Inflammation in Equine Articular Cartilage

The Effect of Cytokines in Chondrocyte Pellets and Explants: Two *in vitro* Models

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Cover: Photomicrograph of a section of microscopically normal articular cartilage obtained from the third carpal bone of a 2-year-old horse. The section has been immunohistochemically stained for growth differentiation factor -5. Reprinted from *American Journal of Veterinary Research*, 2014;75(2), 132-140 by permission of the American Veterinary Medical Association.
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Inflammation in Equine Articular Cartilage. The Effect of Cytokines in Chondrocyte Pellets and Explants: Two *in vitro* Models

Abstract

Osteoarthritis (OA) is the main reason for lameness in racehorses. OA is currently clinically diagnosed late in the disease process, when irreversible damages to the articular cartilage have become evident. Diagnostic biomarkers that can detect the disease before irreversible tissue damage and prior to the onset of clinical signs would be very desirable. These biomarkers could be used to monitor the progression of articular cartilage destruction, repair, and inflammatory status. In order to develop biomarkers, it is necessary to further elucidate the pathogenesis of OA. The initiation and development of OA involves inflammatory processes mediated by pro-inflammatory cytokines. The objective of this thesis was to investigate the influence of cytokines, known to be involved in OA development, on equine articular chondrocytes *in vitro.* The aim was to increase the knowledge of the complex molecular mechanisms of the extracellular matrix (ECM), which may be responsible for the development, and progression of OA.

Healthy equine articular cartilage was stimulated with cytokines (interleukin (IL)-1β, HMGB-1, and IL-6) in two *in vitro* models (explants and three-dimensional pellet cultures). Analyses were performed by: immunohistochemistry, immunoassays (ELISAs, Western blot), biochemical assays (glycosaminoglycan content), quantification of gene expression, and quantitative proteomics. Additionally, synovial fluid collected from horses with healthy or OA joints was analysed with regard to content and glycosylation profile of lubricin.

Our studies showed that IL-1β induced a catabolic response in ECM-related genes and proteins. A time-dependent release of ECM proteins from equine explants was also detected. HMGB-1 stimulation of chondrocyte pellets indicated a promotion of chondrocyte differentiation or increased metabolic activity of chondrocytes. IL-6 stimulation of chondrocyte pellets inhibited the canonical Wnt-signalling pathway and upregulated the gene expression of growth differentiation factor (GDF)-5. The *O*-glycosylation profile of lubricin in synovial fluid was different for equine joints with OA compared to the

normal joints/controls. Additionally, an endogenous cleavage site of lubricin was found both *in vitro* and *in vivo*.

The results from this thesis confirm IL-1β as a master cytokine in equine articular cartilage destruction. Furthermore the results indicate that IL-6 has a regulatory or protective role on articular cartilage metabolism. The results from the *in vitro* studies of equine articular cartilage render novel findings regarding the detailed and time-dependent ECM protein release caused by cytokines involved in OA. This knowledge can be used for the development of diagnostic biomarkers of early OA *in vivo*.

Keywords: articular chondrocytes, *in vitro*, cytokines, osteoarthritis, horse, IL-1β, IL-6, inflammation, extra cellular cartilage matrix

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Dedication

In loving memory of my grandmother Linnéa.

Nog finns det mål och mening i vår färd -
men det är vägen, som är mödan värd.
Karin Boye

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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., Svala, E., Nilton, A., Lindahl, A., Eloranta, ML., Ekman, S. and Skiöldebrand, E. (2011). Effects of high mobility group box protein-1, interleukin-1β, and interleukin-6 on cartilage matrix metabolism in three-dimensional equine chondrocyte cultures. *Connective Tissue Research* 52(4), 290-300.

II Svala, E., Thorfve, A., Ley, C., Henriksson, HB., Synnergren, J., Lindahl, A., Ekman, S. and Skiöldebrand, E. (2014). Effects of interleukin-6 and interleukin-1β on expression of growth differentiation factor-5 and Wnt signaling pathway genes in equine chondrocytes. *American Journal of Veterinary Research* 75(2), 132-140.

III Svala, E., Löfgren, M., Sihlbom, C., Rüetschi, U., Lindahl, A., Ekman, S. and Skiöldebrand, E. (2014). An inflammatory equine model demonstrates dynamic changes of immune response and cartilage matrix molecule degradation *in vitro*. (Submitted)

IV Svala, E*., Jin, C*., Rüetschi, U., Ekman, S., Lindahl, A., Karlsson, N., and Skiöldebrand, E. (2014). Characterization of lubricin in synovial fluid from horses with osteoarthritis. (In manuscript)

Papers I-II are kindly reproduced with the permission of the publishers.

*Authors contributed equally to this work and should both be considered as main authors.

The contribution of ES to the papers included in this thesis was as follows:

I Second author. Active part in the planning and design of the study. Performed the *in vitro* chondrocyte pellet culture, gene expression analysis and immunoassays. Active in presenting data from these experiments, and active in the drafting and revising the manuscript.

II Main author. Active part in the formation of hypothesis and study design. Major responsibility for the planning and organization of the study. Performed the majority of the experiments and analyses, such as *in vitro* cultures, gene expression analyses and immunohistochemistry. Interpreted and summarized the microarray data. Performed the presentation of the data and statistical analyses. Drafted and edited the manuscript.

III Main author. Active part in the formation of hypothesis and study design. Major responsibility for the planning and organization of the study. Performed all experiments (*in vitro* cultures, immunoassays, biochemical analysis, Western Blot) except the mass spectrometry analyses. Interpreted and summarized the mass spectrometry data. Performed the presentation of the data and drafted/edited the manuscript.

IV Main author. Active part in the planning and design of the study, together with the second author. Performed the *in vitro* cultures. Interpreted and summarized the results. Active in presenting the data from these experiments and drafted/edited the manuscript

Abbreviations

3D	three dimensional
ADAMTs	a disintegrin and metalloproteinase with thrombospondin motifs
AgPAGE	agarose polyacrylamide composite gel
BSA	bovine serum albumin
C1, 2C	carboxy terminus neoepitope of the ¾ piece of collagen type I and II
C3	third carpal bone
cDNA	complementary DNA
COMP	cartilage oligomeric matrix protein
CS846	chondroitin sulphate 846
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DMEM/F12	Dulbecco’s modified eagle medium: nutrient mixture F-12
DRF	dorsal radial facet
ECM	extra cellular matrix
EDTA	ethylenediaminetetraacetic acid
GAG	glycosaminoglycan
Gal	galactose
GalNAcol	*N*-acetylgalactosaminitol
GDF-5	growth differentiation factor-5
GlcNAc	*N*-acetylglucosamine
GUSB	beta glucoronidase
HMGB-1	high mobility group box protein -1
HRP	horseradish peroxidase
IL	interleukin
LC/MS-MS	liquid chromatography-tandem mass spectrometry
LTQ	linear trap quadrupole
MCP	metacarpophalangeal
MMP	metalloproteinases

mRNA	messenger ribonucleic acid
NC4	N-terminal non-collagenous domain
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
OA	osteoarthritis
OCF	osteochondral fragment
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	palmar condyle
PEST	pencillin-streptomysin
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real time polymerase chain reaction
RA	rheumatoid arthritis
SDS	sodium dodecyl sulphate
SLRPs	small leucine rich proteins/proteoglycans
SOX-9	sex determing region on the Y chromosome–related high mobility group box-9
TGF-β	transforming growth factor beta
TMT	tandem mass tag
TNF-α	tumor necrosis factor –α

1 Introduction

1.1 General background

Osteoarthritis (OA) is a highly prevalent, chronic, and disabling disease (Johnson & Hunter, 2014) leading not only to articular cartilage destruction but also involving the entire joint and its structures: articular cartilage, subchondral bone, synovial membrane/capsule, and ligaments (Lane *et al.*, 2011). The full pathogenesis for OA is unknown but factors suggested to contribute to the development of the disease are joint injury, mechanical loading, age, obesity, and genetics (Johnson & Hunter, 2014). Inflammation is always involved (Sokolove & Lepus, 2013; Goldring & Goldring, 2007; Saxne *et al.*, 2003; Pelletier *et al.*, 2001).

It is estimated that 12% of the adult human population aged ≥ 18 years have symptomatic OA (Hunter *et al.*, 2014b) and its prevalence increases with age to encompass nearly 34% of those over 65 (Neogi, 2013). The joint most often affected by OA in humans is the knee, and the lifetime risk of developing the disease in this joint is as high as 45% (Neogi, 2013). Considering how many patients that never seek medical advice, the number is probably much higher. The individual burdens, to name a few, include pain, stiffness, and activity limitation leading to a lower quality of life. The socioeconomic cost that follows is tremendously high (Hunter *et al.*, 2014b). Currently there is no disease-modifying drug for structural progression of OA (Jotanovic *et al.*, 2014) and therefore the available treatments aim to relieve pain.

In horses, OA is a main reason for lameness (McIlwraith *et al.*, 2012). The disease can develop at a young age in racehorses and is also a naturally occurring disease in older horses (McIlwraith *et al.*, 2012; Bjornsdottir *et al.*, 2004). The metacarpophalangeal (MCP) (Neundorf *et al.*, 2010), followed by the carpal, are the most commonly OA-afflicted joints in horses (McIlwraith *et*

al., 2012). Racehorses develop OA at a young age due to the heavy and repetitive mechanical load from high-speed training and racing (Poole, 1996). Horseracing is a multibillion industry in Sweden and the economic importance of the horse sector is enormous. It has a direct and indirect total annual turnover of 46 billion Swedish crowns (Remmerth, 2008; Johansson & Andersson, 2004), greater than that for the metal and steel industries.

OA is currently clinically diagnosed late in the disease process, when irreversible damages to the cartilage have become evident. Therefore, there is a need for the development of reliable molecular markers (also called biomarkers) for the early stages of the disease. Optimal biomarkers could be used not only for early diagnosis but also for monitoring progression of the disease and evaluating treatment effects. To develop these biomarkers, the pathogenesis of OA needs to be further elucidated for an understanding of the complex molecular mechanisms responsible for the onset, development, and advancement of the disease.

The work included in this thesis focus on the changes of the extra cellular matrix in articular cartilage caused by inflammation.

1.2 Articular cartilage

Cartilage is a connective tissue consisting of chondrocytes, water, and the extra cellular matrix (ECM) synthesised by chondrocytes. Cartilage can be divided into hyaline cartilage, fibrocartilage, and elastic cartilage. This thesis examines the hyaline articular cartilage outlining the articular surface of bones.

Adult articular cartilage is an avascular, hypocellular, aneural, and alymphatic tissue with limited regenerative capacity (Iwamoto *et al.*, 2013). This complex structure, found in all synovial joints allows pain-free, low-friction joint movement and disperses high mechanical load. Articular cartilage is organized in different zones (Figure 1) on the basis of its morphological features (Pritzker & Aigner, 2010). The superficial zone is immediately subjacent to the joint surface and chondrocytes of this zone are elongated and flattened. Underneath the superficial zone is the middle zone where the morphology of chondrocytes changes to rounder cells. The deep zone is in between the middle zone and the calcified cartilage; here chondrocytes exist as round cells in columns. The boundary between uncalcified and calcified cartilage is called the tidemark and beneath the calcified cartilage is the borderline of subchondral bone called the cement line. The thickness of equine articular cartilage varies in different joints; the average thickness of the articular cartilage in the MCP and carpal joints is similar (0.86 mm) while the

stifle has a thicker articular cartilage (2 mm) (Lee *et al.*, 2014), comparable to that in a human knee (Frisbie *et al.*, 2006).

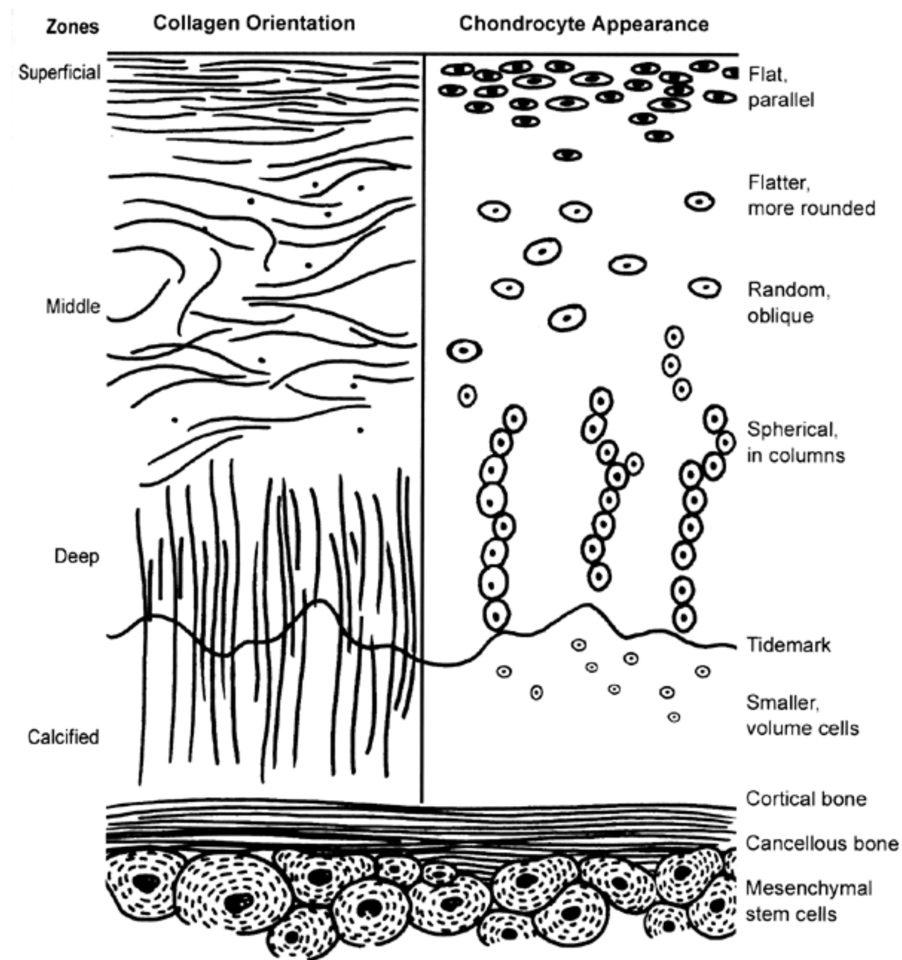

Figure 1. Morphological structure of articular cartilage, organized in superficial, middle, and deep zones. The orientation of the collagen fibres as well as chondrocyte morphology vary in these zones. Originally published in "Surgical Alternatives for Treatment of Articular Cartilage Lesions", *Journal of the American Academy of Orthopaedic Surgeons, (2000;8(3)-180-189),* by Browne, E. Jon and Branch, P. Thomas. Reused with permission and license from Lippincott Williams and Wilkins/Wolters Kluwer Health, Inc. © 2000, American Academy of Orthopaedic Surgeons.

1.2.1 Molecular organization of articular cartilage

Chondrocytes synthesize the ECM as well as produce enzymes responsible for degradation of the ECM. Water is the major constituent (approx. 75%) of ECM and the remainder is composed of several molecular components interacting to form the specialised network – collagens, proteoglycans and non-collagenous proteins. The ECM, which embeds the chondrocytes, is organized into zones based on their distance from the chondrocyte. Closest to the chondrocyte is the pericellular matrix, where molecules from the ECM bind to surface receptors. The territorial and interterritorial zones are found at a further distance from the chondrocyte (Las Heras *et al.*, 2012; Kvist *et al.*, 2008). The distribution of the components in the ECM varies within these zones (Heinegård & Saxne, 2011; Heinegård, 2009) (Figure 2).

