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Proteomic Studies of an Explant Model of Equine Articular Cartilage in Response to Pro-inflammatory and Anti-inflammatory Stimuli

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ABSTRACT

Osteoarthritis (OA) is characterised by cartilage degradation, inflammation and pain within synovial joints. OA is a major cause of morbidity in the elderly human population and in companion animals such as horses. Changes in expression and activity of pro-inflammatory cytokines, chemokines and catabolic mediators contribute towards OA progression, which can be studied using *in vitro* culture models and proteomic approaches. This project studied the secretome from an *in vitro* model of equine articular cartilage, aiming to develop understanding of cartilage biology and degradative processes. These studies also aimed to identify protein markers relevant to this explant model for screening anti-inflammatory properties of novel therapeutics.

To evaluate responses to OA associated pro-inflammatory IL-1β and the non-steroidal anti-inflammatory drug (NSAID), carprofen, time courses of protein release were established in the explant model. The cartilage secretome contained cartilage extracellular matrix (ECM), non-ECM and intracellular proteins, all of which were identified by high-throughput mass spectrometry (MS). Semi-quantitative differences in protein release were reported between untreated control and IL-1β stimulated cartilage by MS. The release of glycosaminoglycans (GAGs) initiated by IL-1β was delayed when carprofen was present.

The proteomic sample preparation method was adapted to deplete high abundance proteins that can hinder the detection of low level proteins in high-throughput MS analysis. Three depletion approaches were applied: CPC precipitation, concanavalin A lectin chromatography and Proteominer™ technology. These approaches provided additional identifications of the non-ECM secreted proteins MMP-10 and IL-9, and of additional intracellular proteins. Further optimization of these methods could further enhance the detection of low level proteins.

Proteins identified by MS analysis of the cartilage secretome were assessed using quantitative western blotting analysis. Carprofen significantly reduced IL-1β stimulated release of MMP-1, MMP-3, MMP-13 and a fibronectin degradation product. Levels of clusterin were reduced by IL-1β and carprofen treatments. These specific proteins were shown to be markers of IL-1β stimulated inflammation and degradative processes, which can be significantly reduced by an anti-inflammatory such as carprofen.

This thesis describes the use of proteomics with other approaches to study the effects of IL-1β and carprofen on release of several important structural, metabolic and inflammatory related components from cartilage. Carprofen was beneficial in decreasing certain aspects of inflammation and degradation, including significantly reducing release of MMPs and their catabolic products (fibronectin and GAGs) from the ECM. The equine explant model can be further studied with high-throughput MS to assess responses to various stimuli and detect released proteins. In conclusion, anti-degradative effects and MMP inhibition can be specifically monitored within this *in vitro* equine cartilage model, to screen efficacy of therapeutics and putative anti-inflammatories to relieve OA.

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PUBLICATIONS

Publications are incorporated in the back of this thesis

Original Papers

A. Williams, J. Smith, D. Allaway, P. Harris, S. Liddell, A. Mobasheri. **Carprofen inhibits the release of matrix metalloproteinases 1, 3 and 13 in the secretome of an explant model of articular cartilage stimulated with interleukin 1beta.** Arthritis Research & Therapy. 2013 Dec 30; 15(6):R223

Review Article

A. Williams, J. Smith, D. Allaway, P. Harris, S. Liddell, A. Mobasheri. **Applications of Proteomics in Cartilage Biology and Osteoarthritis Research** Frontiers in Bioscience 16, 2622-2641, June 1, 2011

Abstracts

A. Williams, J. Smith, D. Allaway, P. Harris, S. Liddell, A. Mobasheri. **Strategies for optimising proteomic studies of the cartilage secretome: Establishing the time course for protein release and evaluating responses of explant cultures to IL-1β, TNF-α and carprofen.** Osteoarthritis and Cartilage, Volume 19, Supplement 1, September 2011, Page S209

A. Williams, J. Smith, D. Allaway, P. Harris, S. Liddell, A. Mobasheri. **High-throughput proteomic analysis of the cartilage secretome for identification of inflammatory biomarkers.** Osteoarthritis and Cartilage, Volume 21, Supplement, April 2013, Page S272

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ABBREVIATIONS

2DE	Two-dimensional electrophoresis
5-LOX	5-lipoxygenase
ACAN	Aggrecan
ACL	Anterior cruciate ligament
ACN	Acetonitrile
ADAMTS	A Disintegrin and metalloproteinase with thrombospondin motifs
AMBIC	Ammonium bicarbonate
APS	Ammonium persulfate
BAALC	Brain and acute leukaemia, cytoplasmic
BIPED	Burden of disease, investigative, prognostic, efficacy of intervention and diagnostic biomarkers
BMP-1	Bone morphogenic protein-1
BSA	Bovine serum albumin
CCL20	Chemokine (C-C motif) ligand 20
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CID	Collision induced dissociation
CILP	Cartilage intermediate layer protein
COL2A1	Collagen, type II, alpha 1
Con A	Concanavalin A
COMP	Cartilage oligomeric matrix protein
COX	Cyclooxygenase
CPAMD8	C3 and PZP-like, alpha-2-macroglobulin domain containing 8
CPC	Cetylpyridinium chloride
CS	Chondroitin sulphate
CT	Cocktail of potential anti-inflammatory ingredients
CTGF	Connective tissue growth factor
CXCL	C-X-C motif chemokine
DIGE	Difference in-gel electrophoresis

DMEM	Dulbecco’s Modified Eagle Medium
DMMB	Dimethylmethylene blue
DMOADs	Disease-modifying osteoarthritis drugs
DTT	Dithiothreitol
ECM	Extracellular matrix
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
emPAI	Exponentially modified protein abundance index
ELISA	Enzyme-linked immunosorbant assay
ESI	Electrospray ionization
ETD	Electron transfer dissociation
GAG	Glycosaminoglycan
GluN	Glucosamine
GRP	Glucose regulated protein
HSP70	Heat shock 70kDa protein 1
HIF-1	Hypoxia inducible factor 1
HPLC	High-performance liquid chromatography
HAPLN1	Hyaluronan and proteoglycan link protein 1
HRP	Horseradish peroxidase
IAA	Iodoacetamide
IGFBP7	Insulin-like growth factor-binding protein 7
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
iTRAQ™	Isobaric tags for relative and absolute quantitation
LAMP	Lysosomal-associated membrane protein
LDH	Lactate dehydrogenase
LECT2	Leukocyte cell-derived chemotaxin-2
LG3BP	Galectin-3-binding protein

LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
MALDI	Matrix-assisted laser desorption/ionization
MIA	Melanoma-derived growth regulatory protein
MIF	Macrophage migration inhibitory factor
M-LAC	Multi-lectin affinity chromatography
MMP	Matrix metalloproteinase
MS	Mass spectrometry
nanoLC-MS/MS	Nano liquid chromatography–mass spectrometry/mass spectrometry
NFκB	Nuclear factor kappa B
NGAL	Neutrophil gelatinase associated lipocalin
NO	Nitric oxide
NSB	Non-specific binding
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
PBS	Phosphate buffered saline
PCOC-2	Procollagen C-endopeptidase enhancer 2
PEDF	Pigment epithelium-derived factor
PGE_2	Prostaglandin E_2
PHLD	Glycosylphosphatidylinositol Specific Phospholipase D
PMF	Peptide mass fingerprint
PRDX6	Peroxiredoxin-6
PVDF	Polyvinyl difluoride
RA	Rheumatoid arthritis
RANTES	Regulated on activation, normal T cell expressed and secreted
Ret A	All-trans-retinoic acid

RO	Reverse osmosis
SAA	Serum amyloid A protein
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SELDI	Surface-enhanced laser desorption/ionization
SFRP	Secreted frizzled related protein
SILAC	Stable isotope labelling with amino acids in cell culture
SIR1	NAD-dependent protein deacetylase sirtuin-1
SOD	Superoxide dismutase
SPARC	Secreted protein acidic and rich in cysteine (also called osteonectin)
STS	Sodium thiosulphate
SZP	Superficial zone protein
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitors of MMP
TNF-α	Tumour necrosis factor-α
TNF-R1	Tumour necrosis factor receptor 1
TRAP1	TNF receptor associated protein 1
TRIS	Tris(hydroxymethyl)aminomethane
TSP	Thrombospondin
VEGF	Vascular endothelial growth factor
WOMAC	Western Ontario and McMaster Universities Arthritis Index
YKL	Chitinase 3-like protein

CHAPTER 1

INTRODUCTION

The aim of the work described in this thesis was to assess a high-throughput proteomic approach to evaluate the secretome of an equine articular cartilage explant model, and to monitor responses to pro-inflammatory stimuli or anti-inflammatories. Studying cartilage biology and degradation *in vitro* will provide scientific understanding that is relevant to and can be applied to osteoarthritis (OA) research. This introductory chapter describes the background to OA, articular cartilage structure, pathogenesis and inflammation in this disease. The introduction will then focus on the advantages of cartilage explant studies models for studying OA and the other research models available. Proteomic and secretome strategies to study OA associated tissues will also be focused upon and reviewed. Available treatments for OA will next be discussed, concentrating on carprofen that was studied throughout this thesis. The details of the aims and hypothesis for the whole thesis and specific chapters will then be explained.

1.1 Osteoarthritis

OA is a prevalent physiological disease affecting large proportions of the elderly population and is expected to become an increasing burden on the healthcare system as people continue to live longer. OA is a major cause of pain, disability and has economic implications through affecting a person's ability to work. It was estimated in 2008 that 26.9 million people had clinical OA in at least one joint in the United States (Lawrence et al., 2008). This condition is also a major cause of morbidity in companion animals like dogs (Henrotin et al., 2005) and horses (Goodrich and Nixon, 2006) leading to lameness and substantial veterinary costs. OA is a degenerative disease of the entire synovial joint, mainly characterised by synovial joint inflammation and progressive destruction of the extracellular matrix (ECM) of articular cartilage (Buckwalter and Mankin, 1998a). A diagrammatic representation of the synovial joint and its tissues is displayed in Figure 1. Synovial joints are located at the

ends of articulating bones and allow controlled movement. The joint capsule contains lubricating synovial fluid and is a defining feature of this type of joint. Articular cartilage covers the ends of bones within the joint, providing a protective surface. Knee joints are most commonly affected but OA can occur in any synovial joint (Cushnaghan and Dieppe, 1991). In general, weight-bearing joints are worst affected. The causes of OA are not currently fully understood but risk factors include age, gender, genetics, obesity, poor nutrition and joint injury or instability (Lee et al., 2013).

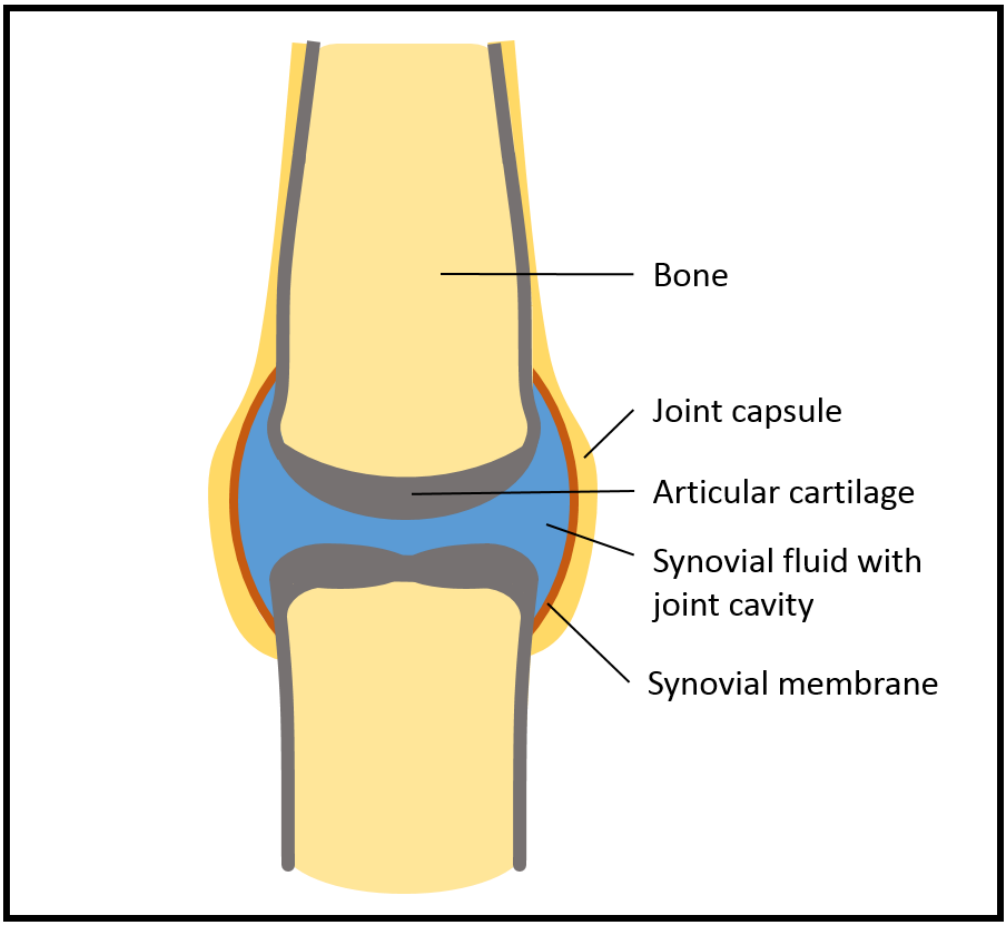

Figure 1. Simplified diagram of a synovial joint

Loss of cartilage will affect the integrity of the smooth articular surface (Figure 2), therefore affecting the usual flowing movement of synovial joints. This ultimately has implications on quality of life, as movement and mobility becomes increasingly painful. Other characteristics of OA can include subchondral bone sclerosis (hardening) and osteophyte (bony outgrowth) formation (Goldring and Goldring, 2007). Treatment for OA relies on symptomatic relief with analgesics, steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) often being prescribed, or the surgical replacement of joints in more serious cases. Current treatments only reduce the levels of pain and inflammation, but have no impact on slowing cartilage loss and destruction. Effective disease modifying OA drugs (DMOADs) continue to be elusive therefore there is extensive interest to advance the discovery of treatments to stimulate cartilage repair and to reverse disease progression. Herbal medicinal products or nutraceuticals with intrinsic anti-inflammatory properties may have potential to provide alternatives to conventional treatments (Cameron et al., 2009). The effectiveness of herbal treatments for OA has not yet been proven and further carefully designed clinical trials are required.

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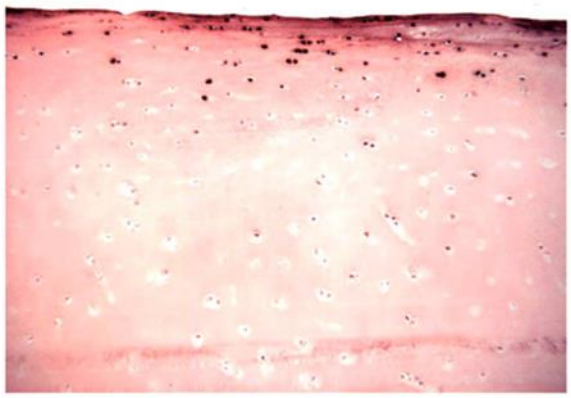

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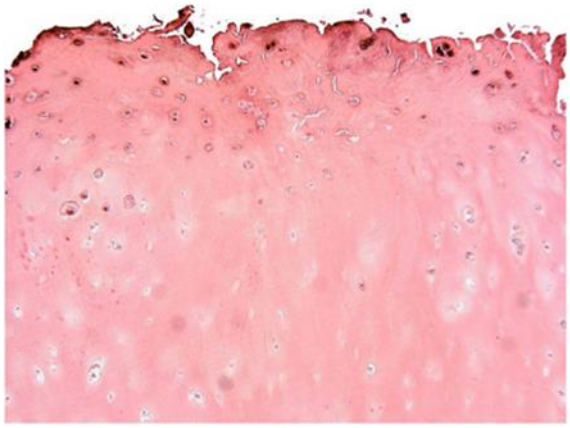


Figure 2. Histological sections of human articular cartilage

(A) Healthy normal articular cartilage with a smooth surface.

(B) OA affected articular cartilage showing signs of degeneration and an irregular surface (Afif et al., 2007).

Cartilage damage occurring in OA can be detected radiographically by a decrease in joint space width, but this is only seen once considerable cartilage degradation has already happened. Collagen and proteoglycan fragments can be detected in the blood, synovial fluid and urine acting as biomarkers after significant degeneration of cartilage (Mobasheri and Henrotin, 2010). These biomarkers can detect changes occurring to cartilage and are consistent with joint space narrowing being observed, although as already mentioned by this point in time the disease has already progressed considerably. Once OA has reached the stage where it can be diagnosed by these methods, any treatment intervention is too late to affect disease progression. Therefore a biomarker (or group of biomarkers) that signifies early OA would be a useful tool to allow screening of individuals, particularly those with a high risk of developing joint disease, so that early detection may facilitate individualized treatment. A protein biomarker specific to early OA that can be easily identified in blood or urine would be ideal since diagnostic tools such as specific antibodies can be developed to proteins that are sufficiently sensitive to allow accurate detection. Diagnosing OA early will allow timelier interventions and lifestyle changes to be introduced, which may be beneficial to patient prognosis.

1.2 Articular cartilage

The ends of long bones within synovial joints are covered with articular cartilage to protect the bone underneath and allow smooth movement of joints (Figure 1). Articular cartilage consists primarily of the extracellular matrix (ECM), water and a single cell type known as the chondrocyte (Archer and Francis-West, 2003) (Figure 3). It is an avascular, aneural and alymphatic connective tissue with unique biological and mechanical properties. These include the ability to be durable and compressed while maintaining tensile strength. The load-bearing function of cartilage is determined by the constituents of the ECM and its interactions with chondrocytes (Buckwalter and Mankin, 1998b). Due to the absence of vasculature, cartilage is maintained and functions within a hypoxic environment as there is a very low oxygen supply to the tissue (Schipani et al., 2001). Chondrocytes are therefore adapted to survive and preserve the ECM in this hypoxic environment (Wilkins et al., 2000).

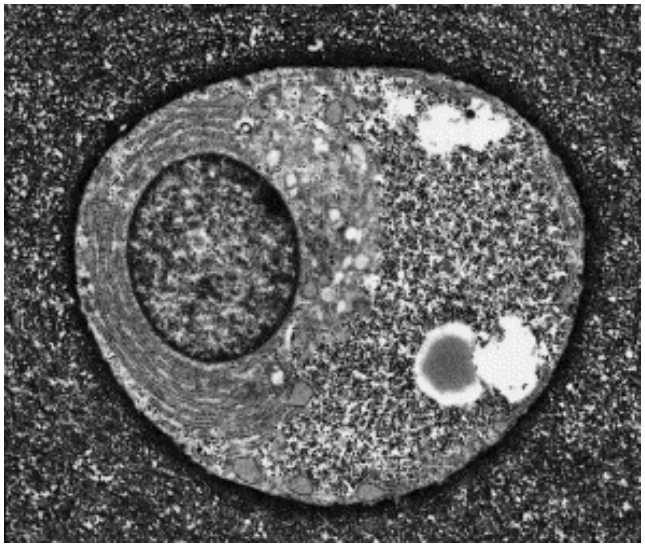


Figure 3. Electron micrograph of an articular chondrocyte

Chondrocytes usually show a rounded morphology and are highly metabolically active, primarily functioning to maintain the ECM and synthesize its specialised proteins (Archer and Francis-West, 2003).

There are four different zones within the full thickness of articular cartilage known as the superficial, transitional, deep and calcified zones (Pearle et al., 2005) (Figure 4). Explant discs cultured throughout this thesis, were taken from full thickness healthy cartilage, and therefore will contain all four cartilage zones. Chondrocytes display different metabolic activities and morphology throughout the individual layers. Closest to the joint surface is the superficial zone (10-20% thickness) that provides a smooth sliding surface vital for synovial joint function and has the ability to resist shear forces. This zone has high collagen content, containing collagen fibrils organised parallel to the surface, and with relatively low proteoglycan concentrations compared to the other layers. Chondrocytes here primarily produce proteins for protection and to aid lubrication. Within the superficial zone, chondrocytes have a histologically elongated appearance (Pearle et al., 2005). Lubricin (which is also known as superficial zone protein (SZP) or proteoglycan 4) is an important protein that functions to lubricate synovial joints. Chondrocytes in the superficial zone can be characterised phenotypically by high levels of lubricin synthesis, compared to chondrocytes in the underlying cartilage layers (Schumacher et al., 1999). Cytokine stimulation can modulate expression of lubricin, therefore inflammation during OA could affect release of this essential protein (Lee et al., 2008).

Beneath the superficial zone, is the transitional (middle) zone that makes up 40-60% of cartilage thickness and has a considerably higher proteoglycan content (Pearle et al., 2005) compared to the superficial zone. The middle zone therefore has increased compressibility than the superficial zone. Collagen content here contains thicker collagen fibres, but these are less densely concentrated and organized than in the superficial zone. Whilst superficial zone chondrocytes are elongated, within the middle zone they have a rounder morphology (Pearle et al., 2005).

Next, the deep zone (30% thickness) has the lowest water concentration and highest proteoglycan concentration of all cartilage zones. Chondrocytes and collagen fibres in deep zone are arranged into columns running perpendicular to the cartilage surface. The final layer is the calcified (tidemark) zone providing direct contact with

subchondral bone. Chondrocytes in this zone are small and the matrix is rich in apatite crystals (Pearle et al., 2005). The calcified zone mostly contains collagen II and hydroxyapatite, along with calcium phosphate, calcium carbonate and collagen type X (Zhang et al., 2012b). Large collagen fibre bundles from the deep zone, cross over into the calcified zone to anchor cartilage in place (Mow et al., 1989). Thickening of the calcified zone and multiple tidemarks are observed in OA, which causes the cartilage to become stiff and unable to absorb compressive forces (Henrotin et al., 2012c).

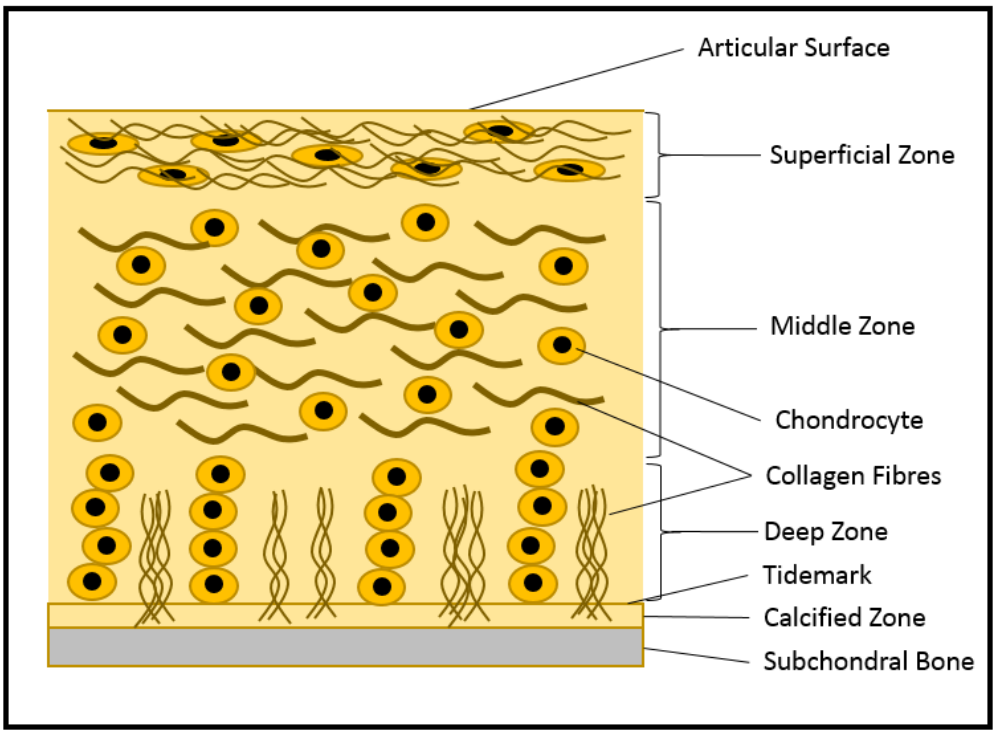


Figure 4. Schematic diagram of the defined layers of full thickness articular cartilage

The load-bearing function of cartilage is achieved by its structural design and interactions between the chondrocytes and ECM (Buckwalter and Mankin, 1998b). Chondrocytes synthesise the macromolecular framework of ECM from three distinct classes of macromolecules: fibrillar and non-fibrillar collagens, proteoglycans and non-collagenous proteins (Muir, 1995). The tensile stiffness and strength of cartilage are provided by a fibrillar meshwork of collagen types II, IX, and XI (Eyre, 2004, Kuettner et al., 1991). Chondrocytes are surrounded by a pericellular/territorial matrix, which contains a high proportion of collagen type VI filaments (Figure 5). Pericellular matrix acts as a transducer of biomechanical and biochemical signals between chondrocytes and the ECM (Guilak et al., 2006, Roughley and Lee, 1994).

Throughout the cartilage ECM are large aggregating proteoglycans (aggrecan), which give cartilage its stiffness to compression, its resilience and contribute to its long-term durability (Kiani et al., 2002, Luo et al., 2000). Hyaluronan chains form the backbone of aggrecan, from which the glycosylated side chains are attached with the support of link proteins. Aggrecan contains numerous side-chains of chondroitin sulphate and keratin sulphate that are highly negatively charged and therefore attract positively charged cations, which in turn draw water molecules into the ECM (Buckwalter and Mankin, 1998b). Cartilage therefore swells and when compressed, water is displaced providing a cushioning effect. Decorin, biglycan and fibromodulin are small proteoglycans that bind other components therefore aiding formation of the ECM (Heinegard, 2009). Decorin and fibromodulin have binding roles in fibrillogenesis and interfibril interactions via connections with type II collagen fibrils in the matrix. Biglycan may interact with collagen type VI (Buckwalter and Mankin, 1998a, Roughley and Lee, 1994) that is mainly located in the immediate surroundings of chondrocytes. Non-collagenous proteins are also found in articular cartilage including cartilage oligomeric matrix protein (COMP), which has been studied as a biomarker of ECM turnover and cartilage degeneration (Di Cesare et al., 1996). Fibronectin and tenascin also have a role in influencing exchanges between chondrocytes and the ECM (Roughley and Lee, 1994). Figure 5 illustrates many

important proteins contained within cartilage ECM that help to produce its specialised tissue structure.

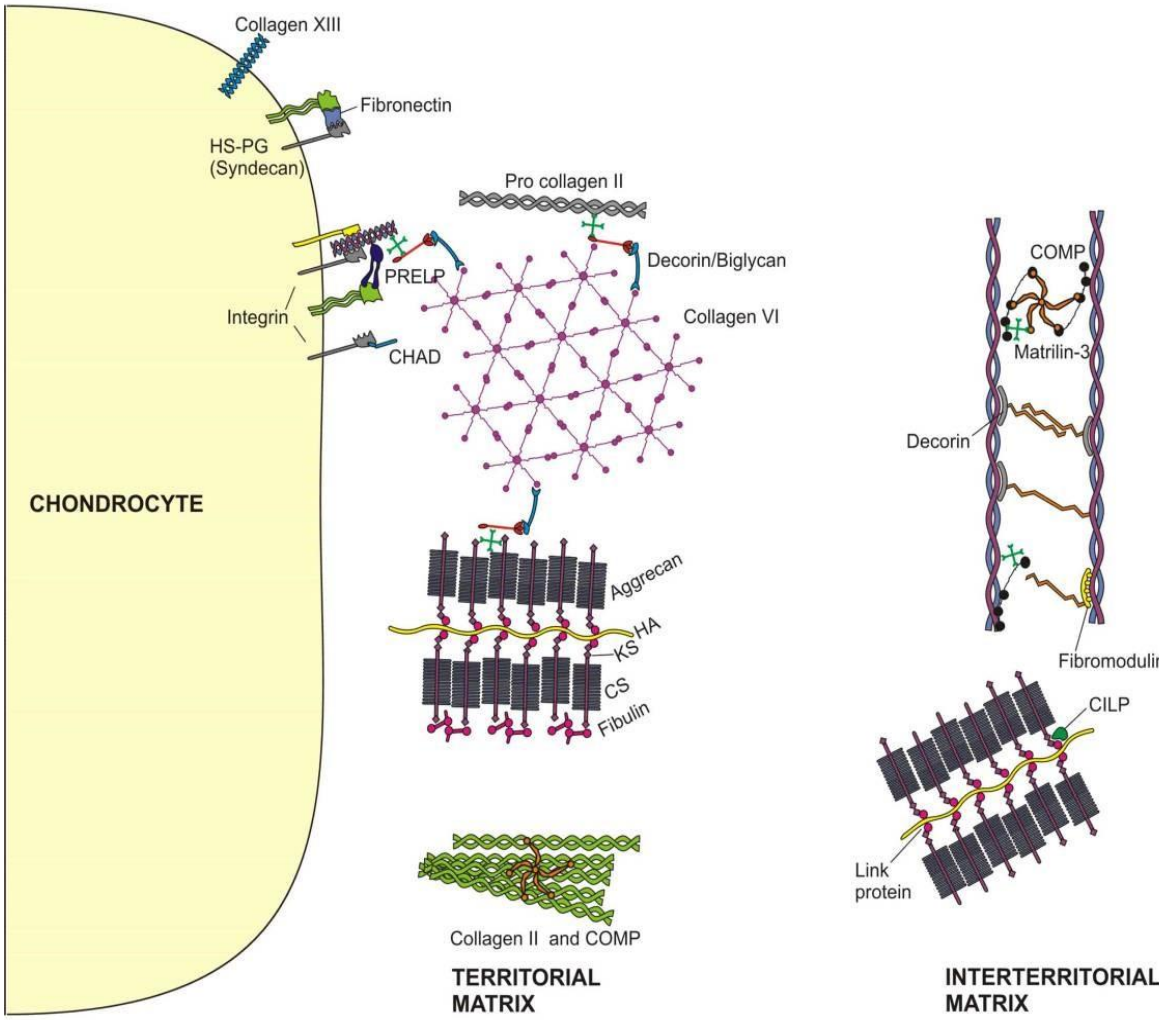


Figure 5. Schematic illustration of the major protein constituents of the ECM of articular cartilage

Chondroadherin = CHAD, proline/arginine-rich end leucine-rich repeat protein = PRELP, chondroitin sulphate = CS, keratan sulphate = KS, hyaluronan = HA, cartilage intermediate layer protein = CILP (Williams et al., 2011).

1.3. Chondrocytes and their role in pathogenesis of OA

The ECM of cartilage is sustained and built by chondrocytes living within this specialised tissue. Chondrocytes have to survive in the ECM environment that is highly hypoxic, acidic and hypertonic (Mobasheri et al., 2005, Mobasheri et al., 2008), whilst also having the ability to carry out their biological functions. This hypoxic environment leads to release of hypoxia inducible factor 1 α (HIF-1α). Activity of HIF-1 is important for differentiation and survival of chondrocytes both during foetal growth and development, and also after skeletal development is complete (Murphy et al., 2009).

Chondrocytes synthesize and secrete macromolecules assembled together into the ECM (Archer and Francis-West, 2003). Appropriate homeostatic balance between the rates of synthesis and degradation of ECM components is required to maintain cartilage. This delicate balance is controlled by both anabolic and catabolic factors expressed by chondrocytes. In OA there is excess degradation of ECM, with chondrocytes implicated in this process because they no longer are maintaining correct levels of cartilage metabolism. Differing patterns of protein expression can be measured in chondrocytes isolated from OA cartilage, compared to healthy articular chondrocytes (Iliopoulos et al., 2008, Lambrecht et al., 2009), which could effectively lead to cartilage metabolism alterations occurring in OA. As chondrocytes age, they have been demonstrated to respond differently to stimuli such as growth factors (Hudelmaier et al., 2001, Loeser, 2009). Growth, regulation, differentiation and protein expression within chondrocytes are influenced by these stimuli, therefore altered responses can cause changes in the maintenance and composition of ECM (Shakibaei et al., 1999). This could contribute to increased cartilage degeneration associated with OA and explain how it develops in older people (Ralphs and Benjamin, 1994). Once cartilage in OA affected joints begins to degenerate, ECM degradation products (for example: type II collagen and aggrecan fragments (Frisbie et al., 2008, Struglics et al., 2006)) are released into surrounding synovial fluid. Both synoviocytes (found in the synovial membrane) and chondrocytes express receptors

to detect these breakdown products stimulating release of proteins, including pro-inflammatory cytokines (IL-1β, TNF-α), and chemokines (IL-8). For example, degraded fragments of fibronectin and hyaluronan can stimulate catabolic signalling (Taylor et al., 2004, Homandberg et al., 1998). As OA progresses, there are decreases in chondrocyte numbers. This impinges on the normal cartilage maintenance processes that occur in healthy cartilage. A cycle of further chondrocyte death, inflammation, ECM degradation and cartilage loss therefore produces advancement of OA.

1.4. Inflammation in articular cartilage

One of the major characteristics of OA is an inflammatory component (Berenbaum, 2013). Acute inflammation is a healthy response in tissues to remove harmful stimuli, remove damaged tissue and help initiate healing processes. Chronic inflammation occurs during OA, where continuous inflammatory signalling causes excess degradation and gradual loss of cartilage. Inflammation associated with OA involves release of pro-inflammatory signalling compounds (prostaglandin E2 (PGE_2), nitric oxide (NO)) and cytokines (IL-1β, TNF-α) from tissues within synovial joints (Abramson and Attur, 2009) (Figure 6). IL-1β and TNF-α bind via specific receptors (i.e. IL-1R and TNF-R) present on the surface of the plasma membrane of chondrocytes and synoviocytes. Downstream signalling after cytokine activation initiates degradation processes including release of catabolic proteins, increased breakdown of ECM, a reduction in synthesis of collagen and proteoglycans, and release of other inflammatory facilitators (Pelletier et al., 2001).

The NF-κB signalling pathway has a significant role in OA inflammation and concludes with active phosphorylated NF-κB being translocated into the nucleus (Rigoglou and Papavassiliou, 2013). This active form of NF-κB induces expression of a variety of pro-apoptotic and pro-inflammatory genes, including further release of IL-1β and TNF-α themselves, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Lianxu et al., 2006). Pro-inflammatory cytokines effectively increase expression and release catabolic proteins including matrix metalloproteinases (MMPs), aggrecanases, cathepsins and ADAMTS (Sutton et al., 2009, Lorenz and Richter, 2006, Martel-Pelletier et al., 2008a). Expression of the proteases MMP-1, MMP-13 and ADAMTS-4 are mediated initially in the synovium and ligament cells (Haslauer et al., 2013).

1.4.1. Interleukin-1β

IL-1β is an important inflammatory mediator associated with OA progression. Synthesis of pro-IL-1β is initiated by NF-κB signalling pathways, before caspase-1 or caspase-8 within an inflammasome activates IL-1β (Martinon et al., 2002, Gringhuis et

al., 2012). Levels of IL-1β are significantly higher in synovial tissue during the early stages of OA, which can contribute to disease development (Benito et al., 2005). Chondrocytes stimulated with IL-1β show reduced expression of cartilage specific collagens type II and IX (Goldring et al., 1988). Stimulation of equine chondrocytes with IL-1β initiates up-regulation of IL-1β itself, IL-6 and IL-8 (David et al., 2007). The expression of catabolic enzymes MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 is increased by IL-1β in equine chondrocytes (Busschers et al., 2010). Similar inflammatory responses have been recorded *in vivo* with horses injected into synovial joints with IL-1β or bacterial lipopolysaccharides (LPS) (Ross et al., 2012). After stimulation, synovium and cartilage tissues produced higher levels of MMP-1, ADAMTS-4 and ADAMTS-5, and synovial fluid contained higher PGE_2, neutrophil counts and MMP activity (Ross et al., 2012).

1.4.2. Tumour necrosis factor-α, IL-6 and additional cytokines

TNF-α is another important pro-inflammatory cytokine that contributes towards OA disease processes, inflammation and cartilage degradation. TNF receptors and TNF-α were observed in affected articular cartilage in a surgically induced canine model of OA (Kammermann et al., 1996). During early stages of OA, TNF-α is expressed at higher levels in synovial joint tissues (Benito et al., 2005). Circulating levels of TNF-α and IL-6 in serum have also been found to be higher in knee OA sufferers (Stannus et al., 2010), while release of GAGs from cartilage explants is induced by TNF-α stimulation (Westacott et al., 2000). Inflammatory signalling mediators COX-2 and NO, chemokines and several MMPs are released in response to TNF-α and IL-6 (Reboul et al., 1996, Lianxu et al., 2006, Rosengren et al., 2012).

Leukaemia inhibitory factor (LIF), IL-11, IL-17 and IL-18 are additional pro-inflammatory cytokines contributing to the complex inflammatory situation within synovial joints (Sutton et al., 2009). Visfatin, an adipokine has been reported to have cytokine like effects that could have a role in OA (Gosset et al., 2008). PGE_2, MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 synthesis in chondrocytes was increased by

visfatin stimulation (Gosset et al., 2008). The role of adipokines in OA adds to evidence of obesity as a disease risk factor. The anti-inflammatory cytokines IL-4, IL-10 and IL-13 are also detected in OA synovial fluid and function to counteract inflammatory processes (Pelletier et al., 2001).

1.4.3. Chemokines

Chemokines are classified into four subgroups based on the arrangement of cysteines and disulphides: C, CC, CXC and CX_3C. They initiate chemotaxis by interaction with G protein coupled receptors, thus causing infiltration of inflammatory cells towards sites of inflammation. Macrophages and neutrophils will migrate into synovial joints and secrete cytokines like IL-1β and TNF-α instigating further inflammation (Bondeson et al., 2006). Chemokines such as IL-8 are secreted by chondrocytes in response to cytokine stimulation (Lotz et al., 1992). Other chemokines related to OA include monocyte chemoattractant protein-1 and RANTES (Patel et al., 1998). OA synovial fluid showed differential levels of chemokines compared to normal synovial fluid (Endres et al., 2010). The effects of specific chemokines on recruitment of inflammatory cells or mesenchymal progenitors will have an impact on inflammatory conditions and progression of OA affected joints.

1.4.4. Matrix Metalloproteinases

Catabolic proteases implicated in cartilage breakdown during OA include the zinc-dependant endopeptidase MMPs (Becker et al., 1995). Activation of MMPs is achieved extracellularly by cleavage of pro-peptide domains from secreted inactive zymogens (Woessner, 1991). MMPs within the stromelysin classification have roles in catalysing removal of the pro-peptide domain to activate other MMPs. The normal function of MMPs is to degrade ECM during embryonic development (Hulboy et al., 1997), bone development (Ortega et al., 2003) and cell migration (Vu and Werb, 2000), along with maintaining healthy levels of cartilage turnover. There are associations with MMPs and disease states including cancer (Bourboulia and Stetler-Stevenson, 2010), central nervous system (Agrawal et al., 2008) and lung diseases (Gueders et al., 2006), as well as arthritic conditions. In healthy cartilage there is a

delicate balance between MMPs and tissue inhibitors of MMPs (TIMPs), ensuring appropriate turnover of ECM proteins (Brew and Nagase, 2010). In OA cartilage, expression of MMPs is increased, while TIMP expression is decreased (Kevorkian et al., 2004). This results in increased breakdown of cartilage therefore contributing to progression of the disease.

MMPs have the ability to degrade a range of ECM components including collagen fibrils, proteoglycans and fibronectin (Woessner, 1991). They are grouped based on their substrate specificity into collagenases, gelatinases, stromelysins, matrilysins, membrane type MMPs (MT-MMPs) and others (Nagase et al., 2006). Collagenases (MMP-1, MMP-8, MMP-13, MMP-18) can cleave collagen types I, II, III, VI and IX, but also have the ability to degrade further ECM proteins (Shiomi et al., 2010). MMP-13 (collagenase-3) is believed to have a major role in collagen type II degradation during OA (Reboul et al., 1996) and has been highlighted as a differentially released protein which is up-regulated in OA cartilage (Iliopoulos et al., 2008, Bau et al., 2002). Stromelysins (MMP-3 and MMP-10) can activate procollagenases by removal of their pro-peptide domains (Nagase and Woessner, 1999). MMP-3 (stromelysin-1) can degrade fibronectin, aggrecan and smaller proteoglycans, gelatins and collagens III, IV, IX and X (Shiomi et al., 2010). As previously mentioned, MMPs are capable of degrading non-fibrillar proteins of the ECM, such as fibronectin. Various MMPs including MMP-1, MMP-3, MMP-13 and MMP-14 are known to degrade fibronectin (Zhang et al., 2012a). It has been shown that cartilage aggrecan degradation is reversible, while collagen loss due to MMPs is not reversible (Karsdal et al., 2008). This suggests that MMP mediated processes in OA could have a critical impact on development of this disease.

1.4.5. ADAMTS

Proteins belonging to the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) class are catabolic enzymes with central roles in mediation of cartilage breakdown. Aggrecan is cleaved at the interglobular domain by MMPs -1, -3 and -13 (Fosang et al., 1991, Fosang et al., 1993, Fosang et al., 1996), but ADAMTS

proteases appear to be responsible for the majority of aggrecan and GAG release in OA (Sandy and Verscharen, 2001). This was deduced because the majority of aggrecan fragments found in OA synovial fluid were cleaved at sites targeted by ADAMTS. The most active ADAMTSs *in vitro* are reported to be ADAMTS-5 and ADAMTS-4 (Gendron et al., 2007).

Constituents of the ECM are therefore broken down by a variety of proteinases including ADAMTSs and MMPs, which generate enzyme specific degradative products released into OA synovial joints. Treatments developed to target metabolic enzymes will therefore need to be effective against several mechanisms that mediate cartilage destruction.

1.4.6. Prostaglandin E2

PGE_2 is produced via the arachidonic acid signalling pathway with the key enzyme responsible for PGE_2 synthesis being COX-2 (Hardy et al., 2002). Elevated expression of COX-2 and PGE_2 production has been observed in chondrocytes from OA affected cartilage (Amin et al., 1997). Potential treatments for OA include COX-2 specific inhibitors to decrease PGE_2 production, therefore alleviating pain and inflammation. Once PGE_2 binds to its receptors (EP1-4), it effects release of MMP-1, MMP-13 and ADAMTS-5, increasing collagen and aggrecan loss, and also reduces proteoglycan synthesis (Attur et al., 2008). Induction of COX-2 and PGE_2 by IL-1β stimulation is one of the main mechanisms of cartilage breakdown associated with OA (Hardy et al., 2002).

1.4.7. Nitric Oxide

NO is a free radical molecule that is involved in cellular signalling. The functions of NO specifically related to OA include control of apoptosis, pain perception and catabolic signalling (Abramson, 2008). NO is produced at significantly higher levels in OA cartilage explants, compared to healthy cartilage (Amin et al., 1995). As previously mentioned, pro-inflammatory cytokines stimulate the release of iNOS, aiding NO synthesis. Apoptosis of chondrocytes can be initiated when exposed to NO, through mitochondrial deregulation. NO can also up-regulate activation of MMPs and

stimulate further activation of pro-inflammatory cytokines IL-1 and IL-18 (Boileau et al., 2002, Abramson, 2008).

1.4.8. Influence of subchondral bone changes in OA

Subchondral bone changes including sclerosis and osteopenia, are also known to have an important role in initiation and progression of OA (Henrotin et al., 2012c). Turnover of the bone is controlled by osteoblasts and osteoclasts. Osteoblasts are responsible for synthesis and organisation of bone matrix proteins, and osteoclasts degrade and breakdown the bone matrix. The phenotype of osteoblasts is altered in OA, with unregulated expression of genes including MMP-13, and increased secretion of proteins such as IL-6. IL-8, TGF-β1 and collagen type I (Sanchez et al., 2008). Inflammatory signalling from IL-6 and PGE_2 initiate differentiation and activation of osteoclasts, increasing bone remodelling in the subchondral bone (Liu et al., 2006). These alterations to pro-inflammatory and pro-angiogenic phenotypes cause release of signalling proteins that could interact with chondrocytes in the cartilage above, causing the changes observed in OA (Henrotin et al., 2012c).

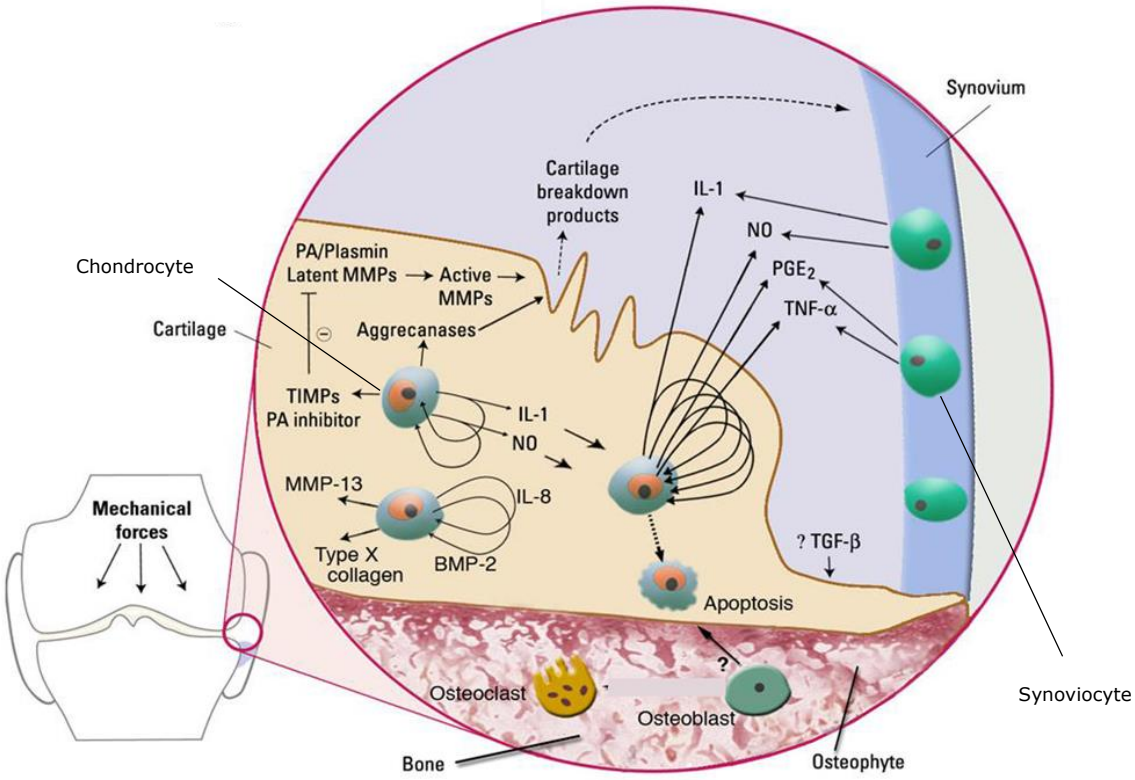


Figure 6. The inflammatory mediators of OA within synovial joints

Cartilage breakdown products stimulate the release of pro-inflammatory signals from synoviocytes. Chondrocytes respond to pro-inflammatory signals by secreting additional cytokines, chemokines and catabolic enzymes, therefore causing further cartilage degradation products (Abramson and Attur, 2009).

1.5. Explant cultures and alternative models available for studying OA

1.5.1. *In vitro* models

1.5.1.1. Advantages and limitations of explant cultures

Relevant *in vitro* models for OA research include chondrocyte, synoviocyte and cartilage explant cultures. These models do not possess the complexity of whole biological systems, but offer a simplistic setting for initial studies. This thesis has utilised a cartilage explant culture model to study cartilage biology, degradation and the secretome. The explant culture model has several advantageous over other models used in cartilage and OA research. Utilizing cultured cartilage explants maintains the chondrocytes within a more physiologically relevant environment than chondrocyte monolayers. Within cartilage ECM, chondrocytes are not in high density cultures or rapidly dividing, as they are during monolayer or alginate bead cultures. Rather chondrocytes are usually distributed alone or in pairs throughout the ECM.

The analysis of proteins released into explant conditioned media is beneficial for providing insights into a variety of degradative processes. ECM constituents and catabolic proteases released will deliver information on cartilage degradation products and insights into the mechanisms involved (Polacek et al., 2010). Further secretion of cytokines will indicate the signalling proteins and pathways utilised during inflammation. Due to the complexity of degradation where many different proteins are involved, proteomic analysis of cartilage explants in culture and proteins released can provide a wealth of biological information (Williams et al., 2011). Measurement of proteoglycan and protein release from explant cultures aids investigations into responses to pro-inflammatory cytokines and anti-inflammatory compounds. Cartilage degradation in response to cytokines TNF-α and IL-1 can be demonstrated by culturing explants (Kobayashi et al., 2005). Comparison of normal and OA affected cartilage explants within culture shows differences in proteoglycan synthesis in diseased tissue (Lafeber et al., 1992). The cartilage explant model can therefore be used to evaluate responses to anti-inflammatories in OA cartilage, or healthy cartilage stimulated with pro-inflammatory cytokines.

Explant models still lack the influence of surrounding synovial fluid, synoviocytes, and immune cells like neutrophils. The explant model applied in this thesis is also a static model therefore the dynamic movement of the joint and synovial fluid are not considered using this approach. Cartilage explant culture therefore provides a simplistic model, to perform initial studies with various experimentally induced perturbations on cartilage and chondrocytes within their natural ECM. Explant models are particularly effective at studying degradation of cartilage proteins, but do not provide significant information on newly synthesised proteins (Polacek et al., 2010). Cartilage explant cultures offer an alternative to *in vivo* studies, and therefore can reduce, refine and replace the use of laboratory animals.

1.5.1.2. Alternative in vitro models

Alternative *in vitro* models for cartilage and OA research include chondrocyte monolayers and high-density alginate bead culture of chondrocytes. Chondrocytes grown in monolayer cultures secrete ECM proteins including collagen type II, proteoglycan and fibronectin (Dessau et al., 1981). One disadvantage to chondrocyte monolayer cultures, is their dedifferentiation into fibroblast-like cells over time (Grundmann et al., 1980). Chondrocytes lose the ability to express collagen type II and instead show increased expression of collagen type I (Benya et al., 1978). Thus, chondrocyte monolayers can only be applied in short term culture studies. Chondrocyte cultures are beneficial for studying newly synthesised proteins in both the chondrocyte secretome (chapter 1.6.2.3) and proteome (1.6.4). The chondrocyte secretome was shown to contain 90% newly synthesised proteins, highlighting the effectiveness of monolayers to study these new proteins in culture (Polacek et al., 2010).

