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Early Cartilage Degeneration: Correlation of Micro-Structural and Proteomic Analysis

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The beginning of wisdom is this: Get wisdom. 
Though it cost all you have, get understanding.
Cherish her, and she will exalt you; embrace her, and she will honor you.

Proverbs 4:7-8
ABSTRACT

Pre-osteoarthritis in the joint remains elusive to the cartilage researcher. To address this, a bovine model of early cartilage degeneration has been used in recent years. In this study the state of early tissue degeneration in bovine cartilage was investigated, correlating subtle microstructural changes in the tissue with its protein profiles.

A total of 44 bovine patellae showing a range of tissue states from completely intact (healthy) to those with localised mild-to-moderate degeneration of the cartilage surface (non-healthy) were used. The degeneration was highly localised to the distal-lateral quarter of the patella and this tissue state has been validated in previous studies to be an early state of degeneration in osteoarthritis. From each patella showing mild-to-moderate tissue degeneration, two samples were used for the analyses, one from the localised site of degeneration and the other from an adjacent intact region. The healthy patellae provided control samples, where two intact cartilage samples were obtained from the distal lateral quarter, and a region adjacent to it.

In the first part of this study, the cartilage samples were systematically characterised based on their microstructural changes using Differential Interference Contrast optical microscopy and Mankin histological scoring. The main changes in the microstructure in early cartilage degeneration were found to be as follows: (1) cartilage surface irregularity with a progressive diminishing of the tangential zone, where in severe degeneration there were clefts and fissures, (2) chondrocyte clustering, (3) fibrillar matrix ‘destructuring’ and re-aggregation, (4) thickening of the zone of calcified cartilage and (5) presence of bone spicules and signs of an advancing cement line. These microstructural changes are consistent to being analogous to a pre-osteoarthritic state in human joints.

Based on the above microstructural variations from early degeneration, the second part of the study reports on the associated protein profile variation. Comprehensive iTRAQ (Isobaric tags for relative and absolute quantitation) labelled LC-MS/MS were performed on whole cartilage tissue samples. A total of
191 proteins were identified. Correlating microstructural variations to known protein functions, 15 proteins, further validated using MRM assays, were identified as potential markers for the early degenerative process. These proteins, including a novel protein WFDC18-like protein, and their specific role in the subtle structural changes in cartilage are discussed in this work. Of interest also is that in the range of tissues with early degeneration, the differences in protein profiles were more pronounced in those from the mildly degenerated tissue group.

The third part of this study involved analysing secretome profiles (unstimulated) of the cartilage explants from the different examined groups. The explants from cartilage with moderate levels of degeneration were found to have enhanced secretory action than samples from mildly degenerated tissues, contrasting to the abundance levels noted in the earlier part of the study (see above). An explanation for this reduction in protein abundance levels in the cartilage tissue with moderate level of degeneration, in which the secretome levels were higher than cartilage with mild degeneration, may be due to the increasingly destructured fibrillar matrix that facilitates protein loss. Also seen in the secretome profile was the novel protein WFDC18-like, with potential anti-protease activity, which was found to be significantly upregulated in the intact cartilage regions of the non-healthy patellae.

Finally, in the last part of the study, a relatively new technique for studying cartilage protein profiles, MALDI-IMS, was explored. This technique allowed for easy quantification of region-to-region peptide variation of localised degenerated sites and adjacent intact tissue.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACAN</td>
<td>Aggrecan</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>A disintegrin and metalloproteinase with thrombospondin motifs</td>
</tr>
<tr>
<td>AMBP</td>
<td>Alpha-1-microglobulin/bikunin precursor</td>
</tr>
<tr>
<td>ANG</td>
<td>Angiogenin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AUC</td>
<td>Area under curve</td>
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<tr>
<td>BML</td>
<td>Bone marrow lesions</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BRN</td>
<td>Brain ribonuclease</td>
</tr>
<tr>
<td>C4A</td>
<td>Complement component 4A</td>
</tr>
<tr>
<td>CCL16</td>
<td>Chemokine (C-C motif) ligand 16</td>
</tr>
<tr>
<td>CHAD</td>
<td>Chondroadherin</td>
</tr>
<tr>
<td>CHCA</td>
<td>α-Cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>CHI3L1</td>
<td>Chitinase-3-like protein 1</td>
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<tr>
<td>CILP</td>
<td>Cartilage intermediate layer protein</td>
</tr>
<tr>
<td>COL</td>
<td>Collagen</td>
</tr>
<tr>
<td>COMP</td>
<td>Cartilage oligomeric matrix protein</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ECSOD</td>
<td>Extracellular superoxide dismutase</td>
</tr>
<tr>
<td>EDIL-3</td>
<td>EGF-like repeat and discoidin I-like domain-containing protein 3</td>
</tr>
<tr>
<td>ENO1</td>
<td>Alpha-enolase</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>F13A1</td>
<td>Coagulation factor XIII A chain</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>FDR</td>
<td>False Discovery Rate</td>
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<tr>
<td>FMOD</td>
<td>Fibromodulin</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GI</td>
<td>Grade I</td>
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<td>GII</td>
<td>Grade II</td>
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<td>GO</td>
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<td>GPI</td>
<td>Glucose-6-phosphate isomerase</td>
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<td>GSTP1</td>
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<td>HA</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HTRA1</td>
<td>High temperature requirement serine peptidase A1</td>
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<tr>
<td>IAM</td>
<td>Iodoacetamide</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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IMS  Imaging Mass Spectrometry
IPI  International Protein Index
iTRAQ  Isobaric tags for relative and absolute quantitation
LAP  Lingual/LAP-like antimicrobial peptide
LC-MS/MS  Liquid chromatography tandem-mass spectrometry
LMNA  Lamin A
LN  Natural log
LYZ1  Lysozyme C, milk isozyme
MALDI  Matrix assisted laser desorption/ionization
MMP  Matrix metalloproteinase
MRI  Magnetic resonance imaging
MRM  Multiple reaction monitoring
MS  Mass spectrometry
NO  Nitric oxide
OA  Osteoarthritis
OCT  Optimal cutting temperature compound
PANTHER  Protein analysis through evolutionary relationship
PBS  Phosphate buffer saline
PCM  Peri-cellular matrix
PCOLCE  Procollagen C-endopeptidase enhancer
PDIA3  Protein disulfide-isomerase
PEDF  Pigment epithelium derived factor
PG  Proteoglycan
PGLYRP1  Peptidoglycan recognition protein 1
PRELP  Proline/arginine-rich end leucine-rich repeat protein (Prolargin)
RBP4  Retinol binding protein-4
ROC  Receiver operating characteristic curve
ROS  Reactive oxygen species
SAA3  Serum amyloid A protein
SCX  Strong cation exchange chromatography
SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM  Scanning electron microscopy
SERPINF1  Serpin family F member 1
SLRP  Small leucine rich proteoglycan
SPARC  Secreted protein acidic and rich in cysteine
TGF-β  Transforming growth factor beta
THBS1  Thrombospondin 1
TIC  Total ion count
TIMP  Tissue inhibitors of matrix metalloproteinase
TLR  Toll-like receptors
TNC  Tenascin C
TNF  Tumour necrosis factor
TOF  Time of flight
VEGF  Vascular endothelial growth factor
<table>
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<th>Description</th>
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<tr>
<td>WFDC18</td>
<td>WAP four-disulfide core domain 18-like</td>
</tr>
<tr>
<td>YWHAQ</td>
<td>14-3-3 protein theta</td>
</tr>
<tr>
<td>ZCC</td>
<td>Zone of calcified cartilage</td>
</tr>
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Co-Authorship Form

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**Chapter 2 and Chapter 3**

**Work to be submitted for publishing**

<table>
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**CO-AUTHORS**

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<td>Ashvin Thambyah</td>
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<tr>
<td>Vijayalekshmi Sarojini</td>
<td>Experiment design+manuscript revision</td>
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<td>Nell Broom</td>
<td>Manuscript revision</td>
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<td>Martin Middleditch</td>
<td>Proteomic analysis</td>
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<td>Mia Jüllig</td>
<td>Data analysis</td>
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<td>Leo Payne</td>
<td>MRM</td>
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**Certification by Co-Authors**

The undersigned hereby certify that:
- the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

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Chapter 5
Work to be submitted for publication

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1.1 Osteoarthritis

Osteoarthritis (OA) is a joint disorder currently afflicting more than 300,000 people in New Zealand according to Arthritis NZ [1]. Estimates provided by World Health Organization show that 9.6% of men and 18.0% of women aged over 60 years have symptomatic osteoarthritis and 80% of those have limitation associated with movement [2]. Osteoarthritis affects joints in hands, knees, hips, shoulder, ankles, feet and spines among other joints. The OA symptoms include joint pain, bone deformity and stiffness which develop over the time. The exact point where OA is initiated is unknown to the patients.

OA is the most common form of arthritis which affects more commonly the older age groups. Unavailability of any cure makes OA patient sufferer for life. Even those treatments providing symptomatic relief are not much effective in managing the disease [3]. In the advanced stage of the disease, total knee replacement surgery is performed [4].

Affecting almost all ethnic groups, OA is the most prevalent disabling disorder for older age groups and more commonly in women [5]. Women with high bone density are more prone to knee OA [6]. Apart from age, other risk factors for OA are genetic predisposition, obesity and over-use of joints as well as factors like hormonal changes and existence of other pathological conditions.

The joint being the point where the bones connect, is facilitated to cater the movements as required. The cartilage covers the bones’ end at the joint and is an aneural and avascular tissue which protects the underlying bone in articulating joints by increasing the loaded contact area. Cartilage itself is a firm, rubbery tissue which provides friction-less movement of the joint. During OA, the cartilage slowly gives
away its resilient structure and over time, the bones at the joint start to come in contact with each other, causing pain and distress.

As there are no nerve endings in cartilage, weight bearing is painless and, likewise, it is reasonable to assume that damage to the cartilage can occur without any pain to the individual. Having no innate blood vessels, the healing and repair of cartilage is extremely limited, and with on-going use of the joint, the cartilage gradually wears away until the underlying bone is exposed. The resultant direct contact loading of the richly innervated bone tissue devoid of cartilage, has thus been implicated in the pathogenesis of pain associated with joint degeneration in osteoarthritis [7, 8].

Diagnosis of OA is mainly via radiographic examination to determine the extent of joint space narrowing, i.e. cartilage loss [9] and by MRI (Magnetic Resonance Imaging) scan; the disease management is typically through non-steroid anti-inflammatory drugs (NSAIDS) and pain relievers to suppress pain and other symptoms of discomfort. In some patients, life-style changes prove to improve the quality of life. In order to give symptomatic relief, surgeons sometimes do perform spongialization [10], subchondral bone penetration [11-13] and osteotomies[14, 15] in patients. Treatment options at times also include physiotherapy and, in more severe cases, total joint replacement.

There is currently no cure for, or reversal of OA. A major hurdle for the OA researcher in finding a cure lies in the difficulty of determining the early onset of the disease [16]. Typically involving pain in the joint, clinical OA symptoms are associated with a relatively late stage of the disease, as the early OA joint is likely to be asymptomatic and thus largely undetectable. It is not unreasonable to argue that future preventive or curative strategies will require the early-to-pre-OA state be better understood.

Extensive research at the genetic, molecular as well as cellular level have been done with the goal to find appropriate therapeutic intervention for OA. Better understanding of pathophysiology of the disease is important for this and also for developing appropriate biomarkers for early diagnosis and proper management of the disease.
A healthy cartilage tissue is resilient and pliant. It has relatively lower number of cells compared to other tissues in our body. Even though these cells are needed for cartilage maintenance, their lower proportion in the tissue may be due to the non-vascular nature of the cartilage, making it hard to provide a larger number of cells with nutrition [17].

Articular cartilage is important for the normal functioning of the joint and hence it is the most studied tissue in relation with OA, even when OA is the disease of the whole joint which include synovium, meniscus, bone and tendons.

**1.2 Articular Cartilage**

Cartilage is a unique connective tissue which provides vast mechanical possibilities for joint movement. Texture-wise, it is tougher than the muscles but flexible and less rigid than the bones. Cartilage is made up of extracellular matrix (ECM) and chondrocytes. A relatively sparse number of chondrocytes maintain the ECM which is made up of water, collagens, proteoglycans and other small molecules [18]. The collagen arrangement in ECM and chondrocyte organization is shown in Figure 1.1. The combination of fluid and extracellular matrix in the articular cartilage contributes to its biomechanical and low friction properties. Cartilage is permanent in a healthy joint i.e., chondrocytes do not undergo proliferation or apoptosis in an adult state [19].
Figure 1.1 Schematic diagram showing cartilage organization in normal health: (A) shows the chondrocyte profile and (B) shows the fibril arrangement in different cartilage zones.
Articular cartilage is inhomogeneous across its depth with four stratified layers or zones based on the chondrocyte morphology and collagen orientation - tangential zone, middle zone, deep zone and zone of calcified cartilage (Figure 1.1). The superficial layer has a higher number of chondrocytes than the radial zone. The radial zone chondrocytes in spite of their lower number have larger surface area and are biologically more active than the superficial zone chondrocytes [20].

The top layer or the tangential zone forms 10-20% of the articular cartilage, providing a smooth articulating surface and overcomes friction during the joint movement. This zone has the lowest compressive modulus where the collagen fibrils are tightly packed and arranged in a highly organized fashion parallel to the cartilage surface [21, 22]. The chondrocytes in this zone are flattened in appearance and are known to secrete very less proteoglycans but a higher level of lubricating proteins [20].

The middle zone (transitional zone) comprises 20-50% of the articular cartilage with the collagen fibres existing in less organized way in this zone. The collagen fibrils are loosely arranged, are thicker and occur oblique to the articular surface. Also, the chondrocytes here appear rounder compared to the superficial zones’. The deep zone measures 40-70% of the cartilage and the characteristics of this zone are the radial alignment of the elongated chondrocytes parallel to the collagen fibres but perpendicular to the cartilage surface [22]. This zone has the highest compressive modulus. The deep zone is followed by the zone of calcified cartilage (ZCC) which lies on the subchondral bone [18]. A tidemark separates deep zone from ZCC and functions as a mechanical buffer between un-calcified cartilage and calcified zone [23].

1.2.1. Extracellular Matrix

Cartilage is a robust and resilient tissue, where the cartilage cells or chondrocytes are embedded in the extracellular matrix (ECM) along with the collagen fibres. ECM consists of an organised arrangement of structural and functional proteins and provides a dynamic microenvironment for the resident chondrocytes. The biochemical and biophysical properties of ECM are responsible for the proper functioning of chondrocytes [24]. In an adult organism, cartilage shows limited
activity and a slow turnover [25]. In the matured state, cartilage maintains balance between the anabolic and catabolic processes. As part of the day-to-day functioning, a healthy cartilage stabilizes any mechanical or biochemical stress encountered by the articular joint. But at the point where the catabolic stress surpasses the anabolic activity, the cartilage matrix starts to degenerate, leading to a faulty joint [26].

Cartilage ECM consists of the interstitial space and the basement membrane. The interstitial space is the region between the cells which is occupied by the networks of collagen and glycosaminoglycan (GAG) and acts as cushion against stress; basement membrane is the attachment on to which the chondrocyte anchors [27].

The mechanical stiffness and elasticity of the cartilage is attributed to the framework composed of collagen fibres and proteoglycans with a high osmotic swelling pressure. About 60-80% of cartilage’s wet weight is due to water [28] which is held in interstitial space by negatively charged proteoglycans. Cartilage dry weight consists of collagen fibres (~60%), proteoglycan (~25-35%) and other non-collagenous proteins (~15-20%) [29, 30].

The ECM is divided into three regions based on its proximity to chondrocyte, namely peri-cellular (PCM), territorial and inter-territorial matrices. Peri-cellular region comprises the thin layer surrounding the chondrocytes; the region acts as the transducer for chondrocytes interpreting biochemical and biomechanical signals [31-33]. Proteoglycans, glycoproteins and non-collagenous proteins are present in PCM. Territorial matrix surrounds PCM in basket-like fashion and is composed of thin collagen fibrils. This region protects chondrocytes from mechanical stress [34]. Inter-territorial region comprises the largest area of the cartilage structure. This region provides structural support and biomechanical properties to the cartilage [35]. The collagen fibrils in this region are comparatively thicker than those from territorial matrix and the orientation of the collagen fibrils varies from superficial to deep zone [36] as discussed previously.
1.2.2. Chondrocytes

Chondrocytes are the only form of cells in cartilage and is found to be embedded in the negatively charged ECM. They are mesenchymal cells which actively divide and differentiate during the developmental stage but show no division in the healthy adult cartilage. Chondrons are basket-like structure formed by collagen fibrils which hold one or more chondrocytes (Figure 1.2). Chondrons have a composition of type II, VI and IX collagens [37].

Chondrocytes act as osmo- and mechano-sensor and they can modify their actions on the basis of various mechanical load and osmotic stress [38, 39]. The changes in gene expression along with production of catabolic factors are the result of chondrocyte sensing mechanical stress [40]. When the integrity of the cartilage tissue is challenged, these cells get back in action and start dividing once again [41].

Chondrocyte morphology varies with the stratified layers of cartilage, and so does its biosynthetic activity. Wong et al. used bovine cartilage sections and measured morphometric as well as biosynthetic parameters across the cartilage depth. They found that number of chondrocytes decreased from the superficial zone to deep zone but the cell volume increased from superficial to deep zone. The biosynthetic activity also increased from top to bottom zones [20].

The chondrocytes in the radial zone are twice the volume of those from the superficial zone. Similarly, the radial zone chondrocytes are ten times more synthetically active than the superficial zone chondrocytes. The radial zone chondrocytes, though big in volume and smaller in number, were placed far from each other than the superficial zone chondrocytes, which were smaller in size but had a larger number and were uniformly present. Wong et al. also observed that most GAG syntheses occur at the upper radial zone whereas the collagen synthesis was maximum at the lower radial zone. The GAG synthesis was concentrated to the peri-cellular region whereas the collagen synthesis were detected at peri-cellular, territorial and inter-territorial matrix regions, suggesting that the newly synthesised GAGs either due to their size or charge, are restricted from diffusing outside the PCM [20].
Figure 1.2 SEM image of (A) the chondrocyte in bovine cartilage and (B) the chondron lacunae. Scale bar: 20 µm. (Author’s own image).
1.2.3. Collagen

Collagens are fibrous proteins which are secreted in copious amounts by the cells of connective tissue. It is made up of three left-handed polypeptide chains and each have a tripeptide sequence of Gly-X-Y. Hydrogen bonds stabilize the three alpha chains and form a right-handed collagen molecule helix. Apart from glycine, the collagen tripeptide sequence incorporates proline or hydroxyproline commonly as well as other amino acid residues. Collagen precursors or procollagen have large C and N terminal end sequences which are necessary for triple helix chain assembly. These extensions are later cleaved off before the fibril formation. The biologically active form of collagen is fibrillar collagen which is the end-product of various post translational modification on procollagen. Collagen fibrils are aligned anti-parallel to each other by the cross links involving lysine residues. An extracellular enzyme in tissues called lysyl oxidase (LOX) deamidates certain lysine and hydroxylysine residues and results in highly reactive aldehyde groups which form covalent bonds with other reactive aldehyde groups or other lysine or hydroxylysine residues [42, 43]. The cross links between collagen fibrils facilitate a network which provide the scope for deformation under compression [44].

The type I and type II collagens are the most common collagens. Type II collagen is the major constituent of cartilage and type I collagen, of the bone. Type II collagen forms 95% of the total collagen component in the adult cartilage. The cartilage tissue also contains 1% type IX and 3% type XI collagens. Likewise, collagens type I, III, IV, V, VI, X, XII and XIV are present but in lower amounts.

Type II collagen forms the fibrils and fibres in the ECM; other collagen molecules help in forming and maintaining the fibril network in the cartilage [45]. Type XI collagens are found inside the fibrils whereas type IX collagen is found on fibril's surface and helps in inter-fibril and fibril-non collagenous protein interaction [45], retaining the articular cartilage stability [46]. Type III collagen is found in association with type II collagen in normal cartilage [47]. Type VI collagen is found around the chondrocytes in peri-cellular matrix and in inter-territorial matrix. In severe OA cartilage, proliferating chondrocytes are seen to be synthesising more type VI collagen [48].
Type X collagen is found in the calcified cartilage region [49] and also synthesised by hypertrophic chondrocytes [50].

Collagens are resistant to cleaving by most of the proteases other than collagenases [51]. Three-alpha helix coil of collagen fibril has one cleavage site where collagenases act [52] and result in a quarter and three-quarter collagen fragments [45]. Though adult cartilage have limited synthesis activity, as part of remodelling a damaged tissue, a slow and often ineffective collagen upregulation can occur [53].

1.2.4. Proteoglycan

Proteoglycan (PG) is the second most abundant protein in the cartilage. It consists of a core protein monomer on to which glycoprotein chains are attached. There are more than 30 different kinds of proteoglycans in the extracellular matrix. The association of these molecules with GAGs enable them to withstand compression [54]. In cartilage extracellular matrix, proteoglycans form gel-like substances by entrapping water molecules and this enable the movement of ions, nutrients and various other factors in the tissue [27].

Among the various proteoglycans, aggrecan is the largest and most prevalent proteoglycan in ECM. Aggrecan is composed of chondroitin sulphate and keratin sulphate glycosaminoglycan chains which are attached to hyaluronic acid (HA) molecule, with the help of link proteins [55]. This structure is called as proteoglycan aggregate. The proteoglycan aggregates with their negative charges are immobilized within the collagen inter-fibrillar space and maintain a high osmotic pressure environment in the cartilage. This arrangement results in high pressurization in cartilage’s fluid phase providing stiffness and viscoelastic properties to the tissue [56]. During mechanical loading of articular cartilage, water moves out of the aggrecan monomers and after the mechanical forces are removed, proteoglycans recapture the water molecules.

Small leucine rich proteoglycans (SLRPs) are smaller PGs but they have almost similar molar level as that of aggrecans in ECM. SLRPs like decorin and fibromodulin interact with collagen type II whereas biglycan is found to interact with collagen type VI.
molecules, in the peri-cellular matrix region [57]. Proteoglycans are also known to regulate cell signalling in the tissue [58].

The degradation of aggrecan takes place when proteases attack hyaluronan and this in turn destabilises the aggrecan's hold on the collagen network [41]. Aggrecan is normally found in the articular cartilage as proteoglycan aggregate but it is also seen in its non-aggregated form in the OA cartilage. This could be the result of proteolytic degradation of the molecules or a result of disruption of normal homeostasis where it is unable to assemble itself into a proteoglycan aggregate.

1.2.5. Glycosaminoglycans (GAGs)

GAGs are long unbranched polysaccharides which are highly polar in nature and hence attract water molecules. The three types of GAGs found to be associated with proteoglycan core molecule are chondroitin/dermatan sulphate (CS), heparan sulphate (HS) and keratan sulphate (KS). GAGs bind to growth factors and cytokines. Hyaluronan or hyaluronic acid (HA) is the simplest form of GAGs and is usually made up of thousands of repeated disaccharide units and helps in resisting compressive forces in the cartilage [59].

In certain cases, HA is used as intra articular injection as part of treating OA, as it suppresses cytokine driven inflammation and reduces monocytes and macrophages triggered during the inflammatory processes [60]. Hyaluronan injections were found to reduce pain, improve the afflicted joint's structure and also control the rate of the OA progression [61].

1.3. Cartilage Function and Homeostasis

In a joint, articular cartilage provides a frictionless surface to the articulating bones and it also cushions the long bones. The spatial organisation of cells and the higher proteoglycan content in the superficial layer of the cartilage help in absorption and distribution of mechanical load.

Mechanical loading of cartilage is very important for its homeostasis since the mechanical impetus induces the fluid movement between cartilage and synovial fluid,
allowing the exchange of nutrients and biochemical factors [62]. Studies have previously shown how immobilization can lead to cartilage degeneration and joint damage [63]. In their work Leong et al., showed that hind limb immobilization in rats resulted in expression of higher levels of matrix metalloproteinase 3 (MMP3) and ADAMTS5 (A disintegrin and metalloproteinase with thrombospondin motifs 5) leading to proteoglycan loss whereas exercising the immobilized joints decreased the elevated protease levels and proteoglycan loss [64]. Moderate level of mechanical loading increases the anabolic activity like aggrecan synthesis [65] at the same time minimizes the expression of catabolic factors [63].

Chondrocytes have a non-motile organelle called primary cilium, which acts as a mechanical sensor with the help of calcium ion channels and integrins on it [66, 67]. Apart from being a mechanical sensor, primary cilium also contributes to cartilage homeostasis [68]. Transgenic mice without primary cilia were shown to have defective cellular organization in their articular cartilage and their chondrocyte differentiation was also affected [69]. Primary cilium is associated with inflammatory pathway, as the mutated cilium-absent mice cannot induce inflammation mediators like prostaglandins and nitric oxide under influence of interleukin 1 beta (IL-1β) [70].

Chondrocytes thrive in a hypoxia-regulated cartilage and hypoxia is important for the chondrocyte homeostasis. Under hypoxic conditions, cartilage synthesise more collagen type II and aggrecan, lowers the synthesis and release of MMP-1 and 13 and also suppresses the cleavage of collagen type II compared to normoxia [71]. The inflammation mediators, prostaglandin and nitric oxide are also lowered under hypoxia [72].

1.3.1 Cartilage Anabolic Factors

The major growth factors for maintaining the homeostasis in cartilage are transforming growth factor beta 1 (TGFβ1), fibroblast growth factors, bone morphogenetic proteins (BMPs), insulin-like growth factor (IGFs), cartilage derived morphogenic factors, and connective tissue growth factors. A few of these proteins have anabolic as well as catabolic role in the cartilage.
In a normal healthy adult cartilage, the level of anabolic factors is relatively low; during OA, these factors are found to be expressed in a comparatively higher level, however they are unable to contain the overall degradation occurring in the cartilage. These upregulated levels in OA, could either be tissue’s attempt at the cartilage repair or could be due to the induction driven by the catabolic factors. In addition, though these anabolic factors are synthesised in higher levels, the intended repair of cartilage is not very effective since their inhibitors or antagonist factors are also upregulated in OA [40].

1.4. Pathophysiology of Osteoarthritis

OA is the disease of an organ involving cartilage, synovium, menisci and bone. Its pathophysiology involves a multifaceted interplay of mechanical, biochemical, biological and enzymatic feedback pathways (Figure 1.3).

Figure 1.3 Signalling pathways and structural changes in the development of osteoarthritis: (a) normal joint and (b) diseased OA joint.
Reproduced from [73] with permission from Elsevier.
1.4.1 Meniscus

The menisci in a knee joint is important for distributing axial forces. Menisci extrusion occurs in OA joints [74] and it changes the knee environment by disturbing the pattern of load distribution, which can lead to subchondral bone changes and also loss of cartilage [75, 76].

1.4.2. Synovial Membrane

Martel-Pelletier et al., suggested that synovial inflammation can be one of the early events of the clinical OA. Synovial membrane is the lining which covers the joint space and is also known as synovium or stratum synoviale. It is a specialised connective tissue which secretes the synovial fluid. Synovium folds into villi and this tissue is well vascularised and innervated to provide the membrane and the cartilage with required nourishment. It consists of two layers- outer layer or sub-intima, joins the fibrous capsule of the joint and the inner layer is called intima. It possesses two types of cells - type A which are macrophages-like synovial cells and type B which are synovial fibroblasts. Type A cells maintain the synovial fluid by getting rid of unwanted debris and type B synovial cells produce extracellular components of the fluid especially the hyaluronan, proteoglycan 4 (PRG4) and lubricin.

Synovial inflammation is one of the clinical symptoms where the patients decide to see a physician and it is also found to occur in early OA at sub-clinical stage [77]. The histology studies reveal that the synovium undergoes changes like hypertrophy and hyperplasia with an increase in the number of cells in those regions. The synovial fluid possesses proteins derived from synovial membrane, cartilage and serum mainly plasma proteins, lubricant molecules, cytokines and growth factors. The cartilage is first thought to release degraded cartilage matrix components into synovial fluid, which activates the type A synovial cells and the already infiltrated inflammatory cells, thus inducing inflammation of synovial membrane. This inflammation results in the synthesis of mediators which diffuse into the cartilage and degrade more components. These mediators and degraded components are then transferred to the systemic circulation, and in this way, the vicious cycle continues.
Patients with knee OA and synovitis are seen to have rapidly progressing OA [78, 79]. Synovial inflammation biomarkers which are used to evaluate the progression of OA are cartilage oligomeric matrix protein (COMP) [80], hyaluronic acid, type II collagen degradation products and C-reactive proteins [81, 82] among others. Increased number of immune cells are also associated with synovial inflammation [83]. Synovitis often contributes to the pain associated with OA.

1.4.3. Subchondral Bone

Subchondral bone and cartilage is believed to be interdependent [40, 84]. Cartilage lies over the bone plate and hence the vascularised and innervated subchondral plate provides the cartilage with required nutrition in addition to synovial fluid. Both articular cartilage and subchondral bone undergoes a series of changes during OA, but it is not clear whether the pathological initiation occurs in cartilage tissue or in the underlying subchondral bone. Hence, there is no agreement as to which occurs first in a degradation pathway – subchondral bone sclerosis or cartilage thinning [85, 86]. The subchondral bone sclerosis and osteophyte formation are seen during clinical OA manifestation [87]. Increased vascular development from the subchondral bone into the calcified zone [88, 89] with or without the thickening of zone of calcified cartilage is seen during the early stages of OA development [90]. Whether microvascular changes in the subchondral bone precede the bone structural changes, is still unclear.

As part of the OA progression, calcified cartilage in a defective joint undergoes substantial mineralization and becomes denser than the subchondral bone [86]. The process of calcification is often associated with chondrocyte apoptosis [91]. In their work, Thambyah and Broom suggested that increased tidemark development in the cartilage and bone spicule formation could be assigned to pre-OA state and that the early bone changes in the subchondral bone could be either initiation of the catabolic occurrences or enabling the disease advancement [90].

1.4.4. Bone Matrix

Bone is a connective tissue made up of mineralized organic matrix, cells like osteoblasts, osteoclasts, osteocytes and non-collagenous molecules like proteoglycans
and collagenous component mainly type I collagen. Mineralization is due to hydroxyapatite crystals which are interspersed among the collagen fibrils [92]. Bone remodelling occurs by the bone matrix synthesis by osteoblasts and bone matrix degradation by osteoclasts. These two cells maintain the homeostatic balance of minerals in circulation. During bone remodelling, the osteoclasts reabsorb the mineralized matrix and then undergo apoptosis and make way for osteoblasts to lay new bone at the site [40].

1.4.5. Subchondral Bone Remodelling

Bone marrow lesions (BML) and cysts are linked to knee OA [93]. Studies have found that the BML-like signalling induces bone cysts formation in OA [94]. BMLs help to determine cartilage loss, which match the extent of BMLs [95]. Presence of BMLs also indicates the risk of OA knee progression in patients [96]. The presence of BMLs results in limited supply of nutrients to the cartilage and at the same time, disrupts the bone’s structural support to the cartilage [94]. Only BML is associated with OA clinical symptoms [97] not bone cysts [98]. OA subchondral bone undergoes abnormal metabolism resulting in increased alkaline phosphatase activity and abnormal levels of proteins like osteocalcin [99], osteopontin [100], TGF-β1, IGF-β1 [101] and various interleukins [102].

OA subchondral bone shows increased stiffness and collagenous content but the bone undergoes hypo-mineralization [103]. One study showed that the tibial plateau in an OA knee expanded, when increased load was applied [104]. When such loads are applied to normal bone, while expanding they deform the cartilage.

Type I collagen is increased in subchondral bone during OA. The ratio of alpha-1 and alpha-2 chain is varied from normal 2.4:1 ratio to 4:1 or 17:1 in OA bone tissue [105]. This lowered level of alpha-2 chain result in tighter packing of the collagen fibrils, lower cross-linking [106] and increased hydroxylation of lysine in collagen fibrils [105]. Increased levels of TGF-β1 has an important role in the increased ratio of collagen alpha 1 and 2 chains and hence production of abnormal collagen and mineralization [107].
Resorption is an important process during bone remodelling, which is increased during OA. Studies have found that during OA, increased production of osteoclasts, proteases like MMPs and cathepsin K occur to aid increased resorption; increased loss of bone is observed during early OA [108]. The osteochondral remodelling also involves the presence of vascular channels with nerve endings, which are the reason for the pain associated with OA [109].

1.4.6 Catabolic Factors in OA

Cartilage tissue has inherent local factors like matrix degrading enzymes, cytokines and inflammatory mediators, which interact with each other and with the chondrocytes via signalling, and maintain the matrix homeostasis. Due to an external or internal interference, at times, these local mediators are triggered to cause more damage to the ECM.

1.4.6.1 Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases are zinc ion containing enzymes expressed by the synovial cells and the chondrocytes and play an important role in tissue remodelling and in maintaining homeostasis. The three main categories of MMPs are collagenases, stromelysins and gelatinases. The collagenases are expressed at a higher level in OA cartilage [106] and they can cleave collagen fibrils disturbing the network, causing more water to be trapped in the tissue and thus resulting in cartilage softening. These enzymes cause irreversible fibrillation of the cartilage tissue. MMP-13 cleaves collagen II and is suggested to be involved in remodelling; it is found in intermediate and deep layer of OA cartilage tissue [110, 111]. The MMP-1 is thought to be involved during the inflammatory process. MMP-3 activates other MMPs, cleaves aggrecan and is also known to be upregulated in early OA and interestingly, downregulated at the later stage [112]. MMP-2 and 9 are called gelatinases. MMP-9 is specifically seen in OA tissue and not in normal [113] and hence could be involved in OA progression. MMP-2 is found to be increased in OA tissues [114]. In MMP-13 knockout mice, the cartilage was found to be protected from erosion when the aggrecan was degraded [115]. Thus,
1.4.6.2 Cytokines

They are the class of mediators which are involved in signal transduction and cell-cell interactions in cartilage and bone tissue. The main types of cytokines are interleukins (IL) and tumour necrosis factors (TNFs). Interleukins are involved in cartilage and bone ECM breakdown and also stimulate other degrading factors involved in ECM remodelling [116]. Interleukin 1 beta (IL-1β) activates the degradation pathways and suppresses the synthesis of macromolecules like type II collagens and aggrecan [117]. Active IL-1β is not synthesized by chondrocytes in OA cartilage [118]. Cytokines stimulate MMP 3 and 13 expression and lowers the level of collagen and aggrecan in a disintegrating cartilage [119]. Other cytokines like TNF, IL-6, IL-17, and IL-18 are also expressed in OA cartilage. TNF-alpha, a pro-inflammatory cytokine is produced by macrophages and by tissue’s resident cells. They appear at the late stage of cartilage degeneration and further influence cartilage breakdown as well as bone resorption.

1.4.6.3 Aggrecanases

The major aggrecanases are ADAMTS-4 and 5. Apart from these, various other serine and cysteine proteases are seen in OA. ADAMTS cleave aggrecan and are secreted as zymogens, which need to be activated [120, 121]. Proteoglycans or aggrecans are cleaved between Glu373-Ala374 by ADAMTS [114]. In ADAMTS5 knocked out mice, the surgical model of OA progress was limited [122].

1.4.6.4 Pro-inflammatory Factors

Nitric oxide (NO) is an inflammatory factor which is synthesized more in OA cartilage and has increased presence in OA synovial fluid and serum. The inducible NO synthase (iNOS) is upregulated in OA and is the reason behind the higher levels of NO. NO has important roles in various degradation associated processes like synovial inflammation and chondrocyte apoptosis [123]. Prostaglandins and leukotrienes are
eicosanoids which are inflammatory factors in cartilage and are largely implicated in the joint inflammation [40, 124, 125].

Adipokines like adiponectin, leptin, resistin and visfatin are soluble mediators secreted by adipose tissues. Obesity is one of the risk factors of OA [126] and, given that the joints are always in a loaded state, this can lead to low grade of inflammation through adipokines, which are associated with immunity and inflammation [127]. A study recently analysed explants from osteoarthritic joint infrapatellar fat pad and found that the tissue secretes higher levels of adiponectin and leptins [128]. Adipokines are synthesised by chondrocytes too and help in cartilage homeostasis and also participate in its degeneration [129].

1.4.6.5 Growth Factors

Growth factors have important roles in tissue homeostasis and remodelling, wherein they stimulate cell proliferation and differentiation. In a distressed tissue, their innate anabolic role takes on a catabolic side and upregulates the synthesis of various ECM components and factors. The important cartilage growth factors are insulin-like growth factor-1 (IGF-1), transforming growth factor beta (TGF-β), bone morphogenetic protein (BMP) and fibroblast growth factors (FGF). IGF contributes to aggrecan and type II collagen synthesis [130]; TGF-β have both anabolic and catabolic roles in damaged tissue where on the one hand it controls interleukins’ actions and protects the tissue, while on the other hand it contributes to cartilage mineralization [131-133] as part of a faulty remodelling. As the tissue degenerates, the IGF acts initially promoting cell proliferation and differentiation. Later, TGF-β and BMPs help with mineralization and bone formation. Most growth factors contribute to a catabolic cycle by promoting the ECM degradation and suppressing further ECM synthesis.

1.4.6.6 Matrix Fragments

Cartilage matrix degradation is one of the foremost events in OA pathology. Many studies have concluded that the fragments generated through the matrix degradation, is capable of sustaining the disease and thus contribute to OA cycle [134]. Once the cartilage suffers a mechanical stress or is stimulated by inflammatory or cytokine
mediators, the cartilage ECM components undergo degradation resulting in fragmentation of many cartilage elements. These fragments are known to further aggravate the cartilage degradation pathway.

As cartilage degradation process occurs, proteins such as fibronectin, SLRPs and collagens are fragmented; these fragments interact with integrin receptors and initiate or amplify feedback mechanism of matrix destruction by production of proteases, cytokines and chemokines [129]. Fibronectin fragments are increased in ECM and synovial fluid during arthritic diseases [135]. Fibronectin fragments stimulate the degradation of other intact fibronectin molecules [136] and is also found to contribute to proteoglycan loss and in suppression of new proteoglycan synthesis [137]. Moreover, cytokines and MMPs production are upregulated by the fibronectin fragments and these catabolic molecules then carry on with further degradation of the cartilage matrix [138, 139].

Collagen type II is another molecule which is fragmented during OA. These molecules are normally found to be associated with proteoglycans in the inter-territorial matrix and hence they are not easily accessible to the proteases. But once the cartilage damage sets in, the proteoglycan loss results in them being more susceptible. Their cleavage into N-telopeptides and C-telopeptides result in upregulating MMP production in cartilage [134, 140], resulting in further GAGs loss and inhibition of collagen synthesis [141].

1.4.7 Cartilage Degeneration in OA

Degeneration of cartilage due to age is an unavoidable mechanical wear of the tissue, which results in irreversible loss of structure and sometimes cartilage function. The cartilage degeneration is most obvious at the site where maximum loading occurs [142]. Yet, superficial fibrillation in cartilage does not always result in OA [139]. The progression of OA in each patient varies and the pain and mobility restriction is not always necessarily proportional to the level of joint degeneration [143]. The disease progresses slowly and affects all organs which form a part of the joint.
Most of the time, OA develops without a known cause (primary or idiopathic osteoarthritis) and other times as a result of joint injury, mechanical stress or some kind of biochemical/biological stimulus (secondary OA). A major challenge in OA research is the variability among the subjects in every experiment. As the disease progresses non-uniformly, with inconsistent repair and remodelling mechanism in individual patient, the rate of degeneration also varies [143]. Primary changes in OA are often cartilage degeneration, subchondral remodelling and osteophyte formation [144, 145].

When articular cartilage is lost, more stress is placed on the subchondral bone, stimulating it to undergo remodelling. Therefore, by the time symptoms develop in OA, both cartilage loss and subchondral bone changes must have already set in. Fibrillation seen in the cartilage surface layer is one of the earliest structural modification in a joint. As OA progresses, these fibrillations run deeper, firstly into the transitional zone and then later into the deep zone. The clefts and the fissures running down from the cartilage surface to the subchondral bone enable elevated cross-talk among the two tissues [146]. These structural changes have been linked to the collagen network breakdown caused by the loss of proteoglycan and is also attributed to the marked increase of the proteases, degradative enzymes and pro-inflammatory factors in the ECM [40, 129, 147, 148]. The cartilage is depleted gradually over time and eburnated bone is left behind [143], leading to enhanced pain and inflammation.

The structural degeneration of cartilage is not fully comprehended and what makes the research more challenging is the lack of knowledge of the "trigger point" in OA. The fact that other joint organs also equally contribute to OA development, makes it even more complicated to identify the disease’s origin [149]. Being a multi-faceted disease, wherein many catabolic actions occur [40] either simultaneously or sequentially in a joint, understanding the OA progression also is a challenge.

The balance between the anabolic and catabolic mechanisms is maintained as long as the joint is healthy. As the chondrocytes fail to maintain the balance between the anabolic and catabolic mechanisms in the cartilage, tissue degeneration sets in.
According to Buckwalter and Mankin, cartilage loss in primary OA can be broadly divided into three stages: changes in cartilage ECM, chondrocyte's reaction towards the cartilage degeneration and finally the decreased level of anabolism and increased catabolic activity by the chondrocytes [143].

Change in cartilage ECM occurs by the breakdown of collagen network [150], whereby the cartilage entraps more water which leads to softening of the tissue [151], making it more susceptible to mechanical wear. The increased water retention is due to loss of proteoglycans in the tissue. The collagen network breakdown is caused when the collagen fibril cross-linking is disrupted either by proteases or other degradative enzymes [152] or inflammatory factors. The concentration of fragments of aggrecan and type II collagen are seen to be elevated in synovial fluid of patients with OA [153, 154].

Second stage begins once the chondrocytes detect the damage happening in their surrounding environment. Chondrocytes can detect the mechanical stress and the change in pericellular matrix (PCM) through receptors like integrin. In normal chondrocytes, these receptors are protected by the unique organisation of PCM. However, biomechanical changes in cartilage bring about the PCM disruption driven by High Temperature Requirement serine peptidase A1 (HTRA1) [155]. So, when the PCM integrity is compromised, it leads to an altered protease expression in the tissue [129]. Additionally, any change in primary cilium in chondrocytes can also lead to loss of chondrocyte homeostasis [129, 156, 157].

During cartilage degeneration, the quiescent chondrocytes are triggered to develop apparent hypertrophic phenotypes like cell clustering, cell proliferation as well as synthesis of anabolic and catabolic factors, leading to matrix remodelling and cartilage calcification [158]. The synthesis of macromolecules and cell proliferation occurring in the tissue, could be viewed as the repair mechanism adopted by the cartilage in the second stage. In some cases, these repair mechanisms stall the rate of progression of OA for a small period but never permanently.

The surface layer disruption in cartilage is caused when the degradation factors destabilize the collagen II fibril network, by degrading molecules like collagen type IX
and XI and SLRPs, which help in cross-linking. Additionally, matrix components like fibronectin which are degraded in the cartilage, stimulate further cartilage degradation by inducing cytokines and proteases [139, 159, 160]. Under mechanical and biochemical stimuli, chondrocytes also produce more nitric oxide [161, 162] which induce cytokines to produce more proteases, resulting in further degradation of the cartilage.

Moreover, mechanical stress due to injury releases various cartilage degradation products, which act similar to cytokines by triggering the inflammation signalling pathways. They induce mitogen activated protein kinase (MAPK) signalling [163] which regulates MMP-13, inducible nitric oxide synthase (NOS2), ADAMTS, cyclooxygenase 2 (COX2) and IL-β1 gene expression. These pathways promote cytokine and chemokine gene expression, suggesting that they have a direct or indirect role in tissue degeneration and in the inept cartilage repair process [158]. Increased level of cytokines and growth factors detected in OA synovial fluid [164-166], suggest the same.