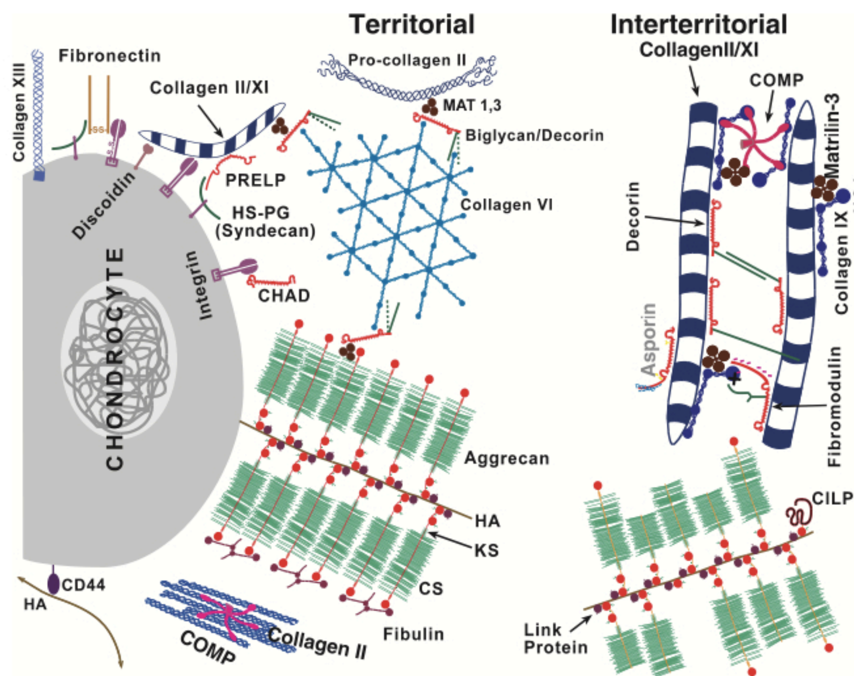

Figure 2. Illustration of molecular components and their organization in the extracellular matrix (ECM) of articular cartilage. The ECM surrounding chondrocytes is arranged into zones described by their distance to the cell. The interterritorial matrix closer to the cells and the interritorial matrix at a further distance from the cell are indicated. Originally published in “Proteoglycans and more - from molecules to biology”, *International Journal of Experimental Pathology (2009),90,575-586* by Heinegård, D. Reused with permission and license from John Wiley & Sons, Inc. ©Dick Heinegård © 2009 Blackwell Publishing Ltd.

Collagens

The collagen network gives cartilage its tensile strength and contributes to approximately 60% of the dry weight of articular cartilage. Articular cartilage comprises of collagen types II, III, VI, IX, XI and XII, and the organization of collagen varies in the different zones of the articular cartilage. In the superficial zone, collagens are arranged parallel to the articular joint surface, in contrast to the deep zone where the collagen fibres are organized perpendicular to the surface. In between these zones (middle zone), the collagen is organized in a random way (Figure 1). The superficial zone has the highest content of collagen and it decreases with the distance from the surface (Eyre *et al.*, 2006).

Collagen type II is the most common form of the collagen network in cartilage, representing approximately 90% of the total collagen content. Collagen type II, which has a fibrillar structure, is synthesized and secreted as procollagen α chains containing amino- and carboxy propeptides that are cleaved away by amino- and carboxy-terminal proteinases in the ECM (Peltonen *et al.*, 1985). Other enzymes, metalloproteinases, (MMPs), cleave the mature collagen type II molecule, leading to a decreased content of collagen type II (Poole *et al.*, 2002). In OA there is evidence of an attempt to repair damage by an increased synthesis (Nelson *et al.*, 1998) of collagen type II, but meanwhile there is also an increased degradation of collagen type II (Hollander *et al.*, 1994) mediated mainly by MMP -1 and -13 (Dahlberg *et al.*, 2000). Fragments resulting from the degradation of collagen type II have been shown to induce cartilage degradation by enhancing release of proteolytic enzymes (Guo *et al.*, 2009) and thereby increasing the cleavage of both collagen type II and aggrecan (Yasuda *et al.*, 2006).

Collagen type III is a trace component of articular cartilage, but can be increased in OA (Tanaka *et al.*, 2013; Eyre *et al.*, 2006).

Collagen type VI, primarily localized in the pericellular matrix (Zhang *et al.*, 2011b), is composed of α-chains (α-1, α-2, α-3) and forms a highly cross-linked microfibrillar network (Gelse *et al.*, 2003). This type of collagen has been found to interact with the chondrocyte by binding to integrins (Marcelino & McDevitt, 1995) of the cell membrane, as well as by interacting with other ECM molecules, such as collagen type II and small leucine-rich proteins/proteoglycans (SLRPs), decorin (Bidanset *et al.*, 1992), and biglycan (Wiberg *et al.*, 2002). Collagen type VI is postulated to strengthen the complete collagen network by binding to other members of the ECM. In OA, the turnover of collagen type VI is increased (Arican *et al.*, 1996) and there is evidence of alteration in the molecular distribution (Wilusz *et al.*, 2013) compared to normal cartilage. The increased concentration seen in the

interterritorial matrix could be the result of degradation of collagen type VI, leading to diffusion of fragments from the pericellular area (Soder *et al.*, 2002).

Collagen type IX can decorate collagen type II fibril surfaces by covalent binding (Vaughan *et al.*, 1988), suggesting that it has a role in improving mechanical restraint. It is hypothesized to act as a glue for the collagen type II network. Collagen type IX consists of three collagenous domains separated by four noncollagenous domains and the N-terminal noncollagenous domain (NC4) that projects out from the fibril surface can interact with other ECM molecules such as cartilage oligomeric matrix protein (COMP) (Holden *et al.*, 2001). *In vivo* studies of collagen IX null mouse cartilage have suggested that proteins such as matrilin-1, matrilin-4, epiphycan, and thrombospondin-4 interact with collagen type IX, (Brachvogel *et al.*, 2013). Degradation of collagen type IX is seen in the early stages of OA (Diab, 1993).

Collagen type XI can covalently crosslink to collagen type II and thereby support the collagen network, but the collagen type XI molecules can also crosslink to each other (Gelse *et al.*, 2003; Eyre, 2002). The importance of this type of collagen is suggested by the recent findings of single nucleotide polymorphism in the collagen type *XIA1* gene that is associated with OA (Rodriguez-Fontenla *et al.*, 2014).

Collagen type XII is a fibril-associated collagen with interrupted triple helices, which can connect to other components in the ECM and is thought to stabilize the organization of the collagen network (Gregory *et al.*, 2001).

Proteoglycans

Proteoglycans contribute to approximately 25-35% of the dry weight of articular cartilage and consist of a core protein with attached glycosaminoglycan (GAG) chains.

Aggrecan is the most common proteoglycan in articular cartilage. Through its negatively charged chondroitin sulphate chains, it has the potential to bind a large amount of water, resulting in a swelling and expansion of the ECM, and thereby enabling the cartilage to sustain high compressive load (Heinegård, 2009).

Aggrecan is a large (>2500 kDa), aggregating proteoglycan residing in the fibrillar collagen network. The superficial zone of articular cartilage has the lowest content of aggrecan and the content increases with the distance from the surface. The structure of the core protein in aggrecan consists of three globular domains called G1, G2 and G3. G1 has the ability to bind to link proteins or hyaluronic acid (Hascall & Heinegård, 1974), thereby producing multi-molecular aggregates. The region between G1 and G2 is called the interglobular domain, which contain sites subject to proteolytic cleavage

(Sandy *et al.*, 1991) by MMPs or aggrecanases (a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)), resulting in several protease-mediated catabolic epitopes (Struglics *et al.*, 2006). MMP-3 and -13, as well as other MMPs, cleave aggrecan in the interglobular domain between Asn 341 and Phe 342, (Flannery *et al.*, 1992) while ADAMTS-4 and -5 also cleave aggrecan in the interglobular domain, but between Glu 373 and Ala 374 (Tortorella *et al.*, 2002; Tortorella *et al.*, 2000b).

Between the G2 and G3 domains, glycosaminoglycan side chains can attach (first about 30 keratan sulphate chains followed by approximately 100 chondroitin sulphate chains). Moreover, aggrecan also contains a variable number of *O*- and *N*-linked oligosaccharides. There are several ADAMTS-4 and -5 mediated cleavage sites in between the G2 and G3 domains (Heinegård, 2009).

Non-collagenous matrix proteins

Non-collagenous proteins contribute to approximately 15-20% of the dry weight of articular cartilage.

The SLRPs family has 18 members to date, including: biglycan, chondroadherin, decorin, epiphycan, fibromodulin, lumican and proline/arginine-rich-end leucine-rich repeat (PRELP) found in cartilage. These horseshoe-shaped proteins have a molecular structure that consists of an N-terminal variable domain and a conserved domain of tandem leucine-rich repeats (Ni *et al.*, 2014). This distinctive structure benefits protein-protein interactions, enabling binding to various growth factors, cytokines, cell surface receptors, and other ECM components, thereby affecting various signalling pathways (Schaefer & Iozzo, 2008). SLRPs exhibit many biological roles; key features of SLRPs in joints are their involvement in tissue development and assembly. The function of SLRPs in collagen fibrillogenesis has been studied in developing mice tendons (Reed & Iozzo, 2002; Ezura *et al.*, 2000). SLRPs are also involved in modulation of fibril formation by interacting with collagens (Kalamajski & Oldberg, 2010), to construct a specific collagen matrix for a functioning ECM.

The involvement of SLRPs in the pathogenesis of OA is hypothesized to occur by several mechanisms. A loss of SLRPs can lead to undesirable changes in the ECM collagen network. The SLRPs can also modulate transforming growth factor (TGF)-β signalling and thereby affect proliferation/differentiation of chondrocytes, or they can interact with the innate immune system by activating or inhibiting the complement cascade. As an example, fibromodulin can bind to C1q and activate the classical component

system (Sjoberg *et al.*, 2005) while the binding of biglycan to C1q inhibits the activation of the classical complement system (Groeneveld *et al.*, 2005)

Matrilins, another type of noncollagenous proteins found in cartilage, are considered to be adaptor proteins, which support the ECM assembly by binding to aggrecan, SLRPs, and collagen fibrils (Klatt *et al.*, 2011).

COMP, also called thrombospondin-5, is a fundamental part of the ECM in articular cartilage. This 524 kDa multidomain glycoprotein consists of five identical units (Oldberg *et al.*, 1992) forming a pentameric structure (Figure 3). COMP can bind to collagen types I and II (Rosenberg *et al.*, 1998) and catalyze fibril formation by interacting with free collagen I and II molecules, bringing these molecules into close proximity with each other to promote further assembly (Halasz *et al.*, 2007). COMP can also interact and bind to collagen type IX (Holden *et al.*, 2001), aggrecan (Chen *et al.*, 2007), matrilins (Mann *et al.*, 2004) and fibronectin (Di Cesare *et al.*, 2002). COMP is found in other structures of the joint such as tendon (Sodersten *et al.*, 2013; Smith *et al.*, 1997). Mutations in COMP can lead to chondrodysplasias, leading to early onset OA and short-limb dwarfism (Briggs *et al.*, 1998). COMP is considered to be a marker of cartilage metabolism. It is studied as a potential diagnostic and prognostic biomarker for OA because serum levels of COMP are elevated in human OA (Valdes *et al.*, 2014; Verma & Dalal, 2013; Clark *et al.*, 1999). COMP has an active role in inflammation by activating the alternative pathway and inhibiting the lectin- and classical-complement pathways in rheumatoid arthritis (RA) patients but not in OA patients (Happonen *et al.*, 2010). This suggests that the specific and likely disease-dependent fragmentation of COMP activates or inhibits inflammation cascades.

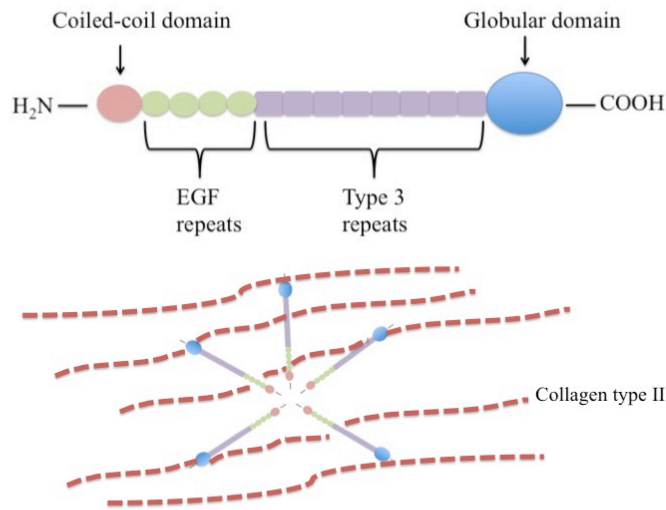

Figure 3. Schematic structure of one subunit of COMP and the interaction between pentameric COMP and collagen type II. COMP consists of five identical subunits, each with epidermal growth factor (EGF) repeats and type 3 repeats (calcium binding domains/calmodulin type units). The coiled-coil pentamerization domain is held together by disulphide bonds to form a pentameric structure. The function of the globular domain is to bind to other members of the ECM. The high-affinity binding between COMP (via the carboxyterminal domain) and native triple-helical collagen type II affects collagen fibril assembly. The COMP molecule can bind to five collagen molecules and facilitate their interaction in accelerating collagen fibril formation (Halasz *et al.*, 2007). COMP can also interact with other collagens of the ECM such as collagen IX, further stabilizing the collagen network. (Illustration by E.Svala)

The mucin-like glycoprotein lubricin, also known as proteoglycan-4, is found in synovial fluid where it acts as a boundary lubricator (Swann *et al.*, 1977) and protector (Rhee *et al.*, 2005b) of articular cartilage. The molecular structure of the protein consists of somatomedin B-like-domains at the N-terminus, followed by several mucin-like repeats which are heavily glycosylated. It ends with a hemopexin-domain at the C-terminus (Rhee *et al.*, 2005b). It is synthesized and secreted by the chondrocytes of the superficial zone of articular cartilage (Schumacher *et al.*, 1994), but is also found in several other structures of the joints such as tendons (Funakoshi *et al.*, 2008) and menisci (Zhang *et al.*, 2011a). The reduction of coefficient-of-friction is associated with inhibition of chondrocyte apoptosis (Waller *et al.*, 2013). Lubricin attaches to denaturated, amorphous and fibrillar collagen at the articular cartilage surface (Chang *et al.*, 2014). Lubricin can present various *O*-glycan structures, which are proposed not only to have a role in lubrication

(Jay *et al.*, 2001), but also in inflammation. They contribute to inflammation by carrying inflammatory oligosaccharide epitopes (Estrella *et al.*, 2010) and binding to peripheral and synovial polymorphonuclear granulocytes (Jin *et al.*, 2012).

1.3 Joint structure changes in osteoarthritis

In racehorses, the MCP joint is the one most commonly affected by OA, followed by the carpal joint (Figure 4) (McIlwraith *et al.*, 2012).
The equine MCP joint is a hinge joint consisting of the distal metacarpal bone III and the proximal phalanx bone (Fails & Kainer, 2011). The MCP has a close-fitting articular surface that can quickly develop linear erosions and wear lines in association with osteochondral fragmentation (McIlwraith *et al.*, 2012). This is possibly due to the small joint surface, wide range of motion, and weight transmission (Pool & Meagher, 1990).

The equine carpal joint is comprised of three joint compartments; the radiocarpal, midcarpal, and carpometacarpal. The middle carpal joint is overextended during the weight-bearing phase of the stride (Bramlage *et al.*, 1988) where it is exposed to high load, particularly on the radial facet of the third carpal bone. During trotting and galloping, it is exposed to forces in both longitudinal and transversal directions (Johnston *et al.*, 1997; Bramlage *et al.*, 1988). The dorsal radial facet (DRF) of the third carpal bone (C3) is a contact area with higher mechanical load compared to the palmar condyle (PC) of this bone which is a noncontact area (Palmer *et al.*, 1994) (Figure 4).

