epitopes of matrix molecules specific for anabolic and catabolic processes will aid in the understanding of factors responsible for joint failure in the equine athletes. Also, studies focusing on cell responses, through integrins (receptors for specific proteins), during cyclic compression at different amplitudes and frequencies, can clarify how mechanical signals are transduced and how the chondrocyte will respond. Cell reaction will depend on the magnitude and rate of load as well as the duration and nature of the loading pattern (constant vs. intermittent).

Future Research

The results presented in this thesis indicate that much research into tissue reactions of mechanical load is needed. Also, the role of the interaction between bone and cartilage at their interface in OA must be clarified. In particular, the following areas should be investigated:

In cartilage, matrix molecules interacting with cell surface receptors and therefore of relevance in understanding cellular responses to events in the matrix should be one focus. An example of such a molecule is chondroadherin. Whether this integrin binding molecule is also involved in modulating responses to mechanical load is another issue of relevance. Other such molecules like fibronectin are less specific for cartilage, although there are specific splice variants. This protein binds a different integrin than chondroadherin. Molecules that appear to provide readout for mechanical responses include COMP and further studies into the role of this protein and its variability should be informative. Molecules like CILP change early in the OA-process and delineating its role should be a part of understanding the biology of joint diseases.

Studies of responses of cartilage to environmental factors, in attempts to understand the apparent altered responses induced by the excessive overload of intense training of racehorses, should be conducted. To control the conditions, initial studies should focus on *in vitro* systems, where cartilage explants can be loaded and details of the responses can be studied. A compression chamber (for unconfined compression) has been constructed, where different load and frequencies can be applied, mimicking the load to tissue when the horse is running at different speeds.

It is clear that the process in the joint include the bone where alterations of sclerosis are observed. Little is known of the molecular events, which represent early phenomenon. One molecule of particular interest is bone sialoprotein (BSP), which is particularly enriched at the bone part of the interface between cartilage and bone, with a role in early bone formation. This molecule is of particular interest since it can specifically bind to the integrin subunits $\alpha 5\beta 3$ and therefore involved in modulating cellular activities.
BSP will be analysed in synovial fluid and serum from 28 young trotters trained and raced during a long-term training period (paper II). The concentration of BSP will be correlated to parameters such as amount of training, lameness, age, bone sclerosis, and bone activity determined by radiographic and scintigraphic examination.

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