Chondrocytes express toll-like receptors (TLRs) which are increased during OA and their ligands are damage-associated molecular patterns (DAMPs) or alarmins. These receptors are increased in cartilage near OA lesion and activate degradative proteases via NF-KB (Nuclear factor kappa-light-chain-enhancer of activated B cells) signalling [167].

Common signalling pathways involved in OA apart from cartilage homeostasis and structure mechanisms are lipid and carbohydrate mechanisms, cytokine signalling and cytoskeleton remodelling [168]. Inhibition of metabolism related proteins is often seen in OA pathology. Proteins related to energy metabolism like aldolase C, lactate dehydrogenase H, enolase-α and glyceraldehyde 3-phosphate dehydrogenase are known to be downregulated in OA tissues [169]. Other proteins like aldehyde reductase and inositol-1-monophosphatase 1 [170] and aldolase-a, superoxidase dismutase 2 [171] among others were also found to be downregulated in OA samples. Immune pathways are also associated with OA degradation. The degradation products of SLRPs like decorin and fibromodulin modulate the classic complement
COMP contributes to tissue degeneration by activating alternate complement pathway by complexing with complement component 3b (C3b) [172]. Abnormal loading and oxidative stress trigger inflammation mediators like cytokines, chemokines, COX-2, soluble phospholipase A2 (sPLA2) and NOS2 which in turn intervene with chondrocyte function, resulting in cartilage loss [129]. The chondrocytes in clonal clusters have receptors that respond to cytokines and chemokines generated in the OA joint [173]. Chondrocytes express chemokine and cytokine receptors in presence of cartilage degradation products and thus, play an important role in activating catabolic pathways and chondrocyte hypertrophy [174]. Chemokines act as chemoattractant and have important role in homeostasis and immune response [175]. Over activation of chemokines lead to inflammation [176]. Increased level of chemokines was also seen in synovial fluid of OA and RA patients [177].

The vascular channels containing blood vessels are present in the osteochondral junctions and they allow the movement of soluble mediators across bone and cartilage. During catabolic phase of the cartilage degeneration, many bone cells derived deleterious factors make their way into cartilage, across the osteochondral junctions [178] through enhanced vascular systems.

In OA cartilage, increased hypoxia is also observed [179] which in turn induces angiogenesis [180], illustrating the stress-related homeostatic nature of this process. During joint disorders, the lowered oxygen level in cartilage alters the level of reactive oxygen species or ROS [181]. ROS have the normal physiological function of acting as signalling molecules in chondrocytes, but their higher levels in cartilage have detrimental effects, leading to cell death [182].

Alarmins, through inflammatory signalling pathways can increase ROS production and thus matrix degradation [183]. Some alarmins like S100A11, enable OA progression through chondrocyte hypertrophy and matrix degradation [184]. Autophagy is the mechanism by which the cell turnover is maintained during cartilage homeostasis, however, when cells are under stress, autophagy declines but the apoptotic cell death is increased [185].
As the disease advances over time, the catabolic mechanisms overtake the cartilage synthesis. If the damage in tissue is not contained by the second stage, it enters the third phase of OA, where the synthesis of matrix components is stopped and the chondrocytes start to undergo apoptosis. With increased level of catabolic activity happening in the cartilage, anabolic factors are also down-regulated. The earliest change observed in the subchondral bone during OA is the increase in bone density due to the addition of new layers on existing bone. Towards the end stage, where the cartilage layer have completely eroded away from the bone, a thickened highly dense subchondral bone is left behind [143].

Enhanced cartilage calcification is seen in OA joints of elderly patients [186]. The calcium pyrophosphate crystals activate TLRs present on the chondrocytes [187] and the hydroxyapatite crystals stimulate interleukins in the cartilage [188]. Thus, increased calcification in cartilage can also contribute to OA pathogenesis through inflammatory pathways.

At the cartilage bone interface, another OA feature is the development of osteophytes. Osteophytes are cartilaginous structures which protrude from the joint surface and limit the movement, causing pain during movements. Since these are the cartilaginous-like features, it can be assumed that osteophytes are the result of increased anabolic activity occurring as the counter mechanism to the cartilage degradation and sub-chondral bone remodelling [189, 190].

After the loss of cartilage during OA, all the organs in the joint attempt to balance out the role of the missing cartilage. This results in an ineffective load distribution in the joint, leading to inflammation of organs like the synovial tissue. The whole joint including muscles, synovial capsules and ligaments are affected in the process. In addition, the advancement of tidemarks and the increased vascularity in subchondral bone, could be the joint’s response to a mechanical insult [90]. But whether these changes in cartilage precede or accompany the subchondral bone changes, is still not clear.
1.5 Cartilage Proteomics

The key molecules involved in the biological processes in cartilage should be understood adequately, before commencing on the treatment for joint diseases like OA. Since the pathophysiology of the disease is not yet fully understood, any new information with regards to molecules involved or its function in cartilage, can prove valuable for the basis of new hypothesis-based researches in OA.

Proteomics involve the comprehensive study and characterization of the proteins in a cell or tissue, to obtain a global view of its biology [191] at a precise time, in a particular environment/condition [192]. It helps to identify the proteins' profile in the healthy as well as in the diseased tissues, broadening our understanding of pathophysiology of the disease. Proteomics studies have an upper hand over the genetic and transcriptome analysis, since it can also consider the post-translational modifications and thus reflect the final constitution of proteins in the tissue.

The proteomic analysis most essentially involves sample preparation, digesting and resolving the proteins into smaller peptides, followed by sample fractionation by LC, data acquisition and finally, analysing the mass spectrometric data through bioinformatics-based tools/software [193]. The samples used in OA research can be whole cartilage tissue, synovial fluid, serum or urine from subjects; or in vitro systems where either primary chondrocytes or tissue explants are cultured. Every analysis method has its own advantages and disadvantages. If the investigation is to understand the cartilage physiology, then analyzing the whole cartilage tissue would prove more beneficial than the primary chondrocyte based cultures as the whole cartilage samples give the exact state of proteins in the tissue.

Analysis of synovial fluid, serum and urine is an important aspect in the biomarker research. The synovium and subchondral bone nourish the cartilage. Cartilage derived proteins could be found in the biological fluids which are in direct or indirect contact with the cartilage. Therefore, investigating these sources would prove more helpful in biomarker identification. However, often discovery-based proteomic/genomic study precedes biomarker search, wherein the molecules
identified as significant during the discovery part of the experiment, is screened further during the biomarker studies.

The highly abundant collagens and proteoglycans pose most challenge in cartilage proteomics, as they make the identification of less abundant proteins difficult [193, 194]. So most proteomic analyses adopt a proteoglycan removal procedure [170, 195] or use a culture based system [196, 197] to analyze the secretome and avoid the most abundant cartilage proteins. Proteoglycan removal procedure can also sometimes remove other proteins of interest which are found associated with PGs [194], hence the resultant expression profile may not show the complete reflection of pathology.

Primary chondrocyte culture based proteomics is another popular approach to study proteome profile, but the main limitation of this method is that the in vitro chondrocytes may not express the same protein profile as that of the in vivo cells, which are always under the influence of other physiological factors present in the cartilage [198]. Under the absence of these conditions, the in vitro chondrocytes may undergo some differentiation and care must be undertaken to avoid such changes [168].

In the quest to identify biomarkers, mass spectrometric techniques and proteomics-based analysis play critical roles. Discussing all proteomic techniques available is beyond the scope of this study, hence the techniques used as part of this study is discussed briefly.

1.5.1 Mass Spectrometry

Mass spectrometry (MS) is an analytical technique where the samples are ionized and then separated on the basis of their mass-to-charge ratios. High throughput, accuracy and robustness of mass spectrometry have made it an invaluable tool in proteomics [199]. Protein MS also offers rapidity and greater coverage of proteins analysed in cells or tissues [200].

There are three basic components in a mass spectrometer namely ion source, mass analyzer and detector. The working principle involves the ion source converting the analytes into gas phase ions, which are then separated based on their mass-to-charge
ratios in the mass analyzer and finally these ions are detected and quantified by the detector system.

Mass spectrometric analysis requires a systematic and efficient work-flow to analyse the sample to produce sensitive and throughput proteome coverage. In mass spectrometry, sample preparation is a very crucial step for the success of an analysis. The ability to resolve proteins from a complex sample mixture like the ones involved in OA research, makes these investigations more challenging [192]. Both gel-based techniques and gel free techniques are most commonly applied to separate the proteins in a sample. Gel-based methods use 1D or 2D SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to separate the peptides/proteins according to their molecular mass and appear as the bands in the gel. These gel bands are then cut out and processed further. The gel free methods can involve the samples to be either tagged with heavy isotopes or label-free. Techniques like iTRAQ isotope labelling is gaining more importance in proteomics. These coupled with fractionating methods like strong cation exchange (SCX) chromatography can resolve the analyte molecules in complex samples and can increase the quality of data identification. In both gel-based and gel-free methods, the samples are digested with trypsin and then separated by liquid chromatography (LC) followed by mass spectrometric analysis.

Less abundant analyte molecules are often lost among the chemical noises in a spectrum, whereas desorbing and ionizing these molecules from the biological specimen to gaseous state can help in extracting information from these less abundant proteins [201]. The two types of ionization methods used in protein mass spectrometry are matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). MALDI method uses matrices like α-Cyano-4-hydroxycinnamic acid (CHCA), sinapic acid (SA), 2, 6-dihydroxyacetphenone (DHA), 2, 5-dihydroxybenzoic acid (DHB), 1, 5-Diaminonapthalene (DAN), etc. for crystallization of analytes, which are then excited by laser application into gaseous state ions. The ESI method uses electrical energy to generate a fine spray of ions from the liquid phase. The ionized molecules are then sent to the mass analyser where they are separated based on their mass-to-charge ratios (m/z). The different types of mass analysers are mainly time-of-flight
(TOF), ion trap, quadrupole and Fourier transform ion cyclotron. These mass analysers are often used in combination as tandem mass spectrometer whereby they can greatly improve the proteomic data acquisition and analysis [202]. The major tandem MS instruments include quadrupole TOF, triple quadrupole, quadrupole ion trap and Fourier transform ion-cyclotron resonance [203].

Proper interpretation of the data acquired by MS techniques is essential for obtaining meaningful results. MS data analysis is a complex multistep process, requiring accurate yet consistent and quality data processing [204]. Programs like Mascot, Sequest among others are used to analyse the MS/MS spectra. Data analysis of MS spectra using database searches involve complex computational algorithms. These software identify the peptide sequences from the mass of the fragment and assigns the peptide to a particular protein with a corresponding score. Each software uses its own scoring system based on different algorithms and hence two different MS data analysis program do not yield identical results [205, 206].

High quality spectral data give more confidence to the peptide identification. In shotgun proteomics where bottom-up proteomic techniques are used in identifying proteins from complex mixtures, the most challenging part of data analysis is the accurate identification of the peptide and assigning it to its parent protein, as each peptide can be common to numerous proteins and hence adding a probability score to it, increases the validity of protein inference [207].

Targeted strategies can confirm the peptide identification with greater sensitivity because they monitor specific product ion spectrum instead of full scale mass spectrum [208]. The multiple reaction monitoring (MRM) is a powerful tool utilising the ability to identify targeted peptides in a complex analyte mixture [209]. This method has evolved to be a major choice for the validation process of the mass spectrometry results over traditional validation methods since it has the ability to monitor several peptides/proteins in a single experiment (Figure 1.4) [210, 211].

Proteins interact with other proteins in vivo and also with DNA and RNA. Software tools like Ingenuity and Gene Ontology helps in categorising the proteins based on their functions [168]. The integration of gene expression, protein, miRNA profiling,
and clinical data to generate “interactome” network would help in understanding the complete network of the disease [168, 212].

Figure 1.4 Comparison of discovery (data-dependent) proteomics and targeted proteomics. Both analyses involve separation of the analytes on a reversed phase column attached to liquid chromatography instrument, which are then converted into gas phase ions. (a) During the full-scan of mass spectrum, the most abundant peptide ions are selected based on their intensity (Q1) and fragmented by collision with inert gas (Q2). The complete summary of peptide fragments are detected in Q3. (b) Peptides of interest are selected (Q1) and fragmented (Q2). Based on user-specified list of transitions (precursors and fragments pairs) the selected fragment ions are detected in Q3.

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1.5.2. Proteomics in Cartilage Research

Proteomic analyses result in vast amount of data which need to be interpreted accurately in order to identify the key players in cartilage on basis of their functions. Much of the earlier work done in cartilage proteomics relate to the comparison between healthy and OA tissues. A study by Wu et.al found 59 differentially expressed proteins in human OA cartilage. Proteins thrombospondin 1 (THBS1), vitrin (VIT), TGF-β1 and HTRA1 were detected in this study [170]. De Seni et al. have detected four novel proteins that have relevance in the pathophysiology of OA, three of which were identified as V65 vitronectin fragment, C3f peptide and CTAP-III. The fourth protein of m/z 3762 was not identified [213].
Vincourt et al. selectively extracted proteins from human cartilages and resolved it over 2DE and found that increased level of matrillln-3 contribute to cartilage degeneration [214]. Wilson et al. did sequential extraction of the cartilage, based on the solubility during fractionation with NaCl and guanidinium hydrochloride, followed by 1DE and immunoblotting. This proteomic analysis showed high inter-sample reproducibility [215]. Human articular cartilage was digested using MMPs and aggreganases in a study and the cartilage degradation products were characterized using MS [216]. Following removal of highly abundant proteins like proteoglycan and collagen from the knee joint cartilages, mass spectrometric analysis resulted in identification of 14 proteins which were differentially expressed in OA cartilages [217]. In another experiment, following proteoglycan aggregation, proteins were selectively extracted from human articular cartilage using cetyl-pyridinium chloride and 127 proteins were identified [218].

Garcia et al., digested human OA cartilage (Kellgren-Lawrence grade 4) with liberase following which the proteins were resolved by 1D-SDS PAGE (one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis), trypsin digestion and nano-liquid chromatography MS/MS. They found that differentially expressed proteins in the human samples were involved in ECM composition, signal transduction, immune response and energy metabolism. They also identified cartilage degrading proteins tenascin C (TNC), lysozyme (LYZ) and triose phosphate isomerase (TPI) from the samples [195].

There is great interest in studying the chondrocytes, being the only cell in tissue, its inability to balance the cartilage metabolism is responsible for the aggravated tissue degeneration. Ruiz Romero et al. isolated and cultured normal articular cartilage chondrocytes and the protein extracts were resolved on 2-D gels and the protein spots were crystallized and analysed by MALDI-TOF to identify the protein profile in the chondrocytes. Their analysis found 93 proteins which were involved in various cartilage functions [198]. Another study by these investigators involved culturing chondrocytes from osteoarthritic and normal knee joints. After 2-DE and MALDI-TOF, they found 28 proteins to be differentially expressed, among which gelsolin, heat
shock protein 90 and vinculin were upregulated in OA [169]. Lambrecht et al. also analysed chondrocytes after 2DE and MS/MS and found cleaved vimentin to be upregulated in OA samples. After discovering the interference from proteoglycans and collagens, Catterall et al. used different methods to remove GAGs and found anion exchange to be most useful. They also found fragmented cartilage molecules including MMPs in their work [219].

Another popular methodology in OA research involves culturing the cartilage tissue explant over a small time period and analysing its secretome. Most culture based OA studies involve in vitro treatment of explants with cytokines. Even though cytokine is known to emulate similar environment as seen in OA tissues in vivo, it is not the best stimulant to understand the pathophysiology of OA [134] considering the inconsistencies in the data from various studies (discussed further in Chapter 4).

Peffers et al. analysed the human cartilage secretome, under cytokine treatment. They employed use of Q-peptides and labelled multiple proteins in the study. This analysis led to identification of a total of 252 proteins, out of which 9 were found to be differentially expressed [197]. Polack et al. were first in using SILAC technology in analysing cartilage proteome. In SILAC or Stable Isotope Labelling of Aminoacids in cell cultures, the culture medium is supplemented with isotope labelled aminoacids and cells/explants are grown in it and later analysed by MS. They analysed both cartilage explants and chondrocyte monolayers and found that secreted proteins were mostly cartilage components and matrix regulators along with inflammation related and metabolic related proteins. However, they could not successfully differentiate the protein profile between the explants and chondrocyte monolayers [220].

Cartilage explants from equine joints were cultured in presence of IL-1β and its inhibitor carprofen, in a study by Williams et al. and it was found that the level of MMPs decreased in the presence of carprofen [221]. In another study, the cartilage explants were grown in a medium containing [35S] labelled methionine/cysteine, to identify the newly synthesised proteins. This experiment found that the collagen type II was strongly upregulated in OA cartilages [222].
Apart from chondrocytes and cartilage, the synovial fluid has also been analysed to determine the protein profile during OA pathology and disease treatment. Synovial fluid remains in direct contact with the physiological and pathological conditions of the cartilage and is found to contain many ECM components.

Also of much interest is the molecular events happening during cartilage breakdown. Based on the visual state of the cartilage researchers have tried to garner more information on cartilage degeneration processes. Lambrecht et al. cultured chondrocytes from intact and lesion regions of the human articular cartilage. They found upregulated vimentin in lesion samples, suggesting distortion of vimentin cytoskeleton at the degenerated site [223] and the same was found through microscopic analysis where the vimentin network was distorted in the OA samples. Comparison of chondrocytes from intact and lesion cartilage sites, as part of a paired analysis to study the synthesis and turnover of ECM, found that the anabolic factors like cell-associated matrix (CAM) aggrecan, type II collagen and fibronectin decreased whereas degradative interleukins were increased in the lesion site samples [224]. Rolauffs et al. identified the change in spatial organization of chondrocytes in the intact sites cartilage lying adjacent to the lesion site and suggested that through reorganization, the chondrocytes in the intact regions are attempting to counteract the adjacent cartilage damage [225]. More recently, Lourido et al. analyzed the secretome from healthy as well as intact and lesion site cartilages and found that the OA tissues differentially expressed 76 proteins. They used the analysis as the basis to study the OA progression and found osteoprotegerin and periostin as potential OA biomarkers [226]. Sato et al. identified significant differences in the gene expression of intact and lesion site human OA cartilages [227]. Geyer et al. also found that genes were expressed differentially between intact and damaged articular cartilages obtained from the same joint of OA patients. In addition, they also found WISP-1, AQP-1 and DNER as novel target molecules and potential biomarkers involved in the pathogenesis of OA [228]. All these studies have added to our understanding of the OA progression and its severity.
1.6. Osteoarthritis and Biomarkers

Proteomic analysis has helped us to broaden our understanding of OA pathology, but advances in regard to the diagnosis and management of disease have been limited. More work needs to be done to see the results at clinical levels [229]. Being a multifactorial disease, several states coexist during OA namely imbalanced biochemical factors, cellular/tissue degeneration and remodelling, immunological mechanisms, inflammatory reactions, catabolic pathways, defective repair mechanism, among many others.

A biomarker is a measurable indicator of risk, presence or stage of a disease. They can be used to diagnose (diagnostic), predict disease progression (prognostic), monitor activity of the disease, assess the therapeutic response (screening) or guide molecular targeted therapy [230]. Molecular biomarkers include genomic, proteomic and transcriptomic biomarkers. Biomarkers for joint diseases may come in many forms—clinical, histological, imaging parameter or molecules [231].

Biomarker discovery is categorised into four phases mainly discovery, qualification, verification and validation. In discovery phase, samples are analysed through various techniques to cover its full proteome, which is then further refined using rigorous criteria and is tested by targeted methods in qualification phase to evaluate its potential as a biomarker. This is followed by verification phase, where the potential markers are tested in large number of samples and applied in clinical trials to check for its specificity. Finally a small set of markers are validated by highly sensitive and specific assays, ideally in thousands of samples [230].

Biomarkers are classified using BIPED where B stands for burden of disease, I for Investigative, P for prognostic, E for efficacy in treatment and D is for diagnostic. In brief, B biomarkers gives the current status of disease, I is marker still under investigation which does not have sufficient data to classify them to other groups, P biomarkers gives the future aspect of the disease either the risk of disease occurring or the prediction of its progression rate, E biomarkers are used to measure the
response to drugs and other treatment methods and D markers are used to diagnose the patients from the general population [229, 232].

Over the years, many research groups have contributed in identifying the potential biomarkers of OA. These biomarkers have added immensely to our knowledge of OA pathophysiology. Fernandez-Puente et al. found 349 proteins in the serum of healthy and moderate to severe OA patients, among which they found COMP, von Willebrand factor, tetranectin and lumican as potential biomarkers [233]. Several proteins including IL8, MMP2, MMP3, IL6, MMP1, MMP9, IL-1β, apolipoprotein A1, apolipoprotein E, decorin, cartilage intermediate layer protein (CILP) among others were tested for their use as biomarker [234, 235].

In another study, serum from knee OA patient were analysed for biomarkers like COMP, HA, C-reactive Protein (CRP) and keratan sulfate (KS), where the disease progression was followed up with radiographs. It was found that higher baseline COMP and HA levels were associated with radiographic changes in knee OA [236]. The effect of SheaFlex70 (a triterpene-rich extract of Vitellaria paradoxa) on OA biomarkers like TNF-α, CTX-II and osteocalcin was investigated in another study where it was found to have beneficial effects in reducing the levels of these biomarkers in plasma, serum and urine samples [237]. Biomarker assays have also been developed to measure the collagen metabolic products in cartilage from both healthy and diseased subjects [238]. Gordon et al. monitored activity related changes in level of HA, COMP, KS, TGF, collagen II-related epitopes (CPII) in serum samples and CTXII and C2C in urine samples of patients with radiographic OA [239]. Steffey et al. studied the potential and limitation of COMP and fibronectin as OA biomarkers, using canine synovial fluid and found that their use as a diagnostic biomarker is limited [240].

Following iTRAQ based proteomics, Ikeda et al. identified 76 proteins which were differentially expressed between OA patient and control, amongst which leukocyte cell derived chemotaxin-2 (LECT2), peroxiredoxin-6 (PRDX6) and brain and acute leukemia, cytoplasmic (BAALC) were proposed as novel biomarkers [241]. Other OA biomarkers reported in the literature include cartilage intermediate layer protein
(CILP) [242], cartilage oligomeric matrix protein (COMP), hyaluronan (HA) [243] and several other extracellular matrix (ECM) components and its degraded fragments [244, 245].

Till date, most of the OA biomarkers were identified based on their role in the disease pathology. The biomarkers identified in OA research mainly pertain to molecules of joint turnover (cartilage and bone) or biochemical factors of (pro-) inflammation [245]. The cartilage turnover biomarkers mainly involve degradation products from the ECM, while the inflammatory biomarkers are cytokines, chemokines and other inflammatory pathway proteins. Other biomarkers fall into the category of signalling molecules, growth factors and auto-antibodies.

Reviewing each of the biomarker identified to date is beyond the scope of this study, since each year more than 100 papers are published in relation to OA biomarkers [229, 245]. The biomarkers are briefly summarised according to the metabolic processes they are involved in as shown in Table 1.1 [246].

The joint turnover biomarkers have an upper hand over other metabolic biomarkers, because of higher stability and sensitivity [247]. OA pathogenesis is not fully understood, this makes it more challenging to employ the biomarker at clinical levels. In addition, the OA progression varies from patient to patient making it difficult to capture various samples in an experiment, at the exact condition/state [248]. Another challenge is the wider use of traditional methods like X-rays to predict the disease status and the joint improvement due to treatment [229]. Future researches based on identifying the molecules which play important role in OA pathophysiology, identification of the early stage of osteoarthritis along with the development of high throughput and sensitive assays, could play important role in biomarker discovery.
Table 1.1 Selected osteoarthritis related biomarkers [246] based on earlier OA researches [245, 249-258]. Reproduced from [246] with permission from BMJ Publishing Group Ltd.

<table>
<thead>
<tr>
<th>Biomarkers related to collagen metabolism</th>
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<tr>
<td>▶ C-terminal telopeptide of collagen type II (CTX-II)</td>
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<td>▶ Type II collagen α chains collagenase neoepitope (α-CTX-II)</td>
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<tr>
<td>▶ Type II collagen propeptides (PIINP, PIIANP, PIIBNP, PIICP, CPII)</td>
</tr>
<tr>
<td>▶ Pyridinoline and Glc-Gal-PYD</td>
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<tr>
<td>▶ Type II collagen cleavage product (C2C)</td>
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<tr>
<td>▶ Collagen type II-specific neoepitope (C2M)</td>
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<tr>
<td>▶ C-terminal telopeptide of collagen type I (CTX-I, α-CTX-I)</td>
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<tr>
<td>▶ N-terminal telopeptide of collagen type I (NTX-I)</td>
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<tr>
<td>▶ Aminoterminal propeptide of collagen type I (PINP)</td>
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<td>▶ Types I and II collagen cleavage neoepitope (C1,C2)</td>
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<tr>
<th>Biomarkers related to aggrecan metabolism</th>
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<tr>
<td>▶ Core protein fragments (aggrecan neoepitopes, ARGS and FFGV fragments)</td>
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<tr>
<td>▶ Chondroitin sulfate epitope 846 and monoclonal antibody 3B3(−)</td>
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<td>▶ Keratan sulfate</td>
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<th>Biomarkers related to other non-collagenous proteins</th>
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<tr>
<td>▶ Cartilage oligomeric matrix proteins (COMP and its deamidated form D-COMP)</td>
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<tr>
<td>▶ Fibulin (peptides of fibulin 3, Fib3-1, Fib3-2)</td>
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<td>▶ Follistatin-like protein 1 (FSTL-1)</td>
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<td>▶ Hyaluronan (hyaluronic acid)</td>
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<tr>
<td>▶ Matrix metalloproteinases (MMP-1, MMP-3, MMP-9, MMP-13 and TIMPs)</td>
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<tr>
<td>▶ YKL-40 (cartilage glycoprotein 39)</td>
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<td>▶ Soluble receptor for advanced glycation endproducts (sRAGE)</td>
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<th>Biomarkers related to other processes</th>
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<tr>
<td>▶ Inflammatory biomarkers: hs-CRP, IL-1β and IL-6 and COX-2</td>
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<tr>
<td>▶ Factors indicating fibrosis and complement proteins</td>
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<tr>
<td>▶ Adipokines (adiponectin, leptin, visfatin)</td>
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<td>▶ Soluble receptor for leptin (sOB-Rb)</td>
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<td>▶ Cellular interactions in bone (periostin)</td>
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<td>▶ Wnt inhibitors (DKKs and SOST)</td>
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<td>▶ Uric acid</td>
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Glc-Gal-PYD, glucosyl–galactosyl–pyridinoline; hs-CRP, high sensitivity C reactive protein; PIIANP, N-propeptide IIA of type II collagen; PIIBNP, N-propeptide IIB of type II collagen; PIICP, C-propeptide of collagen type II; PIINP, N-propeptide II of type II collagen; SOST, sclerostin
1.7. Pre- to Early OA

OA is not diagnosed until very late, when the patients start to experience pain and discomfort. At this stage, the cartilage loss would have already occurred along with the presence of subchondral bone lesions and osteophyte formation [87]. Joint space narrowing in the patients is a diagnostic measure for clinical OA [5, 259]. At this stage, the cartilage would have already entered moderate-to-advanced level of degeneration. While structural changes in OA involve both the articular cartilage and its underlying bone, there is still no consensus as to which precedes which – subchondral bone sclerosis or cartilage thinning [85, 86].

Thambyah and Broom studied the new bone formation occurring in the intact cartilage regions adjacent to the lesion sites. They suggested that the early tidemark development and bony spicule formation in the still intact cartilage region could represent pre-OA state. When the habitually loaded region of the cartilage is degenerated (lesion-like fibrillation), the adjacent intact cartilage helps to re-distribute the load. This puts additional stress onto those still intact regions, triggering certain structural modifications. It was found that for proper bone formation, the distal most tidemark of zone of calcified cartilage (ZCC) will always remain in front of advancing cement-line and newly developing vascular systems, as the ZCC is their source of calcium. These early bone and cartilage changes might play an important role in either initiating the degenerative process or in aiding its progression [90].

Endochondral ossification is the primary means by which subchondral bone changes occur during OA development [260]. In OA joints, the calcified cartilage becomes more mineralized and denser than the underlying subchondral bone [86]. This extensive development of the ZCC in early OA joint, was seen to parallel the corresponding calcification occurring in endochondral ossification in normal bone growth [261, 262]. Increased mineral content during endochondral ossification is attributed to increased chondrocyte apoptosis. This could also be the case with the increased mineralization seen in early OA joints [91, 263]. The newly formed bony spicules is thought to be vascularized at a later stage, nourishing the tissues and thus influencing the progression of the disease [89].
1.8. **Bovine model of pre- to early OA**

Human samples are ideal for OA researches. But due to the difficulty in obtaining and handling them, animal models are often preferred as next alternatives. Another major limitation with human samples are the difference in onset as well as progression of OA in individual subjects [248]. Animal models are widely used in OA research to understand the disease pathology as well as to understand the efficacy of various interventions in both spontaneous and induced OA. The non-invasive primary OA caused by spontaneous changes in animal model, is very valuable in understanding the early modifications in OA.

Moreover, the animal models have the following advantages over human samples [264]:

1) Easily available in large numbers
2) Early stages samples readily obtained
3) Spontaneous degeneration of cartilage
4) Late stages are not tampered with medications which are usually prescribed in human OA patients

Both small and large animal models are popular choice in OA research. However, disadvantages with the small animal models like mice, rats, rabbits and guinea pigs are the difference in their weight, joint anatomy and physiology from the human patients which makes translation of the results more difficult [265]. Widely used naturally occurring large animal models of OA are dogs, pigs, goats, sheep, horses and cows. These larger animal models could be easily related to human samples [265, 266].

A previously published study from our laboratory [266] has used bovine patellae to describe how the structural changes associated with this pre-to-early OA state can be correlated to the structural transformation of mild to full blown OA in human joints (Figure 1.5). Briefly, the study found that the still intact cartilage region next to lesion site in a bovine patella is similar to healthy human sample in terms of cartilage thickness, chondrocyte morphology, histological staining and zonal arrangement. Also, the cartilage degeneration and subchondral bone changes seen in bovine tissues
were similar to those observed during early to mid-OA in humans. Thus, bovine patella was proposed to be a good animal model to study the early changes in OA.

Figure 1.5 Typical histological sections of bovine and human cartilage-on-bone tissue stained with safranin-o and fast-green. Osteoarthritis Research Society International’s Cartilage Histopathology Assessment System (OOCHAS) scoring was done to grade the samples. The samples shown are (A) bovine intact (OOCHAS grade 1), (B) bovine degenerate (OOCHAS grade 4), (C) human intact (OOCHAS grade 1) age 60 male, (D) human degenerate (OOCHAS grade 4), age 62 male. Scale bar: 1 mm

Reproduced from [266] with permission from John Wiley and Sons.
Additionally, previous studies done in our laboratory involving the analyses of several thousands of bovine patellae of varying degrees of degeneration, have identified it as a suitable animal model for investigating the all-important pre-to-early OA changes in the joint [90, 266-271]. Like any other animal model, the major limitation associated with bovine tissues is the translational difference of therapeutic results from animal models to clinical subjects [272]. Another limitation includes the difficulty in data collection in these studies where factors like gender, previous injury, and animal husbandry conditions can cause variation to the results [265].
1.9 Aim

This study attempted to address the following questions/topics in the upcoming chapters:

Chapter 2: Structural Analysis of Articular Cartilage

1. Investigate the early structural changes in bovine osteochondral tissue- how the intact and degenerated cartilage in a non-healthy patella vary in terms of zonal arrangement, histological staining, chondrocyte morphology, matrix texture, ZCC modifications and bone changes?
2. Do these intact and degenerate cartilage samples from tissues showing early degeneration, vary considerably from the healthy tissues?
3. How reliable these changes are in terms of relating to the pathophysiology of OA?

Chapter 3: Generation of Proteome Profile of Bovine Articular Cartilage

1. Do gender variations play significant role in proteome of bovine model of OA?
2. Will cartilage samples taken from two different sites of a healthy patellae show significant proteomic variations?
3. How does the protein profile taken from degenerated region of patellae vary from its adjacent intact site? Are these changes significant and reproducible?
4. If the profile varies between the degenerated regions and intact regions, how well can these changes be correlated to their structural changes?
5. Can the site-specific variations seen in the degenerated cartilage tissue be used to explain the progression of OA?
6. Determining the potential biomarkers which show differential level between healthy samples and tissues with early cartilage degeneration.
Chapter 4: Cartilage Secretome Studies

1. Spontaneous model of OA: Does the non-healthy secretome show significant change from the healthy cartilage explant secretome without any external cytokine intervention?
2. Is significant site-specific secretion seen in the intact and degenerated cartilage explants?
3. Does the secretome analysis results correspond to the whole tissue analysis from Chapter 3?
4. Does the secreted molecules reflect the structural state of tissue - more anabolic proteins in intact region secretome and/or more catabolic proteins in the degenerated region secretome?

Chapter 5: MALDI- Imaging Mass Spectrometry

1. Investigate the healthy and degenerate cartilage tissues using MALDI-IMS.
2. Can MALDI-IMS differentiate the site-specific peptide variations between intact and degenerated cartilage regions in a non-healthy patella?
3. Are there significant site specific variations observed between two cartilage sites of healthy patellae?
4. Identify proteomic biomarkers that could discriminate the intact and degenerated regions of a non-healthy patella.
5. Does the MALDI-IMS results agree with the observations made in previous chapters?
CHAPTER 2

STRUCTURAL ANALYSIS OF ARTICULAR CARTILAGE

2.1 Introduction

Animal models are widely used in OA research to understand the disease pathology as well as to evaluate the efficacy of various interventions in spontaneous and induced OA. The non-invasive primary OA caused by spontaneous changes in animal, is an excellent means to understand the early changes occurring in the disease. Stifle is the most popular joint used in animal model of OA. While the access and control over the smaller animals make them an attractive option as the animal model for OA, the difference in their weight, joint anatomy and physiology from the human patients make the translation of results more difficult [265]. Larger animal models, like bovine samples can be easily related to human samples [266] to this end. The average thickness of human cartilage is reported to be 2.4 mm [273] and is well matched to average bovine cartilage thickness of 2.1 mm [266]. The pattern of joint degeneration in bovine animals is much more similar to that seen in humans.

On the definition of early stage OA, the research team of Thambyah and Broom have extensively studied cartilage, its mechanical and structural properties, and importantly how these properties change with mild to increasingly severe joint degeneration [90, 269, 270, 274]. Of relevance are the thousands of bovine cartilage tissue samples studied by the group, following which they have defined a range of structural states spanning the intact, mild, moderate and severely degenerate OA conditions. From studying the structural changes associated with the progression to the grossly degenerate state, the research team has developed and established a structural model for the all-important pre-to-early OA state of joint degeneration in bovine patella [266].
Two structural changes exist as clues of early cartilage degeneration in osteoarthritic joints. Firstly, is the spicules found in the pre-osteoarthritic joint [90], which suggest that early bone changes take place in conjunction with the cartilage changes. The other indicator of the early OA state concerns the micro-to-nano scale ‘destructuring’ of the collagen fibrillar matrix in cartilage [16, 270, 275, 276]. The highly organised network of type II collagen fibrils restrain the hydrophilic proteoglycan molecules and provide a constraint to the swelling pressure arising from water drawn into the cartilage matrix. Previous studies by Thambyah and Broom’s group have shown that the early degenerative changes in cartilage involve a gradual loss of interconnectivity between fibrils, which are visible at ultra or nano structural levels [267, 268, 271, 277, 278].

DIC microscopy enables the unstained specimen to be viewed by enhancing its contrast. Briefly, the polarised light is made to pass through two prisms- the first prism splits the incident light into two beams which while passing through the specimen is altered due to variation in thickness, slopes and refractive indices, while the second prism combines the two beams, removing the path difference between them and results in an optical image of the specimen with enhanced structural contrast [279, 280]. The main advantage of DIC microscopy is that the researcher can visualize the specimen in a more natural and hydrated state compared to conventional dehydrated and stained histology samples.

In this chapter, the two structural indicators of pre-to-early OA, spicule formation and collagen fibril ‘destructuring’ were analysed using DIC microscopy along with other microstructural features. This can provide a structure-based and functionally relevant direction to look for specific biomarkers associated with the early OA state and this is the rationale for upcoming chapters.


2.2 Methodology

2.2.1 Tissue Source and Classification

The bovine patellae used in this study were obtained from a local abattoir and therefore did not require ethical committee approval. The samples were obtained from cattle reared long for milk and meat and hence, any cartilage degeneration seen in the samples were assumed to be mostly spontaneous changes, at the same time keeping in mind that any history of joint trauma or lameness would be unknown.

The exact age of the female animals used is also unknown but due to their matured state, it is assumed to be 5-9 years at the time of slaughter. The patellae were collected during the boning process after slaughter and hence were from completely random animals. Except for the gender variations study, all the patellae were obtained from female bovine animals.

For convenience, the non-degenerated patellae used in the study are referred to as ‘healthy’ and the patellae with localised mild to moderate degeneration are termed ‘non-healthy’ in the text.

2.2.2 Sample Preparation

All the analysed cartilages came from freshly slain cattle. The grade of cartilage degeneration was assessed using Outerbridge’s scale [281] following India ink staining (Figure 2.1). For control experiments, two intact sites from 3 healthy patellae were taken from cows and bulls (Figure 2.1 A). From each non-healthy patella (n=12), a cartilage-on-bone block was sawn off from the region where the cartilage degeneration was most evident and another one from the region where cartilage was still intact and smooth (Figure 2.1 B and C). Outerbridge’s classification was used to grade the level of degeneration in patellae as shown in Table 2.1.
Figure 2.1 Representative bovine patellae showing the sampling sites – (A) Non-degenerate healthy patella (G0); (B) GI patella; and (C) GII patella. India ink staining to reveal the extent of degeneration showed that G0 (Control) patella contained no surface ink retention, GI patella is stained lightly revealing mild degeneration at the lesion site and GII patella is seen to have slightly intense staining, as the ink is retained in the moderate level fibrillations at lesion site.
Table 2.1 Outerbridge’s classification for scoring cartilage breakdown [281]

<table>
<thead>
<tr>
<th>Outerbridge’s Classification</th>
<th>Description</th>
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<tr>
<td>Grade 0</td>
<td>normal</td>
</tr>
<tr>
<td>Grade I</td>
<td>cartilage with softening and swelling</td>
</tr>
<tr>
<td>Grade II</td>
<td>cartilage fragmentation and fissuring do not exceed 1.5 cm in diameter</td>
</tr>
<tr>
<td>Grade III</td>
<td>fissuring to the level of subchondral bone in an area with a diameter more than 1.5 cm</td>
</tr>
<tr>
<td>Grade IV</td>
<td>exposed subchondral bone</td>
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2.2.3 Chemical Fixation

All samples were equilibrated in 0.15 M saline for two hours at 4 °C. The cartilage-on-bone samples were then immersed in excess formalin (10%) overnight, to fix the tissues. Chemical fixation allows for small cross-linking to take place between the proteins in a cartilage and thus helps to retain its structure and at the same time prevents the enzymatic degradation processes [282].

2.2.4 Decalcification

Following the fixing, formalin was discarded and the tissues were washed under running water, for approximately 1 hour. The samples were then transferred to 10% formic acid and left for mild decalcification for 3 days. Again, the tissues were washed thoroughly under running water for 1 hour. This resulted in softened osteochondral sections, as the calcium salts were removed from the calcified regions [283]. Formic acid is also shown to act rapidly on cartilages with minimal loss of proteoglycans [284].
2.2.5. DIC Microscopy

The chemically fixed and decalcified osteochondral cartilage blocks were trimmed first and glued onto mounting blocks using OCT (Optimal cutting temperature) compound and snap frozen in liquid nitrogen. 20 µm thick osteochondral sections were then microtomed and wet-mounted onto glass slides using 0.15 M saline and covered with a coverslip. Nikon Eclipse 80i fitted with differential interference contrast optics was used to view and image the osteochondral sections. The images were captured using the 4x, 10x, 20x, and 40x objective lenses.

2.2.6 Histology

For histological analysis, cartilage-on-bone samples were formalin fixed overnight and then decalcified in 10% formic acid for a week. The tissue was then washed with 0.15 M saline for 10 mins (3x) and stored in 70% ethanol at 4 °C, until histological analysis. The tissue then underwent standard histological processing [283]. Briefly, the cartilage sections were embedded in paraffin wax and then microtomed to obtain 5 µm-thick sections. The sections were mounted on standard glass slides. To remove wax, the slides were dipped in xylene for 5 mins (2x). The sections were then rehydrated in graded solutions of ethanol through distilled water. Following this, tissue sections were stained for proteoglycans with safranin-O and fast green and were imaged using bright field transmission microscopy. The level of degeneration was assessed using the Mankin cartilage grading system [285]. The scoring used to grade the samples is as shown in Table 2.2.

Safranin-O is a cationic dye which binds specifically and stoichiometrically to the negatively charged chondroitin sulphate or keratan sulphate of glycosaminoglycans (GAGs). The amount of safranin-o staining is directly proportional to the proteoglycan content of the cartilage [285, 286]. Safranin-o stains sulphated GAGs red, whereas fast-green act as the counter stain and stains collagen green.
Table 2.2  Cartilage histopathology grade assessment using Mankin grading [285]

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<thead>
<tr>
<th>Key Feature</th>
<th>Score</th>
<th>Associated Criteria</th>
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<tr>
<td><strong>Structure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>Normal intact surface</td>
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<td>1</td>
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<td>Pannus and surface irregularities</td>
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<td>Clefts to transitional zone</td>
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<td>4</td>
<td>Clefts to radial zone</td>
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<td>Clefts to calcified zone</td>
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<td>Complete disorganisation</td>
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<td><strong>Safranin-O staining</strong></td>
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<td>4</td>
<td>No dye noted</td>
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<td><strong>Tidemark integrity</strong></td>
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<td>Intact</td>
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<td>1</td>
<td>Crossed by blood vessels</td>
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2.2.7 Scanning Electron Microscopy (SEM)

A few of the selected cartilage sections were further analysed by SEM, for studying the fibril arrangement. Cartilage was pared off from the bone and the sections were fixed in 2\% glutaraldehyde (in 10 mM phosphate buffer) for 1 hour at room temperature followed by overnight incubation in refrigerator. The tissue sections were then washed with phosphate buffer saline (PBS) and fixed again in 1\% osmium tetroxide for 1 hour. After fixation, the opaque cartilage sections turned brown black and were washed with PBS, following which the sections were progressively dehydrated using 50\%, 75\%, 95\% and 100\% ethanol, for 20 mins each. The tissues then underwent critical point drying followed by 30 Å gold coating and was imaged using Hitachi SU-70 field emission scanning electron microscope.

2.3 Results

The degenerate patellae used in this study had fibrillation in the distal-lateral quarter (distal from the joint line). The tissue from the degenerated region (Figure 2.1 B and C) was classified as a ‘lesion’ sample. The adjacent tissue, without any fibrillation, was classified as an ‘intact’ tissue sample.

Twelve degenerated patellae were analysed, where 7 showed mild degeneration and were graded as GI; 5 patellae showing moderate level of degeneration were classified as GII in accordance with Outerbridge’s classification [281]. Three patellae showing completely intact surface with no sign of cartilage degeneration, each from cows and bulls were included, to study the gender variations.