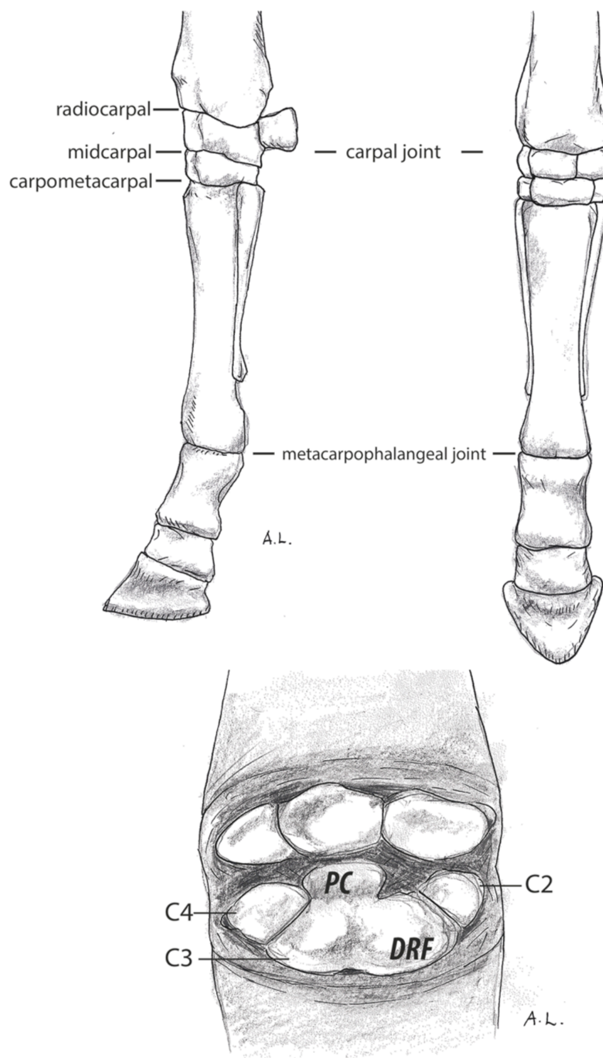

Figure 4. The location of the equine metacarpophalangeal (MCP) and carpal joints, which are the joints most commonly affected by OA (McIlwraith *et al.*, 2012). The dorsal radial facet (DRF) of the third carpal bone (C3) is a contact area with higher mechanical load compared to the palmar condyle (PC) of this bone which is a noncontact area (Palmer *et al.*, 1994). Drawings by Alexandra Leijon.

OA affects not only articular cartilage, but also the other components of the joint (McIlwraith *et al.*, 2010), where it leads to formation of subchondral bone

sclerosis (Grynpas *et al.*, 1991), formation of new bone at the joint margins (osteophyte formation) (Olive *et al.*, 2009), increased bone remodeling (for review see (Li *et al.*, 2013), and synovitis (Myers *et al.*, 1990). These joint tissue changes are similar among species, including man.

Chondrocyte necrosis with adjacent chondrocyte cluster formations (Lotz *et al.*, 2010), superficial fibrillation/fraying and cleft formations are seen in the pathological destruction of articular cartilage (McIlwraith *et al.*, 2010). The mechanism behind the increased number of cell clusters (Poole *et al.*, 1991), seen in OA cartilage, is thought to be proliferation (Pfander *et al.*, 2001) and/or migration (Kouri *et al.*, 1996). There is also a change of phenotype of chondrocytes in OA cartilage, resulting in hypertrophic cells (von der Mark *et al.*, 1992). This hypertrophy is characterized by collagen X and MMP-13 expression (Nurminskaya & Linsenmayer, 1996).

A hallmark of OA is the degradation of components in the ECM (Heinegård & Saxne, 2011). Normal articular cartilage has a low metabolic activity with a low-turnover rate of ECM, delicately regulated through anabolic (production of ECM molecules) and catabolic events (degradation of ECM) (Henrotin & Reginster, 1999). In early OA, there is an initial attempt at repair, reflected by an elevated anabolic activity of the chondrocytes, with cell proliferation and synthesis of ECM molecules and growth factors (Poole *et al.*, 2007). However this activity is later disrupted and overrun by the catabolic events, ultimately leading to a net loss of ECM.

Degradation of collagen type I, II and III molecules occurs through an initial cleavage into ¾- and ¼-length fragments by proteolytic enzymes called collagenases. MMP-13 is an important collagenase in the breakdown of collagen type II (Billinghurst *et al.*, 1997), but chondrocytes are capable of producing and secreting several other proteins in the MMP family. Chondrocytes of human OA cartilage produce MMP-1, -2, -3, -8, -9 and -13 (Tetlow *et al.*, 2001). The general MMP activity is increased in synovial fluid from equine OA joints (van den Boom *et al.*, 2005; Brama *et al.*, 2004; Brama *et al.*, 2000) and messenger ribonucleic acid (mRNA) gene expression of MMP-13 is elevated in synovial tissues and cartilage from equine OA joints compared to normal joints (Kamm *et al.*, 2010). *In vitro* studies have demonstrated this, since cytokine-stimulated bovine (Sondergaard *et al.*, 2006) and equine (Clutterbuck *et al.*, 2011; Clegg & Carter, 1999) articular cartilage cause an increase in the secretion of proteolytic enzymes (MMP-1, -2, -3, and -9). This in turn leads to the degradation and secretion of collagen and proteoglycan fragments into the cell media. Other proteolytic enzymes, ADAMTS-5 (Little *et al.*, 2007; Stanton *et al.*, 2005) and ADAMTS-4 (Tortorella *et al.*, 2000a), also have an important role in the degradation of

equine cartilage. Several *in vitro* studies of articular cartilage explants have revealed a distinct degradation pattern due to inflammatory stimuli, where aggrecan is released early, followed by COMP, fibromodulin and finally, collagen type II (Williams *et al.*, 2013; Heathfield *et al.*, 2004; Dickinson *et al.*, 2003; Sztrolovics *et al.*, 1999).

1.3.1 Osteochondral fragments

Osteochondral fragments (OCFs), also known as chip fractures, are found at the dorsal aspects of the articular margins and facets of bones in the carpal joints and the proximal aspect of the first phalanx. They are thought to arise from fractured osteophytes, degenerated articular cartilage or necrotic subchondral bone in racehorses with OA (Pool & Meagher, 1990). The metabolic activity in the OCFs of the carpal joints appears elevated, as indicated by increased levels of native COMP in synovial fluid from racehorses with OCFs (Arai *et al.*, 2008; Skiöldebrand *et al.*, 2005) and an increased synthesis of aggrecan in serum and synovial fluid (Frisbie *et al.*, 1999) . COMP synthesis is also seen in the chondrocytes, by *in situ* hybridisation, in these OCFs (Skiöldebrand *et al.*, 2005). High levels of interleukin (IL)-6, but not TNF-α are found in the synovial fluid of carpal joints with OCFs (Ley *et al.*, 2007), with the chondrocytes being the potential source of IL-6 (Ley *et al.*, 2009).

1.4 Factors involved in the development of OA

OA is thought to be a multifactorial disease with mechanical loading being one of the factors that promotes it. Normal healthy cartilage has specialized biomechanical properties which allow it to spread force between joints while providing nearly friction-free movement (Kerin *et al.*, 2002). Excessive mechanical load or a major traumatic event alters the physiological biomechanical environment by activating factors involved in the pathogenesis of OA. These factors include inflammatory mediators and proteolytic enzymes, which induce ECM degradation and chondrocyte death (Buckwalter *et al.*, 2013). The extensive training that racehorses undergo provokes metabolic changes in the composition of the ECM, with decreased synthesis and release of COMP (Skiöldebrand *et al.*, 2006). These changes are seen in *in vitro* studies where static compression stimulates a degradation of proteoglycans and collagens in bovine cartilage (Loening *et al.*, 2000) and decreases the synthesis of cartilage matrix proteins. On the other hand, dynamic compression increases the synthetic activity of chondrocytes (Tsuang *et al.*, 2008; Kim *et al.*, 1994)

OA can occur early in equine athletes; however, it can also develop later in older horses (McIlwraith *et al.*, 2012). A prominent risk factor associated with OA is aging; the prevalence of human OA also rises with age. Age-related changes of ECM are degradation of collagen with an increased fibrillation of the articular surface (Hollander *et al.*, 1995) and extensive heterogeneity in the molecular structure of the ECM (Dudhia, 2005). Furthermore, the chondrocyte anabolic activity decreases with age (Aigner *et al.*, 2007). Nevertheless, since not all older horses/humans develop OA, the link between OA and ageing is not fully elucidated.

Another risk factor associated with OA is genetics. Proposed susceptibility candidate genes for human OA are linked to ECM or signalling molecules thereof, e.g., RUNX2, SMAD3 and growth differentiation factor-5 (GDF-5) (Reynard & Loughlin, 2013). There is emerging evidence of a link between obesity and the initiation and progression of human OA with possible contributions from both increased mechanical forces across joints, leading to cartilage destruction, and from systemic factors such as altered adipokine levels (Poonpet & Honsawek, 2014; Zhou *et al.*, 2014; Spector *et al.*, 1994).

1.5 Inflammation

Inflammation is strongly associated with the pathophysiology of both human (Saxne *et al.*, 2003; Pelletier *et al.*, 2001) and equine OA (Kamm *et al.*, 2010); a diversity of inflammatory mediators, such as cytokines, control the inflammatory process. Inflammatory signs seen in the development and progression of OA are clinical local signs such as joint pain, swelling, stiffness, an increased level of C-reactive protein (Jin *et al.*, 2013; Pearle *et al.*, 2007; Sharif *et al.*, 2000), and synovitis (Sellam & Berenbaum, 2010; Sutton *et al.*, 2009) with increased synovial mononuclear cell infiltration (Benito *et al.*, 2005). An inflamed synovial membrane can produce various inflammatory mediators, such as IL-1β and tumor necrosis factor (TNF)-α; the chondrocytes respond by producing proteolytic enzymes. However, OA chondrocytes also produce and secrete several inflammatory mediators themselves including: IL-β, TNF-α, (Tetlow *et al.*, 2001), IL-6 (Ley *et al.*, 2009), IL-7 (Long *et al.*, 2008), IL-18 (Olee *et al.*, 1999), and HMGB-1 (Terada *et al.*, 2011). The inflammatory cytokines are the most important compounds participating in the pathogenesis of OA; their involvement disrupts the normal metabolic homoeostasis of cartilage by favouring catabolic processes, with MMP-activation leading to destruction of cartilage (Wojdasiewicz *et al.*, 2014).

1.5.1 Interleukin-1β and Tumor necrosis factor-α

IL-1β and TNF-α are often regarded as the master cytokines involved in the pathogenesis of OA. IL-1β is synthesized in a pro-form (pro-IL-1β) that is cleaved by casphase-1 (Piccioli & Rubartelli, 2013) to generate an active form before it is secreted by chondrocytes and synovial cells into the joint cavity. Pro-IL-1β can also be found extracellular where it can be cleaved by serine proteases (Wittmann *et al.*, 2011).

Enhanced levels of IL-1β are found in cartilage, bone, synovial fluid and synovial membrane in human OA (Massicotte *et al.*, 2002; Melchiorri *et al.*, 1998; Kubota *et al.*, 1997; Farahat *et al.*, 1993), as well as in articular cartilage (Kamm *et al.*, 2010) and synovial fluid (Morris *et al.*, 1990) in equine OA joints. Increased levels of the membrane bound receptor, IL-1 receptor type I, which activates the intracellular signalling cascade upon binding to IL-1β, is found in human OA chondrocytes (Silvestri *et al.*, 2006). The IL-1 receptor accessory protein is also necessary for signalling (Casadio *et al.*, 2001). IL-1β can also bind to a decoy receptor called IL-1 receptor type II; however, this binding does not generate active signalling, and the abundance of this receptor is reduced in human OA chondrocytes (Wang *et al.*, 2003). Another counterproductive molecule, the receptor antagonist IL-1Ra, can bind to both receptors of IL-1β to inhibit the binding of IL-1β and subsequent signalling. In an established equine OA model, gene transfer of IL-1Ra displays favourable effects with upregulation of IL-1Ra protein expression, improvement in pain and disease activity, and positive effects on histologic parameters in the articular cartilage (Frisbie & McIlwraith, 2000).

There are several effects of active IL-1β signalling in cartilage. The activation of numerous signal transduction pathways leads to an increase in intracellular Ca^{2+}, with activation of protein kinase C, p38, c-JUN N-terminal kinase, and extracellular signal regulated kinase -1 and -2. Active IL-1β signalling also leads to nuclear translocation of nuclear factor-κB (NF-κB), activating transcription factor, and activator protein 1 (Daheshia & Yao, 2008). Activation of these transcription factors results in expression of genes leading to the production of MMPs and ADAMTs (Tetlow *et al.*, 2001), prostaglandin, and nitric oxide. There is also an induction of the production of other cytokines such as IL-6 and to an even greater degree IL-1β, suggesting an autocrine loop (Fan *et al.*, 2007).

Taken together, the effects of IL-1β on chondrocytes induce the expression of catabolic genes. The effects also include downregulation of the expression of anabolic genes involved in the metabolism of chondrocytes and induction of chondrocyte apoptosis (Wojdasiewicz *et al.*, 2014; Daheshia & Yao, 2008).

TNF-α is synthesized as a homotrimeric tramsmembrane protein type II that needs to be cleaved by metallopeptidase TNF-α converting enzyme (also called ADAM-17), for activation (Black *et al.*, 1997). The secretion and presence of TNF-α in human OA is very similar to that of IL-1β, as they are secreted by the same cells in the joint and are elevated in human OA cartilage, subchondral bone, synovial membrane, and synovial fluid (Wojdasiewicz *et al.*, 2014). This suggests that there is a synergistic interaction between them (Henderson & Pettipher, 1989). The concentration of TNF-α is increased in synovial tissue and articular cartilage of equine OA (Kamm *et al.*, 2010). Other studies report that TNF-α concentrations in synovial fluid are not related to equine OA (Ley *et al.*, 2007; Jouglin *et al.*, 2000) suggesting that TNF-α is not a master cytokine in the disease. TNF-α can bind to two receptors, called TNFR-1 and TNFR-II, (Westacott *et al.*, 1994) and active TNF-α signalling can inhibit proteoglycan (Saklatvala, 1986) and collagen synthesis (Seguin & Bernier, 2003) as well as produce MMPs (Wojdasiewicz *et al.*, 2014).

1.5.2 Interleukin-6

IL-1β induces a massive production of IL-6 (Nietfeld *et al.*, 1990). Although IL-6 is called a pro-inflammatory cytokine, this is somewhat misleading because IL-6 has several anti-inflammatory, protective effects such as inhibiting the synthesis of IL-1β and TNF-α and enhancing the production of cortisol (Mölne & Wold, 2007). Classical signalling acts through binding of the membrane-bound IL-6 receptor. The membrane-bound IL-6 receptor system consists of two membrane proteins: a ligand-binding chain (IL-6R) and a membrane-bound β-receptor glycoprotein, gp130. Binding of IL-6 to IL-6R triggers the association of IL-6R and gp130, and gp130 in turn transduces the signal (Kishimoto, 1992). IL-6 can also bind to another receptor, the soluble IL-6 receptor, and the signalling through this receptor is called trans-signalling. In trans-signalling, IL-6 binds to soluble forms of the IL-6R (sIL-6R) and this complex activate cells due to the uniform expression of gp130 (Wolf *et al.*, 2014). A protective role for IL-6 against the pathogenesis of OA has been suggested because IL-6 gene-knockout mice develop more advanced osteoarthritis (de Hooge *et al.*, 2005). Also, high levels of IL-6 have been detected in synovial fluid in canine OA (Venn *et al.*, 1993) and in equine OA synovium (Ley *et al.*, 2009).

1.5.3 High-mobility group box protein-1 (HMGB-1)

HMGB-1 is a 25 kDa chromosomal protein, consisting of two basic boxes (called A and B) responsible for DNA binding, and a highly acidic C-terminus

(Zhang & Wang, 2010). The protein has different functions depending on the intracellular or extracellular location. In the nucleus it facilitates binding of regulatory proteins, with a role as a minor-groove binding enhancer (Lotze & Tracey, 2005). Extracellularly, HMGB-1 acts as a danger-associated molecular pattern (DAMP) molecule, released by necrotic cells or secreted in response to inflammatory stimuli, thereby mediating activation of innate immune response (Castiglioni *et al.*, 2011). HMGB-1 is expressed in synovial tissue from RA patients and from rats with experimental arthritis (Kokkola *et al.*, 2002). HMGB-1 levels in synovial fluid of knee-OA patients are associated with radiographic disease severity (Li *et al.*, 2011) and HMGB-1 is overexpressed in human (Garcia-Arnandis *et al.*, 2010) and equine OA synovium (Ley *et al.*, 2009). Additionally *in vitro* stimulation with IL-1β upregulates the mRNA gene expression of HMGB-1 and promotes the translocation of HMGB-1 from nuclei to cytoplasm in human OA chondrocytes (Terada *et al.*, 2011).

