Chondrocyte and Extracellular Matrix Alterations in Equine Cartilage during Development and Inflammation

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Abstract

Osteoarthritis (OA) is a disease involving inflammation and subsequent degeneration of joint tissues. The disease progresses with time-dependent stages of degeneration, loss and remodelling of the extracellular matrix (ECM) of articular cartilage, and phenotypical changes in the chondrocytes. Currently, OA is diagnosed clinically late in the disease process when irreversible damages to the cartilage are already evident. An understanding of the disease mechanism in the early stage of the inflammatory process is therefore crucial if early stage diagnostic biomarkers are to be identified.

The aim of this thesis was to follow cartilage changes during normal development and interleukin (IL)-1β-induced inflammation. Biomarkers of early OA are needed and a prerequisite for finding unique molecular markers is an understanding of the cellular, genetic and molecular events that occur in chondrogenic development and inflammation. The hypothesis was that inflammation-related events occur at specific time points which can be used to differentiate stages of OA from each other.

The localisation of proteins in developing equine cartilage was studied using immunohistochemistry with the aim of characterising different phenotypes of chondrocytes and their surrounding ECM. The expression of matrix molecules, Notch signalling components, and the stem cell-indicating factor Stro-1 showed spatial changes in expression and localisation during maturation.

Equine articular cartilage explants stimulated with IL-1β were used as an *in vitro* model mimicking the IL-1β-induced inflammation that occurs during the progression of equine OA. The longitudinal release induced by IL-1β of molecules from the explants into the media was studied using quantitative proteomics and the gene expression in the cartilage explants was evaluated using microarray. The secretion of ECM components and inflammatory mediators followed a time-dependent pattern. Microarray analysis also revealed time-dependent differences in gene expression related to inflammation, ECM, and phenotype.

The data from this thesis contribute to the understanding of cellular, genetic and molecular events in the progression of OA. Stimulation of equine articular cartilage explants with IL-1β induces release of proteins and altered gene expression in a time-dependent pattern. This *in vitro* model has the potential to aid in testing medical treatments of equine OA, prior to testing in live animals, thereby minimising the use of research animals.

Keywords: cartilage, osteoarthritis, inflammation, extracellular matrix, growth cartilage, chondrocyte, interleukin-1β, horse

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Dedication

Till minne av min farfar Ernst

Contents

List of Publications

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

I Löfgren, M., Ekman, S., Svala, E., Lindahl, A., Ley, C. and Skiöldebrand, E. (2014). Cell and matrix modulation in prenatal and postnatal equine growth cartilage, zones of Ranvier and articular cartilage. *Journal of Anatomy* 225(5), 548-568

II Svala, E., Löfgren, M., Sihlbom, C., Rüetschi, U., Lindahl, A., Ekman, S. and Skiöldebrand, E. (2015). An inflammatory equine model demonstrates dynamic changes of immune response and cartilage matrix molecule degradation *in vitro. Connective Tissue Research* 56(4), 315-325

III Löfgren, M., Svala, E., Lindahl, A., Skiöldebrand, E. and Ekman, S. Time-dependent changes in gene expression induced by interleukin-1β in equine articular cartilage *in vitro* (manuscript).

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

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

I Main author. Active part in planning and design of the study. Performed all experimental work and active in interpreting the results. Drafted and edited the manuscript.

II Second author. Planned and designed the study together with the co-authors. Collected the material. Active in interpreting the results. Drafted a part of the manuscript. Revised and edited the manuscript.

III Main author. Active part in formation of the hypothesis and study design. Major responsibility for planning and organisation of the study. Performed all experimental work except the microarray analysis. Interpreted and presented the microarray results. Drafted and edited the manuscript.

Abbreviations

ACAN	Aggrecan (gene symbol)
ADAMTS	A disintegrin and metallopeptidase with thrombospondin motifs
ASPN	Asporin (gene symbol)
BGN	Biglycan (gene symbol)
BMP	Bone morphogenic protein
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CHAD	Chondroadherin (gene symbol)
CILP	Cartilage intermediate layer protein
COL	Collagen
COMP	Cartilage oligomeric matrix protein
CSPG4	Chondroitin sulphate proteoglycan 4
CXCL	Chemokine (C-X-C motif) ligand
DCN	Decorin (gene symbol)
Dll	Delta-like
DNA	Deoxyribonucleic acid
DSL	Delta/Serrate/LAG2
ECM	Extracellular matrix
ECM2	Extracellular matrix protein 2
EGFL7	Epidermal growth factor-like domain 7
EPYC	Epiphycan (gene symbol)
FACIT	Fibril-associated collagens with interrupted triple helices
FGF	Fibroblast growth factor
FMOD	Fibromodulin (gene symbol)
GAG	Glycosaminoglycan
GPC	Glypican (gene symbol)
HA	Hyaluronan
Hes	Hairy enhancer of split
HSPG2	Heparan sulphate proteoglycan 2 (also called perlecan)

IHC	Immunohistochemistry
IL	Interleukin
IL1A	Interleukin-1 alpha (gene symbol)
IL-1R	Interleukin-1 receptor
IL-1Ra	Interleukin-1 receptor antagonist
IL1RN	Interleukin-1 receptor antagonist (gene symbol)
IL6	Interleukin-6 (gene symbol)
IL8	Interleukin-8 (also called CXCL8) (gene symbol)
ITG	Integrin (gene symbol)
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LIF	Leukemia inhibitory factor
LUM	Lumican (gene symbol)
MAPK	Mitogen-activated protein kinase
MATN	Matrilin (gene symbol)
MMP	Matrix metallopeptidase
NFκB	Nuclear factor κB
OA	Osteoarthritis
OGN	Osteoglycin (also called mimecan) (gene symbol)
OMD	Osteomodulin (gene symbol)
PLA	Proximity ligation assay
PRELP	Proline/arginine-rich end leucine-rich repeat protein (also called prolargin)
PRG4	Proteoglycan 4 (also called lubricin) (gene symbol)
RNA	Ribonucleic acid
SAA	Serum amyloid A
SDC	Syndecan (gene symbol)
SLRPs	Small leucine rich proteoglycans/proteins
TIMP	Tissue inhibitor of metallopeptidase
TMT	Tandem mass tag
TNF	Tumor necrosis factor
VEGFA	Vascular endothelial growth factor A

1 Introduction

1.1 General background

Lameness is a common clinical problem in horses (Penell *et al.*, 2005; Jeffcott *et al.*, 1982), with impact on economy as well as animal welfare. Osteoarthritis (OA) affecting synovial joints is a common cause of lameness and poor performance in horses (Kidd *et al.*, 2001). The joints most commonly affected by OA are the fetlock joints followed by the carpal joints (McIlwraith *et al.*, 2012; Penell *et al.*, 2005). Multiple factors contribute to the initiation and progression of the disease with inflammation and abnormal mechanical load considered important factors in equine OA (Kidd *et al.*, 2001). Synovial joints include components such as articular cartilage, subchondral bone, synovial fluid, synovial membrane, joint capsule and ligaments (Frisbie, 2006; Archer *et al.*, 2003). OA affects the whole joint and includes features such as inflammation, with degeneration of articular cartilage, and subchondral bone sclerosis. The disease progress from early to late stages of OA including time-dependent stages of degeneration, loss and remodelling of the extracellular matrix (ECM) of articular cartilage, and phenotypical changes in the chondrocytes. The phenotypes of chondrocytes during OA share similarities with those observed during the development of articular cartilage (Goldring & Goldring, 2007). Knowledge about the cellular, genetic and molecular changes during the progression of OA is essential for the possibilities to identify markers of OA stages and to find disease-modifying treatments.

1.2 Articular cartilage

Articular cartilage is hyaline cartilage that constitutes the joint surface. The major function of the articular cartilage is to provide a smooth, frictionless area for transmission of load within the joint. The articular cartilage comprises chondrocytes surrounded by ECM and does not include vessels, nerves or

lymphatics (Sophia Fox *et al.*, 2009; Frisbie, 2006; Buckwalter *et al.*, 2005). The ECM is important for the structure and function of the articular cartilage and has water, collagens and proteoglycans as its main components. Water constitutes up to 70-80% of the cartilage wet weight and is responsible for functions such as transport of nutrients, lubrication and the ability of cartilage to withstand mechanical load. The remaining dry weight is composed of about 50% collagens that provide tensile strength and 35% proteoglycans that provide resistance to compressive forces. The remaining portion of the dry weight is composed of non-collagenous proteins and glycoproteins with various functions in the organisation, assembly and maintenance of the ECM (Sophia Fox *et al.*, 2009; Frisbie, 2006). The articular cartilage can be divided into different zones. The superficial zone, comprising the surface zone and the upper zone, is closest to the joint cavity followed by the middle and the deep zones. The calcified cartilage closest to the subchondral bone is separated from the deep zone by the tidemark (Figure 1) (Pritzker & Aigner, 2010). The average thickness of equine cartilage is 0.86 mm and 0.87 mm in the fetlock and carpal joints, respectively (Lee *et al.*, 2014).

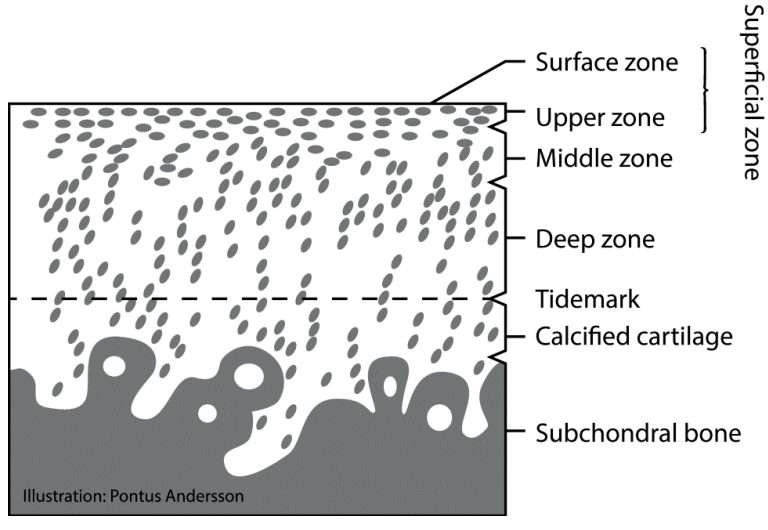

Figure 1. Zones of articular cartilage. The superficial zone comprising the surface zone and the upper zone are located closest to the joint cavity and contain collagen fibres aligned parallel to the joint surface. The surface zone does not contain chondrocytes; however, the upper zone contains flattened chondrocytes aligned parallel to the joint surface. The middle zone contains less-organised collagen fibres and chondrocytes in groups whereas the deep zone is characterised by chondrocytes and collagen fibres perpendicular to the joint surface. The tidemark is the border between uncalcified and calcified cartilage. Chondrocytes and collagen fibres in the calcified cartilage are aligned as in the deep zone and located closest to the subchondral bone (Pritzker & Aigner, 2010).

1.2.1 Chondrocytes

Chondrocytes are the only cell type present in articular cartilage. The major functions of chondrocytes are to synthesise and maintain the ECM and thereby contribute to growth and functionality of the articular cartilage (Archer & Francis-West, 2003; Stockwell, 1978). Chondrocytes respond to mechanical stimuli by altering gene expression and metabolism (McCulloch *et al.*, 2014; Chen *et al.*, 2013a; Guilak *et al.*, 2004; Grodzinsky *et al.*, 2000; Freeman *et al.*, 1994). Rounded chondrocytes are present in normal cartilage, but the shape is more flattened towards the articular surface. The cells contain a nucleus and cytoplasm with rough endoplasmic reticulum and Golgi apparatus as major organelles although lysosomes, mitochondria, filaments, glycogen and lipid deposits are also present. Glycolysis is the major energy source for the chondrocyte and nutrients and metabolites diffuse through the articular surface (Archer & Francis-West, 2003; Stockwell, 1978). Chondrocytes in the articular cartilage express gap junction proteins and can connect to each other via cell-to-cell interactions (Mayan *et al.*, 2013).

1.2.2 Extracellular matrix (ECM)

The chondrocytes are surrounded by the ECM which can be divided into different domains (Figure 2). The pericellular matrix surrounds the chondrocyte and the term chondron refers to one or more chondrocytes and their pericellular matrix. The territorial matrix surrounds the chondrons and the interterritorial matrix is located in between the territorial matrix of different chondrons (Pritzker & Aigner, 2010). The content and organisation of matrix molecules varies with depth from the surface as well as proximity to chondrocytes (Figure 2) (Umlauf *et al.*, 2010; Heinegård, 2009; Buckwalter *et al.*, 2005; Pearle *et al.*, 2005).