2.3.1 Histological Analysis of Osteochondral Sections

The healthy cartilages from cow scored 0-1 (Figure 2.2 A) and were similar to the intact regions (Figure 2.2 C and E). The bull samples scored an overall 1-2 (Figure 2.2 B). With safranin O-fast green staining, the intact cartilage samples from both the GI and GII patellae showed Mankin scores ranging from 0-1 (Figure 2.2 C and E). The lesion sites from the GI patellae showed Mankin scores in the range of 1-4 (Figure 2.2 D), whereas those from the GII patellae scored 2-5 (Figure 2.2 F).
Figure 2.2 Histological sections of cartilage (orange/red) on bone (blue) stained with safranin O-fast green. (A) intact site from healthy cow cartilage (Mankin score 0) with intact surface and normal chondrocytes; (B) intact site from bull cartilage showing smooth surface morphology, undefined bone cement-line and intense staining (Mankin score 1); (C) GI patella (Mankin score 0) showing smooth surface morphology, normal chondrocytes and undisrupted tidemarks; (D) lesion site from GI patella (Mankin score 4) with irregular surface, decreased staining of tangential zone and tidemarks crossed by blood vessel (see white arrow); (E) intact site from GII patella (Mankin score 1); (D) lesion site from GII patella (Mankin score 5) with decreased matrix staining together with radial clefts extending into the mid-zone cartilage. (Scale bar: 500 µm)
2.3.2 Changes in Zonal Arrangement in Cartilage

The normal intact cartilage from healthy cow patella showed four distinct zones namely tangential (superficial), middle, radial (deep) and zone of calcified cartilage (ZCC) (Figure 2.3). Except for the GII lesion samples (Figure 2.4.E), all the other groups showed the four zones of cartilage (Figure 2.4). The bull cartilages (Figure 2.4 A) did not show a well demarcated ZCC as seen in the cow samples (Figure 2.3).

![Figure 2.3 DIC image of an intact cartilage from a healthy cow patella. The articular cartilage surface is smooth and intact and the superficial, middle and deep zone are easily distinguishable, followed by zone of calcified cartilage and subchondral bone. (Scale bar 500 μm)](image-url)
Figure 2.4 DIC images showing zonal arrangement of (A) an intact cartilage from a healthy bull patella with unclear ZCC; (B) intact region of GI cow patella with all four zones; (C) lesion region of GI patella; (D) intact site of GII patella and (E) lesion site of GII patella without a superficial and middle zone. (Scale bar 500 µm)
The horizontally aligned chondrocytes mark the tangential zone in the cartilage (Figure 2.5). The healthy cartilages exhibited a well-defined tangential zone as indicated by the tangentially aligned chondrocytes (Figure 2.5 A). The bull cartilage showed a slightly rounder tangential layer chondrocytes (Figure 2.5 B). The intact regions of GI (Figure 2.5 C) and GII (Figure 2.5 E) patellae exhibited similar tangential layer alignment as the healthy cow controls. The chondrocyte alignment was less pronounced in the GI lesion sites (Figure 2.5 D) suggesting a reduced tangential zone and mostly absent in the GII lesion sites (Figure 2.5 F). Also, in the latter there were localized regions nearer the disrupted surface that exhibited multi-cell clustering or cloning.

The tangential layer was followed by middle zone wherein the chondrocytes had no specific orientation and the radial zone where cells were perpendicularly aligned in vertical chondrons.
Figure 2.5 DIC images of the cartilage surface layer from tissue obtained from (A) healthy cow patella; (B) healthy bull patella; (C) tissue from intact site of GI patella; (D) lesion site of GI patella; (E) tissue from intact site of GII patella and (F) from lesion site of GII patella. Note the irregular surface layer in (D) and completely absent tangential zone and mid matrix in (F). Seen in (F) are radial zone chondrocytes and the arrows indicate chondrocyte cloning. (Scale bar: 50 µm).
2.3.3 Changes in Chondrocyte Morphology in Cartilage

The chondrocytes were found in chondron either singly or in a pair, in the superficial zone of the healthy and all the intact cartilages (Figure 2.6 A). In some of the lesion samples (from both GI and GII patella), chondrocyte clusters (Figure 2.6 B), cell duplication (Figure 2.6 C) and chondrocytes clones (Figure 2.6 D) were seen in the superficial zone or near the cartilage fissures.

Figure 2.6 DIC images showing different chondrocyte morphology seen in the bovine samples (A) normal chondrocytes found in tangential zone where either one or two cells per chondron is present; (B) diffused hyper-cellularity and chondrocytes clustering (circled region) near cartilage damage; (C) chondrocyte duplication (circled region); (D) chondrocyte cloning (black arrows) in tissue from lesion site of GII patella. (Scale bar: 50 µm).
2.3.4 Changes in Matrix Texture of the Cartilage

With higher resolution DIC imaging, the mid-zone matrix in healthy cartilage tissues (both gender) (Figure 2.7 A and B) presented a relatively amorphous texture with little obvious fibrosity. This was similar in the intact samples (Figure 2.7 C and E) of GI and GII patellae.

By contrast, the lesion site cartilages (Figure 2.7 D and F) exhibited a strong directional fibrosity which varied in both intensity and location, the most intense texture with a distinct crimp morphology occurring near the exposed surface of the GII patellae (Figure 2.7 F).
Figure 2.7 DIC images of the mid-zone cartilage matrix for tissues obtained from: (A) healthy cow cartilage; (B) healthy bull cartilage; (C) GI intact site; (D) GI lesion site; (E) GII intact site; and (F) GII lesion site. Note the increased extent of fibrosity in the matrix of lesion site tissue (D and F). In (F) the extent of fibrosity also involves a radial ‘collapse’ of the network that then appears as in-phase crimping. (Scale bar: 50 µm).
2.3.5 Changes in Zone of Calcified Cartilage and Subchondral Bone

The osteochondral junction in the control cow tissues showed a clearly defined zone of calcified cartilage (ZCC) (Figure 2.8 A), with the bone cement line well below the uppermost tidemark. The absence of clear-demarcated ZCC and diffused tidemark was noted in the bull cartilages (Figure 2.8 B).

Progressing from the control to the intact tissue in the GI patellae (Figure 2.8 C), there appeared to be a relative increase in mineralization (as indicated by the ZCC thickness) with the most significant changes occurring at the lesion site (Figure 2.8 D), where the ZCC was disrupted intermittently by bone spicules growing toward the uppermost tidemark. These spicules were less obvious in the intact region of the GII patellae, instead appearing more blended with the bone cement line (Figure 2.8 E). Figure 2.8 F is from a GII lesion site showing remnants of a micro-damage in the ZCC, with what appears to be reactive tissue growth into the articular cartilage, resulting in a slight distortion of the surrounding matrix.

The spicules found in the subchondral bone of intact and lesion site tissues of GI and GII patellae are shown in Figure 2.9.
Figure 2.8 DIC images of the zone of calcified cartilage (ZCC) in (A) tissue obtained from a cow patella with no signs of degeneration; (B) non-degenerate bull cartilage; (C) GI intact; (D) GI lesion; (E) GII intact; and (F) GII lesion site. Black arrow indicates the uppermost tidemark, or boundary between ZCC and articular cartilage above. Yellow arrows point to the cement line or boundary between the ZCC and underlying bone. Note the bone cement line is well below the uppermost tidemark in healthy samples (A). The bull cartilage is devoid of a well demarcated tidemark and ZCC (B). The cement line (yellow arrows) in (C) is far below the uppermost tidemark (black arrow), leaving a wide zone of calcified cartilage compared to the control cow healthy cartilage (A). In (D) bone spicules invade the ZCC space, and in some cases, have disrupted the uppermost tidemark (red arrows). In (E) the bone spicules are less obvious and the cement line appears to have advanced towards the uppermost tidemark. (F) The ZCC contains some obvious micro-damage with the surrounding deep zone articular cartilage matrix appearing to be distorted by reactive tissue growth (Scale bar: 100 µm).
Figure 2.9 DIC images of subchondral bone changes seen in the ZCC from (A) GI intact site; (B) GI lesion site; (C) GII intact site and (D) GII lesion site. The bone spicule is well under a thickened ZCC in (A), whereas in (B) it has almost reached the uppermost distal tidemark, (C) the spicule formed is under the distal most tidemark and in (D) tissue reaction in subchondral bone and ZCC is seen, as a result of micro-damage in the region. The yellow arrows show the central vascular canal in the spicules (Scale bar ~100 μm).
2.3.6 Ultrastructural Changes in Cartilage Regions

SEM analysis of intact and lesion site cartilages showed distinct fibril morphology. The intact site cartilages showed pseudorandom fibril arrangement, whereas lesion site cartilage matrix was seen to possess radial fibrosity with obvious fibril clumping and entwinement (Figure 2.10).

Figure 2.10 SEM image of (A) GII intact site cartilage general matrix with pseudorandom orientation of collagen fibrils; (B) GII lesion site cartilage matrix where the collagen fibrils appear to be in an untangled state, aggregating parallel to the radial direction. (Scale bar 1 µm).
2.4 Discussion

In general, cartilage is considered to be transversely isotropic [287] but with a depth-dependent variation in its tissue structure and composition. This depth-dependent variation is broadly described as having

(A) a tangential zone: the surface layer of the cartilage where the cells lie in a tangential alignment;
(B) a radial zone: the mid-to-deep layers of the cartilage where the cells lie largely in a radial direction, and
(C) a transition zone: in-between the tangential and radial zone, where there is no general preferred orientation of the cells.

In cartilages from bovine patellae, the tangential zone and transition zone typically represents about 40% of the total cartilage thickness and the rest encompasses radial zone. From the previous investigations undertaken in our laboratory, it have been seen that the structural changes observed in the mild to moderate OA bovine samples, are consistent and site-specific. Various morphometric and histological analysis done on bovine tissues, have confirmed these findings [266]. The proteoglycan loss during cartilage degeneration and the changes in cartilage morphology as well as subchondral bone, were found to be similar in both bovine and human degenerate tissues and hence, bovine patella have been validated to be good model to study the early changes in OA. The features like cartilage surface layer erosion, chondrocyte cloning and spicule invasion in bovine cartilage, hints that the degeneration is proliferative and progressive. But the limitations associated with the bovine samples included the lack of knowledge about the age and the joint history of the animal.

2.4.1 Micro-anatomical Variations in Healthy Cartilages

Two visually intact cartilage regions from healthy cow patellae were compared to estimate the level of site-specific structural variations between them. The DIC and histology images did not reveal any major difference between the two intact healthy regions in term of cartilage surface layer structure, chondrocyte morphology, histological staining, matrix texture, ZCC modifications or subchondral bone changes.
This lack of significant region-specific differences between two control regions in the healthy patellae may suggest that the cartilage retain its transverse (parallel to tangential layer) isotropic and homogenous nature [287] across the surface, as long as it upholds its healthy state. Therefore, any structural modification seen in the degenerate tissue is hypothesised to be

- A direct result of the tissue damage or
- An adaptive response to contain the damage and/or
- A reparative action to combat the damage.

2.4.2 Micro-structural Variations in Healthy Bull and Cow Cartilages

On comparing the cartilage from cow to that of the bull, few microstructural variations were found. The bull cartilages showed diffused hyper-cellularity [288] (Figure 2.2 B) compared to healthy cow cartilages (Figure 2.2 A). Another prominent change was the lack of well-demarcated tidemarks and ZCC in the bull cartilages.

The histological staining of bull cartilages with safranin-O and fast-green showed definite cartilaginous tissue islets in the subchondral bone (Figure 2.11). The safranin-O stained tissue in the subchondral bone region suggests that the cartilage is immature and is still developing [289]. The presence of proteoglycan positive tissue in the subchondral bone may indicate that the cartilage in this region is yet to be calcified. Combined with the hyper-cellular nature of the chondrocytes found in the bull articular cartilage, it is suggestive that the tissue is still in its developing stage.

The bull cartilage samples obtained for this study were from animals slain at a comparatively younger age since they were grown mainly for meat production, unlike the dairy cows which were reared longer for milk production and slaughtered only after its maturity. Hence, the differences observed in chondrocyte morphology and in subchondral bone of the bull cartilages could be the result of the age of the animal, rather than its gender. Therefore, for an effective analysis of gender-based cartilage structural changes, age matched animals are vital.
Figure 2.11 Bull cartilage stained with Safranin-O and Fast-Green. The subchondral bone is stained blue-green and cartilage is stained orange-red. Tiny regions stained red with Safranin-O is clearly visible in the subchondral bone and upon magnification, presence of chondrocyte (in circled region of the inset) was confirmed.
2.4.3 Micro-anatomical Variations in Intact and Lesion Site Cartilages

While no significant change in cartilage or subchondral bone from two regions of a healthy patella was found, the non-healthy patellae showed prominent differences between the intact healthy-appearing cartilage and the lesion site cartilages. The changes seen in the intact region which lies adjacent to the lesion cartilage site, are of immense interest to us as these sites offer the information on the structural changes occurring in the pre-OA state [90]. Alternatively, these micro-structural changes could be the reparative response to the adjacent cartilage damage.

The intact region samples (from both GI and GII patellae) resembled the healthy cartilages in their zonal arrangement- with a well-defined tangential layer on the surface followed by middle layer and deep layer. This zonal organization is disrupted in the lesion site cartilages, with GI lesion site cartilages having an irregular surface layer, while the GII lesion sites have nearly absent tangential layer. As seen in an earlier work [44], the surface layer showed minor irregularities in the GI lesion samples while in the GII lesion samples, the surface clefts were seen to extend to the mid to deep zone (Figure 2.12 A), with some samples showing complete absence of surface layer (Figure 2.12 B) leading to further erosion of the underlying cartilage layers as the disease advance.

The safranin-O staining revealed the proteoglycan loss from the lesion site samples in GI and GII patellae. Proteoglycans like aggrecan molecules are lost during OA and as the degeneration advance, the loss of stain from the cartilage is more pronounced as seen in the GII lesion samples (Figure 2.2 F). Proteases and other catabolic enzymes attack the proteoglycan aggregates and disrupt its attachment from the collagen framework [41]. The aggrecan is normally found in the articular cartilage as proteoglycan aggregate but it is seen in its non-aggregated form in OA cartilage [290].
Figure 2.12 DIC images of the GII lesion site tissues showing (A) radial fissure (black arrow) running into deep zone; (B) the presence of radial chondrons on the surface of the tissue indicate the loss of the tangential and middle layer of the cartilage.

The chondrocyte morphology was found to be varying in the degenerate tissues compared to the intact counterparts. In the intact regions, the chondrocytes were flattened, occurring either singly or in pairs, in the superficial zone. At the lesion site, due to the disruptions in cartilage surface layer, the chondrocyte morphology was seen to vary from diffused hyper-cellularity to cell clustering (Figure 2.6 B) and cloning (Figure 2.6 C and D) along the surface fissures running into the deep zone. These large clusters of cells in the chondron of lesion samples, are reported to be involved in chondron peri-cellular remodeling in the degenerating matrix [291]. Rolauffs et al. identified these spatial organization changes of chondrocytes in the intact site adjacent to the lesion sample and suggested that, through chondrocyte reorganization the tissue is trying to repair the cartilage damage [225].

An increased thickness of ZCC at the intact sites of GI tissues was observed (Figure 2.8 C and 2.9 A) and this could be a consequence of the enhanced mineralization taking place at these regions. The new primary bone formation that Thambyah and Broom [90] reported in their structural study of the early or pre-OA joint was shown to commence mostly under the regions of still intact cartilage adjacent to lesion sites in both GI and GII cartilages. The process involves the upward growth of distinct bone spicules containing a central vascular channel from the cement line, and traversing the increased width of the ZCC between the articular cartilage and underlying bone.
Thambyah and Broom showed that the spicules are similar to the cutting cones seen in the fracture healing of bone [292] and these were clearly visible in micro images of the tissue in the present study (Figure 2.8 D and 2.9 B). This would also reinforce the earlier suggestion that the structural transition from the intact to lesion regions of cartilage in a joint, is an analogue for the pathway from initiation to progressive degeneration of the joint tissues in OA [90, 266].

Another prominent structural feature found to be different between the intact and lesion cartilages, was the tissue matrix texture. The intact site showed an amorphous texture which is consistent with the pseudorandom orientation of the fibrils. The collagen fibrillar networks are highly interconnected in the radial direction in middle and deep zone of normal articular cartilage. During cartilage degeneration, the fibril interconnectivity between this network is broken down, resulting in matrix fibril aggregation in radial direction [267]. The lesion site cartilage matrix was seen to possess radial fibrocity with obvious fibril clumping and entwinement in the GII samples (Figure 2.7 D and F). Cartilage matrix fibril aggregation in the radial direction was also seen in the SEM image of the lesion site (Figure 2.10) and could be due to the botched interconnectivity between the fibrils [276].

The two structural indicators of pre-to-early OA, (i) the new bone formation and (ii) collagen type II fibrillar matrix de-structuring at the lesion sites were confirmed in the early degenerated cartilages. The structural variations in the degenerated patella found in this chapter will be the basis for upcoming chapters.
2.5. Conclusions

- The early structural changes in bovine osteochondral samples were investigated.
- On comparing two regions from the healthy patella, no significant structural difference was found.
- The comparison of cow and bull cartilage tissues to identify the gender specific structural variation, should ideally be done in age matched animals. As the bull samples were deemed to be immature, the changes seen in these two groups were inconclusive.
- The intact and lesion cartilages from the GI and GII groups showed considerable differences in zonal arrangement, histological staining, chondrocyte morphology, matrix texture, ZCC modifications and subchondral bone changes.
- The intact tissue samples resembled the healthy tissues in all the structural features except for ZCC thickness and subchondral bone changes.
- All the structural features were consistent with early cartilage changes observed in OA pathology.
- The lack of region-specific structural changes in healthy tissues highlight the importance of changes seen in the intact and degenerated cartilage regions. These structural changes could represent the pre-OA state.
CHAPTER 3

PROTEOME PROFILE OF BOVINE ARTICULAR CARTILAGE

3.1 Introduction

The most common methods used for OA diagnosis and evaluating the progress of the therapeutic intervention in disease are based on either relatively less sensitive radiography and/or MRI. But for assessing the early changes in OA, more sensitive parameters need to be developed. To this end, in recent years the search for OA biomarkers have been actively going on. Proteomics based studies have contributed largely to this development, enabling the detection of sensitive global protein profile of the patient more rapidly and accurately.

The early detection of diseases like OA is vital because as the disease advances, it leads to irreparable damage to the cartilage and other associated tissues. The early structural changes in bovine animal were studied and validated to exhibit similar structural disease progression as those seen in humans [266]. Previously, our laboratory have established two structural indicators of early cartilage degeneration—‘de-structured’ collagen fibril arrangement in cartilage and new spicule formation in subchondral bone [90, 267, 269, 274, 276].

The region-specific structural changes in intact and lesion sites of the non-healthy patella were determined in Chapter 2. These structural changes were found to be significant since the control experiments with healthy patellae did not yield any inherent structural variation between its two intact cartilage sites. More specifically, the structural alteration occurring in the intact regions lying next to the degenerated sites have the potential to provide information to uncover the elusive “pre-OA” state. A better understanding of the early events in OA can be advantageous in identifying
the potential OA biomarkers and developing appropriate targets for early therapeutic mediators.

In the absence of inherent structural changes in different regions of a healthy patella, the microstructural changes identified in intact and lesion sites of non-healthy patella (Chapter 2) is relevant and identifying the proteomic profiles associated with these regions can either provide an explanation for the structural changes or could provide more information on the tissue disease status. Therefore, question of if and how the protein profile of the degenerated and non-degenerated cartilage regions change in accordance with the structural alteration, need to be addressed. Previously it was identified, that these structurally altered cartilages have different secretory patterns compared to healthy tissues [226]. Hence, it can be hypothesised that the structural modifications seen in the intact and lesion site of the bovine patella could be correlated to distinct protein profile associated with these regions.

While structural studies identified significant variation between the healthy tissues and lesion sites of the non-healthy tissues, there were only slight structural variations between the healthy cartilages and the intact sites of the non-healthy patellae. Proteomic analyses of the above sample groups, were carried out to determine whether these patterns of relative structural changes were mimicked when the corresponding protein profiles were compared.

The aim of the study was to identify the specific protein changes in cartilage with different tissue status- healthy vs. non-healthy. Also of interest were the protein changes in intact and degenerated regions of the patella showing early signs of cartilage degeneration. The profile changes of these proteins were assessed with respect to the degeneration severity. Identifying the key proteins which contribute to structural modification in OA can immensely help to expand our understanding of OA pathophysiology.

As a term for convenience, the tissues from G0 patella are henceforth referred in this chapter as ‘healthy’ and those from GI and GII patellae are broadly referred to as ‘non-healthy’. Similarly, the tissue from the degenerated sites in the GI and GII patellae
(Figure 2.1 B and C) are referred to as the ‘lesion’ sample while the adjacent normal appearing tissue, free of any obvious surface disruption, is termed as ‘intact’.

3.2 Methodology

3.2.1 Gel-based Proteomics

As a preliminary investigation to compare protein profiles unique to healthy appearing intact cartilage and early degenerated tissue from bovine patella, cartilage proteome analysis was performed using one dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D-SDS PAGE) followed by in-gel digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

3.2.1.1 Tissue Source

Patellae were obtained from freshly slain mature cows and categorised based on the level of degeneration seen in the distal-lateral facets. Within few hours of obtaining the sample, the patellae were transferred to and stored at -20 °C until further use.

3.2.1.2 Cartilage Sample Preparation

At the time of the experiment, a patella (Figure 3.1) exhibiting a Grade-II lesion according to Outerbridge’s classification [281], was thawed to 4 °C and using a scalpel, the cartilage was pared off the subchondral bone.
Figure 3.1 Bovine patella washed with India ink and revealing regions where cartilage surface disruption has occurred. Here the ‘lesion’ site shows moderate positive staining compared to the relatively intact site showing an absence of ink retention. The black dotted line indicates the separation of tissue for microstructural studies and mass spectrometry analysis.

The cartilage shavings (weighing 150 mg) were then cut into 1 mm$^2$ pieces and were digested in 1 mL serum free Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma D5796) supplemented with 0.033% of liberase TL (Sigma) at 37 °C overnight, under aseptic conditions. After digestion, the medium was centrifuged at 1000 g (Eppendorf Centrifuge 5424R) for 5 min and the supernatant was collected. The centrifuge used for the present work had settings to switch centrifugal speed from rpm (revolution per minute) to rcf (relative centrifugal force expressed in units of gravity (x g)) and vice-versa. When these settings are not available, following formula can be applied for appropriate centrifugal speed settings.

\[ g = (1.118 \times 10^{-5}) R S^2 \]

where g is in rcf

R- Radius of rotor in centimetres (distance from centre of rotor to the sample)

S- Speed of the centrifuge in rpm

The Bradford assay was done on the supernatants to determine the protein concentration and was then adjusted to ~1 mg of protein/mL by concentrating the samples in a vacuum concentrator [195].
3.2.1.3 SDS-PAGE

The protein samples were mixed with the loading buffer in 1:1 ratio and separated on 1D-SDS PAGE precast gels (Biorad) at 150 V for 43 min. Following this, the gels were immersed in and stained with 100 mL of Coomassie blue for 30 min. After the staining, gels were washed with 150 mL of deionised water and 150 mL of 0.1% acetic acid de-staining solution was used to remove the blue colour of the gel. After the overnight de-staining procedure, gels were washed with distilled water (3x) and were ready for protein extraction or stored at 4 °C until further use.

3.2.1.4 In-gel Digestion

Each gel lane was cut into 4 sections (Figure 3.2) irrespective of bands formed and placed into the microcentrifuge tubes and further cut into ~1 mm² small pieces, inside the tubes. These were then washed with 200 µL of 50 mM ammonium bicarbonate buffer (pH 8), then with 200 µL of 50% acetonitrile (3x) and again with 200 µL 50 mM ammonium bicarbonate buffer for 10 mins each until the blue colour was lighter. 100 µL of 100% ACN was then added and the gel pieces turned white in colour. After removing the acetonitrile, the gel pieces were dried at a temperature of 56 °C for 5 to 10 mins in a dry block heater. To reduce the disulphide bonds, 250 µL of 10 mM dithiothreitol (DTT) was added to the gel pieces for 30 mins at 40 °C. After removing the DTT, 300 µL of 50 mM iodoacetamide (IAM) was added for 30 mins under dark conditions, to modify the –SH groups of cysteine residues and prevent the formation of disulphide bonds.
After removing IAM, the gel pieces were then dehydrated with 500 \( \mu \text{L} \) of 100% acetonitrile for 10 mins (2x) and dried at 56 °C for 10 mins. After this, 250 \( \mu \text{L} \) of 12.5 ng/mL of trypsin was added to dried gel pieces at 40 °C and incubated for 30 min under microwave irradiation (CEM discover microwave digestion system, Matthews NC, USA) using 15 W of power, followed by further incubation at 37 °C for 30 min. 10 \( \mu \text{L} \) of 10% formic acid was then added and supernatant was collected and concentrated to 25-30 \( \mu \text{L} \) in a speed vacuum concentrator. To extract the remaining tryptic digests from gel pieces, 150 \( \mu \text{L} \) of 100% ACN was added and after 5 mins the supernatant was collected and concentrated to a volume of 25-20 \( \mu \text{L} \) using a vacuum concentrator. Both the extracts were then combined and underwent LC-MS/MS.

### 3.2.1.5 Mass Spectrometry and Data Analysis

Each digest was diluted 3-fold in 0.1 % formic acid and 10 \( \mu \text{L} \) was injected in the capillary LC-MS/MS system. A 0.3 x 5 mm PepMap cartridge (LC Packings, Dionex Corporation, Sunnyvale, CA, USA) was used for capturing the peptides, which were separated on a 0.3 x 100 mm Zorbax 300SB- C18 column (Agilent) using 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B, at a flow
rate of 6 µl/min over 90 min. The column was equilibrated at 10 % B for 3 min which was then raised to 40 % B over 75 min and further raised to 95 % B over 79 min and retained until 82 min, brought back to 10 % B at 84 min and held there until 90 min. The fraction from HPLC (High Performance Liquid Chromatography) was directed onto the Ion Spray Source of QSTAR XL hybrid Quadrupole- Time-of -Flight mass spectrometer (Applied Biosystems, Foster City CA, USA), with an m/z scan range of 100-1600. From the ion spray, top three multiply charged ions underwent MS/MS analysis. The software package used for HPLC and MS analysis was Analyst QS 1.1 (Applied Biosystems). The data files obtained were saved into Mascot Generic Format (.mgf) files by Mascot.dll script v1.6b13 (Matrix Science, London, UK). These files were then searched against the International Protein Index (IPI) bovine sequence database v3.49 (European Bioinformatics Institute, Hinxton, UK), using an in-house installation of Mascot v2.0.05 (Matrix Science). The different search parameters used to obtain peptide summaries were: Digest agent - trypsin; Maximum number of missed cleavages - 1; Fixed modifications (these modifications are applied universally) - Carbamidomethyl Cysteine (i.e., for all calculations mass of cysteine was used as 161 Da); Variable modifications (are those which may or may not be present) - Deamidation (NQ), Oxidation (M); Peptide mass tolerance - 0.15 Da; Fragment mass tolerance - 0.15 Da. The results were then filtered to require at least one bold red peptide match per protein hit, and to exclude any peptide matches with an ion score < 12 [293, 294].

3.2.2 Gel-free Quantitative Proteomics- iTRAQ-based MS/MS Analysis

Rather than limiting the work by finding the exclusiveness of a protein to the sample, the research was broadened by labelling the samples with iTRAQ based tags, to quantify the difference in their expression. The samples were labelled and then subjected to comprehensive tandem MS analysis. The methodology followed is outlined in Figure 3.3.
Figure 3.3 Schematic overall workflow for cartilage microstructural (Chapter 2) and proteomic study (Chapter 3). The lesion and intact cartilage are taken as shown, from the bovine patella. Black thick line inside the rectangle section indicates division of tissue for microstructural studies and mass spectrometry analysis.
3.2.2.1 Tissue Source

All cartilage samples were harvested from the patellae obtained from 15 freshly slain female bovine animals. Based on the inspection of a preliminary set of patellae stained with India ink [295] to identify the presence and extent of surface fibrillation, each patella was graded according to the Outerbridge’s classification [281]. Three patellae were classified as normal (i.e. G0), 7 mildly degenerate (GI) and 5 moderately degenerated (GII).

Additionally 3 patellae from bulls, showing pristine cartilage surface and with no sign of cartilage degeneration, were included in the study, to determine whether significant gender based proteomic variations were present between the cow and bull cartilages. The bull samples were also classified as G0 according to the Outerbridge’s classification. The rationale for this comparison was to determine whether healthy bull patellae could be substituted for healthy cow patellae during proteomic analysis, as healthy tissues from bulls were more readily available than the healthy cows from the local abattoir.

A comparable pair of samples were taken from each of the 3 normal patellae from cows and bulls (Figure 2.1 A) to determine whether or not there existed a regional variability in protein levels across the normal joint surface.

Likewise from each degenerated patella, a pair of cartilage-bone samples with en face dimensions of ~10x15 mm was sawn from the lesion region and from an adjacent intact region (Figure 2.1 B and C).

Each of the 36 cartilage-on-bone blocks was divided into two equal portions, one half was processed for structural analysis and histology and the other was used for proteomic analysis.

3.2.2.2 Histology Studies

For histological analysis cartilage-on-bone samples were formalin fixed overnight and then decalcified in 10% formic acid, followed by standard histological processing (see section 2.2.6). 5 µm-thick microtomed sections were stained for proteoglycans with
safranin-O and fast green and were imaged using bright field transmission microscopy and then assessed for their level of degeneration using the Mankin cartilage grading system [285].

3.2.2.3 Proteomics Studies

3.2.2.3.1 Sample Preparation

For proteomic analysis, all the 36 cartilage samples were snap-frozen in liquid nitrogen and cryo-microtomed into 10 µm-thick cartilage sections which were then weighed. Twenty milligram of each sample was individually homogenized by sonication in 500 µL of urea-thiourea buffer (7 M urea, 2 M thiourea, 10 mM dithiothreitol in 50 mM ammonium bicarbonate, pH 8) at 12 Hz for 30 s, then microwaved using CEM microwave digestor at 56 °C for 10 min to reduce the disulphides, alkylated with 25 µL of 50 mM iodoacetamide in darkness for 30 min and the reaction was quenched with 5 µL of DTT [241]. Each sample was then centrifuged at 16000 g at 4 °C for 5 min, the supernatant was collected and used for protein analysis. The total protein concentration of each sample was determined by fluorescence-based protein assay (EZQ Protein Quantitation Kit, Invitrogen). A reference pool was created with an equal supernatant volume from all the cartilage samples. 50 µg of each protein sample was then digested with trypsin (Promega, Madison, WI, USA) at 1:25 ratio at 45 ºC using 15 W power microwave irradiation for 3 hours, followed by overnight incubation at 37 °C.

3.2.2.3.2 iTRAQ Labelling

The resulting digests were acidified with 5 µL of 50% formic acid and cleaned by solid phase extraction on 10 mg Oasis HLB cartridges (Waters, Milford, MA, USA) and the proteins were eluted with 300 µL of 40% acetonitrile. Extracts were dried in a speed vacuum centrifuge before being reconstituted with 30 µL of dissolution buffer (AB Sciex, Foster City, CA, USA) and labelled with 8-plex iTRAQ chemical tags (AB Sciex) as per the manufacturer’s instructions. The iTRAQ experiments were conducted by two separate proteomic studies. The labelling scheme adopted for both is shown in Appendix A.
The pools of 8 labelled samples were concentrated in a vacuum centrifuge to ~250 µL, diluted to 2 mL with 0.1% formic acid and loaded onto 30 mg Strata-X SPE cartridges (Phenomenex, Torrance, CA, USA) eluting with 0.9 mL of 50% acetonitrile. The final extracts were concentrated to ~300 µL in a vacuum centrifuge. The resulting peptides were desalted on 10 mg Oasis SPE cartridges (Waters Corporation, MA) and completely dried down using a speed vacuum concentrator (Thermo Savant, Holbrook, NY) [296].

3.2.2.3.3 LC-MS/MS and Database Searches

Aliquots of the labelled pools were fractionated using ten salt steps (5, 10, 15, 20, 25, 30, 50, 75, 150, and 600 mM KCl in 10% acetonitrile, 0.1% formic acid) on a 0.32 x 35 mm BioBasic SCX column (Thermo, San Jose, CA, USA). A 0.3 x 5 mm PepMap cartridge (LC Packings, Dionex Corporation, Sunnyvale, CA, USA) was used for capturing the peptides, which were separated on a 0.3 x 100 mm Zorbax 300SB-C18 column (Agilent), using ~0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B, at a flow rate of 6 µL/min and a linear gradient of 10% - 35% B over 85 min. The HPLC fractions were directed onto the Ion Spray Source of QSTAR XL hybrid Quadrupole- Time-of -Flight mass spectrometer (Applied Biosystems, Foster City CA, USA), with an m/z scan range of 330-1600 m/z. This was followed by three rounds of MS/MS (m/z 75-1200) on the three most intense multiply charged ions in each cycle, giving a total cycle of 6 s. The resulting data were searched against 60806 protein entries in the IPI Bovine database v3.73 (European Bioinformatics Institute, Hinxton, Cambridge, UK) using ProteinPilot v4.0 (Applied Biosystems). Search parameters were: Sample type- iTRAQ 8-plex (Peptide Labelled); Enzyme- trypsin; Special factors- urea denaturation; Cys alkylation- iodoacetamide; Search effort – Thorough (Thorough mode considers about 150 modifications including biological, sample preparation modifications and unexpected cleavages); FDR analysis- Yes.
3.2.2.3.4 Data Processing

The ProteinPilot outputs were processed as previously described in detail [296]. Briefly, data obtained from all the searches were combined and assessed in terms of False Discovery Rate (FDR) score. Any protein below the FDR score, were pruned off. The peptide summary was further trimmed to discard any spectra with

- unused score = 0
- matched to a reversed sequence

The iTRAQ areas were normalized with correction factors established for each label. Any peptide ending with proline or containing a proline (P) immediately followed by glutamine (Q) (since both these have been found to interfere with the 115 (PQ) and 116 (N-terminal P) labels) and peptides found to contain a missed cleavage were processed using a separate set of correction factors. From the peptide summaries, the areas were summed separately for each protein in each label. All the compromised spectra which matched to a peptide containing a PQ or ending with a P were manually deleted across all eight labels or, if only P/PQ spectra were available, the final sums were deleted for 115 only (in case of PQ) or 116 only (in case the matched peptide ended with a P). For further details see Appendix B.

3.2.2.3.5 Statistical Analysis of iTRAQ Results

The sum of areas obtained from peptide and protein summaries in each sample were log transformed. Ratios for the resulting logs were obtained for each protein, between the individual sample and the reference sample pool, the latter of which was repeated between the runs. These ratios represented the relative abundance of each quantified protein in each analysed sample making comparisons between them possible. These ratios from individual samples were then grouped into healthy cow control site A (n=3), healthy cow control site B (n=3), bull site A (n=3), bull site B (n=3), GI intact (n=7), GI lesion (n=7), GII intact (n=5) and GII lesion (n=5).

Three different sets of statistical analyses were done on the samples, to tease apart the comparison between the groups.
(A) **Comparison between healthy and the non-healthy groups.**

To detect statistically significant differences between the diseased group means with respect to the healthy cow cartilage, the ratio obtained for each protein was analysed using One Way ANOVA (Analysis of variance), followed by a Tukey’s multiple comparisons test (if the ANOVA showed that differences among means were statistically significant (p<0.05)).

For the method used to link two separate proteomic analyses, see Appendix C.

The proteome profile variation associated with gender of the animal was also assessed.

(B) **Comparison between two localized regions in a healthy patella.**

The identified proteins from control site A and control site B were compared for determining any significant variation between two regions in a healthy patella, using Two-Way Repeated Measure (RM) ANOVA. The proteins which were found significant (p<0.05), underwent Sidak’s multiple comparisons test.

(C) **Comparison between the intact and lesion region in a non-healthy patella.**

A repeated measure Two-Way ANOVA was performed to look at the overall effect of lesion and grade of degeneration. For any protein showing p<0.05 for AVOVA, a Sidak’s multiple comparison was done.

All statistical analyses were performed using Prism 6 for Windows.

### 3.2.2.3.6 MRM Analysis

Three samples from the following cartilage groups –healthy control site A, healthy control site B, mild (GI) intact, mild (GI) lesion, moderate (GII) intact and moderate (GII) lesion, were assayed during MRM experiment. 50 µg protein aliquots from each sample were reduced, alkylated and digested using trypsin, as stated earlier.

High confidence peptide targets for MRM assays were selected from data collected in the proteomics experiments. Transition lists were built using Skyline [297] and acquisition methods were exported to Analyst TF software. Two to three unique peptides were selected for each target protein.

Data were acquired on a Sciex TripleTOF 6600 instrument with an Eksigent ekspert
nano HPLC system. Solvents used were 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Peptides were loaded onto a Reprosil C18 Aq 3 µm, 0.3 × 10 mm trap column (Dr Maisch GmbH) in 2% acetonitrile at 2 µl/min before being separated on a PicoFrit emitter (New Objective, Woburn, MA with a 75 µm ID and a 15 µm emitter, that had been packed to a length of 20 cm with Reprosil C18 Aq 3 µm). Separations were performed with a gradient of: 2% solvent B for 30 secs; 2-35% solvent B in 46.5 min; 35-98% solvent B in 2 min.

Data obtained as .wiff files were processed using Skyline software and peak areas were exported for subsequent analysis. The identity of peptides were confirmed by comparison of retention times between discovery and targeted analysis and by comparison of the intensities of fragment ions in MS/MS spectra.

For MRM assays, significant differences in relative abundance between cartilage groups were analyzed for all proteins using a RM Two-Way ANOVA followed by a Tukey’s multiple comparisons test (for groups scaled to G0 control where healthy cartilages were compared to the non-healthy cartilages) and Holm-Sidak’s multiple comparison test (for groups scaled to GI intact where intact samples from non-healthy patellae were compared to their adjacent lesion site cartilage).

3.3. Results

3.3.1 Gel-based Proteomics

The patella used in this study, as revealed by India ink staining, was moderately degenerated in the distal-lateral quarter (distal from the joint line). The tissue from the degenerated region (Figure 3.1) was classified as a Grade II lesion sample [281]. The adjacent tissue, without any positive staining, was classified as an intact tissue sample. The protocols for the gel based proteomics were based on Garcia et al. [195] where cartilages from bovine patella were minced and treated with liberase overnight, to break down the collagen network and then it was centrifuged to collect the supernatant. Following this, the proteins in the supernatant were separated by 1D SDS-PAGE. Distinct gel bands as reported by Garcia et al. were not be obtained, hence the gel lanes were cut into four sections (Figure 3.2) and each section was in-gel
digested with trypsin to release the peptides. The digested samples from two corresponding sections from duplicate gel lanes were combined and analysed over individual run.

The peptides from each sample was then separated on a C18 reversed phase HPLC column and detected by QSTAR XL hybrid Quadrupole- Time-of -Flight mass spectrometer.

The data files were searched using MASCOT software against IPI bovine database. A cut-off confidence of ≥ 20 and ion score cut off > 12, was applied to obtain 111 proteins from the intact and lesion samples; and amongst these, 65 proteins were found common to both intact and lesion samples (Table 3.1).

Table 3.1 Common proteins identified in gel-based MS experiment in both intact and lesion samples and the number of unique peptides detected in both categories.

<table>
<thead>
<tr>
<th>IPI Accession #</th>
<th>Protein</th>
<th>No. of unique peptides in intact</th>
<th>No. of unique peptides in lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPI00714673.3</td>
<td>FN1 Embryo-specific fibronectin 1 transcript variant</td>
<td>73</td>
<td>87</td>
</tr>
<tr>
<td>IPI01001422.1</td>
<td>COL6A3 collagen, type VI, alpha 3-like isoform 2</td>
<td>72</td>
<td>83</td>
</tr>
<tr>
<td>IPI01017728.2</td>
<td>COL12A1 Uncharacterized protein</td>
<td>63</td>
<td>72</td>
</tr>
<tr>
<td>IPI00686970.3</td>
<td>COMP Cartilage oligomeric matrix protein</td>
<td>38</td>
<td>34</td>
</tr>
<tr>
<td>IPI00687115.2</td>
<td>ACAN Aggrecan</td>
<td>36</td>
<td>34</td>
</tr>
<tr>
<td>IPI01028455.1</td>
<td>ALB Serum albumin</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>IPI0069504.6</td>
<td>TNC Uncharacterized protein</td>
<td>29</td>
<td>46</td>
</tr>
<tr>
<td>IPI00701014.1</td>
<td>HAPLN1 Hyaluronan and proteoglycan link protein 1</td>
<td>31</td>
<td>30</td>
</tr>
<tr>
<td>IPI00699783.3</td>
<td>CILP2 Uncharacterized protein</td>
<td>26</td>
<td>25</td>
</tr>
<tr>
<td>IPI00713573.3</td>
<td>COL6A1 Uncharacterized protein</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>IPI00712111.1</td>
<td>CHAD Chondroadherin</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td>IPI00840588.2</td>
<td>TGFBI LOC539596 protein</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>IPI01000032.1</td>
<td>CLPX ClpX caseinolytic peptidase X homolog</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>IPI00837992.3</td>
<td>COL6A2 Uncharacterized protein</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>IPI01000857.1</td>
<td>HSPG2 heparan sulfate proteoglycan 2</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>IPI00716123.1</td>
<td>OGN Mimecan</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>IPI00968741.3</td>
<td>COL9A1 collagen, type IX, alpha 1</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>IPI00689035.1</td>
<td>MFG8 Isoform Long of Lactadherin</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>IPI00706002.2</td>
<td>ANXA2 Annexin A2</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>IPI00699333.1</td>
<td>ANXA8L1 Annexin A8</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>IPI00706942.3</td>
<td>TPII Triosephosphate isomerase</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>IPI00717428.1</td>
<td>LOC781146 Lysozyme C, tracheal isozyme</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>IPI00696956.4</td>
<td>EDIL3 Uncharacterized protein</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>IPI00690534.1</td>
<td>TF Serotransferrin</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>IPI01002591.1</td>
<td>LOC100301161 keratin 6A-like</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>IPI00692093.3</td>
<td>ANXA5 Annexin A5</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>IPI00687437.4</td>
<td>COL4A1 collagen alpha-1(IV) chain</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>IPI00760446.1</td>
<td>RNASE4;ANG Ribonuclease, RNase A family, 4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>IPI00912554.1</td>
<td>LOC100138974 keratin 13-like isoform 2</td>
<td>10</td>
<td>8</td>
</tr>
</tbody>
</table>
Twenty-one proteins were found exclusively in the intact samples (Table 3.2), another twenty-five were specific to the lesion sample (Table 3.3). A semi-quantitative approach was adopted in this study by measuring the precursor signals of all peptide sequences from each cartilage protein [298, 299]. This helped to compare the intact and lesion samples on an approximate scale.
Table 3.2 Proteins exclusive to the intact samples and the number of their unique peptides.