2 Aims of the thesis

The overall aim of the thesis was to contribute to knowledge about the influence of inflammatory cytokines on equine articular cartilage.

The specific objectives of this thesis were to:

1) Establish a three-dimensional (3D) culture system (pellets) for equine chondrocytes as an *in vitro* model for OA.

2) Evaluate and compare the effect of the cytokines interleukin (IL) –1β, IL-6 and HMGB-1 on equine articular chondrocytes cultured in an *in vitro* 3D model.

3) Examine if IL-6 could induce an upregulation of GDF-5 in equine chondrocytes and to identify the possible signalling pathways.

4) Investigate the presence of GDF-5 expressing chondrocytes in normal equine articular cartilage and OCFs.

5) Investigate the secretome of equine articular cartilage explants stimulated with IL-1β and cultured *in vitro* with the aim of describing the longitudinal release pattern of ECM molecules.

6) Study the presence and glycosylation profile of lubricin in synovial fluid from horses with OA and normal joints and to examine the secretion pattern of lubricin, after IL-1β stimulation, in equine articular cartilage explants *in vitro*.

Two different *in vitro* models were used with the purpose of mimicking OA *in vivo*. Chondrocyte pellets and explants derived from healthy equine articular cartilage were stimulated with cytokines to induce an inflammation and the analyses were done by: immunohistochemistry, immunoassays (ELISAs, Western blot), biochemical assays (glycosaminoglycan content), quantification of gene expression, and quantitative proteomics.

3 Hypotheses

Hypotheses for the studies on equine chondrocyte pellets *in vitro* were:

- Paper I: Chondrocyte metabolism is affected by exposure to IL-1β, IL-6 and HMGB-1. The response can be different in chondrocytes cultured from differently loaded anatomical sites *in vivo* from the equine third carpal bone.

- Paper II: IL-6 induces an upregulation of GDF-5 in equine OCFs explaining the anabolic processes found in the fragments *in vivo*. Expression of GDF-5 is affected by short and long term stimulation of IL-1β and IL-6 in chondrocytes from the third carpal bone.

Hypotheses for studies on equine cartilage explants *in vitro* were:

- Paper III: Molecular changes in equine cartilage after IL-1β stimulation are time dependent.

- Paper IV: Inflammation in OA influences lubricating properties by altering the level and glycosylation profile of lubricin in equine articular cartilage.

4 Material and methods

This section summarizes the material and methods of the studies for this thesis. Detailed descriptions of the procedures are presented in each of the papers.

4.1 Animals and sample collection (papers I-IV)

Macroscopically normal articular cartilage was aseptically obtained from the DRF (paper I and II) and PC (paper I) of the C3 from euthanized horses without any clinical history of joint disease. Additional osteochondral fragments (OCF) (dorsoproximally of the radial facet of C3, dorsodistally of the radial carpal bone, or dorsoproximally of the first phalanx) were used from three horses that underwent arthroscopy because of lameness (paper II). Full-thickness articular cartilage explants from the weight-bearing part of the distal metacarpal bone III in the MCP joint from three slaughtered horses without any clinical history of joint disease, were used in papers III-IV. All collected samples were transported chilled to the cell culture lab in a sterile saline solution (0.9% sodium chloride) with 50 mg/l gentamin sulphate and 250 µg/ml amphotericin B.

Synovial fluid (paper IV) was aseptically collected from middle carpal joints with macroscopically normal articular cartilage (n=7), structural OA lesions (n=7), and OCF (n=3).

4.2 Isolation and expansion of chondrocytes (papers I-II)

Protocols for isolation and expansion of chondrocytes from articular cartilage were performed according to previously described procedures (Brittberg *et al.*, 1994). In summary, equine articular cartilage shavings were washed with cell culture media and mechanically minced with a scalpel. Enzymatic digestion with 0.8 mg/ml collagenase type 2 was performed for 20-24 h at 37°C in 7%

CO_2/93% air, and a viable count of the single cell solution was performed with 0.4% tryptan blue dye in a Bürker chamber. Cells were seeded into culture flasks at a density of 16,000 cells/cm^2 in complete chondrocyte media (Dulbecco's Modified Eagle medium, i.e., nutrient mixture F-12 (DMEM/F12) supplemented with 1x penicillin-streptomycin (PEST), 2 mM L-glutamine, 0.1 mg/ml ascorbic acid and 10% equine serum) and incubated at 37°C in 7% CO_2/93% air.

At approximately 80% confluence, the chondrocytes were treated with 0.05 % trypsin with 1x ethylenediaminetetraacetic acid (EDTA) at 37°C in 7% CO_2/93 % air until the cells detached from the cell culture flasks. Chondrocytes were seeded into sterile polystyrene cell-culture flasks at a density of 3000 cells/cm^2 in complete chondrocyte media at 37°C in 7% CO_2/93 % air until 80% confluence. Medium was changed twice a week.

4.3 *In vitro* chondrocyte culture models (papers I,II,III,IV)

4.3.1 Three-dimensional chondrocyte pellet cultures (papers I-II)

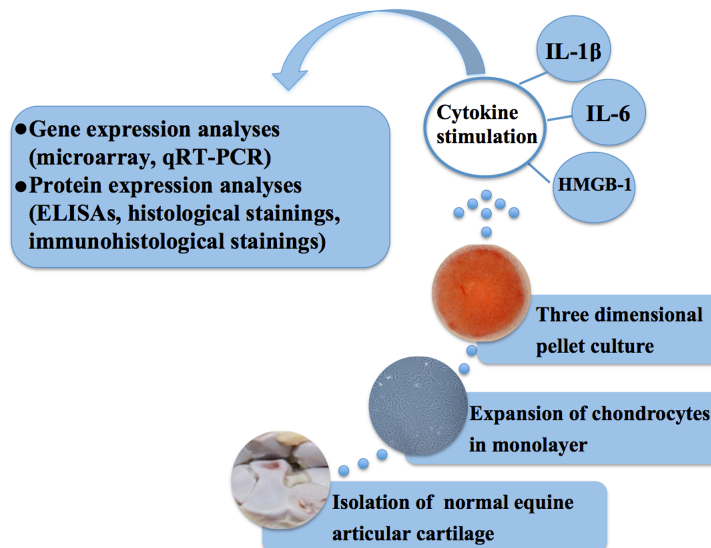

Figure 5. Experimental setup for three-dimensional (3D) pellets cultured *in vitro.* Normal equine articular cartilage was harvested from the third carpal bone in the middle carpal joint. Chondrocytes were isolated and expanded in monolayer until 80% confluence. Chondrocytes were cultured in 3D pellets (200,000 cells/pellet) for 14 days before cytokine stimulation for 1, 2, 12 or 48 hours. Cell media and 3D pellets were analysed for gene and protein expression. Illustration by E.Svala.

The protocol for 3D chondrocyte pellet cultures was slightly modified from previously described procedures (Stenhamre *et al.*, 2008; Tallheden *et al.*, 2004). Expanded chondrocytes from the first subculture (passage 1) were cultured in 3D pellets in a 96-well ultralow attachment plate, for 14 days at 37°C in 7% CO_2/93% air. Pellet medium was changed daily after 3D chondrocyte pellet formation was established (Figure 5).

4.3.2 Explant cultures (paper III-IV)

Cartilage explants (5 mm in diameter) were allocated to groups of 4 explants/well into polystyrene plates. The explants were pre-incubated before cytokine stimulation for 24 h, in 2 ml DMEM/F12 supplemented with 0.1 mg/ml cell-culture-tested bovine serum albumin (BSA), 0.1 mg/ml ascorbic acid and 4% v/v PEST at 37°C in 7% CO_2/93% air (Figure 6).

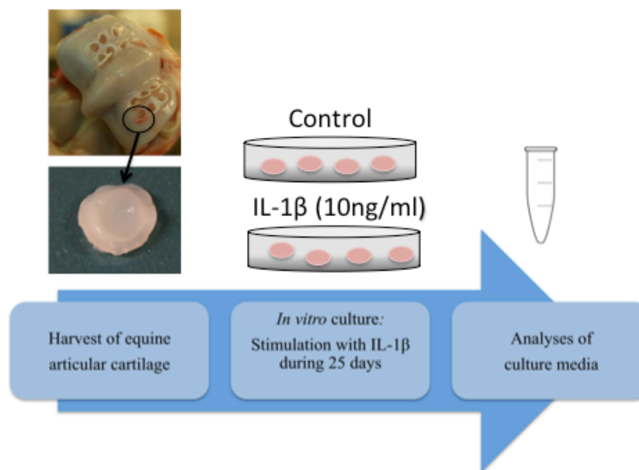

Figure 6. Methodological setup for *in vitro* culture of equine articular cartilage explants. Macroscopically normal cartilage from three horses was harvested from the weight-bearing part of the distal metacarpal bone III in the metacarpophalangeal joints. Cartilage explants (5 mm in diameter) were cultured in culture dishes with or without IL-1β during 25 days. Culture medium was changed and harvested every third day. Analyses performed on cell culture media were: quantitative proteomics, GAG, Western Blot (for COMP and Lubricin) and Elisa (for MMP-13 , C1, 2C and Lubricin). At day 25, the cartilage explants were harvested for histological evaluation (hematoxylin and eosin (H&E), toluidine blue and safranin O). Illustration by E.Svala.

4.3.3 Cytokine stimulation (paper I-IV)

At day 14 of chondrocyte culture, the pellets (papers I, II) were allocated with a randomized procedure to treatment groups and either stimulated with

recombinant equine IL-1β (5 ng/ml), recombinant equine IL-6 (5 ng/ml), recombinant human HMGB-1 (1 μg/ml) or left unstimulated. Medium change was performed daily and pellets were stimulated for 1, 2, 12 or 48 hours.

After the pre-incubation of cartilage explants (papers III, IV) for 24 h, the medium was harvested and the explants were cultured in 2 ml medium in the presence or absence of 10 ng/ml equine recombinant IL-1β (10 ng/ml) at 37°C in 7% CO2/93 % air. The medium was changed and harvested at 3, 6, 9, 12, 15, 18, 22 and 25 days and immediately frozen at -80°C for later analysis.

Pellets or explants were harvested and washed in phosphate buffered saline (PBS) or snap frozen in liquid nitrogen before storage at -80°C or fixed in buffered formalin.

4.4 Histological methods (papers I,II,III)

4.4.1 Fixation

Chondrocyte pellets were fixed in 6% v/v neutral buffered formalin, for a minimum of 24 h. Normal articular cartilage, OCFs, and cartilage explants were immersed in 10 % v/v neutral-buffered formalin and OCFs were decalcified in formic acid before embedment.

4.4.2 Histological stainings (papers I,II,III)

Sections from central parts of pellets or explants, normal articular cartilage and OCFs were dehydrated, embedded in paraffin blocks, cut into 3-6 μm thick sections, and stained with hematoxylin and eosin (H&E), safranin-O or Massons trichrome. A minimum of two sections/samples was evaluated.

4.4.3 Immunohistochemical stainings (paper I-II)

Sections were deparaffinised, rehydrated and immersed in 0.1% v/v detergent solution (GDF-5 in paper II) or immersed in 3% v/v hydrogen peroxide to quench endogenous peroxidase activity. Samples were then incubated with hyaluronidase (COMP in paper I) or subjected to heat-mediated antigen retrieval steps (GSK3β and β-catenin staining in paper II).

Subsequently, non-specific antibody binding was blocked using species-appropriate serum or BSA before incubation with primary antibodies (rabbit-anti-bovine COMP, polyclonal goat GDF-5, rabbit polyclonal GSK3β, mouse monoclonal nuclear-dephosphorylated β-catenin) at 4°C overnight. Washing was performed with PBS or 0.1% v/v detergent solution and an additional blocking were performed before incubation with secondary antibodies at room temperature. Antibodies were either conjugated with horseradish-peroxidase (HRP) (for GSK3β and β-catenin), biotinylated (for COMP) or labelled with

photostable orange fluorescent dye (for GDF-5). Avidin–biotin–peroxidase complexes were visualized using 3,3´diaminobenzidine. HRP conjugated antibodies were detected with a signal amplification kit using fluorophore labelling. Nuclei were stained with the fluorescent dye 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI), or counterstained with Mayer's hematoxylin. Immunostaining was qualitatively assessed using light microscopy or fluorescent microscopy with appropriate filters. Isotype control antibodies, which have no relevant specificity, were used to distinguish non-specific background binding from antigen-specific antibody binding.

4.5 Gene expression analyses

4.5.1 RNA isolation and quantification (papers I,II)

Frozen chondrocyte pellets were disrupted through high-speed shaking for 8 min in 2 ml plastic tubes with tungsten carbide beads. Lysis reagent was added to each sample and the shaking was performed again for 2 min. The homogenate was incubated at 5 min at room temperature before adding chloroform, then shaken vigorously for 15 sec. Samples were incubated at room temperature for 3 min and thereafter centrifuged for 15 min at 4°C. The upper aqueous phase of the sample was transferred into a new sample tube and total RNA was further purified using a commercially available kit and a fully automated sample preparation robot, in accordance with the manufacturer's protocol for animal cells. DNase was added to the isolated total RNA. After quantity and quality assessments, the recovered total RNA was stored at -80°C.

4.5.2 Complementary DNA synthesis

A commercially available reverse-transcription kit was used to prepare complementary DNA (cDNA) from 100 ng of total RNA from each chondrocyte pellet sample. A thermal cycler program of 25°C (10 min), 37°C (120 min), 85°C (5 min), 4°C (∞) was performed and the cDNA (concentration 5 ng/µl) was stored at -20°C.

4.5.3 Quantitative real time polymerase chain reaction (qRT-PCR)

The polymerase chain reaction (Saiki *et al.*, 1985) theoretically amplifies DNA exponentially, doubling the number of target molecules with each amplification cycle. The thermal cycler protocol for the qRT-PCR reaction starts by activating the enzyme uracil-DNA glycosylase at 50°C for 2 min followed by activation of DNA polymerase at 95°C for 10 minutes.
After these initial steps the qRT-PCR consists of 40 cycles of the following steps:

1) Denaturation (95°C for 10 seconds): Double-stranded DNA denatures to single-stranded by disrupting hydrogen bonds between complementary bases.
2) Annealing/extension (60°C for 1 minute): The primers anneal (hybridize) to the complementary DNA sequences. After annealing, DNA polymerase synthesizes a new DNA strand with deoxyribonucleotide triphosphates (dNTPs). The new strand will be complementary to the DNA template strand.

The amount of DNA is measured after each cycle by attaching the template to a fluorescent probe (consisting of an oligonucleotide with a fluorophore (6-carboxyfluorescein FAM)) at the 5′end and a quencher (dihydrocyclopyrroloindole tripeptide minor groove binder, MGB) at the 3′end. As long as the fluorophore and the quencher are in proximity of each other by being attached to the probe, the reporter dye is quenched. The DNA polymerase will cleave this reporter dye off the probe (Holland *et al.*, 1991) during each extension. This increases the fluorescent signal in direct proportion to the number of the number of PCR products (also called amplicons) generated. The qRT-PCR reagent solution contains a passive reference dye (ROX), used to normalize the reporter fluorescence.

qRT-PCR protocol

The quantitative real-time polymerase chain reaction (qRT-PCR) was analysed on 2.5 ng cDNA/sample in duplicate with a solution of polymerase, primers, probes, nucleotides and buffer in a 96-well reaction plate. A negative control without any sample was used to detect contaminating DNA from the reagents. The relative gene expression was determined by the $2^{-(\Delta\Delta CT)}$ method (Livak & Schmittgen, 2001).