Culturing chondrocytes in an agarose gel matrix can reverse the loss of chondrocyte phenotype (Benya and Shaffer, 1982). Alginate beads also provide a culture environment where the chondrocyte phenotype can be preserved, with spherical chondrocyte morphology being maintained (Guo et al., 1989). The ability to synthesize proteoglycan and collagen type II continues throughout long term cultures

of up to 8 months, allowing studies over longer time periods (Hauselmann et al., 1994). Comparison of chondrocyte characteristics in monolayer, alginate or pellet cultures demonstrates that 3D cultures are most suitable for conserving chondrocyte phenotype (Caron et al., 2012). Culture of primary chondrocytes in alginate beads up to 28 days has shown a hypertrophic phenotype in the chondrocytes, which holds potential for studying the angiogenic of OA (Pesesse et al., 2013). Three dimensional scaffolds can be developed within which chondrocytes maintain expression of cartilage specific genes including COMP, aggrecan, collagen types II and XI (Li et al., 2003). This holds future applications and opportunities for cartilage regeneration and replacement surgery in severe cases of OA.

1.5.2. *In vivo* models

Cartilage biology research and investigations into the pathogenesis of OA take advantage of both *in vivo* and *in vitro* models. Potential OA treatments can be examined *in vitro*, but ultimately need to be tested in an appropriate animal model before clinical trials take place. Animals utilized in OA research include mice, rats, rabbits, guinea pigs, dogs, sheep, goats and horses (Gregory et al., 2012). There are advantages and disadvantages to each specific animal model, therefore a model must be chosen meeting the needs of a desired study (Gregory et al., 2012). Animal models of OA can be spontaneous, introduced with surgical procedures or with injection of degradative enzymes. Guinea pigs spontaneously develop cartilage degeneration that is histologically similar to humans (Bendele and Hulman, 1988). Surgical procedures such as anterior cruciate ligament (ACL) transection or meniscectomy cause instability in joints to produce cartilage degradation (Williams et al., 1982, Bendele, 1987). Cartilage breakdown can also be initiated by injection of iodoacetate (Guzman et al., 2003), collagenase (van der Kraan et al., 1990) or papain into synovial joints (Kopp et al., 1983). Genetic mouse models that develop the OA phenotype are available. Gene deletions of collagen types II and IX cause development of OA conditions in transgenic mice (Helminen et al., 1993, Fassler et al., 1994). Animal models provide opportunities to study new therapeutic approaches within the context of a whole living organism. The number of animals used in scientific investigations can be reduced by completing *in vitro* studies assessments prior to animal models.

1.6. The secretome and proteomics in OA research and cartilage biology

There are a wide range of omic approaches being studied in the OA research field, while this thesis concentrates on the secretome, which is a subsection of the proteome. Omic studies aim to characterise all specific biological molecules contained within a defined biological field, such as the genome, transcriptome, proteome or metabolome. The proteome is the complete set of proteins expressed in a cell, tissue or organism under certain defined conditions (Wilkins et al., 1996). This could be a

healthy or diseased state, or after exposure to a certain treatment. Proteomics involves large-scale and multi-dimensional studies of protein structure and function (Williams and Hochstrasser, 1997, Dhingra et al., 2005). Approaches to gain insight into proteomes utilize techniques including electrophoresis, mass spectrometry, bioinformatics and protein arrays. Proteomic techniques have the potential to make a significant contribution to biomarker searches in the OA research field. The majority of current and potential OA biomarkers are degraded ECM fragments and metabolic proteins (Rousseau and Garnero, 2012). Along with biomarker discovery, proteomics can also progress fundamental understanding of cartilage structure.

Specialised analytical techniques are applied in proteomics allowing evaluation of the protein composition of tissues, cells and culture supernatants (De Ceuninck and Berenbaum, 2009, Ruiz-Romero et al., 2010a, Wilson et al., 2009). One dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins in a complex sample according to molecular weight. More detailed expression profiles can be created by separating the proteins in two phases using two dimensional-electrophoresis (2DE) (Rabilloud et al., 2010). During 2DE, isoelectric focusing separates proteins in the first dimension along a pH gradient before second dimension separation by molecular weight, which provides improved profiling over SDS-PAGE approaches. Gels can be fixed and stained with various stains, before scanning with a densitometer to provide a high-resolution image. Image analysis software can measure expression levels of specific protein spots to reveal proteins that differ between two or more sample types (e.g. comparison of healthy vs diseased tissue).

Mass spectrometry analysis can provide identification of proteins corresponding to stained and visualised spots (Ruiz-Romero et al., 2010a, Wilson et al., 2009). Specific spots of interest are excised from gels and enzymatically digested, generating peptides. The peptides are first ionized by various methods including electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI) and surface enhanced laser desorption/ionization (SELDI) (Amstalden van Hove et al., 2010) to create charged peptides. Charged peptides present are separated depending

on the mass/charge ratio (m/z) by a mass analyser. Peptides are detected and ion masses are recorded, producing a peptide mass fingerprint (PMF). Tandem MS (MS/MS) allows fragmentation of selected peptides, which can be compared with predicted/calculated masses of peptides in databases. This allows proteins in complex samples to be identified. Publically available databases include NCBInr and Swiss-Prot.

Proteins present in complex samples can also be profiled using protein antibody microarrays approaches (Borrebaeck and Wingren, 2009, Glökler and Angenendt, 2003). Sample media is incubated with an array membrane with antibodies directed against a range of proteins attached, so proteins present will be specifically bound to antibodies. Attached proteins can be detected by fluorescence or chemiluminescence to provide a quantitative measurement of protein levels. Cytokine antibody array membranes containing inflammatory related proteins relevant to OA could provide information on altered protein synthesis in OA synovial joints.

OA affects the entire synovial joint and so many of the distinct tissues present are affected and potentially contribute to disease progression (Figure 7). Proteomic investigations have therefore been performed on a range of the tissues and cells present including: whole cartilage (Garcia et al., 2006, Wu et al., 2007, Guo et al., 2008, Haglund et al., 2008, Perez et al., 2010, Ruiz-Romero et al., 2010a, Onnerfjord et al., 2012), chondrocytes (Ruiz-Romero et al., 2005, Cillero-Pastor et al., 2010, Iliopoulos et al., 2008, Lambrecht et al., 2008, Lambrecht et al., 2009, Koo et al., 2010, Calamia et al., 2010, Ruiz-Romero et al., 2010b), synovium (Lorenz et al., 2003, Tilleman et al., 2005), synoviocytes (Ruiz-Romero et al., 2010a, Bo et al., 2009), along with studies on the secretome of these constituents. Biological fluids like serum, synovial fluid and urine from OA sufferers have also been analysed in the search for altered protein expression and biomarkers (Sinz et al., 2002, Yamagiwa et al., 2003, Drynda et al., 2004, Kamphorst et al., 2007, Gobezie et al., 2007, Nemirovskiy et al., 2007, Nemirovskiy et al., 2010, Li et al., 2007, Xiang et al., 2004, Jmeian and El Rassi, 2008, Chiaradia et al., 2012, Mateos et al., 2012, Gharbi et al.,

2013, Ritter et al., 2013, de Seny et al., 2011, Fukuda et al., 2012, Fernandez-Costa et al., 2012).

Extensive proteomic studies have revealed various biomarkers to evaluate OA. Current biomarkers being investigated in relation to OA have been reviewed by Lotz and colleagues (Lotz et al., 2013), (Figure 7). The OA biomarkers year review 2013 summarises the range of OA biomarkers being evaluated in serum, plasma, urine and synovial fluid, including COMP, CTX-II, Coll2-1 and MMP-13 (Lafeber and van Spil, 2013). Biomarkers can be classified with the BIPED classification guidelines: burden of disease, investigative, prognostic, efficacy of intervention and diagnostic biomarkers (Bauer et al., 2006). As expected many of the potential biomarkers are degradation fragments of the ECM, including collagen, aggrecan and COMP (Lotz et al., 2013). Other biomarkers that have been investigated include metabolic proteins like MMPs that will influence cartilage turnover, and signalling proteins such as cytokines (Lotz et al., 2013).

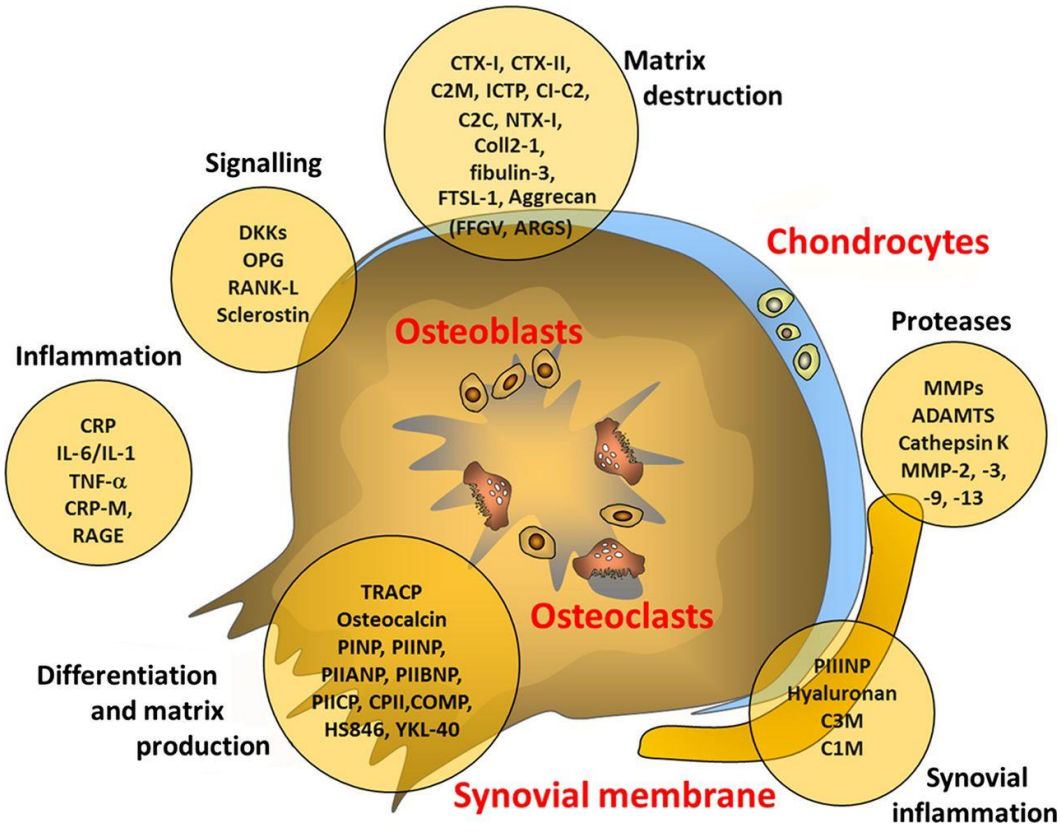


Figure 7. Biomarkers for OA can originate from various tissues and be related to different processes

(Lotz et al., 2013)

Biomarker molecules can be products of cartilage breakdown or other ECM proteins related to matrix turnover. Signalling molecules may also be biomarkers, specifically those involved in pro-inflammatory signalling, along with the catabolic proteases released in response to inflammation could act as biomarkers of OA.

1.6.1. The secretome

This thesis focuses on the secretome of cultured cartilage explants, aiming to develop understanding of cartilage biology and degradation. The secretome is a subsection of the proteome, and is used to describe proteins that are actively secreted from cells or tissues. The chondrocyte secretome is vital in regulation of cartilage, as they secrete all the components of the ECM before matrix assembly. Alterations in ECM or metabolic proteins secreted by chondrocytes will have consequences for cartilage structure, potentially contributing to cartilage loss. The cartilage secretome can be assessed by analysis of cartilage conditioned media, although many ECM proteins will be degraded by catabolic processes or simply leak out into the supernatant during the culture process and therefore are not being actively secreted. Proteomic techniques monitoring release of proteins from cultured cartilage under various conditions/treatments will still provide vital insights into cartilage biology and degradation that are relevant to OA.

1.6.2. Cartilage and chondrocyte secretome

1.6.2.1. OA cartilage secretome

Degenerative processes during OA progression will be enhanced by disruption to the normal levels of protein secretion from chondrocytes within cartilage. By comparing healthy and OA cartilage secretome, several studies have identified proteins with either up-regulated or down-regulated release. OA affected articular cartilage explants secreted inhibin βA that is associated with inflammation (Hermansson et al., 2004). This study also showed connective tissue growth factor (CTGF) and cytokine-like protein C17 were released at higher levels, using medium containing [35S] on methionine and cysteine residues allowing identification of newly synthesized proteins (Hermansson et al., 2004). A antibody microarray study identified 11 secreted proteins in a OA donor sample, and also found 43 secreted proteins using SDS-PAGE and 2DE approaches combined with MS/MS (De Ceuninck et al., 2005). Secreted proteins included chitinase 3-like protein 2 (YKL-39), tissue inhibitor of metalloproteinase-1 (TIMP-1), tumour necrosis factor receptor 1 (TNF-R1),

vitamin D binding protein, pigment epithelium-derived factor (PEDF) and osteoprotegerin (De Ceuninck et al., 2005). Proteins highlighted in these studies on OA cartilage secretome all have roles in signalling pathways and metabolic control therefore differential changes could impact on cartilage maintenance.

Stable isotope labelling with amino acids in cell culture (SILAC) allows existing and newly synthesized proteins to be differentiated, one such study showed that 71% of cartilage explant released proteins were not synthesized during incubation (Polacek et al., 2010). The 29% of proteins containing labelled amino acids (newly synthesized) included YKL-40 and TIMP-1, both of which are involved in ECM remodelling. The majority of the non-labelled proteins released were ECM constituents like COMP, aggrecan core protein and cartilage intermediate layer protein. Most ECM released proteins during explant cultures may simply be leaking out from the tissue rather than being actively synthesized by chondrocytes. Although it is possible that as ECM turnover occurs, newly synthesized ECM components to replace older proteins are incorporated straight into cartilage.

Limited differences were discovered comparing human OA cartilage explant secretome before and after treatment with IL-1β, analysed with a QconCAT quantitative approach (Peffers et al., 2013). Only decreases in TIMP-1 showed statistical alteration with IL-1β, while MMP-1, MMP-3, plasminogen, COMP, fibromodulin and aggrecan were not significantly altered according to QconCAT (Peffers et al., 2013).

1.6.2.2. Secretome of cytokine stimulated cartilage

Studies in this thesis applied healthy equine cartilage that was stimulated with inflammatory cytokines, therefore it is of interest to compare findings with previous studies. MMP mediated degradation of OA cartilage shows release of collagen II neoepitope peptides after stimulation with IL-1β and oncostatin M (Nemirovskiy et al., 2007). Nemirovsky applied a similar method to this thesis, by culturing visually normal (healthy) human cartilage with cytokines before a high-throughput MS analysis, which identified collagen epitopes. Similar collagen degradation products could potentially

be identified in an equine cartilage model. A difference in-gel electrophoresis (DIGE) based study of mouse cartilage stimulated with IL-1β or all-trans-retinoic acid (Ret A), allowed accurate quantification of differentially released proteins (Wilson et al., 2008). Comparison of the protein profiles of untreated control media with the two treatments showed altered levels of a number of ECM components and metabolic proteins, which could have implications in OA. Significant increases in MMP-3, YKL-40, haptoglobin and neutrophil gelatinase associated lipocalin (NGAL) were stimulated by IL-1β (Wilson et al., 2008). Ret A treatment increased COMP, hyaluronan and proteoglycan link protein 1 (HAPLN1), aggrecan G1 domain proteins, matrilin-3, and serotransferrin (Wilson et al., 2008).

Another aspect of OA is load-induced injury leading to damage of cartilage and degradation therefore secreted proteins in a bovine stifle model of cartilage injury with mechanical compression and stimulation with pro-inflammatory cytokines (IL-1β and TNF-α) were investigated (Stevens et al., 2008). Mechanical compression injury mostly caused higher levels of intracellular proteins associated with cell death including vimentin, pyruvate kinase, glucose-regulated protein 58kDa (GRP58) and glucose regulated protein 78kDa (GRP78). The cytokines IL-1β and TNF-α both induced release of MMP-3, clusterin, YKL-39 and YKL-40 (Stevens et al., 2008). This bovine model and stimulatory treatments were further assessed in 2009 with iTRAQ™ and LC-MS/MS quantitative analysis (Stevens et al., 2009). Collagen fibre synthesis was reported to be reduced by all stimuli (IL-1β, TNF-α, mechanical), while aggrecan domain release was increased by cytokines (Stevens et al., 2009). Six MMPs, TIMP-1, -2, chemokines and cytokines also showed differential release due to IL-1β, TNF-α or mechanical compression (Stevens et al., 2009). By applying a LC-MS/MS approach to equine cartilage secretome, this thesis aims to discover if similar or additional proteins are identified in the secretome, to the previously mentioned human (Nemirovskiy et al., 2007) and bovine (Stevens et al., 2009) high-throughput MS analyses.

1.6.2.3. Chondrocyte secretome

In contrast with the cartilage explant secretome, the chondrocyte secretome will contain predominantly newly synthesised proteins and not be complicated by release of pre-existing ECM components into supernatant. An antibody-based microarray identified cytokines, chemokines, angiogenic and growth factors increased by pro-inflammatory cytokine treatment (IL-1β, TNF-α) in the human chondrocyte secretome (De Ceuninck et al., 2004). Eight proteins were identified from 2DE before techniques including CPC precipitation of GAGs, collagenase digestion and a Q sepharose resin batch column were applied (Catterall et al., 2006). Stimulation with IL-1β and oncostatin M caused increases in MMP-1, MMP-3 and cyclophylin A secretion, while calgizzarin, cofilin, YKL40 and β2-microglobin were identified in untreated control and cytokine 2DE gels (Catterall et al., 2006). Poorly resolved higher molecular weight protein regions on the 2DE maps limited the protein identifications possible in this study (Catterall et al., 2006).

Studies have been completed on the chondrocyte secretome from rat articular cartilage treated with LPS, which is thought to be related to septic induced OA processes (Papathanasiou et al., 2010). Toll-like receptor activation by LPS is believed to contribute to OA, RA and crystal-induced joint damage (Ospelt et al., 2009, Su et al., 2005, Liu-Bryan et al., 2005). Proteins related to initiation of cartilage remodelling (MMP-1, MMP-3, YKL-40) and immune response associated proteins were seen to be increased by LPS stimulation (Haglund et al., 2008). Chemokines MIP-3α (CCL20) and LIX (CXCL5) were confirmed to show increased expression using a rat cytokine antibody array (Haglund et al., 2008). These chemokines will recruit inflammatory cells to the synovial joint during OA contributing to inflammatory processes.

SILAC approaches applied to chondrocytes in monolayer culture, displayed 103 newly synthesized proteins were secreted and identified (Polacek et al., 2010). These were mostly ECM constituent proteins, while inflammatory mediators and MMPs were also present. Further work from this group examined chondrocyte and

mesenchymal stem cell secretomes using the SILAC approach, and studied the phenotypes of both cell types, and quantified release of MMPs and TIMPs (Polacek et al., 2011). The SILAC technique has also been applied to chondrocytes treated with IL-1β, to show quantitative and identification of 115 proteins, including pro-inflammatory mediators and proteases, type VI collagen and TGF-β pathway proteins (Calamia et al., 2011).

The main aim of this thesis was to study responses in the cartilage secretome to stimuli, therefore a summary table of secretome studies of cartilage and chondrocyte cultures is presented Table 1.

Table 1. Secretome studies completed on cartilage and chondrocyte cultures

Study	Tissue/Cell	Diseased or treated	Techniques	Proteins highlighted
Studies on OA or normal tissue/cells				
(Hermansson et al., 2004)	Cartilage	OA and healthy	[35S] Methionine/Cysteine radiolabelling, CPC, 2DE, HPLC MS/MS	Inhibin βA higher in OA
(De Ceuninck et al., 2005)	Cartilage	OA	SDS-PAGE, 2DE, MS/MS, Antibody microarray	YKL-39, TNFR1 and TIMP-1 identified
(Polacek et al., 2010)	Cartilage and chondrocyte	Healthy	SILAC quantification, SDS-PAGE, LC-MS/MS	Cartilage: 25-30% proteins newly synthesised. Chondrocytes: 90% proteins newly synthesised.
(Polacek et al., 2011)	Chondrocyte and MSC	Compared normal chondrocytes with MSCs	SILAC quantification, SDS-PAGE, LC-QTOF-MS/MS	Clusterin, mimecan, proteoglycan-4, tenascin, sushi from chondrocytes
Studies with stimuli				
(Nemirovskiy et al., 2007)	Cartilage	IL-1β and Oncostatin M	LC-MS/MS	Collagen II neoepitopes
(Wilson et al., 2008)	Cartilage	IL-1β, Ret A and untreated	2DE, MS/MS	MMP-3, NGAL increased by IL-1β. COMP, matrilin-3 increased by Ret A.
(Stevens et al., 2008)	Cartilage	Untreated, IL-1β, TNF-α and mechanical stress	SDS-PAGE, LC-MS/MS	MMP-3, YKL-40, COMP and collagen VI release increased by treatments

(Stevens et al., 2009)	Cartilage	Untreated, IL-1β, TNF-α and mechanical stress	iTRAQ™, strong cation exchange chromatography, LC-MS/MS	Differential release of aggrecan, collagens, MMPs, chemokines, cytokines
(Clutterbuck et al., 2011)	Cartilage	Untreated, IL-1β and carprofen treatments	nanoLC-MS/MS	Western blotting of clusterin, CILP-1, MMP-1, MMP-3 and TSP
(Peffers et al., 2013)	Cartilage	Cartilage from OA patients IL-1β or unstimulated	QconCAT quantitative approach, LC-MS/MS	Only differential protein: TIMP-1 decreased with IL-1β stimulation
(De Ceuninck et al., 2004)	Chondrocyte	IL-1β, TNF-α treatment compared with non-stimulated	Cytokine protein microarray	Up-regulation of several cytokines and chemokines with treatments
(Catterall et al., 2006)	Chondrocyte	Untreated, IL-1 and oncostatin M	2DE, LC-MS/MS, CPC, Q sepharose, chondroitinase/hyaluronidase	MMP-1, MMP-3, YKL-40, cofilin, S100A11, β2-microglobulin identified
(Haglund et al., 2008)	Chondrocyte and cartilage	LPS stimulated and unstimulated	SDS-PAGE, LC-MS/MS, cytokine antibody array	LPS induced YKL-40, and MMP-3, -13 and various complement proteins.
(Calamia et al., 2011)	Chondrocyte	Normal and IL-1β treated	SILAC quantification, SDS-PAGE, MS/MS	IL-1β increased pro-inflammatory mediators, collagen VI and TGF-β pathway. Decreased secretion of TSP, aggrecan and vitamin K-dependant proteins.

1.6.3. Whole extracts of articular cartilage

Proteomics of whole cartilage contributes to the understanding of structural alterations in cartilage composition occurring during OA. Proteins present in both the ECM and the chondrocytes within it will be studied in whole cartilage proteomics. The "matrisome" defines all proteins that are structural components of extracellular matrices (Hynes and Naba, 2012). The mammalian matrisome is believed to contain around 300 proteins, with the possibly of degraded products of these being OA biomarkers (Hynes and Naba, 2012).

A high percentage of proteins identified in previous whole cartilage studies are ECM components (Garcia et al., 2006) for example, COMP and fibromodulin (Ruiz-Romero et al., 2010a). Altered expression ECM components could ultimately lead to cartilage degradation. Comparison studies between healthy and OA affected cartilage have identified ECM components showing differential expression, including the fibulin family proteins (-1D, -2, -3) (Wu et al., 2007). In another comparison between healthy and OA cartilage, five energy production related enzymes were differentially expressed (alcohol dehydrogenase, adenosine kinase isoenzyme 1, flavin reductase, enolase 1 and pyruvate kinase 3 isoform 2), and 3 signalling proteins were present at differential levels (annexin A1, tubby protein homolog and phosphatidylethanol-binding protein) (Guo et al., 2008). This study also identified higher levels of collagen type I and VI and a reduced level of mitochondrial superoxide dismutase (SOD2) in OA cartilage (Guo et al., 2008). In another study, chitinase 3-like protein 1 (YKL-40) was up-regulated in cartilage by TNF-α and LPS treatment (Haglund et al., 2008). YKL-40 is associated with remodelling of the ECM and therefore could have roles in OA disease progression. During a proteomic analysis of rat articular cartilage, 47 proteins were identified in the cartilage proteome including latexin (Perez et al., 2010). This was the first cartilage proteome study to identify latexin, which is a protein inhibitor of zinc-dependent metallocarboxypeptidases (Perez et al., 2010).

A quantitative proteomic analysis using iTRAQ™ on cartilage from 8 separate anatomical locations, showed differences in protein patterns between the various cartilage types. These differences in protein distribution could contribute to the structure and function of each cartilage type (Onnerfjord et al., 2012). Another study applied quantitative iTRAQ™ on OA and healthy cartilage after Proteominer™ enrichment (Ikeda et al., 2013). This study reported three novel OA biomarkers: PRDX6 (peroxiredoxin-6), BAALC (brain and acute leukaemia, cytoplasmic) and LECT2 (leukocyte cell-derived chemotaxin-2) (Ikeda et al., 2013).

In summary, whole cartilage proteomic studies have helped to provide improved understanding of cartilage biology. These studies have shown that OA changes the protein composition and structure of cartilage ECM.

1.6.4. Chondrocyte proteome

Whole cell lysates of chondrocytes also contribute to OA research, with the first human articular chondrocyte 2DE proteomic profile completed in 2005 with 93 proteins identified (Ruiz-Romero et al., 2005). Inflammatory stimulation of chondrocytes with cytokines IL-1β and TNF-α before 2DE analysis determined expression changes (35 proteins identified) that may occur in the inflamed OA environment (Cillero-Pastor et al., 2010). A reverse phase protein array also identified 76 differentially expressed proteins that were altered by stimulation with the cytokines IL-1β and TNF-α (Iliopoulos et al., 2008). Comparison between chondrocytes isolated from normal cartilage and OA affected cartilage has shown changes in expression of 17 proteins (Lambrecht et al., 2008). Two proteins highlighted as differentially expressed in healthy and OA chondrocytes were vimentin (Lambrecht et al., 2008) and αB-crystallin (Lambrecht et al., 2009).

The ECM provides a hydrophilic and hypoxic environment that chondrocytes must function within, therefore studies have investigated how changes in these conditions effects protein expression. Incubation in culture media of different osmolarities (320 mOsm/kg compared to 400 mOsm/kg) found 20 protein spots with significantly altered expression (Koo et al., 2010). 400 mOsm/kg is the average

osmolarity of articular cartilage (Koo et al., 2010). A comparison of normal and OA chondrocytes cultured under hypoxic conditions found 42 proteins were differentially expressed (Ruiz-Romero et al., 2010b). These included increases in cyclophilin A and TNF receptor associated protein 1 (TRAP1) (Ruiz-Romero et al., 2010b).

The proteome of chondrocytes exposed to popular treatments for OA, glucosamine (GluN) and chondroitin sulphate (CS) was studied to investigate mechanisms behind their reported therapeutic benefits (Calamia et al., 2010). There were 31 proteins with altered expression due to treatment with GluN or CS, alone or in combination, with SOD2 levels reduced by all treatments (Calamia et al., 2010). The chaperone GRP78 was up-regulated by GluN, but not by CS (Calamia et al., 2010).

1.6.5. Chondrocyte mitochondria proteome

SOD2 is a mitochondrial protein involved in superoxide (a reactive oxygen species) metabolism, and has been reported to be reduced in proteomic studies of chondrocyte mitochondrial fractions (Ruiz-Romero et al., 2006, Ruiz-Romero et al., 2009). These studies also identified significant differences in TRAP1, inner membrane mitofillin (IMMT) and optic atrophy 1 (OPA1) that could contribute to chondrocyte functioning disruptions and an increase in cell death. In another examination of proteins in mitochondrial extracts, the expression of dimethylarginine dimethylaminohydrolase 2 was found to be significantly increased when chondrocytes had been exposed to IL-1β (Cillero-Pastor et al., 2012). This enzyme could have a role in production of NO during inflammation and OA (Cillero-Pastor et al., 2012).

1.6.6. Synovial fluid OA biomarker studies

Synovial fluid at joints is encapsulated by the synovial membrane, and is in contact with cartilage surfaces, allowing potential biomarkers of OA to be present here. Fibrinogen β-chain degradation products were found in synovial fluid from patients with OA, rheumatoid arthritis (RA) and reactive arthritis (Sinz et al., 2002). When OA synovial fluid and normal synovial fluid were compared, 18 protein spots were significantly altered in the OA sample, including increased haptoglobin α2 chains

that are associated with inflammation (Yamagiwa et al., 2003). Calcium binding proteins, S100A8 and S100A9, have a diverse range of cellular functions and were found to be significantly higher in synovial fluid in RA compared to OA (Drynda et al., 2004). In another study, 29 proteins were identified in OA synovial fluid with use of ultracentrifugation and solid-phase extraction techniques, with 6 proteins suggested as possible OA biomarkers due to disease association (Kamphorst et al., 2007). Dermcidin, aggrecan and cystatin A all had reduced expression comparing normal and OA synovial fluid from the knee (Gobezie et al., 2007). A proteomic study on equine synovial fluid comparing healthy and OA horses provided identification of 17 differentially regulated proteins, including serotransferrin, anti-thrombin III, vitamin D binding protein and apolipoprotein A-I (Chiaradia et al., 2012). Spectral counting analysis applied to RA and OA synovial fluid after LC-MALDI TOF/TOF analysis, revealed 136 differential proteins (Mateos et al., 2012). Proteins highlighted in OA synovial fluid included COMP, aggrecan core protein 2, YKL-40, complement factor D, tetranectin, cartilage acidic protein 1 and fibronectin (Mateos et al., 2012).

Surgically induced OA with anterior cruciate ligament (ACL) transection caused alterations in the levels of several proteins in canine synovial fluid including those in the complement pathway (Gharbi et al., 2013). Application of 2D-DIGE compared proteins in healthy and OA knee synovial fluid (Ritter et al., 2013). Sixty-six proteins in the following pathways were affected: the complement, response signalling and coagulation pathways. Differential expression of clusterin, lumican and 3 other proteins were validated using selective reaction monitoring (Ritter et al., 2013).

Changes in the complement pathway and proteins have been detected across several OA synovial fluid analyses, depicting a change in the inflammatory status of affected joints.

1.6.7. Synovium/synoviocyte proteome

Proteomic studies have also been completed on synovial membrane and the synoviocytes that maintain the synovium. A western blot array with 260 immobilised antibodies detected 58 proteins showing significant differences in expression between

RA and OA, including SOD2, cathepsin D and STAT1 (Lorenz et al., 2003). A comparison of protein profiles of OA synovium with RA and spondyloarthropathy synovium has also been completed (Tilleman et al., 2005). Significantly differential expression of 25 proteins in synovial fibroblasts was observed in a comparison between normal and RA or OA patients (Bo et al., 2009). Synoviocytes gave a very similar proteomic profile map to chondrocytes (Ruiz-Romero et al., 2010a), which is unsurprising given the function of both cell types is to maintain fibrous structures surrounding them.

1.6.8. Urine and serum OA biomarker studies

The non-invasive collection of urine makes it an ideal biofluid for clinical analysis of biomarkers for early disease identification and monitoring of OA progression. Collagen type II neoepitopes have been investigated in urine and synovial fluid using immunoaffinity LC-MS/MS (Nemirovskiy et al., 2007). The most abundant neoepitope was identified as a 45 amino-acid peptide (uTIINE) and appeared to be an informative biomarker for MMP activity (Nemirovskiy et al., 2007, Nemirovskiy et al., 2010)

Analysis of serum has identified an autoantibody to triosephosphate isomerase as a potential diagnostic OA biomarker during a large population based study (Xiang et al., 2004). Differential protein levels of vitamin D-binding protein precursor along with apolipoprotein A-I and A-IV precursors were reported in a 2D electrophoresis based investigation comparing normal and OA serum (Jmeian and El Rassi, 2008). This study discovered that OA patients taking soy supplements, had serum alterations in kininogen, transthyretin, hemopexin precursor and vitamin D-binding protein precursor (Jmeian and El Rassi, 2008). Analysis of OA serum compared to healthy individuals identified release of two biomarkers higher in OA: V65 vitronectin fragment and C3f peptide (de Seny et al., 2011). Quantitative iTRAQ™ analysis comparing serum from normal and OA patients produced a panel of potential biomarkers for early, moderate and severe OA (Fernandez-Puente et al., 2011). Three glycoproteins were proposed as early biomarkers (lysosomal-associated

membrane protein (LAMP), galectin-3-binding protein (LG3BP) and glycosylphosphatidylinositol specific phospholipase D (PHLD)), whilst in severe OA: COMP, complement proteins and lumican were among biomarker candidates (Fernandez-Puente et al., 2011). In a glycoproteomic 2D-LC-MALDI MS analysis, four proteins were put forward as potential plasma biomarkers for OA progression: clusterin, macrophage stimulating protein, hemopexin and alpha-1 acid glycoprotein-2 (Fukuda et al., 2012). A haptoglobin β chain isoform was increased in OA serum compared to healthy individuals, which was one of fourteen differential proteins. This was discovered in a study utilizing DTT and acetonitrile depletion methods followed by 2D-DIGE (Fernandez-Costa et al., 2012).

1.7. Treatments for OA

Conventional therapeutic treatments for OA are not effective at halting disease progression or repairing damage to cartilage (Goldring and Goldring, 2007). OA is commonly treated clinically with paracetamol in mild cases, or NSAIDs such as carprofen if paracetamol is inadequate. These conventional treatments only target the clinical symptoms associated with OA by reducing pain and inflammation (Goldring and Goldring, 2007). New disease modifying OA drugs (DMOADs) need to be developed to protect chondrocytes and reverse changes occurring in OA affected cartilage. Therapeutics targeting processes involved in cartilage degeneration such as MMPs, the cytokines IL-1β and TNF-α, and iNOS may prove to be effective DMOADs (Mobasheri, 2013). Supplements including GluN and CS are also popular alternatives taken by OA sufferers, while herbal medicines may also be prospective nutraceuticals (Cameron et al., 2009).

1.7.1. Carprofen and other NSAIDs

Pain and inflammation are relieved by NSAIDs, therefore allowing improvements in patient mobility. NSAIDs including phenylbutazone, flunixin, ketoprofen, naproxen and carprofen are available to treat equine OA (Goodrich and Nixon, 2006). The effects of the NSAID carprofen on explant cultures of equine articular cartilage were studied throughout this thesis.

Prescription NSAIDs available for human use include: aspirin, ibuprofen, naproxen, diclofenac, celecoxib and etoricoxib (Zhang et al., 2008). The mechanism of action of NSAIDs is achieved via inhibition of cyclooxygenase enzymes (COX) (Vane and Botting, 1998). Production of bioactive lipids from the arachidonic acid pathway is controlled by the two isoforms: COX-1 and COX-2 (Vane and Botting, 1998). Constitutive expression of COX-1 maintains healthy production of bioactive lipids for biological functions including prostacyclin release, which is cytoprotective to gastric mucosa of the stomach (Griffin, 1998). NSAIDs are associated with adverse side effects on gastrointestinal toxicity due to COX-1 inhibition (Griffin, 1998). COX-2 is the inducible isoform that is up-regulated to during inflammatory processes (Vane et

al., 1994). Selective COX-2 inhibitors have therefore been developed to reduce inflammatory conditions in OA, whilst not causing gastrointestinal side-effects. Cardiovascular and renal side effects are reported with COX-2 specific NSAIDs celecoxib, parecoxib and valdecoxib (Caldwell et al., 2006, Aldington et al., 2005, Hooper et al., 2004), therefore NSAID treatment continues to have associated risks. Even with possible side-effects, OA treatment with NSAIDs continues to be recommended due to their proven analgesic effects (McAlindon et al., 2014).

Carprofen (an NSAID marketed as Rimadyl® by Pfizer) was chosen for studies on IL-1β stimulated cartilage explant secretome in this thesis. This NSAID is used clinically to treat OA associated inflammation and joint pain in horses and dogs (Goodrich and Nixon, 2006). Carprofen was voluntarily withdrawn from human use in 1998 for commercial reasons, but continues to be applied to treat companion animals. It selectively inhibits COX-2 and blocks synthesis of PGE_2 in the arachidonic acid signalling pathway therefore reducing inflammatory processes. Carprofen selectivity for COX-2 over COX-1 has been reported in an equine study (Beretta et al., 2005). Carprofen has been reported to improve clinical symptoms of OA in dogs (Sanderson et al., 2009). Changes in cartilage and subchondral bone that are associated with OA are inhibited by carprofen treatment in dogs (Pelletier 2000). *In vitro* studies on carprofen R and S enantiomers treatment of cartilage explants showed that proteoglycan synthesis was significantly increased by carprofen (Frean et al., 1999) The highest increase in proteoglycan synthesis was associated with the S enantiomer (Frean et al., 1999). PGE_2 production and GAG release can also be reduced by carprofen *in vitro* during equine studies, which shows the potential therapeutic effects of this NSAID in OA treatment (Goodrich and Nixon, 2006).

1.7.2. Intra-articular glucocorticoids and hyaluronate

Injection of glucocorticoids or hyaluronate into OA affected joints is another conventional approach to disease management. OARSI (Osteoarthritis Research Society International) recommends these injections if patients are not responding to NSAID therapy (McAlindon et al., 2014). Glucorticoids possess anti-inflammatory

actions asserted through interaction with cytoplasmic glucocorticoid receptors (van der Velden, 1998). The ability of glucocorticoids to alleviate symptoms of OA in the long-term continues to be debated. *In vitro* evidence from glucocorticoid treatment does not support disease modifying capabilities for OA. For example, IL-1β stimulated GAG degradation and aggrecan cleavage are not decreased in cartilage explants by two glucocorticoids: dexamethasone and triamcinolone (Busschers et al., 2010). Hyaluronate is a large GAG component of cartilage ECM and synovial fluid. Injections of hyaluronate into OA affected joints are considered to provide symptomatic relief. Numerous studies have assessed therapeutic benefits of hyaluronate injections, and show that benefits are seen several weeks after injection (Zhang et al., 2008). A comparative study of glucocorticoids and hyaluronate reported that both treatments were equally effective against OA (Leopold et al., 2003). Both treatments provided higher WOMAC (Western Ontario and McMaster Universities Arthritis Index) scores, but clinical improvements were moderate (Leopold et al., 2003). Although modest alleviation of symptoms can be achieved with intra-articular injections, substantial OA recovery does not occur and new therapeutic approaches are needed.

1.7.3. Alternative biological targets

Fundamental OA disease processes including signalling mediators, inflammatory pathways and metabolic proteins can be targeted for developing new therapeutics. Inducible nitric oxide synthase can be inhibited, which may have positive applications in OA affected joints. An experimental dog model of OA showed iNOS inhibition lowered the levels of IL-1β, COX-2, MMP-1 and MMP-3 (Pelletier et al., 1999). Inhibitors of pro-inflammatory cytokines have been investigated as a potential treatment for OA. Down-regulation of MMP-1, -3 and -13 along with decreases in collagen II cleavage and GAG release were observed in OA cartilage treated with IL-1 and TNF-α antagonists (Kobayashi et al., 2005). Targeting catabolic proteases with anti-MMP therapies could lessen cartilage break down and loss occurring during OA. Pain behaviour, osteochondral vascularity and disease severity were reduced in a rat model of OA treated with an MMP inhibitor (Mapp et al., 2010). All these biological

targets have essential roles in healthy processes; therefore it is disadvantageous if normal processes are affected. Over-activity of catabolic proteins and inflammatory signals is characteristic of OA, therefore specifically targeting these processes could provide advances in OA therapeutics.

1.7.4. Nutraceuticals

The anti-inflammatory and chondroprotective effects of naturally derived compounds suggest nutraceuticals could be beneficial in OA treatment. Nutraceuticals may provide therapeutic effects without the adverse side-effects and lack of disease modifying properties associated with conventional medicine (Cameron et al., 2009). Popular equine nutritional supplements contain a range of potentially beneficial nutraceuticals to support joint health (Trumble, 2005). Nutraceutical alternatives may not totally replace, but could be taken in combination with existing therapeutics such as NSAIDs, to reduce dependence and consumption of traditional pharmaceuticals. Popular nutraceuticals include GluN and CS, while many herbal medicines also have potential valuable properties. Studies on GluN, CS and other potential nutraceuticals including curcumin, resveratrol, rosehips and boswellia have shown beneficial chondroprotective and anti-inflammatory properties that may be beneficial for OA treatment (Henrotin and Lambert, 2013, Clutterbuck et al., 2009, Csaki et al., 2009, Shakibaei et al., 2012, Sengupta et al., 2011).

1.8. Aims of this project using the equine inflammatory articular cartilage explant model

OA is a common cause of joint disease in horses (Clegg and Booth, 2000), therefore enhancing understanding of OA and cartilage degradation in this species is of importance. Therefore studies on equine cartilage secretome were initiated aiming to study cartilage biology and degradation utilising tissue from this species. Studying a time course of the secretome aimed to determine dynamic changes in protein release over time, which has not previously been performed using proteomic approaches. The anti-inflammatory effects of the NSAID carprofen, on cytokine stimulated cartilage explants were studied by high-throughput MS. Catabolic MMPs and ECM proteins identified in the secretome were further investigated in response to IL-1β and carprofen for the first time.

A previous proteomic study on equine secretome reported various ECM, non-ECM and intracellular proteins in the secretome (Clutterbuck et al., 2011). It did not offer a description of which proteins in each treatment group were identified by MS, although there is quantitative western blotting of five proteins. This thesis developed on high-throughput MS initially published (Clutterbuck et al., 2011), by identifying a greater range of proteins and associating proteins with treatment groups. This provided further insights into pro-inflammatory cytokine responses and investigated the capacity of this model as a screening tool for anti-inflammatory compounds. Compared to the previous work, additional protein identifications were made in equine cartilage secretome: MMP-13, collagen type II, secreted frizzled related protein (SFRP) and macrophage migration inhibitory factor (MIF). Inflammation and associated cartilage degradation are fundamental in OA progression, therefore stimulation with cytokines such as IL-1β or TNF-α during these *in vitro* assessments contributed towards understanding of this prevalent disease. While many complex features present in synovial joints and OA inflammation (immune cells, mechanical loading, hypoxia) were absent within the explant model, these studies aimed to improve a suitable alternative to *in vivo* models. These studies advanced biological

understanding of equine cartilage explant cultures, degradation and OA. Biomarkers to monitor IL-1β stimulated processes and for screening anti-inflammatory compounds in this model were also investigated.

There are distinct advantages to utilizing cartilage explants in OA research over chondrocyte monolayers. Investigations with the explant model may therefore give a more accurate response to inflammatory stimulation and anti-inflammatory compound treatment. In explants, chondrocytes will be contained within their natural ECM environment. Chondrocytes within explants should therefore continue to act in a similar manner as they usually would *in vivo*. Chondrocytes in monolayer cultures dedifferentiate into fibroblast-like cells over time and with increasing passage numbers (Benya et al., 1978, Grundmann et al., 1980, Hamilton et al., 2005). Chondrocyte dedifferentiation causes loss of phenotype and a deficiency in the ability to express collagen type II (Benya et al., 1978). Within cartilage explants, chondrocytes are distributed alone or in pairs (Archer and Francis-West, 2003). It should also be taken into consideration that bioavailability of compounds to chondrocytes within explants may be reduced due to hindered diffusion of compounds through the ECM, while monolayers would have instant access to any treatments. Chondrocytes in monolayers are also more metabolically active therefore any responses to treatments would be more easily identifiable with monolayer studies compared to explant cultures. However, chondrocytes within the ECM are not rapidly dividing, highly metabolic or in a monolayer (Archer and Francis-West, 2003). There continues to be several limitations to the cartilage explant model employed in this thesis. The lack of surrounding synovial fluid, immune cells and underlying subchondral bone means that all influencing factors from inflammation and catabolic processes in associated tissues are not being assessed. Use of healthy cartilage stimulated by specific cytokines provides only a simplified picture of catabolic processes. The explant model is also static, and therefore does not introduce dynamic movement of the joint and synovial fluid that would be present *in vivo*. Taking into account these limitations, the explant model continues to provide the opportunity to study degradative processes on cartilage in response to cytokines and therapeutic treatments like carprofen.

In the first experimental chapter, release of cartilage proteins over a time course was investigated to discover if there were dynamic changes in types of proteins released as time passes (Chapter 3). Monitoring responses to IL-1β stimulation and NSAID carprofen aimed to identify potential biomarkers of therapeutic efficacy in this cartilage model. High-throughput MS analysis had previously been used to identify proteins released from this culture model at only one time point (Clutterbuck et al., 2011), therefore the possibility of additional protein identifications being made at different points in the time course was investigated (Chapter 3). This would help determine an appropriate incubation time to proceed with in future proteomic applications. Alterations in release of a number of proteins were observed in response to IL-1β stimulation in chapter 3, but relevant low level proteins may have been masked by abundant ECM proteins within samples. Therefore, the next chapter attempted to gain additional protein identifications by preceding the high-throughput MS with approaches that attempted to remove or deplete the high abundance proteins. These approaches were CPC precipitation, Con A lectin chromatography and Proteominer™ techniques (Chapter 4). The high-throughput MS chapters 3 and 4 identified candidate explant secretome biomarkers that were investigated further using western blotting in Chapter 5. Western blots of ECM components and secreted MMPs, validated responses in candidate biomarkers to IL-1β stimulation and the NSAID, carprofen (Chapter 5). The data obtained showed the explant model can be applied as a tool for assessing cytokine responses and screening potential anti-inflammatory drugs or naturally occurring compounds.

With proteomic studies, this work overall aimed to identify suitable biomarkers within the explant culture model to be used to study anti-catabolic and anti-inflammatory effects of compounds. Deeper understanding at the molecular level of cartilage degradation and protection events will contribute towards research on OA, a prominent disease particularly in the elderly population and in companion animals.

The equine explant model studied here will help provide an alternative to *in vivo* models following the principles of the 3Rs (Replacement, Refinement and Reduction).

CHAPTER 2

MATERIALS AND METHODS

2.1. Animal tissues and statement of ethical approval

All animals used in these studies were sourced from two UK-based abattoirs. Animals were euthanized for non-research purposes having been stunned before slaughter for meat in accordance with Welfare of Animals (Slaughter or Killing) Regulations 1995. Approval for use of abattoir-derived animal tissues was obtained from the local Ethical Review Committee (Ethics Committee of the School of Veterinary Medicine and Science) with input from members of the University of Nottingham's Animal Welfare and Ethical Review Body (AWERB).

2.2. Chemicals and Reagents

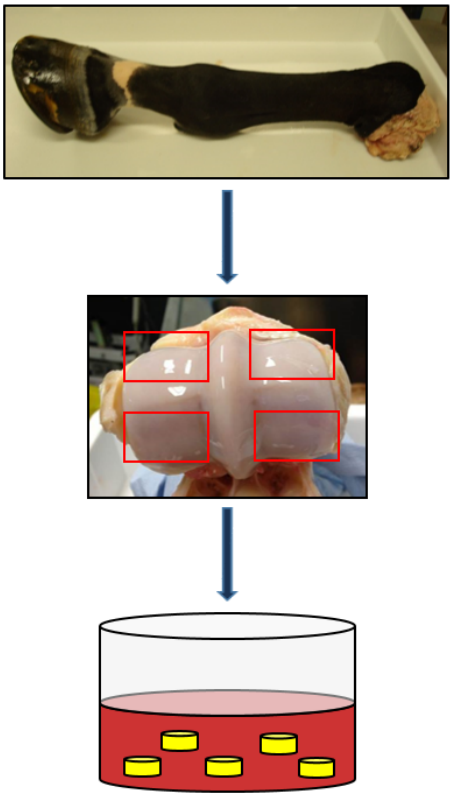
Articular cartilage explants were cultured in culture media containing Dulbecco's Modified Eagles Medium (DMEM) low glucose (Hyclone) + 2% Pen/Strep (Penicillin/Streptomycin solution: (5000 units/ml penicillin, 5 mg/ml streptomycin) (Sigma). Sterile phosphate buffer solution (PBS) was prepared by dissolving 1 tablet (Gibco) in 500 ml reverse osmosis (RO) water before autoclaving. Carprofen (Rimadyl®) stocks were made by grinding down a tablet (containing 50 mg active compound = carprofen (Pfizer)) into a powder with a mortar and pestle. This powder was dissolved into 25 ml of culture media to provide a 2 mg/ml stock solution that was divided into single use aliquots. Protease inhibitor cocktail for general use was purchased from Sigma and contains 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin hydrochloride, N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide, EDTA and leupeptin hemisulfate salt.

2.3. Dissection and explant culture

Cartilage harvested in the following studies was taken from animals euthanized for purposes other than scientific research. Equine metacarpophalangeal joints were utilized during this work and articular cartilage was obtained by the

following dissection protocol. The joints were first shaved to remove excess hair to improve the aseptic conditions for dissection. Joints were disinfected by washing with Hibiscrub and followed by Trigene, before being soaked in bleach for 1 hour. Afterwards, the skin and flexor tendon was carefully removed from the joint without breaking into the synovial structure. A sterile scalpel was used to open up the joint, which was then washed with sterile PBS (Gibco). Only healthy and macroscopically normal articular cartilage shavings of equal thickness were taken from the end surface of the third metacarpal and placed in 50 ml of DMEM (Thermo Scientific) + 4% Pen/Strep (Sigma) (Penicillin/Streptomycin solution: (5000 units/ml penicillin, 5 mg/ml streptomycin)) (pre-warmed to 37 °C). These cartilage shavings were transferred to a Falcon tube containing washing solution (Sterile PBS + 10% Pen/Strep) in which they were twice washed with rotation for 20 minutes.

Cartilage explant discs were prepared using a 3 mm biopsy punch (Kai medical), with discs placed into wells (5 discs per 1 ml DMEM + 2% Pen/Strep) before incubation for 24 hours (37 °C, 5% CO_2). After this equilibration period, the media was removed and replaced with treatment media required for the purposes of each different experiment. Once explants had been treated and incubated for the desired amount of time, the supernatant and cartilage discs were separately placed in labelled Eppendorf tubes and stored at -80 °C. Protease inhibitors (Sigma) were added to the supernatant samples at the time of removal and storage.