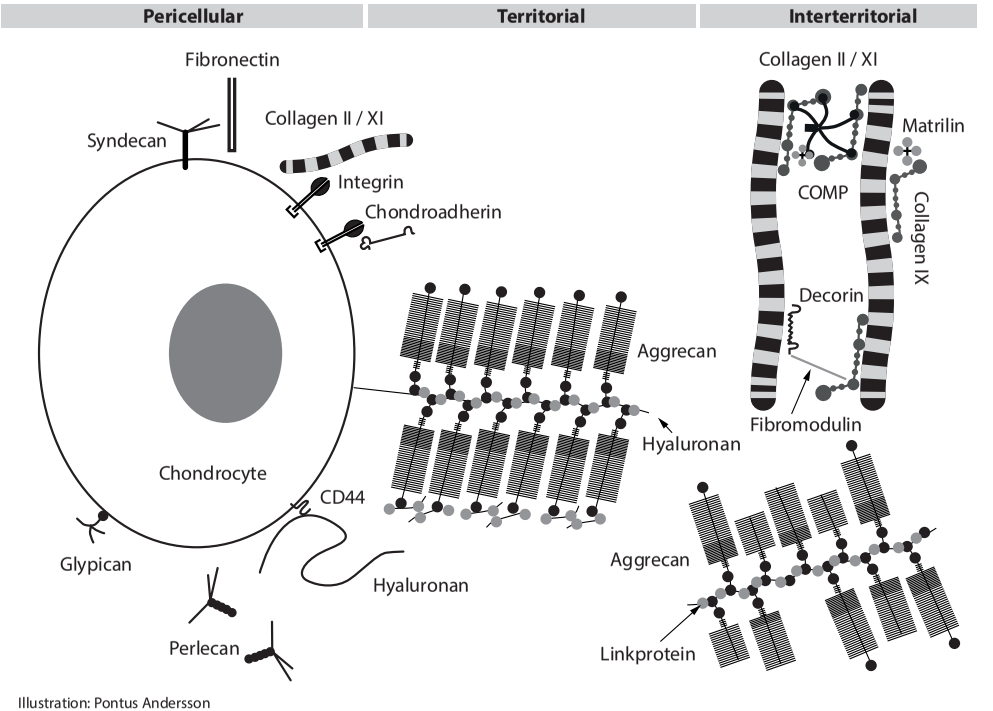

Figure 2. Location and organisation of some of the important extracellular matrix (ECM) molecules in articular cartilage. The pericellular matrix is located closest to the chondrocyte followed by the territorial and interterritorial matrix at further distance. Receptors are located at the cell surface and the distribution of matrix components varies within the surrounding domains. Adapted from Heinegård, D. (2009) Proteoglycans and more – from molecules to biology. *International Journal of Experimental Pathology,* 90(6), pp. 575-586.

Collagens

The major constituent of the ECM dry weight is collagen with several types present in mature cartilage. Collagen type II is the major one in mammals whereas collagen types III, VI, IX, X, XI, XII, and XIV are present in different domains in smaller amounts (Buckwalter *et al.*, 2005; Eyre, 2002). All collagens are triple helices composed of three α-chains which can be similar (homotrimers) or different from each other (heterotrimers). Every third position on the polypeptide chains is a glycine residue with importance for the assembly of the triple helix. The other residues, their modifications, and the lengths of the chains vary between different types of collagens (Gelse *et al.*, 2003). Fibril-forming collagens such as collagen types II, III, and XI form fibrils that are organised into a framework stabilized by other matrix molecules (Buckwalter *et al.*, 2005; Gelse *et al.*, 2003). The organisation of the fibrils differs in different zones of the articular cartilage. The superficial zone contains densely packed, thin collagen fibrils aligned parallel to the surface whereas the middle zone contain thicker fibrils loosely packed in a less-organised oblique arrangement. The alignment of the fibrils in the deep and calcified zones is perpendicular to the articular surface (Pritzker & Aigner, 2010; Pearle *et al.*, 2005; Eyre, 2002).

Collagen type II, composed of three identical α-chains, is the most abundant type of collagen in all zones of articular cartilage where it constitutes about 90% of the collagen content in mature articular cartilage. Collagen types IX and XI bind covalently to collagen type II and form heteromeric fibrils as a part of the ECM framework. Collagen type IX belongs to the fibril-associated collagens with interrupted triple helices (FACIT) and is a heterotrimer of the three chains α1, α2 and α3. Collagen type XI is a fibril-forming collagen composed of three α-chains—α1 and α2 are encoded by the genes *COL11A1* and *COL11A2* and the α3-chain chain is encoded by the same gene as the α-chains of collagen type II (Buckwalter *et al.*, 2005; Gelse *et al.*, 2003; Eyre, 2002). Another collagen found linked to collagen type II is collagen type III which is a minor component of articular cartilage (Eyre, 2002). Collagen type VI is a microfibrillar collagen predominantly located close to the chondrocytes. Other collagens present in articular cartilage are collagen types XII and XIV which are FACIT collagens attached to fibril surfaces. Collagen type X is present around cells in the cartilage bone interface and around hypertrophic cells (Gelse *et al.*, 2003; Eyre, 2002; Poole, 1997).

Proteoglycans

The second largest constituent of the ECM dry weight is proteoglycans which are characterised by glycosaminoglycan (GAG) chains covalently linked to a core protein. Extracellular proteoglycans include proteins such as aggrecan and small leucine rich proteoglycans/proteins (SLRPs) and pericellular proteoglycans, e.g., perlecan (also called heparan sulphate proteoglycan 2, HSPG2). Proteoglycans can also be anchored to the cell surface, either as transmembrane proteins such as chondroitin sulphate proteoglycan 4 (CSPG4) and the syndecans, or anchored to glycosylphosphatidylinositol, e.g., the glypicans. The only intracellular proteoglycan is serglycin (Iozzo & Schaefer, 2015; Heinegård, 2009; Sophia Fox *et al.*, 2009; Roughley, 2006; Hardingham & Fosang, 1992).

Aggrecan is the most abundant proteoglycan forming large aggregates together with hyaluronan (HA). The core protein of aggrecan can be divided into the domains G1, G2 and G3. Interaction between aggrecan and HA occurs at the G1 domain and is stabilized by a link protein. G1 is located in the amino terminus of the core and connected to G2 via an inter-globular domain. The GAG chains are located between the G2 domain and the G3 domain at the carboxy terminus of the core protein. Keratan sulphate side chains are located closest to the G2 region and chondroitin sulphate side chains are located closest to the G3 domain. The G3 domain is important for post-translational processing and secretion of aggrecan (Roughley, 2006; Kiani *et al.*, 2002). Presence of specific cleavage sites allows enzymatic cleavage of the core protein and release of aggrecan fragments (Kiani *et al.*, 2002). The globular domains are highly conserved between species whereas the extending domains are less conserved. (Caporali *et al.*, 2015; Kiani *et al.*, 2002). The overall amino acid identity between human and equine aggrecan is 65.8% (Caporali *et al.*, 2015).

SLRPs are matrix molecules containing repeats of leucine residues which allow binding to collagen fibrils. These molecules are involved in formation and stabilization of the collagen network as well as in interactions between matrix components (Kalamajski & Oldberg, 2010; Heinegård, 2009; Schaefer & Iozzo, 2008). SLRPs can be divided into five classes based on their structural and functional properties. Class I includes biglycan, decorin, asporin, extracellular matrix protein 2 (ECM2) and extracellular matrix protein X (ECMX); class II includes fibromodulin, lumican, prolargin (also called proline/arginine-rich end leucine-rich repeat protein, PRELP), keratocan and osteoadherin; class III includes epiphycan, opticin and osteoglycin; class IV includes chondroadherin, nyctalopin and tsukushi; and, class V includes podocan and podocan-like 1 (Iozzo & Schaefer, 2015; Ni *et al.*, 2014).

Lubricin (also called proteoglycan 4), is a glycoprotein with a protective role in lubrication of articular joints as it reduces friction and chondrocyte apoptosis (Waller *et al.*, 2013; Rhee *et al.*, 2005). Chondrocytes in the superficial zone synthesize and secrete lubricin (Schumacher *et al.*, 1994).

The pericellular proteoglycan perlecan, initially isolated from basement membranes (Hassell *et al.*, 1980), provides cell adhesion to the matrix (Sundarraj *et al.*, 1995), and is involved in mechanotransduction (Wilusz *et al.*, 2012; Vincent *et al.*, 2007), cartilage development (Arikawa-Hirasawa *et al.*, 1999), angiogenesis (Aviezer *et al.*, 1994), and growth factor signalling (Chuang *et al.*, 2010; Aviezer *et al.*, 1994).

Transmembrane syndecans are heparan sulphate proteoglycans with various biological functions such as binding to growth factors, acting as co-receptors, and mediating endocytosis (Iozzo & Schaefer, 2015; Choi *et al.*, 2011). Cleavage by peptidases such as matrix metallopeptidases (MMPs) leads to subsequent shedding of syndecans. This results in a change in surface receptor dynamics and a soluble ectodomain. The ectodomain can exert biological effects or function as a competitive inhibitor of other syndecans (Manon-Jensen *et al.*, 2010).

Glypicans are other heparan sulphate proteoglycans that regulate biological signalling pathways including those involving Wnts, bone morphogenic proteins (BMPs), and fibroblast growth factors (FGFs). Glypicans are attached to the outer surface of the cell by a glycosylphosphatidylinositol anchor and can be released from the surface by lipase cleavage (Filmus *et al.*, 2008).

The intracellular proteoglycan serglycin had been detected in chondrocytes (Zhang *et al.*, 2010). Serglycin allow storage of compounds in granules inside the cells. The stored components can be released through regulated secretion initiated by, for example, inflammatory reactions. Serglycin can be secreted either constitutively or initiated by agents such as inflammatory stimuli (Kolset & Pejler, 2011).

Non-collagenous proteins

In addition to collagens and proteoglycans, there are several other proteins such as non-collagenous proteins and glycoproteins important for the structure and function of the ECM (Sophia Fox *et al.*, 2009).

Cartilage oligomeric matrix protein (COMP, also called thrombospondin 5), consist of 5 subunits linked together at their amino terminus. The carboxy terminus of each subunit can bind to other components of the ECM and thereby mediate matrix assembly and stability (Heinegård, 2009). COMP is considered a catalyst in collagen fibril formation due to its ability to assemble free collagens into fibrils (Halász *et al.*, 2007).

Matrilins, a group of non-collagenous proteins, bind to many other molecules of the ECM such as collagens, COMP, biglycan and decorin, and thereby mediate interactions important for the cartilage integrity (Klatt *et al.*, 2011; Heinegård, 2009; Deák *et al.*, 1999). Additionally it is suggested that matrilins are involved in mechanical stimulation of chondrocytes as the levels of matrilins affect the responsiveness of chondrocytes to mechanical load (Kanbe *et al.*, 2007). Increased gene expression of matrilin has also been seen as a response to mechanical stimulation (Wu & Chen, 2000).

Fibronectin is a glycoprotein protein present in many tissues and involved in interactions between cells and other molecules. Cellular processes such as adhesion, migration, differentiation and proliferation are influenced by signal transduction induced by fibronectin (Romberger, 1997).

Cartilage intermediate layer protein (CILP) is a non-collagenous protein identified in middle and deep zones of articular cartilage. The protein is synthesised by chondrocytes and located in the interterritorial matrix where it is suggested to be involved in maintenance of the tissue structure (Lorenzo *et al.*, 1998a; Lorenzo *et al.*, 1998b).

Receptors for matrix components

Communication between chondrocytes and their surrounding ECM is mediated through cell surface receptors. Signalling initiated by ECM components can affect the behaviour of the cell leading to changes in phenotype and altered metabolism (Loeser, 2014; Kim *et al.*, 2011; Knudson & Loeser, 2002). In addition to the above-mentioned proteoglycans; syndecans and glypicans, there are many other receptors with importance for cartilage homeostasis.

Cluster of differentiation 44 (CD44) is a transmembrane receptor containing a cytoplasmic tail, a hydrophobic transmembrane domain, and an extracellular domain (Goldstein *et al.*, 1989). CD44 is the primary receptor for HA (Aruffo *et al.*, 1990), but can also bind to other molecules such as lubricin (Al-Sharif *et al.*, 2015) and serglycin (Toyama-Sorimachi *et al.*, 1995). CD44 is responsible for endocytosis and degradation of HA in chondrocytes (Hua *et al.*, 1993). Other functions of HA binding to CD44 include inhibition of interleukin (IL)-1β induced production of MMPs (Julovi *et al.*, 2004) and assembly of the pericellular matrix (Knudson, 1993).