<table>
<thead>
<tr>
<th>IPI Accession #</th>
<th>Protein</th>
<th>No. of unique peptides in intact</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPI00689362.1</td>
<td>TTR Transthyretin</td>
<td>2</td>
</tr>
<tr>
<td>IPI00712739.3</td>
<td>S100B Protein S100-B</td>
<td>1</td>
</tr>
<tr>
<td>IPI00727157.3</td>
<td>SOD1 superoxide dismutase-like</td>
<td>2</td>
</tr>
<tr>
<td>IPI00699011.3</td>
<td>IGL@ protein</td>
<td>6</td>
</tr>
<tr>
<td>IPI00727400.4</td>
<td>LOC789113 histone H4 replacement-like, partial</td>
<td>5</td>
</tr>
<tr>
<td>IPI00825007.1</td>
<td>VIT Vitrin</td>
<td>2</td>
</tr>
<tr>
<td>IPI00687360.1</td>
<td>SRPX2 Sushi repeat-containing protein SRPX2</td>
<td>2</td>
</tr>
<tr>
<td>IPI00823954.2</td>
<td>LOC618661 lysozyme C, tracheal isozyme-like</td>
<td>1</td>
</tr>
<tr>
<td>IPI00705558.1</td>
<td>THBS4 Thrombospondin-4</td>
<td>1</td>
</tr>
<tr>
<td>IPI00687375.1</td>
<td>TIMP3 Metalloproteinase inhibitor 3</td>
<td>1</td>
</tr>
<tr>
<td>IPI00698663.2</td>
<td>S100A4 Protein S100-A4</td>
<td>1</td>
</tr>
<tr>
<td>IPI00695489.1</td>
<td>SERPINA1 Alpha-1-antiproteinase</td>
<td>1</td>
</tr>
<tr>
<td>IPI00694142.3</td>
<td>MIF Macrophage migration inhibitory factor</td>
<td>1</td>
</tr>
<tr>
<td>IPI00714446.3</td>
<td>GPI Glucose-6-phosphate isomerase</td>
<td>3</td>
</tr>
<tr>
<td>IPI00709590.5</td>
<td>KRT4 KRT4 protein</td>
<td>4</td>
</tr>
<tr>
<td>IPI00707095.3</td>
<td>ENO1 Alpha-enolase</td>
<td>1</td>
</tr>
<tr>
<td>IPI00712521.1</td>
<td>RARRES2 Retinoic acid receptor responder protein 2</td>
<td>1</td>
</tr>
<tr>
<td>IPI00904206.1</td>
<td>Uncharacterized protein (Fragment) TPA: olfactory</td>
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<tr>
<td>IPI00724838.2</td>
<td>IGK IGK protein</td>
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<tr>
<td>IPI00686092.1</td>
<td>PRDX1 Peroxiredoxin-1</td>
<td>1</td>
</tr>
<tr>
<td>IPI00689304.2</td>
<td>MGC137014 Protein HP-20 homolog</td>
<td>2</td>
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</table>
Table 3.3 Proteins and the number of unique peptides identified only in lesion samples.

<table>
<thead>
<tr>
<th>IPI Accession #</th>
<th>Protein</th>
<th>No. of unique peptides in lesion</th>
</tr>
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<tr>
<td>IPI00908224.1</td>
<td>PREDICTED: Chondroadherin-like protein</td>
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<tr>
<td>IPI01002598.1</td>
<td>TCERG1 transcription elongation regulator 1 isoform 1</td>
<td>1</td>
</tr>
<tr>
<td>IPI01028259.1</td>
<td>Uncharacterized protein Immunoglobulin light chain</td>
<td>7</td>
</tr>
<tr>
<td>IPI01000215.1</td>
<td>LOC782286 heparan sulfate D-glucosaminyl 3-O-sulfotransferase 2-like</td>
<td>2</td>
</tr>
<tr>
<td>IPI00690198.4</td>
<td>HPX Hemopexin</td>
<td>1</td>
</tr>
<tr>
<td>IPI00698285.3</td>
<td>KRT24 keratin, type I cytoskeletal 24</td>
<td>3</td>
</tr>
<tr>
<td>IPI00905420.1</td>
<td>BRDT Uncharacterized protein</td>
<td>2</td>
</tr>
<tr>
<td>IPI00825043.2</td>
<td>COCH Uncharacterized protein</td>
<td>2</td>
</tr>
<tr>
<td>IPI00694684.3</td>
<td>SAFB SAFB protein</td>
<td>1</td>
</tr>
<tr>
<td>IPI00703491.5</td>
<td>CP Uncharacterized protein</td>
<td>1</td>
</tr>
<tr>
<td>IPI00691275.6</td>
<td>PREDICTED: T-lymphoma invasion and metastasis-inducing protein 2 isoform X4</td>
<td>1</td>
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<tr>
<td>IPI00713761.4</td>
<td>IMPG2 interphotoreceptor matrix proteoglycan 2-like</td>
<td>1</td>
</tr>
<tr>
<td>IPI00705441.1</td>
<td>CRP C-reactive protein precursor</td>
<td>1</td>
</tr>
<tr>
<td>IPI00696507.1</td>
<td>AMBP Protein AMBP</td>
<td>1</td>
</tr>
<tr>
<td>IPI00691199.1</td>
<td>LOC525863 Histone H4</td>
<td>3</td>
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<tr>
<td>IPI00685784.3</td>
<td>LCN2 lipocalin 2 (oncogene 24p3)-like</td>
<td>2</td>
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<tr>
<td>IPI00712934.2</td>
<td>VTN Vitronectin</td>
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<td>IPI00843209.1</td>
<td>FGG Uncharacterized protein</td>
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<td>IPI00717369.1</td>
<td>CLEC3B Tetranectin</td>
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<tr>
<td>IPI00714359.3</td>
<td>FBN1 Uncharacterized protein</td>
<td>8</td>
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<td>IPI00712036.3</td>
<td>CA3 Carbonic anhydrase 3</td>
<td>4</td>
</tr>
<tr>
<td>IPI00691819.1</td>
<td>FGA Fibrinogen alpha chain</td>
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<td>LOC537387 collagen type 5 alpha 1-like</td>
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<tr>
<td>IPI01001922.1</td>
<td>CD109 thiolester containing protein II-like</td>
<td>3</td>
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<tr>
<td>IPI00731867.7</td>
<td>KRT6C keratin 6C isoform</td>
<td>3</td>
</tr>
</tbody>
</table>

Many proteins detected in this work were multifunctional in nature. The functions included organisation of the ECM, protein metabolism, immune response, metabolic and energy pathways, signal transduction and transport. Many of these proteins are cited in the literature as responsible for normal cartilage turnover, ECM maintenance or tissue repair, among many others. Quite a few proteins were found to possess specific roles associated with different aspects of OA including initiation of the pathological condition of OA, acting as intermediates in the catabolic cascade during cartilage degradation [300-308] or initiating further damage to the matrix by triggering chondrocyte differentiation, leading up to matrix mineralization and apoptosis in chondrocytes [309].
A few proteins found significant in the preliminary experiment is shown in Table 3.4 along with their specific role in cartilage and subchondral bone as reported in literature.

**Table 3.4** Potential roles of some prominent proteins found in the current study with respect to cartilage and bone, as reported by various literatures.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Role in osteoarthritis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1 Transforming Growth factor-β1</td>
<td>may be involved in endochondral bone formation in cartilage; reported to increase in early OA; boosts anabolic activity of chondrocytes and maintains ECM production.</td>
<td>[310] [300] [311]</td>
</tr>
<tr>
<td>CLEC3B Tetranectin</td>
<td>potential role in mineralization during osteogenesis; impaired regulation of fibrinolysis associated with the inflammatory process in OA; intra articular regulation of fibrinolysis; tissue remodelling and angiogenesis.</td>
<td>[312] [313] [314]</td>
</tr>
<tr>
<td>FBN1 Fibrillin 1</td>
<td>bone formation and growth in youth and bone mineralisation in adult; control bone formation by regulating osteoblast differentiation.</td>
<td>[315] [316]</td>
</tr>
<tr>
<td>CA3 Carbonic anhydrase 3</td>
<td>cartilage homeostasis; pathological role of CA3 in cartilage destruction; cartilage mineralization; cartilage calcification.</td>
<td>[301] [317]</td>
</tr>
<tr>
<td>CD109 thiolester containing protein II-like</td>
<td>inhibits TGF-β signalling; released from the chondrocyte cell surface; “may serve as a protease inhibitor in cartilage or may possibly play a role in complement activity.”</td>
<td>[318] [319]</td>
</tr>
<tr>
<td>FGA Fibrinogen alpha chain</td>
<td>known to be elevated in OA patients; involved in apoptosis; inflammation; immunologic cascade.</td>
<td>[302] [320]</td>
</tr>
<tr>
<td>VTN Vitronectin</td>
<td>involved in the complement cascade; may involve in important role in the pathogenesis of OA; elevated serum levels of vitronectin fragments associated with OA progression; cell adhesion and spreading factor; levels are significantly enhanced in the inflamed joint; influence cell adhesion and modulate tissue repair.</td>
<td>[213] [321]</td>
</tr>
<tr>
<td>C1QC Complement C1q subcomponent subunit C</td>
<td>“collagenous-like subcomponent of the first component of complement .”</td>
<td>[322]</td>
</tr>
<tr>
<td>C1QB Complement C1q subcomponent subunit B</td>
<td>classic complement pathway; reported to degrade matrix component.</td>
<td>[323]</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>LYZ1</td>
<td>Lysozyme C, milk isozyme</td>
<td>found in nonlysosomal component of ECM of cartilage; inflammatory response; its concentration reflecting the aggregate of inflammation and cartilage breakdown; proteoglycan synthesis; found in higher amount in the region undergoing calcification and bone transformation.</td>
</tr>
<tr>
<td>FMOD</td>
<td>Fibromodulin</td>
<td>regulating chondrogenesis and ECM turnover; control ECM structure by interacting with the network of cartilage proteins; mediate cell metabolism by binding to members of the transforming growth factor β superfamily.</td>
</tr>
<tr>
<td>FN1</td>
<td>Embryospecific fibronectin 1 transcript variant</td>
<td>Increased fibronectin fragments in OA; may stimulate cartilage breakdown; cartilage chondrolytic activities; “enhance rates of proteoglycan (PG) loss from cartilage tissue in explant cultures”; increase matrix metalloproteinase (MMP) expression; repress proteoglycan (PG) synthesis in chondrocytes and cartilage; regulates normal cartilage homeostasis; aides cartilage degeneration during OA progression.</td>
</tr>
<tr>
<td>COL6A3</td>
<td>Collagen, type VI, alpha 3-like isoform</td>
<td>maintains integrity of the cartilage matrix; an increased synthesis of type VI collagen in OA; may be responsible for an altered metabolism in osteoarthritic cartilage; changes in type VI collagen distribution and its appearance in osteoarthritic cartilage; “acts as an interface between the rigid inter-territorial cartilage matrix and the chondrocyte; involved in cell anchoring as well as matrix cell signalling.” promotes the formation of chondrocyte clusters.</td>
</tr>
<tr>
<td>COL12A1</td>
<td>Collagen, type XII, alpha 1-like isoform</td>
<td>provide tissue integrity at the fibril perimeter; contribute to the mature matrix; members of the FACIT collagen subfamily; preferentially interacts with proteoglycans fibromodulin and decorin; “stabilize articular cartilage matrix against compressive and shearing forces; may have a role in promoting alignment or stabilizing fibril orientation.”</td>
</tr>
<tr>
<td>COMP</td>
<td>Cartilage oligomeric matrix protein</td>
<td>important degradation product of articular cartilage</td>
</tr>
</tbody>
</table>
Table 3.4 Continued

<p>| <strong>ACAN</strong>&lt;br&gt;Aggrecan | major component of the articular cartilage ECM; aggrecan degradation is considered to play a key role in the progression of osteoarthritis; have “an important role in mediating chondrocyte-chondrocyte and chondrocyte-matrix interactions through its ability to bind hyaluronic;” the progressive degradation of aggrecan in OA leads to cartilage erosion. | [342] |
| <strong>ALB</strong>&lt;br&gt;Serum albumin | “the keratan sulfate rich proteoglycans, prevalent at the surface of joint cartilage are bound to serum albumin by disulfide bonds in the articular cartilage of patients with OA;” albumin shows an increased diffusion coefficient in degenerated cartilage compared to normal. | [345] [346] |
| <strong>TNC</strong>&lt;br&gt;Tenascin | “tenascin is increased in arthritic cartilage and is weakly expressed in normal cartilage;” cartilage degradation and inflammation. | [347] [348] |
| <strong>HAPLN1</strong>&lt;br&gt;Hyaluronan and proteoglycan link protein 1 | key component of the cartilage extracellular matrix; “stabilizes aggregates of aggrecan and hyaluronic acid by binding to aggrecan along the hyaluronic acid chain and contributes to compression resistance and shock absorption in the joints.” | [349] [350] |
| <strong>LCN2</strong>&lt;br&gt;lipocalin 2 (oncogene 24p3)-like | high amount of LCN2 in OA cartilage. | [305] |
| <strong>COL3A1</strong>&lt;br&gt;Collagen, type III, alpha 1 | in late stage of OA, the content of collagen type III increases. | [351] |
| <strong>APOD</strong>&lt;br&gt;Apolipoprotein D | role in aging and age-related diseases. negative regulation of cytokine production. | [352] [353] |
| <strong>MB</strong>&lt;br&gt;Myoglobin | accumulates in inflamed synovial membrane or bone marrow adjacent to cartilage. | [354] |
| <strong>SERPINF1</strong>&lt;br&gt;Pigment epithelium-derived factor | inhibitor of angiogenesis; upregulated in OA. | [306] |
| <strong>BGN</strong>&lt;br&gt;Biglycan | binds to connective tissue and regulates their function; upregulated at late stages of OA to compensate for the general proteoglycan loss. | [355] |
| <strong>UGP2</strong>&lt;br&gt;UTP–glucose-1-phosphate uridylyltransferase | metal binding; protein binding, phosphorylation; UDP glucose-1-phosphate uridylyltransferase activity involved in tissue repair and regeneration. | [356] |</p>
<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COL2A1</strong>&lt;br&gt;Collagen alpha-1(II) chain</td>
<td>“variations in COL2A1 gene may increase the risk of developing OA”; involved in endochondral bone morphogenesis; collagen fibril organization; produced by chondrocytes in response to matrix damage; “tissue sites of proteolysis and denaturation of matrix type II collagen can be observed in normal and OA joint surfaces.”&lt;br&gt;[357] [358] [359] [360] [361]</td>
</tr>
<tr>
<td><strong>CHI3L1</strong>&lt;br&gt;Chitinase-3-like protein 1</td>
<td>increased amount correlates with disease severity in OA; enables growth of human synovial cells; initiate a signalling cascade leading to cell proliferation; accumulation leads to pathogenesis of joint degeneration and inflammation; normally not synthesised in healthy tissues&lt;br&gt;[362] [363]</td>
</tr>
<tr>
<td><strong>ANXA4</strong>&lt;br&gt;Annexin A4</td>
<td>implicated in cytoskeletal or exocytotic and endocytotic pathways; calcium ion binding and signal transduction.&lt;br&gt;[364]</td>
</tr>
<tr>
<td><strong>GSN</strong>&lt;br&gt;Gelsolin</td>
<td>actin cytoskeletal architecture; osteoclast motility; contribute to bone resorption.&lt;br&gt;[365]</td>
</tr>
<tr>
<td><strong>LECT2</strong>&lt;br&gt;Leukocyte cell-derived chemotaxin-2</td>
<td>stimulates proliferation of osteoblasts and chondrocytes in bone remodelling; cartilage repair and stimulates osteoclast differentiation;&lt;br&gt;[366]</td>
</tr>
<tr>
<td><strong>THBS1</strong>&lt;br&gt;Thrombospondin-1</td>
<td>“involved in cell matrix interaction of various tissues particularly in cartilage” anti-angiogenic factor; its content is lower in osteoarthritic cartilage than in normal cartilage; activates TGFβ1; induces endothelial cell apoptosis”&lt;br&gt;[367] [308]</td>
</tr>
<tr>
<td><strong>Protein S100-A1</strong></td>
<td>intracellular calcium binding protein that controls terminal differentiation of chondrocytes; known to be down regulated in OA&lt;br&gt;[368] [369]</td>
</tr>
<tr>
<td><strong>SOD2</strong>&lt;br&gt;Superoxide dismutase [Mn]</td>
<td>down regulated in OA; “Increased level of SOD2 in OA patients indicate increased oxidative stress”; potential biomarker.&lt;br&gt;[370] [371]</td>
</tr>
<tr>
<td><strong>CD9</strong>&lt;br&gt;CD9 antigen</td>
<td>cell differentiation antigen; involved in platelet activation and aggregation; involved in cell adhesion and motility; affects signalling and may be a factor for apoptosis.&lt;br&gt;[372]</td>
</tr>
<tr>
<td><strong>SOD3</strong>&lt;br&gt;Superoxide dismutase</td>
<td>“localised to ECM of tissues by binding to negatively charged proteoglycans and collagen; protects vulnerable proteins and macromolecules of ECM from oxidant injury”; found in large amount in normal cartilage and decreased about 4 fold in OA cartilage.&lt;br&gt;[373]</td>
</tr>
<tr>
<td><strong>COL15A1</strong>&lt;br&gt;Collagen, type XV, alpha 1</td>
<td>influences angiogenesis.&lt;br&gt;[374]</td>
</tr>
<tr>
<td><strong>C3</strong>&lt;br&gt;Complement C3 (Fragment)</td>
<td>higher concentration in SF of individuals with early stage OA.&lt;br&gt;[172]</td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PCOLCE</td>
<td>Procollagen C-Endopeptidase Enhancer</td>
</tr>
<tr>
<td>F13A1</td>
<td>Factor XIII-A blood clotting factor</td>
</tr>
<tr>
<td>KRT14</td>
<td>Keratin, type I cytoskeletal 14</td>
</tr>
<tr>
<td>PRG4</td>
<td>Proteoglycan 4</td>
</tr>
<tr>
<td>ANG</td>
<td>Angiogenin-1</td>
</tr>
<tr>
<td>PGLYRP1</td>
<td>Peptidoglycan recognition protein 1</td>
</tr>
<tr>
<td>MATN2</td>
<td>Matrilin 2</td>
</tr>
<tr>
<td>ANXA1</td>
<td>Annexin A1</td>
</tr>
<tr>
<td>COL11A1</td>
<td>Collagen alpha-1(XI) chain</td>
</tr>
<tr>
<td>CILP2</td>
<td>Cartilage intermediate layer protein 2</td>
</tr>
<tr>
<td>COL6A1</td>
<td>Collagen alpha-1(VI) chain</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
</tr>
<tr>
<td>CHAD</td>
<td>Chondroadherin regulates the collagen fibril assembly and communicates between chondrocyte and the matrix constituents; chondroadherin binds to collagen type II; known to mediate adhesion of isolated chondrocytes</td>
</tr>
<tr>
<td>HSPG2</td>
<td>Heparan sulfate proteoglycan 2 or perlecan</td>
</tr>
<tr>
<td>OGN</td>
<td>Mimecan</td>
</tr>
<tr>
<td>COL9A1</td>
<td>Collagen, type IX, alpha 1</td>
</tr>
</tbody>
</table>
3.3.1.1 Semi Quantitative Analysis of Intact and Lesion Bovine Cartilages

Precursor signals from all the peptides sequences of individual cartilage protein added and their abundance in the sample was measured as relative percentage signal in the sample (dividing individual protein signal to the overall signal in the sample). It was found that despite originating from the same cartilage, the proteome profile of the intact and lesion samples showed significant differences in this preliminary work. As expected, the proteins with high scores (> 5%) were collagens, aggrecan, fibronectin, hyaluronan and proteoglycan link protein (HAPLN1), cartilage intermediate layer protein 2 (CILP2), serum albumin (ALB) and COMP. Tenascin (TNC), chondroadherin (CHAD), EGF-like repeats and discoidin I-like domains 3 (EDIL-3), Mimecan (OGN), MFGE8 isoform long of Lactadherin, Fibromodulin (FMOD) and TGF-β1 were also present in significant amounts (1-5%) (Figure 3.4).

![Figure 3.4](image)

**Figure 3.4** Semi-quantification analysis of 18 abundant proteins present in intact versus lesion regions of GII grade (Outerbridge’s classification) sample.

In comparison to the intact cartilage sample, aggrecan and HAPLN1 content in the lesion sample was almost halved, whereas the collagen, COMP, mimecan and chondroadherin were slightly higher in the lesion sample. COMP is a known degradation product of cartilage in OA [337] and, as expected, was upregulated in the lesion sample. Tenascin and EDIL-3 amounts were doubled in the lesion sample with respect to the intact.

The proteins with comparatively higher differential level in the lesion sample were pigment epithelium-derived factor (Serpin family F member 1 or SERPINF1),
Chitinase-3-like protein 1 (CHI3L1), TGF-βI and THBS1 (Figure 3.4 and 3.5). The anti-angiogenic factor, SERPINF1, was 10-fold higher in the lesion sample compared to intact sample. CHI3L1, which is understood to be associated with the lesion formation, leading to cartilage degeneration in an osteoarthritic cartilage [363, 398] was also increased by 10-fold in the lesion cartilage sample.

TGF-βI is induced by TGF-β and involves in endochondral bone formation in the cartilage [310]. This protein was almost 5-folds higher in the lesion sample than corresponding intact. THBS1 was also detected 5 times more in lesion than the intact region of the patella.

The protein variations observed in the intact and lesion samples are shown in Figure 3.4 and 3.5.
Figure 3.5 Semi-quantification analysis of less abundant proteins present in intact and lesion regions of GII grade (Outerbridge’s classification) sample.
3.3.2 *iTRAQ*-based MS/MS Analysis

For quantitative analysis of the proteins identified in various cartilage groups, *iTRAQ* based tandem mass spectrometry was performed.

3.3.2.1 Scoring based on Histology

Table 3.5 shows the Mankin scores obtained for different sample groups. Following Safranin O-fast green staining, the intact cartilage samples from both GI and GII patellae were graded and showed Mankin scores ranging from 0-1 (Figure 2.2 C and E). The lesion sites from the GI patellae showed Mankin scores in the range of 1-4 (Figure 2.2 D), whereas those from the GII patellae scored 2-5 (Figure 2.2 F). The controls obtained from healthy cow patellae (Figure 2.2 A) were similar to the intact regions. Site A and Site B from G0 bull patellae scored 0-2 on Mankin grading.

**Table 3.5** Mankin scores for different tissue groups used in this experiment.

<table>
<thead>
<tr>
<th>N</th>
<th>Sample Group</th>
<th>Mankin Score Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Healthy cow (Control site A and B)</td>
<td>0-1</td>
</tr>
<tr>
<td>3</td>
<td>G0 bull (Site A and B)</td>
<td>0-2</td>
</tr>
<tr>
<td>7</td>
<td>GI intact</td>
<td>0-1</td>
</tr>
<tr>
<td>7</td>
<td>GI lesion</td>
<td>1-4</td>
</tr>
<tr>
<td>5</td>
<td>GII intact</td>
<td>0-1</td>
</tr>
<tr>
<td>5</td>
<td>GII lesion</td>
<td>2-5</td>
</tr>
</tbody>
</table>
3.3.2.2 Discovery Proteomics

A total of 191 proteins were identified in this study from the 36 samples analyzed over 6 independent iTRAQ experiments. 109 proteins were detected in control samples and 166 proteins in non-healthy samples. Eighty-four proteins were common to both normal and the tissues with early cartilage degeneration, while 25 proteins were exclusively found in normal controls and 82 exclusively in early degenerated cartilages (Figure 3.6).

![Venn diagram showing the number of proteins identified in control cartilage and non-healthy cartilage (early degeneration). 109 proteins were identified in control group and 166 proteins in non-healthy group. The number in the intersecting area of the diagram show the number of proteins that were common to both control and non-healthy cartilage.](image)

**Figure 3.6** Venn diagram showing the number of proteins identified in control cartilage and non-healthy cartilage (early degeneration). 109 proteins were identified in control group and 166 proteins in non-healthy group. The number in the intersecting area of the diagram show the number of proteins that were common to both control and non-healthy cartilage.
Gene ontology (GO) is a bioinformatics tool which categorize the genes and gene products into biologically meaningful annotations [399]. In the present work, GO analysis of the identified proteins (on the basis of their Gene ID) depicted the diverse biological functional roles of these proteins in the cartilage tissue involving cellular and metabolic processes, cellular component organization, stimuli response, development processes, immune and biological responses amongst others (Figure 3.7).

Figure 3.7 Gene ontology analysis of the identified proteins using PANTHER (Protein Analysis through Evolutionary Relationship), which classified the proteins based on their biological processes.
(A) Comparison between healthy and the diseased groups.

The healthy cow group (controls) protein ratios were scaled to zero in log space (1.00 in normal space), and represented as “no change”, while the rest of the ratios from the non-healthy groups (intact and lesion) were scaled relative to that. These scaled ratios were then used to calculate the average and 95% confidence intervals for each protein and the group, in log space. The statistical analysis was then done for each protein separately using the above log ratios.

All the graphs shown in this work are plotted using the natural log ratio values, obtained for the particular protein.

The non-healthy cartilage tissues were compared to control healthy cow samples using One Way ANOVA. This identified 56 proteins to be significantly different (Appendix D) in their group means and a Tukey’s multiple comparisons test was performed to test how the four disease groups compared to the healthy group. Both the ANOVA and Tukey’s test required at least two values for each group, so the analysis was performed on the groups with n>1.
**GI intact vs. Healthy**

A set of 27 proteins showed significant differential presence at the GI intact site when matched against the healthy cartilages. 9 proteins were found to have higher abundance in the intact samples, whereas 18 proteins had a lower level in the GI intact sites than healthy tissues as shown in Table 3.6.

**Table 3.6** Significant differential level of proteins when GI intact cartilages were compared to healthy (cow) cartilages.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Tukey's test Healthy vs. GI intact €</th>
<th>Ratio of GI intact to Healthy †</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYZ1 Lysozyme C, milk isozyome</td>
<td>****</td>
<td>11.24</td>
</tr>
<tr>
<td>PREDICTED: WAP four-disulfide core domain protein 18-like</td>
<td>****</td>
<td>9.23</td>
</tr>
<tr>
<td>PREDICTED: cartilage intermediate layer protein 1</td>
<td>****</td>
<td>5.59</td>
</tr>
<tr>
<td>ANG Angiogenin-1</td>
<td>****</td>
<td>4.22</td>
</tr>
<tr>
<td>FN1 Embryo-specific fibronectin 1 transcript variant</td>
<td>***</td>
<td>3.89</td>
</tr>
<tr>
<td>COMP Cartilage oligomeric matrix protein</td>
<td>***</td>
<td>3.21</td>
</tr>
<tr>
<td>RNASE4:ANG Ribonuclease, RNase A family, 4</td>
<td>****</td>
<td>2.82</td>
</tr>
<tr>
<td>ACAN Isoform 2 of Aggrecan core protein</td>
<td>****</td>
<td>1.98</td>
</tr>
<tr>
<td>CILP2 [Cartilage intermediate layer protein 2]</td>
<td>***</td>
<td>1.85</td>
</tr>
<tr>
<td>COL6A1 Uncharacterized protein</td>
<td>**</td>
<td>0.70</td>
</tr>
<tr>
<td>COL6A2 Uncharacterized protein</td>
<td>**</td>
<td>0.65</td>
</tr>
<tr>
<td>COL6A3 Uncharacterized protein</td>
<td>**</td>
<td>0.65</td>
</tr>
<tr>
<td>COL2A1 Collagen alpha-1(II) chain</td>
<td>**</td>
<td>0.60</td>
</tr>
<tr>
<td>PPIA Peptidyl-prolyl cis-trans isomerase A</td>
<td>*</td>
<td>0.60</td>
</tr>
<tr>
<td>VIM Vimentin</td>
<td>**</td>
<td>0.59</td>
</tr>
<tr>
<td>COL12A1 Uncharacterized protein</td>
<td>*</td>
<td>0.55</td>
</tr>
<tr>
<td>COL9A2 collagen, type IX, alpha 2</td>
<td>*</td>
<td>0.54</td>
</tr>
<tr>
<td>THBS1 Thrombospondin-1</td>
<td>*</td>
<td>0.53</td>
</tr>
<tr>
<td>PCOLCE Procollagen C-endopeptidase enhancer</td>
<td>**</td>
<td>0.49</td>
</tr>
<tr>
<td>COL9A3 collagen, type IX, alpha 3</td>
<td>**</td>
<td>0.48</td>
</tr>
<tr>
<td>LMNA Uncharacterized protein</td>
<td>*</td>
<td>0.47</td>
</tr>
<tr>
<td>COL9A1 collagen, type IX, alpha 1</td>
<td>***</td>
<td>0.41</td>
</tr>
<tr>
<td>TGFB1 [transforming growth factor-beta-induced protein ig-h3 precursor]- LOC539596 protein</td>
<td>*</td>
<td>0.37</td>
</tr>
<tr>
<td>HBB Hemoglobin subunit beta</td>
<td>**</td>
<td>0.33</td>
</tr>
<tr>
<td>HBA1;HBA Hemoglobin subunit alpha</td>
<td>**</td>
<td>0.28</td>
</tr>
<tr>
<td>COL11A1 collagen alpha-1(XI) chain</td>
<td>*</td>
<td>0.28</td>
</tr>
<tr>
<td>CKM Creatine kinase M-type</td>
<td>*</td>
<td>0.23</td>
</tr>
</tbody>
</table>

†For the ease of understanding, log ratios were converted to linear space.
€For the post-test * denotes significant with p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001
**GI lesion vs. Healthy**

This comparison lead to identification of 18 proteins with significant differential levels at GI lesion site compared to healthy cartilages, with 11 proteins showing higher abundance and 7 showing decreased levels at the mildly degenerate cartilage sites in comparison to healthy tissues (Table 3.7).

Table 3.7 Significant differential level of proteins when GI lesion cartilages were compared to healthy (cow) cartilages.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Tukey's test Healthy vs. GI lesion €</th>
<th>Ratio of GI lesion to Healthy †</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYZ1 Lysozyme C, milk isozyme</td>
<td>***</td>
<td>5.06</td>
</tr>
<tr>
<td>PREDICTED: WAP four-disulfide core domain protein 18-like</td>
<td>***</td>
<td>5.00</td>
</tr>
<tr>
<td>VIT Vitrin</td>
<td>**</td>
<td>4.29</td>
</tr>
<tr>
<td>ANG Angiogenin-1</td>
<td>****</td>
<td>3.54</td>
</tr>
<tr>
<td>PREDICTED: cartilage intermediate layer protein 1</td>
<td>**</td>
<td>3.29</td>
</tr>
<tr>
<td>ANXA1 Annexin A1</td>
<td>*</td>
<td>2.81</td>
</tr>
<tr>
<td>RNASE4;ANG Ribonuclease, RNase A family, 4</td>
<td>***</td>
<td>2.26</td>
</tr>
<tr>
<td>CILP2 [Cartilage intermediate layer protein 2]</td>
<td>**</td>
<td>1.59</td>
</tr>
<tr>
<td>FMOD Fibromodulin</td>
<td>**</td>
<td>1.58</td>
</tr>
<tr>
<td>ACAN Isoform 2 of Aggrecan core protein</td>
<td>**</td>
<td>1.48</td>
</tr>
<tr>
<td>PRELP Prolargin</td>
<td>***</td>
<td>1.47</td>
</tr>
<tr>
<td>PGAM1 Phosphoglycerate mutase 1</td>
<td>*</td>
<td>0.62</td>
</tr>
<tr>
<td>COL9A2 collagen, type IX, alpha 2</td>
<td>*</td>
<td>0.58</td>
</tr>
<tr>
<td>VIM Vimentin</td>
<td>***</td>
<td>0.56</td>
</tr>
<tr>
<td>COL9A3 collagen, type IX, alpha 3</td>
<td>*</td>
<td>0.51</td>
</tr>
<tr>
<td>COL9A1 collagen, type IX, alpha 1</td>
<td>**</td>
<td>0.49</td>
</tr>
<tr>
<td>PPIA Peptidyl-prolyl cis-trans isomerase A</td>
<td>***</td>
<td>0.47</td>
</tr>
<tr>
<td>LMNA Uncharacterized protein</td>
<td>***</td>
<td>0.34</td>
</tr>
</tbody>
</table>

†For the ease of understanding, log ratios were converted to linear space.

*For the post-test * denotes significant with p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001.
**GII intact vs. Healthy**

The comparison between the GII intact and healthy cartilages identified 23 proteins to be significantly different after post-hoc test. 10 proteins were seen to have higher levels in grade II intact tissues compared to healthy cartilages and 13 showed decreased levels (Table 3.8).

**Table 3.8** Significant differential level of proteins when GII intact cartilages were compared to healthy (cow) cartilages

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Tukey’s test Healthy vs. GII intact</th>
<th>Ratio of GII intact to Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYZ1 Lysozyme C, milk isoyme</td>
<td>****</td>
<td>13.63</td>
</tr>
<tr>
<td>PREDICTED: WAP four-disulfide core domain protein 18-like</td>
<td>****</td>
<td>7.47</td>
</tr>
<tr>
<td>PREDICTED: cartilage intermediate layer protein 1</td>
<td>***</td>
<td>5.29</td>
</tr>
<tr>
<td>ANG Angiogenin-1</td>
<td>****</td>
<td>4.46</td>
</tr>
<tr>
<td>FN1 Embryo-specific fibronectin 1 transcript variant</td>
<td>***</td>
<td>4.38</td>
</tr>
<tr>
<td>RNASE4;ANG Ribonuclease, RNase A family, 4</td>
<td>****</td>
<td>3.19</td>
</tr>
<tr>
<td>COMP Cartilage oligomeric matrix protein</td>
<td>***</td>
<td>3.10</td>
</tr>
<tr>
<td>CLU Clusterin</td>
<td>*</td>
<td>2.42</td>
</tr>
<tr>
<td>ACAN Isoform 2 of Aggrecan core protein</td>
<td>****</td>
<td>2.18</td>
</tr>
<tr>
<td>CILP2 [Cartilage intermediate layer protein 2]</td>
<td>**</td>
<td>1.65</td>
</tr>
<tr>
<td>COL6A2 Uncharacterized protein</td>
<td>*</td>
<td>0.70</td>
</tr>
<tr>
<td>COL6A3 Uncharacterized protein</td>
<td>*</td>
<td>0.69</td>
</tr>
<tr>
<td>COL2A1 Collagen alpha-1(II) chain</td>
<td>*</td>
<td>0.66</td>
</tr>
<tr>
<td>COL9A2 collagen, type IX, alpha 2</td>
<td>*</td>
<td>0.55</td>
</tr>
<tr>
<td>COL12A1 Uncharacterized protein</td>
<td>*</td>
<td>0.48</td>
</tr>
<tr>
<td>COL9A3 collagen, type IX, alpha 3</td>
<td>**</td>
<td>0.45</td>
</tr>
<tr>
<td>EDIL3 [EGF-like repeat and discoidin I-like domain-containing protein 3]</td>
<td>**</td>
<td>0.45</td>
</tr>
<tr>
<td>PCOLCE Procollagen C-endopeptidase enhancer</td>
<td>**</td>
<td>0.43</td>
</tr>
<tr>
<td>COL9A1 collagen, type IX, alpha 1</td>
<td>***</td>
<td>0.41</td>
</tr>
<tr>
<td>HBB Hemoglobin subunit beta</td>
<td>*</td>
<td>0.37</td>
</tr>
<tr>
<td>HBA1;HBA Hemoglobin subunit alpha</td>
<td>*</td>
<td>0.36</td>
</tr>
<tr>
<td>LMNA Uncharacterized protein</td>
<td>**</td>
<td>0.35</td>
</tr>
<tr>
<td>CKM Creatine kinase M-type</td>
<td>*</td>
<td>0.24</td>
</tr>
</tbody>
</table>

1For the ease of understanding, log ratios were converted to linear space.

*For the post-test * denotes significant with p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001
**GII lesion vs. Healthy**

13 proteins were found to be significantly differential, with 10 having a higher level in the moderately degenerated cartilage regions and 3 having a decreased level of proteins compared to the healthy cartilage samples (Table 3.9).

**Table 3.9** Significant differential level of proteins when GII lesion cartilages were compared to healthy (cow) cartilages

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Tukey's test Healthy vs. GII lesion</th>
<th>Ratio of GII lesion to Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td>PREDICTED: WAP four-disulfide core domain protein 18-like</td>
<td>***</td>
<td>5.22</td>
</tr>
<tr>
<td>LYZ1 Lysozyme C, milk isozyme</td>
<td>**</td>
<td>4.67</td>
</tr>
<tr>
<td>PREDICTED: cartilage intermediate layer protein 1</td>
<td>***</td>
<td>4.58</td>
</tr>
<tr>
<td>FNI Embryo-specific fibronectin 1 transcript variant</td>
<td>**</td>
<td>3.76</td>
</tr>
<tr>
<td>ANG Angiogenin-1</td>
<td>***</td>
<td>3.26</td>
</tr>
<tr>
<td>VIT Vitrin</td>
<td>*</td>
<td>3.01</td>
</tr>
<tr>
<td>COMP Cartilage oligomeric matrix protein</td>
<td></td>
<td>2.36</td>
</tr>
<tr>
<td>RNASE4;ANG Ribonuclease, RNase A family, 4</td>
<td>***</td>
<td>2.36</td>
</tr>
<tr>
<td>CILP2 [Cartilage intermediate layer protein 2]</td>
<td>**</td>
<td>1.79</td>
</tr>
<tr>
<td>ACAN Isoform 2 of Aggrecan core protein</td>
<td>***</td>
<td>1.75</td>
</tr>
<tr>
<td>PPIA Peptidyl-prolyl cis-trans isomerase A</td>
<td>***</td>
<td>0.49</td>
</tr>
<tr>
<td>COL9A1 collagen, type IX, alpha 1</td>
<td>***</td>
<td>0.41</td>
</tr>
<tr>
<td>LMNA Uncharacterized protein</td>
<td>***</td>
<td>0.30</td>
</tr>
</tbody>
</table>

†For the ease of understanding, log ratios were converted to linear space.

‡For the post-test * denotes significant with p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001
To see whether there were any gender variations among healthy groups, the proteome profile of cows and bulls were also compared.

**G0 bull cartilages vs G0 cow cartilages**

A core set of 12 proteins were differentially present on comparing non-degenerated cow and bull cartilages (Table 3.10). Four proteins were found at significantly lower level in bulls compared to healthy cows and eight were significantly higher in bulls. Matrilin 3 was found at 16-fold higher levels in bull compared to cartilage samples from cows.

Since the cartilages from both cow and bull, displayed no indication of degeneration or fibrillation, they were categorized as G0. Hence it could be possible that the protein variation observed between the groups could be partly due to the gender difference. However, as stated in previous chapter, the cow and bull samples were not age matched. Further studies should be performed with age matched samples before confirming the significance of this observation.

**Table 3.10** Significant differential level of proteins when G0 bull cartilages were compared to healthy cow cartilages

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Tukey’s test G0 bulls vs. G0 cows</th>
<th>Ratio of G0 bull to G0 cows</th>
</tr>
</thead>
<tbody>
<tr>
<td>MATN3 matrilin 3</td>
<td>**</td>
<td>16.06</td>
</tr>
<tr>
<td>THBS1 Thrombospondin-1</td>
<td>**</td>
<td>2.47</td>
</tr>
<tr>
<td>COL9A1 collagen, type IX, alpha 1</td>
<td>***</td>
<td>2.39</td>
</tr>
<tr>
<td>VIT Vitrin</td>
<td>*</td>
<td>2.37</td>
</tr>
<tr>
<td>COL12A1 Uncharacterized protein</td>
<td>**</td>
<td>2.22</td>
</tr>
<tr>
<td>COL15A1 collagen, type XV, alpha 1</td>
<td>*</td>
<td>1.76</td>
</tr>
<tr>
<td>COL6A3 Uncharacterized protein</td>
<td>**</td>
<td>1.55</td>
</tr>
<tr>
<td>COL6A1 Uncharacterized protein</td>
<td>*</td>
<td>1.36</td>
</tr>
<tr>
<td>SRPX2 Sushi repeat-containing protein SRPX2</td>
<td>**</td>
<td>0.63</td>
</tr>
<tr>
<td>OGN [Mimecan] OGN protein</td>
<td>**</td>
<td>0.55</td>
</tr>
<tr>
<td>CLU Clusterin</td>
<td>*</td>
<td>0.45</td>
</tr>
<tr>
<td>FN1 Embryo-specific fibronectin 1 transcript variant</td>
<td>*</td>
<td>0.40</td>
</tr>
</tbody>
</table>

*For the post-test * denotes significant with p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001
(B) **Comparison between two localized regions in a healthy patella.**

Significant inherent regional variations were searched for in the control patellae. This led to the finding that except for three proteins (Collagen IX alpha 3 (COL9A3), tetranectin (CLEC3B) and osteoglycin (OGN)), the changes in protein level between control site A and control site B in patellae were not significant (Figure 3.8). Sidak’s multiple comparison showed that only COL9A3 was significantly different between two sites-intact A and intact B, in a healthy cow patella.

![Graph showing protein ratios](image)

**Figure 3.8** Scaled natural log (LN) ratios of proteins found to be differential between two control sites in healthy patellae. For the post-test, ** denotes significant with p<0.01.
(C) Comparison between the intact and lesion region in a non-healthy patella.

The GI group protein ratios were scaled to zero in log space (1.00 in normal space), and represented as “no change”, while the rest of the ratios from the non-healthy groups were scaled relative to that. These scaled ratios were then used to calculate the average and 95% confidence intervals for each protein and the group in log space. The statistical analysis was then done for each protein separately using the above log ratios.

From the intact and lesion sites of the 12-degenerated patella, 166 proteins were analyzed, of which 46 proteins displayed statistically significant regional differences (p<0.05) (Appendix E). Among these, 21 proteins showed lower level and 23 showed higher level in the lesion samples of both GI and GII patellae compared to their respective intact tissues. The Sidak’s multiple comparison test was performed for proteins with p-value<0.05 after RM Two-Way ANOVA. Four different comparisons were made:

- GI intact vs. GI lesion
- GII intact vs. GII lesion
- GI intact vs. GII intact
- GI lesion vs. GII lesion

The post-test identified a stronger difference between GI intact and GI lesion (Table 3.11). Additional cut-off factors of iTRAQ ratios ≥ 1.19 or ≤ 0.83 and protein detection in all the experimental samples, were employed. Of the 24 proteins that were significantly different between GI intact and GI lesion sites, 16 had higher level at lesion sites and 8 had lower level. GII intact and GII lesion also had 10 proteins which were significantly different in that group. Only 2 proteins- EDIL3 (EGF-like repeat and discoidin I-like domain-containing protein 3) and LUM were significantly different among GI intact and GII intact-site cartilages whereas 3 proteins- HTRA1, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and gelsolin (GSN) showed significantly differential level when the lesion site samples from GI and GII patellae were compared.
Table 3.11 Correlation of microstructural and proteomic analysis. Number of proteins with significant difference (p<0.05) after multiple comparison test and corresponding structural changes seen among two groups of patellae (GI and GII) and two tissue types (intact and lesion).