Equine metacarpophalangeal joints

- Hair shaved, washed with Hibiscrub, Trigene and bleach

Joints opened aseptically

- Full thickness shavings of macroscopically healthy cartilage placed into serum free collection media
- Shavings were taken from the weight bearing regions highlighted in red
- Shavings washed in PBS + 10% Pen/Strep

Explant discs made with biopsy punch

- Cultured at 37°C, 5% CO_2
- Equilibrated for 24 hours before culture media replaced with media containing experimental treatment
- Downstream analysis after culture incubated for designated time

Figure 8. Schematic of dissection process to set up equine cartilage explant cultures

2.4. SDS-PAGE with Protean 3 mini-gels

All polyacrylamide gels for SDS-PAGE were prepared within the laboratory using the recipe found in Table 2. When loading gels a volumetric system was used with the same volume of sample (10 µl) loaded in each lane so that relative protein levels between treatments could be distinguished. For each sample, 10 µl collected culture supernatant was added to 10 µl of Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 25% (w/v) glycerol, 0.01% (w/v) bromophenol blue) (Bio-Rad) containing dithiothreitol (DTT) (Bio-Rad) 0.15 M). Samples were boiled for 5 minutes at 95 °C before loading onto 10%T polyacrylamide gels. To reference protein molecular weights Precision Plus Protein™ Standards (Bio-Rad) were also loaded. A voltage of 120 V was applied until the dye front reached the bottom of the gel.

Table 2. Recipes for two mini-gels with Protean 3

	5% Stacking	10% Resolving
Water (18 ohm) (Fisher)	4.1 ml	5.9 ml
30% Acrylamide (Geneflow)	1.0 ml	5.0 ml
1.5M Tris (pH 8.8) (Bio-Rad)	-	3.8 ml
0.5M Tris (pH 6.8) (Bio-Rad)	750 µl	-
10% (w/v) SDS (Bio-Rad)	60 µl	150 µl
10% (w/v) APS (Bio-Rad)	60 µl	150 µl
TEMED (Sigma)	5 µl	8 µl

2.5. Silver staining

Silver staining was used to visualise the proteins once separation had been completed, with an Amersham processor (Amersham Pharmacia Hoefer processor plus) used to aid this process. The silver staining approach used was based on methods described by (Yan et al., 2000). Silver staining was chosen because it is highly sensitive and can detect proteins down in the 2-10 nanogram range (Rabilloud et al., 2009). Silver staining is advantageous because many secretome proteins are present at low levels. This provided detailed SDS-PAGE profiles with improved protein detection over Coomassie blue stains. The precise details of the steps and solution compositions can be found in Table 3. In brief, the gel was first fixed to stop diffusion of proteins in the gel. The gel was exposed to a sensitizer solution before an impregnating solution containing silver nitrate was added. Silver ions were reduced to metallic silver where proteins are present and therefore become deposited in a sensitive manner on the protein bands. A developing solution is added to visualize where silver has become deposited, and when optimal development has occurred a stop solution was added to end the process.

Stained gels were scanned using a densitometer (Bio-Rad GS-800 calibrated densitometer) linked with Quantity One (Bio-Rad) software to create digital images of gels.

Table 3. Silver staining method steps and solutions

Based on method described by (Yan et al., 2000)

Step	**Time (minutes)**	**Solution Function**	**Solution Composition (For 225 ml)**
1+2	2 x 15	Fixation	84.1 ml Methanol (Fisher) 21.03 ml Acetic Acid (Fisher) Dilute to 225 ml with 18 ohm water.
3	30	Sensitizer	8.5 ml 5% Sodium thiosulphate (STS) (Fisher) 14.3 g Sodium acetate (Sigma) Dilute to 225 ml with 18 ohm water.
4+5+6	3 x 5	Wash	18 ohm water
7	20	Impregnate	0.477 g Silver nitrate (Fisher)
8+9	2 x 1	Wash	18 ohm water
10	2 (but variable. Time may need to be extended)	Develop	5.31 g Sodium carbonate (Fisher) 85 µl Formaldehyde (TAAB) 11.9 µl STS (Fisher) Dilute to 225 ml with 18 ohm water.
11	10	Stop	3.1 g EDTA (Fisher) Dilute to 225 ml with 18 ohm water.
12+13+14	3 x 5	Wash	18 ohm water.

5% (w/v) STS = 500 mg in 10 ml 18 ohm water

2.6. Western blotting

Samples were concentrated by lyophilisation (Heraeus-Christ) of 50 µl before resuspension in 20 µl Laemmli buffer (Bio-Rad) prior to completing SDS-PAGE (See SDS-PAGE method). Positive controls were prepared by crushing articular cartilage under liquid nitrogen with a mortar and pestle, and resuspended in Laemmli buffer. Proteins separated on gels were electroblotted onto low-fluorescence PVDF membrane (GE Healthcare) at 80 V for 2 hours. Washing steps with PBS-Tween 20 (0.1% (v/v)) (3 x 5 minutes) were completed between each of the following incubations. Membranes were blocked for 1 hour with 3% (w/v) BSA (Sigma) in PBS-Tween 20 (0.1%) (Fisher). Primary antibodies were diluted as required in blocking solution before overnight incubation at 4 °C. After the overnight primary incubation, membranes were incubated with secondary antibodies for 1 hour. Secondary antibodies in this study were all HRP conjugated and diluted to appropriate concentrations using 3% (w/v) BSA blocking solution.

Ampliflu™ Red Western Blot Kit (Sigma) was used to stain protein bands with red fluorescence, which was detected on a FX-Imager (laser: 532 nm, filters: 555 nm LP, 1,064 blocking) (Bio-Rad) using Quantity One software (Bio-Rad). Densitometry was performed on the images obtained using ImageJ (NIH), with statistical analysis completed and results graphically displayed using GraphPad Prism 6.

2.7. Band excision and tryptic digestion

Protein bands were excised from silver stained SDS-PAGE gels using a scalpel, cut into 1 mm^3 cubes and placed into wells of a microtitre plate each containing 60 µl HPLC grade water (Fisher Scientific). The microtitre plate was placed into the ProteomeWorks MassPREP robotic liquid handling station (Waters, U.K.) for the following procedures; destaining, reduction, alkylation and trypsin digestion. All incubations were performed at 40˚C unless otherwise indicated. A destain solution was freshly made containing a 1:1 mixture of 30 mM potassium ferricyanide (Sigma) and 100 mM sodium thiosulphate (Fisher Scientific). Destain solution was added to

the gel pieces for 3-15 minutes until the silver stain was removed and then the solution was removed. Gel pieces were washed for 20 minutes in 150 µl 200 mM ammonium bicarbonate (Sigma). Supernatant was removed before a further 15 minutes wash in 150 µl HPLC water, before supernatant was again removed. Samples were dehydrated by incubation in 50 µl of acetonitrile (Fisher Scientific). After 5 minutes incubation, acetonitrile was removed and samples were incubated for 10 minutes to allow evaporation.

Proteins were reduced by incubation in 50 µl of a reducing solution containing 10 mM dithiothreitol (Bio-Rad) in 100 mM ammonium bicarbonate. After 30 minutes incubation, reducing solution was replaced with 50 µl of alkylating solution (55 mM iodoacetamide (Bio-Rad) in 100 mM ammonium bicarbonate). Samples were incubated with alkylating solution for 20 minutes (room temperature), then washed for 10 minutes with 50 µl of 100 mM ammonium bicarbonate followed by 50 µl of acetonitrile for 5 minutes, before a dehydrating wash with 50 µl acetonitrile followed by 5 minutes evaporation time. The microtitre plate was cooled to 6˚C for 5 minutes. For digestion, 25 µl of trypsin gold (Promega) at 10 ng/µl in 50 mM ammonium bicarbonate buffer was added per well and incubated at 6˚C for 20 minutes (to allow trypsin entry into the gel with minimal autocatalysis), before incubation at 40˚C for 4 hours. Samples were stored at 4˚C until mass spectrometry analysis was performed.

2.8. High-throughput Mass Spectrometry

2.8.1. Mass Spectrometry sample preparation

To prepare proteins present in secretome samples for MS, reduction of di-sulphide bonds was performed by addition of DTT (Bio-Rad) to a final concentration of 10 mM, followed by vortexing and incubation at 37 °C for 30 minutes. Alkylation of thiol groups performed by addition of IAA (Bio-Rad) to a 55 mM concentration, then vortexed and incubated for 45 minutes at 37 °C (in the dark). Ice cold acetone (1.2 ml) (Fisher) added and incubated at -20 °C for 1 hour, then pelleted by centrifugation at 15,000xg for 5 minutes and acetone was discarded. Proteins in the pellet were digested with 20 ng/µl trypsin gold (1:50 (w/w) – protease: protein ratio) (Promega) at 37 °C overnight (16 hours). The digestion was ended by addition of formic acid to give a 0.1% concentration. To desalt and ensure only soluble proteins were present, samples were ziptipped with C18 resin (Millipore) and eluted with 20 µl 50% methanol and 0.1% formic acid. Samples were heated to 65 °C to evaporate off excess methanol and reduce the volume to 10 µl. Samples were transferred to glass vials and transported to Bruker U.K. prior to being loaded onto the nanoLC column for MS analysis.

2.8.2. Mass Spectrometry analysis on amaZon ETD

High throughput MS analytical runs were made possible by the collaborative support of Dr Julia Smith at Bruker U.K, who performed the data acquisition on MS instruments, providing the raw data for the analysis performed and reported in this thesis. Samples were loaded onto a 15 cm Acclaim® Pepmap RSLC C18 column (Thermo) to be analysed by nanoLC-MS/MS on an amaZon ETD or amazon speed ETD (Bruker) (Figure 9 and Figure 10). A flow rate of 250 nl/minute was used to separate peptides. Solution A (100% H_20 + 0.1% formic acid) and solution B (100% ACN + 0.1% formic acid) were set to create a gradient of 10% solution B to 30% solution B over the course of an hour (0.3% per minute). From each MS scan, the five most abundant peptides were selected for collision induced dissociation (CID) fragmentation.

2.8.3. Mass Spectrometry data processing

Once the raw data had been generated at Bruker U.K., Mascot Daemon software was used to search the NCBInr (All entries) and Swiss-Prot (mammalian entries) databases by submitting the .MGF files (Figure 11). The search parameters were as follows: Instrument: ESI-TRAP, peptide charge: 1+, 2+ and 3+ ions, peptide tolerance: 0.5 Da, ^{13}C = 1, max missed cleavages = 1. Fixed modifications: carbamidomethyl (C) and variable modifications: oxidation (M).

Mascot scores were generated by comparing the peptide masses or fragment ion mass values of entries in the sequence databases with experimental data values. A probability based score is calculated for each peptide match. With increasing amounts of matched peaks, the likelihood of the identification being reliable is increased and this will be associated with a higher Mascot score. The threshold of significance ($p<0.05$) for scores has been selected as a cut-off to determine identification. The specific score for each search is detailed in each chapter because this significant threshold alters depending on the database searched and data submitted.

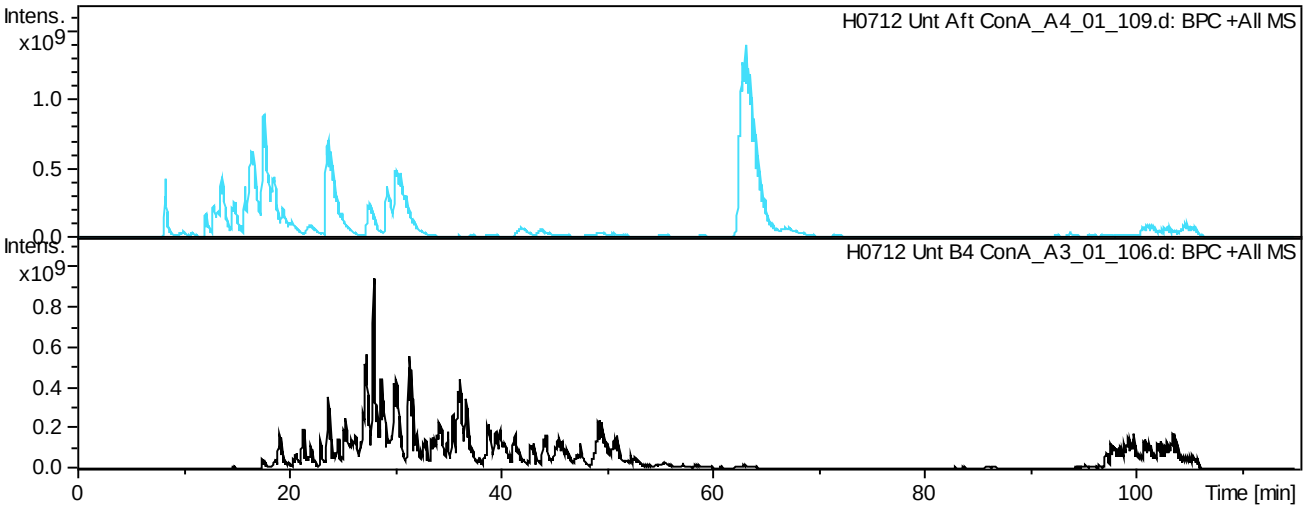


Figure 9. Example of two profiles of peptide elution throughout the mass spectrometry runs

Displayed runs are from untreated control supernatant that has been processed with Con A lectin chromatography (above) and unprocessed (below).

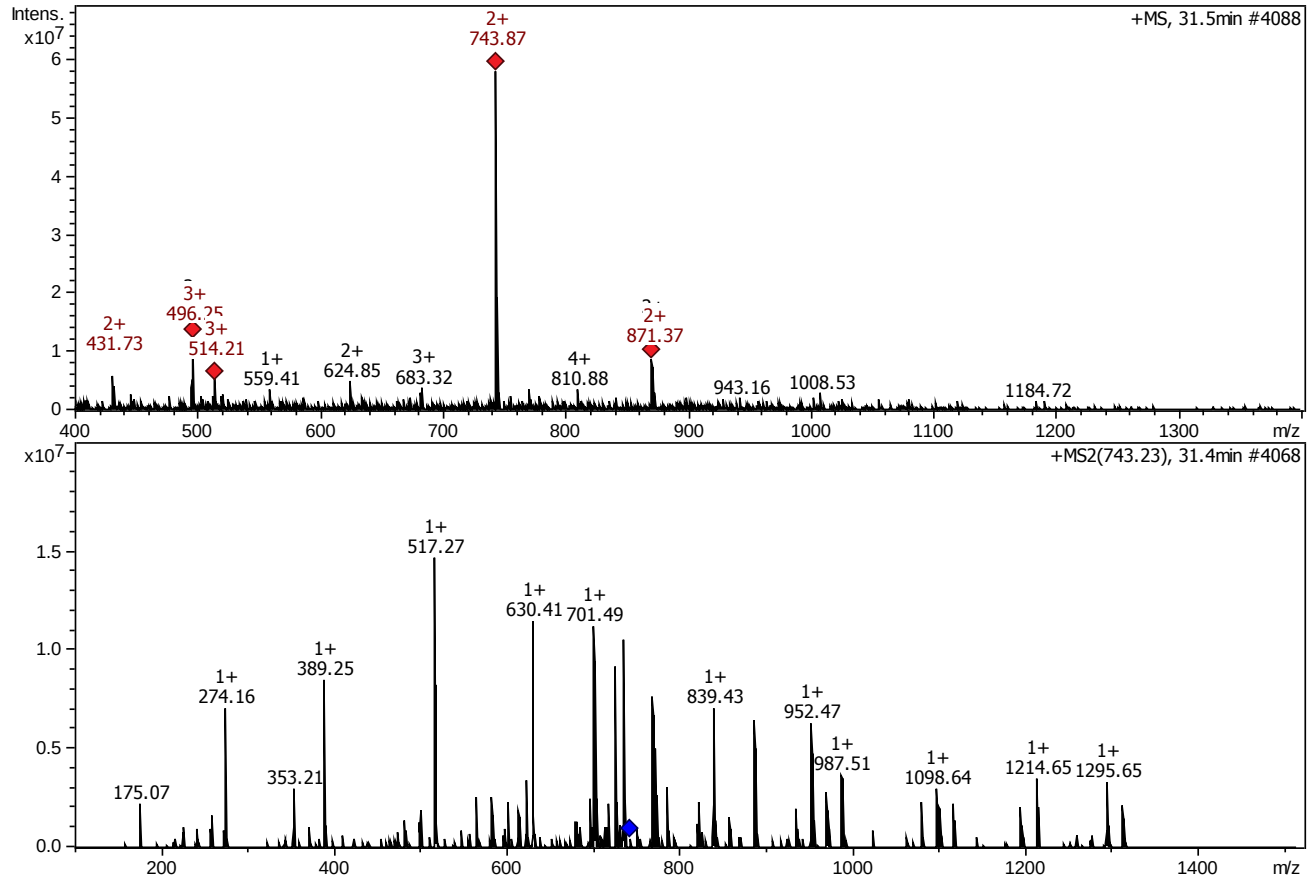
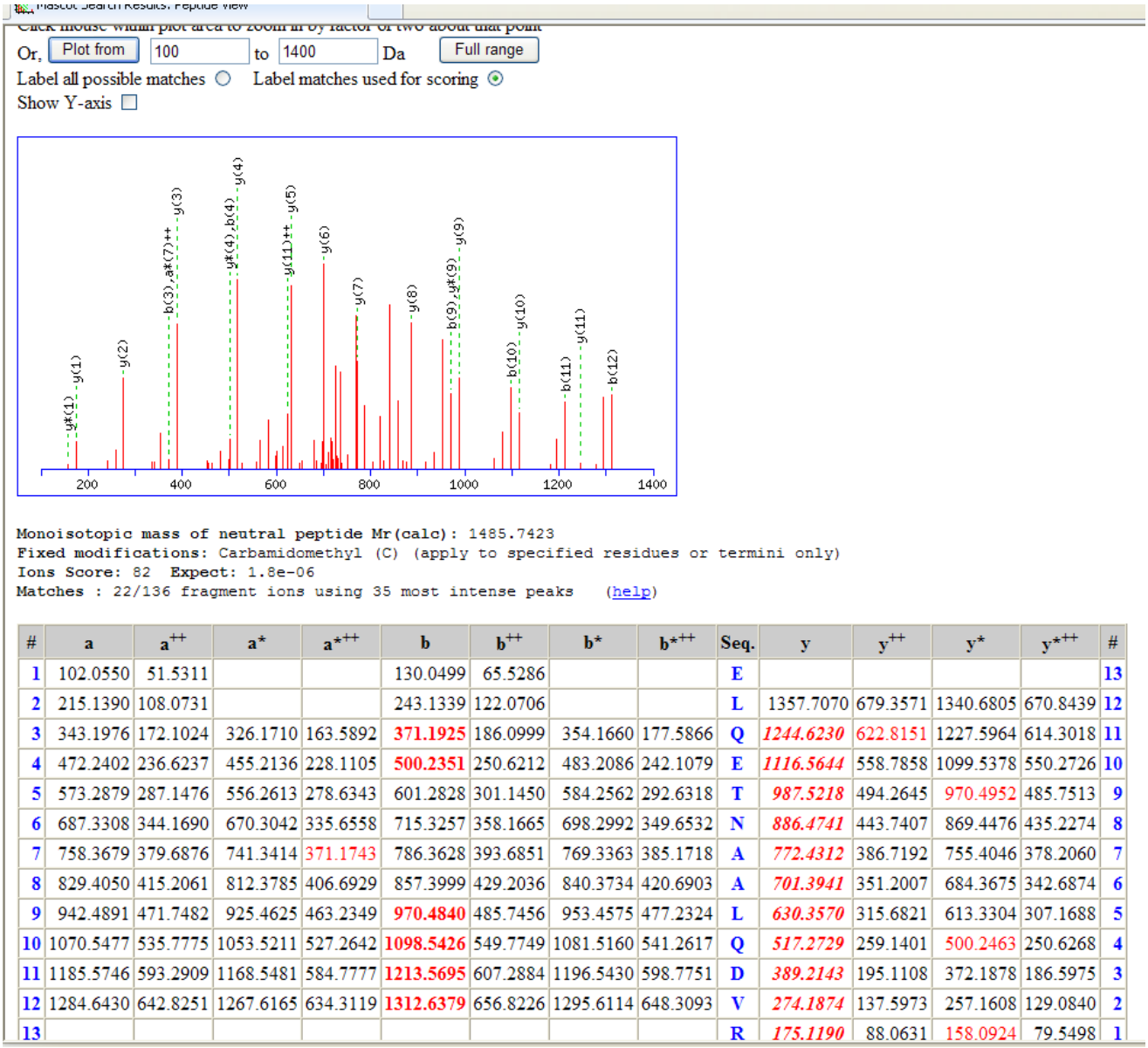


Figure 10. MS (above) and MS/MS (below). Example taken from run of untreated control supernatant

The MS/MS of COMP peptide: ELQETNAALQDVR.



Monoisotopic mass of neutral peptide Mr(calc): 1485.7423
Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)
Ions Score: 82 Expect: 1.8e-06
Matches : 22/136 fragment ions using 35 most intense peaks (help)

#	a	a^{++}	a*	a^{*++}	b	b^{++}	b*	b^{*++}	Seq.	y	y^{++}	y*	y^{*++}	#
1	102.0550	51.5311			130.0499	65.5286			E					13
2	215.1390	108.0731			243.1339	122.0706			L	1357.7070	679.3571	1340.6805	670.8439	12
3	343.1976	172.1024	326.1710	163.5892	371.1925	186.0999	354.1660	177.5866	Q	*1244.6230*	622.8151	1227.5964	614.3018	11
4	472.2402	236.6237	455.2136	228.1105	500.2351	250.6212	483.2086	242.1079	E	*1116.5644*	558.7858	1099.5378	550.2726	10
5	573.2879	287.1476	556.2613	278.6343	601.2828	301.1450	584.2562	292.6318	T	*987.5218*	494.2645	970.4952	485.7513	9
6	687.3308	344.1690	670.3042	335.6558	715.3257	358.1665	698.2992	349.6532	N	*886.4741*	443.7407	869.4476	435.2274	8
7	758.3679	379.6876	741.3414	371.1743	786.3628	393.6851	769.3363	385.1718	A	*772.4312*	386.7192	755.4046	378.2060	7
8	829.4050	415.2061	812.3785	406.6929	857.3999	429.2036	840.3734	420.6903	A	*701.3941*	351.2007	684.3675	342.6874	6
9	942.4891	471.7482	925.4625	463.2349	970.4840	485.7456	953.4575	477.2324	L	*630.3570*	315.6821	613.3304	307.1688	5
10	1070.5477	535.7775	1053.5211	527.2642	1098.5426	549.7749	1081.5160	541.2617	Q	*517.2729*	259.1401	500.2463	250.6268	4
11	1185.5746	593.2909	1168.5481	584.7777	1213.5695	607.2884	1196.5430	598.7751	D	*389.2143*	195.1108	372.1878	186.5975	3
12	1284.6430	642.8251	1267.6165	634.3119	1312.6379	656.8226	1295.6114	648.3093	V	*274.1874*	137.5973	257.1608	129.0840	2
13									R	*175.1190*	88.0631	158.0924	79.5498	1

Figure 11. Example of Mascot database search results matching a COMP peptide

Red indicates significant matches of observed with predicted fragment ion masses.

2.9. Dimethylmethylene blue (DMMB) assays (GAG assay)

Sulphated GAGs in cartilage cultures can be accurately measured by DMMB assays, therefore helping determine loss of GAGs in response to specific stimuli (Farndale et al., 1982). Solutions of 1, 9-dimethylmethylene blue (DMMB) and papain solution were prepared within the laboratory (detailed next page). Supernatant samples were diluted to be within the accurate range (0-75 µg/ml) of a chondroitin sulphate (Sigma) standard curve. On a 96 well plate, 40 µl per well of blank papain solution (duplicate), each standard (duplicate) or sample dilutions (triplicate) were added. Next, 200 µl of DMMB solution was added to each well, before absorbance was read at 540 nm (Labtech LT-4000 plate reader) within 10 minutes. To calculate total % GAG release, corresponding explant discs were digested overnight by incubating with papain solution at 60 °C, before their GAG content was also measured by DMMB assay. Total GAG release levels per well were calculated, before dividing the total GAG release by measurements for individual supernatant samples to provide the % GAG release due to treatments. GraphPad Prism 6 software was used to produce graphical images and complete statistical analysis with a one-way ANOVA using Tukey's multiple comparison test.

DMMB assay solutions

1, 9 – dimethylmethylene (DMMB) blue solution

- 5 ml 100% ethanol (0.5% (v/v)) (Fisher)
- 2ml formic Acid (0.2% (v/v)) (Sigma)
- Made up to 1 litre volume with distilled water
- 16 mg 1, 9 – dimethylmethylene blue (0.046 mM) (Sigma)
- 2 g sodium formate (0.03 M) (Sigma)

Papain Solution

- 1.42 g dibasic sodium phosphate (Sigma)
    - In 90 ml distilled water
- 79 mg cysteine hydrochloride (Sigma)
- 186 mg EDTA (Fisher)
- Adjusted to pH 6.5 using 1M hydrochloric acid (Fisher)
- Volume made up to 100 ml with distilled water
- 105.6 mg papain (Sigma) (1.06 mg/ml)

2.10. Protein assay

To determine the concentration of protein present in supernatant samples, a modified Lowry based method was used (Lowry et al., 1951) (Bio-Rad DC protein assay). In brief, 5 µl of samples were loaded into a 96 well plate, along with 5 µl of blank, control media and BSA standards. To each well 25 µl of reagent A (alkaline copper tartrate) is added followed by 200 µl of reagent B (dilute Folin reagent), before mixing for 5 seconds on a shaker and incubation for 15 minutes at room temperature. The 96 well plate was placed in a Bio-Rad microplate plate reader and absorbance was read at 655 nm wavelength. A BSA standard curve ranging from 0-1.5 µg/µl was produced. Concentration of the protein in the unknown samples could therefore be established by comparison with the standard curve.

2.11. PGE_2 assay

To measure PGE_2 content within culture supernatants a competitive immunoassay was purchased from R&D systems and performed following the manufacturer's protocol. A PGE_2 standard curve was completed ranging from 39-2500 pg/ml, along with zero standard and non-specific binding (NSB) wells. First, 150 µl of each sample was added to each well, followed by 150 µl primary antibody solution added (except for NSB wells) before incubation for 1 hour on a microplate shaker (500 rpm) at room temperature. PGE_2 conjugate (50 µl) was next added to all wells and incubated for 2 hours on the shaker. The plate was then washed with wash buffer four times, before 200 µl substrate solution was added and incubated for 30 minutes in the dark. Finally, 100 µl stop solution was added before optical density of the plate was read at 450 nm and 540 nm (LT-4000 plate reader). The 540 nm reading was subtracted from the 450 nm reading to correct for optical imperfections in the plate. The NSB value was subtracted from all experimental samples before a standard curve was created using GraphPad Prism 6 and PGE_2 values interpolated.

CHAPTER 3

TEMPORAL CHANGES IN THE SECRETOME OF ARTICULAR CARTILAGE EXPLANTS

3.1. Introduction

The term “secretome” refers to proteins actively secreted from cells or tissues into the surrounding environment (Hathout, 2007). Once proteins are synthesised in eukaryotic cells, they can be secreted by classical endoplasmic reticulum (ER) mediated exocytosis or via non-classical secretion pathways (Nickel and Seedorf, 2008). In classical ER secretion pathways, proteins are folded and posttranslational modifications (e.g. glycosylation) occur in the Golgi apparatus. Proteins are then transferred into secretory vesicles that fuse with the cell membrane to release their contents. Several types of proteins are secreted including signalling proteins, hormones, enzymes and ECM components. Chondrocytes are the resident cells of articular cartilage that produce and secrete newly synthesised ECM proteins for incorporation into the matrix (Archer and Francis-West, 2003). They also express a unique set of metabolic proteins for controlling cartilage turnover (Archer and Francis-West, 2003). Secreted catabolic proteins like ADAMTS and MMPs impact on cartilage maintenance, which can lead to ECM breakdown and OA development (Troeberg and Nagase, 2012). Proteins are secreted into the surrounding synovial fluid in OA diseased synovial joints *in vivo* (Sinz et al., 2002). Synovial fluid is consequently a source of OA biomarkers including various ECM components, as well as proteins associated with complement pathways or inflammation (Sinz et al., 2002, Yamagiwa et al., 2003, Drynda et al., 2004, Kamphorst et al., 2007, Gobezie et al., 2007, Chiaradia et al., 2012, Mateos et al., 2012, Gharbi et al., 2013, Ritter et al., 2013). Biomarkers entering systemic circulation are present in blood/serum/plasma or urine. For example, specific peptide breakdown fragments that are prospective biomarkers of OA in serum include: CTX-II, Coll2-1, Coll2-1NO2, Fib3-1 and Fib3-2 (Ishikawa et al., 2004, Henrotin et al., 2012b, Henrotin et al., 2012a).

3.1.1. Studying the cartilage explant secretome

Cartilage explant cultures have advantages over high density cultured chondrocytes, primarily due to preservation of chondrocytes within their native ECM environment (Archer and Francis-West, 2003). Explant models provide opportunities to monitor cartilage turnover in response to pro-inflammatory cytokines, therapeutics or mechanical compression (Saklatvala, 1986, Morris et al., 1992, Sah et al., 1989). For example, IL-1β has been shown to contribute to catabolic processes by up-regulating MMPs, and reducing the synthesis of proteoglycans and collagen type II needed to maintain the ECM (Chevalier, 1997).

Although often referred to as cartilage secretome, not all proteins in explant conditioned media will be secreted. ECM present could be released by degradative processes or simply be leaking out of explants. Additionally, intracellular proteins identified are released during cell death and lysis, are not being truly secreted. Nevertheless, whilst assessing inflammatory or anti-inflammatory stimuli, analysis of cartilage "secretome" has the capacity to show alterations in degradation of existing ECM proteins or effects on chondrocyte health (Clutterbuck et al., 2009, Clutterbuck et al., 2013).

3.1.2. Gel based proteomic studies of the cartilage secretome

Proteomics studies using 2DE have identified differential proteins between healthy and OA affected cartilage. Secreted metabolic and signalling proteins (CTGF, inhibin βA, TIMP-1, PEDF, and TNF-R1) were all identified in OA cartilage secretome (Hermansson et al., 2004, De Ceuninck et al., 2005).

OA relevant cytokine stimulation of healthy cartilage is also a popular approach to compare the responses of cartilage to stimuli. Release of ECM, metabolic and inflammatory associated proteins was significantly higher in cartilage stimulated with IL-1β, TNF-α or Ret A (Wilson et al., 2008, Stevens et al., 2008). Across these studies, IL-1β initiated increases in MMP-3, YKL-39, YKL-40, clusterin, haptoglobin and NGAL release (Wilson et al., 2008, Stevens et al., 2008).

3.1.3. High-throughput and quantitative studies on cartilage secretome

Improvements in HPLC and MS technologies offer the potential to apply high-throughput approaches. In high-throughput methods, digested protein samples are first separated by liquid chromatography which is directly coupled to a mass spectrometer instrument for tandem MS analysis. This allows faster sample analysis, without time consuming two dimensional gels and subsequent protein identification. Quantification strategies can be coupled with high-throughput MS analysis including spectral counting, SILAC, iTRAQ™ and QconCAT (Bantscheff et al., 2012).

Proteins released from bovine cartilage explants treated with IL-1β, TNF-α or mechanical compression have been studied by quantitative iTRAQ™ labelling (Stevens et al., 2009). In that study, the cytokine stimulated increased release of aggrecan G2 and G3 domains, MMPs (-1, -3, -9 and -13), and decreased various collagen subunits (Stevens et al., 2009). Chemokines, cytokines and secreted signalling proteins were also raised in response to all treatments (Stevens et al., 2009). Use of the SILAC technique showed that only 29% of the proteins present in cartilage conditioned media were newly synthesised, whilst most of the proteins identified (71%) were not synthesised during culture (Polacek et al., 2010). Proteins involved in ECM remodelling YKL-40 and TIMP-1 were newly synthesized and secreted (Polacek et al., 2010). A high-throughput analysis of equine cartilage explant secretome examined proteins in untreated, IL-1β and carprofen treated cultures (Clutterbuck et al., 2011). The proteins identified were mostly ECM components, along with secreted MMP-1, MMP-3 and clusterin, or intracellular proteins (Clutterbuck et al., 2011). MMP-1, MMP-3 and TSP were increased by IL-1β stimulation compared to control explants (Clutterbuck et al., 2011). A QconCAT quantitative approach was applied to OA cartilage with or without IL-1β stimulation to compare secretome differences (Peffers et al., 2013). IL-1β stimulation did not significantly alter MMP-1, MMP-3, aggrecan, fibromodulin, COMP or plasminogen levels (Peffers et al., 2013). Differential release of TIMP-1 was the only significantly affected protein with IL-1β stimulation of OA cartilage (Peffers et al., 2013).

One difficulty with high-throughput MS analysis of complex samples, including the cartilage explant secretome, is the dynamic range of proteins released. Cartilage ECM components such as COMP, fibronectin and small proteoglycans (biglycan, fibromodulin, decorin), are abundantly released during cartilage degradation (Wilson et al., 2008). Proteins secreted at lower levels such as MMPs and pro-inflammatory signalling molecules (IL-1β, TNF-α, IL-6) have vital functional roles in cartilage degradation, inflammation and progression of OA (Troeberg and Nagase, 2012). Detection of these lower level proteins can be prevented by the presence of abundant proteins.

3.1.4. Hypothesis and aims

The objective of the studies in this chapter was to expand knowledge on the secretome of an equine cartilage explant model and determine potential biomarkers to monitor efficacy of treatment. The NSAID carprofen was used to evaluate the capability of the IL-1β stimulated model to screen for anti-inflammatory effects, therefore determining proteins as potential biomarkers of treatment efficacy. A previous high-throughput MS study on equine cartilage secretome reported protein identifications over six days of culture (Clutterbuck et al., 2011). It was hypothesised that extending incubation over a time course would result in the identification of additional relevant low abundance proteins, since the release of abundant ECM components from cartilage may be reduced by later time points.

In the first study described in this chapter, a time course up to 27 days (time course A) assessed changes in cartilage secretome. After initial analysis, the time course was altered to collect samples every 6 days over a 24 day time course (time course B), providing samples suitable for downstream processing. Sample quality control was verified using SDS-PAGE, which provided a visual profile of protein release throughout the time courses, before high-throughput MS analysis of the cartilage secretome. Release of proteoglycans and GAGs in response to IL-1β and carprofen was assessed using DMMB assays. Data from high-throughput MS and

DMMB assays taken together provided enhanced insights into cartilage metabolism degradation during IL-1β and carprofen treatment.

In summary, high-throughput MS and conventional protein biochemistry were employed to study the secretome of articular cartilage explants, thereby identifying candidate biomarkers to monitor responses to IL-1β stimulation and efficacy of carprofen treatment. The secretome was also examined throughout time courses to assist selection of a suitable incubation time point for future studies.

3.2. Materials & Methods

3.2.1. Time course A (Initial 27 day time course)

During the first stages of this study, time course experiments were completed up to 27 days (Figure 12). The following experimental design was completed three times. Articular cartilage was harvested from healthy equine metacarpophalangeal joints from separate individual horses on each occasion, with only normal cartilage collected. The dissection and explant culture protocol (Chapter 2.3.) was followed with differences detailed below. Culture media (3 ml) and 15 articular cartilage explants per well, were placed into 6 wells in a 6 well plate and incubated (37 °C, 5% CO_2). Explants were equilibrated for 24 hours in culture media (no treatment) before the treatment phases began. Following equilibration, 3 ml of culture media containing six different treatments was added and incubated (37 °C, 5% CO_2). Supernatant was removed and stored every 24 hours for the first 3 days of this study. The supernatant was replaced with fresh corresponding treatment media each time it was removed. After the first three day sampling had been completed, the media was replaced every 3 days up to 27 days (Figure 12).

Treatments were: untreated control, IL-1β (R&D systems), TNF-α (Roche) + IL-1β, carprofen (Pfizer), carprofen + IL-1β and carprofen + TNF-α + IL-1β. Both IL-1β and TNF-α were at a concentration of 10 ng/ml and carprofen was at 100 µg/ml.

3.2.2. Time course B (24 day time course)

After preliminary experiments had been assessed the secretome over time course A, it was concluded that samples collected with this experimental layout were very dilute and would need additional concentration steps for most downstream processes. Therefore a revised 24 day time course (time course B) was planned to develop analysis of the secretome and provide suitably concentrated samples that could be prepared for high-throughput MS. Each experiment was set up with cartilage from two healthy metacarpophalangeal joints from an individual horse. This was repeated three times with tissue from three separate horses, with collection of only

macroscopically normal cartilage. For time course B, dissection and explant culture protocols were followed (Chapter 2.3.) with three replicate wells (5 explants discs with 1 ml culture media per well) for each treatment. Treatment cultures were incubated (37 °C, 5% CO_2) for 6 days until removal and storage of supernatant, before fresh treatment media was added. This process was repeated to provide a time course with four time points: 0-6 days, 6-12 days, 12-18 days and 18-24 days (Figure 12). Treatments for the time course B were: untreated control, IL-1β (10 ng/ml), carprofen (100 µg/ml) or carprofen (100 µg/ml) + IL-1β (10 ng/ml).

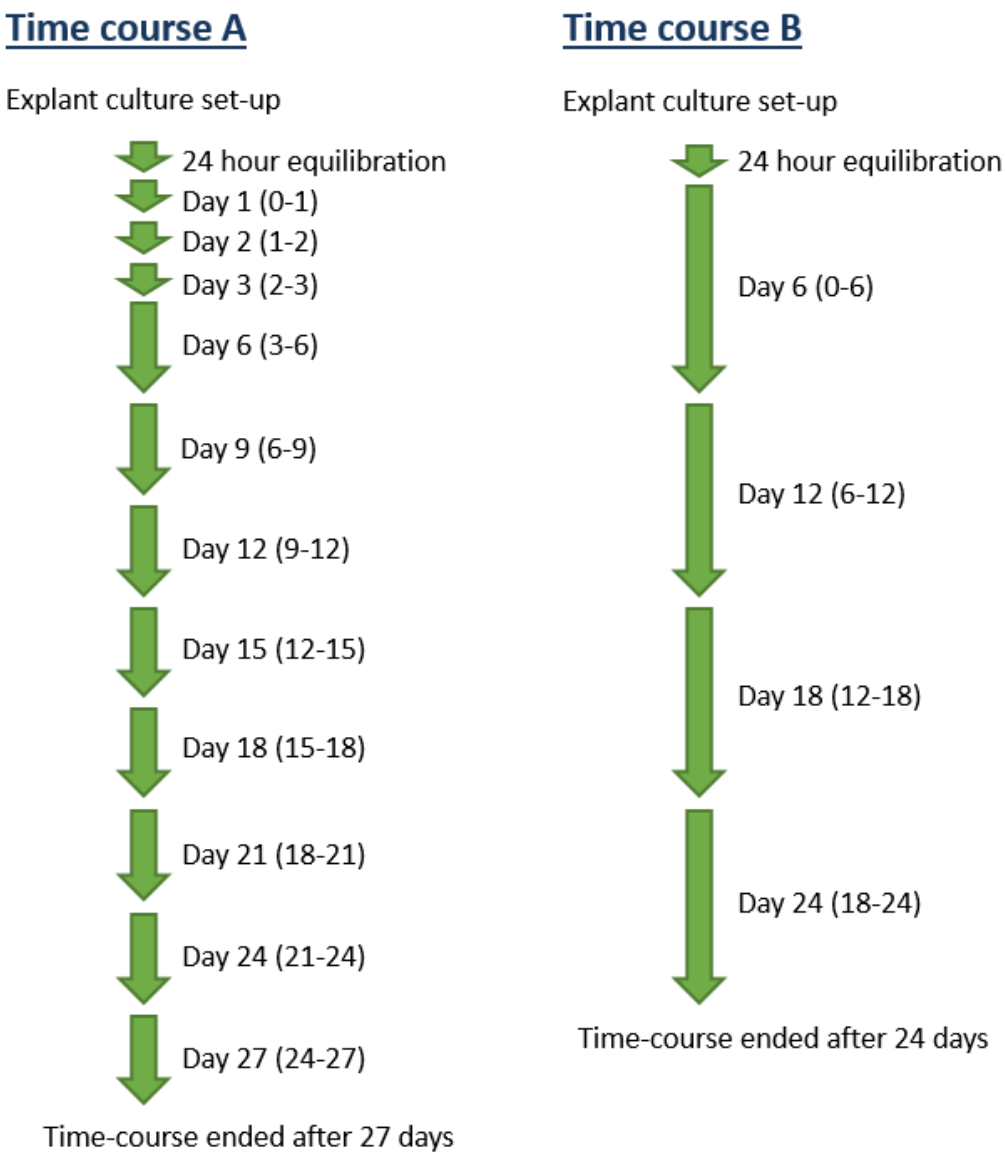


Figure 12. Layout of explant culture time courses described in Chapter 3

Culture supernatants removed after each time point, and treatment media replenished

Time course A treatments: Untreated (control), IL-1β (10 ng/ml), IL-1β (10 ng/ml) + TNF-α (10 ng/ml), carprofen (100 µg/ml), carprofen (100 µg/ml) + IL-1β (10 ng/ml), carprofen (100 µg/ml) + IL-1β (10 ng/ml) + TNF-α (10 ng/ml).

Time course B treatments: Untreated (control), IL-1β (10 ng/ml), carprofen (100 µg/ml), carprofen (100 µg/ml) + IL-1β (10 ng/ml).

3.2.3. SDS-PAGE with Protean 3 mini-gels

See Materials and Methods (Chapter 2.4.)

3.2.4. SDS-PAGE with Protean xi

To provide appropriate protein levels for band excision and analysis by mass spectrometry, electrophoresis was performed using larger gel formats. The recipe for the Protean II xi gels (16 cm x 20 cm) is included in Table 4. Molecular weight markers used with Protean xi gels were pre-stained markers from New England Bio-labs. Samples were loaded into wells and a current of 32 mA (for two gels) was applied until the dye front reached the end of the stacking gel. The current was increased to 42 mA (for two gels) for separation through the resolving gel.

Table 4. Recipe for Protean xi gels

	5% Stacking	10% Resolving
Water (18 ohm) (Fisher)	8.2 ml	8.04 ml
30% Acrylamide (Geneflow)	2.0 ml	6.66 ml
1.5M TRIS (8.8) (Bio-Rad)	-	5 ml
0.5M TRIS (6.8) (Bio-Rad)	1.5 ml	-
10% SDS (Bio-Rad)	120 µl	200 µl
10% APS (Bio-Rad)	120 µl	200 µl
TEMED (Sigma)	10 µl	6.7 µl

3.2.5. Silver staining

See General Materials and Methods (Chapter 2.5.)

3.2.6. Mass Spectrometry

High-throughput MS procedures were described in detail in Chapter 2.8. Figure 13 summarises the identification process.

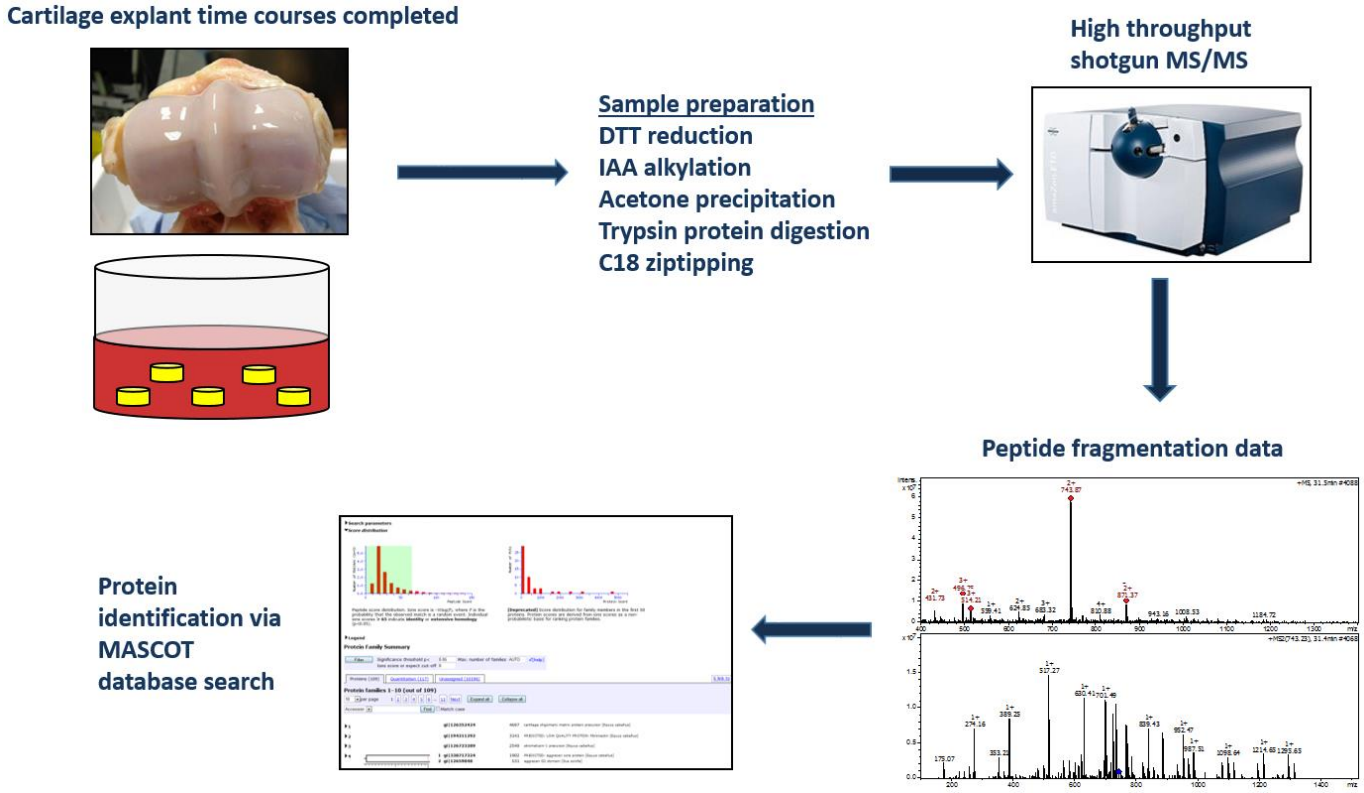


Figure 13. Schematic diagram of high-throughput MS approach to identify secretome proteins

3.2.6.1. MS of time course B - IL-1β and Carprofen + IL-1β at days 6 and 18

All three replicates from one time course B experiment for IL-1β and carprofen + IL-1β treatments at time points day 6 and day 18, were analysed to assess protein release at an early and late time point. Volumes corresponding to 50 µg protein were processed according to the MS sample preparation protocol (chapter 2.7.1.). After ziptipping, samples were heated at 60 °C to remove excess solvent and reduce the volume down to 2 µl, before 20 µl of 0.1% formic acid was added to increase the volume. For these runs, 2 µl of sample was injected into the HPLC column for analysis by an amaZon speed ETD and XtremeScan settings were used, which reduces the MS/MS cycle time. Individual ion scores > 37 (Swiss-Prot Mammals) indicated identity or extensive homology ($p<0.05$).

3.2.6.2. MS of untreated and IL-1β stimulated explant conditioned media

MS analysis was carried out on culture supernatants after 6 days of incubation, from a single horse (untreated control and IL-1β treated) and a pool of supernatants from three animals (untreated control and IL-1β treated). All sample preparation was completed as described in Chapter 2.8 on 90 µl of each sample. Excess solvent was evaporated by heating at 70 °C down to 10 µl and then transferred to glass vials. These samples were analysed on an amaZon speed ETD with 5 µl of each sample loaded onto the column. Individual ion scores > 42 (Swiss-Prot Mammals) or > 65 (NCBInr) indicate identity or extensive homology ($p<0.05$).

3.2.7. Dimethylmethylene blue (DMMB) Assays (GAG assay)

The % GAG release was measured in all time course B samples using DMMB assays as described in chapter 2.9.

3.3. Results

3.3.1. Time course A

3.3.1.1. The profile of proteins released into cartilage explant cultures throughout time course A

Time course A was undertaken to study cartilage explant secretome progression over 27 days. Representative images after protein separation by SDS-PAGE and silver staining are displayed in Figure 14. The profiles contained proteins across a wide molecular weight range, which were present at different levels. Cytokine stimulation of cultures showed a greater number of proteins in the associated profiles. The images also showed carprofen alone did not cause visible effects on overall protein release compared to untreated controls.

This investigation showed that chondrocytes within cartilage explants still respond to pro-inflammatory cytokines up to 27 days. Specific bands induced by cytokines are visible in SDS-PAGE protein profiles when compared to untreated samples. These cytokine induced bands continue to be present even at the later time points), providing evidence of continued cell viability up to 27 days. Cultures on explants frozen to cause chondrocyte death no longer showed any response to IL-1β (data not shown), therefore as responses continue to be associated with this cytokine stimulation, viable cells must remain at the later time points.

Visually comparing the profiles from IL-1β and IL-1β + TNF-α treatments did not reveal additional bands that could be attributed to TNF-α only. However, some cytokine induced bands appeared to be slightly more intense when both cytokines were present, most obviously at ~60-70 kDa. Although certain proteins continue to be released at high levels, some fainter bands lose intensity as the time course progresses. One particular protein band (highlighted in red in Figure 14) was stimulated by IL-1β. As the time course progressed, there was a reduction in this band intensity due to carprofen presence. After 15 days, this band could no longer be visualised showing carprofen had a significant effect on the underlying protein over time. The high-throughput MS analysis would discover if proteins released later in the

time course were ECM components, indicators of cell death or other secreted metabolic proteins able to provide insights into cartilage inflammation and degradation.

The 24 hour equilibration supernatant was also collected and examined during this first experiment as a control to determine any differences between wells before treatment commenced. All of the equilibration supernatants showed similar protein release, which was similar to untreated control wells throughout the rest of the time course.

To provide sufficient protein for reliable MS based identification of particular protein bands, the SDS-PAGE was scaled up onto larger format gels (16 cm x 20 cm). The silver stained image (Figure 15) show the identities of proteins present in selected bands. These include the ECM proteins: COMP, hyaluronan and proteoglycan link protein 1 (HAPLN1) and collagen type II, which were detected across all treatments. One band of note contained MMP-3. Cytokine stimulation of explants provided a substantial increase in the level of this protein. It was notably decreased in amount in cytokine stimulated cultures when the NSAID carprofen was also present. Another band identified as BSA, was introduced as a carrier protein in cytokine reagents. Double the amount of BSA will have been added with TNF-α and IL-1β treatment compared to IL-1β alone.