Integrins are transmembrane glycoproteins composed of α- and β-subunits that mediate interactions between chondrocytes and the ECM. Integrin signalling regulates cellular responses such as proliferation, differentiation, survival, and matrix remodelling (Loeser, 2014; Forsyth *et al.*, 2002; Giancotti & Ruoslahti, 1999). Integrin α10β1 is the primary collagen type II-binding integrin (Camper *et al.*, 1998). Other ECM molecules such as matrilin,

chondroadherin, COMP and fibronectin can also bind to the integrins (Loeser, 2014). Integrins also act as mechanoreceptors involved in mechanotransduction (Chen *et al.*, 2013a; Shimizu *et al.*, 2004; Wright *et al.*, 1997).

1.3 Development of synovial joints

Synovial joints develop as a result of chondrogenesis that starts with formation of a cartilage model. This is followed by endochondral ossification where the cartilage model is gradually replaced by bone. Recruitment and condensation of mesenchymal cells lead to formation of the interzone and differentiation of the cells into chondrocytes during chondrogenesis (Khan *et al.*, 2007; Goldring *et al.*, 2006). A primary ossification centre is formed in the centre of the diaphysis of the cartilaginous bone. This is followed by the formation of secondary ossification centres in the epiphyses, leaving cartilaginous growth plates between the primary and secondary ossification centres. Epiphyseal articular cartilage covers the surface ends of the bone towards the joint cavities (Figure 3) (Mackie *et al.*, 2011).

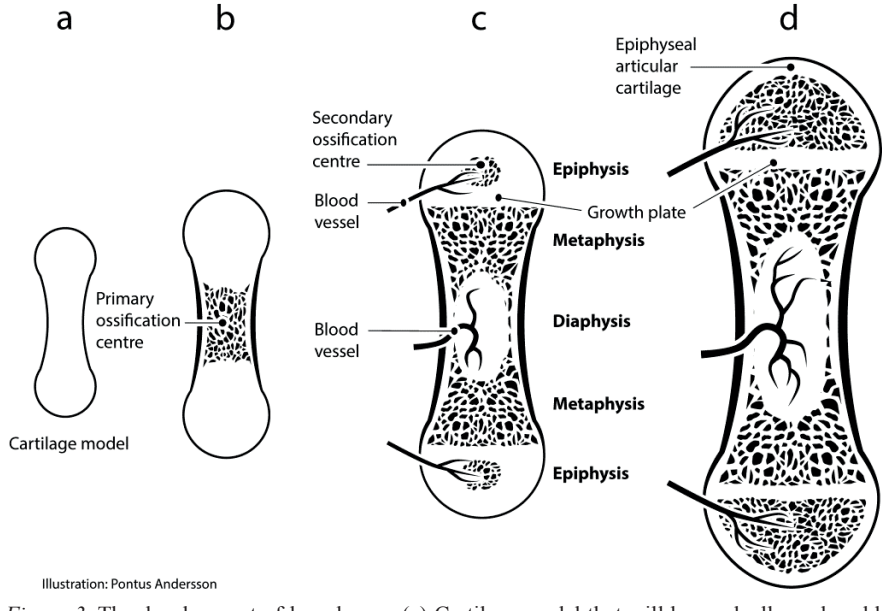

Figure 3. The development of long bones. (a) Cartilage model that will be gradually replaced by bone during endochondral ossification. (b) Formation of the primary ossification centre in the diaphysis followed by (c) formation of the secondary ossification centres in the epiphyses. Blood vessels are invading the tissue. (d) Further ossification gradually replaces the vascular growth cartilage with bone leaving the avascular articular cartilage at the ends of the bone.

The replacement of cartilage by bone occurs as a process of endochondral ossification when resting chondrocytes proliferate and become hypertrophic cells that undergo apoptosis (Khan *et al.*, 2007; Goldring *et al.*, 2006). Hence, the growth plate is divided into resting, proliferative, hypertrophic and ossification zones (Figure 4) (Behonick & Werb, 2003). In contrast to mature articular cartilage, which is avascular, there are vessels invading the tissue during development. Vascularisation is important during endochondral ossification as a source of factors influencing growth and differentiation of cells (Walzer *et al.*, 2014; Gerber & Ferrara, 2000; Bittner *et al.*, 1998). The processes occurring during joint development are regulated by factors such as interactions between matrix and cells, growth and differentiation factors, and various signalling pathways (Goldring *et al.*, 2006; Archer *et al.*, 2003). The ECM molecules are important regulators with the ability to signal directly to the cells in the microenvironment as well as affect signalling molecules and thereby signal to surrounding cells. The chondrocytes in the different zones can additionally produce different types of ECM molecules as a response to various stimuli and thereby affect the organisation of the surrounding ECM. One example is that the hypertrophic cells specifically synthesise collagen type X (Behonick & Werb, 2003).

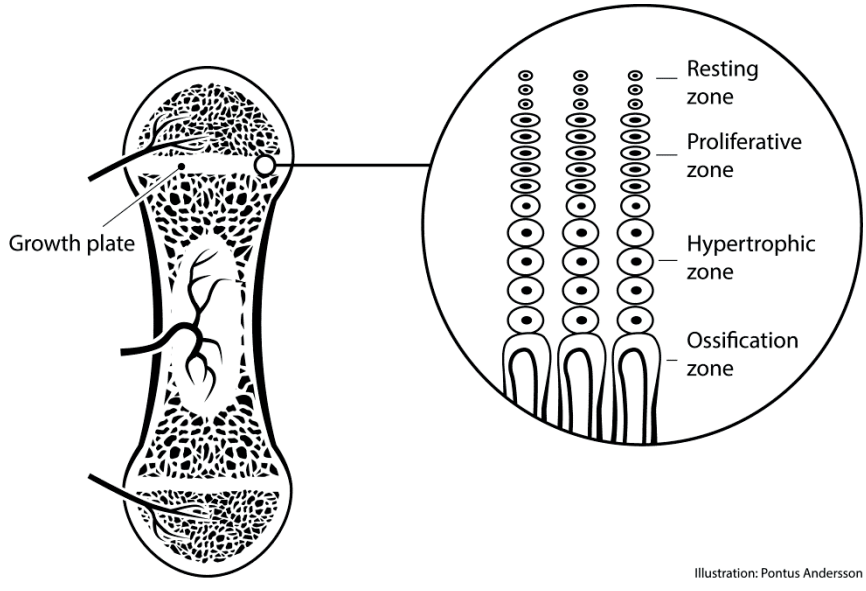

Figure 4. Zones of the growth plate. Chondrocytes in the resting zone start to proliferate, become hypertrophic, and undergo apoptosis leading to ossification of the cartilage into bone.

The perichondrial groove of Ranvier is a fibrochondrosseous structure encircling the growth plate (Figure 5). The structure was first described by Ranvier in 1873 and suggested to be important in the development of bones (Shapiro *et al.*, 1977). Cells with different morphology—including progenitor cells—have been identified in the zone of Ranvier (Shapiro *et al.*, 1977) and further studies have identified a stem cell niche in the same area (Karlsson *et al.*, 2009). A potential migration route—containing transit amplifying cells from the zone of Ranvier towards the articular cartilage—has also been characterised (Henriksson *et al.*, 2013).

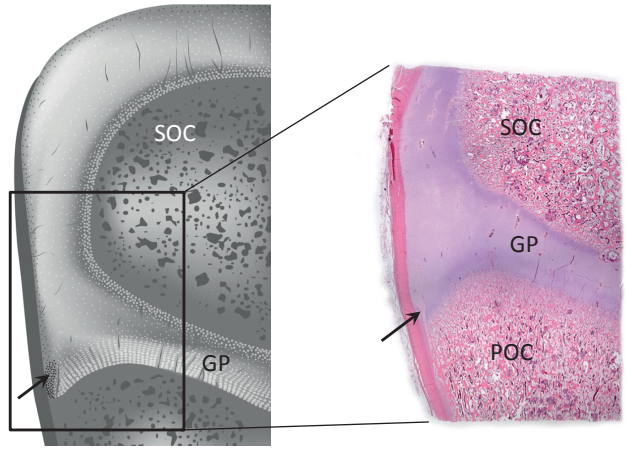

Figure 5. Location of the zone of Ranvier (arrow) in growth cartilage (left) and in equine growth cartilage stained with haematoxylin and eosin (right). SOC, secondary ossification centre; GP, growth plate; POC, primary ossification centre. Schematic drawing (left) by Pontus Andersson, modified from Paper III (Löfgren *et al.*, 2014), and reproduced with permission from the publisher.

Progenitor cells identified in articular cartilage have the potential to be involved in growth and repair of cartilage (Alsalameh *et al.*, 2004; Dowthwaite *et al.*, 2004; Hayes *et al.*, 2001). These cells are not fully characterised and there is no definitive marker for their identification in cartilage tissue. There are, however, a number of stem cell-related-markers that alone or in combination with each other can be used to characterise the progenitor cells (Jiang & Tuan, 2015).

Stro-1 is a potential stem cell-marker which has been identified in human, equine, rabbit and murine articular cartilage (Ozbey *et al.*, 2014; McCarthy *et al.*, 2012; Otsuki *et al.*, 2010; Williams *et al.*, 2010; Grogan *et al.*, 2009; Karlsson *et al.*, 2009), and additionally in the perichondrial groove of Ranvier in rabbits (Karlsson *et al.*, 2009).

Notch1 is a cell surface receptor detected in progenitor cells isolated from bovine, human and equine articular cartilage (McCarthy *et al.*, 2012; Williams *et al.*, 2010; Dowthwaite *et al.*, 2004). Signalling through the Notch1 receptor is involved in the development of various cell types including chondrocytes (Williams *et al.*, 2009; Karlsson *et al.*, 2007; Hardingham *et al.*, 2006; Dowthwaite *et al.*, 2004; Hayes *et al.*, 2003; Watanabe *et al.*, 2003). Notch1 is one of four Notch receptors (Notch1-4) in mammals. The signalling is mediated through binding of ligands to the receptors, leading to translocation of the Notch intracellular domain into the nucleus of the cells and transcription of target genes such as the hairy enhancer of split (Hes) family. The largest family of ligands contains a Delta/Serrate/LAG2 (DSL) domain and includes Jagged1, Jagged2, Delta-like (Dll)1, Dll3 and Dll4 in mammals (Wang, 2011; D'Souza *et al.*, 2010; Kopan & Ilagan, 2009). The DSL ligands signal through canonical Notch signalling in which the ligand is located on a signal-sending cell in proximity to a signal-receiving cell expressing the Notch receptor. However, there are several secreted or membrane bound proteins lacking DSL domains that modify Notch signalling. These proteins are referred to as non-canonical ligands and include several proteins that act in a context-dependent matter (Wang, 2011).

Epidermal growth factor-like domain 7 (EGFL7) is a non-canonical Notch signalling modifier that binds Notch receptors and inhibits Jagged1 mediated signalling (Schmidt *et al.*, 2009). EGFL7 is a secreted protein with known roles in vascular development and cell migration (Campagnolo *et al.*, 2005; Parker *et al.*, 2004; Soncin *et al.*, 2003). Reduced proliferation and self-renewal have been observed in neural stem cells as a result of EGFL7 mediated reduction in Notch signalling (Schmidt *et al.*, 2009).

1.4 Osteoarthritis (OA)

OA involves the entire joint with morphologic changes such as articular cartilage degradation, synovitis, and subchondral bone remodelling with sclerosis and osteophyte formation (Loeser *et al.*, 2012). OA in horses presents similarities as well as differences to human OA. Equine OA often affects synovial joints subjected to high and frequent mechanical load during racing. Single event trauma, as well as poor conformation of the joints causing focal high mechanical load, are other causes of equine OA (Kidd *et al.*, 2001). These factors are also relevant for the initiation of OA in humans, although age is also a major factor (Goldring & Goldring, 2007). The similarities between OA in athletic horses and humans make the horse a suitable model to study the

pathogenesis of naturally occurring OA in humans (McIlwraith *et al.*, 2012; Schlueter & Orth, 2004).