<table>
<thead>
<tr>
<th>PATELLA GROUP Tissue type</th>
<th>GI Intact vs Lesion</th>
<th>GII Intact vs Lesion</th>
<th>GI vs GII Intact</th>
<th>GI vs GII Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of proteins with p&lt;0.05 in post-test</td>
<td>24</td>
<td>10</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Structural changes in cartilage and bone*</td>
<td>Slightly diminished tangential layer in lesion. Amorphous-appearing mid matrix in intact whereas strong directional fibrocity in lesion. Well defined thick ZCC in intact. In lesion samples the ZCC is invaded by numerous bone spicules.</td>
<td>At the lesion site, tangential zone is absent and presence of chondrocyte cloning. Fibrocity as well as crimped matrix texture in the lesion site tissue. Radial fibrillar aggregation at ultra-structure level. Decreased ZCC thickness in intact and lesion. Some micro-damage and tissue remodelling in the cartilage-bone junction of lesion samples.</td>
<td>Well defined tangential layer in both. Same amorphous texture in both. Bone spicules more prominent in GI intact whereas they are more blended into bone cement line in GII intact.</td>
<td>Absence of tangential layer and presence of chondrocyte cloning in GII. Intense fibrocity in GI and GII lesion samples with large scale in-phase crimping easily resolvable at the microscopic level in GII tissue. Bone spicules most prominent and numerous in GI lesion samples while in GII the cement line has advanced towards the uppermost tidemark and in some areas, is accompanied by micro damage at the junction.</td>
</tr>
</tbody>
</table>

*For images showing the structural changes described here, please refer to Chapter 2.
Of interest to us were those proteins that showed a significant differential level in all the lesion samples relative to their respective intact sites in GI patellae (Table 3.12) and GII patellae (Table 3.13). Among these, ACAN, Lysozyme C, milk isozyme (LYZ1), TGFBI and C4A (Complement Component 4A) showed consistent protein variations across both mildly and moderately degenerated patellae. iTRAQ analysis of the bovine cartilages also revealed the significant differential levels of the novel protein, PREDICTED: WAP four-disulfide core domain 18-like (WFDC18) in patellae with regions of mild (GI) cartilage degeneration. The intact cartilage regions in these patellae possessed higher level of the protein compared to the adjacent deteriorated regions.
Table 3.12 GI patellae group. Proteins found in all 7 patellae for this group and which showed significant difference in their lesion and corresponding adjacent intact cartilage samples (p<0.05) after multiple comparison test.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>GRADE I Lesion/Intact(^\dagger)</th>
<th>Sidak's multiple comparisons test(^\varepsilon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALB Serum albumin</td>
<td>Carrier proteins</td>
<td>3.30 **</td>
<td></td>
</tr>
<tr>
<td>TGFBI Transforming growth factor β induced gene product h3</td>
<td>Maintain cartilage and bone matrix</td>
<td>3.23 ***</td>
<td></td>
</tr>
<tr>
<td>THBS1 Thrombospondin-1</td>
<td>Cartilage development</td>
<td>1.99 **</td>
<td></td>
</tr>
<tr>
<td>HBB Hemoglobin subunit β</td>
<td>Oxygen carrier</td>
<td>1.97 *</td>
<td></td>
</tr>
<tr>
<td>IGL Immunoglobulin light chain, lambda gene cluster</td>
<td>Innate immune response</td>
<td>1.88 *</td>
<td></td>
</tr>
<tr>
<td>EDIL3 EGF-like repeat and discoidin I-like domain-containing protein 3</td>
<td>Cartilage development and bone formation</td>
<td>1.38 *</td>
<td></td>
</tr>
<tr>
<td>GSN Gelsolin a</td>
<td>Actin-modulating protein</td>
<td>1.38 **</td>
<td></td>
</tr>
<tr>
<td>COL6A3 Collagen, type VI, alpha 3</td>
<td>ECM organization</td>
<td>1.30 **</td>
<td></td>
</tr>
<tr>
<td>FMOD Fibromodulin</td>
<td>Fibril formation</td>
<td>1.24 *</td>
<td></td>
</tr>
<tr>
<td>COL6A2 Collagen, type VI, alpha 2</td>
<td>ECM organization</td>
<td>1.24 **</td>
<td></td>
</tr>
<tr>
<td>TNC Tenasin C</td>
<td>Angiogenesis</td>
<td>1.23 *</td>
<td></td>
</tr>
<tr>
<td>ANXA2 Annexin A2</td>
<td>Mineralization</td>
<td>1.21 **</td>
<td></td>
</tr>
<tr>
<td>COL6A1 Collagen, type VI, alpha 1</td>
<td>ECM organization</td>
<td>1.19 *</td>
<td></td>
</tr>
<tr>
<td>ACAN Isoform 2 of Aggrecan core protein</td>
<td>ECM structural protein</td>
<td>0.74 **</td>
<td></td>
</tr>
<tr>
<td>COMP Cartilage oligomeric matrix protein</td>
<td>ECM structural protein</td>
<td>0.60 ***</td>
<td></td>
</tr>
<tr>
<td>CILP Cartilage intermediate layer protein 1</td>
<td>ECM structural protein</td>
<td>0.59 *</td>
<td></td>
</tr>
<tr>
<td>WFDC18 PREDICTED: WAP four-disulfide core domain protein 18-like</td>
<td>Anti-protease activity</td>
<td>0.54 *</td>
<td></td>
</tr>
<tr>
<td>LYZ1 Lysozyme C, milk isozyme</td>
<td>Mineralization</td>
<td>0.45 *</td>
<td></td>
</tr>
<tr>
<td>C4A Complement Component 4A</td>
<td>Complement activation</td>
<td>0.43 *</td>
<td></td>
</tr>
</tbody>
</table>

\(^\dagger\)For the ease of understanding, log ratios were converted to linear space.
\(^\varepsilon\)For the post-test * denotes significant with p<0.05, ** p<0.01, *** p<0.001
Table 3.13 GII patellae group. Proteins found in all 5 patellae of this group and which showed significant difference in their lesion and corresponding adjacent intact cartilage samples (p<0.05) after multiple comparison test.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>GRADE II Lesion/Intact †</th>
<th>Sidak's multiple comparisons test €</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB Myoglobin</td>
<td>Muscle oxygen transport</td>
<td>2.68</td>
<td>*</td>
</tr>
<tr>
<td>TGFBI Transforming growth factor β</td>
<td>Ossification and bone formation</td>
<td>2.47</td>
<td>**</td>
</tr>
<tr>
<td>induced gene product h3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACAN Isoform 2 of Aggrecan core protein</td>
<td>ECM structural protein</td>
<td>0.80</td>
<td>*</td>
</tr>
<tr>
<td>C4A Complement component 4A</td>
<td>Complement activation</td>
<td>0.45</td>
<td>*</td>
</tr>
<tr>
<td>LYZ1 Lysozyme C, milk isozyme</td>
<td>Mineralization</td>
<td>0.34</td>
<td>*</td>
</tr>
</tbody>
</table>

†For the ease of understanding, log ratios were converted to linear space.
€For the post-test * denotes significant with p<0.05, ** p<0.01.
3.3.2.3 Targeted Proteomics

Fifteen proteins found interesting from the discovery part of the experiment were selected for validation using MRM assay. These were the proteins which had specific roles (as reported in previous literature) in cartilage degeneration and also showed consistent differences across various state of tissue degeneration. Proteins were monitored by 2-3 peaks respectively, matched to at least 2 peptides selected for targeting in six cartilage groups (n=3 per group) as shown in Table 3.14. Significant differences in relative abundance between the cartilage groups were analyzed for all proteins using a Repeated Measures Two-Way ANOVA followed by a Tukey's multiple comparisons test and Holm-Sidak’s multiple comparison test (GraphPad Prism 6). All the 15 selected proteins were found to differ significantly between the groups and ratios were generally in good accordance with the iTRAQ experiment.
### Table 3.14 MRM transition list. Peptide transitions for proteins optimized for the MRM experiment.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide</th>
<th>Precursor m/z</th>
<th>Precursor charge</th>
<th>Collision energy (V)</th>
<th>Collision energy spread (V)</th>
<th>Fragment ion ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACAN</td>
<td>AC[CAM]LQNSAIATPEQLQAAYEDGFHQ[CAM]DAGWLADQTVR</td>
<td>1037.7</td>
<td>4</td>
<td>64.2</td>
<td>10</td>
<td>y10, y7, y4</td>
</tr>
<tr>
<td></td>
<td>C[CAM]YAGWLADGSLR YPIVTPRPAC[CAM]GGDKPGVR</td>
<td>684.8 2</td>
<td>35.1</td>
<td>10</td>
<td>y10, y9, y7, y4</td>
<td>y11, y5, y4</td>
</tr>
<tr>
<td>ANG1</td>
<td>YGATEDSR SEFQITIC[CAM]K</td>
<td>449.7</td>
<td>2</td>
<td>24.8</td>
<td>10</td>
<td>y7, y6, y2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>563.3 2</td>
<td>29.8</td>
<td>10</td>
<td>y7, y5, y4, y2</td>
<td></td>
</tr>
<tr>
<td>C4A</td>
<td>LGQYTSPVAK EPFL5C[CAM]C[CAM]QFAESLR</td>
<td>532.3</td>
<td>2</td>
<td>28.4</td>
<td>10</td>
<td>y9, y7, y6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>581.9 3</td>
<td>37.8</td>
<td>10</td>
<td>b8, y6, y5, y4</td>
<td></td>
</tr>
<tr>
<td>CCL16</td>
<td>IPESVNPPNPC[CAM]C[CAM]LKL</td>
<td>812.9</td>
<td>2</td>
<td>40.8</td>
<td>10</td>
<td>b6, y8, y7, y6</td>
</tr>
<tr>
<td></td>
<td>LVVGYR</td>
<td>353.7</td>
<td>2</td>
<td>20.6</td>
<td>10</td>
<td>b2, y5, y4, y3</td>
</tr>
<tr>
<td>GSTP</td>
<td>ASC[CAM]LYGQLPK FQDGDLTLQYSNAIRL ALPQLKPFETLLSQNK</td>
<td>568.8</td>
<td>2</td>
<td>30</td>
<td>10</td>
<td>y8, y7, y6, y5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>618.7 3</td>
<td>39.9</td>
<td>10</td>
<td>b8, y8, y7, y6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>491.8 4</td>
<td>32.5</td>
<td>10</td>
<td>y5, y4, y2</td>
<td></td>
</tr>
<tr>
<td>LYZ1</td>
<td>WESNYNTR AWVAWR GVSANWVC[CAM]LAR</td>
<td>535.2</td>
<td>2</td>
<td>28.6</td>
<td>10</td>
<td>b2, y7, y6, y5</td>
</tr>
<tr>
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<td>42.4</td>
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<td>b5, y5, y4, y3</td>
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<tr>
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<td></td>
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<td>Peptide</td>
<td>Precursor m/z</td>
<td>Precursor charge</td>
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<td>Collision energy spread (V)</td>
<td>Fragment ion ID</td>
</tr>
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<td>3</td>
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<td>COL6A3</td>
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<td>32.5</td>
<td>10</td>
<td>b2, y8, y7, y5</td>
</tr>
<tr>
<td></td>
<td>VVESLDVGPDPR</td>
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<td>2</td>
<td>31.1</td>
<td>10</td>
<td>y9, y8, y5, y4</td>
</tr>
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<td>THBS1</td>
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<tr>
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<td>VFGGTTPEDILR</td>
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</tr>
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<td></td>
<td>SITLFVQEDR</td>
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<td>31.6</td>
<td>10</td>
<td>y8, y6, y5, y4</td>
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<td>HTRA1</td>
<td>SAPGAAGCPER</td>
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<td></td>
<td>YNFIAADVVEK</td>
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<td>2</td>
<td>31.4</td>
<td>10</td>
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The post-test showed significant differences in 11 proteins (p<0.05) namely, ACAN, GSTP, LYZ1, VIM, WFDC18-like, LMNA, GSN, TNC, COL6A1, COL6A1 and THBS1 and a further 4 proteins (HTRA1, ANG1, C4A and CCL16) showed near-significant difference (p<0.1) (Figure 3.9 and 3.10). The fact that not all selected proteins showed significant difference between groups in this MRM validation study while they presumably showed a promising behavior in the iTRAQ study may be due to the smaller sample size (n=3). It may therefore be advisable to consider not only p values but the overall trends in terms of directional change when assessing the value of the validation, by MRM experiment.
Figure 3.9 Ratios of LN value of proteins found significant (p<0.05) in iTRAQ and MRM experiment. For each protein the healthy sample (G0) is scaled to 0 in log space and represents "no change", and others are relative to that.
Figure 3.10 Ratios of LN value of proteins found significant in non-healthy cartilages in iTRAQ and MRM experiment. For each protein the GI intact is scaled to 0 in log space and represents “no change”, and other are relative to that.
3.4 Discussion

The objective of this work was to identify and validate the proteins which have differential abundance in structurally altered adjacent cartilage tissues. A set of proteins were identified, whose differential presence during early cartilage degeneration could help to provide more information about the elusive "pre-OA" state and can be potentially used as biomarkers for disease initiation or progression.

Mass spectrometric analysis helps to identify the precise biochemical composition of tissues and fluids and is a powerful technique for biomarker discovery. A MS study combined with tissue structural analysis of OA cartilage help us to comprehend the molecular mechanisms that are at play in an osteoarthritic cartilage and also allow for the correlation of the proteome profile of a specific tissue state to its microstructure, expanding our understanding of the OA pathogenesis. Identifying the proteins which are responsible for pathophysiology of OA, can help us to develop appropriate biomarkers for early disease diagnosis and also identify potential therapeutic targets for disease-modifying osteoarthritic drugs (DMOADs).

The cartilage samples from the patellae of cows were classified on the basis of surface disruption and softening according to Outerbridge’s [281] grading (Table 2.1). From each non-healthy patella, two separate set of cartilage samples were taken– one where the surface appeared completely intact and the other from localised region of degeneration, by visual inspection (confirmed with histological analysis) and from the healthy patellae two corresponding regions were used as controls. This was done to understand the molecular changes which occur at two different sites in a cartilage. The visual interpretation of the samples through DIC imaging confirmed there are considerable changes between the intact and lesion sites of the non-healthy patellae (Chapter 2). Through mass spectrometric analysis, the changes in the proteome profile between these samples were assessed in this chapter.

A gel-based LC-MS/MS was performed during the preliminary study. Though the method proved useful in giving preliminary results suggesting that the intact and lesion proteome varied in accordance with the structural analysis, further investigations were done using iTRAQ labelling for quantitative measurements.
Most of the proteins identified in this work were multifunctional or are known to regulate/involve in more than one biological pathway. The biological processes and molecular functions of the differential proteins give an insight to their potential involvement in the cartilage.

The focus of this discussion will be the correlation of the microstructural variations observed in the previous chapter with the protein profile changes observed here. Proteins found altered significantly between different tissue groups and which are known to be involved in structural variations in cartilage and bone are discussed below.

**3.4.1 Comparisons of Different Non-healthy Tissue States with respect to Healthy**

The tissue status of cartilages was confirmed with histological analysis and it was found that the GI and GII intact were similar to healthy tissues in Mankin scores (0-1) (Table 3.5). The GII lesion samples scored slightly higher Mankin score than GI lesion samples and the corresponding level of degeneration was analysed by DIC optical imaging in the previous chapter.

**3.4.1.1 Cartilage Surface Disruption, Collagen Fibrillar De-structuring and Re-aggregation**

Cartilage is made up of ECM (a network of collagens, proteoglycans like aggrecan and other molecules) and chondrocytes. The interaction between the collagen fibrils and the proteoglycan regulate the fluid phase in the cartilage and thus provides stiffness and visco-elastic properties, characteristic to the cartilage. The early OA changes in cartilage (because of either external or internal impetus) is marked by the surface layer disruption (Figure 2.5) and degradation of collagen fibrils and the inter linked proteoglycans [18]. These changes catapult the chondrocytes from their normal state to a ‘fiercely active state’. In lesion samples, the chondrocytes were seen in various stages of this activation- namely aggregation or clustering, cloning and proliferation (Figure 2.6). The so called ‘active’ form of chondrocytes initiate production of both anabolic and cataboic factors, which inadvertently cause more harm to the affected tissue [23].
Nine separate collagen proteins which differed between healthy controls and the non-healthy groups were detected. COL2A1, COL6A1, COL6A2, COL6A3, COL9A1, COL9A2, COL9A3, COL11A1 and COL12A1 were found to be significantly lowered in both mild (GI) and moderately (GII) degenerated tissues when compared to the controls (Figure 3.11).

Collagen II is the most abundant collagen in cartilage and is found in association with both collagen IX and collagen XI in its fibrillar structure. It provides the tensile strength characteristic of cartilage tissue. Collagen type XII interacts with type I collagen-containing fibrils. COL6A1, A2 and A3 are all constituents of collagen type VI which contributes to chondrocyte microenvironment [45, 400].

Interestingly, in the current study, lesion region of the non-healthy tissues showed more levels of collagen than their intact counterparts. One of the probable explanation for this could be that the chondrocytes at damaged regions are driven to produce more collagen molecules to combat the general loss of the collagens. Hence, this could be a reparative mechanism at the site of degeneration. An earlier investigation by Squire et al. compared intact (remote) and lesion samples from degnerate human condylar cartilage, found an increased synthesis of collagen in the lesion site samples compared to the remote cartilages, suggesting a higher turnover and synthesis rate in the degenerated regions [401]. The same study also assessed the collagen content in these two regions and contrary to present finding, saw a decreased collagen type II at the lesion site compared to the remote samples. A probable reason for this could be the grade of degenerated samples used in both the work. While the intact and lesion samples in the present study had a Mankin score of 0-1 and 1-5 respectively, Squire et al. reported a score of 0-4 for their remote region sample and 2-12 for lesion region samples. It is therefore inferred that, the collagen levels in the present samples indicate the increased levels occurring during early degeneration phase while in Squires et al. study, it is the depiction of collagen levels at a more advanced stage of the degeneration. Looking at the present results, it could be hypothesised that the collagen levels tend to be upregulated at the regions where cartilage surface layer has been disrupted. It is possible that the physical loss/damage of cartilage zone could trigger
the inactive chondrocytes to produce more of this biomolecule at relatively early stages.

Cartilage oligomeric matrix protein (COMP), cartilage intermediate layer protein (CILP) 1 and 2 and the aggrecan, are all typical structural components of cartilage and were found to have distinctly elevated levels in all non-healthy tissues (Figure 3.12).

Interestingly, in comparison to the healthy cartilages, intact regions of the non-healthy patellae possessed comparatively higher levels of aggrecan than the lesion site cartilages. This increased levels of aggrecan can be explained as a trend to compensate the loss of aggrecan from the upper layers as previously observed through the prominent production of aggrecan molecules at the middle and deep layer of human cartilage [402].

Other proteins known to regulate the collagen fibril assembly in cartilage were seen to be differentially present in the non-healthy tissues. The ones which were found to be significant in this work were- a higher level of chondroadherin (CHAD), COMP, decorin (DCN), fibromodulin (FMOD), prolargin (PRELP) in non-healthy cartilage tissues and a comparatively lower levels of thrombospondin 1 and gelsolin in the non-healthy OA cartilages compared to healthy tissues (Figure 3.12 and 3.13). COMP acts as a catalyst in linking the collagen molecules during fibril formation [403]. Previous studies also have reported increased levels of COMP in the human serum during early OA [340]. This increased COMP levels is understood to be an ineffective repair process to compensate the structural damage of the cartilage by aiding the catalysis during collagen fibril assembling.
Figure 3.11 The decreased level of collagens found in non-healthy cartilages (both intact and lesion) compared to healthy cartilages. The GI intact site have most prominent loss of collagens. For the post-test * denotes significant with p<0.05, ** p<0.01, *** p<0.001.
Figure 3.12 Scaled natural log (LN) ratios of proteins found to have potential role in cartilage surface disruption. For each protein, the healthy cartilage is scaled to 0 in log space and represents “no change”, and others are relative to that. For the post-test * denotes significant with $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. 
The decrease in collagen level and upregulation of molecules like COMP give further evidence of the destabilization of fibril orientation [148] observed at the lesion site cartilages in Figure 2.7.

CILP, a non-collagenous ECM protein associated with cartilage scaffolding [404], is also known to be increased in early osteoarthritic cartilage [405]. CILP1, had a five-fold increased level in the GI and GII intact samples compared to healthy tissues (Table 3.6 and 3.8). Also, its level was higher in lesion sites compared to healthy tissues, hinting distinct alteration occurring in the OA cartilage. If and how this increased level of CILP can influence the cartilage degeneration, needs to be better understood.

Fibronectin is a multifunctional glycoprotein which maintains ECM network as part of cartilage homeostasis. A significantly higher level of fibronectin was seen in non-healthy samples (Figure 3.13), which is consistent with the finding that the fibronectin synthesis is increased in OA state even before the PGs are lost from the cartilage surface, as part of an ineffective repair mechanism [139]. The newly synthesised fibronectin accumulating in the degenerate cartilage may or may not possess proteolysis initiation role. But whether the identified fibronectin molecules in the present study are the native cartilage components or the newly synthesised macromolecules produced by chondrocytes is yet to be confirmed. Future studies with growing the present cartilage sample groups in labelled media can provide useful information with respect to this and will help to discriminate between the fragmented inherent molecules and the newly synthesised ones.
Figure 3.13 Scaled natural log (LN) ratios of proteins found to have potential role in collagen fibril de-structuring. For each protein, ratio of the healthy cartilage is scaled to 0 in log space and represents “no change”, and others are relative to it. For the post-test * denotes significance with p<0.05, ** p<0.01, *** p<0.001.
Procollagen C-endopeptidase enhancer (PCOLCE) is involved in collagen turnover. This protein binds to and cleave the type I procollagen and enhances procollagen C-proteinase activity [406]. The present study found its level to be lowered in the non-healthy tissues (intact and lesion sites) when compared to healthy (Figure 3.13). But the effect is more prominent to the GI and GII intact cartilages than their lesion counterparts. This trend here may be the result of a greater collagen turnover at the lesion site and also agrees with collagen level detected in the samples.

Peptidyl-prolyl cis-trans isomerase A (PPIA), influences the rate of collagen folding [407]. A significantly lower level of this protein was found in the present study in cartilage groups of non-healthy patellae, and more so, in samples from lesion region than in intact regions (Figure 3.14). This difference in the abundance level probably have direct implication in disease progression.

![Figure 3.14](image)

**Figure 3.14** Scaled natural log (LN) ratios of PPIA found in the experiment. The healthy cartilage is scaled to 0 in log space and represents ‘no change’, and others are relative to that. For the post-test * denotes significance with p<0.05, ** p<0.01, *** p<0.001.

Overall, the difference in the abundance level of these proteins suggest they are directly or indirectly involved in structural changes observed during early cartilage degeneration. The protein alterations may either be a part of reparative mechanism or the result of tissue degeneration.
3.4.1.2 Mineralization and Bone Formation

Of much interest are the proteins TGFβI, Annexin A1 [408], Lysozyme (LYZ1) [409, 410], EDIL3 [411], Creatine Kinase M-type (CKM) [412] and osteoglycin/mimecan (OGN) [379] (Figure 3.15), which are known to be involved in cartilage mineralization and bone formation associated with osteoarthritis.

Through proteomic analysis of cartilage tissues it was found that TGFβI was decreased by more than half-fold in the GI and GII intact cartilage tissues whereas their corresponding lesion sites from the same patellae, had TGF-βI levels similar to the healthy tissues (Figure 3.15). TGFβI protein is induced by TGF-β and it facilitate cell interactions by binding to various collagens and SLRPs [413]. Experiments conducted on TGFBI knocked out mice revealed that TGFBI helps in maintaining bone and cartilage ECM and also prevents MMPs from attacking the collagen frameworks [414]. Therefore, the lower level of TGFβI at the GI and GII intact site could also be associated with the lower level of collagens found at these sites. TGFBI is also involved in endochondral ossification [415] and is known to be downregulated during osteoblast differentiation[416]. Therefore, the lower levels seen here in the intact regions of non-healthy patellae may be linked to the tissue’s attempt at preventing endochondral bone formation at these sites.

EDIL-3 was found to be lower in non-healthy tissues, especially at the intact regions. EDIL3 is reported to be involved in cartilage development and bone formation [411]. Annexin 1 which is reported to be scarce in normal tissues but upregulated in OA [408], was found to be higher in the non-healthy cartilages (Figure 3.15).

It is clear that at these early stages of cartilage degeneration, the tissue is still attempting to contain the degradative processes and undergo cartilage remodelling. The lower level of TGFBI and EDIL3 at the intact site of non-healthy tissue suggest that these tissue sites are struggling to stop the cartilage degeneration. On the other hand, the increased levels of Annexin A1 could be a step towards the cartilage degeneration.
Figure 3.15 Scaled ratios of natural log (ln) for proteins found to have potential role in mineralization and bone formation. For each protein, its level in healthy cartilage is scaled to 0 in log space and represents “no change”, and others it’s relative values. For the post-test * denotes significance with p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Creatine kinase (CKM) is known to be involved in the energy metabolism in chondrocyte. The inhibition in creatine phosphate metabolism in cartilage is known to affect the anabolic activities in the cartilage. Creatine kinase activity is also associated with endochondral bone formation [412]. A lower level of CKM was detected in non-healthy tissues and significantly so for both GI and GII intact samples
This suggests less efficient/demand for energy conversion (ATP ↔ Creatine phosphate) in intact regions close to cartilage lesion sites.

Another significant differential level was seen in LYZ1 protein; the intact regions of GI and GII cartilages showed 11-13 fold increase (Table 3.6 and 3.8), whereas the corresponding lesion sites showed 5-fold increase compared to healthy cow cartilages (Table 3.7 and 3.9). A significant increase in lysozyme was seen in the non-healthy tissues, both in iTRAQ and MRM experiment (Figure 3.16).

Proteoglycan aggregates are known to prevent calcium phosphate from precipitating in the cartilage [417]. Lysozymes, produced at higher concentrations by chondrocytes [418] during the early stages of OA, cleave the hyaluronan backbone of the proteoglycan aggregates, releasing proteoglycan monomers that no longer hamper the precipitation process [417]. It is possible that the higher level of lysozyme seen at the intact cartilage region is the tissue’s way of getting ready for the calcification processes and is analogous to the increased ZCC thickness seen at the intact regions of non-healthy patellae (Figure 2.8).

![LYZ1](image)

**Figure 3.16** Scaled natural log (LN) ratios of lysozyme in MRM and iTRAQ experiment. The healthy cartilage protein level is scaled to 0 in log space and represents “no change”, and others are relative to that. For the post-test * denotes significance with p<0.05, *** p<0.001, **** p<0.0001.
Lamins (LMN) are components of the nuclear lamina, a fibrous layer on the nucleoplasmic side of the inner nuclear membrane, which is thought to provide a framework for the nuclear envelope and may also interact with chromatin [419]. Lamin A and C are present in equal amounts in the lamina of mammals. LMN A/C is required for normal bone turnover and bone metabolism and a decreased expression of LMN A/C is associated with bone loss seen in aging [420].

It was recently shown that LMNA is upregulated in OA tissues and perturbation of nuclear LMNA causes cell death in chondrocytes [421]. Contrary to this finding, substantially lowered levels of LMNA was detected in all four groups from the degenerated tissues (non-healthy) compared to healthy cartilages and this was further validated with MRM assay (Figure 3.17). The disagreement in result could be due to the source of OA tissue used in both the studies, while the present study analysed relatively early degeneration in the bovine tissues, the samples in Attur et al. study were from well advanced human OA. This is mere speculation and needs to be tested with more advanced stages of bovine cartilage tissues.

**Figure 3.17** Scaled natural log (LN) ratios of lamin A in MRM and iTRAQ experiment. The healthy cartilage protein level is scaled to 0 in log space and represent “no change”, and other are relative to that. For the post-test * denotes significance with p<0.05, *** p<0.001, **** p<0.0001.
The variations in the above mentioned molecules, mirror the advancing bone cement line and spicule formation seen in Figure 2.8 which clearly depicts the microstructural changes in the cartilage and its underlying bone. It is interesting to note that the bone spicules at the lesion sites were advancing more into the tidemark than the bone spicules from intact cartilage region and the abundance level of many bone-related proteins found in this study support this observation.

3.4.1.3 Angiogenesis

Angiogenesis is initiated once the cartilage starts to mineralize and prepares for subchondral bone remodelling. The variable levels of mimecan [194], angiogenin (ANG) [379] and tenascin [422] obtained in the proteome profile (Figure 3.18) indicate that the cartilage is in the process of neovascularisation, and the data is in sync with the DIC images in Figure 2.9 where the bone spicule formed clearly bear vascular canals and bone cuffs with osteocyte lacunae.

Angiogenin levels were found to be 3-4 fold raised in non-healthy tissues (Table 3.6-3.9). They favor the degradation processes in the tissues and is known to be involved in new blood vessel formation in tissues [423]. SERPINF1 (PEDF), an antiangiogenic factor, is known to provide defense against the invasive blood vessels which attack the cartilage as OA progress [306]. SERPINF1 was found to be lowered in the non-healthy cartilages. Another anti-angiogenic factor detected in this work was thrombospondin 1 (THSB1) and it was also found to be lowered in both iTRAQ and MRM experiments.

Also observed was an overall lower level of tenascin in the non-healthy samples in iTRAQ as well as MRM experiments. Increased expression of tenascin in arthritic cartilage [347] is often linked with pro-angiogenic activity [424], cartilage degradation and inflammation [348]. The decreased amount of tenascin C (TNC) seen in the non-healthy samples, could be an intial change in protein level which increases as the severity of the degeneration escalates. This needs confirmation by analysing advanced OA tissues before reaching a conclusion.
Figure 3.18 Scaled ratios of natural log (ln) for proteins found to have potential role in neovascularization. For each protein, its level in healthy cartilage is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test *** denotes significance with $p<0.001$, **** $p<0.0001$. 
3.4.1.4 Proteases and Protease Inhibitors

HtrA1 is a secretory protease which binds to TGF-β and inhibits its anabolic function [425]. It is also known to be upregulated in OA [426] and degrades proteins aggrecan [427], decorin, fibromodulin and collagen II, which maintain cartilage structure and HTRA1 also involve in endochondral ossification [428].

![HTRA1](image)

**Figure 3.19** Scaled natural log (LN) ratios of HtrA1 protease in iTRAQ and MRM experiments. The healthy cartilage protein level is scaled to 0 in log space and represents “no change”, and other are relative to that.

HtrA1 play central role in the joint pathology during several musculo-skeletal disorders [429]. HtrA1 was shown to participate in cartilage degeneration by degrading fibronectin and through fibronectin fragments, indirectly upregulate the MMPs production [430]. Immunohistochemical data showed that this protein is localised to the hypertrophic chondrocytes in deep layer zone [428] and was also proposed to destabilize the PCM surrounding the chondrocytes and thus exposing the cells to further catabolic activities occurring the cartilage [155, 429].

HtrA1 is also associated with the bone remodelling because of their presence in regions of endochondral bone formation and ossification in mice [428] and in areas of new bone formation [431].
An increase in level of HtrA1 in non-healthy cartilages (Figure 3.19) was detected in the present study. The increased protease levels correlate with the structural degeneration in non-healthy patellae (Figure 2.2).

Also identified in this work was a novel protein- PREDICTED: WAP four-disulfide core domain protein 18-like. The non-healthy cartilage tissues showed a significantly raised level of WFDC18; with a more conspicuous amount (7-9 fold increase in linear space) in the intact tissue sites (Figure 3.20).

This protein is poorly characterized at protein level to know its particular biological function in cartilage. But the presence of whey four disulphide core (WFDC) domains indicate that they may possess anti-protease and anti inflammatory activity [432]. WAP four disulphide core (WFDC) domain, a structural motif comprising of four conserved disulphide bridges, was first discovered in whey acidic proteins (WAP) in rodents [433]. The WFDC domain contains around 40-50 amino-acids which have four characteristic intramolecular disulphide bonds, formed by eight conserved cysteine residues [434]. Except for a few proteins, this family of proteins are poorly characterized other than at the gene level. The most studied proteins from WFDC family are secretory leukocyte protease inhibitor (SLPI) and elafin. They both act as serine protease inhibitors [435, 436], targeting endogenous proteases. WFDC proteins also seem to have a role in innate immunity and inflammation [437].

The presence of N-terminal signal peptide suggests that all proteins which contain WFDC domains are secreted from cell into the extracellular space [438]. WAP proteins also have the ability to control the inflammation through anti-protease activity [439] and by regulating the pro-inflammatory factors [440]. It have been shown that spacing of cysteines in WFDC protein is critical for its anti-proteinase activity [432].
The presence of WFDC18-like protein in this study was validated through the MRM experiment. Peptides, GILGACVEMCSGDDSCPRT and QRPGFCPEVPR were monitored in the cartilage samples (Figure 3.20). This protein possess a good potential as the biomarker for early OA and need to be further investigated to fully understand its implication in OA tissues.

**Figure 3.20** (A) Scaled natural log (LN) ratios of WFDC18-like protein in MRM and iTRAQ experiment. The healthy cartilage protein level is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significant with p<0.05, *** p<0.001, **** p<0.0001. (B) MRM traces for GILGACVEMCSGDDSCPRT and (C) QRPGFCPEVPR (peptides of WFDC18-like protein) and their transitions are shown.
3.4.2 Site-specific Changes in Intact and Lesion Cartilage Regions

It is important to understand the molecular changes that take place during the progression of cartilage deterioration. The presence of features such as microfracture, surface cleft or fissure and vascularized channel in the osteochondral region could, in various ways, contribute to elevated levels of communication between the cells via interaction with various biological molecules and signalling pathways [146]. Hence co-relating microstructural changes in cartilage to its protein profile could provide clues to the early degeneration state.

The intact site of non-healthy patellae was found to be very interesting in this work. Although the structural changes were stronger at the later stage of the degeneration i.e., between intact and lesion sites of GII patellae (Figure 3.21), there were less proteins that showed significant difference between these sites, compared to the GI group where the number of protein differences were greater whereas the structural changes were more subtle (Table 3.11). This finding may be indicative of the tissue attempting to overcome the early microstructural changes, and contain the degeneration, at the most earliest stages. Alternatively, the protein levels may be less marked in the advanced degenerated state due to higher protein turnover rates in these damaged areas. Either way, the importance of capturing the pre-to-early OA state is believe to be highlighted by this finding.
Figure 3.21 Histological analysis of osteochondral sections of non-healthy cartilages stained with safranin O-fast green. (A) intact site from GI patella (Mankin score 0) showing smooth surface morphology, normal chondrocytes and undisrupted tidemarks; (B) lesion site from GI patella (Mankin score 4) with irregular surface, cleft down to mid matrix zone and decreased staining of tangential zone and; (C) intact site from GII patella (Mankin score 1); (D) lesion site from GII patella (Mankin score 4) with decreased matrix staining together with surface disruptions extending into the mid-zone cartilage. (Scale bar: 500 μm)
In view of relatively small variation in the protein profiles of the control tissue pairings (Figure 3.8) compared to the array of proteins that exhibited divergent levels in lesion-site pairings in the non-healthy patellae (Table 3.11), the discussion will primarily focus on those proteins which showed differences in earliest cartilage degeneration stage i.e. all GI lesion-bearing patellae. In addition, the significant proteins found in this piece of work have been categorized based on its role in cartilage and bone structural modification (Tables 3.12 and 3.13). The implication of these listed proteins is thus discussed in the next section, in relation to the five microstructural differences observed between the lesion and intact regions, namely cartilage matrix de-structuring, proteoglycan loss, hyper-cellularity, new bone formation and angiogenesis.

3.4.2.1 Cartilage Surface Disruption, Collagen Fibrillar De-structuring and Re-aggregation

Early degenerative changes in cartilage typically include fibrillar disruption of the tangential zone (Figure 2.5), de-structuring of the matrix (Figure 2.7), and proteoglycans loss (Figure 3.21) [48, 276, 441, 442]. In the present study, degradation of the tangential layer, along with the more subtle de-structuring of cartilage general matrix, may be correlated with specific proteins which interact directly or indirectly with collagens. These collagen metabolic- and structure-related proteins were found to be different between the two structurally different regions.

A significant regional difference in the levels of COMP and CILP-1 across mildly degenerated patellae (GI) was found in the current study. These two proteins were found to be significantly lower at the lesion sites compared to intact regions. The lower levels of COMP and CILP at lesion site (Figure 3.22) could be associated as one of the many factors responsible for the reduced tangential zone seen in the corresponding degenerated site (Figure 2.5). Further, these two protein levels between intact and lesion sites were not significantly different in the GII patellae even though their corresponding cartilage surface structure (Figure 2.4 D and E) were distinctly different, with a well preserved tangential layer at the intact site and a mostly absent
one at the corresponding lesion site. Importantly however was the increased number of large chondrocyte clusters in the GII lesion site tissue (Figure 2.5 F and 2.6). These large cell clusters could possibly be responsible for the relative increase in COMP and CILP-1 in GII samples. The increase in these proteins may be interpreted as being a reparative response in ‘rebuilding’ a failing matrix.

Figure 3.22 Scaled natural log (LN) ratios of proteins found to have potential role in cartilage surface disruption and collagen fibrillar de-structuring. For each protein, the GI intact is scaled to 0 in log space and represents “no change”, and others are relative to that. For the post-test * denotes significance with p<0.05, ** p<0.01.

It is suspected that these early stages of degeneration in GI and GII groups are still considerably not advanced to a higher level of degeneration. However this is hypothesis requires testing with more degenerated tissues possibly with GIII and GIV lesions [281] where it is speculated that the site specific differences in COMP and CILP-1 will remain insignificant.
The present study also found increased levels of lumican and fibromodulin in the lesion sites, which belong to the small leucine-rich proteoglycan (SLRP) family. The level of lumican in GII intact site was significantly increased (Figure 3.22). Melrose et al. [443], showed increased level of SLRP fragments in degenerated human cartilage compared with age-matched controls. Lumican and fibromodulin are known to be involved in fibrillogenesis and compete for the same binding site on the collagen fibril and they also influence collagen cross-linking [444]. Cartilage matrix fibril aggregation in the radial direction as seen in the SEM image of the lesion site (Figure 2.10) could be due to the botched interconnectivity between the fibrils [276]. It is possible that these SLRPs are involved in the increased re-aggregation (i.e. following de-structuring) of the fibrils into larger optically resolved bundles (Figure 2.7 D and F). Importantly, SLRPs coat the fibril surface and have been shown to impede the fibril’s access to proteinases such as MMP-1 and MMP-13 [445]. Further, Monfort et al. have shown that SLRPs are degraded by MMP-13 and suggest that the protease-driven cleavage of the SLRP may be a critical initial event in cartilage degeneration [446] preceding collagen cleavage. The increased levels of FMOD and LUM in the lesion site cartilage sample are hypothesized to be a measure to prevent further attack on fibrils by proteases. Alternatively, these could be the product of degeneration itself, which were easily extracted and hence readily detected in the lesion sample.
3.4.2.2 Hyper-cellularity in the Chondrons

The large cluster of cells in the chondron of lesion samples (Figure 2.6) are associated with chondron pericellular remodeling in a degenerating matrix [291]. Type VI collagen is largely involved in the formation of the chondron pericellular matrix (PCM) [291, 447] and by its association with hyaluronan, decorin and fibronectin, it aids in anchoring the chondrocyte to the PCM. Molecules such as cytokines and various growth factors which come in contact with chondrocytes, pass through the PCM which acts as a screening point, modulating the transiting molecules [448].

Mechanically, the PCM acts as a transition between the stiffer matrix outside the chondron and the considerably more compliant chondrocyte, which the PCM effectively cushions [447]. The increased levels of type VI collagen measured at the lesion sites (significant increase seen only in GI patellae) (Figure 3.23) may reflect an early attempt to stiffen the PCM matrix, in order to protect the chondrocytes rendered increasingly vulnerable as a result of a weakened extracellular structural (inter-territorial) matrix, arising from fibril-network de-structuring. This hypothesis is supported by earlier studies showing that the chondron remodeling and cluster formation is sensitive to the stiffness gradients across the peri-cellular, territorial and inter-territorial matrices [291]. Others too have suggested that type VI collagen may affect the mechano-transduction of signals from the ECM into the chondrocytes. In human lesion samples taken from advanced OA cartilage, the proliferating chondrocyte clusters and their surrounding extracellular matrix demonstrated strong staining for type VI collagen mRNA [48]. This supports the hypothesis that the chondrocytes are attempting to adapt to the above structural changes by sequestering the type VI collagen [291].
Figure 3.23 Scaled ratios of natural log (ln) for proteins known to have potential role in chondron hypertrophy in iTRAQ experiment. For the post-test * denotes significant with p<0.05, ** p<0.01, *** p<0.001.

Vimentin, an important cytoskeletal element in chondrocytes was found to have decreased levels at the lesion site of GI and GII patellae compared to their intact regions (Figure 3.24). This finding was validated with MRM and conforms with the earlier studies where reduced expression of vimentin was seen in chondrocytes from rat osteoarthritic model [449] and increased vimentin degradation products were seen in cells from human lesion samples [223].

Figure 3.24 Ratios of LN value of vimentin found significant in iTRAQ and MRM experiment. Here, the GI intact is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significant with p<0.05, ** p<0.01.
3.4.2.3 Proteoglycan Loss

While the smaller proteoglycans (<50kDa) lumican and fibromodulin showed an increase in abundance at the lesion tissue sites compared to the adjacent intact site cartilages (Figure 3.22), there was a significant reduction in the levels of aggrecan (large proteoglycan aggregates >2500kDa) (Figure 3.21 B and D, Figure 3.25) which was confirmed by MRM analysis. This reduction in aggrecan level at the lesion sites may be linked to catabolic mechanisms occurring in cartilage [450], during which, proteases and other degradative molecules attack the aggrecan core protein as well as its hyaluronan (HA) backbone. Any recovery from this initial damage phase, is hampered by the HA-bound aggrecan fragments which prevent the newly synthesized aggrecan molecule from binding at the impaired site [442].

![ACAN Graph](image)

**Figure 3.25** Ratios of LN value of aggrecan found significant in iTRAQ and MRM experiment. Here, the GI intact is scaled to 0 in log space and represents “no change”, and others are relative to that. For the post-test * denotes significance with p<0.05, ** p<0.01.
In non-healthy patellae, the lysozyme levels in the intact cartilage regions were more than double those in the corresponding lesion sites (Figure 3.26) and this finding is validated in MRM experiment.

**Figure 3.26** Ratios of LN value of lysozyme found significant in iTRAQ and MRM experiment. Here, the GI intact is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significance with p<0.05.

Lysozyme, synthesised in cartilage are reported to degrade the proteoglycan aggregates which inhibit calcium phosphate precipitation [409]. It is possible that the increased thickness of ZCC at the intact sites of the GI patellae (Figure 2.8 C) is a consequence of the enhanced mineralization taking place due to the higher lysozyme levels at these regions. It is therefore hypothesized that the significant increase in the lysozyme level in the intact site may be a precursor to the eventual decline in the aggrecan level in the lesion site. This would also reinforce earlier suggestions that the structural transition from the intact to lesion regions of cartilage in a joint is an analogue for the pathway from initiation to progressive degeneration of the joint tissues in OA [90, 266].
3.4.2.4 Mineralization and New Bone Formation

The new primary bone formation that Thambyah and Broom [90] reported in their earlier structural study of the early or pre-OA joint involved the upward growth of distinct bone spicules containing a central vascular channel from the cement line, and traversing the increased width of the ZCC between the articular cartilage and the underlying bone. They found these spicules to be similar to the cutting cones seen in the fracture healing of bone [292]. These spicules were clearly visible in micro images of the tissue in the present study (Figure 2.9).

The present study found that the lesion sites in the non-healthy patella showed varying level of proteins often associated with mineralization and bone formation. TGFBI, EGF-like repeat discoidin I-like domain-containing protein 3 (EDIL3) and Annexin A2 (ANXA2) all increased significantly at the GI patellae lesion sites (Figure 3.27) whereas LYZ1 decreased (Figure 3.26).

![Figure 3.27](image)

**Figure 3.27** Scaled ratios of natural log (ln) for proteins found to have potential role in mineralization and bone formation in iTRAQ experiment. For each protein the GI intact is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significance with \( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \).
EDIL3 showed significant difference between the GI and GII intact regions and also between GI and GII lesion sites (Figure 3.27). Importantly the GI lesion site contained the most abundant level of EDIL3. This abundance is accompanied by the most amount of bone spicule expression in the ZCC (Figure 2.8 D) where in some cases, the spicules have crossed the uppermost tidemark. This association between EDIL3 level and bone spicule formation provide an intriguing direction for future investigations.