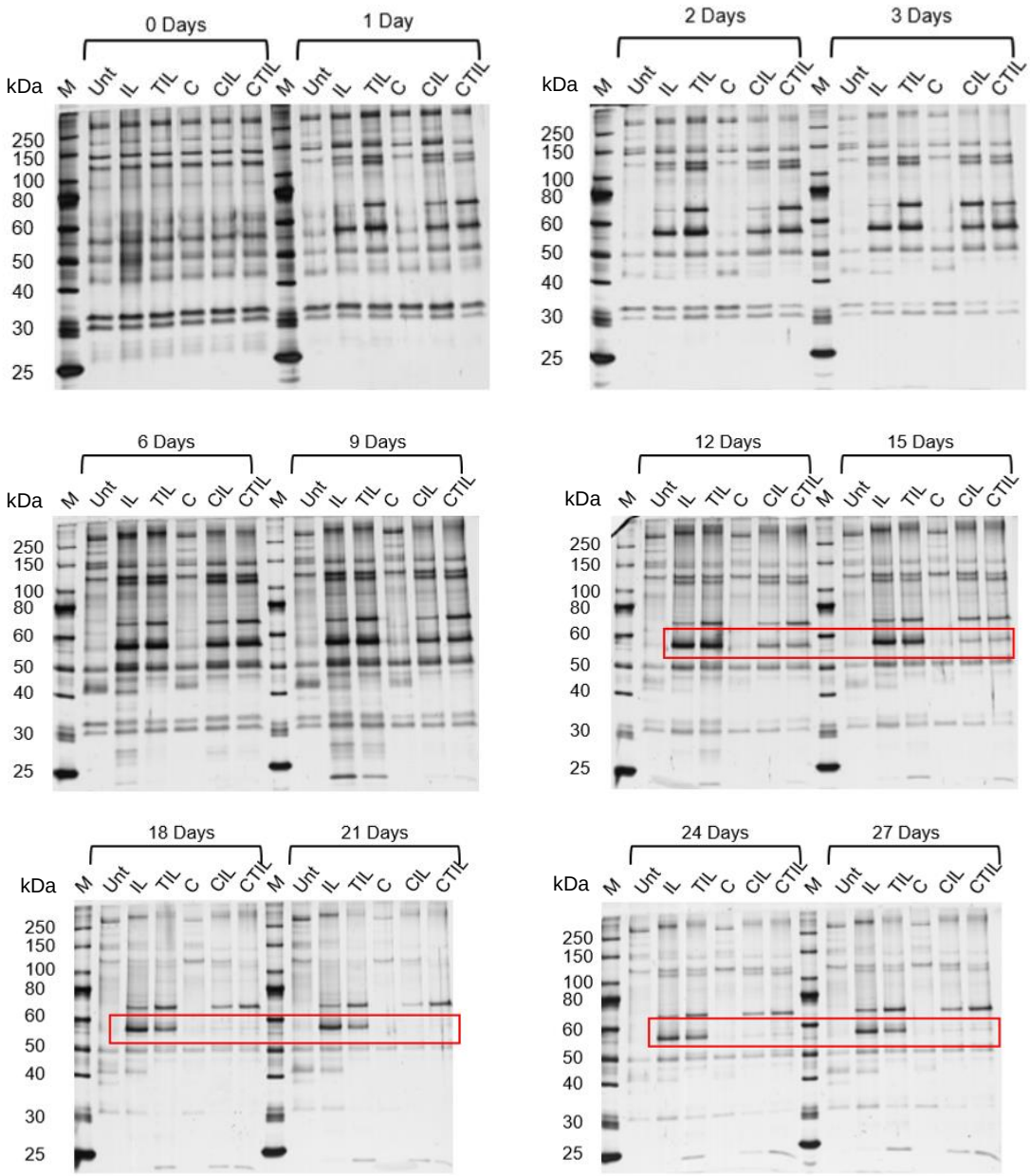


Figure 14. Time course A shows protein release in response to cytokine and carprofen treatments

Silver stained SDS-PAGE profiles of proteins released from articular cartilage explant cultures from a single horse, over 27 days of culture. The band highlighted in the red box was decreased by carprofen treatment contained MMP-3, Unt = untreated Control, IL = IL-1β (10 ng/ml), TIL = TNF-α (10 ng/ml) + IL-1β, C = carprofen (100 µg/ml), CIL = carprofen + IL-1β, CTIL = carprofen + TNF-α + IL-1β. M = molecular weight markers.

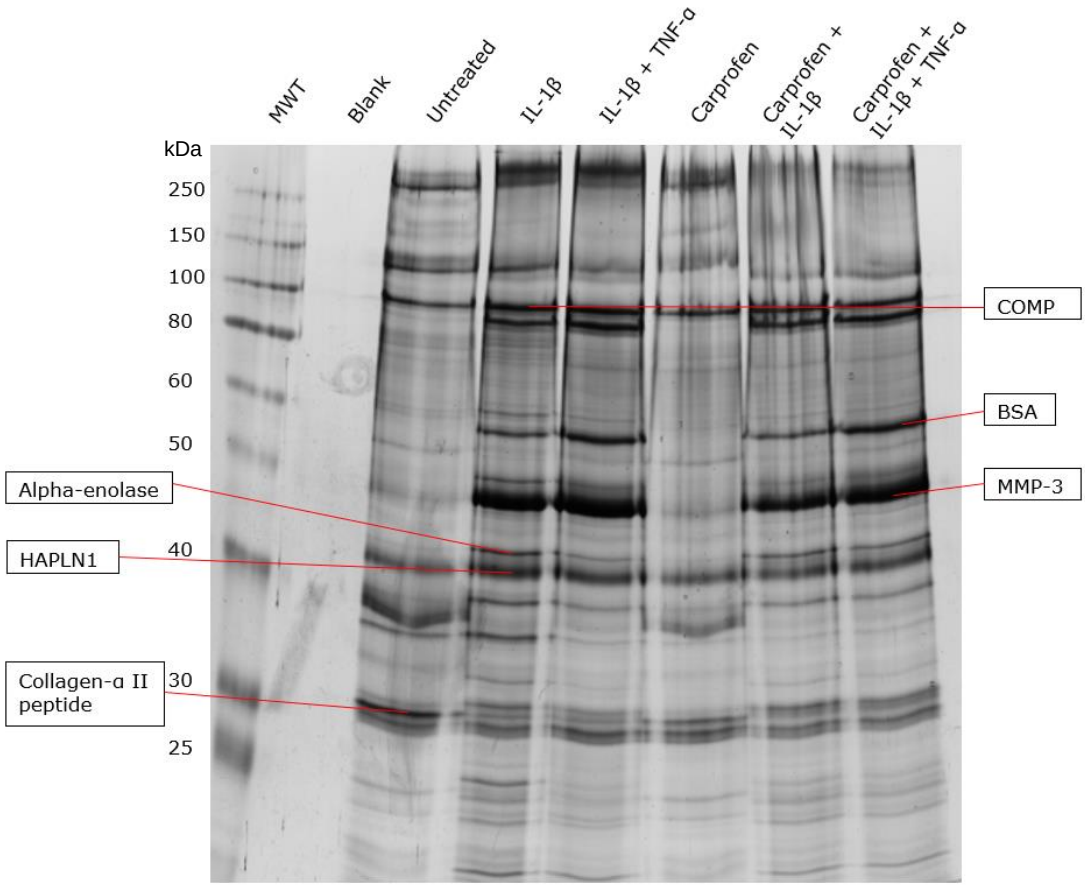


Figure 15. MS of excised bands identified COMP, BSA, MMP-3, alpha-enolase, HAPLN1 and a fragment of collagen type II

Day 6 samples of time course A were separated by SDS-PAGE before bands were excised to identify proteins by MS. Treatment concentrations: IL-1β (10 ng/ml), TNF-α (10 ng/ml), carprofen (100 µg/ml). Selected bands were manually excised and trypsin digested before nanoLC-ESI-MS/MS analysis. M = molecular weight markers.

3.3.2. Time course B

3.3.2.1. SDS-PAGE and silver staining of time course B presents higher yields of proteins in secretome

Previously, time course A provided initial insights into the continual release of cartilage proteins as shown in SDS-PAGE profiles in Figure 14, although later time points provided very dilute samples requiring concentration for most analytical approaches. To allow increased protein yields for downstream processes, a revised 24 day time course B with sample collection every 6 days was therefore undertaken.

Representative SDS-PAGE images of time course B are shown in Figure 16. Incubation for 6 days allowed higher protein concentrations to accumulate. Silver stained visualization of protein profiles was enhanced with elevated secretome content, helping differences between treatments to be more easily distinguished. Greater concentrations of proteins were also beneficial for accurate measurement during downstream assays and high-throughput MS analysis. High-throughput MS analysis of time course A (data not shown) provided some protein identifications, but the amount of proteins identified was considerably less than the eventual MS analysis of time course B. Therefore incubation time points of 6 days can be deemed more suitable to provide samples for high-throughput MS analysis in the search for biomarkers.

The four different treatment groups provided profiles that were consistent across replicates. Untreated control and carprofen alone explants had similar profiles, although certain bands around 30 and 40 kDa may be slightly reduced by carprofen. Later in time course B at days 18 and 24, a band greater than 250 kDa was more intense in untreated and carprofen alone treated samples. This could correspond to an ECM protein being degraded during the early culture stages by IL-1β inflammatory processes, therefore its intact form remains only in untreated samples. Pro-inflammatory IL-1β causes distinct changes with considerably more bands visible due to the cytokines presence. Additional stronger bands in lower molecular weight regions due to IL-1β, may suggest increases in degraded proteins. IL-1β stimulated

the 60 kDa band containing MMP-3, which was visibly decreased when combined with carprofen. Day 6 IL-1β and carprofen + IL-1β shows several bands appear more intensely when only the cytokine is present. The effects of carprofen on IL-1β stimulated processes showed that changes in release of catabolic proteins can be seen in this model. One main objective of this study was to see if anti-inflammatories effects on relevant proteins could be screened using high-throughput MS. Therefore IL-1β and carprofen + IL-1β treated explant supernatants were studied by high-throughput MS in the next section.

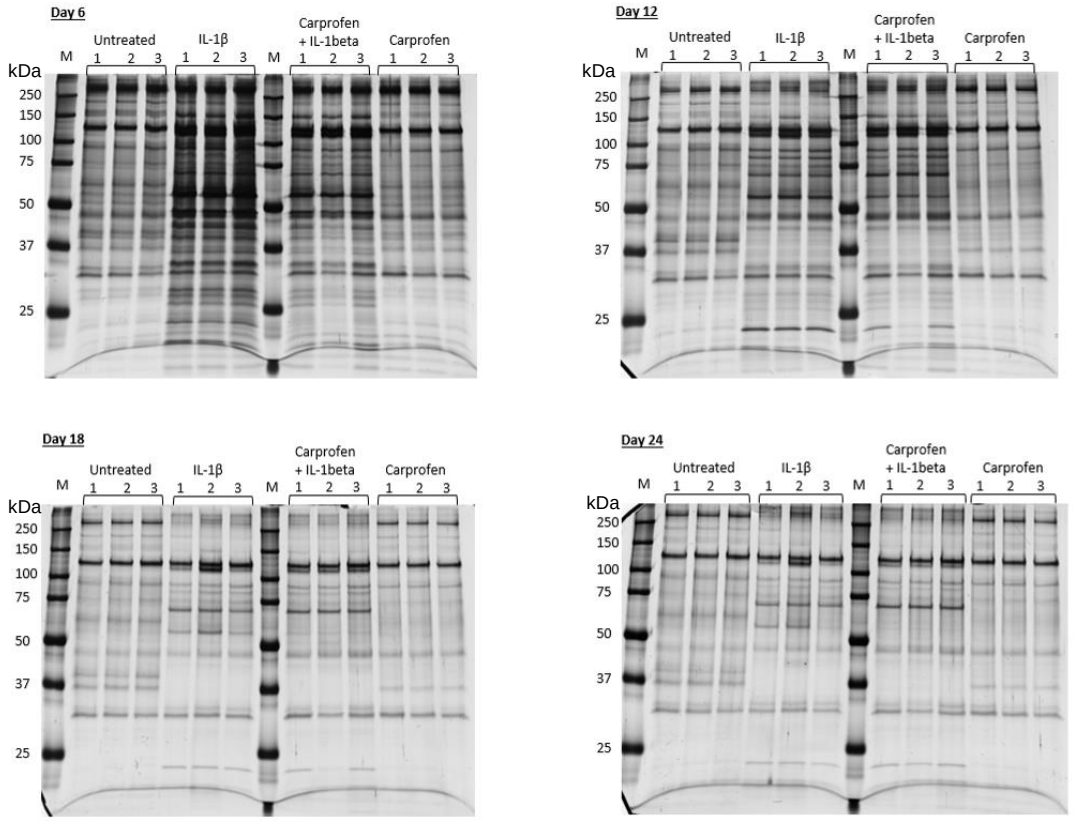


Figure 16. Representative protein profiles of time course B displaying effects of IL-1β and carprofen over 24 days

Images of SDS-PAGE that were silver stained, with each gel corresponding to a specific time point of collection: day 6, 12, 18 or 24. Treatments: untreated control, IL-1β (10 ng/ml), carprofen (100 µg/ml) + IL-1β, or carprofen.

3.3.2.2. Differences in high-throughput MS identifications after 6 day versus 18 days in time course B shows temporal effect on protein identifications

The previous sections of this results chapter determined that differences in the progression of IL-1β induced inflammation could be detected over the time course. Therefore the following high-throughput MS study assessed if the explant model could provide high quality longitudinal data with repeat sampling from the same cartilage culture. A major aim of the work in this thesis was to apply the model to provide a screening tool to assess the effects of anti-inflammatory drugs or natural compounds. Thus, the next objective was to use MS analysis to compare the proteins released during time course B into explant supernatants from IL-1β stimulation at day 6 and day 18 (Table 5), and NSAID carprofen + IL-1β treatment at day 6 and day 18 (Table 6).

Many abundant ECM proteins were released at both early and later time points (Table 5). With IL-1β stimulation, little distinction could be made between time points. Tenascin was an additional ECM protein found after IL-1β incubation for 18 days. The same ECM proteins were identified in carprofen + IL-1β samples (Table 6).

These data could be considered to be semi-quantitative. Increased levels of a protein will increase the probability of its peptides being detected during MS. Thus, a higher Mascot score will be associated with that specific protein. This effect could also be caused by decreases in other secretome proteins, as the relative amount of proteins compared to all the other proteins will affect the likelihood of it being detected. In the comparison of day 6 and day 18 MS analysis, carprofen reduced the Mascot scores of some proteins at day 18 therefore it could be considered that the amount of these proteins was reduced. For example, fibronectin attained scores of 1881, 821 and 1191 at day 6, while these were decreased to 253, 277 and 280 at day 18. Also, cartilage intermediate layer protein 1 (CILP-1) was not detected at day 18 with carprofen + IL-1β, while it was identified in all three corresponding replicates after 6 days.

In terms of non-ECM secreted proteins; nine were identified at IL-1β Day 6 and eleven for IL-1β at day 18 (Table 5). Carprofen + IL-1β analysis identified ten non-ECM proteins at day 6 and only six at day 18 (Table 6). Certain non-ECM secreted proteins were identified at specific time points (e.g. YKL-40 & Procollagen C-endopeptidase enhancer 2 (PCOC-2) in early and C-C motif chemokine 20 (CCL20) in late time points).

A wide range of intracellular proteins were released from cartilage explants in response to IL-1β. The majority of intracellular proteins were identified in IL-1β samples at day 6, with considerably less intracellular proteins identified in IL-1β day 18 samples (Table 5). A number of DNA associated histone proteins were identified at the IL-1β day 18. Comparison of IL-1β stimulation alone versus IL-1β + carprofen treatments at day 18, showed higher amounts of intracellular protein identifications with IL-1β alone. Differences in amounts of intracellular proteins present between treatment groups is demonstrated by identification of twenty-nine intracellular proteins in IL-1β samples (Table 5), with only six identified with carprofen + IL-1β (Table 6). Presence of intracellular proteins suggests cell death and therefore the release of proteins usually contained within chondrocytes. These findings that carprofen reduces intracellular protein identifications is consistent with its ability to decrease inflammation and therefore associated cell death.

Certain proteins identified did not originate from cartilage explants but were added during sample processing or introduced by contamination. Firstly, bovine serum albumin (BSA) is included as a carrier protein for IL-1β. Pancreatic trypsin inhibitor (BPT1_BOVIN) is a component of the protease inhibitor cocktail that was added during sample collection to reduce protein degradation. Trypsin is introduced to digest proteins into peptides prior to MS analysis, therefore autocatalysis fragments of trypsin are expected to be found. Although every attempt was made to reduce exposure during proteomics work, keratin is a source of contamination from dust, skin or hair during sample processing.

Table 5. Proteins identified using high-throughput MS in IL-1β samples at day 6 and day 18 of time course B

Mascot scores are shown for proteins detected in each of three replicates at each time point after searches of Swiss-Prot 2013_01 (all mammalian entries) database. Only Mascot scores >37 indicating identity/extensive homology are included. Identifications organised into ECM proteins, Non-ECM Secreted Proteins and Intracellular Proteins. Proteins in order of highest Mascot score in IL-1β day 6. * indicates proteins where highest homology/Mascot scores corresponded to different species or isoforms therefore only the top scoring Swiss-Prot entry name is mentioned.

		IL-1β Day 6 (3 replicates)						IL-1β Day 18 (3 replicates)					
Protein Name	**Accession**	**1**		**2**		**3**		**1**		**2**		**3**	
		Mascot score	Unique peptides	Mascot score	Unique peptides	Mascot score	Unique peptides	Mascot score	Unique peptides	Mascot score	Unique peptides	Mascot score	Unique peptides
ECM proteins													
COMP	COMP_BOVIN	8118	14	20793	16	9026	17	8253	16	18586	19	11318	16
Fibronectin	FINC_HUMAN	1037	15	1964	22	1042	20	1161	27	926	22	789	21
Aggrecan core protein	PGCA_HUMAN	1117	12	1744	15	1648	13	917	12	975	10	1015	11
Chondroadherin	CHAD_HUMAN	210	4	1610	7	1554	6	1570	11	2064	10	3157	12
Fibromodulin	FMOD_HUMAN	333	5	1444	6	918	7	1324	7	1454	6	1290	8
Biglycan*	PGS1_BOVIN	965	5	1571	7	1045	6	1828	10	4199	12	4247	11
Decorin	PGS2_HORSE	1428	5	1021	5	895	5	906	10	865	6	1445	9
Thrombospondin-1 (TSP-1)	TSP1_HUMAN	773	13	808	12	835	7	571	14	61	6	741	15
CILP-1*	CILP1_MOUSE	69	4	474	7	220	6	156	4	236	8	110	5
Prolargin	PRELP_MOUSE	43	2	88	4	123	2	245	5	405	4	406	4
Proteoglycan 4 (lubricin)	PRG4_HUMAN					86	6						
Asporin	ASPN_BOVIN			46	3			113	3	49	2		
Collagen alpha-1(II) chain	CO2A1_RAT							94	6				
Tenascin	TENA_HUMAN							89	9				
Collagen alpha-2(I) chain	CO1A2_HUMAN							47	4				
Non-ECM Secreted Proteins													
MMP-3	MMP3_HORSE	4280	15	5116	13	4486	12	2459	18	3378	14	1340	11
Clusterin	CLUS_HORSE	1800	6	1347	8	1435	8	481	5	317	6	163	3
MMP-1	MMP1_HORSE	545	5	334	5	235	3	1176	20	712	11	67	4
Serum Amyloid A protein*	SAA_HORSE	285	4	108	4	176	5	177	4	65	4	85	2
TNF receptor 11B*	TR11B_MOUSE			173	1	153	3	94	2				
Chitinase-3-like protein (YKL-40)	CH3L1_BOVIN			40	3	81	2						
Lysozyme C*	LYSC2_HORSE			38	2	80	5	56	2	40	1	116	2

MMP-13	MMP13_HORSE			58	4			1339	13	828	10	254	4
Procollagen C-endopeptidase enhancer 2	PCOC2_HUMAN					44	3						
Annexin A5	ANXA5_HUMAN							93	3	62	1	98	6
C-x-C motif chemokine 6	CXCL6_BOVIN							63	1	40	1		
Coagulation factor XIII A chain	F13A_MOUSE							61	2				
C-C motif chemokine 20	CCL20_BOVIN							44	2				
Intracellular Proteins													
Vimentin*	VIME_MOUSE	574	10	1525	9	1796	16	628	13	248	6	405	9
Enolase*	ENOA_BOVIN					211	5						
Pyruvate kinase isoform M1/M2	KPYM_FELCA			61	5	248	9						
Phosphatidylenolamine-binding protein 1*	PEBP1_BOVIN			122	2	163	1						
Thioredoxin	THIO_HORSE	80	1			133	1						
POTE ankyrin domain family member F	POTEF_HUMAN					115	8						
Phosphoglycerate Kinase 1	PGK1_HORSE					94	6						
L-lactate dehydrogenase A chain	LDHA_BOSMU					81	3						
Transcription termination factor 1	TTF1_HUMAN	67	3	44	6	43	4	37	7	39	5		
Transaldolase	TALDO_HUMAN					49	2						
Glutathione S-transferase P	GSTP1_MACMU					47	2						
Putative polycomb group protein ASXL3	ASXL3_HUMAN					45	4						
Rho guanine nucleotide exchange factor 40	ARH40_MOUSE			44	3								
Sodium/hydrogen exchanger 2	SL9A2_RABIT			41	3	39	2	42	2	59	3	37	2
POTE ankyrin domain family member E	POTEE_HUMAN			39	4								
Phosphoglycerate mutase 1	PGAM1_BOVIN					39	1						
Sperm equatorial segment protein 1	SPESP_MOUSE	39	2										
Inositol 1, 4, 5-trisphosphate receptor type 2	ITPR3_RAT			38	9								
Adenomatous polyposis coli protein 2	APC2_MOUSE			37	13								
Purine nucleoside phosphorylase	PNPH_HUMAN					37	3						
Triosephphosphate isomerase	TPIS_BOVIN					37	2						
Histone H4*	H4_BOVIN							363	7	250	5	459	5
Transmembrane protein 104	TM104_PONAB							45	2				
Metallothionine 1-A	MT1A-BOVIN							44	1				
Rho guanine nucleotide exchange factor 10-like protein	ARGAL_MOUSE							39					
MMP-25	MMP25_MOUSE							38	5				
Solute carrier family 28 member 3	S28A3_MOUSE											38	3
Transmembrane protein 87A	TM87A_HUMAN							37	3				

Other proteins													
Pancreatic trypsin inhibitor	BPT1_BOVIN	192	2	57	3	194	2	316	2	318	2	417	2
BSA	ALBU_BOVIN			49	4	43		1046	26	913	17	1075	17
Trypsin	TRYP_PIG									37	4	78	2

Table 6. Proteins identified using high-throughput MS in IL-1β + carprofen samples at day 6 and day 18 of time course B

Mascot scores are shown for proteins detected in each of three replicates at each time point after searches of Swiss-Prot 2013_01 (all mammalian entries) database. Only Mascot scores >37 indicating identity/extensive homology are included. Identifications organised into ECM proteins, Non-ECM Secreted Proteins and Intracellular Proteins. Proteins in order of highest Mascot score in IL-1β + carprofen day 6. * indicates proteins where highest homology/Mascot scores corresponded to different species or isoforms therefore only the top scoring Swiss-Prot entry name is mentioned.

		IL-1β + carprofen Day 6 (3 replicates)						IL-1β + carprofen Day 18 (3 replicates)					
Protein Name	**Accession**	**1**		**2**		**3**		**1**		**2**		**3**	
		Mascot score	Unique peptides	Mascot score	Unique peptides	Mascot score	Unique peptides	Mascot score	Unique peptides	Mascot score	Unique peptides	Mascot score	Unique peptides
ECM Proteins													
COMP	COMP_BOVIN	8477	17	9934	18	8950	14	12626	16	10080	13	7244	14
Aggrecan core protein	PGCA_HUMAN	2012	12	1946	10	1578	11	2856	10	1592	8	1091	9
Fibronectin	FINC_HUMAN	1881	23	821	22	1191	18	253	19	277	11	280	15
Chondroadherin*	CHAD_HUMAN	1560	7	640	6	1334	5	93	4	48	5		
Fibromodulin	FMOD_HUMAN	1455	6	974	6	646	5	103	4	170	5	52	4
Decorin	PGS2_HORSE	1241	9	1168	8	1382	5	618	6	880	7	661	7
Thrombospondin-1 (TSP-1)	TSP1_HUMAN	1381	17	266	10	1297	13	309	9	61	5	184	10
Biglycan	PGS1_BOVIN	1275	8	861	4	1201	6	206	4	101	4	95	3
CILP-1	CILP1_PIG	275	6	141	6	101	3						
Prolargin	PRELP_MOUSE	199	5	181	3			72	5	79	4	122	3
Collagen alpha-2(VI) chain	CO6A2_HUMAN	56	3										
Collagen alpha-1(II) chain	CO2A1_RAT							72	4			47	5
Non-ECM Secreted Proteins													
MMP-3	MMP3_HORSE	2596	13	1189	11	1552	10	362	9	431	7	343	8
Clusterin	CLUS_HORSE	810	8	646	5	1035	6	53	3	65	2	48	3
TNF receptor 11B	TR11B_RAT	234	4	227	2	83	1	39	1				
TIMP-1	TIMP1_HORSE	91	2										
MMP-13	MMP13_HORSE	57	4	75	1								
Serum Amyloid A protein*	SAA_HORSE			51	2			61	2	57	2	40	2
Chitinase-3-like protein (YKL-40)	CH3L1_BOVIN	39	10	49	1	39	1						
Lysozyme C	LYSCK_SHEEP			48	2								
MMP-1	MMP1_HORSE	47	2					53	2				
Procollagen C-endopeptidase	PCOC2_HUMAN			39	2								

enhancer 2													
C3 and PZP-like alpha-2-macroglobulin domain containing protein 8 (Complement C4-A) (CPAMD8)	CPMD8_HUMAN									52	6	45	4
Intracellular Proteins													
Vimentin	VIME_MOUSE	451	5			221	3					64	4
Enolase	ENOA_BOVIN	45	1										
Transcription termination factor 1	TTF1_HUMAN	39	5	44	3			62	5				
Transmembrane protein 87A	TM87A_HUMAN					39	2						
Rho guanine nucleotide exchange factor 40	ARH40_MOUSE	38	6										
Other proteins													
Pancreatic trypsin inhibitor	BPT1_BOVIN	294	3	304	2								
BSA	ALBU_BOVIN	102	5	37	2	52	3	39	1				
Trypsin	TRYP_PIG			38	1	50	1			81	2	55	1

3.3.3. Comparison of proteins detected in untreated control and IL-1β stimulated explant secretomes

Working towards the aim of applying the explant model for screening of anti-inflammatory compounds, it was important to gather information on proteins affected by IL-1β. Reversal of IL-1β stimulated processes would allow assessment of an anti-inflammatories ability to halt degradation. After time course B evaluation, it was concluded that supernatants collected after 6 days would provide the most appropriate incubation time for secretome studies. An incubation time of 6 days showed increased protein bands visible in SDS-PAGE profiles (Figure 16) and achieved highest quality MS data in the previous section.

In this study, untreated control and IL-1β explant supernatants from an individual horse and a pool of three horses was analysed by high-throughput MS, and organized into groups of ECM, secreted and intracellular proteins (Figure 17 and Table 7). These semi-quantitative data indicates altered levels of release some proteins. For example, MMP-3 was identified in all samples (untreated and IL-1β), but Mascot scores for IL-1β samples were higher (5441, 4580) than for untreated controls (653, 956). This observation is supported by quantitative western blotting reported in Chapter 5.

Fifteen ECM proteins were identified across all samples (Table 7). The only distinct difference in ECM proteins between treatments was the presence of collagen types VI and X, and SPARC (secreted protein acidic and rich in cysteine) in untreated controls. Collagen type II was present in both treatments. Twenty-one non-ECM proteins were detected in untreated supernatant, while seventeen were identified with IL-1β. Many non-ECM proteins were associated with one treatment group. TIMP-2, extracellular superoxide dismutase (SODE), PCOC-2, SFRP-3, connective tissue growth factor (CTGF), augurin, ceruloplasmin, integrin beta-like protein 1, melanoma-derived growth regulatory protein (MIA) and insulin-like growth factor-binding protein 7 (IGFBP7) were identified only in untreated control samples. MMP-1, MMP-13, heat shock 70kDa protein 1-like (HSP70), S-100 protein-A1, MIF, semaphorin-3C and C-X-C motif chemokine 6 (CXCL6) were detected only after IL-1β stimulation. Intracellular

proteins were also identified in both untreated control (8 proteins) and IL-1β (27 proteins) MS analysis, therefore IL-1β stimulation increased numbers of intracellular proteins detected (Figure 17). These intracellular proteins should be contained within chondrocyte cytoplasm and organelles, therefore their detection is indicative of cell death.

Separate databases contain differences in protein entries, therefore the choice of database may affect proteins identified by Mascot searches. Some additional identifications of released explant proteins were made when searching the NCBInr database (Table 8) rather than Swiss-Prot. Glia derived nexin is a serpin, while matrix Gla protein-like is involved with calcium binding. Osteopontin is found in the mineralized matrix, and CILP-2 forms interactions within the cartilage matrix. Beta-defensins are secreted proteins with anti-bacterial functions. Although these extra identifications were made, many more relevant proteins were reliably identified in the Swiss-Prot database searches that were not identified in NCBInr searches. Therefore, the decision was made to concentrate on reporting MS identifications from Swiss-Prot throughout this thesis.

Table 7. Swiss-Prot protein identifications in untreated control and IL-1β cultures with high-throughput MS

Only Mascot scores >38 indicating identity/extensive homology are listed. Proteins identified are grouped into ECM, Non-ECM and Intracellular, and ordered by the highest Mascot scores in untreated samples. Database searched: Swiss-Prot 2013_01 (all mammalian entries).

Protein	**Accession**	**Individual Untreated**		**Pool Untreated**		**Individual IL-1β**		**Pool IL-1β**	
		Mascot score	Unique peptides	Mascot score	Unique peptides	Mascot score	Unique peptides	Mascot score	Unique peptides
ECM Proteins									
Cartilage oligomeric matrix protein	COMP_BOVIN	4090	18	4763	17	7555	20	10590	21
Fibronectin	FINC_BOVIN	4422	41	6222	42	3786	40	3492	34
Aggrecan core protein	PGCA_HUMAN	1815	15	3389	16	2258	13	6412	14
Fibromodulin	FMOD_HUMAN	1307	9	3200	7	1381	6	1930	8
Chondroadherin	CHAD_HUMAN	1022	8	3069	6	1978	8	3689	8
Thrombospondin 1 (TSP-1)	TSP1_BOVIN	741	20	1256	13	3456	28	2782	21
Biglycan	PGS1_HORSE	1099	12	1062	10	2047	11	1720	9
Decorin	PGS2_HORSE	665	13	802	10	513	9	1130	8
Cartilage intermediate layer protein 1 (CILP-1)	CILP1_MOUSE	201	10	789	10	423	19	264	11
Collagen alpha-2(VI)chain	CO6A2_HUMAN	190	8	79	2				
Prolargin	PRELP_MOUSE	124	5	147	5	311	6	301	2
Proteoglycan 4 (Lubricin)	PRG4_HUMAN	139	7	49	6			59	6
Collagen alpha-1(II) chain	CO2A1_BOVIN	137	15	59	10	139	9	82	8
SPARC (secreted protein acidic, cysteine rich) (osteonectin)	SPRC_PIG	108	4	96	5				
Collagen alpha-1(X) chain	COAA1_MOUSE	77	2	102	4				
Non-ECM Secreted Proteins									
Clusterin	CLUS_HORSE	2767	16	4723	16	1785	13	4876	14
Chitinase-3-like protein 1 (YKL-40)	CH3L1_BOVIN	574	6	1178	5	414	6	2018	5
MMP-3	MMP3_HORSE	653	11	956	10	5441	20	4580	17
Alpha-1-antiproteinase 2	A1AT2_HORSE	99	7	342	3	50	3		
TNF-R superfamily, member 11b (Osteoprotegerin)	TR11B_RAT	252	4	319	3	388	6	638	4
Metalloproteinase inhibitor 1	TIMP1_HORSE	83	5	312	4	457	4	146	3
Procollagen C-endopeptidase enhancer 2 (PCOC-2)	PCOC2_HUMAN	163	8	171	4				
Lysozyme C	LYSM_BOVIN	72	1	168	2	179	3	148	2

Secreted frizzled-related protein 3 (SFRP)	SFRP3_BOVIN	74	6	98	8				
Lactadherin	MFGM_PIG	83	1	63	2			54	3
Ceruloplasmin	CERU_RAT			75	6				
Extracellular superoxide dismutase [Cu-Zn] (SODE)	SODE_HUMAN	67	3	72	2				
Integrin beta-like protein 1	ITGBL_MOUSE	56	4						
Serum amyloid A protein	SAA_HORSE			55	2	219	4	436	44
	SAA_CANFA	43	2						
Connective tissue growth factor (CTGF)	CTGF_MOUSE			55	3				
Augurin	AUGN_HUMAN	54	1						
Ribonuclease 4	RNAS4_HUMAN			48	2	46	1	59	2
Metalloproteinase inhibitor 2	TIMP2_CANFA			47	5				
Melanoma-derived growth regulatory protein (MIA)	MIA_HUMAN	47	2						
Insulin-like growth factor-binding protein 7	IBP7_HUMAN	46	5						
MMP-13	MMP13_HORSE					1700	20	699	12
MMP-1	MMP1_HORSE					1678	23	1586	20
S-100 protein-A1	S10A1_BOVIN					383	1		
Macrophage migration inhibitory factor (MIF)	MIF_HUMAN					91	2	86	2
Heat shock 70kDa protein 1-like	HS71L_MOUSE					62	8	74	4
Semaphorin-3C	SEM3C_HUMAN					47	6		
C-X-C motif chemokine 6	CXCL6_HORSE					44	2		
Intracellular proteins									
L-lactate dehydrogenase A chain	LDHA_BOSMU					176	7	134	6
Vimentin	VIME_PIG					2361	24	3837	31
	VIME_PANTR			84	4				
Transcription factor E3	TFE3_HUMAN	47	2						
DnaJ homolog subfamily C member 14	DJC14_BOVIN			43	3				
Sodium/hydrogen exchanger 2	SL9A2_RABIT	42	3			71	4		
Serine-protein kinase ATM	ATM_PIG			42	9				
Alcohol dehydrogenase [NADP(+)] fragment	AK1A1_CRIGR			42	3	72	5		
Phosphoglycerate kinase 1	PGK1_HORSE					792	15	651	8
Alpha-enolase	ENOA_BOVIN					506	13	686	11
Pyruvate kinase isoforms M1/M2	KPYM_HUMAN					488	10	245	8
Phosphatidylethanolamine-binding protein 1	PEBP1_HUMAN					423	4	229	5
Triosephosphate isomerase	TPIS_CANFA					246	5		
14-3-3 protein epsilon	1433E_BOVIN					211	4		
Glyceraldehyde 3-phosphate dehydrogenase	G3P_PIG					208	5	115	3
Purine nucleoside phosphorylase	PNPH_HUMAN					175	6	134	5

Phosphoglycerate mutase 1	PGAM1_MOUSE					170	7	104	7
Thioredoxin	THIO_HORSE					145	2	143	1
Glutathione S-transferase	GSTP1_PIG					119	3		
Superoxide dismutase [Mn], mitochondrial	SODM_HORSE					102	1		
Fructose-bisphosphate aldolase A	ALDOA_RABIT					101	8		
Actin, alpha skeletal muscle	ACTS_BOVIN							88	6
Metallothionein-1A	MT1A_BOVIN							86	2
Metallothionein-1E	MT1E_PIG					85	3		
Gelsolin	GELS_BOVIN					83	4		
Glycogen phosphorylase, brain form	PYGB_PONAB					60	7		
Tripartite motif containing protein 46	TRI46_HUMAN							59	1
Peptidyl-prolyl cis-trans isomerase A	PPIA_AOTTR							51	3
Transmembrane protein	PVRIG_HUMAN					45	2		
Transaldolase	TALDO_CRIGR					43	5		
Ras GTPase-activating protein 4	RASL2_MOUSE							43	4
Other									
Pancreatic trypsin inhibitor	BPT1_BOVIN	283	2	477	2	281	2	703	2
Keratin, type II cytoskeletal 3	K2C3_RABIT	151	4	277	2	110	4	82	6
Trypsin	TRYP_PIG	111	3			106	2	746	3
Serum Albumin	ALBU_BOVIN					283	11	86	8
Uncharacterised protein C12orf55 homolog	CL055_MOUSE							44	3

Table 8. Searching NCBInr database provides certain additional protein identifications in untreated control and IL-1β cultures with high-throughput MS

Mascot scores >65 indicating identity/extensive homology.

Protein	**Accession**	**Individual Untreated**	**Pool Untreated**	**Individual IL-1β**	**Pool IL-1β**
		Mascot score	Mascot score	Mascot score	Mascot score
Osteopontin-like isoform 1	gi|149701529	544	394	131	323
Matrix Gla protein-like	gi|149713779	173	326	-	74
Cartilage intermediate layer protein 2	gi|194223805	195	144	302	-
Glia-derived nexin isoform 2	gi|149711173	81	104	81	-
Beta-defensin-1 precursor	gi|126352458	64	57	44	94

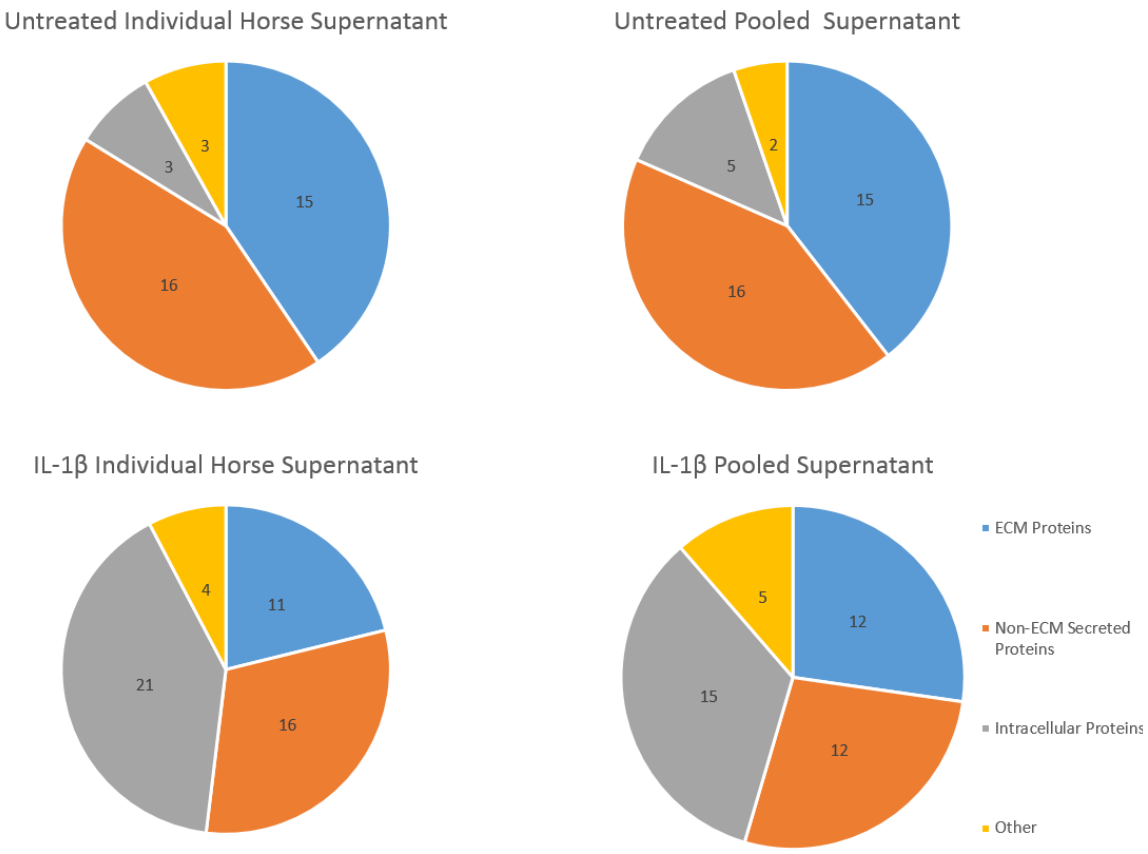


Figure 17. Classification of proteins identified from MS analysis of untreated and IL-1β explant cultures

Protein classifications based on information within the UniProt database (http://uniprot.org). Precise details of protein identifications displayed in Table 7. Numbers represent amount of proteins in each classification group.

3.3.4. IL-1β stimulated GAG release is delayed by carprofen

GAGs are important ECM components that contribute towards cartilage's compressible properties. Release of GAGs from cartilage explants in response to treatment conditions can be measured by DMMB assays. Measurements of % GAG release are displayed for time course B days 0-6 and 6-12 in Figure 18. The majority of inducible GAG release occurred over the first 12 days incubation with subsequently very low levels of GAG release and no significant differences between treatments (data not shown) at the two later time points: day 18 and day 24.

Studies in this chapter showed significant increases in GAG release attributed to IL-1β stimulation (40%) by comparison with untreated controls (13%). There was no effect on % GAG release due to carprofen treatment alone (11%), as parallel levels were recorded compared to untreated cultures (13%). During the first 6 days of incubation, carprofen significantly decreased IL-1β stimulated GAG release from 40% down to 26%. At day 12, IL-1β GAG release was 19% but was significantly higher with carprofen + IL-1β at 27%. GAG release during this time point will have been affected by the amount of GAG already released from explant cultures during the first 6 days of incubation. Carprofen therefore delayed IL-1β stimulated GAG release. Over the time course similar levels of GAGs were eventually discharged by IL-1β stimulation, whether carprofen is present or not.

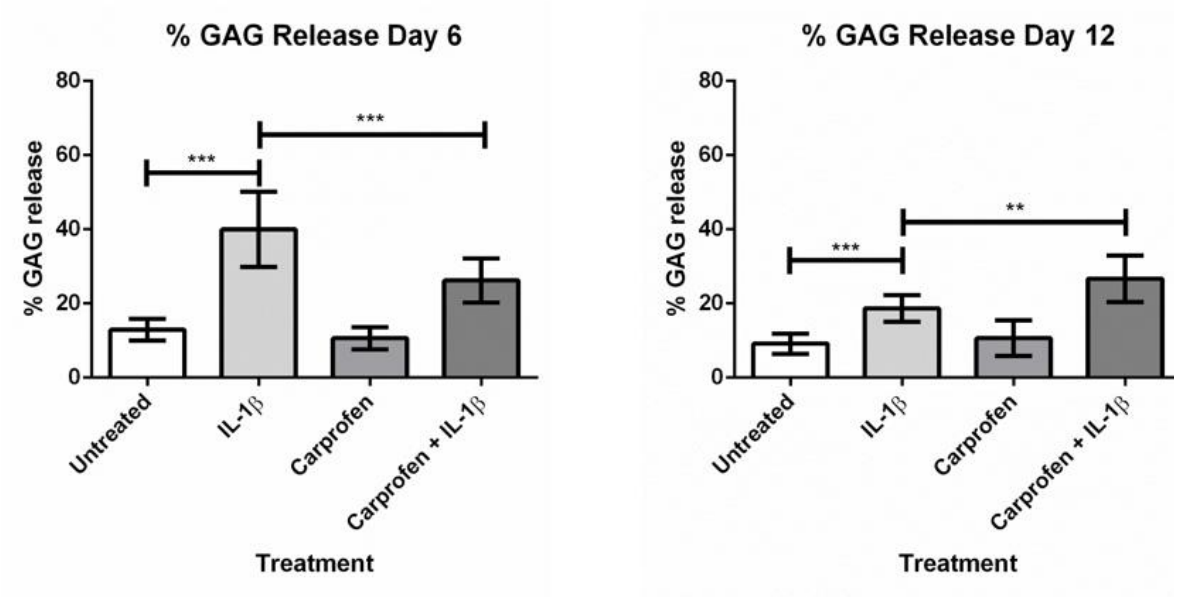


Figure 18. Percentage GAG release at days 6 and 12 from time course B with IL-1β stimulation and NSAID carprofen treatment

The following treatments were applied: untreated control, IL-1β only (10 ng/ml), IL-1β (10 ng/ml) + carprofen (100 µg/ml) or carprofen only (100 µg/ml). The % GAG release was measured at 6 day intervals during time course B, with results up to day 6 and day 12 displayed. Explant culture time courses were completed in 3 separate animals, with 3 treatment replicates for each study. ** = $P < 0.001$, *** = $P < 0.0001$. Error bars indicate Standard Deviation.

3.4. Discussion

The aim of this chapter was to provide new insights into the secretome of equine articular cartilage to identify potential biomarkers for efficacy of treatment. Silver stained SDS-PAGE monitored changes in the secretome over time. Proteins released from untreated control explants and in response to pro-inflammatory cytokine IL-1β and NSAID carprofen were assessed by high-throughput MS. Insights into equine cartilage explant secretome were achieved throughout studies described. Protein identifications made that were not reported by Clutterbuck et al., 2011 included MMP-13, collagen type II, SFRP and MIF. Contributing factors for additional identifications could include biological variation, improvements in technical instruments and updates in the sequence databases. All ECM or non-ECM secreted proteins identified by Clutterbuck et al., 2011 were also identified in studies in this thesis. Identified proteins were organised into corresponding specific treatment groups (e.g. untreated, IL-1β, or carprofen + IL-1β) which was not detailed in previous MS data (Clutterbuck et al., 2011).

3.4.1. The search for biomarkers of treatment efficacy

Proteins identified within the secretome could be investigated as candidate biomarkers of treatment efficacy to study anti-catabolic and anti-inflammatory effects. Potential efficacy biomarkers to highlight include biglycan, fibromodulin, chondroadherin and CILP-1, as release of these ECM proteins was reduced by carprofen treatment (when in combination with IL-1β) compared to IL-1β stimulation alone. IL-1β stimulated non-ECM proteins reduced by carprofen included MMP-1, MMP-3 and MMP-13. These proteins therefore also hold potential as biomarkers and were further investigated by western blotting in chapter 5.

Potential biomarkers require further verification and validation to determine their application as new biomarkers of efficacy according to recognised guidelines (Lee, 2009). The biomarkers highlighted in this thesis may only be relevant to screening in this specific equine model, or may be able to be characterised in the wider OA disease population and clinically relevant. For example, a neoepitope

collagen TIINE was first identified as released from human cartilage *in vitro* in response to cytokine stimulation and MMP activity (Nemirovskiy et al., 2007). An immunoaffinity LC-MS/MS assay was clinically validated to measure levels of collagen TIINE in urine to investigate this neoepitope as an OA biomarker (Li et al., 2007). Biomarkers originating in synovial joints can enter systemic circulation and therefore be detected in serum, plasma or urine (Firestein, 2008). After initial discovery of candidate biomarkers *in vitro*, a targeted approach should be applied to verify and quantify the biomarker in a designed cohort of individuals using an appropriate assay method, such as LC-MS/MS or ELISA. Clinical validation across higher numbers of the population can then be completed to confirm a biomarkers value.

In this chapter, cytokine stimulation of explants was examined over 24 days in time course B using IL-1β, which is associated with ECM breakdown in OA (Goldring and Goldring, 2007). High-throughput MS analysis showed changes in release of various proteins in response to IL-1β stimulation. The NSAID carprofen was selected as an anti-inflammatory treatment throughout the time course because it is used to treat horses and dogs suffering from OA and other painful joint diseases. Synthesis of bioactive lipid PGE_2 is inhibited by carprofen, because it specifically blocks COX-2. The treatments were selected to investigate high-throughput MS to identify biomarkers of anti-inflammatory treatment efficacy. Proteins identified showing semi-quantitative differences based on Mascot scores using MS analysis, were selected for further analysis by western blotting, allowing a quantitative measurement of release described in chapter 5.

3.4.2. ECM proteins identified

3.4.2.1. Non-collagenous ECM proteins

High-throughput MS analysis of explant cultures consistently identified many abundant ECM proteins released into supernatant. The non-collagenous proteins COMP, fibronectin, thrombospondin-1 (TSP-1), CILP-1, chondroadherin and tenascin found in this study, were identified in previous secretome studies, indicating that this model is consistent with similar models from other species (Hermansson et al., 2004,

De Ceuninck et al., 2005, Polacek et al., 2010). Furthermore, these proteins are biologically relevant and vital components of the ECM that could be further studied.

One abundantly released component was COMP, a pentamer protein from the thrombospondin family (Hedbom et al., 1992). COMP binds to collagen fibres and fibronectin within the ECM, directing organisation of the structural matrix (Rosenberg et al., 1998, Di Cesare et al., 2002). COMP fragmentation has been demonstrated *in vitro* by stimulation of cartilage explants with IL-1 (Ganu et al., 1998). It is a potential predictive urinary biomarker of OA in horses after carpal bone fracture (Arai et al., 2008).

During the search for biomarkers of OA, it should be taken into account that specific post-translational modifications may distinguish a protein as a biomarker. Deamidated COMP with native Asn64 (asparagine) converted to Asp64 (aspartic acid), is a biomarker for OA located in the hip (Catterall et al., 2012). To determine if post-translational modifications were required to identify responses to IL-1β, Mascot searches were carried out for deamidated modifications on MS data collected here. None were found, possibly because the explant model utilises healthy cartilage often from younger horses, while deamidation of COMP is associated with accumulation of this non-enzymatic modification in aged human OA cartilage (Catterall et al., 2012).

Fibronectin has the ability to bind collagens and other cell surface proteins (Di Cesare et al., 2002). During OA, fragments of fibronectin are released and induce catabolic signalling through interaction with receptors in synovial joint cells (Homandberg et al., 1998, Homandberg et al., 2006). Tenascin can bind to proteoglycans (Burg et al., 1996) and cell surface integrins (Sriramarao and Bourdon, 1993), and is known to contain a fibronectin III domain (Leahy et al., 1992). CILP-1 is a monomeric glycoprotein that is cleaved into two polypeptides and is mostly found in the intermediate zone of cartilage (Lorenzo et al., 1998). Up-regulation of CILP is associated with both early and later stages of OA (Lorenzo et al., 2004). The IL-1β stimulated explant model is therefore able to simulate certain aspects of OA progression, as it produces release of these important ECM proteins that are released

during OA. The NSAID carprofen was shown to reduce the Mascot scores of many ECM proteins at day 18 of incubation compared to IL-1β stimulated cultures. These included CILP-1 that was not detected at day 18 with carprofen + IL-1β. Fibronectin Mascot scores were also lower with carprofen treatment as described in chapter 3.3.2.2 and Table 6.

3.4.2.2. Collagens

Collagens (primarily type II) are essential for providing cartilage's tensile strength and stiffness. Explant experiments have previously shown that IL-1α initiated loss of collagen causes tensile weakness (Temple et al., 2006). Peptides originating from collagen fibres have been identified across several cartilage secretome studies using 2DE and MS (Hermansson et al., 2004, Stevens et al., 2009, Polacek et al., 2010). Collagen types II, VI and X were identified in MS based proteomic experiments in this thesis.