1.4.1 Initiation and progression of OA

In normal joints, there is a balance between anabolic and catabolic processes. In OA, these processes are in imbalance leading to initiation and progression of the disease. OA always includes inflammation and subsequent degeneration of the articular cartilage in the affected joints. This results in destruction of the connective tissues, with clinical signs such as joint swelling and pain, with subsequent lameness (Goldring & Goldring, 2007; Schlueter & Orth, 2004; Kidd *et al.*, 2001). Macroscopic changes characteristic for OA in articular cartilage include wear lines and erosions ranging from partial- to full-thickness (Figure 6) (McIlwraith *et al.*, 2010).

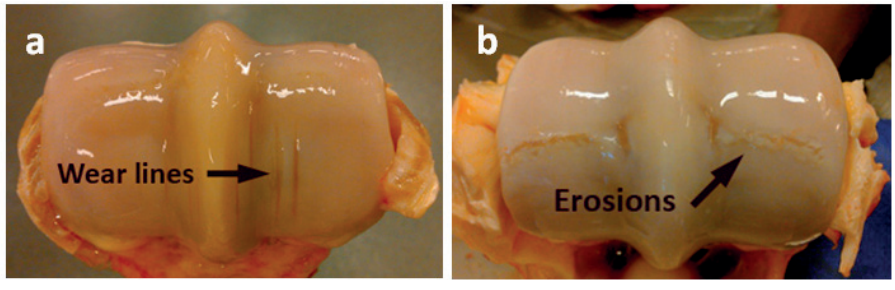

Figure 6. Macroscopical lesions in articular cartilage from the distal third metacarpal bone of the fetlock joint showing a) wear lines, and b) erosions.

Late-stage OA also includes subchondral bone remodelling and osteophyte formation (Loeser *et al.*, 2012). Mechanical load—considered the major factor leading to equine OA—affects the activity of the chondrocytes. Although excessive mechanical load is a risk factor for OA development, some load is necessary to maintain the normal homeostasis of the joints (Guilak *et al.*, 2004). Moderate load leads to increased ECM synthesis and decreases expression of pro-inflammatory and catabolic genes. In contrast, abnormally high load results in apoptosis and necrosis of chondrocytes, ECM catabolism, and synthesis of inflammatory mediators (Sanchez-Adams *et al.*, 2014). The fragments released during ECM degradation, and the inflammatory mediators in combination with continued abnormal load, further progress the disease leading to continued degradation, inflammation and phenotypical changes of the chondrocytes (Goldring & Goldring, 2007). Fragments of collagen type II and fibronectin induce expression of matrix-degrading enzymes; this leads to further degradation of the ECM (Guo *et al.*, 2009; Homandberg *et al.*, 1997). Fragments of fibronectin are also involved in the progression of OA through

induction of pro-inflammatory cytokines (Homandberg *et al.*, 1997). The balance between pro-inflammatory and anti-inflammatory cytokines as well as the level of growth factors and released matrix fragments influence the progression of the disease (Goldring, 2000). See a summary of the progression of OA in figure 7.

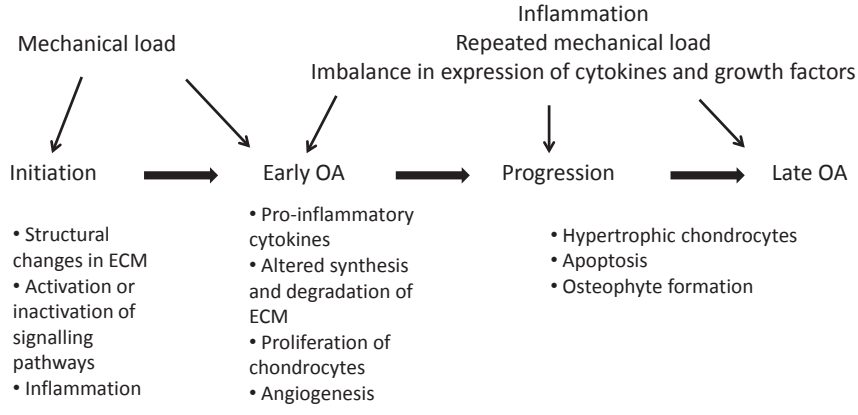

Figure 7. The initiation and progression of OA. Causative factors are listed above and changes occurring during the different stages of OA are listed below. Adapted from Goldring, M. B. and Goldring, S. R. (2007) Osteoarthritis. *Journal of Cellular Physiology,* 213(3), pp. 626-634.

1.4.2 Inflammation

Inflammation during the progression of OA involves several mediators produced by synoviocytes and chondrocytes. The cytokines IL-1β and tumor necrosis factor (TNF)-α are considered key players in the OA-related inflammation. Inflammatory mediators, released matrix products, and external influences such as high mechanical load, stimulate the cells to release cytokines. These cytokines act on cells nearby or on the cells producing them (Goldring, 2000; Westacott & Sharif, 1996). In addition to IL-1β and TNF-α, there are several other pro-inflammatory cytokines involved in OA-associated inflammation. These include IL-1α, IL-6, leukemia inhibitory factor (LIF), oncostatin-M, IL-15, IL-17, IL-18 and IL-21. Some of them, such as IL-6, LIF and oncostatin-M, are regulatory cytokines. There are also a number of anti-inflammatory cytokines such as IL-4, IL-10, IL-11, IL-13, IL-1 receptor antagonist (IL-1Ra) and interferon-γ which counteract the pro-inflammatory actions (Kapoor *et al.*, 2011; Fernandes *et al.*, 2002; Goldring, 2000). The actions of the pro-inflammatory cytokines can also be counteracted by growth factors promoting synthesis of new matrix. These growth factors include proteins in the families of transforming growth factor-β, BMPs, FGFs and insulin-like growth factor-I (Goldring, 2000).

Other mediators of inflammation are the chemokines which increase ECM degradation through MMPs and induce phenotypic changes (Mazzetti *et al.*, 2004; Merz *et al.*, 2003; Borzì *et al.*, 2000). The acute phase response with increased levels of proteins such as C-reactive protein and serum amyloid A (SAA) are also part of the inflammatory process in OA (de Seny *et al.*, 2013; Sipe, 1995). The pathogenesis of OA also involves the complement system triggered by inflammation, and released matrix components. Activation of complement leads to further production of matrix-degrading enzymes and inflammatory mediators (Wang *et al.*, 2011). Fibromodulin, chondroadherin and osteoadherin are matrix molecules that activate the classical complement pathway and, to a lesser extent, the alternative pathway (Sjöberg *et al.*, 2009a; Sjöberg *et al.*, 2005). The matrix molecules also has the ability to inhibit the complement cascade through binding to inhibitors of the signalling (Sjöberg *et al.*, 2009b). COMP regulates the complement cascade by activation of the alternative pathway and by inhibition of the classical and lectin pathways (Happonen *et al.*, 2010).

IL -1β

IL-1β, a member of the IL-1 family associated with inflammation (Van de Veerdonk & Netea, 2013), is detected in synovial fluid, synovial membrane, cartilage and subchondral bone in OA joints (McNulty *et al.*, 2013; Kapoor *et al.*, 2011; Saha *et al.*, 1999; Towle *et al.*, 1997). Active IL-1β is formed after cleavage of a 31 kDa inactive IL-1β precursor to yield the 17.4 kDa active product (Black *et al.*, 1988). The peptidase IL-1β converting enzyme, also called caspase-1, is responsible for the cleavage into active IL-1β (Wilson *et al.*, 1994; Kronheim *et al.*, 1992). There are two types of IL-1 receptors (IL-1R) with the ability to bind IL-1β; these are located on cell surfaces. Binding of IL-1β to IL-1R type I induces a signal transduction resulting in biological effects whereas binding to IL-1R type II does not induce any signalling (Auron, 1998; Dinarello, 1996). The receptors can also be shed from the cell surface; binding of IL-1β to these soluble receptors will not transduce any signalling. Instead, these receptors function as antagonists of IL-1β induced signalling (Dinarello, 1996). IL-1β signalling can also be antagonized by IL-1Ra, another member of the IL-1 family, which interacts with the same receptors as IL-1β and prevents the binding and signalling induced by IL-1β (Arend *et al.*, 1998).

Signalling pathways activated by IL-1β include nuclear factor κB (NFκB) and the mitogen-activated protein kinase (MAPK) pathways (Fan *et al.*, 2007; Auron, 1998). IL-1β initiates phosphorylation of inhibitory κB (IκB) leading to release of NFκB from IκB into the nucleus where NFκB acts as a transcription

factor (Tak & Firestein, 2001; Dinarello, 1996). The three main MAPK pathways activated by IL-1β involve the enzymes c-Jun NH_2-terminal kinase (JNK), 38-kd protein kinase (p38), and extracellular signal-regulated kinase (ERK). The MAPKs activate transcription factors needed for expression of target genes (Fan *et al.*, 2007; Auron, 1998). IL-1β signalling results in increased levels of several factors involved in the progression of OA such as other cytokines, chemokines, inducible nitric oxide synthase, phospholipase A2, cyclooxygenase 2, prostaglandins, and matrix-degrading enzymes (Kapoor *et al.*, 2011; Goldring, 2000).

TNF-α

TNF-α is detected in OA synovial fluid, synovial membrane, cartilage and subchondral bone (Kapoor *et al.*, 2011; Martel-Pelletier *et al.*, 1999). It activates pro-inflammatory and catabolic signalling pathways similar to those activated by IL-1β (Kapoor *et al.*, 2011). Cleavage of membrane-bound TNF-α by the TNF-α converting enzyme yields a biologically active, soluble TNF-α with the ability to bind receptors (Black *et al.*, 1997; Aggarwal *et al.*, 1985). Two cell-membrane bound receptors for TNF-α, TNF receptor type I (TNFRI or p55) and TNF receptor type II (TNFRII or p75) exist. TNFRI is the main receptor for TNF-α in OA (Kapoor *et al.*, 2011; Westacott & Sharif, 1996; Loetscher *et al.*, 1990; Schall *et al.*, 1990). TNF-α can also bind to soluble forms of these receptors resulting in inhibition of the TNF-α signalling pathways (Higuchi & Aggarwal, 1992).

1.4.3 Changes in articular cartilage during OA

Dysregulation of chondrocyte function and remodelling of the ECM are features of OA leading to the destruction of articular cartilage (Loeser *et al.*, 2012; Goldring & Goldring, 2007). Chondrocytes in OA articular cartilage recapitulate a developmental program including phenotypical changes similar to the processes occurring during endochondral ossification (Tchetina, 2011; Sandell & Aigner, 2001). The early stages of OA include phenotypical changes such as proliferation of progenitor cells and the late stages include terminal differentiation towards hypertrophy and apoptosis (Tchetina, 2011; Goldring & Goldring, 2010; Goldring & Goldring, 2007; Sandell & Aigner, 2001). Microscopic changes in OA articular cartilage include focal cell loss, superficial fibrillation, fissures, and erosions (Figure 8) (McIlwraith *et al.*, 2010; Pritzker & Aigner, 2010; Pritzker *et al.*, 2006). Increased number and size of chondrocyte clusters, defined as multiple chondrocytes sharing pericellular matrix (Figure 8) (Pritzker & Aigner, 2010), is a characteristic of

OA cartilage and is assumed to be a result of chondrocyte proliferation towards hypertrophic cells synthesising type X collagen (Lotz et al., 2010).

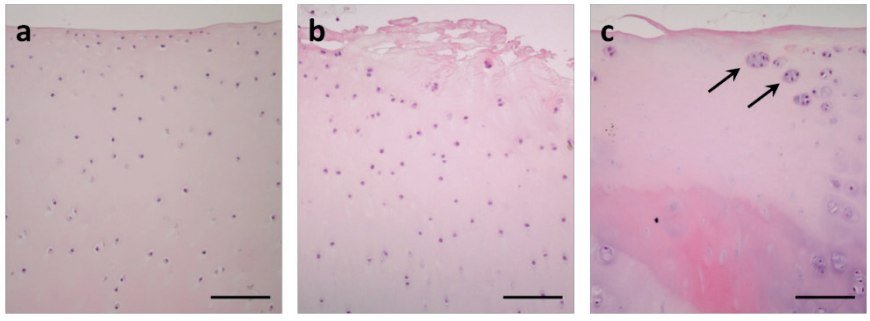

Figure 8. Light microscopic sections of equine articular cartilage stained with haematoxylin and eosin showing a) normal articular cartilage, b) fibrillated surface of articular cartilage, and c) focal cell loss and chondrocyte clusters (arrows). Scale bar: 100 µm.