Of particular interest too, is the significant increased level of TGFBI in the GI and GII lesion sites compared to their respective intact sites (Figure 3.27). It is possible that this trend may be due to TGF-βI being (a) expressed more at the lesion site (b) produced less at the intact regions or (c) degraded more at the disease initiation stage.

Though the proteins involved in mineralization and bone formation were analysed, a definite pattern of protein level was not found in the sample. Therefore, it is suggested that the unpredictable levels of proteins in the tissue can contribute to the OA pathogenesis. Whether this is due to causation or manifestation of the disease needs to be investigated in detail.

3.4.2.5 Angiogenesis

The avascular cartilage is sustained metabolically by the subchondral bone and the synovium. Normal cartilage actively maintains its avascularity by balancing pro- and anti-angiogenic factors [451]. However, it was found that THSB1, an anti-angiogenic factor [424], was significantly increased at the lesion site of GI patellae (Figure 3.28). THBS1 binds to angiogenic factor vascular endothelial growth factor (VEGF), preventing their binding to receptors [452] and thus inhibits vascularization of tissues.
Further, pro-angiogenic CCL16 (Chemokine (C-C motif) ligand 16) is a cytokine that promotes endothelial cell migration required for vascular formation. It is also known to induce the release of other pro-angiogenic factors [453] and was seen to be elevated in the intact cartilage regions and confirmed by MRM assay (Figure 3.29).

Figure 3.28 Scaled ratios of natural log (ln) for proteins associated with angiogenesis found significant in iTRAQ experiment. For each protein the GI intact is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significant with p<0.05, ** p<0.01.

Figure 3.29 Ratios of LN value of CCL16 in iTRAQ and MRM experiment. The GI intact is scaled to 0 in log space and represents “no change”, and other are relative to that.
Finally, angiogenin (ANG1), another pro-angiogenic molecule associated with neovascularization [423], was found to be increased at the intact site both in iTRAQ and MRM experiments (Figure 3.30). Although at first sight, these changes in angiogenic factors seem confounding, there was also a significant decrease in pro-angiogenic tenasin (TNC) in the intact site (Figure 3.28), a factor known to promote vascularisation [424].

This rise and fall of pro- and anti-angiogenic factors across two regions in the early OA patella may indicate a complex interplay of factors attempting to sustain some level of homeostasis in a failing tissue.

**Figure 3.30** Ratios of LN value of angiogenin in iTRAQ and MRM experiment. The GI intact ratio is scaled to 0 in log space and represents “no change”, and others are relative to that.
3.4.2.6 Cartilage Response to Catabolic Influences

Even though OA is the result of chemical imbalance of anabolic and catabolic factors in cartilage, investigations have reported that the deteriorating cartilage attempts to control the situation by producing anti-catabolic factors.

In osteoarthritic cartilage, an increased hypoxic condition is observed [179] which in turn induces angiogenesis [180], illustrating the stress-related homeostatic nature of this process. During joint disorder, the lowered oxygen level in cartilage alters the level of reactive oxygen species or ROS [181]. ROS have the normal physiological function of acting as signalling molecules in chondrocytes but their higher level in cartilage have detrimental effects leading to pathological results.

To tackle excess ROS, antioxidants are present in the cartilage- Glutathione S-transferase P (GSTP) is one such enzymatic antioxidant. GSTP scavenge for the degradative radicals generated in the cartilage [454]. A decrease level of GSTP was found at the lesion site, which may suggest that the degenerated tissues are more susceptible to ROS (Figure 3.31) and hence overall cartilage degeneration.

Figure 3.31 Ratios of LN value of GSTP1 in iTRAQ and MRM experiment. The GI intact ratio is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significant with p<0.05.
C4A, released during the classical complement pathway, have been found to increase in osteoarthritic synovial fluid [455]. The excessive complement activation by the cartilage ECM fragments is suggested to cause cartilage degeneration, inflammation and chondrocyte apoptosis [456]. Reports also suggest that inflammation may precede the structural changes in early OA and that the complement activation can trigger further cartilage damage [124]. Significantly lower level of this protein was detected at the lesion sites compared with the adjacent intact sites in both GI and GII patellae (Figure 3.32) in the present study. To explain this contrasting finding, it is suggested that firstly, previous studies [455] having sampled from the synovial fluid may not have been able to resolve on the actual source for the increase in C4A. Secondly, that the intact site with a relatively higher level of C4A could also be indicative of the site’s response to the adjacent lesion, such that tissue degeneration progresses to the intact regions through complement activation.

**Figure 3.32** Ratios of LN value of complement component 4A (C4A) in iTRAQ and MRM experiment. The GI intact ratio is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significance with p<0.05.
Also of interest is the novel protein- PREDICTED: WAP four-disulphide core domain protein 18-like. Since the protein is still uncharacterized, its particular biological function in cartilage is still largely unknown. But the presence of whey four disulphide core (WFDC) domains indicate that they may possess anti-protease and anti-inflammatory activity [432]. Its prominent presence in intact cartilage of mildly degenerated patellae (GI) (Figure 3.33) adds to its potential as the biomarker for early OA and also suggests a possible therapeutic role. This site dependent variation of WFDC18 protein was further validated by MRM method. Future studies involving WFDC18 knockout mice can help us to identify the protein’s role in cartilage homeostasis. The functional role of the protein WFDC18-like can also explain the cartilage degeneration and OA progression and could be able to assist in the development of improved structure-based mechano-biological models of early OA.

Figure 3.33 Ratios of WFDC18-like protein in non-healthy cartilages in iTRAQ and MRM experiment. The GI intact ratio is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significance with p<0.05.
3.4.3 Biomarker Panel

Through this study, a series of proteins were identified, many of which have specific biological roles in cartilage and bone development/maintenance, and also with the degeneration of cartilage in association with OA. This emphasises the need to focus on early pre-OA state for the identification of reliable biomarkers of the disease, since the cartilage undergoes certain biochemical variations in the intact region much before these changes manifest themselves as structural variations or disease symptoms [224].

The intact tissue samples (from both mildly and moderately degenerated patellae) showed more prominent protein variations than the corresponding degenerated cartilages. This can be an indication of the active tissue response to the degeneration, where specific proteins differing to a larger degree in the intact samples than the lesion specimens, could be the potential key players in the mounted response. Their role may be central in tissue’s attempt at overcoming the pathological changes. Alternatively, these differences may be directly linked to the ‘cause’ rather than protection, such that the protein levels may be less marked in the lesion samples due to high turnover rates in these damaged areas.

Through this work, it is hypothesized that the protein variations occurring in intact cartilage regions lying next to the localized early degenerated sites (mildly degenerated GI lesion) have a prominent in role in early cartilage pathology. Speculating that these proteins could give key information with regard to the pre-OA state, the following proteins in Table 3.15 have been identified in this study to have potential to be early OA biomarkers.

Nine proteins namely, lysozyme C, WFDC18-like protein, CILP-1, CILP-2, angiogenin-1, fibronectin, COMP, ribonuclease 4 (angiogenin) and aggrecan were found to be increased in the intact regions of non-healthy patellae compared to healthy tissues. Thirteen proteins including six collagen molecules were found to have decreased level at the intact sites compared to healthy tissues.
Table 3.15 Potential biomarker panel identified in bovine cartilage tissue analysis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Post hoc analysis</th>
<th>Fold change (Intact/healthy)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Higher level in GI intact</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LYZ1 Lysozyme C, milk isozyme</td>
<td>****</td>
<td>11.24</td>
</tr>
<tr>
<td>PREDICTED: WAP four-disulfide core domain protein 18-like</td>
<td>****</td>
<td>9.23</td>
</tr>
<tr>
<td>CILP1 Cartilage intermediate layer protein 1</td>
<td>****</td>
<td>5.59</td>
</tr>
<tr>
<td>ANG Angiogenin-1</td>
<td>****</td>
<td>4.22</td>
</tr>
<tr>
<td>FN1 Embryo-specific fibronectin 1 transcript variant</td>
<td>***</td>
<td>3.89</td>
</tr>
<tr>
<td>COMP Cartilage oligomeric matrix protein</td>
<td>***</td>
<td>3.21</td>
</tr>
<tr>
<td>RNASE4;ANG Ribonuclease, RNase A family, 4</td>
<td>****</td>
<td>2.82</td>
</tr>
<tr>
<td>ACAN Isoform 2 of Aggrecan core protein</td>
<td>****</td>
<td>1.98</td>
</tr>
<tr>
<td>CILP2 Cartilage intermediate layer protein 2</td>
<td>***</td>
<td>1.85</td>
</tr>
<tr>
<td><strong>Lower level in GI intact</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COL6A1 Collagen alpha-1(VI) chain</td>
<td>**</td>
<td>0.7</td>
</tr>
<tr>
<td>COL6A2 Collagen alpha-2(VI) chain</td>
<td>**</td>
<td>0.65</td>
</tr>
<tr>
<td>COL6A3 Collagen alpha-3(VI) chain</td>
<td>**</td>
<td>0.65</td>
</tr>
<tr>
<td>COL2A1 Collagen alpha-1(II) chain</td>
<td>**</td>
<td>0.6</td>
</tr>
<tr>
<td>PPIA Peptidyl-prolyl cis-trans isomerase A</td>
<td>*</td>
<td>0.6</td>
</tr>
<tr>
<td>VIM Vimentin</td>
<td>**</td>
<td>0.59</td>
</tr>
<tr>
<td>COL12A1 Collagen alpha-1(XII) chain</td>
<td>*</td>
<td>0.55</td>
</tr>
<tr>
<td>THBS1 Thrombospondin-1</td>
<td>*</td>
<td>0.53</td>
</tr>
<tr>
<td>PCOLCE Procollagen C-endopeptidase enhancer</td>
<td>**</td>
<td>0.49</td>
</tr>
<tr>
<td>LMNA Lamin A</td>
<td>*</td>
<td>0.47</td>
</tr>
<tr>
<td>TGFB1 Transforming growth factor-beta-induced protein ig-h3 precursor - LOC539596 protein</td>
<td>*</td>
<td>0.37</td>
</tr>
<tr>
<td>COL11A1 collagen alpha-1(XI) chain</td>
<td>*</td>
<td>0.28</td>
</tr>
<tr>
<td>CKM Creatine kinase M-type</td>
<td>*</td>
<td>0.23</td>
</tr>
</tbody>
</table>

The changes in level of these proteins correlate well with the microstructural changes observed in this work. Further study in this direction will provide us with more concrete evidence on the roles of various proteins in the microstructural variations seen across the degenerated osteoarthritic cartilage.
3.4.4 Proposed Cartilage Degeneration Mechanism

A proposed model of early cartilage degeneration is depicted as a schematic diagram in Figure 3.34. Shown are the histological analyses of a range of cartilage tissue states occurring during the degeneration process. The present study involved samples predominantly from first three tissue states wherein the cartilage retains most of its thickness, as well as the proteoglycan loss is not too severe.

The two early microstructural indicators of cartilage degeneration, as mentioned earlier, are shown in the schematic—firstly, the collagen fibrillar de-structuring followed by fibril re-aggregation and secondly, the bone spicule growth into the zone of calcified cartilage. The proteins which were found to be significantly influencing the transformation of dense fibrillar network into a less tangled collagen network, where fibrils appear to aggregate parallel to each other in radial direction, were identified as lumican, fibromodulin, thrombospondin 1, collagen type II alpha 1, collagen type VI, collagen type XI alpha 1, collagen type XII alpha 1, COMP, CILP1, CILP2, PCOLCE, fibronectin, PPIA and vimentin. The proteins identified to be involved in mineralization and bone formation during early cartilage degeneration were TGFβI, CILP1, lysozyme, EDIL3, annexin 2, CKM, thrombospondin 1 and lamin A. Also shown are the proteins tenascin C, angiogenin 1, CCL16 and RNASE4 which could involve in vascularization of the newly evolving bone spicules.

It is unclear whether cartilage matrix alteration brings about the changes in subchondral bone or vice-versa. But it is noteworthy that these microstructural changes are also reflected in the changed protein profiles of two regions with different level of degeneration. This study found that correlation of the matrix protein changes to the specific patterns of microstructural modification is a promising future direction for research, to explore the hypothesis of early OA initiation and progression.
Figure 3.3 Schematic illustrating the proposed early progression of OA involving significant changes in tissue structure, histological staining response and protein levels. The samples from the present study fall largely within the first four stages where there appears to be an adaptive change in the zone of calcified cartilage (ZCC) related to changes in the fibrillar structure of the articular cartilage matrix. Several key proteins specifically found to be relevant to the microstructural changes reported in this study are highlighted.
3.5 Conclusions

- The protein profile between two intact cartilages from the healthy patellae showed little variations.

- Whereas a set of 56 proteins were found to vary significantly between the healthy and non-healthy cartilage tissues.

- On comparison with healthy tissues, the intact cartilage lying next to mildly degenerated cartilages showed significant difference in abundance level of 27 proteins, and these could have vital role in pre-to-early OA state.

- Comparison of protein profile from the degenerated region of patellae was shown to vary from its adjacent intact site. A set of 46 proteins were found to be differentially present between the intact and lesion tissues in the non-healthy bovine patellae.

- The patellae with mild level of degeneration (GI) were found to have more protein differences than patellae with moderate level of degeneration. This may suggest that the cartilage puts up an initial repair/defence process and as the disease advances, the degenerative mechanisms overtake the repair/defence activity.

- The variations seen at the biochemical level in the intact and lesion sites of the cartilage, explains how various protein molecules are differentially expressed across the different tissue states and also provide a good parallel for the microstructural variations seen at the two sites.

- This work brings forward the idea that the intact region behaves as a relatively early stage of the disease (pre-OA) whereas the lesion tissue region gives the glimpse of the aftermath of disease.

- By correlating the early microstructural variations to the protein profiles, a set of proteins were obtained which have the potential to be biomarker candidates for OA disease progression and pathophysiology.
CHAPTER 4
CARTILAGE SECRETOME STUDIES

4.1 Introduction

The load given to a joint is spread across to its entire surface by a healthy well-functioning cartilage. This protects these focal point regions, where the mechanical force is applied, from being overused and worn out. Therefore, the influence of mechanical impetus on cartilage is an important feature to be examined, while considering the risk factors contributing to various joint disorders. The cartilage health is most often stated by its structural composition. The breakdown of the cartilage structure leads to loss of function, in providing a frictionless gliding surface for load distribution and as a resilient structure which absorbs and dissipates energy during loading.

The cartilage resilience is attributed to the interaction between the fluid phase and the solid phase in the cartilage. Negatively charged GAGs trap water molecules and regulate osmotic pressure in cartilage, through its electrostatic interactions with cartilage structural molecules [457]. The constant movement of the fluids to and from cartilage ECM during mechanical loading, facilitate the joint movement [458, 459]. During early cartilage degeneration, the loss of GAGs lead to accumulation of water molecules in the tissue and this result in the cartilage softening [460]. The clinical OA is diagnosed when the cartilage structure has been compromised and the patient starts to experience discomfort associated with the disease.

Mass spectrometry (MS) is one of the emerging tools in diagnostic and biomarker discovery and it has the advantage of speed, accuracy, and sensitivity for identifying and quantifying proteins in a sample. MS, along with other investigative biochemical techniques have been used to study the cartilage matrix degradation processes [195, 461]. Cartilage proteomics studies based on the analyses of whole cartilage tissue as
well as cartilage secretome, with or without external catabolic initiation, have expanded our understanding of OA [168, 193, 195, 197, 221, 264, 462-464]. Cartilage secretome is analysed in these experiments to identify the proteins secreted by the chondrocytes/explants into the medium, along with the degradation products from the tissue as well as the products of tissue death.

Being the only cells in cartilage and with limited metabolic activity, chondrocytes play a major role in cartilage. They respond to mechanical and biological stimuli through molecular signalling, and by regulating gene expression and protein synthesis in the cartilage. The pathophysiology of OA is often illustrated through the involvement of pro-inflammatory and catabolic cytokines like interleukins-1β (IL-1β) and tumour necrosis factor-α (TNF-α) [147]. These cytokines are involved in downregulating the collagen and proteoglycan production and at the same time drive the catabolic mechanisms by augmenting the inflammatory signalling and protease synthesis in the tissue [125].

Most culture based OA studies involve in vitro treatment with IL-1β and/or other cytokines [197, 221, 465-467]. Even though IL-1β is known to emulate similar environment as seen in the in vivo OA tissues, it is not the best stimulant to understand the pathophysiology of OA [134] considering the inconsistencies in data from various studies.

The in vitro studies involving IL-1β and TNF-alpha suppresses the chondrocyte’s anabolic activity [468, 469]. IL-1β doses used in vitro studies are often several folds higher than what is actually seen in OA synovial fluid. These cytokines increase catabolism by inducing MMPs and aggrecanases. Even though good at stimulating an OA-like environment in vitro, there are limitations to use of cytokines as a stimulant. Earlier work has found that IL-1β level increase in RA patients but not in OA sufferers [470]. There is also inconsistency in detectable levels of IL-1β detected in osteoarthritic synovial fluid, serum or synovial tissue [471-478]. Orita et al. analysed synovial fluid from patients with progressing grade of OA and found an inverse relationship between IL-1β and grade of OA degeneration [479]. This finding
interpreted IL-1β’s catabolic role to be most potent during the early stages of degeneration and found that it decreases as the disease advances [134]. Therefore, current OA research based on the IL-1β’s role in stimulating catabolism in vitro should be approached with caution considering the contradictions in data from various studies. Importantly, more focus should be on determining the actual initiation factor in OA using other spontaneous/in vivo models.

The rationale for the present study is to analyse the secretome protein profile of bovine cartilages showing early cartilage degeneration, in order to determine the spontaneous changes similar to pre-to-early OA state.

4.2 Methodology

4.2.1 Optimization of Culture Conditions

A preliminary experiment was performed to ascertain the conditions required for the cartilage culture.

4.2.1.1 Tissue Source

A bovine patella showing mild cartilage degeneration at the distal lateral surface was obtained from the abattoir soon after the slaughtering. With Outerbridge’s classification [281] it was classified as Grade I patella.

4.2.1.2 Preparation of Samples

Under sterile conditions, the patella was washed with PBS to remove the synovial fluid and blood traces from the cartilage surface. Eight to ten cartilage discs each from intact and lesion region were punched out using 3 mm biopsy punches (Miltex). The discs were then pared-off the subchondral bone using scalpel.

Five discs from each sample group, were washed in pre-warmed 1 mL DMEM (with 4500 mg/L glucose, L-glutamine and sodium bicarbonate, Sigma) supplemented with 4% penicillin/streptomycin at 37 °C. They were then transferred into 5 mL of PBS containing 10% penicillin, washed under rotation for 20 mins (2x).
4.2.1.3 Preconditioning of the Samples

Five cartilage discs per sample group were incubated in 1 mL DMEM containing 2% Penicillin/Streptomycin, for 24 hours at 37 °C and 5% CO₂ in 12 well plate. Two conditions were maintained for each sample- one where the media was supplemented with 10% v/v of foetal calf serum (FCS) and another without it.

4.2.1.4 Optimization of Incubation Time

After the equilibration step, the cartilage discs were taken out of the 12 well plate and washed twice with 5 mL DMEM for 20 mins each, under rotation. After washing, 5 discs per sample were transferred to 1 mL DMEM and was incubated at 37 °C and 5% CO₂. The explants were cultured for two time points - one for 48 hours and other for 96 hours respectively.

After the incubation period, the media was collected and centrifuged at 14500 rpm for 5 mins. The supernatant was then transferred in to new microcentrifuge tubes and stored at -80 °C until further use.
Figure 4.1 Schematic representation of overall methodology used in secretome study. Cartilage explants were punched out from the intact and lesion site of the patella, followed by incubation in culture media for 96 hours, the secretome was then collected and underwent proteomic analysis.
4.2.2 Cartilage Explant Culture

The methodology followed for this section is outlined in Figure 4.1.

4.2.2.1 Tissue Source

Patellae were obtained from mature bovine animals soon after slaughter and based on the extent of cartilage degeneration on their surface, 3 were scored G0, 3 as GI patellae and 3 as GII patellae based on Outerbridge’s classification (Figure 4.2).

![Sampling sites for cartilage explant culture from (A) G0 patella (control), (B) GI patella and (C) GII patella. Shown here is the gross appearance of the patellae, which were graded based on visual and tactile inspection followed by histological analysis. The boxed region depicts the representative site for each cartilage group in this study.](image)

4.2.2.2 Sample Preparation

The sample was prepared as stated in section 4.2.1.2. From each patella, 12-13 cartilage discs were punched out from the intact and lesion regions respectively. For individual sample, 5 explants per well were cultured in replicates of two and 1-2 cartilage discs were cryo-frozen in liquid nitrogen and 1 was chemically fixed.

4.2.2.3 Histology Analysis of Cartilage Tissues

In order to determine the extent of cartilage/proteoglycan loss from different tissue groups and their regions, toluidine blue staining was performed. The cartilage explant was fixed in 4% para-formaldehyde, overnight. After chemical fixation, the cartilage disc was washed in 2 mL of PBS for 10 mins (3x). Each sample was then placed in
labelled embedding cassette and stored in 70% ethanol until histological analysis was performed.

Following serial dehydration in graded solutions of ethanol, the cartilage plugs were embedded in paraffin wax. 5 μm-thick sections were then obtained from the explants which were mounted on the glass slides. The slides were dipped in xylene (2x), to remove the wax and rehydrated in graded solutions of ethanol and finally washed through distilled water. Each slide was then dipped in toluidine blue for 10 mins and then the excess stain was removed by immersing the slide in water for 1 min. Following which, the slides were air-dried for 30 mins, DPX mounted and left to dry for 24 hours. The tissues were imaged using bright field transmission microscopy and scored using Mankin cartilage grading system [285].

4.2.2.4 Equilibrating the Cartilage Explants

Before culturing, the explants were pre-incubated in 1 mL of serum-free DMEM (phenol-red free) containing 2% Pen/Strep for 24 hours at 37 °C and 5% CO₂. This equilibration step was performed to ensure that any stress to the cartilage explants is avoided.

4.2.2.5 Cartilage Culture

The pre-equilibrated explants were washed in 5 mL DMEM for 20 minutes twice. Following which five cartilage discs were placed per well containing 1 mL DMEM and they were cultured for 96 hours at 37 °C and 5% CO₂. Each sample was cultured in replicates of two.

After the incubation period, media was collected from the wells and centrifuged. Carefully the supernatant was transferred to the labelled tubes and stored at -80 °C until further use.
4.2.3 Proteomics

4.2.3.1 Sample Processing

The conditioned media were thawed out and concentrated using VivaSpin 500 centrifugal concentrators fitted with a 3,000 Da cut-off membrane. The concentration of secreted proteins in the concentrated conditioned media samples was determined by EZQ fluorescent protein assay (Invitrogen).

The concentrated proteins in the media were then reduced with DTT for a final concentration of 10 mM for 1 hour at 56 °C. Iodoacetamide (IAM) was added to a final concentration of 50 mM, and samples were incubated in the dark, at room temperature for another 1 hour. DTT was added to a final concentration of 20 mM before proteins were digested overnight with trypsin (in 50 mM ammonium bicarbonate buffer) at 1:25 ratio (Promega, Madison, WI, USA) at 37 °C.

4.2.3.2 iTRAQ Labelling

Once the proteins were digested, they were acidified using 5 µL of 50% formic acid and then desalted on 10 mg Oasis HLB cartridges (Waters, Milford, MA, USA). The eluted peptides were dried in a speed vacuum concentrator. The peptides were then resolubilized in 30 µL of 0.5 M triethylammonium bicarbonate (TEAB). Aliquots were taken from each sample to create a reference pool for normalisation of iTRAQ signals across different runs. Individual samples and the reference pools were labelled with iTRAQ 8-plex isobaric tags (Ab Sciex) for each run, as per manufacturer’s instruction. Labelled samples were pooled and desalted on an HLB column prior to analysis by LC-MS/MS.

4.2.3.3 LC-MS/MS Analysis

Samples were analysed on a TripleTOF 6600 Quadrupole-Time-of-Flight mass spectrometer (Ab Sciex) coupled to an Eksigent NanoLC 400 UHPLC system and controlled via Analyst TF 1.7 software (Sciex). Peptides were loaded onto a 0.3 x 10 mm trap column packed with Reprosil C18 media (Dr Maisch) and desalted for 6
minutes at 2 µl/min before being separated on a 0.075 x 200 mm picofrit column with a 15 µm integrated electrospray emitter (New Objective) packed in-house with Reprosil C18 media. Solvents for HPLC were 0.1% formic acid (buffer A) and 0.1% formic acid in acetonitrile (buffer B). Separations were performed at a flow rate of 250 nl/min using a gradient of: 100 min from 5% B to 40% B; 5 min from 40% B to 98% B; 5 min at 98% B; 2 min from 98% B to 5% B; 8 min at 5% B.

For MS/MS analysis an initial MS survey scan from 350 – 1600 m/z was collected for 250 ms, followed by MS/MS scans of 80 – 1600 m/z and 40 ms for the 40 most abundant precursor ions detected with a charge state of between 2 and 5. Previously selected precursor ions were excluded for 30 seconds.

4.2.3.4 Data Analyses

The resulting data were searched against a database containing the Uniprot bovine proteome (32,233 entries) using ProteinPilot version 5.0 (Sciex). Search parameters were: Sample Type- iTRAQ 8-plex (Peptide Labelled); Search Effort- Thorough; Cys Alkylation- Iodoacetamide; Digestion- Trypsin. Peptide and Protein summary files were then exported for further bioinformatics/statistical analyses.

4.2.4 Statistical Analysis

The data were processed as stated before (see section 3.2.2.3.4). Briefly, data obtained from each search were combined and trimmed. For each iTRAQ label, the sum of all peak areas for each protein was computed separately. The sum of areas obtained from the iTRAQ runs were then log transformed and ratios were obtained for each protein between individual sample and the sample reference pool.

As a term for convenience, the tissues from G0 patella are henceforth referred in this text as “healthy” and those from GI and GII patellae are broadly referred to as “non-healthy”.

As with previous chapter, the log ratios were then categorised based on the sample groups healthy control site A (n=3), healthy control site B (n=3), GI intact (n=3), GI lesion (n=3), GII intact (n=3) and GII lesion (n=3).
The following comparisons were made to understand the significant differential expression of proteins between different tissue groups.

4.2.4.1 Comparison between the Secretome from Healthy Cartilages and Non-healthy Tissues

The average value of the ratios calculated for the healthy control A and control B sites were used to make up a final set of three healthy sample values. For each protein, log ratios obtained for healthy group means were scaled to 0 (1 in normal space) and the non-healthy groups were scaled to the above mentioned healthy samples.

Each protein was then tested using a One-Way ANOVA and if the test turned significant, the difference of the protein in each non-healthy group was tested against the healthy group using Dunnett's multiple comparisons test.

4.2.4.2 Comparison of Secretome of Healthy Control Site A and Control Site B

Using a two-tailed t tests, the secretome profile of two control sites obtained from the healthy patella were compared to detect the presence of any significant difference between these two regions.

4.2.4.3 Comparison of Cartilage Secretome from Intact and Lesion Sites of the Non-healthy Cartilage Tissues

Two-Way ANOVA was used to make comparison between all four non-healthy groups. The GI intact sample were scaled to 0 and represented ‘no change’ and the rest of the group means were scaled to that of GI intact samples. All proteins with n>1 for each group were tested. Of the tested proteins, those found to have significant differences were tested further using Tukey's multiple comparisons test.
4.3 Results

4.3.1 Optimization of Secretome Analysis

Intact and lesion site cartilage samples were collected from a GI bovine patella and underwent optimization to determine appropriate conditions for culturing. Incubation time period required for the cartilage explant secretome and the need for serum supplementation to the culture media for explant maintenance were evaluated in the optimization stage of the experiment. The results from the optimization stage were adopted for the main cartilage secretome study.

4.3.1.1 Optimized Incubation Time

The cultures were maintained over a period of 48 hours as well as for 96 hours. The amount of proteins secreted after 48 hours were found to be relatively less compared to the 96 hours’ incubation period. After 48 hours, the level of proteins secreted by the explants into the medium were less than the detectable level of the protein assay. Even with the 96 hours’ incubation period, the total protein concentration were in the range of 20-30 µg. Therefore, to increase the total protein content in the secretome analysis, each sample was cultured in replicates of two, each with 5 discs per well, for 96 hours and after the incubation, the replicates were pooled together to obtain final secretome.

4.3.1.2 Effects of Serum Supplementation

In order to assess the importance of serum for the explant culture maintenance, the present study attempted to grow cartilage explants both with and without supplementation with the foetal calf serum for 24 hr. Bovine serum is usually supplemented to media during cell/explant culture to achieve optimal growth in an in vitro environment [465]. But after pre-incubation with serum, complete removal of the serum components from the explant and its secretome, is difficult to achieve. Even with stringent washing protocols, highly abundant serum proteins are often found in secretome, masking the actual proteome profile of the tissue. This interference of serum could affect the studies which aim at identifying the proteins secreted into the media affecting the less abundant protein's identification confidence [480].
Earlier work by Williams et al. have shown that the explants can survive in the culture media for 96 hours without any increase in the cytosolic activity [221]. Cartilage explants have been shown to grow in serum-free media for almost 3 weeks’ time where the cell viability, proteoglycan synthesis, content and loss, collagen II content and synthesis [481] remained stable over the entire incubation period. Bian et al. showed that chondral and osteochondral sections can be maintained in serum free media for long term for up to 6 weeks. The mechanical testing after the incubation time period showed the cartilage explants remained stable as in day 0 and so did its GAG content and cell viability. They found lower levels of COMP, GAG and MMP3 in serum-free media and suggested that serum-free media provides a more supportive environment for cartilage explants to retain their structural composition by lowering catabolic activities [482].

The serum supplemented (serum+) and serum free (serum-) secretome was analysed using TripleTOF 6600 Quadrupole-Time-of-Flight mass spectrometer (Ab Sciex) coupled to an Eksigent NanoLC 400 UHPLC system. A total of 247 and 215 proteins were found in serum+ and serum- sample in this preliminary experiment. The peptide summary from serum+ sample showed that 7.6% of the detected peptides were derived from serum whereas serum- sample showed that only 0.24% of the peptides were serum-derived. The comparative abundance of these serum-derived peptides in serum+ sample confirmed that they could suppress the intensity of detection of lower abundant proteins. Therefore, for the quantitative analysis of samples, serum-free DMEM media was used in this body of work.

4.3.1.3 On the Absence of External Catabolic Factors

Comparison performed on the basis of signal intensity of proteins [298, 299] obtained from the intact and lesion site of the GI patellae in the preliminary study showed that there were considerable difference in the proteome profiles even in the absence of external catabolic factors like interleukins and cytokines, which are often supplemented to media to induce OA-like environment. Hence, an unstimulated media was chosen, devoid of any external catabolic factors (which can interfere with the inherent characteristics of the secretome) to culture the cartilage explants.
4.3.2 Cartilage Secretome Analyses

Along the lines of the previous studies, a pair of samples were taken from a set of 9 patellae graded as control (G0), mildly degenerate (GI) and moderately degenerate (GII) and were cultured in serum-free DMEM media for 96 hours. Cartilage explants were taken from intact sites of patellae showing no signs of cartilage fibrillation as controls, whereas in GI and GII patellae, explants were included from both intact and lesion sites. Thus, six cartilage sample groups comprising control site A (N=3), control site B (N=3), GI intact (N=3), GI lesion (N=3), GII intact (N=3) and GII lesion (N=3) were analysed in this work.

4.3.2.1 Scoring of Chondral Sections based on Histology

Keeping up with the previous works, this study included 6 different sample groups in this secretome analysis- control site A and control site B from healthy patellae, intact and lesion sites from the patellae showing mild degeneration (Outerbridge’s Grade I OA classification): GI intact and GI lesion and finally intact and lesion sites from patellae showing moderate level of degeneration (Outerbridge’s Grade II OA classification): GII intact and GII lesion.

The histologically processed sections underwent toluidine blue staining to assess the level of cartilage degeneration and proteoglycan loss [483]. Modified Mankin scoring system were used to score the cartilage surface structure, chondrocyte morphology and extent of toluidine blue staining. Any modification associated with tidemark integrity was disregarded during scoring as the samples were obtained as cartilage plugs (chondral sections), instead of osteochondral sections.

The Mankin score obtained for sample groups are as shown in Table 4.1. The images of respective samples are shown in Figure 4.3.
Table 4.1 Mankin score obtained for the samples in cartilage secretome study.

<table>
<thead>
<tr>
<th>N</th>
<th>Sample Group</th>
<th>Mankin Score Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Control site A</td>
<td>0-1</td>
</tr>
<tr>
<td>3</td>
<td>Control site B</td>
<td>0-0.5</td>
</tr>
<tr>
<td>3</td>
<td>GI intact</td>
<td>0-1</td>
</tr>
<tr>
<td>3</td>
<td>GI lesion</td>
<td>1.5-3</td>
</tr>
<tr>
<td>3</td>
<td>GII intact</td>
<td>0-1</td>
</tr>
<tr>
<td>3</td>
<td>GII lesion</td>
<td>3-5</td>
</tr>
</tbody>
</table>
Figure 4.3 Histological sections of cartilage stained with toluidine blue. (A) control site from healthy cartilage (Mankin score 0) with uniform staining, intact surface and normal chondrocytes (control site B was similar therefore not shown here); (B) GI patella (Mankin score 0) showing smooth surface morphology, uniform staining and normal chondrocytes; (C) lesion site from GI patella (Mankin score 3) with irregular surface and decreased staining of tangential zone; (D) intact site from GII patella (Mankin score 1); (E) lesion site from GII patella (Mankin score 5) with decreased matrix staining together with radial clefts extending into the mid-zone cartilage. Scale bar ~500 µm.
4.3.2.2 Proteomic Analysis of Cartilage Secretome

A total of 251 unique proteins were identified across three iTRAQ runs from the 6 sample groups. Of these, 194 proteins with \( n > 1 \) in all the six groups were subjected to the statistical tests.

Using Gene Ontology algorithm, the identified proteins were categorized based on its molecular function, biological process and cellular component as shown in Figure 4.4 using PANTHER (Protein Analysis through Evolutionary Relationship).

The majority of the identified proteins belonged to the extracellular regions and ECM and were involved in catalytic activities and binding in the cartilage. Further discussion on functional aspects of the identified proteins in this study is based on their association with cartilage and bone (see discussion).
Figure 4.4 Gene ontology analysis of the identified proteins using PANTHER (Protein Analysis through Evolutionary Relationship), which classified the proteins based on their molecular functions, biological processes and cellular component.
4.3.2.2.1 Differential Secretion of Proteins from Healthy and Non-healthy Cartilage Tissues

Data scaled to the healthy samples were used to compare all the four non-healthy groups (GI intact, GI lesion, GII intact and GII lesion) to the healthy baseline using One-Way ANOVAs. Following One-Way ANOVA, the significant difference of each non-healthy group against the healthy group was analysed, using Dunnett’s multiple comparisons test.

Of the 194 tested proteins, 53 showed significant differences in One-Way ANOVA (Appendix F). The outcome of the post-tests is given further.
GI Intact Secretome vs. Healthy Secretome

A group of 18 protein were found to be significantly different in the secretome of GI intact sample compared to the healthy cartilage explants. Only two proteins- COMP and WFDC18-like protein were found to be upregulated in the intact region of GI patellae. The rest of the proteins were downregulated in GI intact secretome (Table 4.2) compared to healthy samples.

Table 4.2 Significant differential expression of proteins when GI intact cartilage secretomes were compared to healthy cartilage secretomes.

<table>
<thead>
<tr>
<th>Accessions</th>
<th>Protein Names</th>
<th>Dunnett's test (p value)</th>
<th>Ratio of GI intact to Healthy †</th>
</tr>
</thead>
<tbody>
<tr>
<td>tr</td>
<td>F1N6D1</td>
<td>F1N6D1_BOVIN</td>
<td>WFDC18 WAP four-disulfide core domain protein 18-like</td>
</tr>
<tr>
<td>sp</td>
<td>P35445</td>
<td>COMP_BOVIN</td>
<td>COMP Cartilage oligomeric matrix protein</td>
</tr>
<tr>
<td>tr</td>
<td>B0JYM5</td>
<td>B0JYM5_BOVIN; sp</td>
<td>Q35Z4I</td>
</tr>
<tr>
<td>tr</td>
<td>F1MRZ6</td>
<td>F1MRZ6_BOVIN</td>
<td>TNC Tenasin</td>
</tr>
<tr>
<td>sp</td>
<td>Q28008</td>
<td>CLC3A_BOVIN</td>
<td>CLEC3A C-type lectin domain family 3 member A</td>
</tr>
<tr>
<td>tr</td>
<td>F1N401</td>
<td>F1N401_BOVIN</td>
<td>COL12A1 Collagen alpha-1(XII) chain</td>
</tr>
<tr>
<td>sp</td>
<td>Q05443</td>
<td>LUM_BOVIN</td>
<td>LUM Lumican</td>
</tr>
<tr>
<td>tr</td>
<td>F1MCU5</td>
<td>F1MCU5_BOVIN</td>
<td>CHADL Chondroadherin-like</td>
</tr>
<tr>
<td>tr</td>
<td>F1N3A1</td>
<td>F1N3A1_BOVIN</td>
<td>THBS1 Thrombospondin-1</td>
</tr>
<tr>
<td>tr</td>
<td>Q2HJB6</td>
<td>Q2HJB6_BOVIN</td>
<td>PCOLCE Procollagen C-endopeptidase enhancer</td>
</tr>
<tr>
<td>tr</td>
<td>Q08E14</td>
<td>Q08E14_BOVIN; tr</td>
<td>F1MXS8</td>
</tr>
<tr>
<td>sp</td>
<td>Q28069</td>
<td>C1QB_BOVIN</td>
<td>THBS1 Thrombospondin-1</td>
</tr>
<tr>
<td>tr</td>
<td>Q1RMH5</td>
<td>Q1RMH5_BOVIN</td>
<td>AMBP Protein AMBP</td>
</tr>
<tr>
<td>tr</td>
<td>A7WTB6</td>
<td>A7WTB6_BOVIN</td>
<td>TGFBI LOC539596 protein</td>
</tr>
<tr>
<td>sp</td>
<td>P02459</td>
<td>CO2A1_BOVIN</td>
<td>COL2A1 Collagen alpha-1(II) chain</td>
</tr>
<tr>
<td>sp</td>
<td>Q2KIV9</td>
<td>C1Q8_BOVIN</td>
<td>C1QB Complement subcomponent subunit B</td>
</tr>
<tr>
<td>tr</td>
<td>Q1RMH5</td>
<td>Q1RMH5_BOVIN</td>
<td>C1QC Complement subcomponent subunit C</td>
</tr>
<tr>
<td>sp</td>
<td>Q5E93E</td>
<td>C1QA_BOVIN</td>
<td>C1QA Complement subcomponent subunit A</td>
</tr>
</tbody>
</table>

†For the ease of understanding, log ratios were converted to linear space.
**GI Lesion Secretome vs. Healthy Secretome**

When the GI lesion samples were compared to healthy control secretome, a set of 12 proteins were found to be differentially present in the media, of which only expression of fibronectin was upregulated in the GI lesion secretome compared to healthy and the rest of the protein’s expression was downregulated in the mildly degenerated cartilage region (Table 4.3).

**Table 4.3** Significant differential expression of proteins when GI lesion cartilage secretomes were compared to healthy control samples.

<table>
<thead>
<tr>
<th>Accessions</th>
<th>Protein Name</th>
<th>Dunnett’s test (p value)</th>
<th>Ratio of GI lesion to Healthy †</th>
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</thead>
<tbody>
<tr>
<td>tr</td>
<td>B8Y9S9</td>
<td>B8Y9S9_BOVIN</td>
<td>FN1 Embryo-specific fibronectin 1 transcript variant</td>
</tr>
<tr>
<td>tr</td>
<td>A7YWB6</td>
<td>A7YWB6_BOVIN</td>
<td>TGFBI LOC539596 protein</td>
</tr>
<tr>
<td>tr</td>
<td>F1MB08</td>
<td>F1MB08_BOVIN;</td>
<td>ENO1 Alpha-enolase</td>
</tr>
<tr>
<td>sp</td>
<td>Q9XSJ4</td>
<td>ENOA_BOVIN</td>
<td>PCOLCE Procollagen C-endopeptidase enhancer</td>
</tr>
<tr>
<td>tr</td>
<td>Q2HJ6</td>
<td>Q2HJ6_BOVIN</td>
<td>AMBP Protein AMBP</td>
</tr>
<tr>
<td>tr</td>
<td>F1MMK9</td>
<td>F1MMK9_BOVIN</td>
<td>CHADL Chondroadherin-like</td>
</tr>
<tr>
<td>sp</td>
<td>Q28008</td>
<td>CLC3A_BOVIN</td>
<td>CLEC3A C-type lectin domain family 3 member A</td>
</tr>
<tr>
<td>sp</td>
<td>Q28178</td>
<td>TSP1_BOVIN</td>
<td>THBS1 Thrombospondin-1</td>
</tr>
<tr>
<td>sp</td>
<td>P39873</td>
<td>RNBR_BOVIN</td>
<td>BRN Brain ribonuclease</td>
</tr>
<tr>
<td>tr</td>
<td>A5D7E8</td>
<td>A5D7E8_BOVIN;</td>
<td>PDIA3 Protein disulfide-isomerase</td>
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<td>PDIA3_BOVIN</td>
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</tr>
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<td>YWHAQ 14-3-3 protein theta</td>
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<tr>
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<td>1433T_BOVIN</td>
<td></td>
</tr>
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<td>P02459</td>
<td>CO2A1_BOVIN</td>
<td>COL2A1 Collagen alpha-1(II) chain</td>
</tr>
<tr>
<td>tr</td>
<td>Q32L14</td>
<td>Q32L14_BOVIN;</td>
<td>RBP4 Retinol binding protein 4</td>
</tr>
<tr>
<td>tr</td>
<td>G1K122</td>
<td>G1K122_BOVIN;</td>
<td></td>
</tr>
<tr>
<td>sp</td>
<td>P18902</td>
<td>RET4_BOVIN</td>
<td></td>
</tr>
<tr>
<td>sp</td>
<td>P13213</td>
<td>SPRC_BOVIN</td>
<td>SPARC Osteonectin</td>
</tr>
</tbody>
</table>

For the ease of understanding, log ratios were converted to linear space.
**GII Intact Secretome vs. Healthy Secretome**

A group of 27 proteins were found differentially expressed when the GII intact cartilage secretome was compared to the healthy controls. As shown in Table 4.4, 10 proteins were significantly upregulated in the intact site secretome of the GII patellae compared to the healthy secretome and 17 were found to have lower expression at the GII intact samples.

**Table 4.4** Significant differential expression of proteins when GII intact cartilage secretomes were compared to healthy control samples.