Primers and probes

A commercially available qRT-PCR assay mix of primers and probes intended for the detection of human genes was used for detection of *GDF-5*, Sry-related high-mobility group box 9 (*SOX-9*) and *collagen type II*. A basic local alignment search tool was used to verify homology between the human and equine genes. A database and genome browser were used to select equine-specific primer and probe sequences for *collagen type I*, *versican*, *aggrecan, COMP*, *MMP-9*, *MMP-13*, *ADAMTS-5*, *TIMP-1*, and beta glucuronidase (*GUSB*). A validation experiment was performed for each equine-specific primer and probe set with a dilution series of cDNA template for the target

genes. Several potential reference genes were validated (unpublished data) and equine *GUSB* was used as a reference gene.

4.5.4 Microarray (paper II)

High-throughput gene expression microarrays have been developed during the last twenty years and the technology is based on the ability of DNA (on an array) to specifically bind/hybridize to complementary sequences (RNA or DNA target molecules) from samples (Forster *et al.*, 2003). In paper II we used Affymetrix® human gene 1.1 ST microarrays on which millions of short strands (approximately 25 bases) of DNA (called oligonucleotide probes) are built onto a glass chip, one base at a time. A solution of RNA or DNA from samples, labelled with biotin, and a fluorescent stain is distributed over the array. When RNA or DNA from the samples binds to the probe on the array this results in a fluorescence signal; screening for this hybridization shows if a gene is expressed. Highly expressed genes give stronger fluorescence signals than genes with lower expression.

At the time of the experiments in paper II, no equine specific arrays were commercially available. Human microarrays had previously been used to study the transcriptome of equine chondrocytes in monolayer (Graham *et al.*, 2010) and therefore a similar approach was used in paper II.

Total RNA (250 ng) from chondrocyte pellets was processed and analysed with a human microarray for global transcriptome quantification at the Nottingham Arabidopsis Stock Centre (University of Nottingham, United Kingdom). Briefly, target preparation from total RNA used T7 primers in the cDNA synthesis step, followed by *in vitro* transcription to produce cRNA. Random primers were then used to synthesize cDNA from the cRNA. The cDNA was fragmented, biotin-labeled, and mixed into a hybridization cocktail before application to the array. The high-throughput array was scanned (Genechip 3000 7G scanner) and expression signals were extracted and normalized by applying the Robust Multichip Average (RMA) normalization method. A web-based probe-match tool was used to compare equine nucleotide sequences against individual probes on the human array, with the purpose of confirming that the human probe sets matched the equine query sequence of the corresponding gene. The data set was used to screen for novel putative target genes that displayed a change in expression when IL-6 stimulated samples were compared to their unstimulated controls. The data were only analysed on the gene-level and not on the exon-level.

4.6 Biochemical analyses (paper III)

4.6.1 Measurement of glycosaminoglycans.

Dimethylmethylene blue assay was used to estimate the proteoglycan content by quantification of sulphated GAGs (Farndale *et al.*, 1986). Samples of 20 µl (cell culture media from explant cultures), standards, and controls were added to a 96-well microtiter plate. 100 ul of reagent solution (16 mg dimethylmetylene blue, 2,5 ml 99% v/v ethanol, 2 g sodium formate, 2 ml formic acid, 500 ml ultrapure water) was added to all wells. Spectrophotometric analysis was immediately performed at an absorbance wavelength of 530 nm. Chondroitin sulphate was used as a standard (50, 40, 30, 20, 10, 5, 2.5 µg/ml) as well as an internal control (4, 10, 20 µg /ml); water was used as a blank control. All samples were analysed in duplicate and mean values were used for calculating ratio-to-cartilage explant wet weight.

4.7 Protein expression analyses (papers III-IV)

4.7.1 Proteomic analyses

Figure 7. shows the workflow for the quantitative proteomics analysis. Tandem mass tags (TMTs) are small chemical molecules that covalently attach to the free amino termini of lysine residues, thereby labelling various peptides in a sample. All TMTs have the same mass but internally they are different (Figure 8). This results in a single MS peak, but when fragmented (in MS/MS mode), the reporter group falls off, producing a unique reporter ion signature that can be used for relative quantification of the samples.

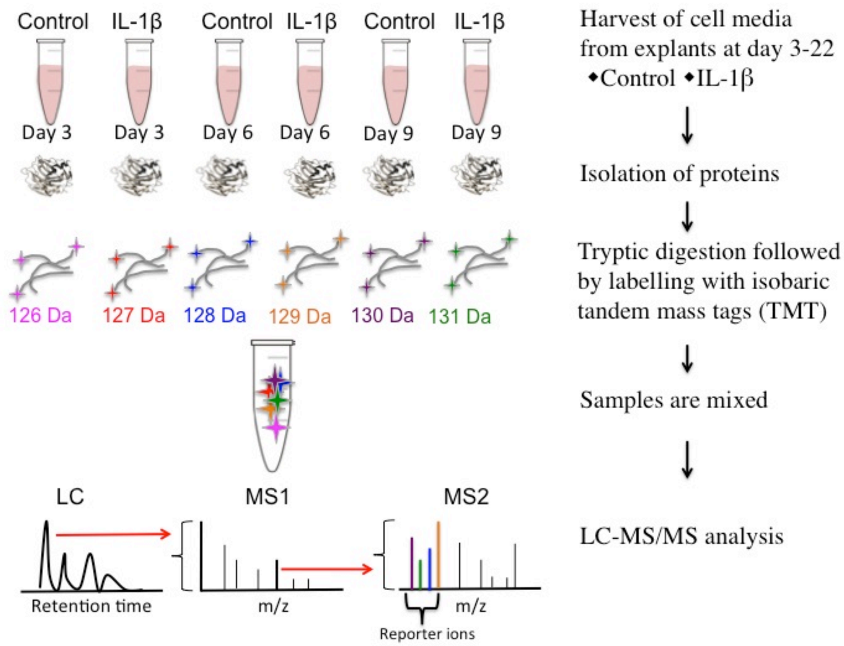

Figure 7. Schematic workflow for quantitative proteomics analyses of the secretome of equine cartilage explants. Cell culture media were harvested from control and interleukin (IL)-1β stimulated explants. Proteins were isolated before tryptic digestion. Tandem mass tag (TMT) reagents 126,127,128,129, 130 and 131 were added to the samples and mixed before liquid chromatography-tandem mass spectrometry (LC-MS/MS). For a quantitative comparative analysis of the secreted proteins from the IL-1β-stimulated and unstimulated explants, an aliquot from each time point was used as a reference pool. Illustration by E.Svala.

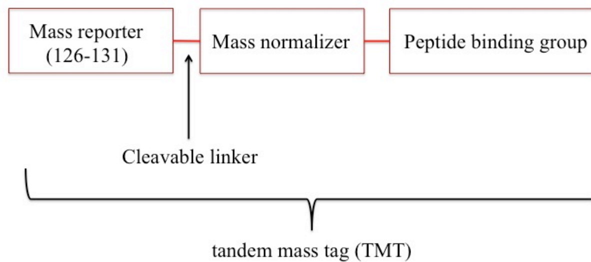

Figure 8. Simplified structure of a tandem mass tag. The peptide-binding group covalently attaches to the free amino termini of lysine residues. The mass reporter consists of one of the following isobaric TMT reagents 126,127,128,129, 130 or 131. The mass normalizer is a balance group for the mass reporter, with the result that all tags have the same mass. During MS/MS mode the tags fragments, and subsequently the mass reporter, cleave off at the cleavable linker site, making relative quantification possible. Illustration by E.Svala

Sample preparation

Culture media, harvested at days 3-22, (excluding days 0 and 25), were concentrated on 3 kDa molecular weight cut-off filters. The retentates were washed with water and triethyl ammonium bicarbonate until a pH value >8 was achieved. Sodium dodecyl sulphate (SDS) solution was added to the retentates and the proteins were reduced and alkylated before digestion with trypsin overnight at 37°C. In parallel, reference pools consisting of an aliquot from each sample were prepared as described above.

Labelling of culture media with isobaric mass tags

Samples were labeled with TMT reagents (according to the manufacturer's protocol), combined and concentrated, yielding three sets of samples (see experimental setup in paper III). TMT-labelled peptides were separated by strong-cation exchange chromatography (SCX) on an ÄKTA purifier system. The concentrated peptides were acidified, diluted and injected onto a polySULFOETHYL A™ SCX column. During separation, ultraviolet absorbance was monitored and peptide-containing fractions were evaporated and desalted.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

The desalted and dried fractions were reconstituted and analysed on a linear trap quadrupole (LTQ)-Orbitrap™-Velos mass spectrometer interfaced with a nano-LC column constructed inhouse. The peptides were trapped on a pre-column and separated on a reversed-phase column packed inhouse with 3-µm Reprosil-Pur C18-AQ particles. The LTQ-Orbitrap™ Velos was operated in data-dependent mode with a single MS1 fourier-transform mass-spectrometer precursor-ion scan. This step was followed by collision-induced and high-energy collision dissociation MS2 scans of the five most abundant doubly or triply protonated ions in each precursor scan. All samples were analysed a second time with an exclusion list of all m/z values corresponding to peptides identified at 1% false discovery rate from the first MS-analysis.

Database search and TMT quantification

MS raw data files from all 15−20 peptides containing strong-cation exchange fractions within a single TMT set were merged for protein identification and relative quantification using Proteome Discoverer version 1.3. Database searches were performed using the Mascot search engine against the ENSEMBL horse protein and Uniref100 mammalian protein databases. For specific parameters see paper III. The detected protein threshold in the software was set to a confidence level corresponding to a false discovery rate

of 1% at the peptide level. TMT reporter ion intensities in MS/MS spectra were divided by their reference reporter ion intensities, yielding a ratio for each quantified peptide. A protein ratio was calculated as the median value for all unique peptides for a given protein. No normalisation was applied, and the albumin ratio was used to validate the variation in sample preparations.

Characterization of endogenous proteolytic peptides of lubricin in synovial fluid

Synovial fluid from one horse with normal articular cartilage and from one horse with an OCF was individually depleted from high abundant serum proteins by the multiple affinity removal system according to the manufacturer's instructions. Synovial fluid was diluted with MARS buffer A and injected onto the column. Absorbance was recorded at 280 nm and fractions with absorbance levels above background were collected. The collected fractions, approximately 2.5 ml each, were pooled and concentrated to <50 µl using 3kDa cut-off ultrafiltration. Protein separation was performed on NuPAGE. Following Coomassie blue staining each gel lane was cut into 15 equally sized pieces that were individually digested by trypsin following the protocol previously described (Stenberg *et al.*, 2013). Each fraction was individually analyzed by nanoLC-MS/MS on an Ultimate 3000 separation unit coupled in-line to an QExactive mass spectrometer. The resulting data files were merged into two data sets representing the two horses. The proteins were identified by performing searches using the parameters described in detail in paper IV.

4.7.2 Western Blot

COMP

For COMP detection, aliquots (12 µl) of each medium harvested between days 0−25 from explants originating from a single horse, were mixed with electrophoresis buffer (0.125 M Tris-HCl, 2% w/v SDS, 0.002% w/v bromophenol blue, 20% v/v glycerol) in the absence of 2-mercaptoethanol. The samples were applied to a 4-20% w/v SDS-polyacrylamide gel (PAGE) and run according to the Laemmli protocol (Laemmli, 1970). After electrophoresis, the proteins were electrotransferred onto a nitrocellulose membrane in Tris-glycine buffer (25 mM Tris-HCl, 192 mM glycine, pH 8.3, 20% v/v methanol) at 120 V for 2 h. Following the transfer, the membranes were blocked with 5% w/v skimmed milk in Tris-buffered saline pH 7.4 with 0.05% v/v Tween 20 (TBS/T). Antigenic COMP was detected with an anti-bovine polyclonal

antibody (1:2000, kindly provided by Professor Dick Heinegård) in 5% w/v skimmed milk in TBS/T. Membranes were washed with TBS/T prior to incubation with the secondary antibody, goat anti-rabbit IgG-HRP. Immunoreactive bands were detected using chemiluminescence detection reagents.

Lubricin

For lubricin detection, 20 µl of collected medium was separated by gradient SDS-AgPAGE after reduction with 10 mM dithiothreitol and alkylation with 25 mM iodoacetamide. Proteins were blotted onto a PVDF membrane using a semi-dry method, as previously described (Schulz *et al.*, 2002). After blocking with 1% w/v BSA in TBS/T, horse lubricin was detected with monoclonal mouse anti-human lubricin and HRP-conjugated rabbit anti-mouse immunogoblulins. After washing, immobilized antibodies were visualized by an ultrasensitive enhanced chemiluminescent substrate for low-femtogram-level detection.

4.7.3 ELISAs

Chondroitin sulphate 846 (CS846)

To monitor aggrecan synthesis, a commercially available ELISA assay was used for the detection of CS846 in culture media from pellets (stimulated and unstimulated). The antibody has a strong cross-reactivity for horse. The assay was performed according to the manufacturer's protocol, although cell culture medium was used instead of serum. Analyses were performed on duplicate samples (diluted 1:300) and the lower detection limit was 20 ng/ml.

MMP-13

A commercially available ELISA assay was used for the detection of active MMP-13 in culture media from pellets and explants (stimulated and unstimulated). Culture medium was diluted 1:10 for pellets and 1:50-1:100 for explants. Adding p-aminophenylmercuric acetate (APMA) to the samples activated the pro-forms (by removal of the C-terminal domain) but not the latent or mature forms bound to their inhibitors, TIMPs. The assay was performed according to the manufacturer's protocol. Analyses were performed on duplicate samples and the lower detection limit was 8 pg/ml.

C1, 2C

A commercially available ELISA assay was used for the detection of the carboxy terminus neoepitope of the ¾ piece (C1, 2C) (Billinghurst *et al.*, 2000;

Billinghurst *et al.*, 1997). This piece is generated by the cleavages of type I and type II collagen by collagenases. The antibodies in the assay, which have a strong cross-reactivity with horse, recognize an α-chain fragment containing an approximately 8-amino acid sequence on the carboxy terminus of the ¾ length piece. This fragment is produced by collagenase (MMP-1, MMP-8 and MMP-13) cleavage of type II collagen. These antibodies show affinity for similarly cleaved human type I collagen α-chains. The assay was performed according to the manufacturer's protocol, except that cell culture medium was used instead of serum. Analyses were performed on duplicate samples of culture medium from explants (diluted 1:2 or 1:4) and the lower detection limit was 10 µg/ml.

Lubricin

25 µL of synovial fluid or cell culture media from explants were coated on 96-well microtiter plates in 0.1 M sodium carbonate buffer, pH 9.5. After washing and blocking with 1% w/v BSA in TBS-T buffer, bound lubricin was detected by mAb13 and HRP-conjugated rabbit anti-mouse immunoglobulins. Tetramethyl benzidine buffer was used as substrate for 20 min before a stop solution was added (1M H_2SO_4). Color development was measurement at 450 nm.

4.8 Glycomic analysis

Acidic proteins in horse synovial fluids were purified as previously described for the human acidic proteins (Jin *et al.*, 2012). Reduced and alkylated acidic proteins were separated by SDS-agarose polyacrylamide composite gel (AgPAGE) and blotted onto a polyvinylidene fluoride (PVDF) membrane. Alcian blue-stained protein bands, corresponding to the size of lubricin, were excised and subjected to reductive β-elimination. Released *O*-linked oligosaccharides were desalted and dried for capillary graphitized-carbon LC-MS and LC-MS/MS in negative-ion mode using an LTQ Ion Trap (Jin *et al.*, 2012; Schulz *et al.*, 2002). Oligosaccharides were identified from their MS2 spectra using the UniCarb-DB (2013 version) (Hayes *et al.*, 2011) and validated manually.

4.9 Statistics

All statistical analyses were performed with software; values of $P < 0.05$ were considered significant.

In paper I, the statistical model ANOVA was used to analyze the difference in chondrocyte pellet gene expression as well as the concentrations of MMP-13

and CS846 in the culture medium. The statistical model included the fixed effects of animal, site, stimulation, and interactions between site and stimulation. Pairwise comparison of least square means between sites and between cytokine-stimulated and untreated pellets was tested for significance.