Chondrocyte activity is altered during the different stages of OA. Anabolic activity leads to synthesis of ECM proteins, whereas catabolic activity leads to synthesis of matrix-degrading enzymes. Degradation of articular cartilage occurs when the anabolic activity cannot withstand the actions of the degrading enzymes. The degradation includes a gradual depletion of proteoglycans followed by disruption of the collagen network (Goldring & Goldring, 2010).

Matrix-degrading enzymes such as a disintegrin and metallopeptidase with thrombospondin motifs (ADAMTS) and the MMPs are increased in OA cartilage and are responsible for degradation of the ECM (Troeberg & Nagase, 2012; Heinegård & Saxne, 2011; Goldring & Goldring, 2010). Inflammatory stimuli such as cytokines induce the expression of matrix-degrading enzymes but these can also be induced by factors such as released matrix fragments (Goldring & Otero, 2011) and mechanical load (Kurz *et al.*, 2005). The activity of MMPs and ADAMTS can be inhibited by tissue inhibitors of metallopeptidases (TIMPs) (Brew & Nagase, 2010). ADAMTS-4 and 5 are the major enzymes responsible for cleavage of aggrecan (Troeberg & Nagase, 2012). MMP-13, also called collagenase-3, is the primary collagenase involved in the OA process (Troeberg & Nagase, 2012). MMP-13 is involved in the cleavage and denaturation of collagen type II (Billinghurst *et al.*, 1997; Knäuper *et al.*, 1996) but can also cleave aggrecan (Fosang *et al.*, 1996). Other MMPs such as MMP-1 (collagenase-1), MMP-3 (stromelysin-1), MMP-8 (collagenase-2), MMP-9 (gelatinase B) and MMP-14 (membrane-type-1 MMP) are also involved in the degradation of cartilage, either by degradation of ECM directly or by activation of other matrix-degrading enzymes (Troeberg & Nagase, 2012; Billinghurst *et al.*, 1997).

1.4.4 Diagnosis of equine OA

Lameness examination of horses includes a physical palpation of the joint for pain, presence of swelling, heat and effusion of the joint, and evaluation of how the horse moves (Kidd *et al.*, 2001; Trotter & McIlwraith, 1996). Subjective evaluation of the movements is routinely performed although objective methods for measuring asymmetry are emerging as a tool for lameness evaluation (Keegan, 2007). Flexion tests and intra-articular anaesthesia aid in the localisation of the specific joint responsible for the lameness (Kidd *et al.*, 2001; Trotter & McIlwraith, 1996). The diagnosis of OA is based on different imaging techniques or arthroscopy. Radiology is the most common imaging technique. It is user-friendly and non-invasive, but the disadvantage is that early OA cannot be diagnosed due to the limited possibility to visualize non-mineralised tissue. Hence the technique cannot detect cartilage degeneration (Kidd *et al.*, 2001; Park *et al.*, 1996). Arthroscopy is an invasive method that allows direct visualisation of the joint structures and thereby detection of superficial cartilage fibrillation, fissures, and erosions (Kidd *et al.*, 2001; Trotter & McIlwraith, 1996).

Biomarkers

The use of biomarkers as a tool for diagnosing OA is of interest in humans as well as equines. Soluble biomarkers in synovial fluid, blood or urine would be useful for the diagnosis of OA, classification of disease stages, prognosis, and monitoring of treatment response. The markers are often inflammatory mediators or molecules involved in ECM remodelling, degradation or repair (Bay-Jensen *et al.*, 2016; Felson, 2014; Mobasheri, 2012). COMP and carboxy-terminal cross-linked fragment of type II collagen (CTX-II) are the best validated biomarkers in humans (Bay-Jensen *et al.*, 2016). There is a need for further development and validation of biomarkers that accurately reflect the disease stages since the current biomarkers cannot differentiate normal ECM metabolism from pathological processes (Felson, 2014; Mobasheri, 2012).

2 Aims and hypotheses of the thesis

The overall aim of the thesis was to follow cartilage changes resulting from cellular, genetic and molecular events during inflammation and development of cartilage.

The overall hypothesis was that specific events occur at distinct time points that could be used to distinguish the different stages of OA.

The aim of Paper I was to study the expression and localisation of proteins in developing equine cartilage comprising chondrocytes of different phenotypes.

The hypothesis was that the phenotypes could be characterised based on expression of matrix molecules, Notch signalling components, and the stem cell-indicating factor Stro-1.

The aim of Paper II was to follow the progression of inflammation in equine OA longitudinally. An *in vitro* inflammatory model was used to mimic the progression of inflammation in equine articular cartilage longitudinally to follow secretion of matrix molecules and inflammatory mediators induced by IL-1β.

The hypothesis was that matrix molecules and inflammatory mediators would be secreted in a time-dependent pattern, thereby allowing differentiation between early and late stage inflammation.

The aim of Paper III was to follow the progression of inflammation in equine OA longitudinally. An *in vitro* inflammatory model was used to mimic the progression of inflammation in equine articular cartilage longitudinally to follow changes in gene expression induced by IL-1β.

The hypothesis was that gene expression of inflammatory mediators, matrix molecules, and phenotypic markers would change in a time-dependent pattern allowing differentiation between early and late stage inflammation.

3 Material and methods

This section summarises the material and methods used in the studies included in this thesis. Detailed descriptions of the procedures are presented in each of the papers.

3.1 Sample collection

Collection of equine cartilage samples was approved by the Ethical Committee on Animal Experiments, Stockholm, Sweden (Dnr: N378/12) and the Ethical Committee on Animal Experiments, Uppsala, Sweden (Dnr: C62/13).

3.1.1 Equine growth cartilage (Paper I)

Equine osteochondral slabs of the distal radius were collected post-mortem from two foetuses (6.5 and 10 months gestational age) and from two young horses (2-day-old and 1-year-old) without clinical signs of orthopaedic disease and no macroscopic or microscopic lesions of the sampled joints. Growth-plate cartilage was sampled from all four individuals and articular cartilage was collected from the 6.5-month-old foetus, the 2-day-old and the 1-year-old horse. The slabs were fixed in 10% neutral buffered formalin, decalcified in 3.4% (w/v) sodium formiate and 15.1% (v/v) formic acid, and embedded in paraffin.

3.1.2 Equine articular cartilage explants (Papers II and III)

Full-thickness cartilage explants (5-mm in diameter) were harvested aseptically from the distal third metacarpal bone post-mortem. The joints were macroscopically and microscopically normal and the horses had no clinical history of joint disease. Three horses with mean age 3 years were included in Paper II and 3 horses with mean age 6 years were included in Paper III.

3.2 Histology (Papers I-III)

Paraffin-embedded tissues were sectioned and stained with haematoxylin and eosin (Papers I-III) for morphological evaluation; with picrosirius red (Paper I) for detection of collagen fibers; and safranin O (Paper II), and toluidine blue (Paper III) for evaluation of proteoglycan content.

3.3 Immunohistochemistry (IHC) (Paper I)

Protein localisation was delineated with IHC using primary antibodies for the surface antigen Stro-1, Notch signalling components Notch1, Jagged1, Dll4 and Hes1, the Notch dysregulating protein EGFL7, and the matrix molecules COMP, fibromodulin, matrilin-1 and chondroadherin. The sections were digested with hyaluronidase and chondroitinase ABC. Endogenous peroxidase activity was quenched using hydrogen peroxide. Isotype controls were run in parallel with the primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies were used for detection of bound primary antibodies. Visualization was done with a TSA-Direct Cy3 kit and nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) dihydrochloride. Subjective evaluation of the sections was performed using a Nikon Eclipse E600 fluorescence microscope and NIS Elements Basic Research software, version 3.22.11. The expression of Notch signalling components and Stro-1 was graded subjectively from the staining as: absent, a few, many, or a majority of cells stained. The matrix molecules were graded as follows: absent, weak, moderate, or intense staining.

3.4 Proximity ligation assay (PLA) (Paper I)

Proximity between Notch1 and EGFL7 in equine growth cartilage was studied using Duolink™ *in situ* PLA technology. The sections were treated as for IHC until application of primary antibodies, and thereafter according to the manufacturer's instructions. In brief, the two antibodies were added to the same section and proximity in location (<40 nm) resulted in amplification of a detectable fluorescent signal. Negative controls omitting one of the antibodies were run in parallel. Subjective evaluation of the sections was performed using a Nikon Eclipse E600 fluorescence microscope and NIS Elements Basic Research software, version 3.22.11.

3.5 Culture of articular cartilage explants (Papers II and III)

In vitro studies of cartilage can be performed using different models such as chondrocytes grown in monolayer or three-dimensional pellet cultures. Explants from articular cartilage used in papers II and III allowed studies of chondrocytes within their surrounding ECM. The overall workflow is shown in figure 9 and the more specific details are described below.

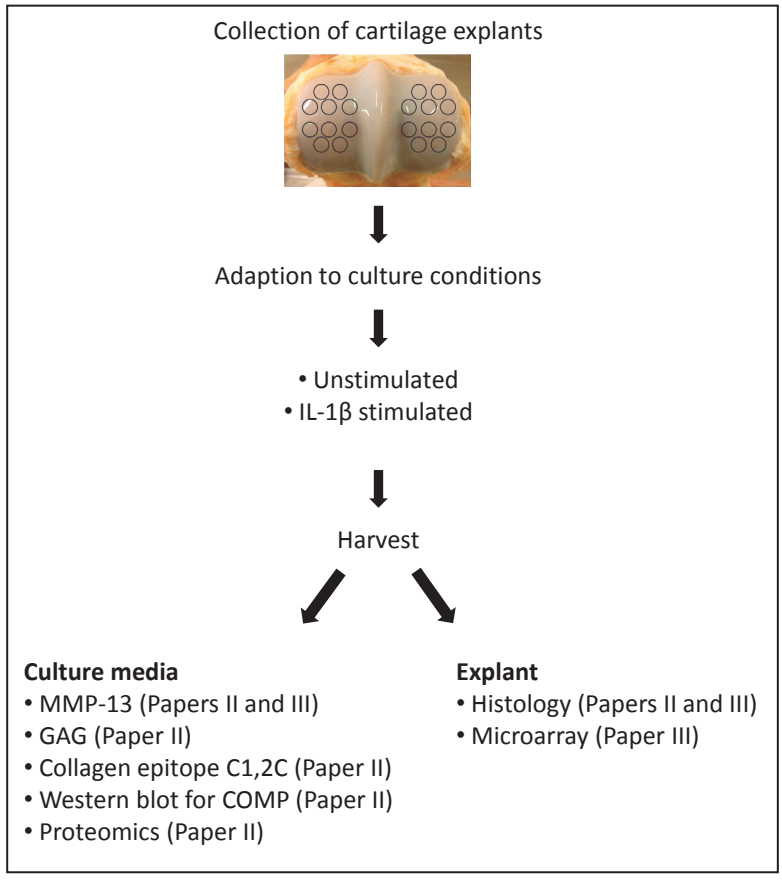

Figure 9. Workflow of cartilage explant culture including harvest of explants, adaption to culture conditions for 24-48 hours, stimulation with interleukin-1β (IL-1β) or with culture media as unstimulated controls followed by harvest of explants and culture media. Culture media was collected at day 0, 3, 6, 9, 12, 15, 18, 22 and 25 (Paper II) or at day 0, 3, 9, 15, 21 and 27 (Paper III). Cartilage explants were harvested at day 25 (Paper II) or at day 0, 3, 9, 15, 21 and 27 (Paper III). This was followed by analysis of culture media and explants.

3.5.1 Stimulation of cartilage explants (Papers II and III)

Equine cartilage explants were incubated in cell culture media containing Dulbecco's modified Eagle medium Nutrient Mixture F-12 supplemented with 0.1 mg/ml cell culture-tested bovine serum albumin, 0.1 mg/ml ascorbic acid, and 4% penicillin/streptomycin for 24 h (Paper II) or 48 h (Paper III) at 37°C in 7% CO_2/93% air to allow adaption to culturing conditions.

The medium was removed from the explants and new cell culture medium containing 10 ng/ml recombinant equine IL-1β was added to the wells. Medium without IL-1β was added to the unstimulated controls. The explants were cultured at 37°C in 7% CO_2/93% air and the medium was changed every third day.

3.5.2 Harvest of cartilage explants and media (Papers II and III)

Media were collected at day 0, 3, 6, 9, 12, 15, 18, 22 and 25 (Paper II) or at day 0, 3, 9, 15, 21 and 27 (Paper III) after start of stimulation. The media were stored at -80°C until further analysis. Cartilage explants were harvested from one well containing medium with, and one well without, IL-1β at 25 days (Paper II) or at all time points (Paper III). The explants were fixed in buffered formalin (Papers II and III) for 24h followed by paraffin embeddment. Two cartilage explants from each group were harvested at all time points (Paper III) and stored in sterile phosphate buffered saline (PBS) until isolation of ribonucleic acid (RNA). The total number of explants harvested from each well was weighed prior to different analyses.