Collagen type II identification (Table 7) had not previously been reported by MS in equine cartilage explant supernatant (Clutterbuck et al., 2011). Collagen release is increased after longer incubation times of cartilage with pro-inflammatory stimuli, as assessed by collagen ELISA and hydroxyproline assays (Sztrolovics et al., 1999, Gabriel et al., 2010). Collagen type II identification was also made in untreated healthy cartilage explant supernatant as shown in Table 7. The half-life of collagen in cartilage is reported to be around 117 years (Verzijl et al., 2000). Release of collagen fragments from the healthy cartilage explants described in this thesis may be a result of stresses occurring during the experimental culture process. Analysis of time course B day 18 did not show increased identification of collagen peptides (Table 5) compared to day 6 samples. Collagen types VI and X were only identified in untreated cultures (Table 7), indicating that IL-1β stimulation may affect the identification of these collagen fibres. This could be due to inhibition of synthesis of collagen types VI and X, or because they are only detected in the absence of abundant proteins released in response to IL-1β. The pericellular matrix located around chondrocytes contains a high proportion of type VI collagen, helping to bind cells with the

surrounding ECM (Poole et al., 1988). Collagen type X is produced by chondrocytes and is usually found within the calcified zone of cartilage (Gannon et al., 1991). Synthesis of six fibrillar collagen types is reported to be decreased by IL-1β and TNF-α within explant culture (Stevens et al., 2009). Collagen consists mostly of proline, hydroxyproline and glycine, therefore Mascot searches with hydroxyproline as a modification were completed on some MS data (from time course A day 18 - data not shown). This identified increased Mascot scores and hits to collagen from many species including equine collagen type II,

3.4.2.3. Proteoglycans

Analysis of explant cultures shows a range of proteoglycans are released from cartilage (Steinberg et al., 1979), including small proteoglycans that are commonly detected (Polacek et al., 2010, Clutterbuck et al., 2011). In the studies described in this chapter, small leucine-rich proteoglycans like biglycan, decorin, fibromodulin, chondroadherin and prolargin were identified, as well as asporin which is not widely reported in secretome studies. Asporin inhibits TGF-β signalling in human articular cartilage, having consequences on expression of chondrogenesis and cartilage specific genes (Nakajima et al., 2007). Increased expression of an asporin polymorphism in cartilage is associated with OA (Kizawa et al., 2005).

The effect on carprofen on IL-1β stimulated explant cultures aided the search for potential ECM biomarkers of NSAID treatment efficacy such as proteoglycans biglycan, chondroadherin and fibromodulin. Decreased release of these proteoglycans was observed after MS analysis of carprofen + IL-1β samples compared to IL-1β alone after 6 days incubation, and more noticeably after 18 days of incubation. Proteoglycans including decorin, biglycan, fibromodulin, chondroadherin and prolargin, bind to collagen fibrils to support correct fibre formation and maintain the rate of assembly (Hedbom and Heinegard, 1989, Oldberg et al., 1989, Mansson et al., 2001, Bengtsson et al., 1995). These small proteoglycans continue to bind collagen fibres after formation and play an important role in cross-linking to other ECM components (Heinegard, 2009).

Lubricin (also known as proteoglycan 4) is secreted at the superficial zone of cartilage, aiding maintenance of a smooth and lubricated surface for joint movement (Schmidt et al., 2004). The link proteins that are responsible for binding together large chains of glycosylated side chains in aggrecan were also identified, namely: aggrecan core protein and HAPLN1. The proteoglycan lumican, was not identified in this study, but has been in previous secretome studies using gel fractionation prior to MS (Polacek et al., 2010). High-throughput MS used in this thesis cannot identify all proteins present in the secretome, due to the complexity of samples and the presence of high abundance proteins. Attempts of increase additional MS identifications were therefore made in chapter 4, using abundant proteins depletion approaches.

The proteoglycans identified in equine cartilage explant supernatant studies are consistent with previous cartilage secretome studies (Chapter 1, Table 1). Small proteoglycans have vital roles in the ECM constituent binding and organisation (Heinegard, 2009). Therefore it may be expected that IL-1β stimulated catabolism of the ECM would increase release of these proteoglycans. This was not observed in these studies using MS proteomic analysis. Detectable levels of small proteoglycans were continually present in untreated control cultures therefore these may be leaking out of the ECM, or being secreted straight into the supernatant.

3.4.2.4. ECM proteins modulated by stimuli

Certain collagen proteins displayed differences between untreated and IL-1β stimulated explants (Table 7). Collagen type II was identified across all explant cultures, but collagen types VI and X were only identified in untreated cultures. Chondrocytes control collagen synthesis, therefore IL-1β appears to have a negative impact on their ability to synthesize and turnover these fibrous proteins. Another ECM protein only identified in untreated controls was SPARC. SPARC has roles in calcium mineralization, signalling during development and cellular processes, and binds with collagen (Motamed, 1999).

3.4.3. Non-ECM proteins

Limited variation in ECM protein release was shown comparing untreated and IL-1β treatment, while there were more obvious alterations in secreted non-ECM proteins (Table 7). Additional insights into alterations of non-ECM secreted proteins may have been available if abundant ECM proteins did not hinder additional MS identifications. Approaches were taken in chapter 4 of this thesis to remove abundant ECM proteins in an attempt to increase secreted protein identifications.

Decreases in a MMP-3 containing band due to carprofen were shown by SDS-PAGE and silver stained images from both time course studies. MMP-3 is a catabolic protease induced during inflammation by IL-1β, with the ability to degrade collagens and proteoglycans (Shiomi et al., 2010). Activation of procollagenases MMP-1 and MMP-13 is also initiated by MMP-3 activity (Nagase and Woessner, 1999).

Serum amyloid protein (SAA) has consistently been present in untreated and cytokine treated supernatants, and is an inflammatory related component (Sipe, 1995). Studies have measured SAA in serum and synovial fluid as a diagnostic marker of inflammatory activity in degenerative joint diseases in dogs (Kjelgaard-Hansen et al., 2007). Another identified protein, YKL-40 that was detected in several IL-1β associated samples in this thesis, has been suggested as a biomarker in a range of different diseases (Prakash et al., 2013). This glycosylated protein is thought to be involved in tissue remodelling and signalling, although the precise mechanisms of its biological function require further research. Increased levels of YKL-40 in serum are associated with OA (Conrozier et al., 2000). Interestingly, increased expression of YKL-39 was recorded in OA cartilage while YKL-40 expression was not increased (Steck et al., 2002). Macrophages and synovial cells are the likely source of YKL-40, therefore providing rises of YKL-40 in OA serum (Steck et al., 2002).

3.4.4. Untreated control secretome associated secreted proteins

Proteins identified in untreated control supernatants, but not after IL-1β stimulation included: TIMP-2, SODE, PCOC-2, SFRP, CTGF, augurin, ceruloplasmin,

integrin beta-like protein 1, MIA and insulin-like growth factor-binding protein 7 (IGFBP7). Extracellular space is protected from reactive oxygen species by SODE, which catalyses breakdown of superoxide radicals into H_2O_2 and O_2. A reduced ability of chondrocytes to protect themselves from radicals could increase cell death and have negative implications on maintenance of cartilage.

PCOC-2 binds to procollagen C-terminal propeptide and aids its cleavage by bone morphogenetic protein-1 (BMP-1) into the mature collagen form, allowing incorporation into the ECM (Steiglitz et al., 2002). New cartilage is processed into its final fibrillar form to provide the ECM with strength and stability, therefore IL-1β may be interfering with collagen turnover.

Another protein only identified in untreated controls was SFRP, a modulator of Wnt signalling pathways. SFRP inhibits Wnt activation through blockage of frizzled receptors, therefore stopping activation of this signalling pathway (Luyten et al., 2009). This has implications on cell development, migration and proliferation (Luyten et al., 2009). IL-1β interruption of ordinary cellular signalling through decreased release of SFRP and other signalling factors such as CTGF, MIA and IGFBP7 will affect chondrocytes ability to maintain healthy cartilage.

3.4.5. IL-1β stimulated secretome associated secreted proteins

Certain important catabolic proteins in OA associated cartilage degradation including ADAMTS and cathepsin family members (Troeberg and Nagase, 2012) were not detected in the IL-1β stimulated explant secretome. However, release of several additional proteins was initiated by IL-1β stimulation of cartilage explants. Catabolic proteases MMP-1 and MMP-13 were only identified with IL-1β stimulation, while Mascot scores for MMP-3 were noticeably higher. Further in depth western blot analysis of MMP-1, MMP-3, and MMP-13 release in this explant model is detailed in chapter 5 of this thesis. Secretion of the pro-inflammatory cytokine MIF, was also detected with IL-1β stimulation. This cytokine can increase expression of MMP-1 and MMP-3 in synovial fibroblasts (Onodera et al., 2000). Increasingly higher levels of serum and synovial MIF are observed in OA knee patients compared to normal

controls (Liu and Hu, 2012). The chaperone HSP70, calcium binding protein S-100 protein A-1, semaphorin-3C and chemokine CXCL6 were identified in IL-1β stimulated samples. The identifications of IL-1β associated inflammatory proteins in these studies, has demonstrated the utility of the cartilage explant model in combination with high-throughput MS. These approaches have identified inflammatory related proteins as potential biomarkers including MMP-1, MMP-3, MMP-13, MIF, HSP70 and CXCL6, which could be applied to monitor anti-inflammatory effects.

3.4.6. Effects of carprofen on non-ECM secreted proteins

Carprofen also affected release of several IL-1β stimulated non-ECM proteins, which included reducing the Mascot scores of MMP-3 and clusterin at later stages of the time course. MMP-13, YKL-40 and lysozyme C were not observed after 18 days when carprofen was present. Interestingly, CPAMD8 (C3 and PZP-like, alpha-2-macroglobulin domain containing 8) was only detected after 18 days of incubation with carprofen + IL-1β. This secreted complement protein is shown to bind heparin to become membrane associated (Li et al., 2004). CPAMD8 has likely functions in innate immunity like other complement family proteins (Li et al., 2004). Complement family proteins in synovial fluid have been associated with OA in a number of studies (chapter 1.6.6.), therefore further studies on CPAMD8 may provide insights into OA processes.

3.4.7. Intracellular protein release from cartilage explants

Identification of intracellular proteins increased with IL-1β stimulation, compared to untreated cultures. IL-1β is known to cause cell death therefore increased death/lysis of chondrocytes will release proteins expected to be located in the nucleus and cytoplasm (Caramés et al., 2008). Western blotting of β-actin was used as an indirect assessment of levels of chondrocyte lysis in these explant culture studies (data described in appendix 1). β-actin release was only detected in cultures with treatments containing IL-1β after 6 days of incubation, while carprofen treatment alone did not cause an increase in β-actin release. Release of another intracellular protein, lactate dehydrogenase (LDH) can also be used as a measure of cytotoxicity.

LDH measurements on explant cultures treated with IL-1β showed increased LDH release with cytokine stimulation, which was not significantly altered by carprofen treatment (data not shown).

The cytoplasmic proteins vimentin and enolase are often observed during proteomic investigations and tend not to be specific to any particular disease or species (Wang et al., 2009b, Petrak et al., 2008). Enolase has diverse biological roles and is suggested to be a marker of pathological stress in several diseases (Diaz-Ramos et al., 2012). Due to the high sensitivity of MS approaches, proteins released due to minor levels of cell death may be identified. Indeed, the presence of intracellular proteins has been reported in other proteomic studies of cartilage conditioned media (Wilson et al., 2008, Stevens et al., 2008, Polacek et al., 2010, Clutterbuck et al., 2011). Small amounts of cell lysis may release sufficient intracellular proteins to hinder MS identification of some low level secreted proteins. Commonly identified intracellular proteins include metabolic enzymes such as pyruvate kinase and phosphoglycerate kinase, which were identified in this chapter and the studies mentioned above.

The MS analysis of the day 18 IL-1β stimulated explant supernatant revealed the presence of histone proteins. Histone aids formation of nucleosomes that enable DNA to be compacted within chromosomes. Release of histones specifically at this time point could indicate that prolonged IL-1β exposure causes release of these proteins. Another nuclear protein found in the explant cultures was transcription termination factor 1. Comparison of the data in Table 5 and Table 6, shows IL-1β and carprofen + IL-1β samples identified twenty-nine and six intracellular proteins respectively. Since carprofen decreased the amounts of cytoplasmic and nucleus associated proteins, it may have the effect of reducing IL-1β stimulated cell death.

3.4.8. Changes in protein release at different time points

There were advantages to high-throughput analysis at both early and late time points. Only IL-1β treated cultures without the addition of carprofen showed the presence of YKL-40 and PCOC-2, and only at the day 6 in time course B. Both of

these proteins are of substantial interest to cartilage turnover and metabolism, therefore may represent key features to detect and monitor changes in the secretome under different experimental conditions.

At day 18 of time course B with IL-1β stimulation, additional identifications were made of two chemokines: C-x-C motif chemokine 6 (CXCL6) and C-C motif chemokine 20 (CCL20), and two blood coagulation related proteins: Annexin A5 and coagulation factor XIII A chain.

The CXCL6 protein was not identified at early time points during time course B comparison analysis, but was subsequently identified after 6 days of IL-1β incubation in study 3.3.3. (Table 7). CXCL6 is a chemokine known to attract neutrophil granulocytes to sites of inflammation (Proost et al., 1993). CCL20 (also known as macrophage inhibitory protein-3A) is strongly chemotactic to lymphocytes. CCL20 has a weaker attraction for neutrophils (Hieshima et al., 1997). The presence of chemokines at this stage of the cultures may represent increased release of chemokines later in the time course as inflammatory processes progress, or an enhanced ability to identify these by MS at 18 days of culture.

The release of proteoglycans (decorin, biglycan, lumican, fibromodulin) from IL-1β stimulated explants over a 14 day time course has previously been assessed by western blotting (Sztrolovics et al., 1999). Only fibromodulin showed increased degradation product release as time progressed (Sztrolovics et al., 1999). Extended time courses may therefore not produce alterations in release of most ECM proteins. This is consistent with continued identification of proteoglycans and ECM components in high-throughput MS analysis at day 18 described in this chapter (Table 5 and Table 6).

3.4.9. IL-1β and carprofen affects GAG release throughout the time course

Stimulation with IL-1β causes higher levels of MMPs and other proteases to be activated (Busschers et al., 2010). Subsequently this initiates increased release of GAGs and proteoglycans, which can be measured by DMMB assays (Figure 18). The

COX-2 specific inhibition by carprofen reduces bioactive lipid signalling of PGE_2, thereby decreasing levels of catabolic MMPs, which has effects on cartilage degeneration (Goodrich and Nixon, 2006). In the present study, carprofen significantly reduced IL-1β associated GAG release up to six days (Figure 18). GAG release was not halted during the entire time course as seen at day 12, but carprofen did delay loss of GAGs. Measurements of GAG content in explant supernatant at 18 and 24 days, showed that most of the inducible GAG loss occurred in the first 12 days. Another time course study of IL-1β stimulated explants, showed the majority of GAG release occurred within 4 days over a 14 day experiment (Sztrolovics et al., 1999). Previous studies on equine cartilage stimulated with IL-1β indicated increased levels of GAG release over 5 days, compared to untreated control explants, whilst carprofen was reported to reduce IL-1β induced GAG release (Clutterbuck et al., 2009, Clutterbuck et al., 2013). Treatment of canine chondrocytes in 3D agarose culture with IL-1β and carprofen demonstrates IL-1β stimulated GAG release was lower with carprofen up to 6 days (Dvorak et al., 2002), which agrees with findings in this thesis. However, a study investigating another NSAID meloxicam on OA affected canine explants, showed it did not cause a decrease in GAG or collagen release (Budsberg et al., 2013).

Other aggrecanases not affected by carprofen also contribute towards GAG release. The ADAMTS enzymes are responsible for the majority of aggrecan, GAG and proteoglycan degradation (Sandy and Verscharen, 2001). Therefore while carprofen blocks one route of GAG breakdown via MMP inhibition, ADAMTS continue to degrade the ECM. A study has shown, inhibition of ADAMTS-4 and ADAMTS-5 prevented aggrecan degradation in human OA cartilage explants (Malfait et al., 2002). ADAMTS presence was not detected within this explant model, therefore no assessment was made on its release due to IL-1β or carprofen. The study in this chapter showed that although IL-1β stimulated GAG release was reduced by carprofen, it did not completely inhibit all mechanisms of GAG release. This is likely to be due to continued activity of ADAMTS that were not affected by carprofen.

3.4.10. Conclusions

The studies in this chapter have developed understanding of this equine articular cartilage explant model and explored changes in protein release throughout a time course of incubation. It was hypothesised that high-throughput MS analysis would show differences in untreated control, IL-1β and carprofen treated explant cultures. This aimed to identify biomarkers of treatment efficacy that could be monitored in this model to screen anti-inflammatory compounds such as carprofen.

This chapter has found that NSAID carprofen has various effects on the release of IL-1β stimulated proteins. Carprofen decreased release of ECM proteins including biglycan, fibromodulin, chondroadherin, CILP and fibronectin. DMMB assays also showed that IL-1β stimulated GAG release was decreased when carprofen was present. Inhibition of proteoglycan synthesis by carprofen could potentially account for the reduced release observed, although carprofen has been shown to be capable of increasing proteoglycan synthesis by measuring incorporation of $^{35}SO_4$ into equine cartilage explants (Frean et al., 1999). A similar approach could be applied to assess proteoglycan synthesis in the experimental culture conditions utilised in this thesis. Various NSAIDs effect proteoglycan synthesis in different ways, either increasing proteoglycan synthesis, causing no change or inhibiting synthesis (Dingle, 1999, Mastbergen et al., 2006). Celecoxib, a COX-2 specific inhibitor (as is carprofen), increased proteoglycan synthesis *in vitro* (Mastbergen et al., 2006). Catabolic proteins stimulated by IL-1β such as MMP-1, MMP-3 and MMP-13 were reduced by carprofen. The number of intracellular proteins identified by MS on cultures stimulated with IL-1β were lowered due to carprofen treatment, suggesting this NSAID reduced chondrocyte death.

A number of candidate biomarkers were released in response to IL-1β. These included MMP-1, MMP-3, MMP-13, MIF, HSP70 and CXCL6. As carprofen decreased release of the following matrix proteins, potential ECM biomarkers of treatment efficacy were identified including proteoglycans (biglycan, fibromodulin and chondroadherin) and CILP were identified by high-throughput MS. Selected candidate

proteins including MMPs, TSP and fibronectin, were further studied in chapter 5 to assess their capability to be biomarkers in this model. These may help in the screening of anti-inflammatory effects of novel treatments in the equine cartilage *in vitro* model.

High-throughput MS analysis reported that few additional proteins were uncovered by MS analysis at 18 days of incubation as compared with 6 days, as the majority of identifications continued to be ECM constituents. The day 18 cultures were examined to determine whether extending the time of culture would produce samples with reduced levels of ECM proteins, since the leaking out of these proteins from cut cartilage discs may be expected to reduce over time. Cultures containing lower levels of the abundant ECM components would aid high-throughput MS identification of additional secreted proteins involved in cartilage degradation and inflammatory processes. Silver stained profiles of later time points and MS identification of excised bands showed proteins present were mostly still ECM proteins including COMP, fibronectin and aggrecan core protein. Additional protein identifications were insufficient in number to support modifying the culture model to include MS analysis of later incubation time points. In addition, only limited information was gained by DMMB assay analysis at later time points, since explant GAG measurements were influenced by release during earlier incubation. Incubating cartilage cultures over longer periods of time also required additional costs of resources and time that did not warrant this approach in subsequent secretome studies. An incubation time of 6 days was therefore chosen for the next studies in this thesis, which involved abundant protein depletion approaches prior to high-throughput MS analysis detailed in chapter 4.

Overall, the series of studies described in this chapter identified physiologically relevant cartilage proteins released into untreated, IL-1β and IL-1β + carprofen treated explant cultures. Proteins identified provided insights into ECM components released during cartilage degradation and the secreted proteins involved in inflammatory related degradative diseases like OA.

CHAPTER 4

UNCOVERING ADDITIONAL PROTEINS RELEASED FROM ARTICULAR CARTILAGE BY DEPLETION OF HIGH ABUNDANCE PROTEINS

4.1. Introduction

High-throughput MS analysis described in chapter 3 previously identified a range of abundantly released ECM and non-ECM secreted proteins in an equine cartilage explant model. It was hypothesised that depletion of the highly abundant cartilage proteins would allow additional identification of low level proteins and a further in depth analysis of the secretome. In chapter 3, non-ECM proteins including clusterin, MMP-1, MMP-3, MMP-13, SAA and MIF were released in response to IL-1β, which have been implicated in inflammatory processes and cartilage degradation in OA (Kjelgaard-Hansen et al., 2007, Fukuda et al., 2012, Liu and Hu, 2012, Troeberg and Nagase, 2012). Low abundance proteins previously unidentified in the cartilage secretome could be of interest in cartilage biology and also candidate biomarkers of treatment efficacy, allowing greater understanding of cartilage degradative processes and OA disease. The aim of this chapter was to identify additional secreted proteins associated with IL-1β stimulation and inflammatory processes occurring in cartilage explant culture.

Analysis of complex protein samples with a large dynamic range of protein levels by high throughput mass spectrometry approaches provides reliable identifications of high abundance components, but identification of low abundance proteins that could provide additional biological information is hindered by the high signal levels from the abundant proteins (Millioni et al., 2011). Therefore, depleting the secretome of high abundance proteins could provide additional protein identifications of lower abundance proteins by MS analysis. In this chapter, three different approaches to deplete the high abundance proteins present in cartilage explant culture fluids were investigated. In two of these, the characteristic of glycosylation of the abundant ECM proteins was exploited. Abundant ECM proteins are highly

glycosylated therefore this property can be targeted by cetylpyridinium chloride (CPC) precipitation (Hermansson et al., 2007) and concanavalin A (Con A) lectin chromatography depletion methods. Another potential technique to deplete high abundance proteins for high-throughput proteomics approach is Proteominer™ protein enrichment technology (Fernandez et al., 2011). This approach involves application of samples to a diverse bead-based ligand library, which subsequently reduces the dynamic range of complex protein samples.

4.1.1. GAG depletion with CPC precipitation

CPC is a cationic detergent that interacts with negatively charged groups inherent in glycosylated ECM proteins and GAGs. This approach has previously been used to increase resolution during 2DE proteomics of cartilage tissues because GAGs and proteoglycans interfere with the separation and isoelectric focusing of other proteins in gels (Catterall et al., 2006, Ruiz-Romero et al., 2010a, Hermansson et al., 2007, Hermansson et al., 2004). Indeed, a comparison of proteins released from healthy or OA cartilage found it necessary to remove proteoglycans with CPC to allow resolution by 2DE (Hermansson et al., 2004). The same study showed increases in collagen type II and inhibin βA release from OA cartilage (Hermansson et al., 2004). In 2006, a study aimed to increase resolution of higher molecular weight proteins during 2DE used CPC precipitation, enzymatic digestion or anion exchange techniques on proteins secreted from human chondrocytes stimulated with IL-1β and oncostatin M (Catterall et al., 2006). On that occasion, only eight proteins were identified from 2DE gels without CPC precipitation including MMP-1, MMP-3, YKL-40 and S100A11 (Catterall et al., 2006). Although CPC precipitation produced reduced protein smearing during 2DE, high losses of biologically relevant proteins and insufficient high molecular weight resolution was reported (Catterall et al., 2006). In another study, the ability to distinguish and identify individual spots with MS from 2DE based investigations on cartilage extracts was improved using CPC precipitation (Ruiz-Romero et al., 2010a). Proteins identified after CPC precipitation included collagen type VI, COMP, fibromodulin and ferritin light chain (Ruiz-Romero et al.,

2010a). Application of CPC precipitation prior to high throughput MS analysis of cartilage explant secretome has not been reported in the literature. Therefore the investigations described in this chapter evaluated if this approach would uncover additional low abundance proteins with high-throughput MS.

4.1.2. Glycoprotein depletion with concanavalin A lectin chromatography

Lectin chromatography techniques are used to selectively bind glycosylated proteins. Specific lectins are chosen that bind specific types of carbohydrate structures for example, galactose, mannose, N-acetylglucosamine or fructose residues. A multi-lectin affinity chromatography (M-LAC) approach has been developed that can bind a wide range of side chains types. Plasma has often been processed with M-LAC to assist analysis of the plasma proteome (Plavina et al., 2007, Kullolli et al., 2010, Dayarathna et al., 2008), and in searches for biomarkers of diseases like RA (Zheng et al., 2009) and breast cancer (Zeng et al., 2011). In 2009, the RA human plasma peptidome and proteome were studied after M-LAC depletion via tryptic digestion and nanoLC-MS/MS (Zheng et al., 2009). Calgranulin A, B and C were identified only in M-LAC bound RA fractions, while C-reactive protein and actin were associated with RA plasma both with and without M-LAC depletion (Zheng et al., 2009).

The lectin Con A is derived from the jack bean (*canavalia ensiformis*). Con A has a high affinity for α-D-mannosyl and α-D-glucosyl residues (Bryce et al., 2001) found throughout ECM GAGs and proteoglycans. In a model of chondrocyte maturation, early stage chondrogenesis differentiation markers have recently been reported after Con A chromatography and MS (Ishihara et al., 2013). In that study, glycoproteins were fractionated from lysate of mouse chondroprogenitor cells that had been matured by insulin presence (Ishihara et al., 2013). Selected glycoproteins from MS identification in glycosylated fractions were then subjected to gene expression analysis in human MSCs, to assess the relevance of applying chondroprogenitor cell markers to a human chondrogenesis model. Transcription of collagen type III, collagen type XI, aquaporin-1, netrin receptor unc-5 homolog B and ectonucleotide

pyrophosphatase/phosphodiesterase family member 1 were shown to be up-regulated during early chondrogenesis (Ishihara et al., 2013). In this chapter, Con A lectin chromatography was applied to deplete explant culture media of high abundance proteins and produce a glycoprotein depleted fraction for high-throughput MS analysis.

4.1.3. Proteominer™ technology to reduce the dynamic range of secretome

Proteominer™ protein enrichment kits (Bio-Rad) were developed to decrease the dynamic range of complex biological samples, thereby allowing improved analytical coverage of the proteome (Figure 19). The principle involves applying complex protein samples to a diverse bead-based combinatorial peptide ligand library. High abundance proteins will saturate their available binding ligands. Proteins of lower abundance are concentrated (relative to high abundance proteins) on their high affinity ligands. Excess proteins are washed away and therefore once proteins are eluted off the ligand beads, there will be a reduction in the dynamic range of all proteins. Proteominer™ technology has been reported to assist the proteomic analysis of whole OA cartilage (Ikeda et al., 2013), serum (Fernandez et al., 2011), cell lysates (Fonslow et al., 2011), urine (Candiano et al., 2012), egg white extract (D'Ambrosio et al., 2008) and beer (Fasoli et al., 2010). To discover new biomarkers for OA, Proteominer™ technology has been applied to healthy and OA serum before 2DE, which showed OA serum to be associated with an altered haptoglobin (Fernandez-Costa et al., 2012). In another study searching for OA biomarkers in whole cartilage, the use of Proteominer™ depletion before iTRAQ™ quantification revealed three novel candidate OA biomarkers: leukocyte cell-derived chemotaxin-2 (LETC2), BAALC (brain and acute leukaemia, cytoplasmic) and peroxiredoxin-6 (PRDX6) (Ikeda et al., 2013). The Proteominer™ technology was assessed as an approach to deplete the cartilage explant secretome of high abundance proteins, and therefore potentially uncover additional protein identifications using high-throughput MS.

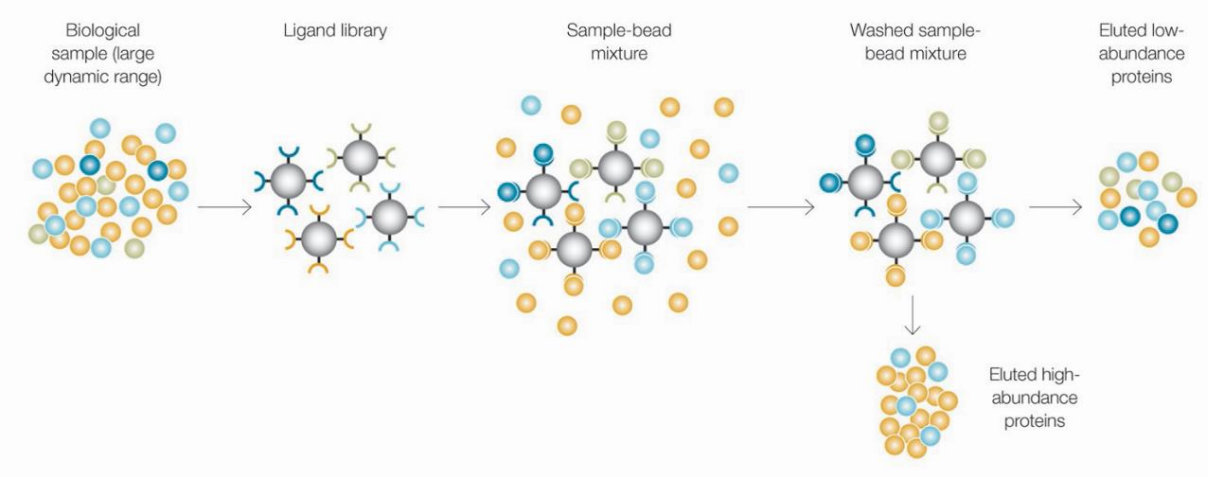


Figure 19. Proteominer™ protein enrichment technology decreases the dynamic range of proteins in biological samples, allowing increased identification of low abundance proteins

(http://www.bio-rad.com/en-uk/product/proteominer-protein-enrichment-kits)

4.1.4. Hypothesis and aims

High-throughput MS on cartilage explant conditioned media was studied within Chapter 3. Released proteins were identified including ECM components, secreted metabolic and intracellular proteins. This data provided a comprehensive foundation to assess the capabilities of using the previously described high-throughput MS approach (chapter 3), but extra steps need to be implemented into experimental procedures for analysis to aid identification of additional proteins in the explant model. The hypothesis addressed in the work of this chapter was that by depleting the cartilage explant supernatant of highly abundant ECM proteins, the detection of additional low-level proteins may be achieved. Therefore CPC precipitation, Con A lectin chromatography or Proteominer™ enrichment, were carried out before high-throughput MS techniques were applied with a view to expand the range of proteins identified within the explant culture secretome. This aimed to discover if any of these procedures prior to high-throughput MS would allow further identification of low abundance proteins.

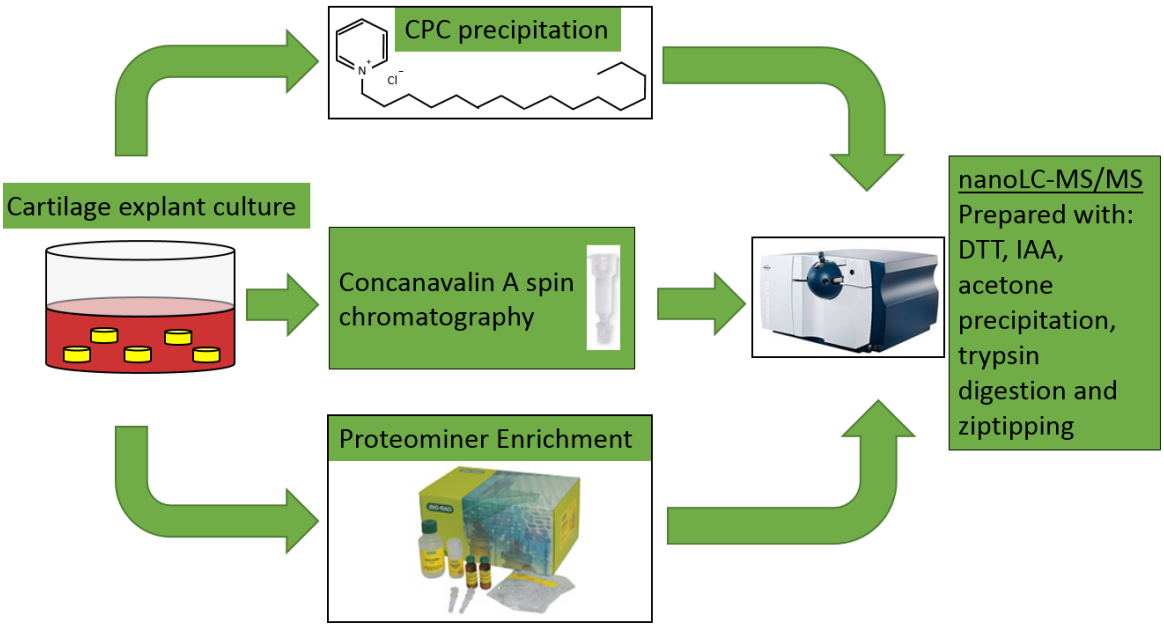


Figure 20. Methods to deplete high-abundance proteins trialled in Chapter 4

Three methods were applied to cartilage secretome prior to high-throughput MS: CPC precipitation, Con A chromatography or Proteominer™ enrichment.

4.2. Materials and Methods

4.2.1. CPC precipitation

CPC precipitation was based on the approach developed by (Hermansson et al., 2007) for 2DE analysis of cartilage secretome. A stock solution of 5% CPC (Sigma) was prepared in HPLC grade water (Fisher), and appropriate volumes were added to samples to give a ratio of 3 mg CPC/mg GAG. Typically, samples contained between 100-400 µg/ml GAG as estimated by DMMB assays (Appendix 2, Figure 36). Samples with CPC were incubated at room temperature for 30 minutes to precipitate the GAG/CPC complex, before centrifugation at 13,000xg for 10 minutes. The GAG depleted supernatant was transferred to a fresh Eppendorf tube. To resuspend the proteins in the CPC pellet, it was first washed with 0.4 M sodium acetate (Sigma) in 90% (v/v) ethanol (Fisher) and then washed with ethanol alone (to remove sodium acetate). It was then resuspended in Laemmli buffer for SDS-PAGE or AMBIC (+10% ACN) for MS analysis processing.

4.2.1.1. Preliminary CPC precipitation mass spectrometry

During preliminary investigations, 1 ml of an untreated and an IL-1β treated explant culture supernatant sample (from time course A, day 21-24 (chapter 3)) were CPC precipitated. The CPC/GAG pellet was resuspended in 50 µl AMBIC (+10% ACN). The GAG depleted samples (350 µl of each supernatant) were taken for the next stages.

A methanol/chloroform precipitation method was applied to remove salt and detergent. Per 100 µl of sample, 400 µl of methanol (Fisher), 200 µl chloroform (Fisher) and 300 µl of H_20 were added. Samples were centrifuged for 1 minute at 14,000xg, before the top aqueous layer was removed and an additional 300 µl methanol was added. Again, samples were centrifuged at 14,000xg for 5 minutes, before as much methanol as possible was removed without disturbing the pellet. Pellets were briefly allowed to air dry and then resuspended in 20 µl AMBIC (+10% ACN).

Samples were prepared for MS runs with reduction, alkylation and acetone precipitation as described in Chapter 2.8.1. Prior to loading onto the column, 10 µl of these samples were diluted 1:10 in 0.1% formic acid and was loaded and analysed by nanoLC-MS/MS on an amaZon ETD (Bruker).

4.2.1.2. Final CPC precipitation mass spectrometry

CPC/GAG depletion was carried out (chapter 4.2.1.) on IL-1β stimulated explant conditioned media that had been incubated for 6 days. This time, free CPC and salts were removed using dialysis overnight against 20 mM Tris-HCl (pH 7.4) (slide-A-Lyzer, 10 kDa cut off, Pierce). The GAG depleted sample and an unprocessed aliquot of the same culture supernatant (also dialysed) were reduced, alkylated, acetone precipitated and trypsin digested as previously described in Chapter 2.8.1. After digestion, the samples were desalted and concentrated using C18 resin ZipTips (Millipore) according to the manufacturer's instructions and using 20 µl 50% methanol and 0.1% formic acid to elute the bound peptides. Excess solvent was evaporated off by heating at 70 °C to leave 10 µl of samples, which was transferred to glass vials ready to be loaded onto the nanoLC column. During this analysis, 5 µl was loaded onto the column for nanoLC-MS/MS analysis on an amaZon speed ETD (Bruker).

4.2.2. Con A lectin chromatography

4.2.2.1. Con A column fractionation method

Binding buffer was prepared with 20 mM Tris (Bio-Rad)-HCl pH 7.4 + 0.5 M NaCl (Sigma) + 1 mM $MnCl_2$ (Sigma) + 1 mM $CaCl_2$ (Sigma). Concanavalin A (Con A) (GE Healthcare) was prepared into a slurry by washing in 10 bed volumes of binding buffer to remove preservative. This was centrifuged for 2 minutes at 8000xg in a 50 ml falcon tube before excess buffer was removed. An equal amount of fresh binding buffer was added to the settled medium (i.e. 1 ml settled medium + 1 ml binding buffer), which was allowed to equilibrate to room temperature and degassed. Prepared Con A slurry was placed in Micro Bio-Spin® Chromatography Columns (Bio-Rad) (0.5 ml of slurry per column), centrifuged at 1,000xg for 2 minutes and excess

binding buffer was removed. Samples (200 µl) were added to the centre of the column and centrifuged at 1000xg for 4 minutes. Bound proteins were recovered by introducing 0.5 M methyl α-D-mannopyranoside (200 µl) to the column before centrifugation at 1000xg for 4 minutes.

4.2.2.2. Con A MS analysis

To ascertain if the Con A approach would provide additional protein identifications, selected samples were analysed by MS before and after lectin chromatography. Untreated and IL-1β treated explant culture media (after 6 days of incubation) from an individual horse and a pool of three separate horses went through the Con A column fractionation. Dialysis against 18 ohm water was performed overnight on samples using a Tube-O-Dialyzer MWCO 1000 Da (G-Biosciences). Corresponding unprocessed samples were also MS analysed for comparison with the Con A technique. Reduction, alkylation, acetone precipitation, trypsin digestion and C18 ZipTip desalting were carried out as previously described (chapter 2.8.1.), before nanoLC-MS/MS analysis on an amaZon speed ETD.

4.2.3. Proteominer™ protein enrichment

Explant cultures were set up (chapter 2.3.) with 8 explant discs per 1 ml DMEM + 2% Pen/Strep, with the aim of collecting highly concentrated secretome supernatants. Explants were either left untreated (12 wells) or treated with 10 ng/ml IL-1β (12 wells). After 6 days incubation, supernatants for each treatment were concentrated from 12 ml down to ~0.5 ml using Centriplus YM-3 (Millipore) and Centricon-3 (Millipore) centrifugal filter units. The concentrated samples (500 µl) were processed with a Small Capacity Proteominer™ Protein Enrichment Kit (Bio-Rad) following the manufacturer's instructions. After elution, 120 µl was recovered, from which 20 µl was protein assayed (DC Bio-Rad) to allow trypsin to be added at an appropriate amount during later digestion. The remaining 100 µl was reduced, alkylated, acetone precipitated and trypsin digested overnight, before digested peptides were applied to C18 spin columns (Pierce), followed by nanoLC-MS/MS analysis on an amaZon speed ETD (Bruker) (chapter 2.8).

4.2.4. MS data processing

MS data was processed as described in chapter 2.8.3.

4.3. Results

4.3.1. CPC Precipitation

Fractionation using CPC precipitation provided separation of components of the cartilage explant secretome which could be visualized by SDS-PAGE and silver staining (Figure 21). Some of the most strongly staining bands corresponding to abundant proteins were effectively located in the CPC pellets. These abundant proteins were depleted in both untreated control and IL-1β treated explant supernatants. In untreated control supernatants, the majority of protein bands were removed by CPC precipitation, suggesting that most proteins released from untreated explants are ECM proteins containing negatively charged side groups. A greater number of proteins remained in IL-1β treatment supernatants after CPC precipitation compared to untreated control samples, including bands known to contain MMP-3 and COMP. Although some of the abundant proteins remain in IL-1β GAG depleted samples, it was anticipated that removal of the majority of abundant proteins would allow identification of additional proteins by nanoLC-MS/MS in the next phase of the assessment of the CPC precipitation technique.

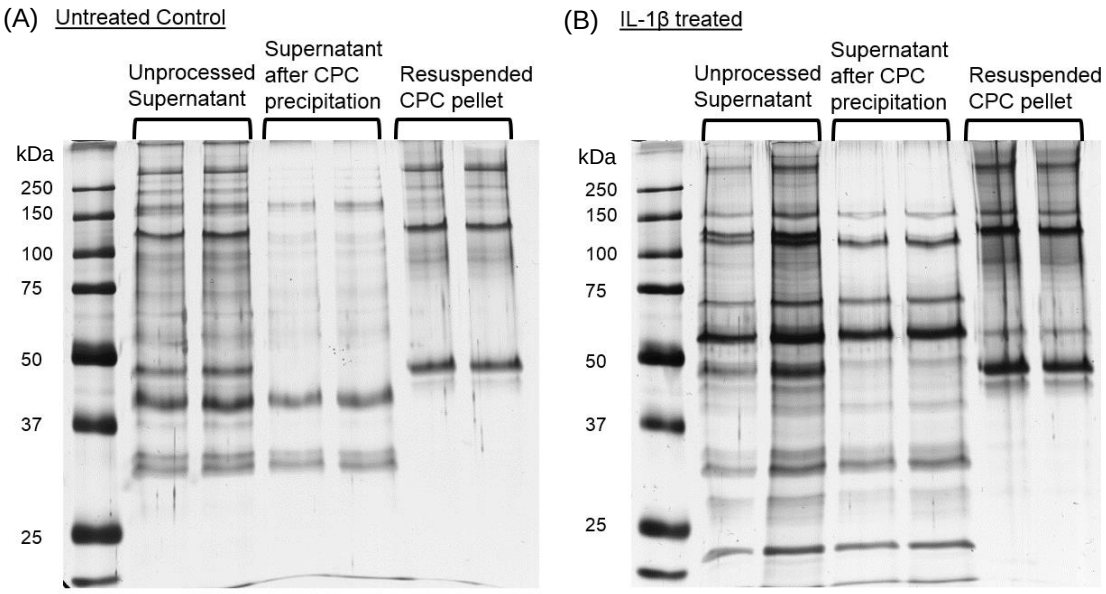


Figure 21. One dimensional gel protein profiles of untreated control (A) and IL-1β treated (B) cartilage explant supernatants showing the protein fractionation achieved by the CPC precipitation

Unprocessed supernatant shows profile before CPC precipitation. The supernatant profiles after CPC precipitation (GAG depleted) are visible along with profiles of proteins contained within resuspended CPC pellets that had been extracted during the process. All samples were run in duplicate.

4.3.1.1. Predominantly ECM proteins are identified in resuspended CPC pellet

Preliminary MS analysis of GAG depleted supernatant by CPC precipitation was unsuccessful, most likely because the protein amount recovered after trypsin digestion of samples was very low. Figure 22 and Table 9 show the proteins identified in the high-throughput MS of proteins present in the CPC pellets from untreated controls and IL-1β explant culture supernatants. ECM proteins including COMP, fibronectin and small proteoglycans decorin and biglycan, were identified in the pellets from both treatment types. Aggrecan core protein and HAPLN1 which form links within large aggregating proteoglycans in the ECM were also detected in both treatment pellets. In addition, the predominant collagen type in articular cartilage (type II) was present in the IL-1β pellet. Identification of extra ECM proteins in the IL-1β samples could be due to increased release of these proteins after cytokine stimulated catabolic processes, as the protease MMP-3 was also found. Almost all the proteins in the pellets were ECM constituents that are released at high levels during cartilage explant culture. This suggested that CPC precipitation could be an effective method to deplete high abundance proteins from explant culture supernatants.

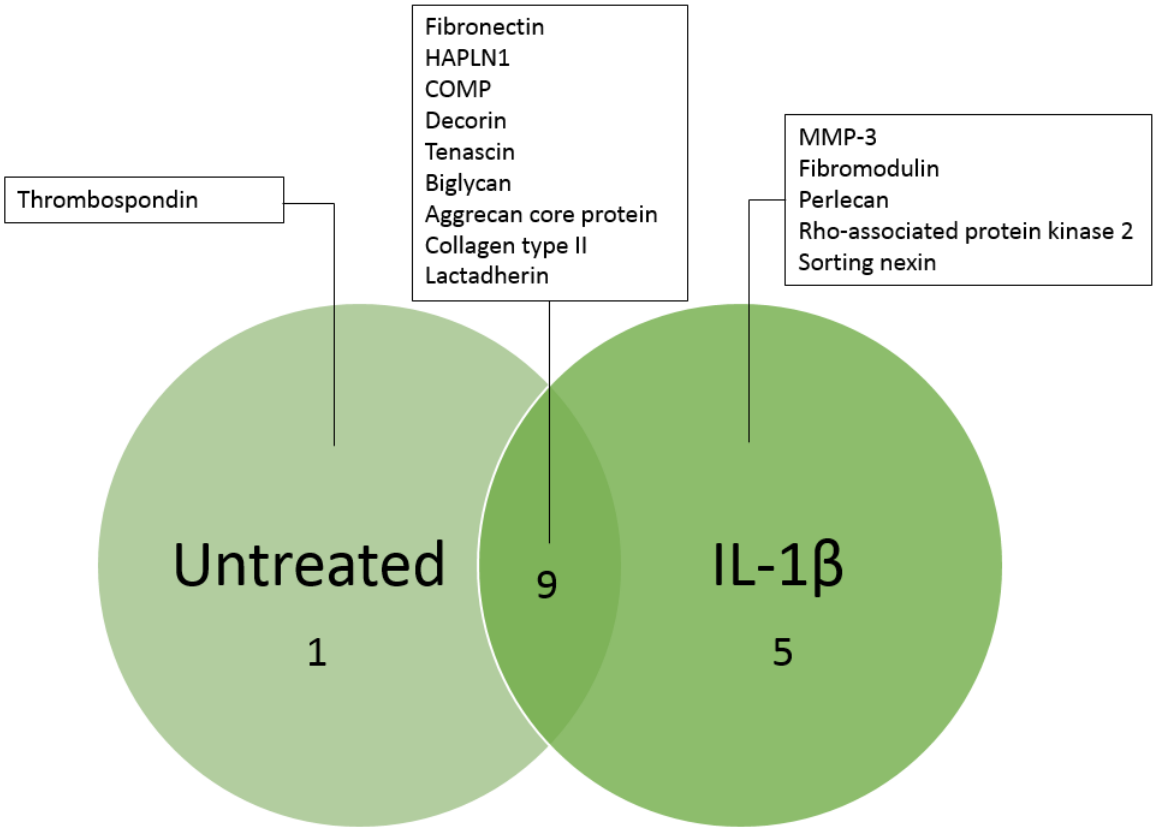


Figure 22. Venn diagram showing proteins identified in CPC precipitated pellets from untreated control and IL-1β cartilage explant supernatant

Details of protein identifications made by high-throughput MS are displayed in Table 9.

Table 9. Protein identifications from CPC precipitated pellets from untreated control and IL-1β cartilage explant supernatants

Pellets were resuspended after CPC precipitation and prepared for MS analysis. Swiss-Prot 2013_01 (all mammalian entries) database search performed using Mascot search engine. Only Mascot scores >39 indicating identity/extensive homology are reported.

Protein	Accession	Mascot Score	Unique Peptides	Sequence Coverage (%)
Untreated				
Fibronectin	FINC_BOVIN	1467	26	12
HAPLN1	HPLN1_HORSE	495	12	30
COMP	COMP_MOUSE	233	6	10
Decorin	PGS2_HORSE	224	15	43
Thrombospondin	TSP1_BOVIN	199	18	17
Tenascin	TENA_HUMAN	79	4	6
Biglycan	PGS1_HORSE	70	7	19
Aggrecan core protein	PGCA_CANFA	65	8	3
Collagen type II	COA1_BOVIN	62	12	9
Lactadherin	MFGM_PIG	48	3	8
IL-1β				
Fibronectin	FINC_BOVIN	1408	29	4
Biglycan	PGS1_HORSE	516	7	20
HAPLN1	HPLN1_HORSE	374	13	
Decorin	PGS2_HORSE	118	14	26
MMP-3	MMP3_HORSE	246	7	4
Tenascin	TENA_HUMAN	108	16	9
COMP	COMP_BOVIN	105	6	7
Collagen type II	COA1_BOVIN	92	9	8
Fibromodulin	FMOD_HUMAN	76	2	6
Lactadherin	MFGM_PIG	60	4	10
Aggrecan core protein	PGCA_PIG	56	8	11
Perlecan	PGBM_HUMAN	46	12	3
Rho-associated protein kinase 2	ROCK2_MOUSE	41	6	5
Sorting nexin 2	SNX2_BOVIN	41	5	12

4.3.1.2. Depletion of GAG from the secretome by CPC precipitation

A further CPC precipitation experiment was attempted on IL-1β stimulated supernatant after alterations to technical aspects of the preparation methods and in selection of appropriate samples. High-throughput MS analysis was completed on IL-1β treated explant supernatant from an individual horse before (unprocessed) and on the supernatant fraction from the CPC precipitation (GAG depleted), with the identifications shown in Table 10. Overall, 39 proteins were identified in the unprocessed sample and 36 unbound proteins were detected in the GAG depleted sample. Although not truly quantitative, clear differences in Mascot scores can give indications on proteins affected by CPC precipitation. Small proteoglycans decorin and fibromodulin were not identified after CPC precipitation, while biglycan gave a considerably lower score, suggesting these were successfully removed. Thrombospondin-1 and aggrecan core protein also gave higher scores before the depletion step was applied. Two ECM proteins appeared to be enriched after the process: HAPLN1 and CILP-1.

It was hypothesised that removal of abundant ECM proteins with CPC precipitation would facilitate identification of additional secreted proteins involved in cartilage turnover and maintenance in the unbound fraction, but large numbers of additional proteins were not found. Proteins with roles in cartilage breakdown and inflammation were lost: MMP-13 and macrophage migration inhibitory factor (MIF), while MMP-10 and HAPLN1 were detected in unbound samples after CPC precipitation. The overlap of ECM and secreted protein detected in unprocessed samples or unbound fractions after CPC precipitation are displayed in Figure 23.

The intracellular proteins that were detected are consistent with chondrocyte cell death caused by IL-1β cytokine (Table 10). A number of these proteins were only identified in either the unprocessed sample or in the GAG depleted sample after CPC precipitation. The mitochondrial form of SOD and ferritin heavy chain protein detection was aided by precipitation, while scores for alpha-enolase and triosephosphate isomerase were considerably higher. Phosphoglycerate kinase 1, L-lactate

dehydrogenase A chain and sodium/hydrogen exchanger 2 were identified only in unprocessed supernatants.