In paper II, least square mean data were analysed by means of 2-way ANOVA with the variables time, stimulation, and the interaction between time and stimulation included in the model. Time was considered as a categorical variable to determine the stimulation time that would be important for changes in GDF-5 mRNA expression. Significant variables identified in the 2-way ANOVA were analysed with a multiple-comparisons procedure (Holm-Sidak method) to further identify the groups with significant differences within the variable. To evaluate the effect of cytokine stimulation, logarithmic gene expression values for 1- to 2-h (short-term) and 12- to 48-h (long-term) stimulations were compared with values for unstimulated chondrocytes using a pairwise *t*-test. Due to the low transcriptional effect and the limited number of animals, statistical tests were not performed for the microarray analyses in paper II.

For paper III no statistical analyses was performed due to the limited number of animals in the study.

In paper IV, all quantitative data from the glycomic analysis of synovial fluid were presented as mean± standard deviation (SD). Two-way ANOVA (multiple comparisons) were performed to compare synovial fluid from normal joints and joints with OA or OCF.

5 Results

A summary of the main results follows; please see the individual papers for details.

5.1 Paper I

The effects of cytokine stimulation (IL-1β, IL-6 and HMGB-1) on chondrocyte metabolism in chondrocyte pellets were evaluated with gene expression analyses, ELISA assays, and immunohistochemistry. For the chondrocyte pellet culture, chondrocytes from either the noncontact area of the less-loaded PC or the high-loaded area of DRF were used to evaluate the effect of gene expression in anatomical sites exposed to different loading *in vivo*.

There was a significant increase in mRNA gene expression of *versican* ($P<0.0001$), *MMP-9* ($P=0.0137$) and *TIMP-1* ($P=0.0059$) in response to IL-1β stimulation compared to unstimulated controls. In contrast, there was a significant decrease in *aggrecan* ($P<0.0001$), *collagen type I* ($P=0.0077$), and *collagen type II* ($P=0.0082$) gene expression in the IL-1β stimulated chondrocyte pellets compared to unstimulated controls. *MMP-13* and *ADAMTS-5* gene expressions were also clearly increased in IL-1β-stimulated pellets, but since the residual was not normally distributed, a test of significance was not performed. A trend towards decreased COMP expression ($P=0.0543$) was also detected. Response to cytokines rarely differed between DRF and PC chondrocyte pellets.

Additionally, IL-1β stimulated pellets resulted in a decreased safranin O staining and an increased COMP immunostaining. Media from IL-1β-stimulated pellets showed the lowest concentrations of aggrecan epitope CS 846; however, the results were not statistically significant. Concentrations of active MMP-13 were markedly increased from 0.28 ng/ml in control media, to

62.5 ng/ml in IL-1β-stimulated pellets from DRF; the value in the control medium was 0.03 ng/ml and 53.4 ng/ml in IL-1β-stimulated pellets from PC.

The effects of IL-6 and HMGB-1 on gene expression varied in different horses. There was an indication of a slight anabolic effect due to the upregulatory effect on *SOX-9*, which was seen by both cytokines, but it was only statistically significant for HMGB-1-stimulation (P=0.0120). The COMP immunostaining and safranin O staining, in IL-6- and HMGB-1-stimulated pellets, were similar to the staining in the control.

A notable difference was seen between the unstimulated chondrocyte pellets of DRF and PC in the three youngest horses (1-, -2 and 3-years old). A relatively higher expression of *SOX-9*, *aggrecan*, and *collagen type II* was evident in the PC, which is a less-loaded area *in vivo* compared to the highly-loaded area of DRF. There was a higher *versican* and *collagen type I* gene expression found in DRF compared to PC.

5.2 Paper II

The presence of GDF-5 in normal articular cartilage and OCFs was evaluated with immunohistochemistry. The effects of cytokine stimulation (IL-1β and IL-6) on chondrocyte pellets were evaluated with immunohistochemistry and gene expression analyses.

Macroscopically normal articular cartilage showed prominent cellular and pericellular staining of GDF-5 in the superficial and upper-third of the middle zones of the articular cartilage. GDF-5 expression was localized to the chondrocytes and their pericellular matrix in the middle and deep parts of the articular cartilage from the OCF samples.

Significantly (P = 0.041) lower gene expression of *GDF-5* was detected in IL-1β stimulated pellets compared to the unstimulated chondrocyte pellets. Furthermore, a significant (P = 0.002) difference was detected in mean *GDF-5* expression between chondrocyte pellets stimulated with IL-1β and those stimulated with IL-6, with the higher expression being in the IL-6-stimulated chondrocyte pellets.

When analyzing the effect of cytokine stimulation at the individual time points, IL-6 stimulation for 2 h showed a significant (P = 0.007) upregulation of *GDF-5* mRNA expression versus unstimulated chondrocyte pellets. Significance was also found between IL-6 stimulated and IL-1β stimulated chondrocyte pellets at 48 h, with a higher expression in the IL-6 group (P = 0.011).

The global transcriptional analysis (microarray) for chondrocyte pellets stimulated with or without IL-6 for 1h or 2 h, identified 59 and 21 genes, respectively, regulated by IL-6 (mean fold change in expression > 1.5).

IL-6 stimulation resulted in an induction of gene expression for a negative regulator of the canonical Wnt-signalling pathway, called *CCDC88C*, after 1 h of stimulation. A screen of the genes in the canonical Wnt-signalling pathway identified 23 affected genes in the IL-6 stimulated pellets compared to controls. IL-6 stimulation resulted in an increase of several inhibitors and negative regulators of the canonical Wnt-signalling pathway and a decreased expression of receptor and ligands (summarized in figure 9).

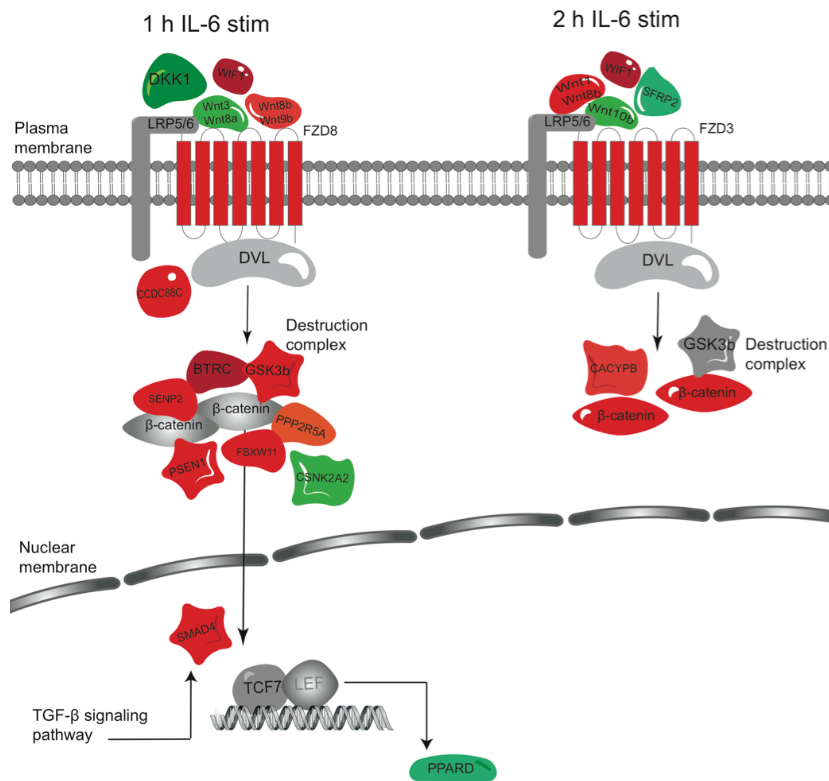

Figure 9. Illustration showing a summary of the results from the microarray analysis of genes in the canonical Wnt pathway, based on their known function in combination with the altered gene expression by IL-6 stimulation (1, 2 h) in paper II. The regulated expression of genes by IL-6 marked red leads to an inhibited canonical pathway while genes marked green leads to an activation of the canonical Wnt pathway. As an example, *DKK1*, which is an extracellular negative regulator of the canonical pathway, was downregulated by IL-6 at 1 h, indicating an activation of the pathway, and therefore marked green. The key activator, *β-catenin*, was downregulated at 2h by IL-6, indicating an inhibited pathway, therefore marked red. llustration by Anna Thorfve.

Immunohistochemistry was used to evaluate the expression of nuclear dephosphorylated β-catenin and GSK3β in chondrocyte pellets stimulated with IL-6 or IL-1β (for 1 h). Results showed prominent nuclear dephosphorylated β-catenin staining in chondrocyte pellets stimulated with IL-1β, but only a few cells stained for β-catenin in chondrocyte pellets stimulated with IL-6. This result was similar to findings for unstimulated chondrocyte pellets. Cellular immunostaining of GSK3β was detected in a diffuse pattern in IL-6-stimulated chondrocyte pellets; only a few cells had GSK3β staining in IL-β-stimulated chondrocyte pellets, again similar to findings for unstimulated chondrocyte pellets. The results suggest that the inhibition of the canonical Wnt-signalling pathway is caused by IL-6.

5.3 Paper III

The secretome of equine articular cartilage explants cultured in the presence or absence IL-1β was studied with quantitative proteomics, ELISA assays, and Western Blot during three-day intervals for a total of 25 days.

The content of GAG was highest at day 3 in media from both stimulated and unstimulated cartilage explants. The concentration was higher (1.5-2.9 times) in the medium from IL-1β stimulated cartilage explants at all time points except at day 15 where it had the same concentration as the control. Intact COMP was observed by Western Blot analysis in media from unstimulated cartilage explants at each time point, while five distinct bands (correlated to the calculated mass of monomeric to pentameric COMP) were observed at each time point in the media from IL-1β-stimulated cartilage explants.

The concentration of C1, 2C peptide in media from IL-1β-stimulated cartilage explants increased from day 15 to day 25 (4.1 μg/ml) whereas in the unstimulated cartilage explants, it was low throughout the culture period (highest concentration 0.04 μg/ml). The concentration of activated MMP-13 was first detected at day 3 (544 ng/ml) in media from IL-1β-stimulated cartilage explants. Concentrations during the culturing period remained high until a distinguished decrease on day 22. Very low concentrations of active MMP-13 were detected in media from unstimulated cartilage explants; the highest concentration was detected at day 22 (100 ng/ml).

The quantitative proteomics identified a total of 126 proteins in media. IL-1β stimulation resulted in an abundance of proteins related to inflammation, including MMP-1, -3, -13 which were higher in media from IL-1β-stimulated than unstimulated cartilage explants at any given time point. Acute phase

proteins (SAA), complement components (complement C1s, complement factor C1r, complement factor B), and IL-6 were also identified.

A time-dependent degradation of the molecular components of the ECM was evident in media from cartilage explants stimulated with IL-1β (Figure 10). ECM molecules were released at different time points, with a high levels in IL-1β-stimulated cartilage explants at days 3-6 for aggrecan, COMP, chondroadherin, osteomodulin, lubricin (proteoglycan-4), PCOLCE, hyaluronan and proteoglycan link protein-1, lumican, thrombospondin-1, epiphycan, and collagen type XI-α1. A semitryptic peptide originating from the G1 and G2 region of aggrecan was identified close to the previously described MMP cleavage site PEN↓FFG, supporting endogenous processing.

High levels of ECM molecules from IL-1β-stimulated cartilage explants at day 9-12 were found for collagen type XII, XI-α2 and at days 15-22 for collagen type-II, -VI, -IX, fibronectin, biglycan, CILP, fibromodulin and decorin. Lumican, fibronectin, CILP, fibromodulin, and decorin displayed a diphasic release in the media from IL-1β-stimulated cartilage explants, with two peak levels at early (days 3 and 6) and late (day 22) time points.

The procollagen type II C-terminal propeptide was identified as a semitryptic peptide in markedly higher levels in media from unstimulated explants than in the media from IL-1β-stimulated explants. A high level of procollagen C endopeptidase enhancer (PCOLCE) — a glycoprotein that binds to newly synthesised procollagen molecules and catalyzes the enzymatic cleavage of the C-terminal propeptide—was identified in the media of unstimulated explants.

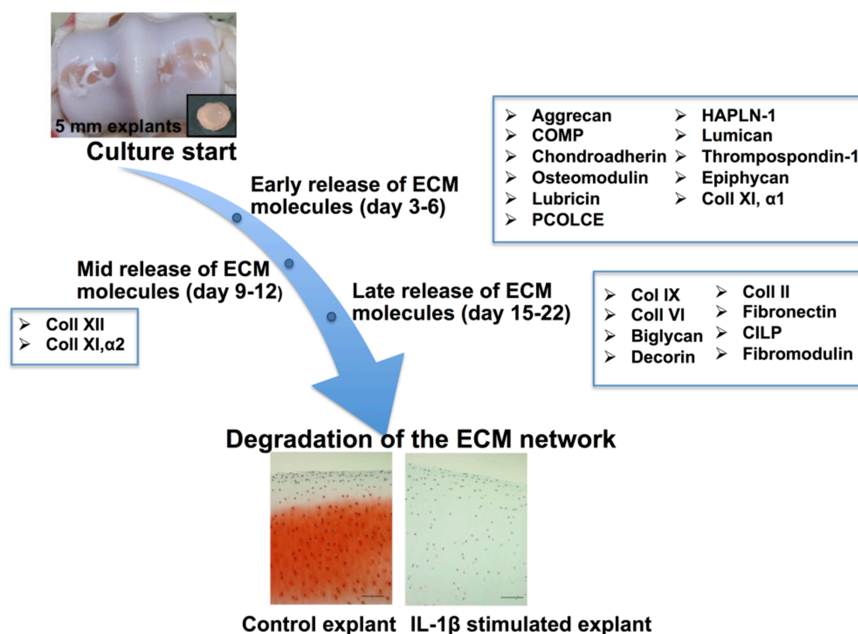

Figure 10. Time-dependent release of ECM molecules from cartilage explants into the medium. The pro-inflammatory cytokine IL-1β was used to induce degradation of equine articular cartilage explants *in vitro* with the purpose of mimicking OA *in vivo.* The release of ECM related proteins at different time points was detected with quantitative proteomics. Fragmentation of aggrecan and COMP was detected already at days 3-6 while the degradation of the collagen network was observed at days 18-22, similar to OA progression *in vivo*. At the end of the culture period, depletion of GAGs was seen in the IL-1β stimulated explant (safranin O staining). Histological pictures by Maria Löfgren. Illustration by E.Svala.

5.4 Paper IV

The secretion and glycosylation pattern of lubricin in synovial fluid from joints with normal articular cartilage, with structural OA lesions and with OCF, was studied with proteomics, glycomics, and Western Blot. Lubricin levels in media and extract of cartilage explants stimulated with or without IL-1β were also studied with quantitative proteomics, ELISA assays, and Western Blot.

Both core 1 [Galβ1-3GalNAcol] and 2 *O*-glycans [Galβ1-3(GlcNAcβ1-6)GalNAcol] were identified on equine synovial lubricin; however, core 1 *O*-glycans were predominant in all purified lubricin (synovial fluids from joints with normal cartilage, structural OA lesions and OCF) (Figure 11).