3.5.3 Analysis of culture media

Analysis of MMP-13 in media (Papers II and III)

MMP-13 content in culture media was measured using Fluorokine® E Human Active MMP-13 Fluorescent Assay. The assay was performed according to the manufacturer's instructions using p-aminophenylmercuric acetate (APMA) which activates any potentially active forms of MMP-13. All samples were assayed in duplicate and the lower limit of detection was 8 pg/ml.

GAG measurements (Paper II)

Sulphated GAG content was measured in cell culture media using the 1,9-Dimethylmethylene blue assay (Farndale *et al.*, 1986). Samples were compared to a standard curve containing chondroitin sulphate, assayed in duplicate and normalized for cartilage wet weight.

Assay for collagen epitope C1,2C (Paper II)

Cleavage of type I and type II collagens by collagenases generates a three-quarter length carboxyterminal neoepitope fragment (C1, 2C) which was measured using a commercial enzyme-linked immunosorbent assay (ELISA). Samples were assayed in duplicate and the assay performed according to the manufacturer's instructions. The limit of detection was 0.03 µg/ml.

Western blot for COMP (Paper II)

COMP detection in culture media was done using an anti-bovine polyclonal antibody in a western blot. The samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli protocol (Laemmli, 1970) and thereafter transferred to a nitrocellulose membrane. The membrane was blocked in skimmed milk, followed by incubation with the primary antibody and thereafter addition of the secondary antibody. Chemiluminescence detection reagents were used for detection of immunoreactive bands.

3.5.4 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of tandem mass tag (TMT) labelled culture media (Paper II)

Shotgun proteomics was used to identify proteins released into the culture media of equine cartilage explants. Labelling with TMT-reagents was performed to quantify the levels of proteins and to compare the protein content in media from IL-1β stimulated and unstimulated explants. The TMTs are tags composed of a reporter group, a mass-normalization spacer, and a chemically reactive group. The tags are chemically identical and have the same overall mass, but contain different isotopes. After fragmentation, the isotopes can be distinguished from each other and thereby allow relative quantification of the peptides (Dayon & Sanchez, 2012; Thompson *et al.*, 2003). The overall workflow is shown in figure 10 and the more specific details are described below.

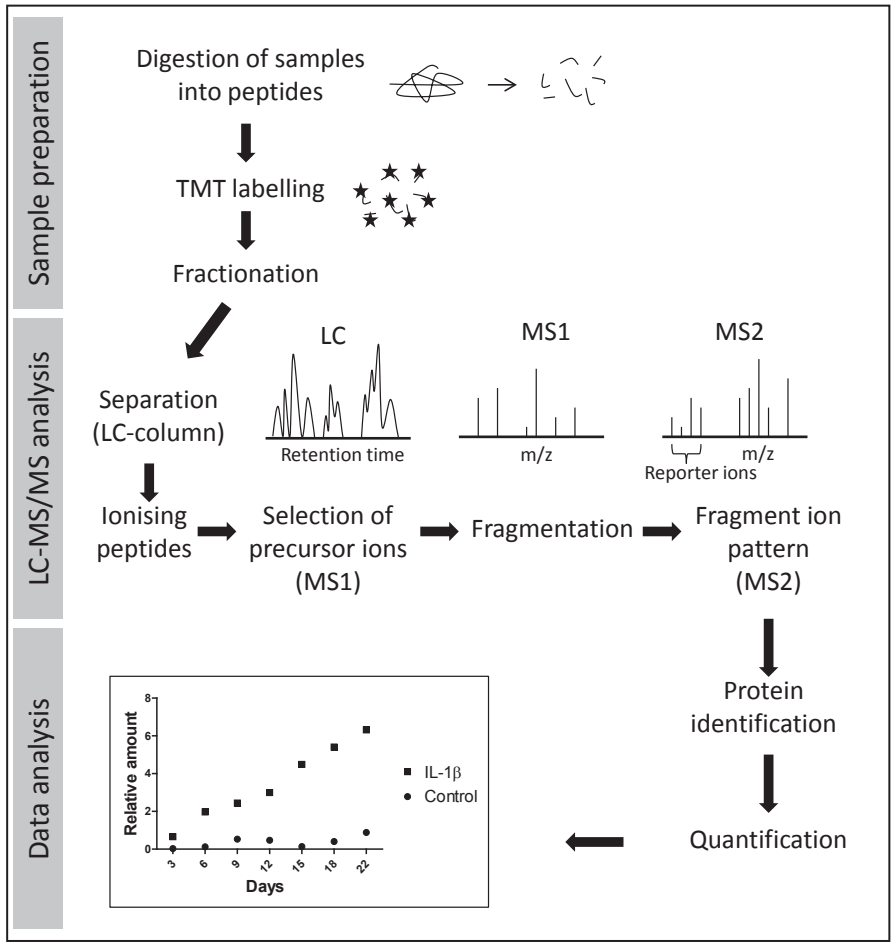

Figure 10. Workflow of proteomic analysis of culture media including sample preparation with tandem mass tag (TMT) labelling, liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, and data analysis.

Sample preparation

Culture media were concentrated on 3 kDa molecular weight cut-off filters and thereafter washed with water and 0.5 M triethyl ammonium bicarbonate until a pH >8 was reached. Sodium dodecyl sulphate was added to the samples and the proteins were reduced and alkylated prior to digestion with trypsin. A reference sample containing an aliquot of each sample was prepared in parallel with the samples.

TMT labelling

Samples were labelled with TMT reagents 126, 127, 128, 129, 130 or 131 according to the manufacturer's instructions. The reactions were quenched and the labelled samples were combined and concentrated. This was followed by separation by Strong Cation Exchange Chromatography (SCX) on an ÄKTA purifier system. The peptides were acidified and injected into a PolySULFOETHYL A™ SCX column. The peptide-containing fractions were desalted on PepClean C18 columns and evaporated to dryness.

LC-MS/MS analysis

The fractions were analysed on an LTQ-Orbitrap-Velos mass spectrometer interfaced with an in-house constructed nano-LC column. The peptides were trapped on a pre-column and separated on a reverse-phase column. The LTQ-Orbitrap-Velos was operated in data-dependent mode with one MS1 FTMS precursor ion scan followed by collision-induced dissociation (CID) and high-energy collision dissociation (HCD) MS2 scans of the five most abundant doubly or triply protonated ions in each precursor scan. All samples were analysed a second time excluding *m/z* identified at 1% false discovery rate (FDR) on the first MS-analysis.

Database search and TMT quantification

Protein identification and relative quantification was performed using Proteome Discoverer version 1.3. Database searches were performed using the Mascot search engine against the ENSEMBL equine protein database version 2.63 as well as the Uniref100 mammalian protein database. The TMT reporter ion intensities were divided by their reference reporter ion intensities yielding a ratio for each quantified peptide. Protein ratios were calculated as the median value for all the unique peptides for the given protein. No normalisation was applied and the albumin ratio was used to validate the variation in sample preparations.

3.5.5 RNA isolation (Paper III)

Isolation of RNA from the chondrocytes was performed using a modified method (Ali & Alman, 2012). The method included isolation of the chondrocytes from their surrounding ECM through mincing with a scalpel prior to enzymatic digestion using trypsin and collagenase. The cells were washed and filtered during the procedure and finally lysed with TRIzol® reagent. RNA was extracted by addition of 1-bromo-3-chloropropane followed by purification using a Qiagen RNeasy Mini Kit. Genomic DNA was removed using a Qiagen RNase-Free DNase Set and the samples were cleaned using a

Qiagen RNeasy MinElute Celanup Kit. All kits were used according to the manufacturer's instruction. Concentration and integrity of RNA were analysed using a NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer.

3.5.6 Microarray (Paper III)

Whole-transcriptome analysis was performed using the Affymetrix Equine Gene 1.0 ST Array. The chip on which the samples are analysed contain probes able to detect the entire equine transcriptome. The probes are selected across the entire genes and the array contains a median of 21 probes per gene. RNA from explants treated with and without IL-1β from all time points was analysed for the three horses in the study.

Microarray expression analysis

In brief, complementary DNA was generated from 2 ng of total RNA using a GeneChip® WT Pico Kit, followed by hybridization to a GeneChip® Equine Gene 1.0 ST Array. The arrays were washed and stained and finally scanned using the GeneChip® Scanner 3000 7G.

Microarray data analysis

The raw data were normalized in an Expression Console, provided by Affymetrix, using the robust multi-array average (RMA) method that was first suggested by Li and Wong in 2001 (Irizarry *et al.*, 2003; Li & Wong, 2001). Subsequent analysis of the gene expression data was carried out in the statistical computing language R. A paired empirical Bayes moderated t-test was applied using the 'limma' package (Smyth, 2005; Smyth, 2004) to find differentially expressed genes between the IL-1β-stimulated and the unstimulated explants. To address the problem with multiple testing, the p-values were adjusted using the method of Benjamini and Hochberg (Benjamini & Hochberg, 1995). Differences between the IL-1β stimulated and the unstimulated explants was considered significant if the logarithmic fold change (log FC) was ≥ 1 or ≤ -1 with $p<0.05$.

The Database for Annotation, Visualization and Integrated Discovery (DAVID), v6.7 was used to identify enriched Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in the data sets (Huang *et al.*, 2009; Huang *et al.*, 2008).

4 Results and discussion

This section briefly describes and discusses the results from the studies included in this thesis. Detailed information is presented in each of the individual papers.

4.1 Paper I

The matrix molecules COMP, fibromodulin, matrilin-1, and chondroadherin showed spatial changes in expression and localisation during maturation. The most prominent change was the relocalisation of COMP, fibromodulin and chondroadherin in the hypertrophic zone of the growth plate from their prenatal interterritorial to postnatal pericellular position. This localisation change of the proteins was also observed in the other zones of the growth plate and in the articular cartilage; however, the shift occurred at different stages of maturation for the proteins. The studied matrix molecules are involved in collagen fibril formation and organisation of the ECM (Heinegård, 2009), with the pericellular matrix being the most important in the response to mechanical load (Guilak *et al.*, 2006). Pericellular presence indicates an increased production of the molecules. The changes during maturation indicated involvement in the joint development, potentially induced by mechanical load after birth. It was not, however, possible to characterise the phenotypes of the chondrocytes based on the studied ECM molecules since the expression and localisation varied with the age of the horses rather, than with specificity to a certain phenotype.

The localisation also changed during maturation of the Notch signalling components Notch1, Dll4, Hes1, the Notch dysregulating protein EGFL7 and the stem cell-indicating factor, Stro-1. Active Notch signalling is known to influence cell proliferation and inhibit chondrogenic differentiation (Karlsson *et al.*, 2007; Dowthwaite *et al.*, 2004; Watanabe *et al.*, 2003). Thus the presence of Notch signalling components in equine growth cartilage suggests

that the studied proteins are involved in the development of equine joints. A juxtapositioning between Notch1 and EGFL7—detected as positive signals using *in situ* PLA—was seen in perivascular areas and hypertrophic cells. No clear association between EGFL7 and the target gene Hes1 was observed in our material. This may be because there were effects on other ligands or target genes than those studied. EGFL7 is also known to affect vessel infiltration (Parker *et al.*, 2004); the presence of EGFL7 in perivascular chondrocytes and cells of the cartilage bone interface indicates a role for EGFL7 in vascular infiltration of equine growth cartilage. Hence EGFL7 could potentially be involved in the early stages of osteochondrosis latens in which there is a premature closure of vascular channels leading to chondronecrosis (Olstad *et al.*, 2015).

Notch1 was detected in all zones of the articular cartilage, a result consistent with studies of human articular cartilage (Grogan *et al.*, 2009; Ustunel *et al.*, 2008; Hiraoka *et al.*, 2006). It also indicates that Notch1 is not exclusively expressed by progenitor cells and is therefore not an optimal marker for those cells. The stem cell-indicating factor Stro-1 was detected in all zones of the prenatal articular cartilage whereas the expression was restricted to the superficial and middle zones of the postnatal articular cartilage. Stro-1 was also detected in the zone of Ranvier (Figure 11) and in an area extending from the zone of Ranvier towards the articular cartilage along the perichondrium that has previously been suggested as a migration route for cells (Henriksson *et al.*, 2013). This may strengthen Stro-1 positive cells as a potential source for repair of damaged cartilage.