Table 10. Proteins detected in IL-1β treated explant supernatants with or without CPC precipitation

Identifications included in the table only if the protein was found above identity cut-off in either unprocessed or CPC precipitated sample. Swiss-Prot 2013_01 database (all mammalian entries) searched with Mascot Daemon software. Identification cut-off determined by Mascot scores >42 indicates identity/extensive homology.

Protein	**Accession**	**Unprocessed Supernatant**		**Supernatant after CPC Precipitation**	
		Mascot score	Unique peptides	Mascot score	Unique peptides
ECM Proteins					
COMP	COMP_BOVIN	18133	17	13737	18
Aggrecan core protein	PGCA_HUMAN	5791	14	785	9
Fibronectin	FINC_HUMAN	2810	32	4474	34
Decorin	PGS2_HORSE	2063	8		
Biglycan	PGS1_HORSE	5766	12	689	3
Chondroadherin	CHAD_HUMAN	4834	9	5273	8
Fibromodulin	FMOD_HUMAN	2439	6		
Thrombospondin 1	TSP1_BOVIN	1197	20	168	11
Collagen alpha-1(II) chain	CO2A1_BOVIN	99	7	140	7
Cartilage intermediate layer protein 1 (CILP-1)	CILP1_MOUSE	78	4	506	6
Cartilage intermediate layer protein 2 (CILP-2)	CILP2_HUMAN	55	8		
Tenascin	TENA_HUMAN	522	13	51	10
Proteoglycan 4 (lubricin)	PRG4_HUMAN	75	5	101	11
Hyaluronan and proteoglycan link protein 1 (HAPLN1)	HPLN1_HORSE			48	5

Secreted Proteins					
Clusterin	CLUS_HORSE	3130	12	120	3
Chitinase-3-like protein	CH3L1_BOVIN	2344	6	2732	5
Serum amyloid protein A	SAA_HORSE	400	4		
Lysozyme C	LYSM_BOVIN	367	2	450	4
Metalloproteinase inhibitor 1	TIMP1_HORSE	213	2	1358	5
MMP-1	MMP1_HORSE	1481	17	3074	23
MMP-3	MMP3_HORSE	5480	19	5159	17
MMP-10	MMP10_HUMAN			710	1
MMP-13	MMP13_HORSE	96	6		
Macrophage migration inhibitory factor	MIF_HUMAN	69	2		
Lactadherin	MFGM_PIG	69	3	54	1
Ribonuclease 4	RNAS4_HUMAN	63	1		
Intracellular proteins					
Vimentin	VIME_MOUSE			427	12
	VIME_PIG	1899	21		
Alpha-enolase	ENOA_BOVIN	179	3	2152	12
L-lactate dehydrogenase A chain	LDHA_MOUSE	68	4		
Phosphoglycerate kinase 1	PGK1_HORSE	57	5		
Superoxide dismutase [Cu-Zn]	SODC_HORSE	63	7	64	5
Superoxide dismutase [Mn], mitochondrial	SODM_HORSE			175	3
Metallothionein-1G	MT1G_HUMAN	66	2		
Metallothionein-1A	MT1A_BOVIN			119	2
Ferritin heavy chain	FRIH_HORSE			118	3
Triosephosphate isomerase	TPIS_BOVIN			825	3
Ras GTPase-activating protein 4	RASL2_MOUSE			64	5

Tripartite motif containing protein 46	TRI46_HUMAN			72	1
Sodium/hydrogen exchanger 2	SL9A2_RABIT	151	3		
Zinc finger protein 643	ZN643_HUMAN	48	2		
Cytoplasmic dynein 1 heavy chain 1	DYHC1_HUMAN	44	12		
Class E basic helix-loop-helix protein 23	BHE23_MOUSE	42	1		
Tetratricopeptide repeat protein 19, mitochondrial	TTC19_MOUSE			50	1
Solute carrier family 28 member 3	S28A3_RAT			50	5
Putative malate dehydrogenase 1B	MDH1B_BOVIN			43	2

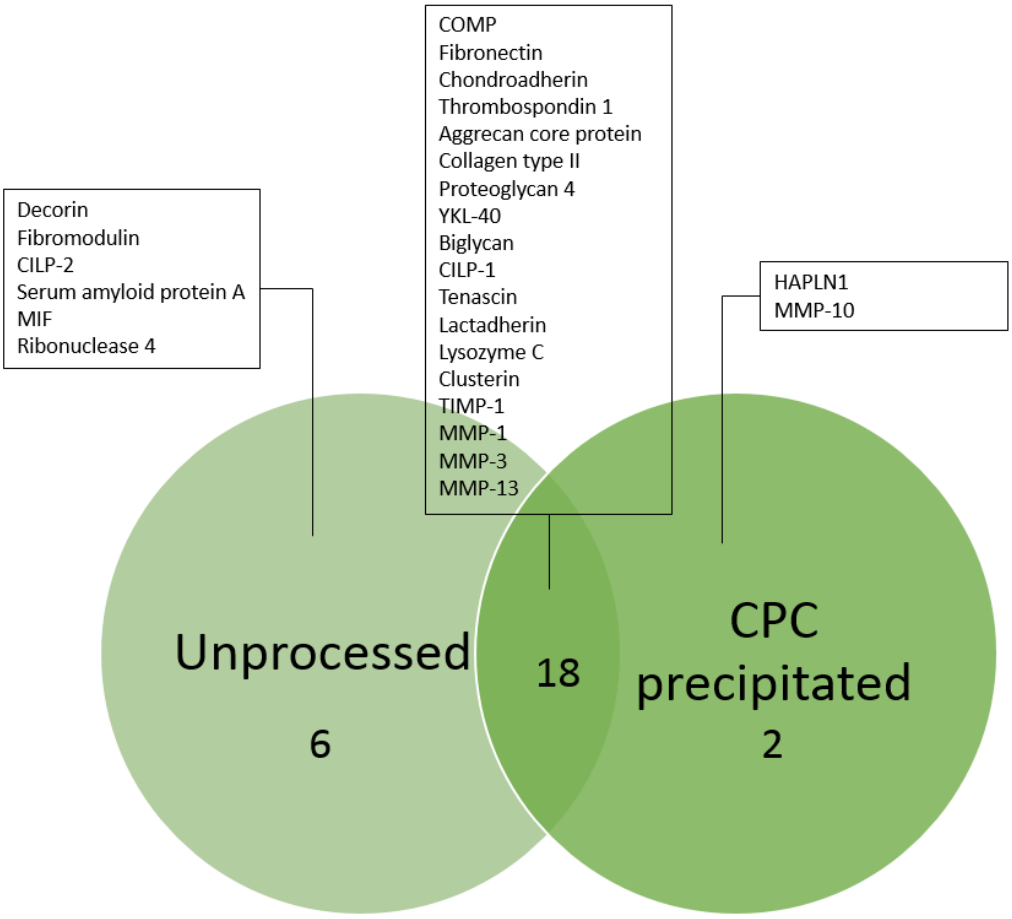


Figure 23. Venn diagram showing the number of ECM and secreted proteins detected in unprocessed samples and unbound fractions

nanoLC-MS/MS analysis was completed on IL-1β stimulated explant supernatant. Details on specific proteins are provided in Table 7.

4.3.2. Concanavalin A Lectin Chromatography

4.3.2.1. Depletion of proteins with Con A shown on SDS-PAGE

In a second approach to produce a glycoprotein depleted fraction of the cartilage explant secretome, Con A lectin chromatography was investigated. Silver stained SDS-PAGE displayed distinct bands after supernatant was centrifuged through Con A spin columns (Figure 24). A strong band was introduced to samples at 30 kDa corresponding to Con A itself. Additional elution steps were completed in an attempt to reduce the presence of this band, but this did not alter the amount of Con A present in the samples (data not shown). Introducing high levels of Con A to samples will cause high signal detection from this abundant protein that will hinder the ability of MS to identify additional proteins.

The unprocessed SDS-PAGE profiles were similar to previous explant supernatant profiles (Figure 24). The Con A lectin has high affinity for glycosylating carbohydrate groups, therefore only non-glycosylated proteins should pass through the column and appear in the unbound fraction. Attempts were made to elute bound proteins from the lectin with methyl α-D-mannopyranoside. Figure 24 shows that the proteins were not eluted and remained directly or indirectly bound to Con A.

The majority of proteins were bound to Con A within the spin column, especially in untreated controls fractions. This suggests most proteins in untreated secretome are ECM proteins that tend to be highly glycosylated. Higher numbers of unbound proteins are visible in the IL-1β stimulated fractions. To provide protein identities, bands were excised and analysed by trypsin digestion and nanoLC-MS/MS. An intense band in both untreated and IL-1β unbound fractions at ~50 kDa was identified as HAPLN1 (Figure 24). Some of the lower intensity bands excised from unbound IL-1β fractions were MMP-3 and BSA, which were proteins already reliably identified with high-throughput MS in Chapter 3. A higher molecular weight band ~250 kDa contained two ECM constituents: fibronectin and aggrecan core protein.

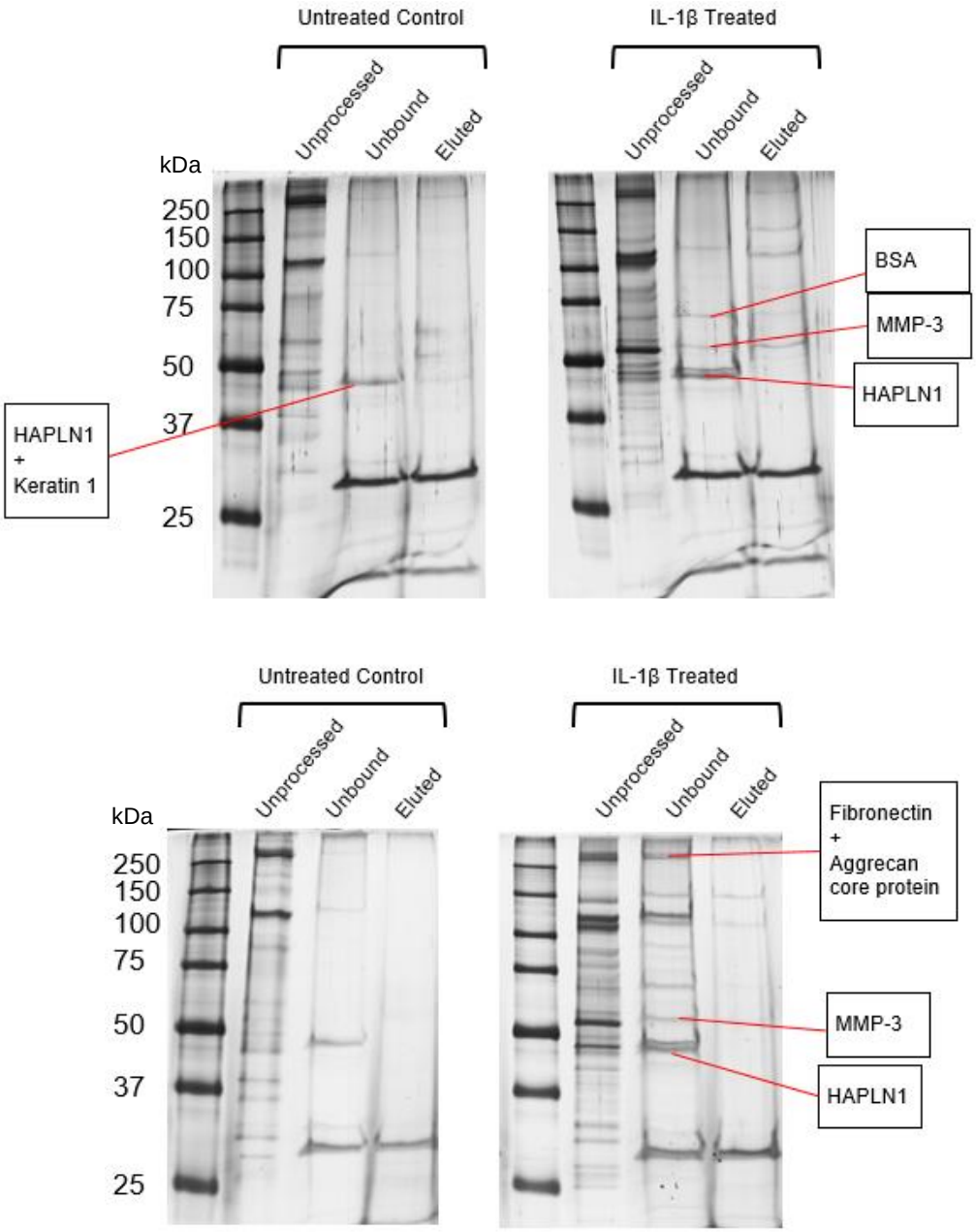


Figure 24. SDS-PAGE and silver staining of untreated and IL-1β stimulated supernatants from explant cultures with two different horses show the protein band profiles before and after Con A chromatography

Excised bands identified using nanoLC-MS/MS and NCBInr database searches. Unprocessed supernatant shows profile before Con A chromatography. The profiles of supernatant after Con A chromatography (unbound) are shown along with a lane containing eluted proteins from the Con A lectin spin column.

4.3.2.2. High-throughput analysis of Con A chromatography glycoprotein depleted secretome

A comparison of proteins identified by nanoLC-MS/MS was completed on unprocessed explant supernatants and Con A unbound fraction supernatants from the same experimental culture. Both untreated and IL-1β samples were analysed to ascertain any differences between treatment types. Protein identifications in untreated explant cultures before or after Con A processing are shown in Table 11, and IL-1β stimulated culture identifications are shown in Table 12. The classification of the proteins identified are summarised in Figure 25.

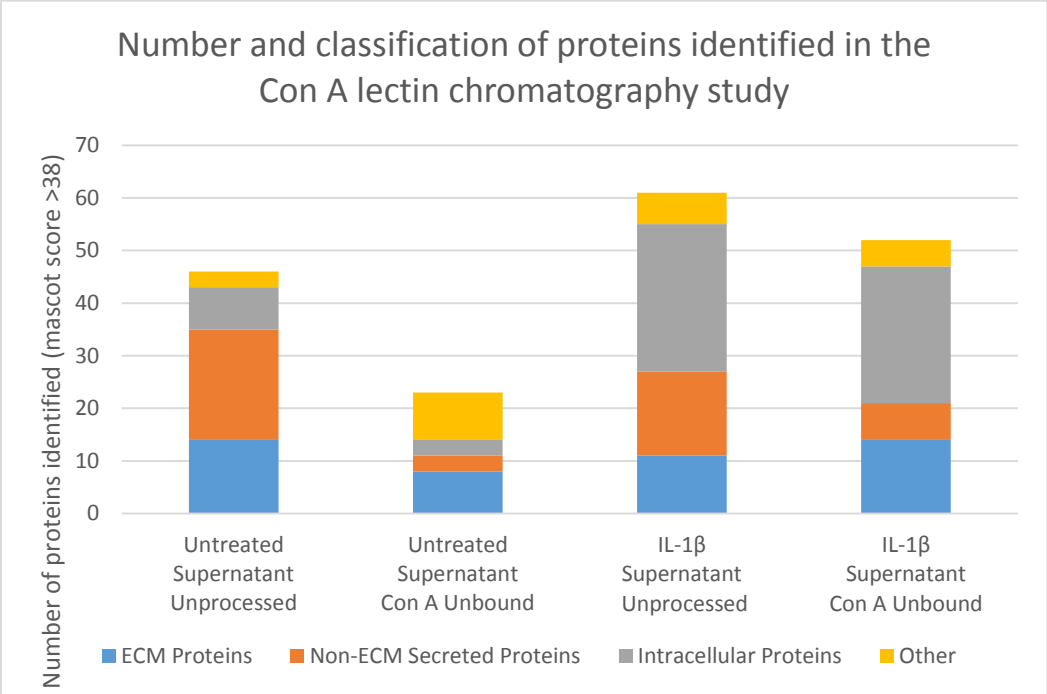


Figure 25. Bar graph summarizing the number and classification of proteins identified by MS during the Con A lectin chromatography study

Classification of the proteins described in detail in Table 11. A reduction in the number of proteins identified after Con A chromatography is observed, particularly secreted non-ECM proteins. Information on protein classification was from protein entries in the UniProt database (www.uniprot.org).

There was a decrease in the number of ECM proteins found in both untreated and IL-1β supernatants (unbound) after Con A techniques had been applied, and a substantial decrease in Mascot scores of some of those proteins that remained. MS analysis on untreated cultures identified 14 ECM proteins without Con A processing, and only 8 ECM proteins afterwards in unbound supernatants. IL-1β sample MS analysis followed a similar pattern, with 12 unprocessed and 8 unbound ECM protein identifications. It is noticeable that HAPLN1 appeared to be highly enriched by Con A, as it achieved high Mascot scores for each sample after Con A. This protein was already known to be released into explant cultures, but was below the set identification threshold in these runs on unprocessed samples. HAPLN1 remained unbound during chromatography, therefore making up a high proportion of the depleted fraction. This allowed HAPLN1 to be consistently and reliably identified with less interference from other previously abundant proteins.

Detection of non-ECM secreted proteins was reduced after Con A approaches. In untreated cultures only 3 non-ECM proteins were identified in Con A unbound supernatant, while 21 were detected in unprocessed supernatant (Table 11). Therefore Con A chromatography appears to be detrimental to an in depth analysis of the explant model. Interleukin-9 (IL-9) was identified only in unbound supernatants and was the only non-ECM secreted protein uncovered.

A range of intracellular proteins were found both in unprocessed and Con A unbound supernatants, especially in IL-1β samples once again indicating an increase in cell death (Table 12). Proteins involved in metal binding and storage such as ferritin heavy and light chains, metallothionine and selenium binding protein were identified after Con A, but not in unprocessed samples. Although numerous intracellular proteins were identified, these are of limited interest because they are unlikely to yield any insights on cartilage biology or inflammatory processes. Con A of untreated cultures also showed higher scores for many contaminating keratin proteins. These could have been introduced during the additional sample processing involved with the Con A procedure, or the process may have been selective for this type of protein.

The number of ECM and secreted proteins associated with Con A processing are shown for untreated and IL-1β stimulated cultures in Figure 26. In both cases, introducing Con A chromatography prior to MS contributed limited additional or novel protein identifications.

Table 11. Proteins identified in untreated explant supernatants, unprocessed (no Con A) or unbound after Con A lectin chromatography

Identifications provided searching MS data within the Swiss-Prot 2013_01 (all mammalian entries) database. Mascot scores >38 indicate identity/extensive homology.

Protein	**Accession Number**	**Unprocessed Individual**		**Unprocessed Pool**		**Con A Unbound Individual**		**Con A Unbound Pool**	
		Mascot score	Unique peptides	Mascot score	Unique peptides	Mascot score	Unique peptides	Mascot score	Unique peptides
ECM Proteins									
Cartilage oligomeric matrix protein	COMP_BOVIN	4090	18	4763	17	274	7	399	6
Thrombospondin 1	TSP1_BOVIN	741	20	1256	13				
Fibronectin	FINC_BOVIN	4422	41	6222	42			137	8
Chondroadherin	CHAD_HUMAN	1022	8	3069	6	306	7	2728	7
Aggrecan core protein	PGCA_HUMAN	1815	15	3389	16	4846	13	11335	15
Proteoglycan 4 (Lubricin)	PRG4_HUMAN	139	7	49	6				
Decorin	PGS2_HORSE	665	13	802	10			44	3
Biglycan	PGS1_HORSE	1099	14	1062	10			82	4
Fibromodulin	FMOD_HUMAN	1307	9	3200	7				
Collagen alpha-1(II) chain	CO2A1_BOVIN	137	15	59	10				
Collagen alpha-2(VI)chain	CO6A2_HUMAN	190	8	79	6				
Collagen alpha-1(X) chain	COAA1_MOUSE	77	2	102	4				
Prolargin	PRELP_MOUSE	124	5	147	5				
Cartilage intermediate layer protein 1 (CILP-1)	CILP1_MOUSE	201	10	789	10				
	CILP1_PIG					1501	9	3451	7
Hyaluronan and proteoglycan link protein 1 (HAPLN1)	HPLN1_HORSE					2110	20	4968	21
Secreted Proteins									
Clusterin	CLUS_HORSE	2767	16	4723	16	53	5	261	3

Serum amyloid A protein	SAA_CANFA	43	2						
	SAA_HORSE			55	2				
Chitinase-3-like protein 1 (YKL-40)	CH3L1_BOVIN	574	6	1178	5			134	1
Metalloproteinase inhibitor 1	TIMP1_HORSE	83	5	312	4				
Metalloproteinase inhibitor 2	TIMP2_CANFA			47	5				
Alpha-1-antiproteinase 2	A1AT2_HORSE	99	7	342	3				
Extracellular superoxide dismutase [Cu-Zn]	SODE_HUMAN	67	3	72	2				
MMP-3	MMP3_HORSE	653	11	956	10				
Procollagen C-endopeptidase enhancer 2	PCOC2_HUMAN	163	8	171	4				
TNF-R superfamily, member 11b (Osteoprotegerin)	TR11B_RAT	252	4	319	3				
SPARC (secreted protein acidic, cysteine rich)	SPRC_PIG	108	4	96	5				
Secreted frizzled-related protein 3	SFRP3_BOVIN	74	6	98	8				
Lysozyme C	LYSM_BOVIN	72	1	168	2				
Lactadherin	MFGM_PIG	83	1	63	2				
Connective tissue growth factor	CTGF_MOUSE			55	3				
Augurin	AUGN_HUMAN	54	1						
Ceruloplasmin	CERU_RAT			75	6				
Interleukin-9	IL9_HUMAN					49	1	71	1
Ribonuclease 4	RNAS4_HUMAN			48	2				
Insulin-like growth factor-binding protein 7 (IGFBP7)	IBP7_HUMAN	46	5						
Integrin beta-like protein 1	ITGBL_MOUSE	56	4						
Melanoma-derived growth regulatory protein	MIA_HUMAN	47	2						
Intracellular proteins – Overall number		3 proteins		5 proteins		4 proteins		3 proteins	
Other – Overall number		3 proteins		2 proteins		6 proteins		5 proteins	

Table 12. Proteins identified in IL-1β treated explant supernatants, unprocessed (no Con A) or unbound after Con A lectin chromatography

Identifications provided searching MS data within the Swiss-Prot 2013_01 (all mammalian entries) database. Mascot scores >38 indicate identity/extensive homology.

Protein	**Accession**	**Unprocessed Individual**		**Unprocessed Pool**		**Con A Unbound Individual**		**Con A Unbound Pool**	
		Mascot score	Unique peptides	Mascot score	Unique peptides	Mascot score	Unique peptides	Mascot score	Unique peptides
ECM Proteins									
Cartilage oligomeric matrix protein	COMP_BOVIN	7555	20	10590	21			254	8
	COMP_RAT					839	9		
Thrombospondin 1	TSP1_BOVIN	3456	28	2782	21	115	2		
Fibronectin	FINC_BOVIN	3786	40	3492	34				
	FINC_HUMAN					893	14		
Chondroadherin	CHAD_HUMAN	1978	8	3689	8	1683	6	3518	6
Aggrecan core protein	PGCA_HUMAN	2258	13	6412	14	4521	16	17599	14
Proteoglycan 4 (Lubricin)	PRG4_HUMAN			59	6				
Decorin	PGS2_HORSE	513	9	1130	8				
Biglycan	PGS1_HORSE	2047	11	1720	9	169	5		
Fibromodulin	FMOD_HUMAN	1381	6	1930	8				
Collagen alpha-1(II) chain	CO2A1_BOVIN	139	9	82	8				
Prolargin	PRELP_MOUSE	311	6	301	2				
Cartilage intermediate layer protein 1 (CILP-1)	CILP1_MOUSE	423	19	264	11	2320	7	4235	6
Hyaluronan and proteoglycan link protein 1 (HAPLN1)	HPLN1_HORSE					2204	20	2915	18
Secreted Proteins									
Clusterin	CLUS_HORSE	1785	13	4876	14	200	7		
Serum amyloid A protein	SAA_HORSE	219	4	436	6				
Chitinase-3-like protein 1 (YKL-40)	CH3L1_BOVIN	414	6	2018	5	127	3		
Metalloproteinase inhibitor 1	TIMP1_HORSE	457	4	146	3				

Alpha-1-antiproteinase 2	A1AT2_HORSE	50	3						
MMP-1	MMP1_HORSE	1678	23	1586	20	89	8	52	3
MMP-3	MMP3_HORSE	5441	20	4580	17	421	7	118	8
MMP-13	MMP13_HORSE	1700	20	699	12				
Interleukin-9	IL9_HUMAN							51	1
TNF-R superfamily, member 11b (Osteoprotegerin)	TR11B_RAT	388	6	638	4				
Heat shock 70kDa protein 1-like	HS71L_MOUSE	62	8	74	4				
Macrophage migration inhibitory factor	MIF_HUMAN	91	2	86	2	60	2		
Lysozyme C	LYSM_BOVIN	179	3	148	2				
	LYSCK_SHEEP					42	2		
Lactadherin	MFGM_PIG			54	3				
Ribonuclease 4	RNAS4_HUMAN	46	1	59	2				
Semaphorin-3C	SEM3C_HUMAN	47	6						
C-X-C motif chemokine 6	CXCL6_HORSE	44	2						
Intracellular proteins – Overall number		22 proteins		15 proteins		22 proteins		18 proteins	
Other – Overall number		4 proteins		5 proteins		5 proteins		2 proteins	

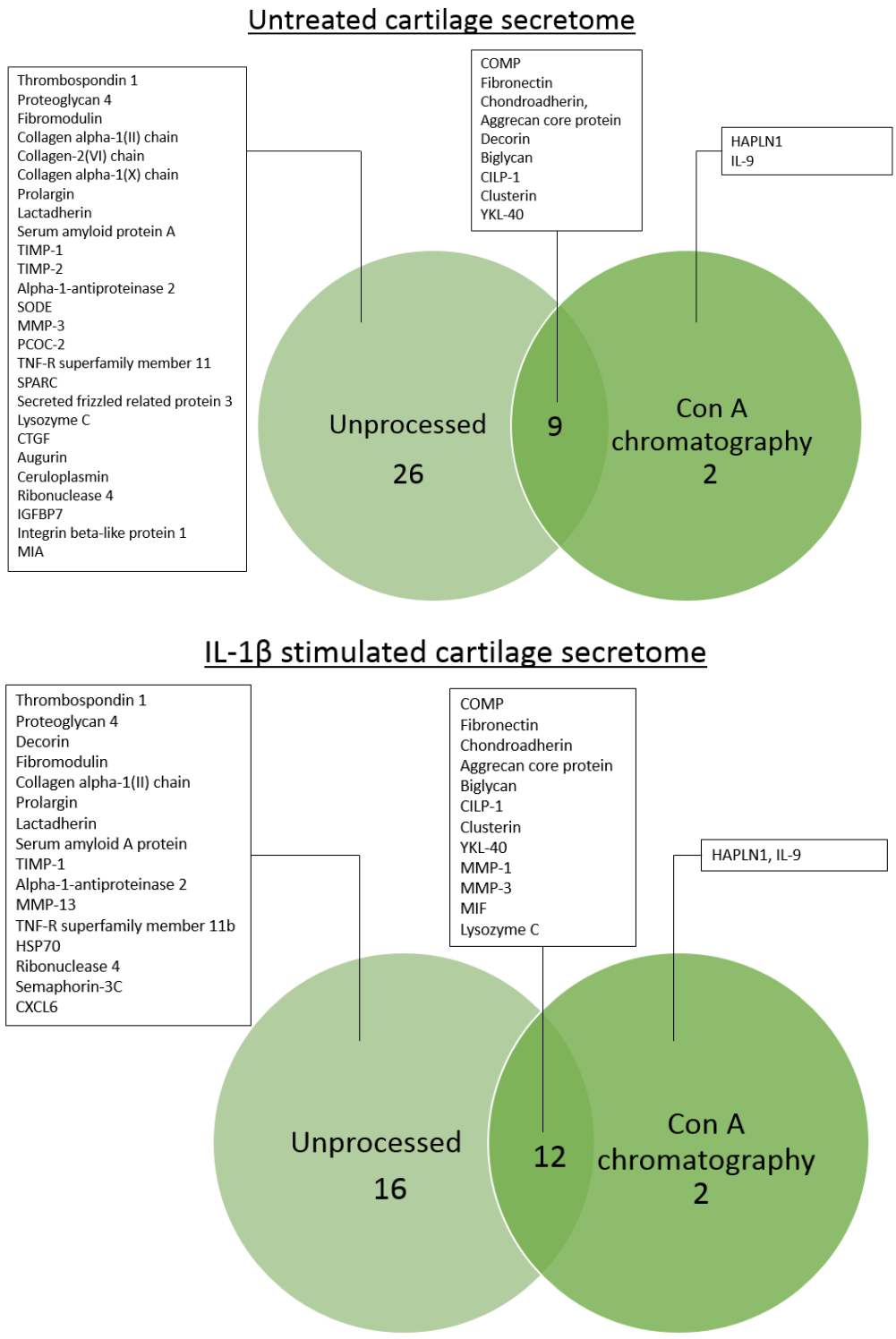


Figure 26. Venn diagram displaying the number of ECM and secreted protein identifications associated with unprocessed and Con A chromatography on untreated and IL-1β stimulated cartilage secretome

nanoLC-MS/MS analysis was completed on untreated and IL-1β cartilage explant conditioned media. Details on specific proteins provided in Table 8 and Table 9.

4.3.4. Proteominer™ enrichment technology

The final approach which was assessed for reducing the dynamic range of proteins present in the cartilage explant culture secretome was the Proteominer™ enrichment technology. Proteins identified after Proteominer™ processing are recorded in Table 13 for untreated cultures and Table 14 for IL-1β stimulated explant cultures. Only 10 proteins were identified in untreated supernatant after Proteominer™ enrichment, with 3 of these being trypsin or protease inhibitor cocktail components. These included high abundance ECM proteins like COMP, fibronectin, fibromodulin and chondroadherin. A secreted component of the complement pathway, complement C4-A was also identified here.

In IL-1β stimulated cultures 15 proteins were identified overall after Proteominer™ enrichment. These included several inflammatory related proteins usually observed like clusterin, SAA, HSP70 and MIF, but unfortunately many of the expected identifications (i.e. MMP-1, -3 and -13) were not made. Four highly abundant ECM proteins continued to be identified and 6 intracellular proteins were present. Intracellular proteins found in these samples that were not already mentioned in this thesis were actin, nestin (intermediate filament, neural development) and erlin-2 (endoplasmic reticulum associated protein).

One limitation to Proteominer™ analysis was the presence of CHAPS within the elution buffer (8M urea, 2% (w/v) CHAPS). This interfered with peptide identification from about half way through MS runs, at which point CHAPS came off the column and produced a strong signal that overwhelmed the signal of any peptides. Attempts were made to reduce the concentration of this detergent with the acetone precipitation and C18 spin columns, but this did not deplete CHAPS significantly enough. Time constraints did not allow further development of the method to explore alternative elution buffers or complementary clean up attempts, during which CHAPS concentrations may have been reduced to improve MS analysis.

Table 13. Protein identifications in untreated explant cultures after Proteominer™ enrichment

Database searches were performed with Mascot Daemon on the Swiss-Prot 2013_01 database (all mammalian entries). Mascot scores >38 indicate identity/extensive homology.

Protein	**Accession**	**Mascot Score**	**Unique Peptides**	**Sequence Coverage (%)**	**Biological Function**
Clusterin	CLUS_HORSE	300	9	12.50	Implicated in various functions, primarily an extracellular chaperone
COMP	COMP_BOVIN	141	4	4.50	Extracellular matrix protein
Trypsin	TRYP_PIG	121	3	16.50	Introduced by trypsin digestion of proteins
Pancreatic trypsin inhibitor	BPT1_BOVIN	103	2	22.00	Introduced in protease inhibitor cocktail
Fibromodulin	FMOD_BOVIN	82	2	6.90	Small proteoglycan in extracellular matrix
Fibronectin	FINC_RAT	73	2	1.00	Extracellular matrix protein
Chondroadherin	CHAD_BOVIN	68	2	4.40	Extracellular matrix protein
Fibronectin (Fragment)	FINC_CANFA	61	2	6.10	Extracellular matrix protein
Eukaryotic translation initiation factor 2-alpha kinase 4	E2AK4_HUMAN	50	1	0.50	Cytosolic – promotes phosphorylation of eukaryotic translation initiation factor
Complement C4-A	CO4A_HUMAN	50	1	0.60	Secreted component of complement pathway
Spleen trypsin inhibitor I	BPT2_BOVIN	48	1	9.00	Introduced in protease inhibitor cocktail

Table 14. Protein identifications in IL-1β treated explant cultures after Proteominer™ enrichment

Database searches were performed with Mascot Daemon on the Swiss-Prot 2013_01 database (all mammalian entries). Mascot scores >38 indicate identity/extensive homology.

Protein	**Accession**	**Mascot Score**	**Unique Peptides**	**Sequence Coverage (%)**	**Biological Function**
Vimentin	VIME_HUMAN	278	6	10.10	Cytoplasmic intermediate filament protein
Clusterin	CLUS_HORSE	263	6	9.60	Implicated in various functions, primarily an extracellular chaperone
Serum amyloid A protein	SAA_HORSE	145	3	30.90	Major inflammation acute phase reactant, induced by cytokines
Serum amyloid A protein	SAA_BOVIN	143	2	20.80	Major inflammation acute phase reactant, induced by cytokines
Actin, aortic smooth muscle	ACTA_BOVIN	108	2	5.60	Cytoskeletal protein, aids cell mobility
Macrophage migration inhibitory factor	MIF_BOVIN	81	1	9.60	Pro-inflammatory cytokine
Pyruvate kinase isozymes M1/M2	KPYM_HUMAN	69	2	5.50	Glycolytic enzyme
COMP	COMP_RAT	68	2	4.10	Extracellular matrix protein
Fibromodulin	FMOD_BOVIN	63	2	6.90	Small proteoglycan in extracellular matrix
Trypsin	TRYP_PIG	58	1	3.50	Introduced by trypsin digestion of proteins
Heat shock 70 kDa protein 1-like	HS71L_BOVIN	54	1	2.30	Stress induced chaperone
Nestin	NEST_MOUSE	51	1	0.50	Neural development protein, intermediate filament
Alpha-enolase	ENOA_HUMAN	50	2	3.90	Multifunctional enzyme, glycolysis
Thrombospondin-1	TSP1_HUMAN	46	1	1.20	Extracellular matrix protein
Erlin-2	ERLN2_HUMAN	41	1	2.70	Endoplasmic-reticulum associated protein, IP3R degradation
Fibronectin	FINC_MOUSE	40	1	0.70	Extracellular matrix protein

4.4. Discussion

The previous chapter covered a time course of proteins released from cartilage explant cultures with high-throughput nanoLC-MS/MS. This identified proteins that could be utilized to study aspects of cartilage biology. Coverage of the explant secretome could be expanded with further in depth studies to identify additional biologically relevant proteins. Knowledge of additional proteins will provide biomarkers for monitoring responses to inflammatory mediators and potential anti-inflammatory compounds. In complex samples, highly abundant proteins are known to "mask" identifications of low-level proteins (Millioni et al., 2011). In the MS analysis described in this thesis, only the 5 most abundant peptides from each MS survey scan are selected for fragmentation, therefore low abundance peptides will generally fail to be analysed. Three different approaches, CPC precipitation, Con A chromatography and Proteominer™ were assessed for their ability to deplete high abundance proteins prior to MS. For both CPC precipitation and Con A approaches, SDS-PAGE and silver staining were used as an additional assessment of the depletion of samples. After it was confirmed abundant proteins were being removed, the depleted supernatants were analysed by high-throughput MS.

4.4.1. CPC precipitation in cartilage proteomics

CPC was effective at precipitating highly abundant ECM components including proteoglycans, as certain small proteoglycans like decorin and fibromodulin were no longer detected after CPC precipitation, but were present in the CPC pellet (Table 9). Unexpectedly, two ECM proteins HAPLN1 and CILP-1 appeared to be enriched by the CPC processing. It is possible that degraded fragments of these proteins may not interact with CPC. While YKL-40 and MMP-13 identifications were lost, MMP-10 was identified after this approach. Of the three depletion processes described in this chapter, CPC precipitation was least detrimental with respects to reduction of the overall number of proteins detected by MS.

One protein that was found only in the glycoprotein depleted secretome after CPC precipitation was MMP-10 (stromelysin-2), which is a member of the stromelysin

group of MMPs as is MMP-3 (stromelysin-1). MMP-10 can activate other procollagenases like MMP-1 (Nakamura et al., 1998) and has a weak degradative action on collagens type III, IV and V (Nicholson et al., 1989). Presence of MMP-10 was detected in synovial fluid and cartilage from RA and OA patients (Barksby et al., 2006). That study also demonstrated MMP-10's role in proMMP activation, specifically MMP-1, MMP-8 and MMP-13 (Barksby et al., 2006). Synoviocytes expressing MMP-10, -1 and -3, display significantly more invasive characteristics contributing to RA and OA progression (Tolboom et al., 2002). A Wnt-1-inducible signalling pathway protein 3 ($WISP_3$/CCN6) gene mutation associated with OA and cartilage breakdown can increase MMP-10 expression in chondrocytes (Baker et al., 2012). Stimulation with OA related cytokines can also affect MMP-10 secretion, including increased release from IL-1 stimulated OA cartilage detected by protein antibody arrays (Jarvinen et al., 2008). Chondrocytes and synovial fibroblasts can be induced to express MMP-10 by the pro-inflammatory cytokines IL-1, TNF-α and oncostatin (Barksby et al., 2006). Confirmation of the presence of MMP-10 in the secretome of IL-1β treated equine cartilage could allow this OA associated protease to be studied within this explant model.

4.4.2. Con A and M-LAC aided analysis in cartilage proteomics

The highly glycosylated status that attracts water molecules and swells the ECM is an important characteristic of cartilage (Buckwalter and Mankin, 1998b). Con A lectin chromatography was chosen to target glycosylated residues on abundant ECM components to provide depleted fractions for MS analysis. One limitation to this approach was introduction of Con A leaching out into elution material, observed in the SDS-PAGE image in Figure 24. A band at 30 kDa corresponding to Con A after sample processing, is not present in unprocessed supernatants. Introducing an additional abundant protein will be detrimental to detection of low level proteins. Attempts to reduce Con A release with wash steps before processing were made, but these did not decrease levels of Con A leakage. Mascot searches of the Con A processed samples against all taxonomic entries in the NCBInr database revealed

Con A to be the top protein hit (data not shown). Con A presence at such high levels will have hampered identification of additional low level proteins in these experiments.

Lectin chromatography techniques have not yet been widely used for OA research. No reports have been published on Con A fractionation of proteins from cartilage conditioned media (PubMed search terms “proteomics”, “cartilage” or “osteoarthritis”, Dec 2013). The plasma peptidome and proteome of RA have been studied with M-LAC fractionation being used prior to high-throughput MS analysis (Zheng et al., 2009). Actin and C-reactive protein were associated with RA plasma, and calgranulin A, B and C were detected only in M-LAC bound fractions (Zheng et al., 2009). The glycoproteome of early chondrogenesis and accompanying differential markers has been investigated with Con A (Ishihara et al., 2013). Lysate of differentiating mouse chondroprogenitor cells was fractionated to identify the glycoproteome, before gene expression of selected proteins in human MSCs (Ishihara et al., 2013). Collagens type III and XI, aquaporin-1, netrin receptor unc-5 homolog B and ectonucleotide pyrophosphatase/phosphodiesterase family member 1 all showed potential as early chondrogenesis markers (Ishihara et al., 2013). The types of proteins found in cell lysate will be fundamentally different to those found in cartilage secretome, which may explain why Ishihara et al achieved successful application of Con A methods. In this chapter, Con A processing did increase detection of metal binding proteins like ferritin heavy/light chains, metallothionine and selenium binding proteins, which are all involved in intracellular metal storage (Proulx-Curry and Chasteen, 1995). The presence of intracellular proteins in the cartilage explant secretome suggests that there is some chondrocyte cell death during culture. However, detecting such proteins in greater numbers after depleting the levels of abundant ECM proteins demonstrates that using Con A for glycoprotein depletion has facilitated the MS detection of proteins present at lower levels.

Excised bands from SDS-PAGE provided MS identifications of high abundance ECM proteins such as fibronectin and aggrecan core protein, and catabolic MMP-3 in Con A unbound samples (Figure 24). Therefore targeted removal

of glycosylated proteins was not totally effective at removal of abundant ECM proteins. HAPLN1 achieved higher Mascot scores after this Con A processing, indicating it was comparatively enriched compared to the other proteins present. Con A did not bind this ECM protein, therefore it remained in the unbound fraction as observed in Figure 24. This link protein is responsible for attachment of hyaluronic acid chains with proteoglycan aggregates (Poole et al., 1982), which help to form a highly hydrated ECM. A high proportion of HAPLN1 must be unbound from the aggregating proteoglycans to not interact and be depleted by Con A chromatography. This could be newly synthesised HAPLN1 that has not been incorporated into the ECM.

The cytokine IL-9 was identified in the secretome of untreated and IL-1β stimulated explants, only in unbound supernatants after Con A processing. IL-9 stimulates expression of IL-22 in T lymphoma cells, which in turn will contribute towards release of pro-inflammatory factors (Ikeuchi et al., 2005, Dumoutier et al., 2000). STAT1, STAT3 and STAT5 transcription factors are activated by IL-9 leading to gene expression that inhibits apoptosis (Demoulin et al., 1999). The potential role of IL-9 in OA has not been extensively studied and therefore the implications of this identification are unclear and warrant further research. Further proteomic and other studies will assess the reliability of IL-9 identification because sample numbers were limited, and only one significant peptide was repeatedly detected to contribute towards reported Mascot scores. Con A chromatography has aided identification of IL-9 in the explant model, but additional work is needed to determine effects of cytokines or anti-inflammatory factors on this protein.

4.4.3. Applications of Proteominer™ in proteomics and OA

Proteominer™ technology is designed to enrich low abundance proteins and decrease the dynamic range of complex samples. This technique has been applied analysis of serum for a range of diverse diseases to discover potential biomarkers. Proteominer™ analysis of serum from lung cancer patients aided identification of SAA1 (Milan et al., 2012). Gelsolin association with hepatitis B-associated liver

cirrhosis was also found with Proteominer™ enrichment of serum (D'Amici et al., 2012). In the search for novel biomarkers for osteoarthritis, serum analysis with Proteominer™ technology has also been completed (Fernandez et al., 2011, Fernandez-Costa et al., 2012). An altered haptoglobin profile in serum showed this protein to be increased in OA patients by 2DE (Fernandez-Costa et al., 2012).

In chapter 3 of this thesis, coverage of the secretome was limited by the presence of highly abundant proteins, therefore a trial study using Proteominer™ before MS was completed. The high-throughput MS analysis of cartilage explant secretome after Proteominer™ application did not prove beneficial to low abundance protein identification. Optimisation of this kit by the manufacturers focused on plasma and serum therefore it was not guaranteed to work for cartilage explant conditioned media, although whole OA cartilage has been analysed with Proteominer™ with three novel OA biomarkers proposed: LECT2, BAALC and PRDX6 (Ikeda et al., 2013). While many proteins may be found both in whole cartilage or cartilage supernatant, there are fundamental differences in composition of these samples. Throughout the literature, many other sample types have been aided by Proteominer™ including urine (Candiano et al., 2012), egg white extract (D'Ambrosio et al., 2008), cell lysates (Fonslow et al., 2011) and beer (Fasoli et al., 2010).

Proteominer™ kit instructions stated best results would be achieved with 10 mg protein being processed by the procedure. With knowledge that suitable volumes and concentrations from cartilage explant culture supernatant would provide considerably lesser amounts of proteins, two changes were introduced to previous explant culture methods. Firstly, the number of explants was increased from 5 to 8 discs per 1 ml media. Secondly, centrifugal filter units were utilised to concentrate 12 ml of secretome samples down to ~0.5 ml. Protein assays confirmed that concentrated samples contained protein amounts that would be more suitable for starting this procedure. Although these steps were introduced to concentrate material, it is possible that cartilage explant supernatants still provided sample material that was not ideal for Proteominer™ techniques. Analysis of whole cartilage benefited from

Proteominer application™ (Ikeda et al., 2013), but achieving suitably higher concentrations of protein would be possible with whole cartilage samples. Another limitation to this trial was the presence of CHAPS in elution buffer. Sample preparation with acetone precipitation and C18 spin columns did not decrease this detergent sufficiently, and therefore high levels were present in the MS runs. The first half of runs provided peptide peaks within the mass spectrum, but once CHAPS began to come off the column, signals from it obscured the peptide signals. The protein identifications that were made with data before the peptide signals were lost, indicated that known high abundance ECM proteins continued to achieve the highest Mascot scores and limited additional low-level proteins were found (Table 13, Table 14). Alternative elution buffers (not containing CHAPS) could be investigated to separate bound proteins from the hexapeptide bead library, but this was not followed up due to time constraints. Identifications from data received also gave no indication of effective depletion of abundant proteins. The majority of Proteominer™ studies used 2DE afterwards to assess improvements in proteomic coverage, in which CHAPS did not disrupt the process. One study on cell lysate from HeLa cells did show improvements in enrichment of low abundance proteins using an alternative elution buffer before high-throughput MS (Fonslow et al., 2011). Therefore high-throughput MS applications can be achieved via this technology with further method optimisation.

4.4.4. Challenges of abundant protein depletion in cartilage explant culture proteomics

There are several possible explanations for the minimal yield of additional low abundance proteins identified in high-throughput MS using depletion methods reported in this chapter. The functional roles of many cartilage related proteins involves binding to other proteins within the ECM (Heinegard, 2009). Therefore ECM constituents contain a variety of binding sites for smaller proteins, growth factors and cytokines. It is possible that binding interactions remained during the depletion/enrichment procedures therefore low abundance proteins were co-extracted along with the selectively-removed ECM components. Therefore the depleted sample remaining was also depleted of low abundance proteins, hence the lack of additional

identifications reported. This effect could be influential on the current results because there are excess abundant ECM proteins to bind with lower level proteins. Secreted low abundance proteins may also be glycosylated themselves so would interact and be removed by the Con A lectin or CPC precipitation. Further refinement of these or alternative high abundant protein depletion approaches may aid additional proteins identifications in cartilage secretome. Enzymatic treatment of samples prior to CPC precipitation/Con A chromatography could aid analysis with the techniques. For example, trypsin digestion before Con A chromatography and MS was completed on ATDC5 cells (Ishihara et al., 2013). Enzymatically digesting proteins or reducing proteins with DTT may interrupt the binding abilities of abundant proteins and therefore develop effective depletion methods. Unfortunately due to time constraints of this project, no further improvements were made to report in this thesis.

4.4.5. Conclusion

The hypothesis of this chapter was that by depletion of highly abundant ECM proteins, coverage of low level proteins in explant secretome could be achieved. Three different methods were trialled on explant conditioned media before nanoLC-MS/MS. CPC precipitation targeted negatively charged groups inherent in proteoglycans and GAGs. Con A lectin chromatography specifically binds to glycosylation groups, allowing abundant ECM components to be bound, leaving behind a glycoprotein depleted fraction. Proteominer™ enrichment aimed to equalise the dynamic range of secretome, thereby enriching low level proteins and decreasing abundant proteins. MMP-10 and IL-9 identification was aided by CPC precipitation and Con A chromatography respectively. Uncovering these low level proteins that are relevant to the cartilage explant model and OA, demonstrates the potential of applying these depletion approaches prior to high-throughput MS. The most effective depletion method was CPC precipitation due to effective removal of certain proteoglycans and ECM proteins, whilst being least detrimental to numbers of secreted proteins identified, including MMP-1, MMP-3, MMP-10 and TIMP-1. Although, the data achieved in this thesis showed high-throughput MS analysis on unprocessed cartilage

supernatant (without any depletion methods as reported in chapter 3) provided the most definitive coverage of the secretome. Whilst these current studies only revealed a few additional low abundance proteins, further optimisation of the experimental approaches described here may allow increased identifications. Once an appropriate depletion technique is developed, larger scale studies will allow greater coverage of the cartilage secretome to be achieved.

CHAPTER 5

APPLYING AN IL-1β STIMULATED CARTILAGE EXPLANT MODEL TO ASSESS ANTI-INFLAMMATORY EFFECTS OF CARPROFEN

5.1. Introduction

Progression of OA is a complex process, leading to loss of cartilage ECM facilitated by inflammatory mediators and metabolic processes (Goldring et al., 2008). Excess release of catabolic proteins within synovial joints affected by OA contributes towards cartilage breakdown and loss (Abramson and Attur, 2009). Degradative fragments of ECM proteins such as fibronectin, are released into surrounding synovial fluid where they initiate further inflammatory signalling (Homandberg et al., 1998). These degradative products are also released into culture supernatant whilst incubating cartilage explants. In Chapter 3, released ECM components and catabolic MMPs were identified in cartilage secretome by high-throughput MS. These candidate proteins identified in the secretome were chosen for quantitative western blotting. These proteins can act as biomarkers within this model for assessment of cartilage breakdown and metabolism in response to selected treatments. This chapter describes the ability of the cartilage explant model to monitor selected candidate proteins MMP-1, MMP-3, MMP-13, TSP, COMP, fibronectin and clusterin.

5.1.1. IL-1β and carprofen treatments within explant culture

The pro-inflammatory cytokine IL-1β, was chosen to stimulate cartilage explants. IL-1β is implicated in OA cartilage inflammation and progression of the disease, and is found at significantly higher levels in early OA stages (Benito et al., 2005). In chondrocytes it stimulates release of cytokines, chemokines and catabolic proteins, and inhibits synthesis of collagen type II and IX (David et al., 2007, Busschers et al., 2010, Goldring et al., 1988). Injection of IL-1β into synovial joints of horses demonstrates comparable responses *in vivo* (Ross et al., 2012). Stimulation of cartilage explants with IL-1β also caused changes in SDS-PAGE profiles and MS analysis identifications throughout previous thesis chapters.

The NSAID, carprofen (marketed as Rimadyl® by Pfizer) was chosen to evaluate the potential for screening anti-inflammatory effects on IL-1β stimulated cartilage explant secretome. Joint pain and inflammation associated with OA in dogs and horses are treated clinically using this NSAID (Goodrich and Nixon, 2006). Carprofen was available as a prescription medication for humans from 1988 to 1998 but was voluntarily withdrawn for commercial reasons. It is a selective COX-2 inhibitor that blocks synthesis of PGE_2 in the arachidonic acid signalling pathway. Selectivity for COX-2 (over COX-1) at IC_{50} has been confirmed in horses (Beretta et al., 2005). Carprofen has been reported to hold back progression of OA related changes in cartilage and subchondral bone in dogs (Pelletier 2000). Beneficial effects include decreased PGE_2 production and GAG release, and increased proteoglycan synthesis in equine *in vitro* studies (Goodrich and Nixon, 2006). Carprofen was selected to study the explant models ability to screen anti-inflammatory compound effects on ECM degradation and secretion of proteases.