$[M\text{-}nH]^{n-}$	Normal	Fracture	OA
384^{-}	10.1±1.9	11.1±4.5	12.5±5.1
675^{-}	1.3±1.0	1.7±1.3	2.9±3.6
675^{-}	49.5±1.9	53.8±7.8	57.3±8.8
691^{-}	1.9±0.9	2.2±1.1	2.1±1.2
966^{-}	26.7±6.7	23.0±5.0	21.2±4.9
982^{-}	1.3±0.6	1.4±0.5	0.6±0.6
1040^{-}	1.4±1.5	0.8±0.9	0.5±0.2
1040^{-}	1.6±1.6	1.1±1.0	0.8±0.6
1331^{-}	3.3±1.8	4.1±3.5	2.2±2.1
705^{2-}	1.8±1.9	0.8±1.1	ND
847^{2-}	1.1±2.4	ND	ND

Figure 11. *O*-glycan profiles of equine synovial lubricin isolated from equine joints with normal articular cartilage, osteochondral fractures, and structural OA lesions. All observed mass was indicated as $[M\text{-}nH]^{n-}$. 10-15% of core 1 *O*-glycans were neutral *O*-glycans (*m/z* 384) while more than half of core 1 *O*-glycans were mono-sialylated (*m/z* 675 and 691) and 20-30% of core 1 *O*-glycans were disialylated (*m/z* 966 and 982). Less than 10% of total *O*-glycan was core 2 O-glycans. All of them were sialylated with disialylated as predominant structures (m/z 1331, 705^{2-} and 847^{2-}) and monolylated as minor ones (*m/z* 1040). One sulphated *O*-glycan (m/z 7052-) was detected in both normal and OCF samples. *O*-glycan structures are depicted using Consortium for Functional Glycomics (CGF) symbol nomenclature. Symbol represents: yellow circle, galactose; yellow square, *N*-acetyl galactosamine; blue square, *N*-acetyl glucosamine; purple diamond, *N*-acetyl neuraminic acid; shallow purple diamond, *N*-glycolyl neuraminic acid.
Illustration by Chunsheng Jin.

The majority of core 1 *O*-glycans were sialylated (mainly Neu5Ac and less extent of Neu5Gc), and the most common form was mono-sialylated core 1 *O*-glycans (60% of all core 1 *O*-glycans), followed by di-sialylated (20-30%) and neutral (10%) core 1 *O*-glycans. Mono-sialylated core 1 *O*-glycans consist of two type *O*-glycans, Neu5Ac(Gc)α2-3Galβ1-3GalNAcol (linear structure; *m/z* 675 and 691 in Figure 11) and Galβ1-3(Neu5Acα2-6)GalNAcol (branched structure; *m/z* 675 in Figure 11). The linear is predominant in a range of 51-59% (of all *O*-glycan); branched, however, is minor only in a range of 1-3%.

Significant differences were found for the mono-sialylated core 1 *O*-glycans from the synovial fluid of normal joints compared to that of joints with OA (P=0.0283) or OCFs (P=0.001); the lower amount in synovial fluid came from the normal joints. Moreover, significant differences were found for the di-sialylated core 1 *O*-glycans [Neu5Ac(Gc)α2-3Galβ1-3(Neu5Acα2-6)GalNAcol; *m/z* 966 and 982 in Figure 11] in synovial fluid from normal joints compared to that of joints with OCFs (P=0.0297), with the higher level in synovial fluid coming from the normal joints.

The highest level of lubricin was detected with quantitative proteomics analysis and ELISA at day 3 in media from both IL-1β stimulated and unstimulated cartilage explants; nevertheless, the level of lubricin was higher in media from IL-1β stimulated cartilage.

Western Blot analysis confirmed the presence of lubricin in media from IL-1β stimulated cartilage explants, at all time points, but with a faint staining at day 22. Media from unstimulated cartilage explants, however, revealed the presence of lubricin only at days 0, 3 and 6. The Western Blot analysis of extraction from the unstimulated and stimulated cartilage explants showed no lubricin.

The proteomic analysis for non-tryptic cleavage sites identified 17 cleavage sites at the C-terminal sequence in synovial fluid from one horse with normal articular cartilage and from one horse with OCF. These sequences were compared to those identified in media from cartilage explants. One identical C-terminal non-tryptic cleavage site at position LRPH|VLTP was identified in synovial fluids and media from both IL-1β-stimulated explants and control media (Figure 12).

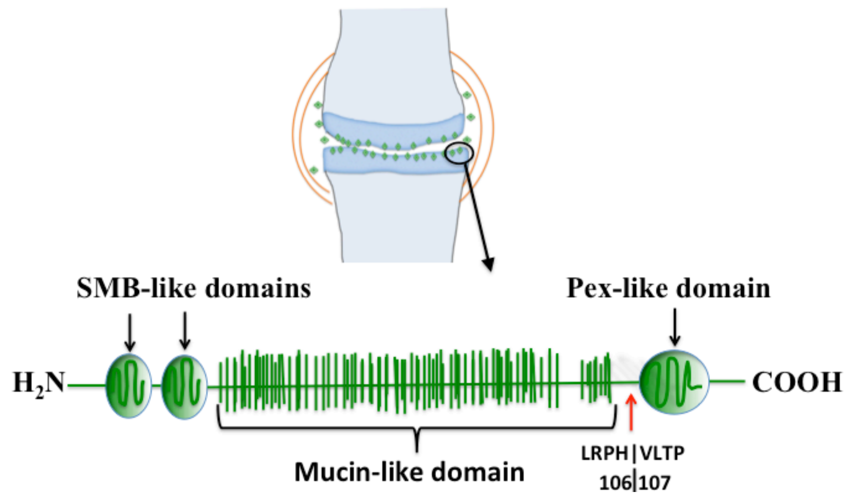

Figure 12. Lubricin acts as a boundary lubricant in articular cartilage present in synovial fluid and superficial zone of cartilage. Lubricin is secreted by the chondrocytes and cells in the meniscus into the superficial zone of articular cartilage, or by synoviocytes in synovium into synovial fluid. Lubricin consists of somatomedin B-like-domains (SMB) at the N-terminus, followed by a mucin-like domain that is heavily glycosylated and ends with a hemopexin-domain (Pex) at the C-terminus (Rhee *et al.*, 2005b). In paper IV we demonstrated an identical proteolytic cleavage site in both *in vivo* (synovial fluid) and *in vitro* (media from IL-1β-stimulated and from control cartilage explants). The cleavage site LRPH|VLTP was identified in the C-terminal end of lubricin. Illustration by E. Svala.

6 General discussion

6.1 *In vitro* methods: chondrocyte pellets and cartilage explants

To study *in vitro* articular cartilage that resembles the chondrocytes of the *in vivo* articular cartilage, 3D cell-culture pellets (Tallheden *et al.*, 2004) were used. However, when the chondrocytes expand in monolayer, they de-differentiate into a fibroblast-like phenotype (von der Mark *et al.*, 1977). In this phenotype there is a gene downregulation of cartilage specific markers such as *collagen type II*, *SOX-9* and *aggrecan,* and an upregulation of *RUNX*, *MMP-13*, *ALP*, and *OPN*, these being associated with a hypertrophic phenotype (Caron *et al.*, 2012). In *in vitro* 3D cell-culture pellets (Ma *et al.*, 2013; Tallheden *et al.*, 2004), chondrocytes are re-differentiated. This results in a gene upregulation of cartilage specific markers such as *collagen type II*, *SOX-9*, and *aggrecan*, and a down regulation of the hypertrophic markers such as *collagen type X* (Caron *et al.*, 2012), rendering the pellets with a chondrogenic phenotype. The studies in papers I and II established pellets of equine chondrocytes and further developed and validated the *in vitro* system as a high throughput approach. Replacement of 15-ml tubes with 96-well plates facilitated the culturing of large amount of chondrocyte pellets.

However, culturing cartilage explants with the intact tissue comprising chondrocytes and its ECM will provide an even more *in vivo* like model of the articular cartilage tissue, compared to chondrocyte pellet cultures. The chondrocytes in cartilage explants display intact chondrogenic features, instead of going through the de-differentiation and subsequent re-differentiation that occur in chondrocyte pellets (Moo et al., 2011). Hence, articular cartilage explants, cultured *in vitro*, were used (papers III and IV) to study the release and degradation pattern of the ECM.

The use of *in vitro* experiments aims to mimic the *in vivo* situation; nevertheless *in vitro* experiments have many limitations. OA involves all the structures of the joint (including bone, ligaments and synovial tissues). Because the microenvironment (including mechanical load and the cross-talk between tissues during the initiation and progression of OA) is important, co-cultures of tissues in the joint could improve the system by mimicking the *in vivo* situation of OA. The induction of inflammation by addition of IL-1β in cell culture media could also be improved by using biomaterials that provide a constant release of cytokines over time (Murab et al., 2013). This would more closely resemble the pathological condition *in vivo*.

Nevertheless, the results from *in vitro* studies of inflamed articular cartilage can render important and novel findings regarding the ECM molecule release and fragmentation that is only present in diseased cartilage. Fragments of ECM molecules can be evaluated as potential biomarkers of early OA and for monitoring the progression of OA *in vivo.*

6.2 Effects of the cytokines IL-1β, IL-6 and HMHB-1 on chondrocyte pellets *in vitro* (papers I and II)

The effect of cytokine stimulation on gene and protein expression of molecules related to the ECM metabolism was studied (paper I). Gene expression (an increase in *versican*, *MMP-9*, *-13*, *ADAMTS-5* and a decrease in *aggrecan* and *collagen type II*) and protein analyses (decreased glycosaminoglycan content, increased MMP-13 activity and decreased aggrecan synthesis) demonstrated a catabolic effect of IL-1β stimulation on cartilage metabolism. These results confirm those of other studies on IL-1β-stimulated equine cartilage (Neil *et al.*, 2005; Richardson & Dodge, 2000). Additionally, *SOX-9* gene expression was reduced by IL-1β stimulation, suggesting an anti-chondrogenic effect, since *SOX-9* is required for chondrocyte differentiation and in chondrogenesis (Bi *et al.*, 1999). The effects of IL-6 and HMGB-1 on equine cartilage metabolism *in vitro* were variable; inter-individual differences had no major effect on ECM degradation, and caused only minor alterations in the gene expression of *MMPs* and *ADAMTs*. In contrast, an upregulation of *SOX-9* was observed for both IL-6 and HMGB-1. The HMGB-1 stimulation was statistically significant, indicating a mild anabolic effect.

The response to these cytokines (IL-1β, IL-6 and HMGB-1) was also evaluated, using chondrocytes harvested from two different anatomical areas of the carpal bone–the less-loaded (PC) area and the highly loaded area (DRF) of C3 (Palmer *et al.*, 1994). The highly loaded DRF is more prone to develop

cartilage lesions compatible with OA (Pool & Meagher, 1990); it has been suggested that the effect of IL-1β differs in topographically defined regions in human OA joints (Barakat *et al.*, 2002). However, our results showed that chondrocytes harvested from DRF and PC responded in a similar manner to cytokine stimulation. Our results are supported by another study of cytokine-stimulated equine cartilage (Little *et al.*, 2005), suggesting that site-associated cartilage destruction in equine carpal OA is not explainable by topographical differences in the response to inflammatory mediators. Some site-associated changes were, however, found in the youngest horses, for certain genes, which might be explained by an age-related cell response (Morris & Treadwell, 1994).

Furthermore the results suggested a more hyaline cartilage phenotype of unstimulated cells harvested from the less loaded area of PC, with higher *Sox9*, *aggrecan*, and *collagen type II* gene expression compared to the highly loaded area of DRF. However higher *versican* and *collagen type I* gene expression, suggesting a more fibrocartilaginous gene profile was found in DRF compared to PC. This topographical difference in the articular cartilage phenotype in the young horses may indicate an altered metabolism, after frequent mechanical load. A topographic variation in proteoglycan synthesis in articular cartilage from sheep has been related to mechanical stress (Little *et al.*, 1996). There is significantly less proteoglycan synthesis in regions of high mechanical load (Little *et al.*, 1996) and the variation in chondrocyte metabolism is suggested to be determined by post-natal loading (Little & Ghosh, 1997).

GDF-5, also known as bone morphogenetic protein-14, is one of the earliest known markers for joint formation (Storm & Kingsley, 1996). It is an essential protein in chondrogenesis, chondrocyte proliferation, appendicular skeletal patterning, and longitudinal bone growth (Settle et al., 2003). Mutations in the GDF-5 gene result in abnormal joint development and skeletal malformation diseases (Hellman et al., 2012). An association between a functional polymorphism (T/C; rs143383) in the 5′-untranslated region of GDF5 and susceptibility to OA has been confirmed in European as well as Asian population studies, suggesting that reduced GDF-5 expression is involved in the pathogenesis of knee and hip OA (Chapman et al., 2008; Miyamoto et al., 2007). The catabolic effect after IL-1β stimulation of chondrocyte pellets, (paper I), was further supported by the down regulation of *GDF*-5 mRNA expression (paper II). Similar findings of regulation of GDF-5 by IL-1β-stimulation have been reported in human chondrocytes cultured in monolayer (Liu et al., 2010) and in 3D cultures of human intervertebral annulus cells (Gruber et al., 2014).

IL-6 is a regulatory or modulatory mediator in OA (van der Kraan & van den Berg, 2000; Goldring, 1999), but also crucial for IL-1β-induced matrix degradation (Westacott & Sharif, 1996). It was found to upregulate the gene expression of *GDF-5* within 2 hours after stimulation of chondrocyte pellets (paper II). This is in line with the trend of an upregulation of *SOX-9* gene expression after 48 hours of stimulation (paper I), suggesting a prochondrogenic effect by IL-6. IL-6 stimulation of human chondrocytes *in vitro* increases the production of GAGs (Tsuchida *et al.*, 2012) and expression of *collagen type II*, *BMP-7*, *BMP receptors* (Namba *et al.*, 2007) and *TIMP-1* (Silacci *et al.*, 1998). Using a global microarray analysis, we further studied the short-term effect of IL-6, in order to investigate by which mechanisms and pathways IL-6 exerts its effects. One pathway affected by the IL-6 stimulation of chondrocyte pellets, is the canonical Wnt-signalling pathway, which has an essential role in embryonic joint specification, formation and chondrogenesis; it is also associated with OA pathogenesis (Corr, 2008). Upon binding of extracellular Wnt ligands to receptors (Frizzled) and co-receptors (LRP 5/6), the key protein β-catenin is translocated into the nucleus, while in the absence of Wnt ligands, β-catenin is phosphorylated by GSK3β and subjected to proteosomal degradation. Nuclear translocation of β-catenin is a sign of activation of the canonical Wnt-signalling pathway (Willert & Nusse, 1998). Active canonical Wnt-signalling maintains chondroprogenitor cells in a proliferative state, inhibiting their development into mature chondrocytes (Tamamura *et al.*, 2005). In contrast, activation of β-catenin in articular chondrocytes induces hypertrophy, expression of ECM degradation proteins, and stimulates matrix mineralization (Enomoto-Iwamoto *et al.*, 2002; Ryu *et al.*, 2002). Activation of β-catenin signalling leads to an OA-phenotype in mice, with cell cloning, surface fibrillation, vertical clefting, osteophyte formation and reduced articular cartilage area (Zhu *et al.*, 2009).

After IL-6 simulation, the inhibitors *CCDC88C*, *GSK3β* and *Wnt inhibitory factor 1* were upregulated (paper II). Furthermore there was an increased expression of genes involved in the ubiquitin-mediated degradation of β-catenin and an indication of down regulation of *β-catenin*. Together with the decreased expression of ligands (*WNT8B*, *WNT9B*) and receptors (*FZD8*) belonging to the canonical Wnt signalling pathway, this suggests an inhibitory effect by IL-6. These results were supported by the immunohistochemistry staining of GSK3β and β-catenin in the chondrocyte pellets. The IL-1β-stimulated chondrocyte pellets displayed nuclear β-catenin, a clear sign of Wnt-signalling activity. In contrast, GSK3β, which phosphorylates β-catenin, was clearly present in the IL-6 stimulated chondrocyte pellets.

GDF-5 stimulation of human chondrocytes has been shown to inhibit the canonical Wnt-signalling pathway by increased gene expression of the inhibitors *DKK1* and *FRZB* (Enochson *et al.*, 2014). A possible explanation for the upregulation of *SMAD4* and *GDF-5* (and consequent downregulation of the canonical Wnt pathway) by IL-6 (paper II) could be due to cross-talk between the two pathways (Hiyama *et al.*, 2011). This would further strengthen the role of IL-6 as a regulatory cytokine with possible prochondrogenic effects. Taken together the data from paper II suggest that IL-6 inhibits the canonical Wnt-signalling pathway in chondrocyte pellets.