<table>
<thead>
<tr>
<th>Accessions</th>
<th>Protein Names</th>
<th>Dunnett's test (p value)</th>
<th>Ratio of GII intact to Healthy †</th>
</tr>
</thead>
<tbody>
<tr>
<td>tr</td>
<td>Q5W5H8</td>
<td>Q5W5H8_BOVIN;</td>
<td>LAP LAP-like antimicrobial peptide</td>
</tr>
<tr>
<td>sp</td>
<td>Q28880</td>
<td>LAP_BOVIN</td>
<td>CCL16 C-C motif chemokine</td>
</tr>
<tr>
<td>tr</td>
<td>E1BIM2</td>
<td>E1BIM2_BOVIN</td>
<td>C4 Complement C4</td>
</tr>
<tr>
<td>sp</td>
<td>P01030</td>
<td>CO4_BOVIN</td>
<td>HIST1H1D Histone H1.3</td>
</tr>
<tr>
<td>tr</td>
<td>G3MWV5</td>
<td>G3MWV5_BOVIN;</td>
<td>WFDC18 WAP four-disulfide core domain protein 18-like</td>
</tr>
<tr>
<td>sp</td>
<td>P02253</td>
<td>H12_BOVIN;</td>
<td>CILP Cartilage Intermediate Layer Protein</td>
</tr>
<tr>
<td>tr</td>
<td>E1BKL4</td>
<td>E1BKL4_BOVIN</td>
<td>PGLYRP1 Peptidoglycan-recognition protein</td>
</tr>
<tr>
<td>sp</td>
<td>P35445</td>
<td>COMP_BOVIN</td>
<td>ACAN Aggrecan core protein</td>
</tr>
<tr>
<td>tr</td>
<td>F1MCU5</td>
<td>F1MCU5_BOVIN</td>
<td>CILP2 Cartilage Intermediate Layer Protein 2</td>
</tr>
<tr>
<td>sp</td>
<td>P21809</td>
<td>PGS1_BOVIN</td>
<td>COMP Cartilage oligomeric matrix protein</td>
</tr>
<tr>
<td>sp</td>
<td>Q28008</td>
<td>CLC3A_BOVIN</td>
<td>CHADL Chondroadherin-like</td>
</tr>
<tr>
<td>sp</td>
<td>A6H737</td>
<td>LOXL2_BOVIN</td>
<td>BGN Biglycan</td>
</tr>
<tr>
<td>tr</td>
<td>Q2HJB6</td>
<td>Q2HJB6_BOVIN</td>
<td>CLEC3A C-type lectin domain family 3 member A</td>
</tr>
<tr>
<td>tr</td>
<td>F1N401</td>
<td>F1N401_BOVIN</td>
<td>LOXL2 Lysyl oxidase homolog 2</td>
</tr>
<tr>
<td>tr</td>
<td>E1BI98</td>
<td>E1BI98_BOVIN</td>
<td>PCOLCE Procollagen C-endopeptidase enhancer</td>
</tr>
<tr>
<td>tr</td>
<td>F1MUC5</td>
<td>F1MUC5_BOVIN</td>
<td>COL12A1 Collagen alpha-1(XII) chain</td>
</tr>
<tr>
<td>tr</td>
<td>Q08E14</td>
<td>Q08E14_BOVIN;</td>
<td>COL6A1 Collagen alpha-1(VI) chain</td>
</tr>
<tr>
<td>tr</td>
<td>F1MXS8</td>
<td>F1MXS8_BOVIN</td>
<td>COL15A1 Collagen alpha-1(XV) chain</td>
</tr>
<tr>
<td>tr</td>
<td>F1MXS8</td>
<td>F1MXS8_BOVIN</td>
<td>COL3A1 Collagen alpha-1(III) chain</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Gene Name</td>
<td>Log Ratio</td>
<td>Fold Change</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
<td>-----------</td>
<td>-------------</td>
</tr>
<tr>
<td>tr</td>
<td>B0JYM5</td>
<td>B0JYM5_BOVIN;</td>
<td>YWHAQ 14-3-3 protein theta</td>
</tr>
<tr>
<td>sp</td>
<td>Q3SZI4</td>
<td>1433T_BOVIN</td>
<td>TGFBI LOC539596 protein</td>
</tr>
<tr>
<td>tr</td>
<td>A7YW66</td>
<td>A7YW66_BOVIN</td>
<td>AMBP Alpha-1-microglobulin protein</td>
</tr>
<tr>
<td>tr</td>
<td>F1MK9</td>
<td>F1MK9_BOVIN</td>
<td>TNC Tenascin</td>
</tr>
<tr>
<td>tr</td>
<td>F1MRZ6</td>
<td>F1MRZ6_BOVIN</td>
<td>COL5A1 Collagen alpha-1(V) chain</td>
</tr>
<tr>
<td>sp</td>
<td>Q5E9E3</td>
<td>C1QA_BOVIN</td>
<td>C1QA Complement C1q subcomponent subunit A</td>
</tr>
<tr>
<td>tr</td>
<td>Q1RMH5</td>
<td>Q1RMH5_BOVIN</td>
<td>C1QC Complement C1q subcomponent subunit C</td>
</tr>
<tr>
<td>tr</td>
<td>Q8SQ28</td>
<td>Q8SQ28_BOVIN;</td>
<td>SAA3 Serum amyloid A protein</td>
</tr>
</tbody>
</table>

†For the ease of understanding, log ratios were converted to linear space.

GII Lesion Secretome vs. Healthy Secretome

While comparing the GII lesion site cartilage secretome to the healthy controls, a set of 21 proteins were found to be significantly differential (Table 4.5). Six proteins were upregulated and 15 were downregulated at the GII lesion site in comparison to healthy cartilage secretome.
Table 4.5 Significant differential expression of proteins when GII lesion site cartilage secretomes were compared to healthy control samples.

<table>
<thead>
<tr>
<th>Accessions</th>
<th>Protein Names</th>
<th>Dunnett’s test (p value)</th>
<th>Ratio of GII lesion to Healthy †</th>
</tr>
</thead>
<tbody>
<tr>
<td>tr</td>
<td>F1MW44</td>
<td>F1MW44_BOVIN</td>
<td>F13A1 Coagulation factor XIII A chain</td>
</tr>
<tr>
<td>tr</td>
<td>G3MWV5</td>
<td>G3MWV5_BOVIN</td>
<td>HIST1H1D Histone H1.3</td>
</tr>
<tr>
<td>sp</td>
<td>P02253</td>
<td>HI12_BOVIN; A7MAZ5</td>
<td>HI3_BOVIN</td>
</tr>
<tr>
<td>tr</td>
<td>A6QQQ3</td>
<td>A6QQQ3_BOVIN</td>
<td>FBLN1 Fibulin-1</td>
</tr>
<tr>
<td>tr</td>
<td>F1MYN5</td>
<td>F1MYN5_BOVIN; A5D7S8</td>
<td>A5D7S8_BOVIN</td>
</tr>
<tr>
<td>tr</td>
<td>F1N368</td>
<td>F1N368_BOVIN; F1N367</td>
<td>FN1 Embryo-specific fibronectin 1 transcript variant</td>
</tr>
<tr>
<td>tr</td>
<td>B8Y959</td>
<td>B8Y959_BOVIN</td>
<td>TGFBI LOC539596 protein</td>
</tr>
<tr>
<td>tr</td>
<td>Q2HJB6</td>
<td>Q2HJB6_BOVIN</td>
<td>PCOLCE Procollagen C-endopeptidase enhancer</td>
</tr>
<tr>
<td>tr</td>
<td>F1MCU5</td>
<td>F1MCU5_BOVIN</td>
<td>COL12A1 Collagen alpha-1(XII) chain</td>
</tr>
<tr>
<td>tr</td>
<td>F1N401</td>
<td>F1N401_BOVIN</td>
<td>TNC Tenasin</td>
</tr>
<tr>
<td>tr</td>
<td>Q08E14</td>
<td>Q08E14_BOVIN; F1MXS8</td>
<td>COL3A1 Collagen alpha-1(III) chain</td>
</tr>
<tr>
<td>sp</td>
<td>Q28008</td>
<td>CLC3A_BOVIN</td>
<td>CLEC3A C-type lectin domain family 3 member A</td>
</tr>
<tr>
<td>sp</td>
<td>P20414</td>
<td>TIMP1_BOVIN</td>
<td>TIMP1 Metalloproteinase inhibitor 1</td>
</tr>
<tr>
<td>tr</td>
<td>E1B98</td>
<td>E1B98_BOVIN</td>
<td>COL6A1 Collagen alpha-1(VI) chain</td>
</tr>
<tr>
<td>tr</td>
<td>A0A0M3T9B6</td>
<td>A0A0M3T9B6_BOVIN; P35096</td>
<td>SPP1 Osteopontin</td>
</tr>
<tr>
<td>sp</td>
<td>Q2KIV9</td>
<td>C1QB_BOVIN</td>
<td>C1QB Complement subcomponent subunit B</td>
</tr>
<tr>
<td>tr</td>
<td>Q1RMH5</td>
<td>Q1RMH5_BOVIN</td>
<td>CIQC Complement subcomponent subunit C</td>
</tr>
<tr>
<td>sp</td>
<td>Q5E9E3</td>
<td>C1QA_BOVIN</td>
<td>CIQA Complement subcomponent subunit A</td>
</tr>
<tr>
<td>sp</td>
<td>P31213</td>
<td>SPRC_BOVIN</td>
<td>SPARC Osteonectin</td>
</tr>
</tbody>
</table>

†For the ease of understanding, log ratios were converted to linear space.
4.3.2.2.2 Comparison of Secretome of Healthy Control Site A and Control Site B

Using a two-tailed t tests, 4 proteins were found to be significantly different between secretome of healthy control site A and healthy control site B. Collagen alpha-2(XI) chain (COL11A2), epididymal secretory protein E1 (NPC2), thrombospondin-1 (THBS1) and vitrin (VIT) were found to be differentially expressed by the cartilage explants from two different regions in a healthy patella (Figure 4.5).

**Figure 4.5** Scaled natural log (LN) ratios of proteins found to be differential between the secretome of two control cartilage sites from healthy patellae.
4.3.2.3 Comparison of Cartilage Secretome from Intact and Lesion Site of the Non-healthy Cartilage Tissues

The comparison was made between the four non-healthy groups, with all the other three groups scaled to GI intact samples. Two-way ANOVA was used to identify significant difference between the groups. A total of 68 proteins were found significantly different (Appendix G) and was tested further with Tukey’s multiple comparisons tests.

Four different comparisons made were:

- GI intact vs. GI lesion
- GII intact vs. GII lesion
- GI intact vs. GII intact
- GI lesion vs. GII lesion

A set of nine proteins were found to significantly vary between the intact and lesion site secretomes of GI cartilages (Table 4.6) whereas 11 proteins were differentially expressed between the intact and degenerate site secretomes of GII patellae (Table 4.7). WFDC18-like protein, superoxide dismutase (ECSOD), tenascin and PGLYRP1 were found to be differentially expressed in between intact and lesion sites of both GI and GII patellae.
Table 4.6 GI patellae group secretome comparison. Proteins which showed significant difference in their lesion and corresponding adjacent intact cartilage secretome samples (p<0.05) after Tukey’s multiple comparison test.

<table>
<thead>
<tr>
<th>Accessions</th>
<th>Protein Names</th>
<th>Tukey’s test (p value)</th>
<th>GRADE I Lesion/Intact†</th>
</tr>
</thead>
<tbody>
<tr>
<td>tr</td>
<td>F1N401</td>
<td>F1N401_BOVIN</td>
<td>COL12A1 Collagen alpha-1(XII) chain</td>
</tr>
<tr>
<td>tr</td>
<td>A7YW6</td>
<td>A7YW6_BOVINN</td>
<td>TGFBI LOC539596 protein</td>
</tr>
<tr>
<td>tr</td>
<td>F1MRZ6</td>
<td>F1MRZ6_BOVIN</td>
<td>TNC Tenascin</td>
</tr>
<tr>
<td>tr</td>
<td>A3KLR9</td>
<td>A3KLR9_BOVIN</td>
<td>ECSOD Superoxide dismutase [Cu-Zn]</td>
</tr>
<tr>
<td>sp</td>
<td>P48616</td>
<td>VIME_BOVIN</td>
<td>VIM Vimentin</td>
</tr>
<tr>
<td>tr</td>
<td>H2CNR1</td>
<td>H2CNR1_BOVIN</td>
<td>PGLYRP1 Peptidoglycan-recognition protein</td>
</tr>
<tr>
<td>tr</td>
<td>B5T254</td>
<td>B5T254_BOBOX;</td>
<td>WFDC18 WAP four-disulfide core domain protein 18-like</td>
</tr>
<tr>
<td>sp</td>
<td>Q8SPP7</td>
<td>PGP1_BOVIN</td>
<td>PDIA3 Protein disulfide-isomerase</td>
</tr>
<tr>
<td>tr</td>
<td>Q3L14</td>
<td>Q3L14_BOVIN</td>
<td>RET4 Retinol binding protein 4</td>
</tr>
</tbody>
</table>

†For the ease of understanding, log ratios were converted to linear space.
Table 4.7 GII patellae group secretome comparison. Proteins which showed significant difference in their lesion and corresponding adjacent intact cartilage secretome samples (p<0.05) after Tukey’s multiple comparison test.

<table>
<thead>
<tr>
<th>Accessions</th>
<th>Protein Names</th>
<th>Tukey's test (p value)</th>
<th>GRADE II Lesion/Intact†</th>
</tr>
</thead>
<tbody>
<tr>
<td>tr</td>
<td>A5PJE3</td>
<td>A5PJE3_BOVIN; sp</td>
<td>P02672</td>
</tr>
<tr>
<td>tr</td>
<td>F1MYN5</td>
<td>F1MYN5_BOVIN ; tr</td>
<td>A5D7S8</td>
</tr>
<tr>
<td>tr</td>
<td>F1MRZ6</td>
<td>F1MRZ6_BOVIN</td>
<td>FBLN1 Fibulin-1</td>
</tr>
<tr>
<td>tr</td>
<td>A6QQQ3</td>
<td>A6QQQ3_BOVIN</td>
<td>TNC Tenascin</td>
</tr>
<tr>
<td>tr</td>
<td>A3KLR9</td>
<td>A3KLR9_BOVIN</td>
<td>PRELP Proline/arginine-rich end leucine-rich repeat protein</td>
</tr>
<tr>
<td>tr</td>
<td>E1BIR5</td>
<td>E1BIR5_BOVIN</td>
<td>ECSOD Superoxide dismutase [Cu-Zn]</td>
</tr>
<tr>
<td>tr</td>
<td>E1BL4</td>
<td>E1BKL4_BOVIN</td>
<td>CILP Cartilage Intermediate Layer Protein</td>
</tr>
<tr>
<td>tr</td>
<td>H2CNR1</td>
<td>H2CNR1_BOVIN</td>
<td>CILP2 Cartilage Intermediate Layer Protein 2</td>
</tr>
<tr>
<td>tr</td>
<td>B5T254</td>
<td>B5T254_BOBOX; sp</td>
<td>Q8SPP7</td>
</tr>
<tr>
<td>tr</td>
<td>Q5W5H8</td>
<td>Q5W5H8_BOVIN ; sp</td>
<td>Q28880</td>
</tr>
<tr>
<td>tr</td>
<td>F1N6D1</td>
<td>F1N6D1_BOVIN</td>
<td>WFDC18 LOC100847724</td>
</tr>
</tbody>
</table>

†For the ease of understanding, log ratios were converted to linear space.
7 proteins were significantly different when the secretome from intact sites of GI and GII samples were compared (Table 4.8) and 12 were found to be different between the lesion site secretomes of GI and GII patellae (Table 4.9).

**Table 4.8** Differential expression of proteins when the intact sites secretome of GI and GII groups were compared.

<table>
<thead>
<tr>
<th>Accessions</th>
<th>Protein Names</th>
<th>Tukey’s test (p value)</th>
<th>GII intact: GI intact</th>
</tr>
</thead>
<tbody>
<tr>
<td>tr</td>
<td>Q5W5H8</td>
<td>Q5W5H8_BOVIN ; sp</td>
<td>Q28880</td>
</tr>
<tr>
<td></td>
<td>LUM Lumican</td>
<td></td>
<td>0.0089</td>
</tr>
<tr>
<td></td>
<td>ACAN Aggrecan core protein</td>
<td></td>
<td>0.0174</td>
</tr>
<tr>
<td>tr</td>
<td>H2CNR1</td>
<td>H2CNR1_BOVIN; tr</td>
<td>B5T254</td>
</tr>
<tr>
<td></td>
<td>PGLYRP1 Peptidoglycan-recognition protein</td>
<td></td>
<td>0.0036</td>
</tr>
<tr>
<td></td>
<td>TNC Tenacin</td>
<td></td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td>COL15A1 Collagen alpha-1(XV) chain</td>
<td></td>
<td>0.0386</td>
</tr>
</tbody>
</table>

*For the ease of understanding, log ratios were converted to linear space.*
Table 4.9 Differential expression of proteins when the lesion sites secretome of GI and GII groups were compared.

<table>
<thead>
<tr>
<th>Accessions</th>
<th>Protein Names</th>
<th>Tukey’s test (p value)</th>
<th>GII lesion: GI lesion†</th>
</tr>
</thead>
<tbody>
<tr>
<td>tr</td>
<td>E1BH06</td>
<td>E1BH06_BOVIN</td>
<td>WFDC18 LOC100852118</td>
</tr>
<tr>
<td>sp</td>
<td>Q3ZBD7</td>
<td>G6PI_BOVIN</td>
<td>GPI Glucose-6-phosphate isomerase</td>
</tr>
<tr>
<td>tr</td>
<td>G3MWV5</td>
<td>G3MWV5_BOVIN;</td>
<td>HIST1H1D Histone H1.3</td>
</tr>
<tr>
<td>sp</td>
<td>P02253</td>
<td>H12_BOVIN;</td>
<td></td>
</tr>
<tr>
<td>sp</td>
<td>A7MAZ5</td>
<td>H13_BOVIN</td>
<td></td>
</tr>
<tr>
<td>tr</td>
<td>Q32L14</td>
<td>Q32L14_BOVIN;</td>
<td></td>
</tr>
<tr>
<td>tr</td>
<td>G1K122</td>
<td>G1K122_BOVIN;</td>
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</tr>
<tr>
<td>sp</td>
<td>P18902</td>
<td>RET4_BOVIN</td>
<td></td>
</tr>
<tr>
<td>tr</td>
<td>F1MW44</td>
<td>F1MW44_BOVIN</td>
<td>F13A1 Coagulation factor XIII A chain</td>
</tr>
<tr>
<td>tr</td>
<td>F1MB08</td>
<td>F1MB08_BOVIN;</td>
<td>ENO1 Alpha-enolase</td>
</tr>
<tr>
<td>sp</td>
<td>Q9XSJ4</td>
<td>ENOA_BOVIN</td>
<td></td>
</tr>
<tr>
<td>sp</td>
<td>Q28178</td>
<td>TSP1_BOVIN</td>
<td>THBS1 Thrombospondin-1</td>
</tr>
<tr>
<td>tr</td>
<td>H2CNR1</td>
<td>H2CNR1_BOVIN;</td>
<td>PGLYRP1 Peptidoglycan-recognition protein</td>
</tr>
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<td>tr</td>
<td>B5T254</td>
<td>B5T254_BOBOX;</td>
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</tr>
<tr>
<td>sp</td>
<td>Q8SPP7</td>
<td>PGRP1_BOVIN</td>
<td></td>
</tr>
<tr>
<td>sp</td>
<td>P48616</td>
<td>VIME_BOVIN</td>
<td>VIM Vimentin</td>
</tr>
<tr>
<td>tr</td>
<td>A6QQQ3</td>
<td>A6QQQ3_BOVIN</td>
<td>PRELP Proline/arginine-rich end leucine-rich repeat protein</td>
</tr>
<tr>
<td>tr</td>
<td>F1MRZ6</td>
<td>F1MRZ6_BOVIN</td>
<td>TNC Tenascin</td>
</tr>
<tr>
<td>tr</td>
<td>F1N401</td>
<td>F1N401_BOVIN</td>
<td>COL12A1 Collagen alpha-1(XII) chain</td>
</tr>
</tbody>
</table>

†For the ease of understanding, log ratios were converted to linear space.
4.4 Discussion

Secretome is the repertoire of proteins secreted by cells, tissues or organs in healthy or disease condition or under any therapeutic intervention. Analysing the secretome can be vital in obtaining information regarding the proteins, which potentially promote the disease condition in a tissue [484, 485]. Identifying these proteins from the explant secretome can be advantageous in determining the potential diagnostic biomarkers as they can turn up in blood stream [486]. Mass spectrometry based proteomics can be used to analyse the secreted protein profile of chondrocytes or cartilage explants and differentiate between the healthy and disease states [487].

The limitations often associated with the secretome analysis are

(a) Contamination

Care must be taken to limit the serum contamination while culturing the explants, as the serum proteins are difficult to get rid of from the samples and can mask the protein identification. Proper washing protocols or serum-free medium should be adopted to limit such problems. Metabolic stress can occur in the serum free media so adequate optimization of incubation time is also vital. In the present work, culture conditions for bovine cartilage explants were optimized to 96 hours incubation period in serum-free media.

(b) Low concentration of secreted proteins

The relative quantity of secreted proteins is low under the normal conditions, which is further diluted when collected along with the media. In this work, in order to avoid induction of any form of stress in vitro, replicates of 5 cartilage plugs/well were used and later pooled together (to obtain ~40 µg of secreted proteins).

(c) Common limitations which often accompany the in vitro studies

The physiological factors which are present in vivo, is absent during the culture period, for example, chondrocytes in articular cartilage is under loaded stress in vivo, which is absent when the cartilage explants are grown in the media. These in vitro conditions can modulate some secretory pattern in the cells. So, absence of these changes should be taken into account while analysing the results and correlating to the secretory profile in vivo.
More recently, Lourido et al. analysed the cartilage secretome from wounded (lesion) and unwounded (intact) sites of human cartilage with the aim of identifying the proteins which could be putative markers for cartilage degradation [226]. Though the study was much similar to the present work, the tissues used were at a much advanced level of degeneration with a Mankin score of 7-9 for the wounded tissues while the bovine tissues for the present study scored a maximum of 3-5 in Mankin scale for the GII lesion site samples (others sample group showed even lower Mankin score, please refer section 4.3.1).

The aim of the present work was to identify the biochemical and biological factors released by the cartilage tissue when the degeneration is at a relatively earlier stage.

**4.4.1 Comparison of Cartilage Secretome from Different Non-healthy Tissue States with respect to Healthy Controls**

Secretome from four non-healthy cartilage groups namely GI intact, GI lesion, GII intact and GII lesion were compared to healthy controls to identify the differential expression of proteins in each group. Those proteins which were found significant in this study is briefly discussed with regard to their potential role in cartilage structural modification (Chapter 2) studied previously.

**4.4.1.1 ECM Organization and Cartilage Structure**

Extracellular matrix of cartilage is composed of a network of macromolecules predominantly made up of collagens, proteoglycans, non-collagenous glycoproteins and other small molecules. Chondrocytes are the only cell present in the cartilage and they maintain the cartilage by balancing the anabolic and catabolic biological processes occurring in the ECM.

The differential level of collagens were detected in the non-healthy group secretome compared to the healthy controls. The collagens were significantly downregulated in the non-healthy group (Figure 4.6). This result is similar to what was observed when the whole cartilage tissue was analysed (Figure 3.11). The decrease in collagens can explain the structural degeneration of the cartilage occurring in the GI and GII patellae.
Figure 4.6 The decreased level of collagens found in non-healthy cartilage secretome (both intact and lesion) compared to healthy cartilage secretomes. The GII intact site have most prominent loss of collagens. For the post-test * denotes significance with $p<0.05$, ** $p<0.01$. 
ECM structural proteins like COMP, fibronectin, CILP, CILP2, aggrecan and Proline/arginine-rich end leucine-rich repeat protein (Prolargin or PRELP) were found to be significantly upregulated in the non-healthy cartilage groups (Figure 4.7). The upregulated levels of these proteins can either be the result of an ineffective repair mechanism or alternatively, the result of increased degeneration seen in the non-healthy tissues.

**Figure 4.7** Scaled natural log (LN) ratios of proteins known to associate with ECM structure and was upregulated in the bovine OA tissue secretomes. For each protein, the healthy cartilage is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significant with p<0.05, ** p<0.01, *** p<0.001.
Other ECM proteins like lumican, PCOLCE, Alpha-enolase (ENO1) and biglycan were found to be predominantly downregulated in the non-healthy group (Figure 4.8). The reduced expression of these proteins could destabilize the collagen network and can result in cartilage’s loss of structure and function.

**Figure 4.8** Scaled natural log (LN) ratios of proteins known to associate with ECM structure and was found to downregulate in the bovine OA tissue secretomes. For each protein the healthy cartilage is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significant with p<0.05, ** p<0.01.

CHADL or chondroadherin-like is a novel protein found to be expressed in the pericellular matrix of the adult chondrocytes. It can interact with collagen and can influence collagen fibrillogenesis. They act as negative regulators of chondrocyte differentiation and thus maintains cartilage homeostasis [488]. The expression of CHADL was seen to be downregulated in the non-healthy cartilage secretome (Figure 4.9) and it can be hypothesised that the lower expression of these proteins in the non-healthy cartilage can affect the collagen fibrillogenesis resulting in a weaker fibrillar network as seen in Figure 2.7.
Figure 4.9 Scaled natural log (LN) ratios of CHADL found in the cartilage secretome. The healthy cartilage is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significance with p<0.05, ** p<0.01.

Lysyl oxidase-like 2 (LOXL2) is a member of lysyl oxidase (LOX) family and facilitate formation of collagen crosslinks [489, 490]. LOXL2 expression was earlier seen in the chondrocytes at healing fracture and also in growth plate where it aids in chondrocyte differentiation [491]. A significant downregulation of the enzyme was found in the GII intact site secretome (Figure 4.10) in the present study. These lower levels of LOXL2 may be linked to the weaker fibrillar network in cartilage and could also affect the incorporation of any newly synthesised collagen into the collagen network.

Figure 4.10 Scaled natural log (LN) ratios of LOXL2 found in the cartilage secretome. The healthy cartilage is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significant with p<0.05.
Similar to Lourido et al., the present study also detected an upregulated level of fibulin 1 in the lesion site secretome sample (Figure 4.1). Fibulin is a plasma and ECM glycoprotein, which binds to fibronectin and can associate with other fibulin molecules in ECM [492]. It was previously identified in the osteoarthritic synovial fluid [493]. This secretory protein is also reported to be incorporated into extracellular matrix, forming dense fibrillar network [494]. The upregulated level of this protein in GII lesion site may suggest the attempt of remodelling occurring in cartilage.

![Figure 4.1](image)

**Figure 4.1** Scaled natural log (LN) ratios of fibulin-1 found in the cartilage secretome. The healthy cartilage is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significant with p<0.05.

The overall imbalance of these cartilage structure-related and collagen-related proteins in non-healthy cartilages, may be linked to the destructured and re-aggregated fibrillar network (Chapter 2). It is interesting to note that while a set of proteins were upregulated, the others showed the opposite trend as the degeneration progressed.

**4.4.1.2 Mineralization and Bone Formation**

The differential expression of a set of bone-related proteins was found in bovine cartilage secretome in this work. The expression level of these proteins in early cartilage degeneration can provide more information on the OA pathology.

As discussed in previous chapters, a subtle indicator of the early-to-pre OA state in bovine patellae tissue is the formation of microscale bone spicules that emerge from
the cement line into the zone of calcified cartilage (ZCC) [90]. Reported as the evidence that early bone changes take place in conjunction with cartilage changes, these spicules have also been shown to occur in human OA tissue [270].

**Figure 4.12** Scaled ratios of natural log (ln) for proteins found in the cartilage secretome with potential role in mineralization and bone formation. For each protein, its level in healthy cartilage secretome is scaled to 0 in log space and represents “no change”, and others are relative to that. For the post-test * denotes significance with p<0.05, ** p<0.01, *** p<0.001.
SPARC (secreted protein acidic and rich in cysteine or osteonectin) are often suggested to be involved in mineralization of the bone and cartilaginous tissue due to their close affinity towards hydroxyapatite and was also shown to be expressed in hypertrophic chondrocytes [495, 496]. A significant downregulation of SPARC in the lesion site cartilage secretome was observed (Figure 4.12). Previously, SPARC was found to be secreted into culture medium both by freshly harvested cartilage explants and by chondrocytes [497]. Under the influence of IL-1, the secretion of protein was found to be diminished and further activation with growth factor like TGF beta and IGF-1 were able to partially reverse the IL-1 effect on SPARC.

AMBP (Alpha-1-Microglobulin/Bikunin Precursor) was found to be downregulated in the non-healthy cartilage secretome (Figure 4.12), unlike in previous studies by other groups where they found that the synovial fluid showed upregulated levels of AMBP in OA state [320, 498]. But it should be noted that these earlier detections of the upregulated AMBP were from late OA patients. AMBP/Bikunin also inhibits expression of TGF-β1 as well as certain calcium mediated channels [499].

Looking at the lower levels of SPARC and AMBP in non-healthy patella, which is quite contrary to earlier reports, it could be hypothesised that these decreased levels are tissue’s response to contain the degenerative processes at the earliest stages. Whether these levels will be reversed as the degeneration advance is something to be investigated upon. It would be interesting to analyse the severely degenerated grades of bovine tissues to verify these findings.

PDIA3 (Protein disulfide-isomerase) is another protein involved in bone homeostasis. A lower expression of this protein was observed in the GI lesion secretome compared to healthy secretome (Figure 4.12). PDIA3 binds to calcitrol (Vitamin D metabolite) and inhibit chondrocyte differentiation [500]. During endochondral bone formation and cartilage degeneration, the inactive chondrocytes starts to differentiate again [501]. The lower expression of PDIA3 in the lesion sample may suggest that the region favours tissue remodelling.

HIST1H1D encodes the protein Histone H1.3 [502] and is known to modulate skeletal size [503] and height in humans [504]. H1 is a linker histone which interacts with linker
DNA between nucleosomes and aid in condensing DNA material into higher order structures. A higher expression of this protein was found in the GII secretome (both intact and lesion samples) compared to healthy tissues (Figure 4.12). A possible explanation would be the increased metabolic activity occurring in the tissue when the chondrocytes perceive the cartilage distress.

Serum amyloid A protein or SAA3 belongs to the group of apolipoproteins and its expression is increased during the inflammation episode in the tissue [505]. SAA3 is found to inhibit the parathyroid hormone (PTH) signalling in osteoblasts thus affecting the bone formation [506]. A significant decreased expression of the protein was seen in the GII intact secretome (Figure 4.12).

Also, lower expression of TGFBI was observed in the non-healthy cartilage secretome (Figure 4.13). Previous experiments conducted on TGFBI knock out mice showed that TGFBI is involved in bone and cartilage ECM maintenance and have a protective role in cartilage by preventing metalloproteinases from attacking the collagen frameworks [414]. The downregulated expression of this protein in non-healthy samples justifies the degeneration seen in the tissues, as they fail to maintain the anabolic processes in the cartilage.

![Graph of TGFBI expression](image)

**Figure 4.13** Scaled natural log ratios of TGFBI found in the experiment. The protein’s expression in healthy cartilage secretome is scaled to 0 in log space and represents “no change”, and other non-healthy groups are relative to that. For the post-test * denotes significant with p<0.05, *** p<0.001.
These marked differences in the expression of the above mentioned bone-related proteins further evidences the early micro-structural changes occurring in ZCC and subchondral bone, with the intact samples possessing thickened ZCC (Figure 2.8 C) and the lesion site samples having bone spicules disrupting their distal most tidemarks (Figure 2.8 D).

4.4.1.3 Angiogenesis

The bone spicule formation seen at the sites of early degeneration (Figure 4.14) is similar to the increased vascularity seen at the osteochondral junction in OA patients [89]. New bone formation at the sites of early degeneration indicates the pre-OA changes [90], which can precede before the clinical symptoms of OA is evident. Both positive and negative regulators of angiogenesis can contribute to the neovascularization occurring in the newly formed bone spicules.

Figure 4.14 DIC image of a bone spicule growing into the zone of calcified cartilage at the osteochondral junction in the GI intact site. The central vascular canal is visible in the spicule (red arrow). Scale bar ~100 µm.
The analysis of secretome from early degenerated cartilage revealed a series of angiogenesis/vascularity-related proteins which were found be differentially expressed in comparison to the healthy control cartilages (Figure 4.15).

Retinol binding protein-4 (RBP4) can aid in angiogenesis, through its interaction with retinoid signalling and VEGF [507]. It can also stimulate matrix degrading enzymes like MMPs in the tissue and can aid in bone growth through secondary ossification. A significantly downregulated expression of RBP4 was observed in GI lesion samples when compared to the healthy cartilage secretome. Surprisingly, the DIC images of the osteochondral sections from GI lesion sites showed the bone spicules bearing clear vascular canals (Figure 2.9B) invading the ZCC. Considering the microstructural and proteomic data together it can be speculated that the lower expression of RBP4 and BRN in the GI lesion site samples could be a means by which further cartilage degeneration is controlled in the tissue. This downregulation is not upheld when the cartilage advances to GII grade of degeneration (Figure 4.15), so it is imperative to assume that these downregulation of the proteins are restricted to the early degeneration states.

A significantly lower level of brain ribonuclease (BRN) protein was detected in GI lesion cartilage secretome. Bovine BRN was shown to be homologous to human RNase1, with a potential role in vascular homeostasis [508]. The lower level of the protein in the degenerate samples can be an indication of the loss of homeostatic equilibrium in the tissue.

Tenascin C, another pro-angiogenic factor was found to be decreased significantly in the non-healthy secretomes (Figure 4.15). This finding is identical to the previous observation when the whole cartilage was proteomically analysed (Figure 3.16). Others have also shown that the tenascin deficiency progressed during cartilage degeneration in spontaneous and induced OA models [509].
Figure 4.15 Scaled ratios of natural log (ln) for proteins found to have potential role in angiogenesis. For each protein, its level in healthy cartilage secretome is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significant with p<0.05, ** p<0.01, *** p<0.001.

CCL16 which is known to be involved in pathologic angiogenesis [453] was found to be highly expressed in the non-healthy cartilage secretome (Figure 4.15). On the other hand, anti-angiogenic factor thrombospondin-1 was found to be significantly downregulated in the GI cartilage secretomes (Figure 4.15). These evidences suggest that both positive and negative regulators of angiogenesis are altered in the non-healthy cartilages and these alterations could either be a part of the degeneration process or the resultant effect.
4.4.1.4 Catabolic Factors

Interestingly, 14-3-3 protein theta (YWHAQ) was found to be significantly downregulated in all the non-healthy tissue groups (Figure 4.16). This protein have been previously detected in the secretome of human articular chondrocytes isolated from the intact regions of cartilage showing degeneration [220]. 14-3-3 proteins are found to have pro-catabolic activity in the cartilage by stimulating MMP activity [510, 511] and also partake in the bone-cartilage communication during OA process [512].

The lower protein expression of YWHAQ found in the non-healthy group in this work could be due to the relatively early state of the degeneration in the bovine cartilages. Further investigation with severely degenerate cartilages can clarify whether the protein expression will be upregulated with the increased level of degeneration.

Figure 4.16 Scaled natural log (LN) ratios of YWHAQ found in the cartilage secretome. The protein’s expression in healthy cartilage is scaled to 0 in log space and represents “no change”, and other non-healthy groups are relative to that. For the post-test * denotes significant with p<0.05, *** p<0.001.

A significantly increased expression of LAP-like antimicrobial peptide (LAP) was found in the GII intact cartilage secretome (Figure 4.17). LAP belongs to broad spectrum antimicrobial peptides beta-defensin family. β-defensin has been shown to be present in cartilage without any bacterial intervention [513]. β-defensin can be induced by cytokines TNF-α and IL-1. Increased expression of β-defensin was seen in mouse model of early OA but not in advanced stage. They were shown to have a role
in catabolic activity of the cartilage by activating MMPs and downregulating TIMPs (tissue inhibitors of matrix metalloproteinases) and in tissue remodelling processes [514].

![Figure 4.17](image)

Figure 4.17 Scaled natural log (LN) ratios of LAP in the cartilage secretome. The protein’s expression in healthy cartilage is scaled to 0 in log space and represents “no change”, and other non-healthy groups are relative to that. For the post-test * denotes significant with p<0.05.

The relatively opposite trend of YWHAQ and LAP proteins, both with catabolic activity, is notable suggesting how erratic the protein profiles are during early stages of cartilage degeneration, where the cartilage keeps attempting to contain the degeneration.

4.4.1.5 Inhibitors of Catabolic Factors

Osteopontin have been reported to be overexpressed in OA joints and suggested to correlate with the disease severity [515-517]. But there have been studies which reported contradictory results. Dong et al. found a lower level of osteopontin in serum from OA patients compared to the controls [518]. Osteopontin is also found to negatively regulate the MMP-13 expression through integrin receptors [519]. Additionally, in osteopontin-deficient mice, the MMP-13 expression is upregulated during cartilage degradation. These findings elucidate the inhibitory effect of osteopontin on MMP-13 expression and therefore inhibition of the OA progression [520]. The presence of cytokine IL-1β was also found to downregulate the osteopontin expression [521]. In this present study osteopontin (SPP1) was found to be
downregulated in the non-healthy cartilage secretome compared to the healthy tissue secretome. This differential expression was more significant at GII sample secretome (Figure 4.18). This downregulation of SPP1 in non-healthy cartilages appears to be favourable to cartilage degeneration, with GII cartilages having much lower levels of SPP1 than the GI cartilages.

Peptidoglycan recognition protein 1 (PGLYRP1) is 19 kDa protein which binds to peptidoglycan [522]. PGLYRP1 have anti-inflammatory role in tissues and were found to control the induction of pro-inflammatory cytokines in mouse arthritis models [523]. PGLYRP1 gene expression was seen to be upregulated in the RA patients and was suggested to have significant role in inflammation signalling [380]. An upregulation of PGLYRP1 protein was seen in intact site sample and more significantly in GII intact site secretome (Figure 4.18). This could be a protective action of the intact cartilages, on discerning the adjacent tissue degeneration.

Several proteins involved in classical complement pathway were found to be differentially expressed in the non-healthy secretome compared to healthy samples. Complement cascade is known to be triggered in an osteoarthritic cartilage, by various degrading ECM components. Complement activation leads to formation of membrane attack complex (MAC) on chondrocytes, which induce the production of inflammatory mediators, catabolic enzymes and other complement components, which lead to further joint damage [455]. An upregulation of complement component 4 was detected in the non-healthy secretome, the expression of three components (A, B and C) of c1q were downregulated when compared to healthy controls (Figure 4.18). This suggests that the complement activation is altered during early cartilage degeneration.
Figure 4.18 Scaled ratios of natural log (ln) for proteins found to have potential role in inhibiting catabolic activities in cartilage secretome. For each protein, its level in healthy cartilage secretome is scaled to 0 in log space and represents “no change”, and other are relative to that. For the post-test * denotes significant with \( p<0.05 \), ** \( p<0.01 \).

Tissue inhibitors of matrix metalloproteinases (TIMPs) are the endogenous inhibitor of MMPs and ADAMTs in the cartilage. A significantly reduced expression of TIMP-1 was seen in the non-healthy cartilage secretome (Figure 4.18). This decrease is progressive with the level of degeneration seen in the tissue and suggests that with
increased degeneration anabolic processes are unable to overturn the damage taking place.

The novel protein predicted WFDC18-like (LOC100847724) was found to be significantly upregulated in the non-healthy cartilage secretome especially at the intact sites (Figure 4.19). This protein is yet to be characterized at the protein level and this may be the first time WFDC18 is reported in relation with cartilage secretome and osteoarthritis.

![Figure 4.19](image)

**Figure 4.19** Scaled natural log (LN) ratios of WFDC18 in the cartilage secretome. The protein’s expression in healthy cartilage is scaled to 0 in log space and represents “no change”, and other non-healthy groups are relative to that. For the post-test ** denotes significant with p<0.01.

It contains WAP four disulphide core (WFDC) domain and the three diverse functions associated with WFDC proteins are

(i) Protease inhibition
(ii) Anti-infective activity
(iii) Immunomodulatory activity [432].

The above stated functions of WAP proteins suggests that the higher expression of WFDC18 in the intact site cartilage secretome is a protective action by which the tissue is trying to combat the impending degeneration. A similar trend was seen in previous chapter where whole cartilage tissues were analysed (Figure 3.20) and it was further validated by MRM assay. The reinforcement of the result with secretome samples underscores the calibre of this protein as a potential biomarker candidate.
4.4.2 Comparison of Cartilage Secretome from the Intact and Lesion site of Degenerate Patellae

Given the extensive work in the cartilage research, the very early OA state, where only subtle micro-level cartilage and bone changes have begun, has yet to be studied. This is largely due to the difficulty in identifying and obtaining cartilage tissue that is in the early-to-pre clinical state of OA. Squire et al. (2003) stressed the importance of investigating early OA, and did so by studying tissue with degenerative focal lesions seen in aging and compared them with the same characteristics as those observed in OA [401].

The bovine patellae cartilage with GI (mild) positive India ink staining [281], is a very early state of degeneration within the OA process [266], one where there is full thickness cartilage and a gross appearance that shows no overt surface disruptions. In such tissue, the gross appearance of the cartilage is no different from the healthy tissue. However, while India ink washes off healthy cartilage tissue, in the GI grade bovine patella tissue, there is mild uptake of the ink leaving a slight stain. Such positive staining and its intensity are further correlated with ultrastructural fibrillar-scale network destructuring [90, 266-271]. Only detectable via microscopy, this destructuring refers to the fibrillar-level disconnecting of the highly inter-connected network of type II collagen fibrils that constrain the high swelling potential of the hydrophilic proteoglycans. This early fibril-level de-structuring in bovine GI tissue has been suggested as a preceding event to further larger-scale matrix disruption, and analogous to very early stages of human OA [270].

Therefore in this study, the secretome protein profiles of bovine cartilage explants from sites of localized GI (mild) to GII (moderate) degeneration versus adjacent sites of intact tissue were compared. Nine proteins were found to significantly vary between the intact and lesion cartilage explants in the GI patellae and 11 were found to be differentially expressed between these sites in the GII patellae.

Interestingly, this finding is contradictory to the earlier observation (Chapter 3), where the analysis of whole cartilage tissue from GI intact and GI lesion sites detected more number of proteins to be differential in comparison to tissues from GII intact and GII
lesion cartilages. It is hypothesised that the secretome of GII cartilage is a reflection of more prominent loss (secretion) of proteins from the explants and in comparison, the GI cartilage secretome is the manifestation of a relatively lower secretory pattern. The following discussion encompasses the potential roles of these site-specific proteins in the early microstructural changes observed in the cartilage.

4.4.2.1 ECM organization/ Cartilage Structure

Based on the histology work and DIC imaging (Chapter 2), it was found that the intact sites of cartilage (from both GI and GII patellae) retained an undisturbed surface layer with uniform toluidine blue staining whereas the lesion site cartilage represented various surface disruptions ranging from minor fibrillation to cleft up to the middle zone and a slightly less intense staining with toluidine blue at the tangential layer (Figure 4.3).

The impact of these early microstructural alterations on the secretion pattern of proteins was assessed in this current work. A set of proteins, often associated with the ECM structural organization, were found to be differentially expressed in the intact and lesion site cartilage explants (Figure 4.20).

On comparing, COL12A1 and COL15A1 were found to be significantly upregulated in the lesion site cartilage secretome to their corresponding intact site secretome. As found previously by Lourido et al., the increased expression of these collagen molecules by the damaged cartilage explants may be an indication of catabolic activities occurring in cartilage ECM [226] or it could be an early phase change wherein the chondrocytes are synthesising more collagens to compensate the loss of ECM proteins in cartilage [524].
Figure 4.20 Scaled ratios of natural log (ln) values for proteins found to have potential role in ECM organization and cartilage structure. For each protein, its level in GI intact site cartilage secretome is scaled to 0 in log space and denotes “no change”, and other are relative to that. For the post-test * denotes significant with p<0.05, ** p<0.01.