5.1.2. Candidate protein biomarkers monitored in the explant model - MMPs, ECM proteins and clusterin

MMPs -1, -3 and -13 were all identified in IL-1β stimulated cartilage explant secretome and showed semi-quantitative differences in release (Chapter 3). These three MMPs have all been implicated in progression of OA. Collagenases (MMP-1 and MMP-13) will degrade collagen fibres along with other non-collagenous ECM proteins. MMP-1 is known as interstitial collagenase, which efficiently cleaves collagen types I, II and III (Chung et al., 2004). MMP-1 has the vital ability to unwind triple-helical collagen fibres to facilitate collagen enzymatic degradation (Manka et al., 2012). The C-terminal hemopexin domain enables this unwinding, whilst a separate N-terminal catalytic domain cleaves the collagen chain (Manka et al., 2012). IL-1β treatment causes significant increases in MMP-1 secretion from chondrocytes *in vitro* shown by quantitative SILAC analysis (Calamia et al., 2011). IL-1β and LPS intra-articular injections up-regulate gene expression of MMP-1 in equine articular cartilage *in vivo* (Ross et al., 2012).

MMP-13 has an important role in collagen type II degradation, which is the most abundant collagen type found in cartilage (Knauper et al., 1996, Reboul et al., 1996). Collagens types VI and IX, aggrecan and other proteoglycans can also be degraded by MMP-13 (Shiomi et al., 2010). Transgenic mice have shown that constitutive active MMP-13 causes OA *in vivo* (Neuhold et al., 2001). Delayed development of OA occurs with deletion of the MMP-13 gene from a meniscal-ligamentous injury (MLI)-induced mouse model (Wang et al., 2013). Proteomic investigations have also identified MMP-13 as a differentially up-regulated protein in OA cartilage (Iliopoulos et al., 2008).

MMP-3 (stromelysin-1) can degrade fibronectin, collagens including types I, IV and IX, and proteoglycans (Chin et al., 1985, Okada et al., 1989). MMP-3 has a vital role in producing collagen degradation by cleavage of pro-peptide domains to activate collagenases including MMP-1 and MMP-13. The secretome of OA cartilage contains higher levels of MMP-3 compared to healthy tissue (Hermansson et al., 2004, De Ceuninck et al., 2005). Its release is also stimulated by pro-inflammatory cytokine treatment of chondrocytes and cartilage (Stevens et al., 2008, Calamia et al., 2011, Catterall et al., 2006). Treatment with an MMP inhibitor on a rat meniscal transection model of OA, showed a reduction in perceived pain, joint damage and osteochondral vascularisation (Mapp et al., 2010). Semi-quantitative differences in MMP-1, -3 and -13 were consistently reported in the secretome. The physiological relevance of these MMPs in cartilage degradative and OA make these proteins suitable candidates for western blotting analysis.

ECM proteins made up a large proportion of the cartilage secretome identifications after MS analysis (Chapter 3). Pro-inflammatory cytokines increase release of catabolic enzymes including ADAMTS and MMPs, which leads to degradation of the ECM (Goldring, 1999). Here, western blots of ECM constituents COMP, TSP and fibronectin assessed if pro-inflammatory or anti-inflammatory stimuli caused differential release of degraded fragments. This helped determine if changes in ECM proteins correlated with alterations occurring in MMP secretion levels.

COMP is a non-collagenous pentameric protein made up of 5 glycosylated subunits around 100 kDa (Hedbom et al., 1992). It binds to collagen fibres and is important in organisation of the cartilage ECM (Rosenberg et al., 1998). COMP has been studied as a diagnostic and prognostic biomarker of OA, and a deamidated epitope of COMP in serum is associated with hip OA specifically (Catterall et al., 2012). TSP is an adhesive glycoprotein that helps mediate cell to matrix interactions in cartilage, interacting with integrins in the plasma membrane (Frazier et al., 1999).

Fibronectin is a dimeric glycoprotein (440 kDa) found in cartilage ECM, which interacts with collagens and cell surface proteins (Potts and Campbell, 1996). Its main roles involve contributing to cell adhesion in the ECM and motility (Potts and Campbell, 1996). Fragments of fibronectin released during osteoarthritis contribute towards cartilage degradation by up-regulating catabolic signaling (Homandberg et al., 1998). These degradation fragments are produced by MMP-1, MMP-3 and MMP-13 enzymatic actions (Zhang et al., 2012a).

Clusterin consistently achieved high Mascot scores in chapter 3. Clusterin is a secreted chaperone that takes part in a variety of different functions including inflammation and apoptosis, which could contribute to diseases like OA (Wilson and Easterbrook-Smith, 2000). One study has shown that clusterin mRNA was expressed at higher levels in early OA compared to healthy cartilage (Connor et al., 2001). Whilst in late OA, clusterin mRNA expression was reduced (Connor et al., 2001). In early stages of OA there could have been an increased attempt to protect cartilage from damage, while in advanced disease stages there is a loss of ability to express clusterin (Connor et al., 2001).

Synovial fluid and synoviocyte expression of clusterin from RA and OA patients has revealed differences in expression levels (Devauchelle et al., 2006). The full-length and spliced isoform of clusterin mRNA were lower in RA samples compared to both OA and normal synovial tissues (Devauchelle et al., 2006). Clusterin was proposed as a marker to distinguish between RA and OA (Devauchelle et al., 2006). Intracellular studies showed siRNA targeting clusterin caused an increase in IL-6 and

IL-8 synthesis, and that clusterin can affect NF-κB signalling within synoviocytes (Devauchelle et al., 2006). A comparison of serum and synovial fluid from healthy individuals and patients suffering from knee or hip OA reported elevated levels of secreted clusterin (Fandridis et al., 2011). In 2012, clusterin was identified as a candidate plasma biomarker for OA progression after 2D-LC-MALDI glycoprotein analysis (Fukuda et al., 2012). Unfortunately, validation of clusterin as a biomarker for OA progression was not supported by ELISA or western blot data completed (Fukuda et al., 2012). This chapter monitored clusterin release from cartilage explants in response to IL-1β and carprofen treatments, due to clusterin abundance in the secretome and potential association with OA.

5.1.3. Nitric Oxide (NO) measurement

OA cartilage explants produce increased levels of NO release associated with cartilage inflammation (Amin et al., 1995). Cytokine stimulation increases iNOS release, which synthesises NO and leads to higher NO levels in inflammatory conditions (Taskiran et al., 1994). NO has a variety of functional roles related to OA including pain perception, catabolic signalling and apoptosis (Abramson, 2008). NO released into cartilage conditioned media will eventually be converted to nitrite, which can be measured by Griess assays. This allowed comparison of NO released from untreated cartilage in response to IL-1β and carprofen stimulation.

5.1.4. Hypothesis and aims

In previous chapters, high-throughput MS analysis on cartilage secretome identified released ECM proteins, along with secreted chemokines, metabolic proteins and inflammatory stress related proteins. Studies in this chapter describe assessment of the effects of IL-1β and carprofen treatments on quantitative release levels of the proteases MMP-1, -3 and -13, clusterin, selected ECM constituents and NO. It was hypothesised that carprofen would decrease IL-1β stimulated release of catabolic MMPs and inflammatory related products, due to its inhibition of COX-2 and PGE_2 production. Decreases in MMPs could therefore cause reduced release of degradative ECM components monitored by western blotting. Examining effects of carprofen on

IL-1β treated explant cultures showed this approach can be used to screen other anti-inflammatory compounds.

5.2. Methods

5.2.1. Dissection and explant culture

See Materials & Methods for dissection and explant culture protocols (Chapter 2.3.). All articular cartilage explant cultures in this chapter were incubated for 6 days at 37 °C, 5% CO_2.

5.2.2. Western blotting

Western blotting protocol was described in Chapter 2.6. Primary antibody dilutions were made up as follows in 3% (w/v) BSA: MMP-1 (1:800 dilution, Aviva), MMP-3 (1:1000 dilution, Aviva), MMP-13 (1:1000 dilution, Aviva), TSP (1:1000, Abcam), fibronectin 1 (1:1000 dilution, Aviva), clusterin (1:1000 dilution, Aviva), COMP (1:500 dilution, kindly donated from the Misumi laboratory).

Secondary antibodies were diluted in 3% (w/v) BSA to the following concentrations: 1:100,000 anti-rabbit (Bio-Rad) for MMP-1, MMP-3, MMP-13, fibronectin and clusterin or 1:40,000 anti-mouse (Bio-Rad) for COMP. All secondary antibodies were HRP conjugated to be compatible with Ampliflu™ Red detection.

5.2.3. Griess assay

Measurement of nitrite concentration was achieved using a Griess Reagent System (Promega) (Griess, 1879). A reference curve was prepared for each assay, starting with a 100 µM nitrite concentration before 6 serial two-fold dilutions were completed. This provided nitrite concentrations of 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0 µM, all diluted in DMEM + 2% Pen/Strep. Each experimental sample was assayed in triplicate with 50 µl added into a 96 well plate. Sulfanilamide solution (50 µl) was added to all wells and incubated for 5 minutes protected from light. Next, naphthylethylenediamine dihydrochloride (NED) solution (50 µl) was added and incubated for 5 minutes protected from light. Phenol red in culture media interferes with the ability to measure quantitative nitrite levels. Thus, interpolated levels of untreated and carprofen samples reported negative values, therefore optical density

(540 nm) values were recorded to compare treatments. Absorbance at 540 nm was measured within 30 minutes on a LT-4000 plate reader using Manta software (Labtech). Graphical and statistical analysis (Tukey's multiple comparisons) was completed with GraphPad Prism 6.

5.3. Results

5.3.1. Carprofen reduces IL-1β stimulated MMP-1 release

Qualitative differences in MMP-1 identifications between untreated and IL-1β supernatants analysed by MS were reported in Chapter 3, with Mascot scores up to 1678 reported in IL-1β samples after 6 days, while no score was associated with untreated samples (Table 7). To quantify MMP-1 release, western blotting and densitometry were completed as displayed in Figure 27. Release of MMP-1 was not detected in cartilage conditioned media from untreated or carprofen only treated extracts. In IL-1β and carprofen + IL-1β treated supernatants, bands visible at around 53 kDa corresponded to the expected molecular weight of MMP-1. The positive control provided an additional band at a slightly lower molecular weight, as well as the 53kDa band observed in secretome samples. Intense MMP-1 bands were initiated by IL-1β treatment. A decrease in IL-1β stimulated MMP-1 release was observed when carprofen is present alongside the cytokine. Densitometry reveals this decrease to be statistically significant, therefore carprofen is able to reduce release of this catabolic protein. This may indicate beneficial effects of carprofen treatment to lessen cartilage breakdown catalysed by MMP-1 during OA.

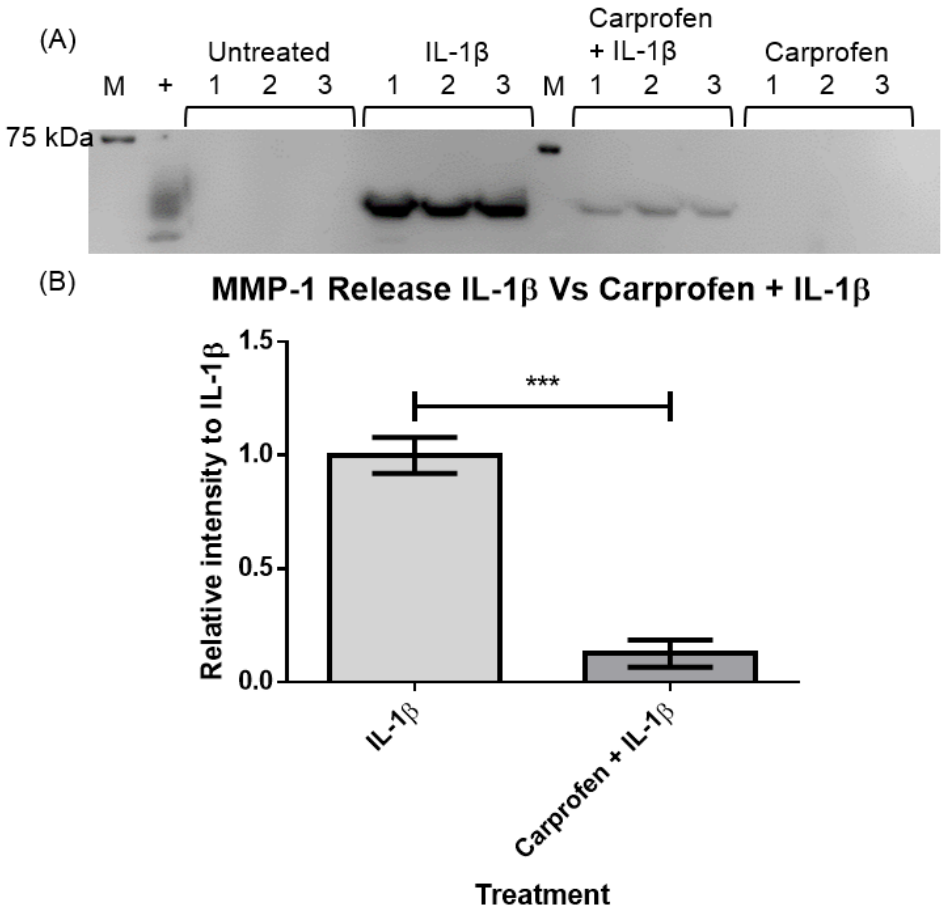


Figure 27. IL-1β stimulated release of MMP-1 is significantly decreased with carprofen treatment

(A) Representative image of western blot against MMP-1 on cartilage explant supernatant after 6 days of incubation. Treatments were as follows: untreated, IL-1beta (10 ng/ml), carprofen (100 µg/ml) + IL-1beta (10 ng/ml) or carprofen (100 µg/ml). 50µl of each cartilage supernatant sample was lyophilised, then resuspended in 20 µl Laemmli buffer and loaded onto gels. (B) Densitometry analysis on western blots (ImageJ, GraphPad Prism 6) from three horses, each with three treatment replicates (*n=9*). Error bars indicate standard deviation. M = molecular weight markers (Bio-Rad), + = positive control prepared by crushing articular cartilage with a mortar and pestle in liquid nitrogen and resuspending in Laemmli buffer, before being 5-fold diluted for loading.

5.3.2. Carprofen decreases IL-1β stimulated MMP-3 release

In previous chapters, MMP-3 was identified by nanoLC-MS/MS across all explant treatments. An increase in MMP-3 release was suggested by higher Mascot scores with IL-1β stimulation (956 in untreated samples, 5441 in IL-1β samples (Table 7)). To accurately quantify MMP-3 release across treatments, this chapter set out to western blot this protein in response to IL-1β and carprofen. At 54 kDa, MMP-3 bands are visible across all treatments studied (Figure 28). An additional band is stimulated in samples where IL-1β is present. MMP-3 is synthesized in a non-active zymogen form where cleavage of pro-peptide domain activates the enzyme (Woessner, 1991, Grossetete et al., 2009). The lower band therefore should correspond to the active form of MMP-3, which is activated by IL-1β signalling. Carprofen significantly decreased the cytokine stimulated MMP-3 when densitometry on western blots was completed. As MMP-3 has roles in ECM breakdown and activation of related procollagenases, carprofen treatment could affect MMP mediated degradation in OA.

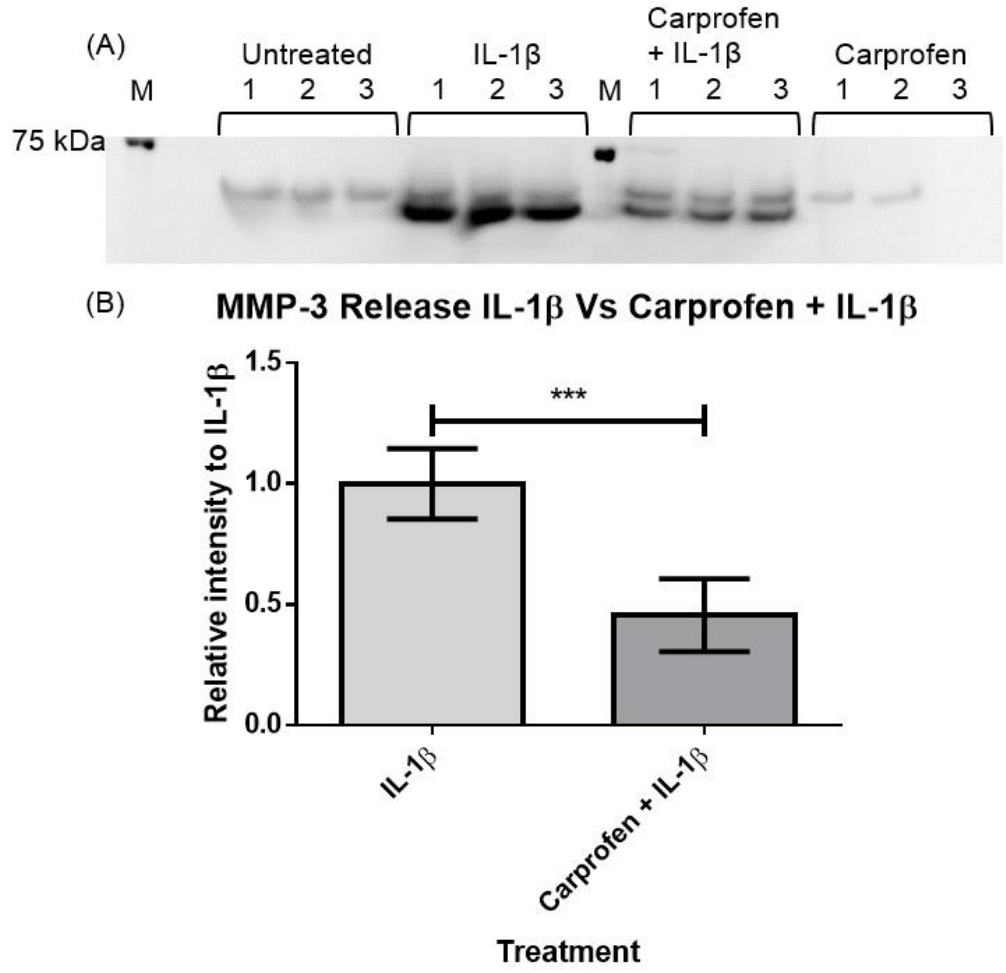


Figure 28. IL-1β stimulated release of MMP-3 is significantly reduced by carprofen

(A) Representative image of western blot against MMP-3 on cartilage explant supernatant after 6 days of incubation. Treatments were as follows: Untreated, IL-1beta (10 ng/ml), carprofen (100 µg/ml) + IL-1beta (10 ng/ml) or carprofen (100 µg/ml). 50µl of each cartilage supernatant sample was lyophilised, then resuspended in 20 µl Laemmli buffer and loaded onto gels. (B) Densitometry analysis on western blots (ImageJ, GraphPad Prism 6) from three horses with three technical replicates (*n=9*). Error bars indicate standard deviation. M = molecular weight markers (Bio-Rad).

5.3.3. IL-1β stimulated MMP-13 release is decreased by carprofen

MMP-13 was selected as a candidate protein to western blot after identification by MS of the cartilage secretome (Chapter 3) and its known roles in ECM degradation and OA (Knauper et al., 1996, Shiomi et al., 2010). In chapter 3, Mascot scores up to 1700 were reported with IL-1β stimulated samples, while untreated samples did not provide MMP-13 identification (Table 7). Throughout western blotting analysis in the current chapter, MMP-13 was not detected in untreated or carprofen only samples (Figure 29). When IL-1β was present, it caused MMP-13 bands to be observed at 54 kDa (Figure 29). Fainter bands are visible with carprofen + IL-1β treatment, compared to intense bands with IL-1β alone. Densitometry confirmed statistically higher levels of MMP-13 for IL-1β, and lower levels with carprofen + IL-1β.

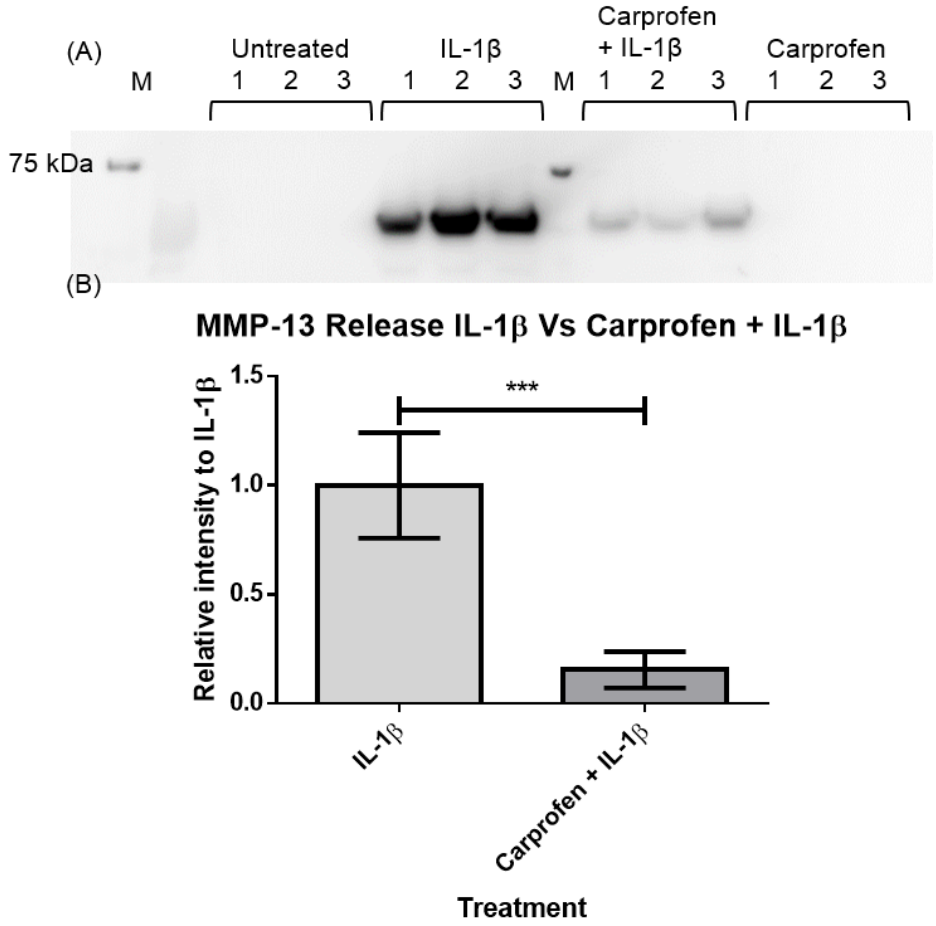


Figure 29. Carprofen decreases IL-1β stimulated MMP-13 release from cartilage explants

(A) Representative image of western blot against MMP-13 on cartilage explant supernatant after 6 days of incubation. Treatments were as follows: Untreated, IL-1beta (10 ng/ml), carprofen (100 µg/ml) + IL-1beta (10 ng/ml) or carprofen (100 µg/ml). 50µl of each cartilage supernatant sample was lyophilised, then resuspended in 20 µl Laemmli buffer and loaded onto gels. (B) Densitometry analysis on western blots (ImageJ, GraphPad Prism 6) from three horses with three replicates per treatment (*n=9*). Error bars indicate standard deviation. M = molecular weight markers.

5.3.4. Thrombospondin release stimulated by IL-1β is not affected by carprofen

Western blotting against TSP was completed on selected MMP-3 blotted membranes. MMP-3 along with other MMPs is known to target ECM substrates (Troeberg and Nagase, 2012), therefore TSP release was assessed by western blotting. TSP bands were detected around the expected 125 kDa in IL-1β and carprofen + IL-1β conditioned media (Figure 30). No TSP was detected in untreated and carprofen only samples. In Chapter 3, TSP was reliably identified across all treatments with high Mascot scores, but only IL-1β was capable of releasing TSP to detectable levels in western blots. Although IL-1β induced secretion and activation of MMPs can be decreased by carprofen, this does not have an impact on levels of TSP released from the ECM.

Figure 30. Thrombospondin release stimulated by IL-1β is not significantly decreased following carprofen + IL-1β treatment

Thrombospondin western blots produced a band at 125 kDa only observed when IL-1β stimulation was present. An unpaired t-test comparing IL-1β and carprofen + IL-1β bands reported no significant difference ($p>0.05$). Explant cultures were completed for 6 days with 3 treatment replicates from the same individual animals ($n=3$). Explant treatment: untreated control, IL-1beta (10 ng/ml), carprofen (100 µg/ml) + IL-1β (10 ng/ml) or carprofen (100 µg/ml). 50µl of each cartilage supernatant sample was lyophilised, then resuspended in 20 µl Laemmli buffer and loaded onto gels.

5.3.5. IL-1β stimulation of explant culture initiates degradation of COMP

One of the most abundant proteins in the cartilage explant secretome is COMP, which achieved high Mascot scores across all sample types in MS analysis (Chapter 3). Western blotting provided insights into COMP degradation occurring during cytokine stimulation of cartilage explants (Figure 31). The highest band (120 kDa) was released into supernatant in all treatments, and was the definitive band in the cartilage positive control. Cultures with IL-1β and carprofen + IL-1β contained an intense band slightly below (at 110 kDa). A fainter band is seen at 90 kDa across all samples, while another band around 10 kDa smaller (at 80 kDa) is observed in samples associated with IL-1β. Bands at 110 kDa and 80 kDa were only visible after explants are IL-1β stimulated, which suggests these bands correspond to degradation products. It was not possible to complete densitomeric quantification for COMP due to streaking and the close proximity of bands. Application of an ECL detection approach during future western blotting could allow development times to be altered, which could improve the ability to accurately quantify specific bands.

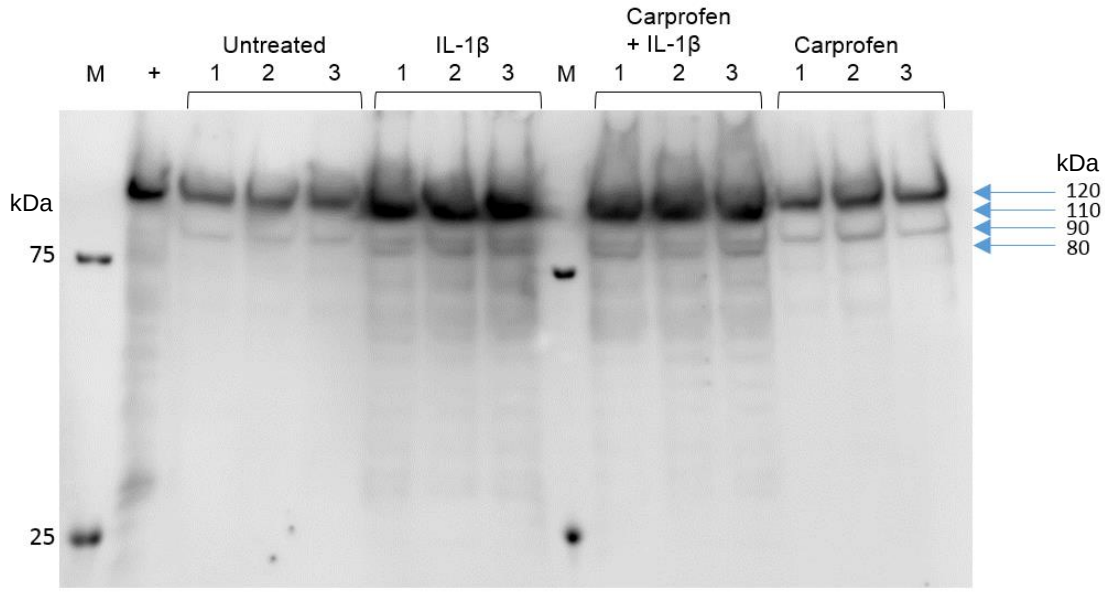


Figure 31. IL-1β stimulation of explant cultures initiates release and degradation of COMP

Western blot image against COMP shows four main bands corresponding to glycosylated COMP monomer (120 kDa) and lower COMP products (110, 90, 80 kDa). Explant culture was completed for 6 days with 3 replicates per treatment (*n=3*). Explant treatments: untreated control, IL-1β (10 ng/ml), carprofen (100 µg/ml), or carprofen (100 µg/ml) + IL-1β (10 ng/ml). M = molecular weight markers (Bio-Rad), + = positive control prepared by crushing articular cartilage with a mortar and pestle in liquid nitrogen and resuspending in Laemmli buffer. A 10-fold dilution of positive control stock was performed and 20µl was loaded. 50µl of each cartilage supernatant sample was lyophilised, then resuspended in 20µl Laemmli buffer and loaded onto gels.

5.3.6. Release of a fibronectin 60 kDa degradation product stimulated by IL-1β is reduced by carprofen

High-throughput MS identified fibronectin released into the cartilage secretome in chapter 3, reporting Mascot scores up to 6222 in untreated and 3786 in IL-1β samples (Table 7). Therefore it was selected as a candidate protein to assess differential release in response to IL-1β and carprofen. Fibronectin western blotting showed bands at 230 kDa, 60 kDa and 30 kDa. The glycosylated monomer of fibronectin corresponds to the highest molecular weight band at 230 kDa, the intensity of which was lower when IL-1β is present. This pro-inflammatory cytokine caused an intense band at 60 kDa to be detected, which is probably a degradation product released due to cytokine stimulation of catabolic enzymes. Carprofen caused a significant reduction in intensity of this IL-1β induced 60kDa band, suggesting this NSAID could potentially reduce certain mechanisms of ECM degradation.

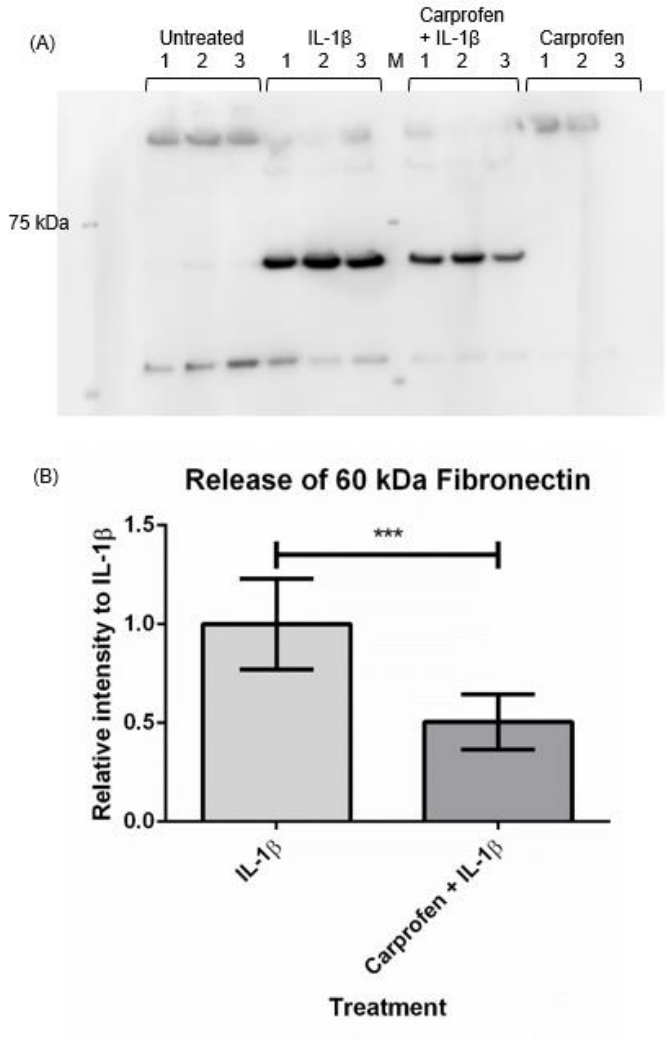


Figure 32. Fibronectin degradation induced by IL-1β was decreased by carprofen

(A) Representative image of fibronectin western blot. The band at 230 kDa corresponds to the glycosylated monomer of fibronectin. IL-1β stimulates release of a 60 kDa degradation product of fibronectin. 50µl of each cartilage supernatant sample was lyophilised, then resuspended in 20 µl Laemmli buffer and loaded onto gels. (B) Densitometry results for the 60 kDa fibronectin band. Explant cultures were carried out in 3 animals with 3 technical replicates per animal *(n=9)*. Standard deviation is shown by error bars. ***= P<0.001. Culture treatments: untreated (control), IL-1β (10 ng/ml), carprofen (100 mg/ml) or carprofen (100 mg/ml) + IL-1β (10 ng/ml).

5.3.7. IL-1β and carprofen treatments cause alterations in clusterin release

As well as catabolic proteins (MMP-1, -3, -13) and ECM components (TSP, fibronectin), the explant secretome contains secreted inflammatory related proteins. Clusterin is a chaperone that functions to protect proteins during inflammatory stress, therefore its secretion into explant supernatant was studied. The positive control of crushed cartilage produced bands in two positions: 70 kDa and 35 kDa, while detected bands in supernatants only gave bands at 35 kDa. The 70 kDa band in positive controls represents the full form before it is cleaved (de Silva et al., 1990). Clusterin is cleaved when secreted, hence no observation of the 70 kDa band and presence of the 35 kDa band in all secretome samples (Wilson and Easterbrook-Smith, 2000). Clusterin bands were most intense for untreated culture samples. IL-1β stimulated explants displayed reduced levels of clusterin release. Carprofen treatment alone also significantly decreased clusterin levels. When carprofen + IL-1β treatments were combined, there was a further decrease in clusterin levels. With this combination clusterin was almost undetectable, and was significantly lower compared to IL-1β alone.

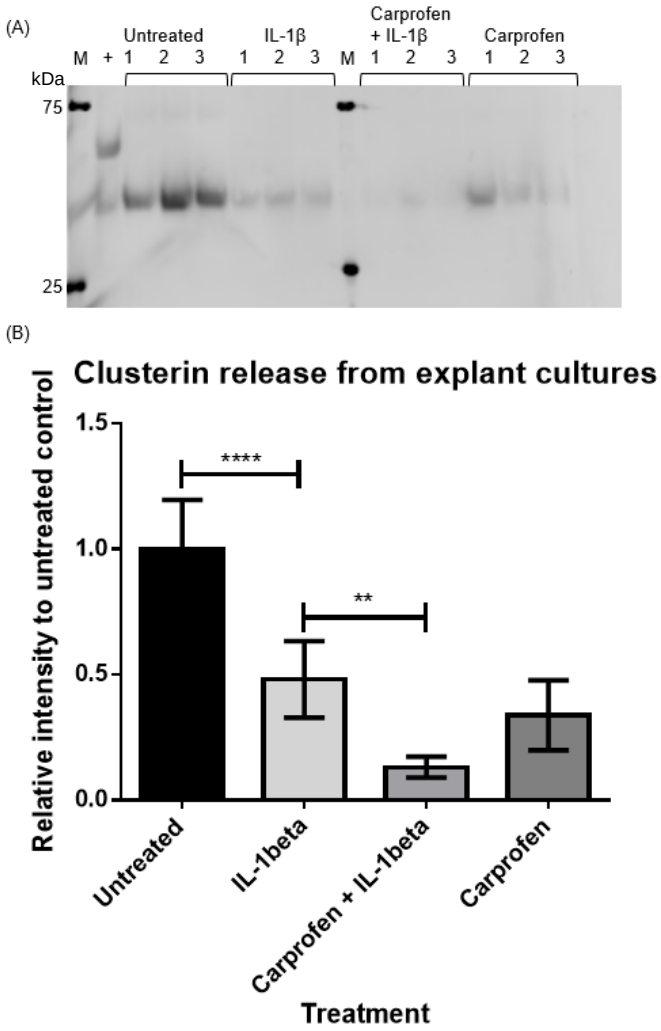


Figure 33. Clusterin release from cartilage explants is decreased by IL-1β stimulation and carprofen treatment

(A) Representative western blot image with clusterin precursor band at 70 kDa and 36-39 kDa representing secreted form. 50µl of each cartilage supernatant sample was lyophilised, then resuspended in 20 µl Laemmli buffer and loaded onto gels. (B) Graphical representation of densitometry for clusterin. Explant cultures were completed for 6 days in 3 separate animals, with 3 treatment replicates on each occasion (*n=9*). Explant treatments: untreated control, IL-1β (10 ng/ml), carprofen (100 µg/ml), or carprofen (100 µg/ml) + IL-1β (10 ng/ml). Error bars indicate Standard Deviation. A one-way ANOVA using Tukey's multiple comparison test was applied to assess significance. **** = $P < 0.0001$, *** = $P < 0.001$. M = molecular weight markers (Bio-Rad), + = positive control prepared by crushing articular cartilage with a mortar and pestle in liquid nitrogen then resuspending in Laemmli buffer. Positive control stock was diluted 5-fold before loading.

5.3.8. Nitric Oxide (NO) release is induced by IL-1β treatment and carprofen does not decrease NO release

Nitrite levels in explant conditioned media were measured across all treatment groups. NO is a free radical known to be produced by stimulation with pro-inflammatory cytokines (Taskiran et al., 1994). NO synthesized will be converted into nitrite, which is measured by the Griess assay (Griess, 1879). Treating explants with IL-1β or carprofen + IL-1β caused significantly higher levels of NO synthesis, compared to untreated and carprofen alone. Carprofen alone showed a slight non-significant increase in NO levels compared to untreated explants. There was no significant difference in NO levels between IL-1β and carprofen + IL-1β, therefore carprofen's anti-inflammatory mechanism did not reduce cytokine stimulated NO release.

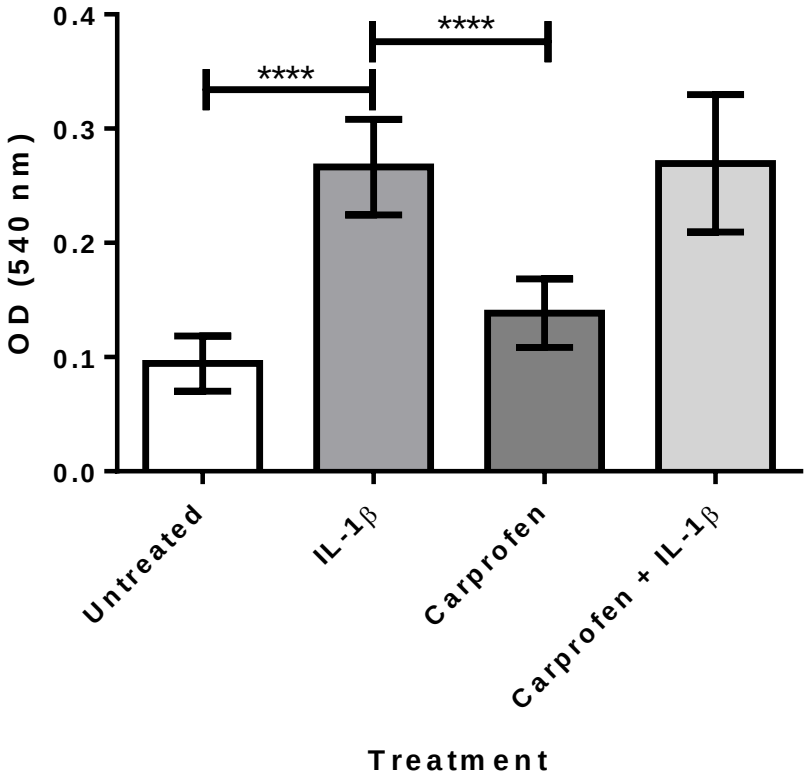


Figure 34. IL-1β treatment causes an increase in the presence of nitrites in cartilage explant supernatant. Carprofen did not significantly decrease cytokine stimulated nitrite levels

Griess assays were completed on explant supernatants collected from three individual horses with three treatment replicates per animal (*n=9*). Explant treatments: untreated control, IL-1β (10 ng/ml), carprofen (100 µg/ml), or carprofen (100 µg/ml) + IL-1β (10 ng/ml). Absorbance at 540 nm was measured in triplicate per sample. Error bars indicate Standard Deviation. One-way ANOVA using Tukey's multiple comparison test used to assess significance. **** = $P < 0.0001$.

5.4. Discussion

This chapter describes use of an equine cartilage explant model to screen anti-inflammatory therapeutics. Release of candidate biomarkers were monitored with western blotting, along with NO measured indirectly via Griess assays. Carprofen is applied here as an example therapeutic to assess anti-inflammatory effects on IL-1β stimulated explants. Inflammation of articular cartilage is stimulated by pro-inflammatory cytokines such as IL-1β, thereby causing degradation and loss of ECM in diseases like OA (Goldring, 2000). IL-1β binds to receptors on cell surfaces, initiating inflammatory signalling pathways including active NF-κB translocation into the nucleus. Transcription of pro-inflammatory and pro-apoptotic genes causes the release of catabolic proteins including MMPs, cathepsins and ADAMTS (Martel-Pelletier et al., 2008b). To simulate the environment within OA synovial joints, IL-1β stimulation is therefore often applied to culture models (Goldring et al., 2008). Studying cartilage explant models can provide mechanistic insights into inflammatory cartilage responses whilst providing alternatives to *in vivo* models. Candidate proteins (MMPs, ECM components, clusterin) identified in chapter 3 were selected for further western blot quantification. This provided further details on IL-1β stimulation of cartilage, and the abilities of explant models to screen anti-inflammatories such as carprofen.

5.4.1. MMP -1, -3 and -13 secretion from cartilage cultures and the effects of carprofen on these MMPs

MMP-1, MMP-3 and MMP-13 were released into the secretome of the equine articular cartilage model. Western blots confirmed release levels of all three MMPs were significantly raised after IL-1β treatment. These findings are consistent with iTRAQ™ reported increases in MMP-1, -3 and -13 release after cytokine stimulation of bovine explants (Stevens et al., 2009). IL-1β and MMP mediated enzymatic breakdown of cartilage explants contributes towards proteoglycan and collagen type II degradation (Mort et al., 1993, Billinghurst et al., 1997). The delicate balance between ECM synthesis and degradation could therefore be tipped in favour of OA cartilage degradation during inflammatory conditions.

Western blotting detected release of MMP-3 from untreated explants, but significantly higher levels and a band 10 kDa lower were stimulated by IL-1β. Inactive proMMP-3 is cleaved into the active form via protease activity (Nagase et al., 1990). The MMP-3 band 10 kDa lower is likely to be active MMP-3 released by IL-1β stimulation. This highlights the ability of western blotting approaches to potentially identify active forms of catabolic proteins.

This study shows that measurements of MMP-1, MMP-3 and MMP-13 released from IL-1β stimulated explants *in vitro* can help screen anti-inflammatory compounds. Significant decreases in IL-1β stimulated release of all three MMPs were associated with carprofen presence. The effects of new and existing therapeutics on MMP release can therefore be screened via this western blotting approach. Researchers have shown in previous equine explant studies that carprofen decreases IL-1β stimulated PGE_2 and GAG release *in vitro* (Benton et al., 1997, Armstrong and Lees, 1999). Inflammatory stimulated MMPs were also decreased by hyaluronan treatment in a similar human cartilage explant model (Julovi et al., 2004). Degradation products of ECM components such as fibromodulin can be specifically attributed to MMP-13 (Heathfield et al., 2004). Thus, carprofen's inhibition of MMP-13 could decrease release of certain ECM degradation products.

Carprofen's actions as a COX-2 inhibitor have been shown here to have downstream effects on MMP release in equine cartilage explant cultures. Prostaglandins including PGE_2 are produced via COX-2 enzymatic activity in the arachidonic acid signalling pathway (Hardy et al., 2002), and PGE_2 will induce release of MMP-1, MMP-13 and other inflammatory effects (Attur et al., 2008). Carprofen may therefore reduce catabolic processes during OA and slow down loss of cartilage.

5.4.2. Nitric Oxide release from equine cartilage culture

This chapter also monitored levels of NO production in response to IL-1β and carprofen, via Griess assay measurements of nitrite. NO has complicated roles in mediation of apoptosis, catabolic signalling and pain perception (Abramson, 2008). Progression of OA is associated with death of chondrocytes, therefore NO control of

apoptosis could have implications in the disease. Significantly higher levels were recorded due to IL-1β signalling, whilst carprofen did not appear to decrease levels of NO in cartilage conditioned media. Increases in NO release are observed from OA cartilage explants (Amin et al., 1995), therefore this cytokine causes similar inflammatory processes to be initiated. IL-1β stimulated NO release from equine chondrocytes has also been reported (Benton et al., 2002). As carprofen inhibition of COX-2 does not decrease NO levels, alternative pathways activated by IL-1β contribute to production of NO. This has consequences in OA as synthesis of proteoglycans in cartilage is inhibited by increased levels of NO (Mastbergen et al., 2008). An alternative pathway involving MAPK/ERK signalling can be activated by cytokines and causes expression of various pro-inflammatory processes during OA (Loeser et al., 2008). While this chapter concentrated on inhibition of COX-2 signalling, explant models can be utilized to distinguish between different inflammatory/anti-inflammatory mechanisms. For example, ERK inhibitors applied to bovine explants assessed this signalling pathways role in responses to mechanical compression (Ryan et al., 2009).

5.4.3. ECM proteins and release of degradation fragments in response to IL-1β

After finding that carprofen decreases levels of MMPs secreted, the consequences on release of specific ECM proteins: fibronectin, TSP and COMP were assessed. In this study there was no convincing evidence that IL-1β stimulated release of TSP or COMP was effected by carprofen treatment. TSP was undetectable by western blotting in untreated cultures, yet was identified in untreated cultures by MS in chapter 3. IL-1β stimulation could therefore be causing increased release of a specific form of TSP that the primary antibody recognises. Alternatively, a more accurate quantitative MS approach may have demonstrated differential release of TSP comparing untreated or IL-1β stimulated cultures. Four bands detected at 120, 110, 90 and 80 kDa molecular weights were visible with COMP western blots. COMP is known to be glycosylated, therefore this relates to the 120 kDa band. The 90 kDa band is potentially non-glycosylated COMP. IL-1β stimulation was responsible for

presence of 110 and 80 kDa bands, which are likely to be degradation projects of COMP. Additional COMP fragments have been reported in western blots of diseased equine synovial fluid, including OA (Misumi et al., 2001). As carprofen has significantly inhibited MMP secretion, IL-1β stimulated release of TSP and COMP may be primarily caused by other catabolic proteins.

Studies on human OA cartilage explants have demonstrated that release of collagen neoepitopes is reduced by MMP inhibition, but GAG release is not decreased (Dahlberg et al., 2000). This demonstrates that degradation of certain ECM components are controlled by different catabolic proteins, which has been observed in this thesis. Inhibition of MMPs in bovine explants can reduce IL-1β stimulated COMP fragmentation (Ganu et al., 1998). ADAMTS-4 has also been identified as another mediator of COMP degradation (Dickinson et al., 2003). Therefore multiple catabolic proteins can contribute towards COMP breakdown. Novel therapeutics inhibiting specific catabolic processes including ADAMTS family proteins, could be screened within an explant model to assess effects on TSP and COMP release.

Fibronectin proved a useful candidate marker for assessing IL-1β stimulation and the beneficial properties of carprofen treatment. Cartilage explants stimulated with IL-1β released significant amounts of a 60 kDa fibronectin degradation product, indicating ECM degeneration *in vitro*. Bands at 230 kDa representing the fibronectin monomer were visually reduced with cytokine stimulation. This suggests degradation of this monomer form is occurring. Carprofen was able to significantly decrease generation of 60 kDa fragments when added along with IL-1β. Fibronectin is therefore an appropriate marker for assessing certain anti-inflammatory processes within the explant model.

This western blotting approach offered additional details on the degradation profile of fibronectin induced by IL-1β. Distinct bands at 230 kDa and 60 kDa displayed differences associated with treatment types. In chapter 3, MS analysis alone did not provide this degradation information on fibronectin. The merits of western blotting ECM proteins to assess degradative information from cartilage

explants have also been shown in a study by (Monfort et al., 2006). In that study, MMP-13 induced specific degradative profiles of small leucine-rich proteoglycans including decorin, biglycan, fibromodulin and lumican (Monfort et al., 2006). Fibronectin data in this chapter has also demonstrated an advantage of western blotting analysis of cartilage conditioned media over MS analysis.

In previous studies, explant models have shown the role of fibronectin fragments in IL-1β stimulation of MMP-13 release and collagen type II degradation (Yasuda and Poole, 2002). Fibronectin fragments also cause MMP-3 and MMP-13 release, and decreases in proteoglycan synthesis in explant models (Homandberg et al., 1997, Homandberg et al., 2006). Therapeutic screening of hyaluronic acid, glucosamine-HCL and chondroitin sulphate demonstrated reduced MMP activity in these fibronectin stimulated models (Homandberg et al., 1997, Homandberg et al., 2006). Stimulation of equine cartilage with two fibronectin fragments has also reported differences in release of NO, COMP and chondroadherin (Johnson et al., 2004). These studies and findings in this chapter, emphasize the importance of fibronectin degradation in OA, and therefore the utility of cartilage explant models for monitoring its release.

5.4.4. Clusterin release within explant models and OA

The equine cartilage explant model exhibited highest clusterin secretion in untreated cultures. IL-1β or carprofen treatment alone caused a reduction in clusterin secretion, whilst in combination these treatments caused another significant decrease. Clusterin acts as a chaperone to aid protein refolding in situations of stress and is constitutively secreted by mammalian cells (Wilson and Easterbrook-Smith, 2000). IL-1β and carprofen both appear to interrupt clusterin secretion and therefore the protection it offers from healthy functioning cells. Normal COX activity may have a role in constitutive clusterin secretion, therefore inhibition of COX by carprofen may affect its release. An additive inhibition by actions of IL-1β and carprofen combined contributed to a more significant reduction of clusterin release.

Previous studies have reported that clusterin levels were decreased by IL-1β treatment compared to untreated controls in mouse femoral head cartilage culture media (Wilson et al., 2008). In another study, increases in clusterin levels in culture media were observed with IL-1β and TNF-α treatments on bovine articular cartilage (Stevens et al., 2008). During investigations on chondrocyte mitochondrial deregulation, clusterin levels appeared decreased in OA chondrocyte mitochondrial fractions compared to healthy cartilage (Ruiz-Romero et al., 2009). As indicated in this chapter's introduction, clusterin association with OA needs to be further investigated. This thesis provides some insights on clusterin release during *in vitro* cartilage cultures.