6.3 Effects of IL-1β stimulation on the secretome of articular cartilage explants cultured *in vitro* (paper III)

Previous studies (Williams *et al.*, 2013; Clutterbuck *et al.*, 2011) have explored the secretome of equine articular cartilage from the MCP joint. However our results are the first describing a time-dependent, relative quantification of equine ECM proteins in an *in vitro* inflammation model.

In order to develop diagnostic tools and an evaluation of treatments for OA, it is necessary to elucidate the exact series of events with the specific fragmentation patterns of ECM and the enzymes responsible for the degradation of articular cartilage. An approach used to find potential biomarkers of various diseases, including OA, is high-resolution proteomic analyses by mass spectrometry (Stenberg *et al.*, 2013; Takinami *et al.*, 2013; Ruiz-Romero & Blanco, 2010). LC-MS/MS, a highly specific and sensitive technique, is used not only in basic research but also in routine laboratory diagnosis such as drug monitoring and endocrinology/metabolism applications (Leung & Fong, 2014). In studies III and IV we used quantitative proteomics to study the secretome of equine articular cartilage explants over time after IL-1β stimulation.

The explants were harvested by cutting, which consequently initiates an inflammation (Gruber *et al.*, 2004). Thus, the first release of ECM proteins from unstimulated explants into the medium could be explained by the inflammation caused by harvest of the cartilage and the effect of long-term culturing. However, the unstimulated explant recovers from this inflammation with a release of COMP (proteomics) and also native COMP (Western Blot). Additionally, at days 3-6, we detected high levels of procollagen type II C-pro peptides, PCOLCE, and PCOLCE2, suggesting a type II collagen synthesis. At the same time, there was little or no evidence of MMPs. This suggests that the quantitative proteomics results in the medium from the unstimulated explant represent both newly synthesized molecules and degradation.

In the IL-1β stimulated explants, the degradation process continued throughout the period. Numerous *in vitro* studies of IL-1 stimulated articular cartilage explants show a time-dependent pattern in both the release and degradation of ECM, starting with aggrecan, followed by COMP, fibromodulin and finally collagen type II, with complete dissolution of the collagen network (Williams *et al.*, 2013; Heinegård, 2009; Heathfield *et al.*, 2004; Dickinson *et al.*, 2003; Sztrolovics *et al.*, 1999). Our results were in agreement with these findings and also revealed a release pattern for other ECM proteins in equine cartilage. Media from IL-1β-stimulated explants showed a high concentration of active MMP-13 during the entire culture period, similar to what is seen in OA cartilage (Goldring *et al.*, 2011). The high levels of MMP-1, -3 and -13 and lack of ADAMTs during the *in vitro* culture are also reported by other studies of cytokine-stimulated equine articular cartilage (Williams *et al.*, 2013; Clutterbuck *et al.*, 2009).

IL-1β significantly upregulates the mRNA gene expression of IL-6 in equine chondrocytes (David *et al.*, 2007) and we detected high levels of IL-6 in the media from IL-1β stimulated explants. Moreover, other mediators of inflammation were detected, such as serum amyloid A (which may play may a key role in inflammatory process in OA (de Seny *et al.*, 2013)) and chemokine (C-C motif) ligand 20, an important chemokine in RA (Kawashiri *et al.*, 2009). Complement components, involved in the initial stages of the activation of the classical (C1r, C1s) and the alternative (CFB) pathway, increased in the media from IL-1β stimulated explants, consistent with a study of IL-1β-stimulated bovine cartilage explants (Stevens *et al.*, 2008). Deregulation of the complement system has a key role in the pathogenesis of OA; the expression and activation of complement is high in human OA joints and the membrane attack complex is crucial to the development of OA in mice models (Wang *et al.*, 2011). COMP fragments can activate the alternative pathway (Happonen *et al.*, 2012), while SLRPs (fibromodulin, osteoadherin, and chondroadherin) can activate the classical pathway (Sjoberg *et al.*, 2009). This implies that there is an inflammation-related release of ECM molecules linked to the regulation of complement components. The SLRPs interact with collagens to form a proper ECM network (Kalamajski & Oldberg, 2010); their biphasic release (early and late stage of culturing) may reflect increased synthesis and release of the protein, in intact or fragmented form.

The degradation of the collagen network progressed in a time-dependent pattern, with release of collagen types -XII, -IX, -VI and type II. A high level of collagen type XII was detected in media from IL-1β-stimulated explants, at day 9-12, before the release of collagen types -IX, -VI and –II. Collagen type XII is involved in the organization of collagen fibrils in developing rat

cartilage (Gregory *et al.*, 2001) and competes or collaborates with SLRPs (Eyre, 2002). The NC4 domain of Collagen IX is the segment of the molecule that projects out from the fibril surface, connecting to ECM molecules while the *C*-terminal part can interact with other collagens. Peptides originating from the NC4 domain were identified in media from IL-1β-stimulated explants at days 15-18 but no peptides were identified from the C-terminal part of Collagen IX. This suggests that the NC4 domain was cleaved by proteolytic enzymes, resulting in a loss of collagen type IX from type II fibrils as the initial step of destruction of the fibrillar network. Increased release of collagen type VI, which supports the collagen network, was found at day 18, preceding the peak levels of SLRPs in media from IL-1β-stimulated explants. Collagen type II release was highest at the end of the culturing period (day 22) and collagen degradation was identified by C1, 2C (ELISA), in agreement with late-stage OA (Goldring & Otero, 2011). The low levels of procollagen type II C-propeptide, a product of endogenous proteolytic processing of newly synthesised proprotein, in the media from the stimulated explants suggest that collagen type II synthesis was not prominent.

6.4 Presence of GDF-5 in normal articular cartilage and OCFs (paper II)

The presence of GDF-5 in human articular cartilage has been found in all cartilage zones (Bobinac *et al.*, 2008) but also restricted to the upper zones (Erlacher *et al.*, 1998). Our results are in agreement with the latter observation, with the localization of GDF-5 restricted to the superficial and middle zones of normal equine articular cartilage. The presence of chondrocytes, positive for GDF-5 in the superficial zone, coincides with the presence of progenitor cells in this area in normal equine (McCarthy *et al.*, 2012) and human (Dowthwaite *et al.*, 2004) cartilage. The GDF-5 positive chondrocytes, in the OCFs, were localized in most zones of the articular cartilage. Previous studies have shown that chondrocytes from equine OCFs synthesize COMP (Skiöldebrand *et al.*, 2005), collagen type II (Frisbie *et al.*, 1999), and express IL-6 (Ley *et al.*, 2009; Ley *et al.*, 2007). These results, taken together with the presence of GDF-5, indicate an anabolic process in the cartilage of the OCFs, maybe as an attempt to repair the damage.

6.5 Characterization of lubricin (paper IV)

The reduced expression of lubricin in human OA menisci and synovial fluid (Musumeci et al., 2014) is suggested to change the boundary-lubricating ability

of the articular cartilage surface, subsequently leading to the development of OA. Therefore, we wanted to investigate possible modifications in the levels of equine synovial lubricin and alterations in its glycosylation pattern from horses with OA. The *O*-linked glycosylation of lubricin is essential for the biological lubrication of the superficial zones of articular cartilage (Jay, 1992) but glyco-epitopes on core 1 and 2 *O*-glycans also have the potential to interact with, for example selectins and galectins, to facilitate inflammation (Jin *et al.*, 2012; Liaqat *et al.*, 2012). Studies of human lubricin have shown that, in addition to the level of lubricin, both the glycosylation and its degradation products are potential markers for inflammation and disease progression of OA and RA (Liaqat *et al.*, 2012).

Our results showed that the core 1 *O* glycans were the most dominating structure on equine lubricin, similar to the findings for human synovial fluid lubricin (Estrella *et al.*, 2010). Additionally lubricin in synovial fluid from horses with structural OA lesions and joints with OCFs, had overall a reduced amount of sialylation of core 1 *O*-glycans compared to synovial fluid from normal joints. The removal of sialic acid and core 1 oligosaccharides has been shown to cause loss of boundary lubrication (Jay *et al.*, 2001).

Synovial fluid from normal joints contained a slightly elevated level of core 2 *O*-glycans compared to synovial fluids from horses with OA or OCFs. This lack of complex core 2 *O*-glycan on lubricin may increase the susceptibility to endogenous proteolysis and decrease the carbohydrate-dependent interaction with other components in both cartilage and synovial fluid (Liaqat *et al.*, 2012). Synovial lubricin from RA patients expresses core 2 *O*-glycans which comprise sialyl Lewis x, an epitope as well as sulfate substitution. Sialyl Lewis x is involved in binding to L-selectin ligands on polymorphonuclear granulocytes, indicating that lubricin also may be involved in polymorphonuclear granulocyte-mediated inflammation (Jin *et al.*, 2012). However in our study, no fucosylated core 2 *O*-glycans were detected in any of the synovial fluid samples, indicating an absence of sialyl Lewis epitopes on equine synovial lubricin.

Both quantitative proteomics and ELISA showed higher levels of lubricin in the medium from IL-1β stimulated explants. Western Blot analysis confirmed the high release of lubricin in this medium during the culturing period; however, medium from non-stimulated explants only showed immune detection at days 0-6 (Western Blot). IL-1β has been suggested to have multiple roles in controlling new synthesis of lubricin, as well as promoting degradation of the cartilage, and releasing lubricin into the media (Jones & Flannery, 2007). Other studies have found both *N*- and *C*-terminal fragments of lubricin in normal bovine synovial fluid (Schmidt *et al.*, 2009; Rhee *et al.*,

2005a), and in human OA synovial fluid samples (Kamphorst *et al.*, 2007; Rhee *et al.*, 2005a).

We also identified peptides containing non-tryptic cleavage sites in synovial fluid from healthy and diseased joints and in media from stimulated and unstimulated explants. We detected a shared non-tryptic site at position LRPH|VLTP in lubricin from both the synovial samples and the media. This confirms the explant model as a suitable system for future studies on the role of inflammation in lubricin processing and decreased lubrication of the joints.

6.6 Horse as a model system for translational OA research

A recent population-based study in Sweden estimates that at least an additional 26,000 individuals per 1 million in the population aged ≥45 years have consulted a doctor for OA in a peripheral (knee or hip) joint in year 2032 compared to 2012 (Turkiewicz *et al.*, 2014). Hence new tools for early diagnosis of OA with the aim of prevention and early treatment are warranted.

There are many animal models of OA attempting to resemble the human disease. Genetically engineered mice studies have contributed valuable knowledge into the pathobiology of OA and identified disease-modifying targets (Little & Hunter, 2013). Another new approach for studying genomics of OA uses zebra fish (Mitchell *et al.*, 2013) as a model; this animal model is suggested to be a link between *in vitro* cell or tissue cultures and *in vivo* studies with rodents (van der Kraan, 2013).

The horse, as a model for OA, has many advantages; the prevalence of *in vivo* OA is high among racehorses and consequently there is a widespread knowledge in diagnosing clinical equine OA. The genome of the domestic horse shows a large synteny with the human genome, making the horse a suitable model for studying human diseases (Wade *et al.*, 2009). Equine and human cartilage has similar geometrical and organizational structures with respect to cartilage thickness and GAG, DNA and collagen content (Malda *et al.*, 2012). However it is much easier to obtain healthy equine articular cartilage for chondrocyte pellets or explant culturing than from human individuals. Moreover, results from the *in vitro* cultures of cartilage explants (paper III) showed a battery of ECM proteins paralleling some of those previously observed in human OA cartilage explants (Stenberg *et al.*, 2013).

6.7 Change in diagnostic procedure of osteoarthritis

Equine OA is clinically characterized by lameness including soft tissue swelling, and/or synovial effusion (McIlwraith *et al.*, 2010). An increased degree of lameness is often seen after flexion of the joint. For lameness diagnosis of a specific joint, intra-articular anaesthesia in combination with flexion tests is used. The traditional diagnosis of OA relies heavily on imaging techniques such as radiographs, ultrasound, computed tomography and magnetic resonance imaging (MRI) to detect structural changes of the joint tissues. However the challenge for the future is to develop other diagnostic markers that could detect early molecular changes before irreversible joint damage. An assortment of cartilage-associated proteins is evaluated for this purpose. These molecular markers, also known as biomarkers, can be degraded proteins with specific catabolic epitopes, that preferably exists in OA joints, or anabolic neo-epitopes which correlate to synthesis/turnover of ECM molecules (Goldring & Goldring, 2007). The "OA biomarker consortium" study originated by the Foundation for the National Institute of Health is currently evaluating 12 different biomarkers in clinical studies of human knee and hip OA. The evaluations are based on ELISA assays and five of these markers are associated with collagen type II degradation while two are connected to bone resorption and the remaining ones with aggrecan turnover, cartilage degeneration, synovitis, type II collagen synthesis, and articular cartilage degradation (Hunter *et al.*, 2014a). Suggested synovial fluid or serum biomarkers for early equine OA are CS846, CPII, Col CEQ (a marker of type II collagen degradation), C1, 2C, osteocalcin, Col I, and prostaglandin E2 (Frisbie *et al.*, 2008). The detection of degraded and/or new synthesis of COMP has been proposed as a marker of equine OA in several studies (Yamanokuchi *et al.*, 2009; Arai *et al.*, 2008; Arai *et al.*, 2005; Skiöldebrand *et al.*, 2001). There are positive correlations between synovial fluid levels of bone-specific alkaline phosphate and keratan sulphate with cartilage damage (Fuller *et al.*, 2001). A suitable approach to identify new cleavage sites of candidate proteins is quantitative proteomics of articular cartilage (Peffers *et al.*, 2014) and of the secretome (Peffers *et al.*, 2013). The future goal of this research should be to achieve a biomarker panel based on unique protein fragments, representing different stages of OA. An optimal biomarker panel could be used in diagnosis, monitoring the progression of disease and in treatment evaluation.

7 Conclusions

- Stimulation by IL-1β induces a catabolic response on ECM-related genes and proteins in chondrocyte pellets and cartilage explants *in vitro*, confirming IL-1β as a master cytokine in cartilage pathology.
- IL-1β stimulation of cartilage explants leads to a release of ECM molecules at different time points with a final release of components of the collagen network.
- HMGB-1 stimulation of chondrocyte pellets *in vitro* induces an upregulation of SOX-9, indicating a promotion of chondrocyte differentiation or increased metabolic state of chondrocytes.
- Site-associated articular cartilage carpal deterioration is not explained by a topographically dependent altered response to cytokines in equine carpal OA.
- IL-6 stimulation of chondrocyte pellets *in vitro* does not lead to a similar response as IL-1β; instead, IL-6 has a regulatory role in the inhibition of the canonical Wnt-signalling pathway, leading to a subsequent upregulation of GDF-5 mRNA gene expression.
- GDF-5 protein is expressed in both normal articular equine cartilage and in OCFs, possibly indicating a repair attempt in the latter.
- A change in *O*-glycosylation profile of lubricin is seen in synovial fluid from equine joints with OA/OCFs compared to synovial fluid from normal joints, with lower amounts of mono-sialylated core 1 *O*-glycans and higher amounts of di-sialylated core 1 *O*-glycans in the normal synovial fluid.
- An endogenous proteolytic cleavage site of lubricin was found *in vitro* and *in vivo*.

8 Future research

Basic research, delineating the pathological framework from the earliest molecular aspects of the disease until the late stage clinical OA, is important for future specific diagnostic techniques, drug development, and a correct prognostic evaluation. A new generation of biomarkers based on unique cleavage sites generated in the early stages of OA will give the opportunity to achieve early diagnosis of OA before joint destruction. Specific biomarkers could also be used in evaluating treatments and in assessing new training programs for the racehorse to prevent joint damage.

To follow up the work with the *in vitro* models in this thesis, it would be of importance to:

- Develop biomarker assays with the aid of the *in vitro* studies of cartilage explants (paper III) by producing monoclonal antibodies against specific neo-epitopes. These assays can then be used to detect specific cleavage sites of the ECM molecules in synovial fluid and serum from horses with different stages of OA.
- Characterize the molecular changes in equine cartilage explants after IL-6 stimulation to further explore the possible regulatory effect of this cytokine.
- Evaluate the effect of IL-6 on the secrection, glycosylation and localization of lubricin in equine articular cartilage *in vitro*.

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