The zone of Ranvier has not previously been described in equine cartilage; in our study it was identified as an area comprising densely packed cells and in addition to Stro-1 also positive for EGFL7 and chondroadherin. It was further distinguishable from the surrounding tissue due to the absence of COMP. In summary, the markers used did not visualise exact phenotypic definition of the chondrocytes in the developmental growth cartilage.

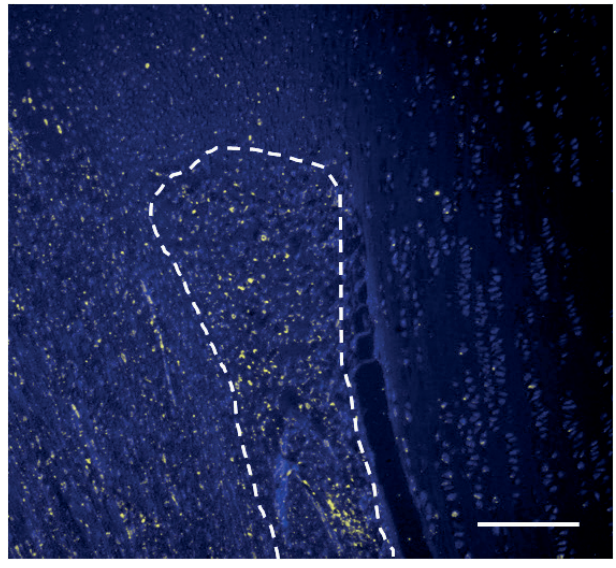

Figure 11. Immunohistochemical staining of Stro-1 positive cells in the zone of Ranvier (encircled area) of a 10-month-old foetus. Positive labelling shown in yellow and nuclei in blue. Scale bar: 500 μm.

4.2 Paper II

Equine cartilage explants stimulated with IL-1β *in vitro* during 25 days displayed a longitudinal release of molecules into the media. 127 proteins including ECM molecules and proteins related to inflammation were detected and quantified using quantitative proteomics.

The release of GAGs into the media was highest day 3 and the release of collagen C1,2C peptides increased successively from day 18 to 25 in media from the IL-1β stimulated explants. The release of these two correspond with the known degradation of the ECM during OA whereby proteoglycans are degraded prior to disruption of the collagen network (Goldring & Goldring, 2010). MMP-13—the major collagen type II degrading enzyme (Billinghurst *et al.*, 1997; Knäuper *et al.*, 1996)—increased over time in media from the IL-1β stimulated explants. Thus, it is presumably involved in the degradation of the collagen network also in this *in vitro* model. MMP-1 is another collagen degrading peptidase which also increased in media from IL-1β stimulated explants. MMP-3 is a peptidase known to be involved in the OA progression through activation of MMP-1 and MMP-13 (Troeberg & Nagase, 2012). MMP-3 increased in media from IL-1β stimulated explants.

Inflammation was confirmed through detection of IL-6 (which has a regulatory role in OA with involvement in the IL-1β induced matrix

degradation (Westacott & Sharif, 1996)), the chemokine (C-C motif) ligand 20 (CCL20) (which is involved in pro-inflammatory and degradative responses in cartilage (Alaaeddine *et al.*, 2015)), and through detection of SAA (which is involved in acute phase response triggered by IL-1β (de Seny *et al.*, 2013)). Also components of the initial stages of the activation of the complement system were increased at days 3 and 6 in media from IL-1β stimulated explants indicating an inflammation related increase.

Components of the ECM were released in a specific time pattern with early release of aggrecan, COMP, hyaluronan and proteoglycan link protein-1 (HAPLN-1), chondroadherin, thrombospondin-1, and proteoglycan-4 (also called lubricin) (Figure 12). This was followed by release of collagen type XIIαI and a late release of biglycan, fibromodulin, fibronectin, prolargin, and the collagens type II, VIαIII, IXαI and XIαI (Figure 12). The release of lumican, decorin and CILP consisted of two peaks (one early and one late) (Figure 12). This trend was also observed for fibromodulin and fibronectin, however the late peaks were the most prominent. The early release of aggrecan, identified by quantitative proteomics, was further confirmed by high levels of GAGs in media at day 3 and release of a semitryptic peptide (YDAICYTGEDF) originating from the G1 and G2 regions of the aggrecan molecule. Aggrecan is known to be degraded by aggrecanases in early stage OA (Caterson *et al.*, 2000). The aggrecanases ADAMTS-4 and ADAMTS-5 was not detected in our proteomic study. However ADAMTS may have been present, but undetected for several reasons. They may have been below the dynamic range of detectable concentrations; they may have been bound to the cartilage; they may not have been secreted at the studied time points; or, they may have had a short half-life *in vitro*.

The release of matrix molecules involved in the organisation and stabilization of the collagenous network indicates that the collagen network is degraded in a time-dependent pattern. The dynamic changes observed *in vitro* may be useful for identifying components released during the progression of OA *in vivo.* If different time-specific components can be measured in synovial fluids from equine joints with OA, it may be possible to identify the duration of the joint pathology in that particular joint. These potential biomarkers could also aid in evaluating different treatments, including training regimes.

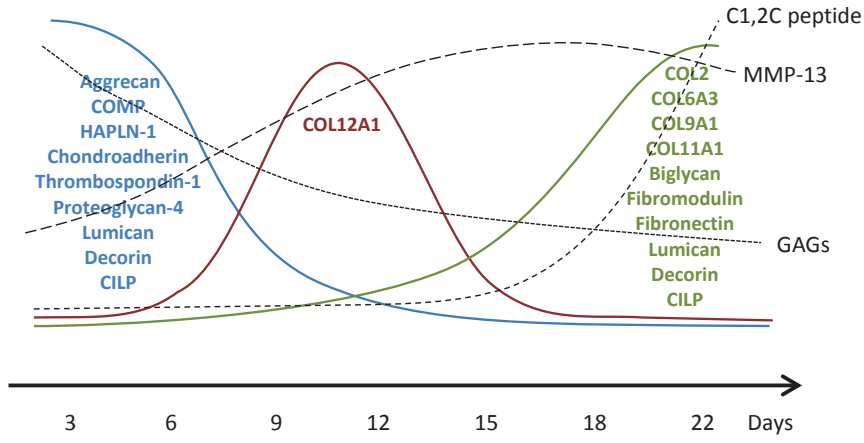

Figure 12. Molecules identified by quantitative proteomics during 22 days of IL-1β stimulation. Molecules released early in blue, released intermediate in red and released late in green. Levels measured by assays for collagen C1,2C peptide, matrix metallopeptidase-13 (MMP-13) and glycosaminoglycans (GAGs) are denoted in black. COMP, cartilage oligomeric matrix protein; HAPLN-1, hyaluronan and proteoglycan link protein-1; CILP, cartilage intermediate layer protein; COL, collagen.

4.3 Paper III

Stimulation of equine cartilage explants with IL-1β resulted in significant changes in gene expression during 27 days of culturing. Microarray analysis revealed differences related to inflammation, ECM and phenotype.

IL-1β induced ECM degradation was seen as a decrease in toluidine blue staining, indicating a loss of proteoglycans in the stimulated explants. Results from the microarray analysis confirmed this as a downregulation of aggrecan (*ACAN*) from day 9, reaching significant downregulation by day 21. Collagen type IIαI (*COL2A1*), *COL9A2* and *COL9A3* were downregulated from day 15 in stimulated explants, suggesting disruption of the collagenous network late in the culturing period. *COL11A1* was downregulated at all time points after IL-1β stimulation, indicating that the collagenous network is affected already early after IL-1β stimulation. The ECM degradation was further confirmed as upregulation of matrix-degrading enzymes. MMP-1, -8 and -13 are collagen type II-degrading enzymes found in OA joints (Troeberg & Nagase, 2012; Goldring *et al.*, 2011; Billinghurst *et al.*, 1997; Knäuper *et al.*, 1996; Reboul *et al.*, 1996). Genes for these were upregulated in stimulated explants together with *MMP2* and *MMP3* involved in activation of other MMPs. This suggests increased synthesis of these enzymes and activation through MMP-2 and MMP-3 leading to subsequent degradation of the collagenous network.

ADAMTS4 was upregulated in stimulated explants during the whole culturing period and *ADAMTS5* at day 27. Both of these genes express proteins that are considered to be the major aggrecan degrading enzymes in OA joints (Troeberg & Nagase, 2012; Bondeson *et al.*, 2008).

Induction of an inflammatory response after IL-1β stimulation was identified by upregulation of *IL1A, IL6* and *LIF* during the whole culturing period. IL-1α is a pro-inflammatory cytokine whereas IL-6 and LIF are regulatory cytokines. IL-6 is involved in upregulation of MMPs (Rowan *et al.*, 2001), downregulation of collagen type II (Porée *et al.*, 2008) and induction of an acute phase response (Tilg *et al.*, 1997). The protein LIF increases the production of IL-1β, IL-6 and IL-8 (Henrotin *et al.*, 1996; Villiger *et al.*, 1993). The only anti-inflammatory cytokine differentially expressed in the stimulated and unstimulated explants was interleukin-1 receptor antagonist (*IL1RN*). It was upregulated at day 3 in stimulated explants, indicating an attempt to inhibit the inflammatory actions by IL-1β early in the culturing period. Chemokines are other mediators of inflammation upregulated during the culturing period. Among these were chemokine (C-X-C motif) ligand 1 (*CXCL1*) and *IL8* which induce chondrocyte hypertrophy and calcification and may contribute to the phenotypical changes occurring during the progression of OA (Merz *et al.*, 2003). The complement system is suggested to be a key component of OA pathogenesis (Wang *et al.*, 2011) and the downregulation of the inhibitors C4 binding protein alpha (*C4BP*), complement factor I (*CFI*), and *CFH* may affect the complement mediated inflammation.

Non-collagenous molecules and SLRPs are important for the organisation and structure of the collagenous network (Heinegård, 2009). There was a downregulation in stimulated explants of the non-collagenous molecules *COMP* from day 9, matrilin-2 (*MATN2*) at days 3, 9 and 27 and *CILP* at all time points. The SLRPs extracellular matrix protein 2 (*ECM2*), lumican (*LUM*), osteomodulin (*OMD*), epiphycan (*EPYC*), mimecan (also called osteoglycin, *OGN*) and chondroadherin (*CHAD*) were downregulated at all time points, fibromodulin (*FMOD*) from day 9, biglycan (*BGN*) from day 15 and decorin (*DCN*), asporin (*ASPN*) and prolargin (*PRELP*) at day 27 (Figure 13). This may have affected the collagenous network during the inflammatory process in a time-dependent manner.

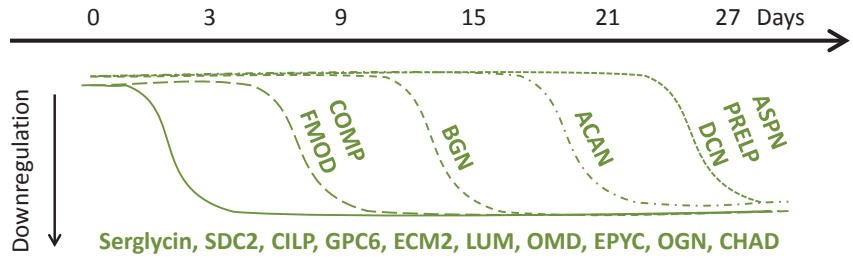

Figure 13. Gene expression of downregulated non-collagenous matrix molecules and proteoglycans in stimulated explants compared to unstimulated ones during the culturing period. Serglycin, *SDC2, CILP, GPC6, ECM2, LUM, OMD, EPYC, OGN* and *CHAD* downregulated at all time points of IL-1β stimulation; *COMP* and *FMOD* from day 9; *BGN* from day 15; *ACAN* from day 21; and *ASPN, PRELP,* and *DCN* at day 27.

Lubricin is important for boundary lubrication of articular joints where it reduces friction and chondrocyte apoptosis (Waller *et al.*, 2013). The upregulation of lubricin (*PRG4*) during days 9 to 21 in the stimulated explants indicates an attempt to protect the articular cartilage. Lubricin has recently been suggested to interact with the receptor CD44 (Al-Sharif *et al.*, 2015) which is the primary receptor for HA (Aruffo *et al.*, 1990). *CD44* was upregulated at all time points in the IL-1β stimulated explants, thereby enabling CD44 mediated interactions between chondrocytes and their surrounding ECM during the inflammatory process. The integrins are other receptors involved in interactions between cells and their surrounding ECM (Loeser, 2014). Upregulation of integrin α3 (*ITGA3)* from day 9 to 27 in stimulated explants; *ITGA5* at day 15 to 27; and the downregulation of *ITGA10* and *ITGA11* from day 9 to 27, may affect the interaction between cells and matrix with subsequent changes in proliferation, differentiation and matrix remodelling.