5.4.5. Conclusions

Studies in this chapter described the applications of equine cartilage explant models to monitor degradation of ECM proteins, catabolic processes and inflammatory related proteins in response to stimuli. Carprofen decreased release of candidate biomarkers MMP-1, MMP-3, MMP-13 from IL-1β stimulated explants. The reduced release of MMPs was correlated with the decrease of an IL-1β stimulated 60 kDa fibronectin product. Although, carprofen did not affect COMP or TSP release from IL-1β explant cultures. Certain processes instigated by IL-1β are not affected by carprofen, such as raised NO levels that were not lowered with carprofen treatment. Secretion of the protective chaperone clusterin was decreased by IL-1β and carprofen, which could have implications on protein folding and aggregation in OA affected joints. New anti-inflammatory drugs or nutraceuticals can be screened for their effects on MMP activity in this *in vitro* approach. Effects on other essential proteins involved in cartilage metabolism could also be monitored with this model if effective and specific antibodies are available. This model provides an alternative to *in vivo* animal models of OA, delivering insights into cartilage degeneration, responses to inflammatory stimuli and assessing the potential effects of new therapeutics.

CHAPTER 6

GENERAL DISCUSSION

6.1. Introduction

The aim of this thesis was to apply high-throughput proteomic approaches to the secretome of cultured equine articular cartilage explants. This approach investigated protein release from untreated healthy cartilage and in response to inflammatory stimulation with IL-1β and anti-inflammatory treatment with carprofen. It was hypothesised that proteomic investigations would identify candidate biomarkers associated with OA, to then monitor treatment efficacy and degradative processes in this explant model using more sensitive, quantitative approaches. Carprofen was shown to have advantageous effects on IL-1β stimulated cartilage, such as decreasing release of GAG and ECM proteins as well as decreasing secretion of catabolic proteins (e.g. MMPs). This suggests there are beneficial properties of this NSAID for OA treatment. These studies advanced biological understanding of cartilage explant cultures and OA, allowing biomarker discovery and screening of anti-inflammatory compounds.

These studies were completed on an equine derived cartilage model and were initiated because of an interest in veterinary and equine health. Moreover, joint disease in horses is a common clinical issue with lameness often associated with OA (Clegg and Booth, 2000). Equine OA therefore has a significant economic impact, through veterinary costs to owners of companion animals, or via financial losses in the horse racing industry (Neundorf et al., 2010). The ability to screen putative anti-inflammatory compounds for treatment of horses is therefore of importance. For scientific study purposes, equine joints allow high numbers of homologous explant discs to be processed. Horses are active animals compared to other large animals, therefore cartilage harvested will have been exposed to an active lifestyle like humans. Sourcing human tissue has added ethical complications and normal/healthy cartilage is not easily available. The thickness of articular cartilage from humans and

horses was reported to be most similar compared to cartilage from rabbits, sheep, dogs and goats, therefore studying OA pathological processes in equine cartilage could provide pre-clinical insights that are also relevant to humans (Frisbie et al., 2006).

6.2. The secretome of articular cartilage

Proteomic studies on the secretome of articular cartilage have been reported on human (Hermansson et al., 2004, De Ceuninck et al., 2005, Nemirovskiy et al., 2007, Polacek et al., 2010, Peffers et al., 2013), murine (Wilson et al., 2008, Haglund et al., 2008), bovine (Stevens et al., 2008, Stevens et al., 2009), equine (Clutterbuck et al., 2011) and canine (Swan et al., 2013) derived tissues. Highly abundant ECM proteins reported here in equine secretome, such as COMP, aggrecan core protein and various small proteoglycans are consistently identified across all these studies. In chapter 3, time course studies were performed to gain a better understanding of progression of pro-inflammatory and anti-inflammatory processes, and their effects on the cartilage secretome. Interrogation of time courses aimed to assess the most suitable time to maximise differentiation between treatments. An initial time course over 27 days (time course A) demonstrated altered protein release from cartilage between untreated and pro-inflammatory cytokine (IL-1β and TNF-α) stimulated cartilage. The NSAID, carprofen caused gradually more substantial decreases in a cytokine stimulated MMP-3. Initial results suggested that further optimisation of the time course was required. Therefore as described in chapter 3, a 24 day time course (time course B) was completed and high-throughput MS analysis was performed on day 6 and day 18 time points. Proteomic high-throughput MS analysis of a time course of cartilage secretome has not previously been reported in the literature.

A previous study was published using high-throughput MS on cultured equine articular cartilage (Clutterbuck et al., 2011). In the mentioned publication, IL-1β stimulation and carprofen treatment were applied to the cultured explants, but no correlation with treatment groups was reported in the MS data, whilst MS data in this thesis showed specific treatment group effects. All ECM and non-ECM secreted

proteins previously found were also identified throughout this thesis, with additional identifications attained here including MMP-13, TIMP-1, collagen type II, PCOC-2, CXCL6 and SFRP. Various different intracellular proteins were reported in the secretome of equine cartilage compared to this thesis (Clutterbuck et al., 2011). The secretome studies in chapter 3 have expanded the range of protein identifications and provided further information on proteins released in an equine model of articular cartilage.

Release of collagen has been reported to occur at higher levels after extended incubation times of bovine explants up to 14 days (Sztrolovics et al., 1999), and feline explants up to 28 days (Gabriel et al., 2010)). In equine cartilage explants, collagen peptides were identified in supernatant after both 6 and 18 days, although no specific quantitative measure of collagen release was employed in this thesis. The continued identification of small proteoglycans and collagen later in the time course, agrees with the study by (Sztrolovics et al., 1999), which showed release of these ECM components up to 14 days. IL-1β stimulated release of biglycan, decorin and lumincan had previously been shown to occur at consistent levels over a time course using bovine cartilage explants (Sztrolovics et al., 1999), whilst this thesis also reported similar release after 6 and 18 days of culture. Monitoring carprofen treatment on IL-1β stimulated cultures showed that carprofen reduced levels of small proteoglycan release, especially after 18 days (chapter 3). This suggests that these small proteoglycans may be useful biomarkers of treatment efficacy.

Analysis of cartilage secretome of untreated and IL-1β treated explants revealed differences in protein release (chapter 3.3.3.). These differences between treatments could benefit from quantitative analytical approaches including iTRAQ™, SILAC and QconCAT, which are described later in this discussion section. In chapter 3, a variety of ECM components, inflammatory related proteins, cytokines, chemokines, catabolic proteases and intracellular proteins were identified. Previous 2DE human OA cartilage secretome analysis highlighted the identification of activin A, cytokine-like protein 17 and CTGF (Hermansson et al., 2004), whilst data in this thesis

only identified CTGF in untreated culture (Table 7). Another study on human OA cartilage secretome using 2DE and protein microarrays identified several ECM proteins and secreted proteins MMP-3, TIMP-1 and PCOC (De Ceuninck et al., 2005), which were also detected in the equine secretome MS in chapter 3. The OA associated cytokine IL-6 and chemokine IL-8 were not identified in this thesis, but were found in human OA secretome (De Ceuninck et al., 2005). Comparison with these previous studies demonstrates the differences in studying OA diseased cartilage and healthy cartilage cultures.

Identification of collagen types VI and X only in untreated controls suggests that IL-1β stimulation interferes with normal collagen synthesis. Alternatively, increased release of other degraded proteins due to IL-1β, obscured detection of these collagen types. Future studies with increased sample numbers could further support this observation, as previous studies have reported that collagen type VI release into supernatant is increased by cytokine treatment of cartilage (Stevens et al., 2009) and chondrocytes (Calamia et al., 2011).

Catabolic factors contribute to OA within synovial joints (Troeberg and Nagase, 2012) and the main MMPs secreted by IL-1β stimulated cartilage were MMP-1, MMP-3 and MMP-13. It has been suggested that MMP-2 is secreted from cartilage explants that were analysed via gelatin zymography (Clegg et al., 1997), but gelatin classified MMPs -2 and -9 were not identified in the cartilage secretome in this thesis. Overall, these secretome studies identified expected ECM proteins and metabolic proteins as reviewed in (Williams et al., 2011). Certain proteases associated with OA such as cathepsins and ADAMTS (Troeberg and Nagase, 2012) were not identified in any secretome MS analysis in this thesis.

To increase the depth of coverage of the secretome, depletion strategies were considered to remove abundant proteins from cartilage cultures. It was hypothesised that by targeting and depleting abundant proteins, identification of additional low level proteins could be achieved. Three methods were applied prior to high-throughput MS analysis: CPC precipitation, Con A lectin chromatography and

Proteominer™ technology. Abundant proteins were removed with these applications, and comparison with unprocessed samples concluded that identification of certain proteins was facilitated. CPC precipitation assisted identification of HAPLN1, CILP-1 and MMP-10, while Con A chromatography supported HAPLN1 and IL-9 detection. CPC precipitation has previously been applied to cartilage proteomic studies to increase resolution with 2DE (Hermansson et al., 2004, Catterall et al., 2006, Hermansson et al., 2007, Ruiz-Romero et al., 2010a), while there are no reports in the literature of depletion methods being applied to cartilage secretome before high-throughput MS analysis. Various intracellular proteins were identified after the depletion processes, but these are most likely indicators of cell death and have limited value to understanding cartilage metabolism. Proteominer™ analysis did not produce additional identifications that had not been previously reported in the thesis. The Proteominer™ technique has potential in cartilage proteomics but requires further development for secretome analysis. Co-extraction of low abundance proteins along with the abundant ECM proteins could be responsible for the difficulties in application of the depletion methods. ECM constituents can bind to smaller proteins such as growth factors and cytokines (Heinegard, 2009), therefore low abundance proteins may remain bound during depletion procedures and also be removed.

6.2.1. Quantitative secretome MS analysis

Secretome studies have identified the most abundant proteins being released in cartilage explant cultures (Table 1). To improve proteomics analysis and comparisons of different treatment conditions, future studies could benefit from quantitative analysis. Traditional 2DE analysis or DIGE techniques can differentiate between protein levels within acrylamide gels, while there are several options to quantify high-throughput MS approaches (Bantscheff et al., 2012). Discussed below, are the latest studies on cartilage secretome that have applied quantitative techniques such as label-free spectral counting and bioinformatics methods (Mateos et al., 2012, Swan et al., 2013), or labelled approaches including iTRAQ (Stevens et al., 2009) and QconCAT (Peffers et al., 2013).

Application of bioinformatics approaches to generate data would aid interpretation of these complex data sets. Throughout this thesis, MS data from Mascot searches was manually organised into tables for comparison between different treatments and/or time points. Semi-quantitative analysis suggested by Mascot scores alluded to differences between treatment groups, but there is no statistical comparison of these differences. Peptide spectral counting and exponentially modified protein abundance index (emPAI) scores could be used for bioinformatics approaches to provide statistically verified relative quantitative measures of proteins in different sample types. An emPAI score is calculated to provide a relative quantitative score of proteins in a mixture (Ishihama et al., 2005). Larger data sets are likely to be required for application and validation of bioinformatics methods of label-free quantification. An analysis of synovial fluid from RA and OA patients which quantified peptides with spectral counting discovered differential ECM proteins associated with OA, including COMP, fibronectin and YKL-40 (Mateos et al., 2012). Fibronectin release was shown to be a useful marker of carprofen efficacy in chapter 5 of this thesis, whilst COMP western blots were inconclusive. Machine learning and network analysis on MS data generated from a canine explant model has been published (Swan et al., 2013). These bioinformatics approaches classified samples into treatment groups: untreated control, IL-1β, carprofen or carprofen + IL-1β (Swan et al., 2013). MMP-3, IL-8 and matrix Gla protein were highlighted as important proteins for treatment classification (Swan et al., 2013). The explant model applied in this study was essentially the same as within this thesis, apart from the use of canine cartilage rather than equine tissue. The absence of IL-8 and matrix Gla protein identification in all Swiss-Prot searches of equine derived secretome in this thesis may underline the influence of species differences. It should be noted that the equine IL-8 sequence is present in the Swiss-Prot database. The matrix Gla protein was identified in NCBInr searches of data in this thesis (Chapter 3, Table 8), but the equine sequence to this protein is currently not in the Swiss-Prot database. Such apparent species differences could be due to variability in peptide sequences causing protein identifications to be missed if there is insufficient

sequence identity in the peptides detected by MS. Alternatively there may be genuine species specific biological responses. Follow-up western blot studies on canine and equine cultured cartilage could be used to confirm or disprove these observations. The bioinformatics approaches described by (Swan et al., 2013), could be applied to equine explant secretome studies if suitable sample numbers are available.

Labelling peptides with isobaric tags such as iTRAQ™ to provide relative quantitation of peptide levels has been applied to cartilage secretome (Stevens et al., 2009), as well as whole cartilage (Onnerfjord et al., 2012, Ikeda et al., 2013) and serum (Fernandez-Puente et al., 2011). Many of the proteins identified by MS analysis in this thesis were also reported in another study, which found cartilage stimulated with IL-1β, TNF-α or mechanical stimulation showed quantitative differences in release of aggrecan, collagens, MMPs, cytokines and chemokines (Stevens et al., 2009). As reported in this thesis, differential release of MMP-1, -3, -9 and -13 also occurred upon cytokine stimulation (Stevens et al., 2009). Only one peptide from ADAMTS-4 was detected with cytokine stimulation (Stevens et al., 2009). High-throughput analysis of equine cartilage secretome did not detect ADAMTS family proteins in this thesis. Chemically synthesised peptide standards such as QconCAT allow absolute quantification of proteins in complex samples, and this approach has been applied to the OA cartilage secretome (Peffers et al., 2013). Comparison of human OA cartilage stimulated with IL-1β or unstimulated reported that IL-1β increased MMP-3 and decreased TIMP-1 levels (Peffers et al., 2013). Absolute quantitative analysis did not report significant differences in release of COMP, fibromodulin, plasminogen, aggrecan and MMP-1 when OA cartilage was stimulated with IL-1β (Peffers et al., 2013). This previous QconCAT study utilized OA diseased cartilage, whilst the equine model in this thesis used healthy cartilage, therefore this may account for certain differences observed between these studies.

In chapter 3, the effects of carprofen on IL-1β cultures analysed by MS, gave semi-quantitative indications that ECM proteins like fibronectin were reduced by the NSAID. Screening of anti-inflammatories on IL-1β stimulated cartilage explant cultures

could be assisted with quantitative analysis such as iTRAQ or a similar approach, coupled with appropriate bioinformatics analysis.

6.2.2. Consequences of differences in species protein sequence

Relatively few antibodies directed specifically against equine proteins are available as compared to the vast array of antibodies that recognise human or mouse proteins. The majority of available primary antibodies produced are directed against human derived protein sequences, rather than equine proteins. Cartilage ECM proteins and MMPs have similar sequences in humans and horses, allowing successful western blotting against these proteins. However, a number of potentially relevant secreted proteins may not be detected unless equine-specific primary antibodies are available. For example, a primary antibody against PCOC-2 did not recognise this protein in equine cartilage conditioned media (data not shown). PCOC-2 is involved in collagen maturation and its release was suggested to be inhibited by IL-1β in data shown in chapter 3. With a lack of quantifiable MS data, cartilage metabolism insights could have been provided by western blots of PCOC-2, but this was not possible using equine cartilage models. This limitation could apply to other proteins therefore this represents a challenge in studying proteins in equine derived tissues. Human protein directed primary antibodies are more readily available, therefore utilising human cartilage models could improve opportunities to study cartilage degradation and OA. Alternatively, equine protein specific antibodies could be raised for use in equine cartilage explant models.

Proteins identified during MS analysis relied on detected peptide sequences being searched against databases of known proteins/peptides. Human and other species proteins have been entered into these databases more extensively than equine entries. Conserved protein sequences across many species allowed equine derived proteins to be matched to other species entries. Species differences in protein sequences will impact on Mascot scores achieved, as just one different residue will cause variation in Mascot scores. Proteins could potentially be missed if sequence differences are substantial and equine entries are missing. If only a few peptides of a

certain protein are detected, even small differences in the sequence of equine proteins could cause failure to match, when otherwise protein identification would have occurred if there was no species sequence variation.

6.3. The effects of carprofen on IL-1β stimulated cartilage and biomarkers of treatment efficacy

This thesis studied release of candidate biomarkers of efficacy within the explant model in response to pro-inflammatory cytokine IL-1β and NSAID carprofen. These included catabolic MMP-1, MMP-3, MMP-13, ECM components fibronectin, TSP, COMP, and clusterin. IL-1β stimulation increased release of MMP-1, -3 and -13, and degradative products of cartilage ECM proteins. ECM degradation products such as fibromodulin have been associated with MMP-13 activity (Heathfield et al., 2004). Therefore, the observed carprofen reduction of MMP-13 could decrease release of certain ECM proteins that could be efficacy biomarkers. Here, the IL-1β initiated release of MMPs and a fibronectin degradation fragment were all significantly decreased by carprofen treatment. This data therefore showed these three MMPs can help screen therapeutic effects on IL-1β stimulated explants *in vitro*. MMP-1 and MMP-3 have been shown as prognostic biomarkers of OA, as levels of these two MMPs in serum were correlated with cartilage volume loss in human patients (Pelletier et al., 2010). A previous study reported stimulated release of MMPs was reduced by hyaluronan treatment in an explant model using human cartilage (Julovi et al., 2004). In chapter 5, a fibronectin degradation fragment was assessed as a biomarker of treatment efficacy, with carprofen able to significantly decrease generation of the 60 kDa fragment stimulated by IL-1β. Fibronectin proved to be an appropriate marker for assessing certain anti-inflammatory processes in equine explant cultures.

Carprofen decreased the release of other IL-1β stimulated proteins from the ECM including biglycan, fibromodulin, chondroadherin and CILP. This observed effect in response to carprofen, suggests these ECM proteins could be further investigated as biomarkers of treatment efficacy. Other potential biomarkers of efficacy include

PCOC-2, HSP-70, CXCL6 and MIF as release of these proteins was effected by IL-1β stimulation. Carprofen was also shown to have beneficial effects on chondrocyte survival as the numbers of intracellular proteins identified by MS on cultures stimulated with IL-1β were lower when carprofen was present. Thus, cultured equine cartilage explants provide a useful model to evaluate potential anti-catabolic and anti-inflammatory effects *in vitro*, in accordance with the 3Rs.

The effect of carprofen and IL-1β on other factors associated with pro-inflammatory cytokine stimulation and OA were also assessed in the explant model, including measurements of sulphated GAG release and NO synthesis. Measurement of GAG release evaluated degradation of cartilage, while NO free radical production gave an indication of inflammation. The explant model therefore provided evidence of additional aspects of IL-1β and carprofen treatment. Increased levels of GAG were detected with IL-1β stimulation, indicating breakdown of cartilage components. Carprofen has previously been shown to decrease IL-1β stimulated PGE_2 release from equine explants (Benton et al., 1997), which will reduce inflammatory signalling and associated degradation of the ECM. A study published in 2013, has also shown that carprofen (at the concentration applied in this thesis of 100 µg/ml), significantly decreases IL-1β stimulated release of GAGs and PGE_2 from equine cartilage explants (Clutterbuck et al., 2013). Increases in proteoglycan synthesis have also been revealed in equine cartilage explants treated with carprofen enantiomers (Frean et al., 1999). In this thesis, carprofen significantly delayed IL-1β initiated GAG release over 6 days. Monitoring GAG release through a time course revealed this effect to be transient.

NO synthesis in explant culture was increased by IL-1β treatment compared with untreated cultures. Carprofen treatment had no effect on NO levels, suggesting IL-1β initiation of certain signalling processes is not influenced by COX inhibition. Other researchers also found that treatment of cartilage explants with the NSAID celecoxib did not decrease cytokine stimulated GAG and NO release, but inhibited

PGE_2 levels (Mastbergen et al., 2008). This shows that NSAIDs do not inhibit all cytokine stimulated processes that cause inflammation and cartilage break down.

Overall, these studies have contributed to understanding of equine cartilage secretome and the capability of this model for screening anti-inflammatory effects. By studying the effects of carprofen on IL-1β stimulated protein release, a number of potential biomarkers of treatment efficacy were identified. There continues to be certain limitations to the work described and several amendments could assist future studies with cartilage cultures are discussed in more detail below.

6.4. Prospective adaptations to the explant model

The explant model was chosen to study responses to stimuli because it maintains chondrocytes within an ECM environment. This is closer to the *in vivo* environment when compared to monolayer and high density cultures. It also provides the opportunity to study metabolic effects on the existing cartilage matrix that would otherwise be absent. Explant models have been historically applied to study cartilage degradation in response to pro-inflammatory cytokines (Saklatvala, 1986, Hubbard et al., 1988). Culture of healthy and OA affected cartilage is also suitable to demonstrate metabolic changes in diseased tissue (Lafeber et al., 1992). Additional treatment with proposed therapeutics on IL-1 stimulated explants can then assess beneficial properties. For example, sodium hyaluronate or glucosamine HCL can reduce IL-1 stimulated proteoglycan degradation in explant cultures (Morris et al., 1992, Fenton et al., 2000). This thesis has shown effects on release of selected ECM proteins and MMPs by utilizing an IL-1β stimulated equine cartilage model. The high-throughput MS analysis showed variation in proteins released due to treatment with IL-1β as compared with IL-1β + carprofen, although it would benefit from quantitative approaches (discussed above). The explant model applied throughout this thesis remains a relatively simple model, which is missing several components that contribute natural conditions in synovial joints. Adaptations to the explant model that

could further advance the studies in this thesis and the OA literature are discussed below.

6.4.1. Alternative stimuli

Inflammation of the synovial joint has an important role in OA progression (Scanzello and Goldring, 2012). Toll-like receptors, complement activation and pro-inflammatory mediators are all associated with OA (Scanzello and Goldring, 2012). Alternative pro-inflammatory stimuli TNF-α (Chapter 3) and LPS (which activates Toll-like receptors) (Appendix 3) were also introduced to equine cartilage cultures during this project. The responses to these stimuli were assessed on silver stained SDS-PAGE gels. The protein profiles produced after treatment with TNF-α or LPS were visually indistinguishable from those after IL-1β stimulation. Other studies on bovine cartilage secretome (Stevens et al., 2009) and human chondrocyte proteome (Cillero-Pastor et al., 2010) have reported differential protein responses to IL-1β or TNF-α. Stimulating the equine cartilage explant model with alternative stimuli such as IL-6 could highlight differences in initiated protein release.

Obesity is a risk factor associated with OA (Coggon et al., 2001). Adipokines secreted from adipose tissue potentially have roles in OA progression. Serum adipokines: adiponectin, leptin and resistin have been associated with inflammation in OA patients (de Boer et al., 2012). Another adipokine, visfatin caused increased synthesis of MMP-3, MMP-13, ADAMTS-4, ADAMTS-5 and PGE_2 in chondrocyte monolayers (Gosset et al., 2008). The equine model utilized in this thesis proved successful for measuring release of several MMPs, therefore a similar approach could monitor responses to adipokines.

Growth factors that initiate ECM synthesis and cartilage repair include IGF-1, BMPs, TGF-β and CTGF (Alcaraz et al., 2010). Responses to these growth factors could be studied on IL-1β stimulated cartilage. MS analysis identified CTGF in untreated explant supernatants (Table 7) but it was not detected in IL-1β stimulated cultures. Release of anabolic growth factors could be reduced by IL-1β, while addition of additional growth factors to the model may instigate cartilage repair processes.

6.4.2. Mechanical loading

Mechanical loading has not been applied in this equine explant model, but is known to have important effects on healthy chondrocyte and cartilage metabolism (Grodzinsky et al., 2000). Injurious compression of cartilage explants causes increased loss of GAGs, and stimulates chondrocyte apoptosis and catabolic MMP release (Loening et al., 2000, Stevens et al., 2009). Dynamic loading of cartilage and chondrocytes affects the turnover and synthesis of aggrecan and smaller proteoglycans (Sah et al., 1989, Valhmu et al., 1998). Beneficial anti-catabolic effects of moderate dynamic compression has been shown on mechanically injured, TNF-α and IL-6/soluble IL-6 receptor stimulated explants (Li et al., 2013). Integrating mechanical loading into the equine explant model could simulate moderate forces usually present in synovial joints. Alternately, excessive mechanical load could initiate cartilage degradation, which therapeutic compounds could be screened against.

6.4.3. Co-culture models

Multiple tissues contribute to the environment within synovial joints and OA progression, therefore co-culture models have been developed to incubate cartilage explants and synovial cells together (Haupt et al., 2005, Pretzel et al., 2009). Transduction of insulin-like growth factor-1 and IL-1 receptor antagonist protein together into synoviocytes co-cultured with cartilage explants showed protection against IL-1 catabolic induction (Haupt et al., 2005). A co-culture model containing cartilage and synovial fibroblasts stimulated with TNF-α and IL-1β has demonstrated increases in aggrecanase, MMP-1 and MMP-3 activity, and up-regulation of IL-6 and IL-8 (Pretzel et al., 2009). Significantly higher responses were reported in co-cultures compared to mono-cultures of these tissues (Pretzel et al., 2009). These models still lack inflammatory cells like macrophages that will be present *in vivo*, but are a step towards improved models to replace live animal usage. Screening of therapeutics within co-culture models represents a potential strategy for future studies.

6.4.4. Physiological oxygen conditions

Articular cartilage and the chondrocytes within it are physiologically exposed to hypoxic conditions, which are not taken into account in the current equine model. Chondrocyte survival and differentiation is maintained by hypoxia-inducible factors (HIFs), which are up-regulated within a hypoxic environment (Schipani et al., 2001). In hypoxic conditions, HIF-1α and HIF-2α protect against cartilage deterioration by inhibiting catabolic proteins, and promote anabolic processes by up-regulating SOX-9 (Thoms et al., 2013). Future studies on cartilage explant responses to cytokines and anti-inflammatories could be completed in hypoxic conditions to provide a more accurate and realistic environment.

6.5. Additional analysis approaches to study inflammatory processes and degradation in cartilage cultures

The equine cartilage explant model has provided cartilage conditioned media after incubation with various treatments. In future studies, the following techniques could be applied to analyse collected samples to improve understanding of cartilage degradation and biological processes in response to stimuli or therapeutics.

6.5.1. Hydroxyproline assays

Hydroxyproline assays can provide a measurement of collagen concentrations and could assess release of collagen into cartilage conditioned media. The reported anti-MMP effect of carprofen could have been assessed with these assays. Significant collagen release due to cytokine stimulation may require extended culture time over 14 days (Gabriel et al., 2010, Sztrolovics et al., 1999). Analysis of collagen release throughout the 24 day time course B in response to IL-1β and carprofen could contribute further information on cartilage degradation within this model. Unlike aggrecan loss, collagen degradation is not reversible therefore the collagenase events mediated by MMPs are especially important for long term cartilage damage and OA (Karsdal et al., 2008).

6.5.2. PGE_2 Assays

During this project, PGE_2 assays were also attempted on cartilage explant cultures to assess release of this bioactive lipid from untreated, IL-1β stimulated and carprofen treated cultures, as described in Chapter 2.11. Carprofen is known to reduce PGE_2 production in horses *in vivo* (Beretta et al., 2005) and *in vitro* studies (Goodrich and Nixon, 2006), and it would have been useful to have reported PGE_2 levels in this thesis. Despite several attempts to quantify PGE_2 concentrations in supernatant samples, the immunocompetitive ELISA method used did not provide measurements that were consistently within the values of the standard curve. Whilst the kit protocol stated that all samples must be diluted, even with undiluted cultures from untreated, carprofen alone and most carprofen + IL-1β samples, PGE2 levels were below the detection threshold. Samples from IL-1β stimulated cultures gave readings too high to be accurately quantified. Although this indicated carprofen could reduce IL-1β stimulated PGE_2 release, data generated could not provide accurate measurements that could be graphically displayed or statistically analysed. PGE_2 measurement kits are available from alternative manufacturers, but due to time restraints and costs these could not be applied for this thesis.

6.5.3. Zymography

Presence of MMP-1, -3 and -13 in the cartilage secretome has been confirmed by western blotting. Additional information on the activity of these MMPs within the model could be achieved with zymography. An MMP substrate such as gelatin can be incorporated into polyacrylamide gels, and subsequent staining can display areas of digestion and catabolic activity (Vandooren et al., 2013). The cartilage secretome has been shown to contain various catabolic proteins, therefore the mixture of proteases will all contribute towards different digestion regions. Data in this thesis suggests release of active MMP-3 with its pro-region cleaved (chapter 5), although presence of MMPs does not necessarily equate to enzyme activity. Zymography would provide an assessment of enzymatic activity, allowing studies to

monitor the effects of therapeutics such as carprofen within samples generated from cytokine stimulated explant models.

6.5.4. Microarrays and ELISAs

Antibody microarrays are another alternative that could provide high-throughput quantitative measurements of specific proteins in secretome samples. OA cartilage (De Ceuninck et al., 2005) and LPS stimulated cartilage (Haglund et al., 2008) secretomes have been studied with protein microarrays, but comparison of normal and cytokine stimulated tissues may provide new insights (Marzoq et al., 2013). Specifically targeting aggrecan and collagen fragments, or MMPs with ELISAs, could also provide information on degradative activities in cartilage culture (Wang et al., 2009a). ELISAs can be designed with specific degradation product neoepitopes that could be used to assay specific OA biomarkers. MMP inhibition was shown with ELISA approaches to have no effect on aggrecanase activity on cartilage (Wang et al., 2009a). This agrees with findings in this thesis that MMP inhibiting activity of carprofen cannot totally suppress loss of GAG and aggrecanase activity.

Arrays used to study MMP profiles of synovial fluid show differences between advanced OA compared with normal or early OA (Heard et al., 2012). No differences in MMP profiles could be distinguished between normal or early OA (Heard et al., 2012). Significant increases in MMP-1, MMP-3 and MMP-13 were reported in this thesis on IL-1β stimulated cartilage secretome, which suggests the model represents late OA, rather than early OA.

6.5.5. Metabolomics and Lipidomics

Whilst studying proteins as biomarkers of OA provides a significant contribution to OA research, insights could also be gained through metabolomic and lipidomic studies. The metabolic profile of end-stage OA and early/no OA was compared in media conditioned by synovial tissue samples (Adams et al., 2012). Significant differential alterations in pro-hydroxyproline, branched chain amino acids catabolic products and energy production associated metabolites were reported

(Adams et al., 2012). Hydroxyproline is abundant in collagen fibres therefore changes in its release could be of functional importance. Urinary and serum profiles of metabolites associated with arthritic diseases have been studied using NMR spectroscopy (Lamers et al., 2005, Young et al., 2013). Metabolites identified will give an indication of the cellular products generated by OA diseased cartilage. Metabolites will be produced by protein-regulated mechanisms, therefore providing another dimension to proteomic studies. It is worth noting that analysis of these systemic fluids may be complicated by many other factors such as a person's nutritional status impacting on their metabolome.

Bioactive lipids play an important role in inflammatory signalling through arachidonic acid signalling pathways during OA, therefore differential lipidomic analysis will provide relevant insights. Lipidomic analysis of equine synovial fluid has assessed eicosanoid profiles of normal and LPS treatment with/without meloxicam (de Grauw et al., 2011). Fourteen eicosanoids were identified in synovial fluid, some of which were significantly increased in inflamed joints (de Grauw et al., 2011). LPS induced increases in eicosanoids including PGE_2 were reduced by meloxicam displaying *in vivo* anti-inflammatory effects of this NSAID (de Grauw et al., 2011). Serum analysis from OA patients has also detected alterations in lipids involved in the arachidonic acid pathway (Castro-Perez et al., 2010). Immunoassays detected a range of eicosanoids in cartilage explant conditioned media (Attur et al., 2012). OA cartilage released significantly higher levels of PGE_2 and LTB4, while further accumulation of LTB4 was recorded with COX-2 inhibitors (Attur et al., 2012).

Extra information into mechanisms of OA progression can be achieved by studying the metabolome and lipidome of OA relevant tissues. Changes in metabolite or lipid profiles may occur more quickly than alterations in the protein secretome, allowing changes to be seen before chondrocyte lysis. The explant model of articular cartilage could be adapted to collect and prepare samples appropriately so that they would be compatible with metabolite or lipid analysis in the future.

6.6. Summary

Work described in this thesis studied the secretome of equine cartilage explants in response to inflammatory and anti-inflammatory stimuli. The secretome was assessed over a time course by SDS-PAGE with silver staining and high-throughput MS proteomic approaches. Identifications of ECM, non-ECM secreted and intracellular proteins were made in equine cartilage explant secretome by MS analysis. These identifications provided information on cartilage degradation, release and secretion of proteins within the explant model. This model has potential to be adapted to study alternative pro-inflammatory stimuli, mechanisms of cartilage degeneration and actions of anti-inflammatory treatments.

Exploratory candidate biomarkers including proteases MMP-1, MMP-3 and MMP-13 were identified from high-throughput MS analysis, and showed semi-quantitative differences between untreated control and IL-1β stimulated cartilage. These three MMPs are associated with OA and contribute towards cartilage catabolism and disease progression (Troeberg and Nagase, 2012). The suitability of the model to study effects on MMPs was supported by western blotting and reported quantitative differences due to carprofen treatment of IL-1β stimulated explants. Fibronectin western blotting showed a specific degradation product in response to IL-1β. Carprofen treatment significantly reduced this IL-1β stimulated product; therefore this fibronectin fragment is a suitable marker to assess certain anti-inflammatory properties. Monitoring release of these MMPs and fibronectin is indicated to be a useful approach for screening efficacy of novel therapeutics. One future hypothesis is that multiplexing approaches will be able to measure multiple biomarkers of OA. Evaluation of numerous biomarkers could indicate changes occurring in the various tissues of the synovial joint such as bone, synovium and cartilage. Multiple biomarkers may be quantified that also indicate the cause of OA disease (e.g. traumatic or overuse), helping to provide increasingly accurate prognosis knowledge and treatment options.

The main original findings of this thesis are summarised here:

- High-throughput MS analysis with nanoLC-MS/MS indicated differences in equine cartilage explant secretome in response to IL-1β, IL-1β + carprofen treatments and in untreated controls.
- Depletion of abundant secretome proteins by CPC precipitation, Con A lectin chromatography or Proteominer™ approaches were assessed by high-throughput MS. Identification and coverage of intracellular proteins was increased by depletion. Additional identifications of MMP-10 and IL-9 were made with CPC precipitation and Con A lectin chromatography respectively.
- Carprofen was shown to decrease the release of MMPs in IL-1β stimulated equine cartilage explants. Release of a fibronectin degradation product associated with IL-1β stimulation was also reduced by carprofen treatment.
- Carprofen delayed GAG release and did not affect NO production in IL-1β stimulated equine cartilage cultures.

This thesis has explored the capability of an equine explant model to study release of proteins in response to cytokine stimulation and NSAID treatment. It has demonstrated that assessment of catabolic processes and properties of anti-inflammatory therapeutics can be screened and assessed within an *in vitro* model. Utilising equine cartilage has shown these effects in a model for addressing horse joint health, which may ultimately benefit therapy of this companion animal.

APPENDICES

Appendix 1. β-actin western blotting

β-actin western blotting

See Materials and Methods for general western blotting protocol (Chapter 2). These investigations utilised a primary antibody against β-actin (1:7000 dilution, Sigma) and secondary anti-mouse HRP conjugate (1:100,000, Cell Signalling).

β-actin release initiated by IL-1β shows no significant difference with carprofen present

Chondrocyte death and lysis within cartilage explants was indirectly measured by assessing release of β-actin into cartilage conditioned media. Untreated control, IL-1β and carprofen treated supernatants were analysed by western blotting against β-actin (Figure 35). β-actin was detected in IL-1β and carprofen + IL-1β stimulated explant supernatants after the first 6 days incubation. Explants cultures with carprofen treatment alone or untreated controls did not release detectable levels of β-actin over 0-6 days. Western blotting of time course supernatants at 6-12 and 12-18 did not reveal detectable levels of β-actin with any treatment groups. These western blot investigations indicate that IL-1β has cytotoxic effects leading to chondrocyte lysis. Carprofen did not increase cytotoxicity and chondrocyte lysis as levels of β-actin were reduced, although not significantly.

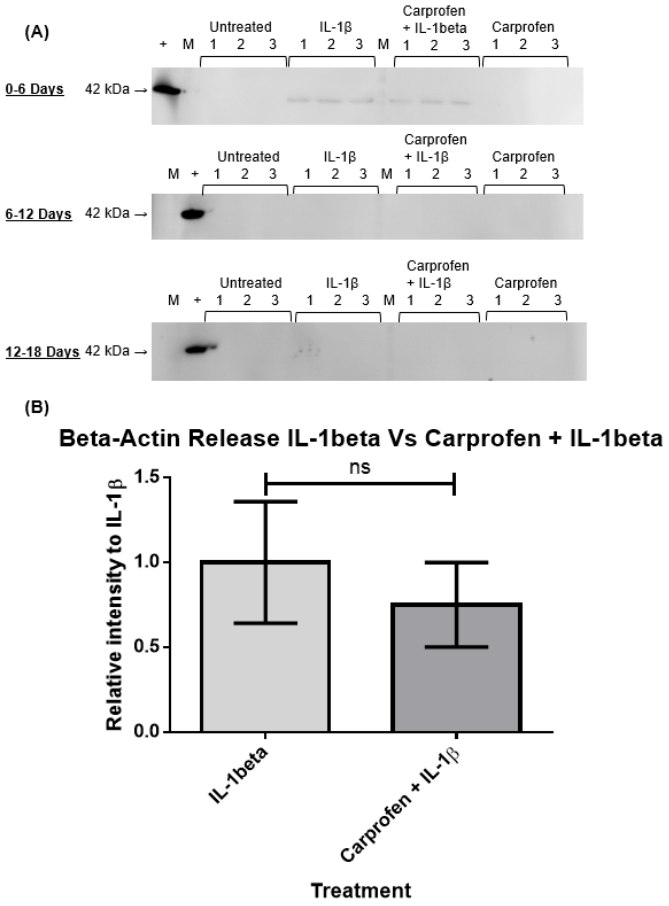


Figure 35. Cartilage explant cultures treated with IL-1β or carprofen + IL-1β do not show significant differences in release of β-actin

(A) β-actin western blotting images for time course supernatants from 0-6, 6-12 and 12-18 days. There was no detection of β-actin from explant cultures untreated or treated with carprofen alone. Positive control produced an expected band corresponding to β-actin ~42 kDa. Bands can be detected for 0-6 days with IL-1β and carprofen + IL-1β treatments. β-actin was not detected after 6-12 or 12-18 days. (B) Graphical representation of densitometry of β-actin bands. An unpaired t-test was applied to assess significance with ns = $P > 0.05$. + = positive control of chondrocyte lysate. Explant cultures were completed for 6 days with 3 treatment replicates from two animals (*n=6*). Explant treatments: untreated control, IL-1β (10 ng/ml), carprofen (100 µg/ml), or carprofen (100 µg/ml) + IL-1β (10 ng/ml).

Appendix 2. CPC precipitation depletes GAGs in explant culture supernatant

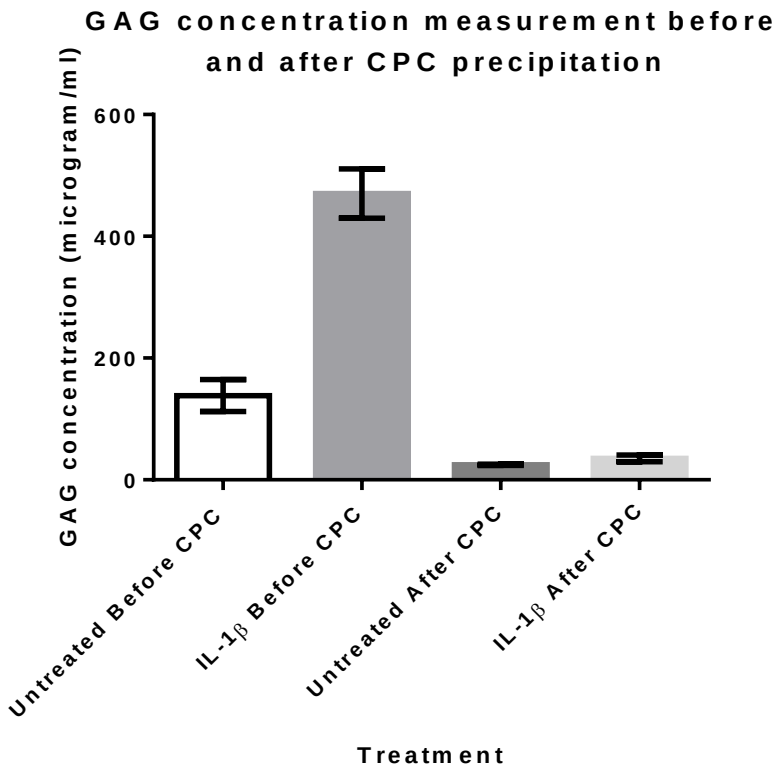


Figure 36. DMMB assay showing depletion of GAG from cartilage explant conditioned media

GAG concentration was measured before and after CPC precipitation, showing that GAG levels are reduced after CPC precipitation. Technical replicates from an individual animal explant culture assayed (*n=2*). Error bars = standard deviation.

Appendix 3. LPS and IL-1β stimulate similar protein release from cartilage explant cultures

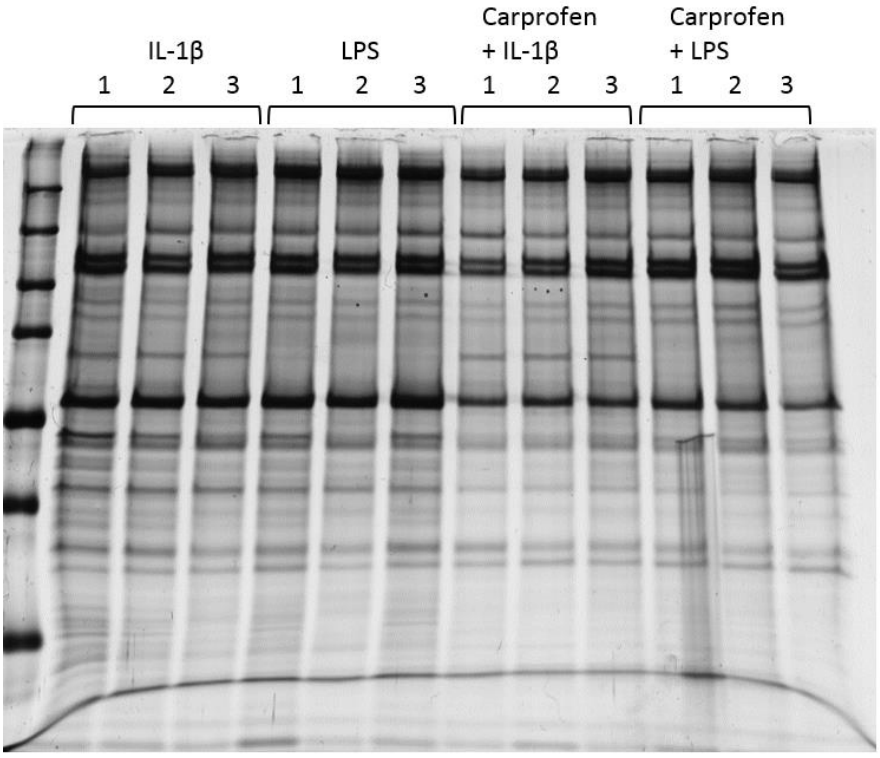
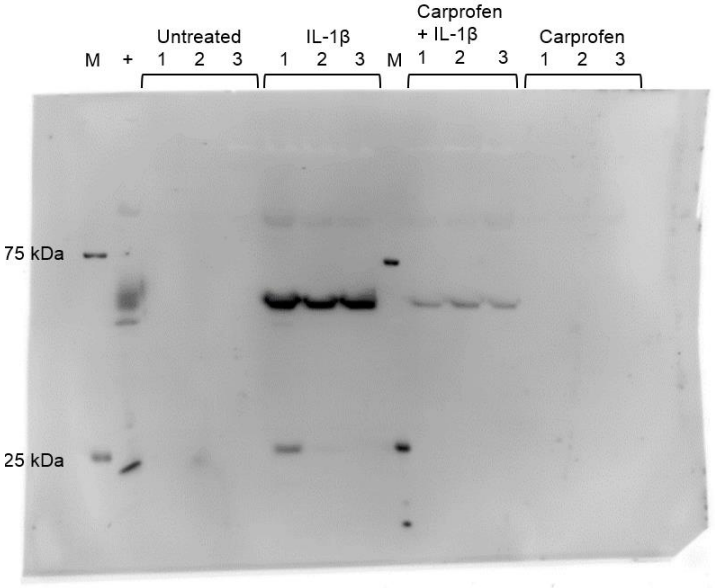


Figure 37. Representative image of cartilage conditioned media after IL-1β, LPS and carprofen treatments

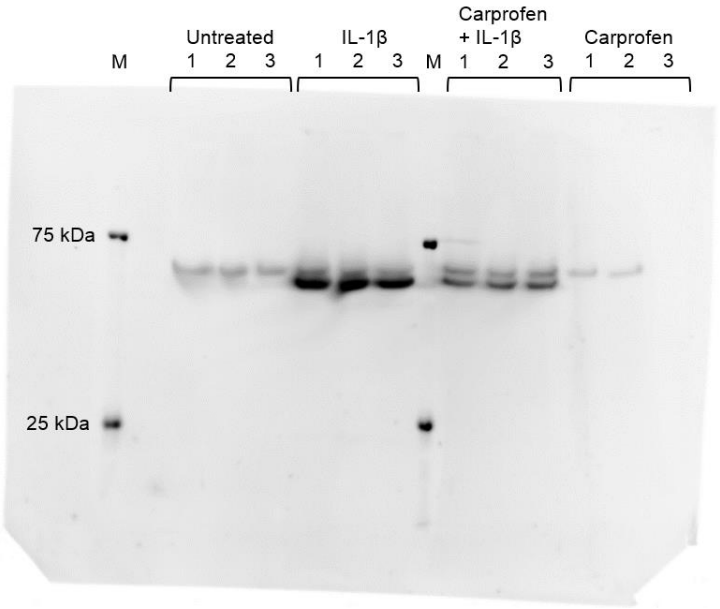
Protein release profiles were similar in explant cultures set up on two separate occasions from two individual horses. Treatments: IL-1β (10 ng/ml), LPS (10 ng/ml), carprofen (100 µg/ml).

Appendix 4. Full images of western blotting images contained in figures in Chapter 5

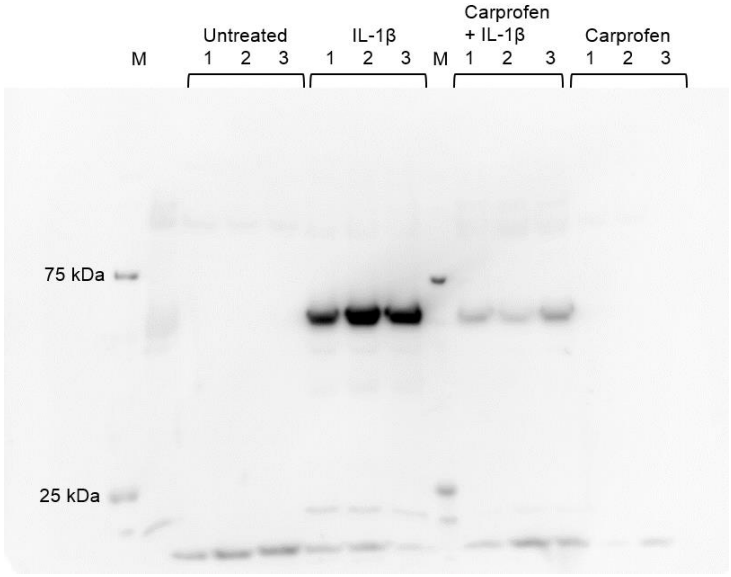
Full image of MMP-1 western blot from Figure 27



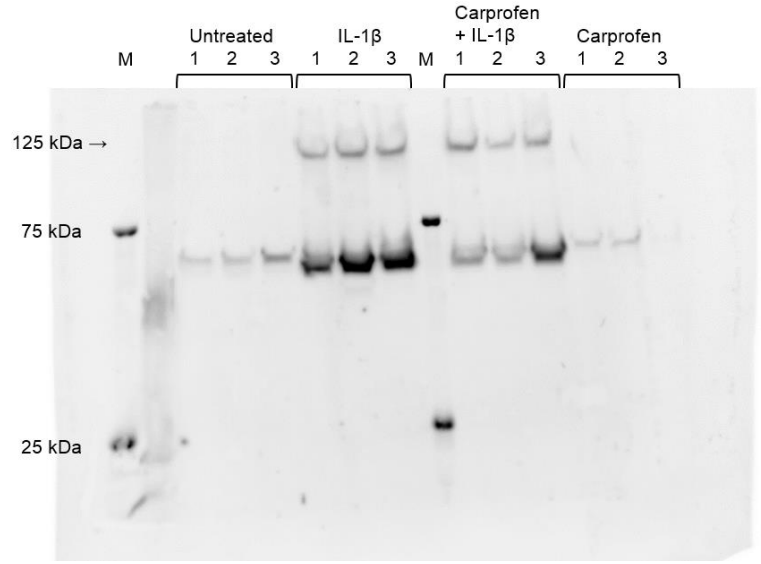
Full image of MMP-3 western blot from Figure 28



Full image of MMP-13 western blot from Figure 29

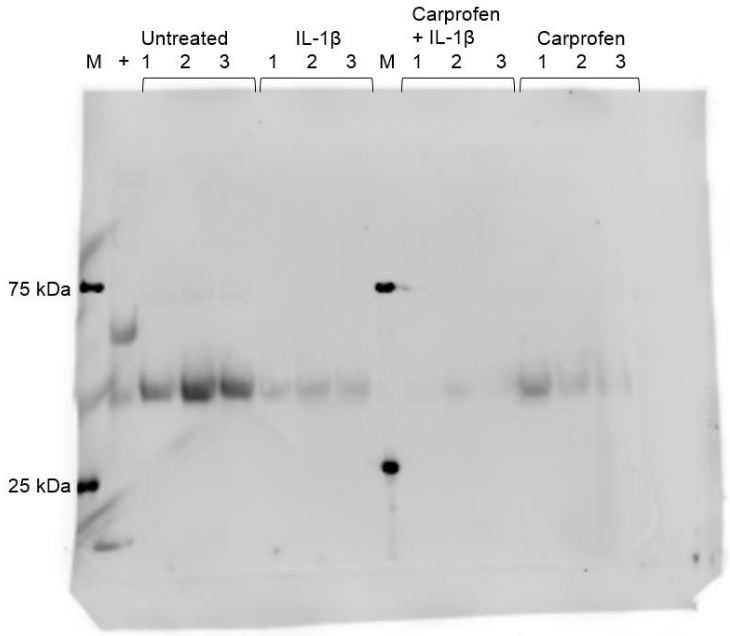


Full image of thrombospondin western blot from Figure 30



As stated in the results section (chapter 5.3.4), thrombospondin blotting was completed on a membrane where MMP-3 had previously been blotted, therefore only the band at band at 125 kDa was attributed to thrombospondin. All other bands visible were previously associated with MMP-3 detection.

Full image of clusterin western blot from Figure 33



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