Syndecans are transmembrane heparan sulphate proteoglycans with various biological functions such as binding to growth factors, acting as co-receptors, and mediating endocytosis (Iozzo & Schaefer, 2015; Choi *et al.*, 2011). Syndecan 4 (*SDC4*) was upregulated and *SDC2* was downregulated at all time points after IL-1β stimulation. Glypicans are other heparan sulphate proteoglycans anchored to the outer surface of the cell. Glypican 6 (*GPC6*) was downregulated at all time points in the IL-1β stimulated explants which may have affected the chondrocyte. The pericellular proteoglycan perlecan (*HSPG2*) was upregulated at days 3 and 9 in the IL-1β stimulated explants. Perlecan is involved in ECM stability, mechanotransduction, angiogenesis, chondrocyte metabolism and phenotypical changes that are important events during OA progression (Wilusz *et al.*, 2012; Whitelock *et al.*, 2008; Vincent *et*

al., 2007; Arikawa-Hirasawa *et al.*, 1999; Aviezer *et al.*, 1994). Perlecan can also enhance FGF signalling by formation of complexes with FGFs and FGF receptors (FGFRs) (Chuang *et al.*, 2010). Enhancement of FGF2 signalling through FGFR1 leads to a catabolic response (Yan *et al.*, 2011), whereas FGF18 signalling through FGFR3 initiates an anabolic one (Davidson *et al.*, 2005). *CSPG4,* a transmembrane cell surface proteoglycan upregulated at days 15 and 21, is also involved in FGF signalling (Cattaruzza *et al.*, 2013).

Genes related to a chondrogenic phenotype include *COMP, COL2A1,* v-ets erythroblastosis virus E26 oncogene homolog (*ERG*), melanoma inhibitory activity (*MIA*), and *ACAN* (Richardson *et al.*, 2015; Schroeppel *et al.*, 2011). These were downregulated from day 9, 15, 15, 21, and 21 respectively. This indicates a chondrogenic phenotype at the early time points of the culturing period, that was lost at the late time points of IL-1β stimulation. In addition *FGF18*, involved in chondrogenic differentiation of mesenchymal cells (Davidson et al., 2005), was upregulated from day 3 to 21. Upregulation of runt-related transcription factor 2 (*RUNX2*) (at days 15 and 27) and vascular endothelial growth factor A (*VEGFA*) (at days 15 and 21) indicates a transition towards hypertrophy at the late time points of stimulation. However, no evidence of a hypertrophic phenotype was detected histologically and there were no upregulation of *COL10A1* suggesting that the 27 days of culturing was not sufficient time to change the chondrocytes into hypertrophic cells. Transition of chondrocytes into hypertrophy can be inhibited by sex determining region Y-box 9 (*SOX9*) (Akiyama *et al.*, 2002; Zhao *et al.*, 1997), and this gene was not differentially expressed between the stimulated and unstimulated explants. Notch signalling is involved in phenotypical changes including inhibition of differentiation towards chondrogenic cells (Chen *et al.*, 2013b; Karlsson *et al.*, 2007; Watanabe *et al.*, 2003). The receptor *NOTCH1* was upregulated at day 21 in the IL-1β stimulated explants, the target genes *HES1* at days 21 and 27, and hairy/enhancer-of-split related with YRPW motif protein 2 (*HEY2*) from day 15. This indicate activation of Notch signalling potentially activated by the non-canonical ligand delta/notch-like EGF repeat containing (*DNER*) upregulated from day 15.

The *in vitro* model shows time-dependent changes in gene expression related to inflammation, ECM, and phenotypical alterations of relevance for IL-1β induced inflammation in OA. Knowledge about gene expression during inflammation is crucial in the search for unique biomarkers that can identify relevant anabolic and catabolic processes in the equine articular cartilage *in vivo*.

4.4 Papers II and III

The combined studies of gene expression and protein release from equine articular cartilage explants will increase the knowledge of the events during IL-1β induced inflammation in articular cartilage. The release of a protein into the culture media, together with downregulated gene expression in the cartilage tissue, indicate a release of ECM proteins without new synthesis to compensate this loss. This was true for many ECM molecules such as the collagen types II, IX and XI. Release of aggrecan and COMP into culture media of stimulated explants was seen early in the culturing period, followed by a downregulation of gene expression. Protein release prior to downregulation of gene expression was also observed for decorin and prolargin; however, it occurred late in the culturing period. Downregulation of gene expression during the whole IL-1β stimulation, and an early release of proteins into media, was observed for lumican, osteomodulin, epiphycan, chondroadherin and CILP. A downregulation of gene expression before release of protein into media was seen for fibromodulin and biglycan. A release of ECM proteins, combined with downregulated gene expression may lead to depletion of the molecules in the cartilage explants as a result of IL-1β stimulation. Lubricin, however, was released early into the culture media and followed by an upregulation of gene expression, which indicates an attempt to compensate for the loss of this important surface protein.

The peptidases MMP-1, -3 and -13 were increased in culture media of stimulated explants and also upregulated at the gene level, indicating an IL-1β induced production. This was also the case for the cytokine IL-6 and the chemokine CCL20. Release of ADAMTS was not detected in the culture media even though an early upregulation of gene expression was seen for *ADAMTS1, 4,* and *9*. However, the upregulation indicates an involvement of these ADAMTS in the IL-1β induced inflammation.

In summary, IL-1β induces production of IL-6, CCL20 and MMPs, seen as upregulation at the gene level and increased release of the gene products into culture media. Time-dependent release of many ECM molecules was observed together with downregulated gene expression, suggesting depletion of the proteins in articular cartilage without new synthesis during the inflammation.

5 Conclusions

- The ECM molecules COMP, fibromodulin, matrilin-1 and chondroadherin as well as the Notch signalling components show spatial changes in localisation in developing equine cartilage, suggesting their involvement in maturation of synovial joints.
- Stro-1 positive cells detected in the zone of Ranvier, along the possible migration route, and in the articular cartilage suggest presence of cells with stem cell-like characteristics in equine cartilage.
- It was not possible to characterise the phenotypes of the chondrocytes during maturation based on IHC of the ECM molecules.
- Proteins related to inflammation and ECM were released into cell culture media of IL-1β stimulated explants in a time-dependent pattern with a late release of components of the collagenous network. The majority of released ECM molecules showed a downregulation of gene expression, suggesting release without new synthesis to compensate the loss.
- Gene expression of inflammatory mediators and matrix-degrading enzymes was upregulated in IL-1β stimulated explants during the culturing period, confirming the catabolic effects of IL-1β on articular cartilage.
- Genes related to a chondrogenic phenotype were downregulated late in the culturing period of IL-1β stimulated explants and genes related to a hypertrophic phenotype were upregulated late in the culturing period. This suggests, a chondrogenic phenotype early with transition towards hypertrophy at the late time points of stimulation, however not reaching a morphology of hypertrophy during the 27 days of culturing.

6 Future research

Increased knowledge about the cellular, genetic, and molecular events during the progression of the inflammation involved in OA is essential for diagnosis, prognosis and treatment of the disease. Studies using *in vitro* methods will provide knowledge applicable in clinical studies. The results achieved from this thesis can be used as a basis for further studies.

- Relating the genes and proteins to different stages of OA can aid in diagnosis and prognosis of the disease. Evaluating the genes and proteins included in this thesis in articular cartilage and synovial fluid from OA-affected joints will connect the *in vitro* results to *in vivo* conditions.
- The inflammation in OA progression includes other inflammatory mediators than IL-1β. Hence *in vitro* studies with other mediators, e.g., TNF-α, IL-6 and IL-8, will be of value.
- *In vitro* studies of co-cultures of cartilage and synovial membrane explants will further increase the knowledge about how inflammation affects the joint structures.
- The *in vitro* model presented in this thesis is an important tool to evaluate effects of OA medical treatments prior to studies *in vivo.* Pharmacological substances can be tested in this model prior to testing in live animals. This would minimize the use of research animals and be of great value for animal welfare.

7 Sammanfattning på svenska

Osteoartrit (OA) är en vanlig orsak till hälta hos hästar. Sjukdomen innefattar inflammation i leden samt successiv nedbrytning av ledbrosket, subkondral benskleros med mikrofrakturer, osteofyter och en inflammatoriskt förtjockad ledkapsel (synovit och kapsulit). Ledbrosket består av kondrocyter omgivna av ett extracellulärt matrix (ECM) som påverkas under sjukdomens förlopp. Progressionen av OA från tidigt till sent stadium innefattar nedbrytning och omstrukturering av ECM med kollaps av det kollagena nätverket samt fenotypiska förändringar i kondrocyterna. Inflammatoriska mediatorer såsom cytokiner påverkar initiering och progression av OA bland annat genom att öka mängden matrixnedbrytande enzymer. Klinisk diagnos av OA ställs oftast sent i sjukdomsförloppet då det redan finns irreversibla skador i ledens vävnader. Det är därför viktigt att öka kunskapen om sjukdomens förlopp för att utveckla metoder som kan diagnosticera sjukdomen i ett tidigt stadium då det går att stoppa det destruktiva förloppet i ledbrosket.

Syftet med avhandlingen var att följa förändringar som sker i brosket vid dess normala utveckling samt vad som sker under tidsintervallet 0-27 dagar när inflammation induceras av cytokinen interleukin (IL)-1β. Ökad kunskap om hur celler, gener och molekyler förändras kan användas för att hitta markörer för tidig OA. Hypotesen var att det sker specifika händelser under broskets utveckling samt under inflammationsförloppet som skulle kunna användas för att identifiera tidiga stadier av OA före kollaps av ECM.

I arbete I studerades lokalisationen av olika proteiner under broskets utveckling. Syftet var att karaktärisera kondrocyternas fenotyp samt dess omgivande ECM. Studien visade att uttrycket samt lokalisationen av matrixmolekyler förändras under broskets utveckling. Signalering via receptorn Notch1 är involverad vid utveckling av olika celltyper och för komponenter involverade i signaleringen sågs förändringar under broskets utveckling. Även lokalisationen av stamcellsmarkören Stro-1 förändrades. Resultaten i studien indikerar att de studerade proteinerna har betydelse för

broskets utveckling, men någon exakt definition av kondrocyternas fenotyp kunde inte göras med dessa proteiner.

För att studera hur inflammation inducerad av cytokinen IL-1β påverkar ledbrosket användes en *in vitro*-modell där ledbrosk stimuleras med IL-1β. Modellen innebär att intakt ledbrosk avlägsnas från ledytan och efter tillsatts av IL-1β studeras ledbrosket samt dess omgivande medium vid olika tidpunkter. Detta sker för både IL-1β stimulerat ledbrosk samt ostimulerade kontroller för att följa inflammationsförloppet som startas av IL-1β. I arbete II kartlades de proteiner som utsöndras i mediet med hjälp av kvantitativ proteomik. Resultaten visade att proteiner relaterade till inflammation och ECM utsöndrades i ett tidsrelaterat mönster med kollaps av det kollagena nätverket efter 25 dagar. I arbete III studerades uttrycket av gener i brosket med hjälp av microarray. Gener relaterade till inflammation, ECM samt fenotyp förändrades under stimuleringstiden. Gener för inflammatoriska mediatorer som cytokiner och kemokiner uppreglerades vid IL-1β stimulering. Även gener för matrixnedbrytande enzym uppreglerades och gener för ett flertal ECM molekyler nedreglerades vid IL-1β stimulering. Gener relaterade till en kondrogen fenotyp var nedreglerade i slutet av stimuleringsperioden vilket indikerar att kondrocyternas fenotyp förändras under inflammationsförloppet.

Sammanfattningsvis bidrar resultaten i avhandlingen till ökad kunskap om kondrocyter och dess omgivande matrix samt hur dessa påverkas av inflammationen under sjukdomsförloppet vid OA. En tidig förändring av ECM-molekyler, som i de senare tidsperioderna resulterade i det kollagena nätverkets kollaps, var tydlig. Resultaten från *in vitro*-modellen kan användas för att upptäcka nya potentiella kandidater avseende biomarkörer för tidig OA. Modellen är även användbar för att studera effekter av nya farmakologiska substanser och dessas potential att hejda sjukdomsförloppet vid OA utan att använda försöksdjur.

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