CILP1 and CILP2 were found to be significantly upregulated in the intact region cartilage secretome especially in the GII patellae. CILP is a secretory protein which
has been found to be upregulated during early OA and with age [404, 525]. Contrary to the current finding, a previous analysis of the total content of CILP from the normal appearing cartilage (intact) and fibrillated (lesion) cartilage found that the fibrillated cartilages possessed and also synthesised more CILP compared to normal appearing cartilages [405]. But it should be noticed that the regions (fibrillated and non-fibrillated) were from different patients increasing the inter-sample variability.

Fibulin (FBLN1), a secreted glycoprotein, is known to form ECM fibrillar network by self-associating and also by binding to the ECM fibronectin [492, 494]. The secretome of the lesion site samples had a higher expression of fibulin compared to the intact sites, with a more significant upregulation in secretome from the GII lesion site. This upregulation in the expression of fibulin at the GII site could be either due to an increased synthesis of the protein to repair the collapsed fibrillar network or alternatively, could be the result of the degradation itself.

Lumican and PRELP belong to SLRP family and they interact with collagens and modulate fibril formation [444]. A higher expression of LUM and PRELP were observed in the lesion site secretome compared to the intact site secretome, suggesting an attempted ‘repair’ process in the region [526]. Lumican was found to be progressively upregulated in the secretome from GI intact to GI lesion through GII intact and finally highest level in GII lesion. This may suggest that the tissue deciphers the degeneration occurring around it and responds accordingly.

Vimentin, a cytoskeletal protein found in chondrocytes, was seen to be downregulated in the lesion site secretome. This observation is contrary to Lourido et al. study, where they found increased vimentin level in the lesion site secretome [226]. However, this imbalance in the level of vimentin at the two structurally different regions in a patellae indicate a disruption in normal cartilage homeostasis due to loss of cells’ cytoskeletal interaction with the matrix [527]. But, the slight upregulation of this protein in the secretome of GII samples compared to GI samples in the present study, indicates that this upregulation may continue further as the degeneration advances.
4.4.2.2 Energy Metabolism

The metabolic synthesis of glucose for energy and proteoglycan synthesis in chondrocytes takes place by anaerobic pathway [528]. Glucose-6-phosphatase isomerase (GPI) and enolase (ENO1) are enzymes which are involved in the anaerobic glycolytic pathway in a cell. GPI helps to catalyse the reaction, where glucose-6-phosphate is interconverted into fructose-6-phosphate. Enolase catalyses the reversible conversion of 2-phosphoglycerate and phosphoenolpyruvate (PEP). The present study identified an initial downregulation of the glycolytic enzymes at the GI lesion site secretome but a significant upregulation of GPI and enolase in the secretome of cartilage explants from the GII lesion site (Figure 4.21). This upregulation support the earlier observations, where enolase was seen to be increased in the lesion site samples [223, 226]. This upregulation of glycolytic enzymes suggest increased energy demand at the regions where cartilage degradation have already advanced. Interestingly, another work have previously shown the downregulation of glycolysis related enzymes in the OA chondrocytes when compared to normal chondrocytes [169].

The analysis of the present work reveals that ENO1 and GPI are downregulated in tissues where the degeneration is at its earliest phase. As and when this degradation intensifies, it is probable that the chondrocytes detect the catabolic stress in the tissue. During degeneration, as the oxidative gradient maintained in the cartilage (6% oxygen tension at the surface layer and 1% the deep layer) [529] is disrupted and this may trigger the altered levels of energy-related metabolic proteins in cartilage.
**Figure 4.21** Scaled ratios of natural log (ln) values for ENO1 and GPI in cartilage secretome. For each protein, its level in GI intact site cartilage secretome scaled to 0 in log space and denotes “no change”, and other are relative to that. For the post-test * denotes significant with p<0.05.

### 4.4.2.3 Bone Metabolism Related Proteins

TGFBI was upregulated in the lesion site secretome compared to their corresponding intact site samples (Figure 4.22). These observations mirror the earlier proteomic analysis of whole cartilage tissue from the respective sites. TGFBI is induced by TGF-beta and maintains bone and cartilage ECM [414]. As it is also involved in endochondral ossification [415]. The comparative higher expression of TGFBI in the lesion site secretome suggests that it may promote of endochondral bone formation at these sites.

PDIA3, involved in bone homeostasis was found to be downregulated in lesion samples. HIST1H1D, known to modulate skeletal size [503] and F13A1 (Coagulation factor XIII A chain), which is reported to increase in the hypertrophic chondrocytes and in the regions where mineralization occur [376, 530], were found to be downregulated in the GI lesion site secretome (Figure 4.22). These downregulation is presumed to be an initial occurrence, as they were seen to be comparatively upregulated in GII cartilage secretomes suggesting some form of bone remodelling mechanism taking place in these tissues.
Figure 4.2. Scaled ratios of natural log (ln) values for proteins found to have bone-related functions. For each protein, its level in GI intact site cartilage secretome is scaled to 0 in log space and denotes “no change”, and other are relative to that. For the post-test * denotes significant with \( p < 0.05 \), ** \( p < 0.01 \).

4.4.2.4 Angiogenesis

RBP4, THBS1, and TNC were found to be significantly altered in the secretome of various degenerated cartilage groups (Figure 4.23). The lack of familiar expression pattern among these three angiogenesis-related proteins in the examined cartilage group is noteworthy and indicates a highly unstable homeostatic mechanism taking place, at the very early stage of cartilage degeneration. RBP4 and TNC are pro-angiogenic molecules which were found to be significantly downregulated in the GII secretome whereas the anti-angiogenic factor thrombospondin 1 was upregulated in the GII cartilage secretome. Though it is difficult to pinpoint the exact mechanism by which these proteins contribute to angiogenesis, while correlating the microstructural images of respective sites (Figure 2.9), it is possible that these are counter-mechanism by which the cartilage is trying to control the invasion of vascular system from further deteriorating the tissue state.
Scaled ratios of natural log (ln) values for proteins found to have potential role in angiogenesis. For each protein, its level in GI intact site cartilage secretome is scaled to 0 in log space and denotes “no change”, and other are relative to that. For the post-test * denotes significant with $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

### 4.4.2.5 Catabolic and/or Inflammation Factors

LAP proteins belonging to β-defensin family, which have role in in tissue remodelling processes [514], were found to be upregulated in the intact cartilage secretome compared to adjacent lesion site secretomes (Figure 4.24). Fibrinogen alpha chain precursor (FGA) is an inflammatory marker [531] and is known to cause cartilage damage [532]. A significant upregulation of the FGA was seen in the GII lesion site secretome compared to corresponding intact site secretome. The significant differential level of expression of LAP and FGA in GII grade samples indicate that as the health of the tissue deteriorates, there is significant alteration in secretory pattern of tissues which are affected and which lies next to the affected area. Alternatively, these levels could also represent the degenerated proteins in these regions. Whether these inflammatory mediators contribute towards the ‘healing’ or promote further damage is unclear from the present results and need to be investigated further in order to understood the early inflammatory mechanism taking place in the tissues.
Figure 4.24 Scaled ratios of natural log (ln) values for proteins found to have potential role in cartilage catabolic activities and/or inflammation. For each protein, its level in GI intact site cartilage secretome is scaled to 0 in log space and denotes “no change”, and other are relative to that. For the post-test * denotes significant with p<0.05.

4.4.2.6 Response against Catabolic Factors

Extracellular superoxide dismutase (ECSOD) scavenges for the reactive oxidative species (ROS) which cause matrix dysregulation in cartilage [533] and was found to be lowered in the osteoarthritic joints. Similarly, a significantly lowered level of this protein was found in lesion site cartilage secretome compared to corresponding intact site secretome (Figure 4.25) and reflects how the lesion site samples are left vulnerable to the catabolic factors like ROS.

In addition, PGLYRP1 which have anti-inflammatory role in cartilage [523], was seen to be downregulated in the lesion site secretome. Similarly, complement component 4 was found to be decreased in the lesion secretome compared to the intact cartilage secretome. The lowered expression of these proteins suggest that once degeneration advances, the tissue is left more susceptible to the catabolic factors leading to irreversible damage to cartilage structural and functional components.
Figure 4.25 Scaled ratio of natural log (ln) values for proteins found to respond to cartilage’s catabolic activities and/or inflammation. For each protein, its level in GI intact site cartilage secretome is scaled to 0 in log space and denotes “no change”, and other are relative to that. For the post-test * denotes significant with p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

SERPINF1 or pigment epithelium derived factor (PEDF) is an antiangiogenic factor, which controls the vascular invasion into the cartilage [306]. A significant increase of SERPINF1 protein in the lesion site secretomes (Figure 4.25) was found and this corresponds to the earlier observation by Lourido et al. where they detected a higher expression of PEDF in the wounded zone (lesion) cartilages [226]. Also seen was the increased expression of C4 complement factor in the GII secretome and suggests heightened complement activation in the degenerated tissues.

The differential level of WFDC18-like protein between the intact and lesion site cartilage secretome (Figure 4.26) was compared. Consistent with the previous analysis of whole cartilage tissue sites (Figure 3.31), the expression of this potential anti-protease protein was downregulated in the lesion cartilage secretome. This decrease may allude to the disruption of cartilage integrity at the lesion sites. Further, it is
suggested that the intact sites, the tissue still uphold its structural constitution because of the protective proteins like WFDC18. But again this is just a hypothesis assuming WFDC18 have a protective role in cartilage. This needs to be verified by further investigations involving WFDC18 gene knockout mice and identify its potential role in cartilage and subchondral bone.

**Figure 4.2** Scaled natural log (LN) ratios of WFDC18 like protein in cartilage explant secretome. The GI intact cartilage secretome protein level is scaled to 0 in log space and denotes “no change”, and other are relative to that. For the post-test ** denotes significance with p<0.01.
4.4.3 Biomarker Panel

On the basis of the cartilage secretome study, a set of proteins were identified to be differentially altered during the early cartilage degeneration. These proteins, secreted into the medium during the culture period, were found to associate with cartilage and bone-related functions. Of much significance were those proteins, which were found to be differentially present in the secretome from intact regions of GI patellae (where the cartilage degenerations were particularly mild). These changes could potentially represent the pre-to-early OA state, which is often left unexplored.

Presented in Table 4.10 is a panel of 17 proteins which were differentially present in GI cartilage secretome compared to the healthy cartilage secretome. Of these seventeen proteins, WFDC18-like protein, COMP, COL2A1, COL12A1, THBS1, PCOLCE and TGFBI were identified as potential biomarker in earlier cartilage whole tissue studies (Table 3.15), highlighting their importance in early cartilage degeneration. These proteins could help us to capture the elusive pre-OA state and should be investigated in future works involving other early animal model (spontaneous or invasive models) or ideally using human samples.
Table 4.10 Potential biomarkers identified in cartilage secretome studies.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Post hoc analysis</th>
<th>Fold change (GI Intact/healthy)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Higher level in GI intact</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WFDC18 WAP four-disulfide core domain protein 18-like</td>
<td>0.0028</td>
<td>2.05</td>
</tr>
<tr>
<td>COMP Cartilage oligomeric matrix protein</td>
<td>0.0048</td>
<td>1.51</td>
</tr>
<tr>
<td><strong>Lower level in GI intact</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YWHAQ 14-3-3 protein theta</td>
<td>0.0062</td>
<td>0.59</td>
</tr>
<tr>
<td>TNC Tenascin</td>
<td>0.0143</td>
<td>0.59</td>
</tr>
<tr>
<td>CLEC3A C-type lectin domain family 3 member A</td>
<td>0.0014</td>
<td>0.57</td>
</tr>
<tr>
<td>COL12A1 Collagen alpha-1(XII) chain</td>
<td>0.0087</td>
<td>0.57</td>
</tr>
<tr>
<td>LUM Lumican</td>
<td>0.0396</td>
<td>0.56</td>
</tr>
<tr>
<td>CHADL Chondroadherin-like</td>
<td>0.0026</td>
<td>0.53</td>
</tr>
<tr>
<td>THBS1 Thrombospondin-1</td>
<td>0.0021</td>
<td>0.53</td>
</tr>
<tr>
<td>PCOLCE Procollagen C-endopeptidase enhancer</td>
<td>0.0037</td>
<td>0.52</td>
</tr>
<tr>
<td>COL3A1 Collagen, type III, alpha 1</td>
<td>0.0116</td>
<td>0.51</td>
</tr>
<tr>
<td>AMBP Protein AMBP</td>
<td>0.0038</td>
<td>0.43</td>
</tr>
<tr>
<td>TGFBI LOC539596 protein</td>
<td>0.0001</td>
<td>0.4</td>
</tr>
<tr>
<td>COL2A1 Collagen alpha-1(II) chain</td>
<td>0.033</td>
<td>0.39</td>
</tr>
<tr>
<td>C1QB Complement C1q subcomponent subunit B</td>
<td>0.0135</td>
<td>0.38</td>
</tr>
<tr>
<td>C1QC Complement C1q subcomponent subunit C</td>
<td>0.0056</td>
<td>0.3</td>
</tr>
<tr>
<td>C1QA Complement C1q subcomponent subunit A</td>
<td>0.0085</td>
<td>0.28</td>
</tr>
</tbody>
</table>
4.5 Conclusions

- The culturing of cartilage explants in serum-free DMEM for a period of 96 hours without any cytokine stimulation, was adopted for the present study.
- On comparing the protein profiles between two control cartilage secretomes from the healthy patellae, only 4 proteins were found to show differential variations.
- A set of 53 proteins were found to significantly vary between the healthy and non-healthy cartilage tissue secretomes.
- The secretome from GI intact cartilage showed significant differential expression of 17 proteins. These proteins could be the representation of pre-to-early OA state.
- Considerable variation was seen in the secretomes from degenerated and non-degenerated cartilage of the patellae. A set of 68 proteins were found to be differential expressed in intact and lesion samples in the present study.
- Contrary to earlier finding with the bovine whole cartilage tissues, in secretome analyses, the patellae with moderate level of degeneration (GII) were found to have more protein differences than patellae with mild level of degeneration (GI). This may suggests that as the cartilage degeneration advances, the loss (or secretion) of proteins from the tissue is more acute. Hence, the earlier whole tissue analyses from GII samples represent a cartilage state, where the protein loss have already set in. This points to the importance of analysing the early-OA state of the cartilage samples. The increased secretion/loss of proteins from GII cartilages could be further facilitated by the fibrillar network disassembly as seen in Chapter 2.
- This study also suggests that secretion pattern of the intact region could be the relatively early stage of the disease (pre-OA) whereas the lesion tissue secretome is the representation of the disease state where the tissue’s protein secretion is relatively higher.
CHAPTER 5

MALDI-IMAGING MASS SPECTROMETRY

5.1 Introduction

Matrix Assisted Laser Desorption Ionization- Imaging Mass Spectrometry (MALDI-IMS) is an evolving technique which facilitates measurement of the spatial distribution of various molecules across the sample surface, based on their mass to charge (m/z) ratios [534]. This powerful label free method allows for a comprehensive analysis of a large number of m/z channels in a single experiment [535, 536].

MALDI-IMS results in molecular images, which provide visual information regarding the biological/biochemical specimens. Over the years, investigation of on-tissue analyte distributions have helped to add to our knowledge of various biological processes occurring in different regions of a tissue. Though traditional methods such as histological staining and immunohistochemistry provide similar information, their applications have the limitation of availability of specific antibodies and also that only a small number of analytes can be identified in a single experiment. Using IMS, region-specific molecular distributions can be studied directly from fresh or fixed tissues [537-541]. The most widely investigated analytes in IMS are peptides, proteins, lipids, metabolites and drugs to provide visual information in the form of molecular images of samples [542].

MALDI-IMS can be used to image an entire sample or can be used to image only regions of interest, after histological analysis or other imaging techniques. Either approach provides region-specific m/z information which can be used as part of a diagnostic analysis or therapeutic approach.

MALDI-IMS can also be used as a discovery tool or to focus on specific analytes of interest. For most discovery-related investigations, the whole sample is imaged to study the distribution of (e.g. disease-related) molecules in tissue, whereas histology-
directed molecular imaging often addresses a specific question, by analysing a specific molecule or region in the sample [542, 543].

5.1.1 Basic Workflow of MALDI-IMS

IMS has come a long way since the development of Secondary Ion Mass Spectrometry (SIMS) imaging [544, 545], which has a similar working principle. Briefly, the samples (thin tissue sections) are coated with matrix molecules which help in enabling their desorption from the sample surface on application of a UV laser, ionising them into gas phase [546]. These ions are then separated based on their mass-to-charge, and detected in the mass spectrometer [547]. During imaging, mass spectra are collected from the tissue surface at various spatial points, in an x/y raster pattern. The resultant summary mass spectrum shows the relative abundance of the ionisable molecules at each analysed spot [535].

5.1.2 Important Considerations for MALDI-IMS

A typical MALDI-IMS instrument has the following three components [542]:
(a) MALDI source and mass analyzer
(b) IMS data acquisition software
(c) Data visualization and analysis software

The main features which must be considered for IMS analysis are the following [542]:

1. Spatial Resolution

Spatial resolution needs to be optimized for the acquisition of high quality images. At higher spatial resolutions, the ablated regions are smaller and sensitivity is therefore lower, such that only analytes that are abundant or ionise well, are detected. The higher resolution also produces large data sets, making data management difficult. The range of laser focus for normal commercial MALDI instruments, the main determinant of spatial resolution, varies from 10 µm to 200 µm.

2. Tandem MS performance

With appropriate instrumentation, MALDI-IMS can fragment a specific m/z, and when coupled with a second mass analysis step, an ion fragmentation pattern is detected and analysed, to confirm the identity of the parent molecule.
3. Mass accuracy
An increase in mass resolving power often results in higher mass accuracy. Accurate mass measurement can help in the identification of precursor ions during tandem MS.

4. Throughput
The speed of acquisition of images has improved considerably in recent years, largely due to the increased laser repetition rates and associated detection electronics. High throughput of data is desirable in MALDI-IMS.

5. Calibration
A shift in m/z can cause difficulty in correctly identifying the peak areas. Therefore for accurate molecular mass measurement calibration using standard molecules of known masses is often performed.

5.1.4 Proteomics in Cartilage Research

Little is known about the early degeneration occurring in cartilage. Protein markers are yet to be identified, which could regionally differentiate the degenerated cartilage site from the adjacent intact site. This understanding of the regional variations occurring in a compromised cartilage, could shed more light on the nature and progression of OA pathology and is essential, if effective curative strategies are to be developed.

Most proteomics research generates oversimplified protein profiles, as they fail to comprehend the complexity associated with individual tissue morphology [548]. The cartilage tissue structure varies across the diseased joint, with some regions showing early cartilage structural degeneration while others appear intact and pristine. In many cartilage investigative works, the highly degenerated cartilage regions are often taken as the diseased sample. In these studies, the comprehensive proteomic profile is analysed using various proteomics techniques, yielding information regarding the protein markers, which helps to differentiate between the healthy and the diseased tissue [229]. In recent times, researchers have focussed on understanding the proteomic implications of various region-specific changes in cartilage [223, 225, 226], but they still provide little information regarding the spatial distribution of these
proteins in tissue. To address this limitation, proteomic techniques which can provide the spatial information of OA cartilage, will prove beneficial.

So far the application of MALDI-IMS in analysing cartilage is underutilized. Using the technique, the distributions of lipids [549], peptides and proteins [550] in healthy and OA human cartilage have been compared and studied. In addition, markers for aging and OA cartilage were identified in horses [551] and more recently the N-glycome of cartilage and subchondral bone was spatially resolved in the knee of OA patients [552]. In their work, Cillero-Pastor et al., [550] investigated the spatial distribution of proteins in human control and OA articular cartilage. They found that OA-related peptides showed a stronger presence in the deep zone of the articular cartilage. Their research also showed the exclusive presence of COMP and fibronectin in OA tissues.

In this study, the region-specific peptide variations in bovine patellae showing early signs of cartilage degeneration, were investigated. MALDI-IMS was used to map the distribution of a variety of different proteins, within normal and degenerated cartilage. Further, advanced MALDI-IMS analysis software was applied to differentiate between the intact regions and the degenerated regions in these patellae.

5.2 Methodology

For convenience, the term ‘healthy’ is used in this text to refer the samples from G0 patellae and ‘non-healthy’ to broadly address samples from GI and GII patellae.

5.2.1 Tissue Source

In an earlier study [266], bovine patella was used to describe the structural changes associated with the pre-to-early OA state, showing how well it fits within the structural transformation of mild to full blown OA in human joints.

All cartilage samples used in this work, were harvested from the bovine patellae obtained from 14 adult cows. Soon after slaughter, based on visual inspection to identify presence and extent of surface fibrillation, each patella was graded according to the Outerbridge’s classification for cartilage degeneration [281] and stored at -20 °C until further use. Four patellae that were classified as control (i.e. G0), 5 mildly
degenerated (GI) and 5 moderately degenerated (GII) were used in this study (Figure 5.1).

**Figure 5.1** Representative bovine patellae showing the sampling sites used in IMS– (A) Control patella (G0); (B) GI patella; and (C) GII patella. India ink staining to reveal the extent of fibrillation showed G0 (Control) patella contained no surface ink retention, GI patella is stained lightly revealing mild degeneration at the lesion site and GII patella is seen to have slightly intense staining as the ink is retained in the moderate level degenerations at lesion site.

### 5.2.2 Histology

For histological analysis cartilage tissues were fixed in 4% para-formaldehyde overnight. The tissues were then washed with 2 mL of PBS for 10 mins (3x) and stored in 70% ethanol at 4 °C until histological analysis. The tissues then underwent standard histological processing [266]. Briefly, the cartilage samples were microtomed to obtain 5 µm-thick sections. The sections were mounted on standard glass slides. After rehydrating the sections in graded solutions of ethanol and finally with water, these were then stained in toluidine blue for 10 mins and were imaged using bright field transmission microscopy. The level of degeneration was assessed using the Mankin cartilage grading system [288], without considering the scoring for tidemarks. No tidemark or subchondral bone was present in the samples used in the current study.

### 5.2.3 Sectioning of the Sample for MALDI-IMS

A comparable pair of full thickness cartilage samples was punched out from each of the 4 normal patellae (Figure 5.1 A) using an 8 mm biopsy punch, in order to determine whether or not there existed a regional variability in protein levels across the normal joint surface – referred to as controls. Similarly, from each non-healthy (GI and GII) patella, pairs of cartilage discs were punched out from the lesion region and from an adjacent intact region (Figure 5.1 B and C).
8 µm-thick sections (cut through the cartilage depth- superficial layer to deep zone) were collected, from each of the 28 frozen cartilage plugs, using a disposable blade stage-equipped cryostat (Leica CM3050 S, Germany) with both cryostat and object temperature at -20 °C. Cartilage was pared off from the underlying bone beforehand, to avoid microtome blade damage and subsequent difficulty with sectioning.

A typical embedding medium for cryosectioning, OCT, is often used to attach frozen tissue to chucks, but is known to produce strong ion signal in the low mass range and can suppress other ions [553, 554]. To avoid any possibility of contamination of the relatively thin cartilage tissue (~2-3 mm), cartilage sample was mounted using a binder’s clip. The frozen tissue block was first clipped on a binder clip and after removing its handles, the binder clip’s underside was affixed on the cryo-microtome chuck using OCT (Figure 5.2). This solved the OCT contamination issue as well as helped to obtain neat serial sections from the cartilage without damaging the blade. The cutting temperature was maintained at -20 °C, and after careful optimization, 8 µm thickness was chosen as ideal for further tissue sectioning.

![Figure 5.2 Mounting of cartilage tissue on the chuck using binder clip for cryo-sectioning the cartilage samples without OCT contamination.](image)
5.2.4 Mounting Cartilage Sections for MALDI-IMS

A thaw mounting technique was used to mount the cartilage sections onto the slides, wherein the slides were first brought to the same temperature as that of the sections (-20 °C) and after careful placement of the cartilage section on the cold slide surface as flat as possible, the tissue was thaw mounted by warming the cold glass slide beneath each section, with the fingers [555].

The surface onto which the tissue sections are mounted must be conductive, in order to accelerate the ions in the source of MALDI-TOF mass spectrometer [542]. Initial attempts using gold plated targets to mount the cartilage sections during optimization process were unsuccessful, as the tissues did not adhere well onto the plates, perhaps due to their unique tissue properties. Therefore, Indium-tin oxide (ITO) coated glass slides (HST Inc., Newark, NJ, USA) were used for further MALDI-IMS work as they were conductive as well as appeared to adhere the cartilage sections better. Each glass slide was used to mount 3-4 cartilage sections from each of the two different regions (intact and lesion) from a patella.

5.2.5 Pre-treatment of Samples

5.2.5.1 Tissue Washing

Most interfering species in any biological sample are physiological salts, small molecules, and lipids. These molecules can form adducts with biological components of interest and suppress their ionisation. To increase the sensitivity of the image, these interfering molecules must be removed before any further processing [555].

In an optimal washing procedure, the interfering molecules are removed from the tissues, while causing the least variation to the sample's molecular distribution. In the present experiment, the wash was standardized to 30 s in 100% ethanol, 30 s in 70% ethanol, 2 min in Carnoy's fluid (100% ethanol (60%), chloroform (30%) and acetic acid (10%)) and finally 30 s in 100% ethanol. Carnoy's fluid helps to delipidate the sections [556, 557].
After vacuum dessicating the cartilage sections for 30 mins following collection, the cartilage sections were rinsed sequentially in the 4-step bath. The samples were then dried completely in vacuum desiccator for 15 mins or more until further use.

5.2.5.2 On-tissue digestion

Trypsin digestion of the cartilage samples was done before imaging them, since the undigested cartilage tissue showed few signals of poor quality in the spectra (data not shown). Due to the higher cost of trypsin and unavailability of appropriate loop volumes to make trypsin spraying cost effective, instead of automated spraying of the trypsin as reported before [550, 551], in the present work the cartilage sections were incubated with 50 ng/µl of trypsin (proteomics grade, Sigma, T6567) soaked filter paper [558]. Ten microliters of the trypsin solution was applied on a clean surface, a small piece of filter paper (Advantec, grade 2, size 42.5 mm) was used to adsorb the trypsin solution and it was immediately placed on the cartilage section and lightly pressed to obtain a uniform contact of trypsin on the tissue. Making sure that the sections were completely covered by the trypsin soaked filter paper, the entire system was maintained in a moist chamber at 37 °C. After several trials, 5 hr incubation was deemed optimal for the cartilage sections trypsin digestion, since it resulted in good quality peptide mass spectra. After in situ digestion, the filter paper was carefully removed from the top of each cartilage section, while taking care not to dislodge the tissue off the slide and then the samples were left to dry in a vacuum desiccator for 10 mins. This was followed by matrix application on the digested tissues.

5.2.6 Matrix Selection and Solvent Conditions

The commonly used MALDI-matrices are shown in Table 5.1 [542]. Choosing the right solvent is equally important as choosing the right matrix. The analytes need to be soluble in the solvent and uniform recrystallization should occur after matrix application. The amount of organic solvent to be used and its pH are important aspects of matrix preparation as it can influence the diffusion of analytes across the sample surface. CHCA matrix was chosen for the present study and a series of acetonitrile gradients with 0.1% TFA tested for increasing the quality of spectra. The final
optimized matrix preparation for the present study involved dissolving 10 mg/ml of CHCA in 70% acetonitrile and 0.1% TFA.

Table 5.1 Commonly used matrices in MALDI-IMS†

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Common Analytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,5-dihydroxybenzoic acid (DHB)</td>
<td>peptides, proteins, lipids, drugs</td>
</tr>
<tr>
<td>α-cyano-4-hydroxycinnamic acid (CHCA)</td>
<td>peptides, proteins, lipids, drugs</td>
</tr>
<tr>
<td>3,5-dimethoxy-4-hydroxycinnamic acid (SA sinapinic acid)</td>
<td>proteins</td>
</tr>
<tr>
<td>3-hydroxypicolinic acid (3-HPA)</td>
<td>peptides, oligonucleotides</td>
</tr>
<tr>
<td>2,4-dinitrophenylhydrazine (2,4-DNPH)</td>
<td>peptides</td>
</tr>
<tr>
<td>4,6-trihydroxyacetophenone (THAP)</td>
<td>lipids, oligonucleotides, drugs</td>
</tr>
<tr>
<td>2,6-dihydroxyacetophenone (DHA)</td>
<td>lipids</td>
</tr>
<tr>
<td>1,5-diaminonaphthalene (DAN)</td>
<td>lipids</td>
</tr>
</tbody>
</table>

† (Reproduced with permission from [542]. Copyright (2013) American Chemical Society).

5.2.7 Matrix Application for Imaging

The initial experiments were based on manual spraying of matrix on the tissues using a glass reagent sprayer. This lead to over-wetting of the tissues at times and difficulty in reproducing the data. Matrix application by vacuum sublimation [559] was attempted, but the resultant images obtained were not of very high quality. Fortunately, during the course of present work, an automated matrix solution sprayer (TM sprayer (HTX Technologies)) was obtained, allowing computer controlled, reproducible matrix spraying conditions. The matrix is sprayed as a controlled fine mist and the following conditions were used to apply the matrix uniformly across the samples: Flow Rate - 200 µl/min, Spray Nozzle Velocity - 700 mm/sec, Spray Nozzle Temperature - 75 °C, Track Spacing - 4 mm, Number of Passes - 6. The program was standardized and saved and used throughout MALDI-IMS data collection.
5.2.8 MALDI-IMS

For the present work, MALDI-IMS was performed on an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in positive ion reflector mode. The instrument was equipped with a 2 kHz Smartbeam II™ UV MALDI laser and 60–70 µm laser beam size. All samples were imaged in replicates of two.

5.2.8.1 Data Acquisition

FlexImaging™ 4.1 software from Bruker was used for MALDI-IMS data acquisition in the present work. This software package allows for MALDI-IMS data acquisition as well as offering MALDI image production and limited analysis tools. To set up acquisition, an optical image of the specimen mounted on a slide is matched with the sample as it sits in the x/y stage in the MALDI source, using three teaching points. The region to be imaged is specified in flexImaging, and when begun, data acquisition is automated, with instrument parameters set using the instrument control program flexControl (version 3.4). The following measurement settings were used for imaging: Detection range of m/z 700-3500, 150 laser shots per spot, raster spacing of 150 µm. An external calibration was done using peptide calibration standard (Bruker Daltonics) before each imaging data acquisition run.

5.2.8.2 Data Pre-processing

The spectral data is processed first to remove noise that could affect the relevant m/z signals [560-563].

The data pre-processing involves the following [542]:

1. **Baseline correction**

   At the lower mass ranges in a spectrum, the chemical noise is more prominent. So, certain algorithms are built in to remove these without disturbing the actual relevant information.

2. **Data normalization**

   Total ion count method was used to normalize the spectral data in all experiments.
3. **Spectral Realignment**

Sometimes small variations in the experiment can bring about slight shifts in m/z which can make further analysis difficult. Therefore, a common calibration peak list can be employed to align the peaks correctly [562].

4. **Peak detection**

Following spectral realignment, manual correction of the peaks must be done, if needed.

The data acquired in this work underwent spectral realignment using an internal peptide calibrant list obtained from LC-MS/MS analysis of bovine cartilage samples. A pre-installed algorithm (*PAC_ExternalCalibration.FAMSMethod*) was used to apply the new internal peptide peaks automatically to each spectrum in the raw imaging data. These realigned spectra were used for further analysis.

5.2.8.3 **Data Analysis Using SCiLS Software**

The aligned data files were imported into SCiLS Lab software (SCiLS Lab 2015b). The spectra were pre-processed by TopHat baseline subtraction and normalized to total ion count (TIC). The data sets were grouped into ‘regions’ of interest: two regions (samples from two sites) from control cartilage (grouped as healthy) - Control Site A and Control Site B, GI intact region, GI lesion region, GII intact region and GII lesion region, before applying ‘Find Discriminative m/z Values’ tool. All the data points in individual spectra were analysed separately across two grouped ‘regions’. Since each grouped region had a different spectra subset size, the parameter tab was set to process ‘Random subset’ where the subset size was selected according to the lower number of spectra in the two groups. The minimal interval width was set to 0.05 Da. The threshold was set to 0.7, when detecting discriminative m/z values [535, 564]. The data were exported from the SCiLS report table as the differential m/z signals identified across the selected groups.
5.2.9 On-tissue Tandem MS Analysis Using LIFT Mode

In a separate set of experiments aimed at gaining some peptide sequence information on imaged peptides, to confirm their identity, the cartilage samples were processed as above and digested with trypsin. The CHCA matrix was applied on the digested tissue as manual spots as this was seen to increase the quality of the spectrum. Each m/z of interest was analysed using LIFT method in positive reflector ion mode using LID. In the LIFT mode, the selected precursor ion is fragmented and the fragments are further accelerated in the LIFT module, increasing their potential energies [565]. The mass of these fragments were then measured in the TOF analyser, and the resulting tandem mass spectra were first processed in flexAnalysis version 3.4. The data files obtained were then saved into Mascot Generic Format (.mgf) files. The peak lists obtained from each tandem mass spectrum were searched using MASCOT software online version. The different search parameters used to obtain peptide summaries were: Digest agent - trypsin; Mass values - Monoisotopic; Maximum number of missed cleavages - 1; Peptide mass tolerance - ± 0.2 Da; Fragment mass tolerance - ± 1.2 Da. For the peptide mass fingerprint search, the significance threshold was chosen as p < 0.05. To add confidence to the MASCOT data search, the fragment ion peak lists were further searched against bovine entries using ProteinPilot software (AB Sciex).
5.3 Results

In this work, the presence or absence of specific peptide variations in two different regions of a patella were investigated using MALDI-IMS (workflow shown in Figure 5.3). The mild-to-moderate ‘lesion’ region in a non-healthy patella and its adjacent intact cartilage regions were examined. Similarly, two sites from a healthy patella which was devoid of any signs of degeneration were included as control, to ascertain whether any pre-existing site specific variation would be detected by MALDI-IMS in the control group (Figure 5.1).
Figure 5.3 Schematic outlining the overall methodology used for MALDI-IMS and tandem mass spectrometric analysis for protein identification from bovine cartilages used in the present study.
5.3.1 Histology Analysis of Cartilage Samples

The grades of degeneration were confirmed by histological staining of the cartilage tissues. Intact cartilage samples from both the GI and GII patellae showed Mankin scores ranging from 0-1 (Figure 5.4). The lesion sites from the GI patellae showed Mankin scores in the range of 1-4, whereas those from the GII patellae scored 2-5. The controls were similar to the intact regions (Figure 5.4). The tidemark integrity was not assessed during Mankin scoring as the samples lacked tidemarks and subchondral bone.
Figure 5.4 Histological sections of cartilage stained with toluidine blue to assess the proteoglycan loss. Control G0 cartilage (Mankin score 0) showing smooth surface morphology, normal chondrocytes and uniform toluidine blue staining; intact site from GI patella (Mankin score 1) with undisturbed surface layer and uniform staining; lesion site from GII patella (Mankin score 3) with irregular surface and slightly decreased staining of tangential zone (asterisks); intact site from GII patella (Mankin score 1); lesion site from GII patella (Mankin score 5) with decreased matrix staining (asterisks) together with obvious cartilage disruption (Scale bar ~500 µm).
5.3.2 Peptide Distributions using MALDI-IMS

The realigned spectral data (Figure 5.5) were exported into SCiLS, for further data analysis. The datasets from multiple samples yield a huge amount of information, requiring a computational approach to analyse the data in an unbiased and timely manner. SCiLS software allowed for an automated workflow, which could discriminate between the m/z signals from different samples. The software was used to analyze the spatial distribution of the biological molecules within each sample, and to identify m/z signals that had different relative abundances in different sample groups. This approach provided insight into the pathophysiological state of the sample. The m/z signals that discriminated between different samples could be used as potential biomarker candidates.

The spectral data points were collected for 6 groups - two sites from the non-degenerated ‘healthy’ patellae: control site A and B; one sample each from lesion site and intact site of patellae showing mild degeneration: GI intact and GI lesion; and cartilage sample each from lesion and intact site of patellae containing moderate level of degeneration: GII intact and GII lesion. The summary spectrum of each group is shown in Figure 5.6. The control site A and B showed very similar summary spectra across the cartilage tissue Figure 5.7.
Figure 5.5 Realigning the spectra. These images show representative mass spectra acquired from different acquisition runs. (A) The raw data where summary spectrum from each sample run is misaligned. (B) The recalibrated spectra after using pre-installed external calibration method (PAC_ExternalCalibration. FAMSMethod) in FlexAnalysis software to update the internal calibration list to a new list chosen from the previous MS analysis of bovine cartilage samples.
Figure 5.6 Mean spectrum of various cartilage region groups—Control shows spectrum summary of all samples taken from non-degenerated patellae; GI intact shows spectrum of intact cartilage samples from mildly degenerated patellae and GI lesion shows mean spectrum from its corresponding lesion site; GII intact is the spectral summary from intact region of moderately degenerated patellae and GII lesion is the mean spectral data from its lesion site samples.
Figure 5.7 Mean spectral comparison between two intact cartilage regions taken from the non-degenerated control patella.
5.3.3 Comparison of the Mass Spectra across Different Regions

Using SCiLS, an unsupervised clustering was attempted applying the segmentation pipeline on all the samples, to cluster each group in accordance with its tissue status. This approach was unsuccessful at distinguishing different groups and hence the comparison in the present study was based on the less automated way of ROC (Receiver Operating Characteristic curve) plots.

Various group regions were compared pairwise and the discriminative m/z values between the pair were found out. Using ROC analysis, the discriminative capability of each m/z signal was computed. The ROC curve depicts the graph of calculated true positive rate (sensitivity) versus the false positive rate (1 - specificity) at each intensity. The area under curve (AUC) measures the discrimination such that when the level of m/z is significantly different between two groups, the ROC curve generated will have an AUC close to 1 (Figure 5.8). An AUC > 0.7 is considered a fair test to discriminate between two outcomes of a test [566] was employed to discriminate m/z in the present work.

![Figure 5.8](image.png)

**Figure 5.8** A representative image showing receiver operating characteristic (ROC) curve for m/z 839.7 in bovine cartilage. The AUC value for the m/z is 0.99 which suggests that there is significant difference between the two compared groups.
5.3.3.1 Comparison of the Mass Spectra from Two Control Regions

Comparisons were made between different cartilage groups, pair-wise, to understand the distribution profile of m/z signals across the tissues. To determine the inherent intra-discriminative m/z signals present in the control tissues, two cartilage regions from each of the four non-healthy bovine patellae were analyzed. Only one signal (m/z 913.8) was significantly different between control tissues (Figure 5.9), indicating very little variability in the relative abundance of m/z signals in control tissues detected through IMS.

![Image of Site A and Site B](image-url)

**Figure 5.9** Spatial distribution of m/z 913.8 Da found significantly discriminative between the two control sites (shown as Site A and B) in G0 patella.
5.3.3.2 Comparison of Intra- and Inter- differential m/z Across Various Groups

Both intra- and inter- differential m/z signals were analysed across the degenerated and non-degenerated cartilages. The comparison of intact and lesion region cartilage from mildly degenerated samples (GI) resulted in identifying no discriminative m/z signals, whereas the moderately degenerated (GII) patellae resulted in 6 discriminative m/z signals (m/z 770.1, 846.2, 866.2, 884.2, 892.2, and 1032.3).

To identify the signals that were different in non-healthy samples, each region (i.e., intact and lesion) of the degenerated patellae was compared to the control cartilage sections. Nine m/z signals were found to be different between the intact tissues from the GI patellae and control cartilages, however, the lesion region cartilage from the same patellae showed only 2 discriminative m/z signals. Similarly, the intact region of GII patellae showed 29 discriminative m/z signals and its corresponding lesion site showed 8 m/z signals which were differentially expressed compared to the non-degenerated control bovine tissues (Table 5.2).

Since the intact region of the non-healthy patellae had more m/z signal differences than the lesion site, the intact regions from GI patellae and GII patellae were compared, and 24 m/z signals were found to be different. This difference was more than what was seen when the lesion sites from GI and GII samples were analyzed, where 10 m/z signals were found to be discriminative (Table 5.2).
Table 5.2 The discriminative m/z found to be differentially present in non-healthy cartilage regions when compared pairwise to the control samples.

<table>
<thead>
<tr>
<th>GI intact vs Control Centroid [m/z]</th>
<th>GI lesion vs Control Centroid [m/z]</th>
<th>GII intact vs Control Centroid [m/z]</th>
<th>GII lesion vs Control Centroid [m/z]</th>
<th>GI intact vs GII intact Centroid [m/z]</th>
<th>GI lesion vs GII lesion Centroid [m/z]</th>
</tr>
</thead>
<tbody>
<tr>
<td>718.66</td>
<td>773.07</td>
<td>715.10</td>
<td>732.04</td>
<td>718.64</td>
<td>718.63</td>
</tr>
<tr>
<td>737.04</td>
<td>1362.62</td>
<td>718.07</td>
<td>734.09</td>
<td>720.65</td>
<td>732.13</td>
</tr>
<tr>
<td>770.03</td>
<td>866.09</td>
<td>737.10</td>
<td>760.10</td>
<td>732.12</td>
<td>839.74</td>
</tr>
<tr>
<td>884.20</td>
<td>892.20</td>
<td>759.07</td>
<td>773.11</td>
<td>760.21</td>
<td>855.74</td>
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<tr>
<td>907.75</td>
<td>930.15</td>
<td>760.16</td>
<td>782.07</td>
<td>770.13</td>
<td>871.73</td>
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<td>773.19</td>
<td>907.72</td>
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<td>1050.90</td>
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<tr>
<td></td>
<td>1066.87</td>
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</table>
5.3.4 Identification of Differentially Abundant Proteins

On-tissue tandem MS analysis was performed on selected peptides from peak-list generated after LIFT mode tandem MS analysis, to identify the proteins they were derived from. A total of 12 m/z were manually selected and analysed, out of which six were successfully identified. The online MASCOT software positively identified peptides detected at m/z 779.4, 1044.5 and 831.4 belonging to fibromodulin (FMOD), prolargin (PRELP) and aggrecan core protein (ACAN) respectively. In order to further validate these findings, the tandem MS peak-list was also analyzed by ProteinPilot and it identified m/z 735.4 and 1031.6 as fibronectin (FN1), m/z 831.4, 1044.5, and 1061.5 as ACAN, PRELP and cartilage intermediate layer protein 2 (CILP2) respectively (Table 5.3).
Table 5.3 MALDI-IMS via identified proteins which were found to be discriminative across various localized regions in non-healthy and control cartilages

<table>
<thead>
<tr>
<th>IMS Obs m/z (SCiLS)</th>
<th>Localized region</th>
<th>TOF/TOF Obs m/z</th>
<th>TOF/TOF Theor m/z</th>
<th>Score</th>
<th>Sequence</th>
<th>Gene Symbol</th>
<th>Protein name</th>
<th>Search Engine</th>
</tr>
</thead>
<tbody>
<tr>
<td>734.09</td>
<td>Control</td>
<td>735.39</td>
<td>735.41</td>
<td>51.42</td>
<td>YLQGVR</td>
<td>FN1</td>
<td>Fibronectin</td>
<td>ProteinPilot</td>
</tr>
<tr>
<td>778.15</td>
<td>Control, GII Intact</td>
<td>779.45</td>
<td>778.44</td>
<td>28</td>
<td>IPSPLPR</td>
<td>FMOD</td>
<td>Fibromodulin</td>
<td>MASCOT</td>
</tr>
<tr>
<td>832.20</td>
<td>GII Intact</td>
<td>831.44</td>
<td>831.47</td>
<td>73.01/27</td>
<td>YPIVSPR</td>
<td>ACAN</td>
<td>Aggrecan</td>
<td>ProteinPilot, MASCOT</td>
</tr>
<tr>
<td>1032.31</td>
<td>GII Intact</td>
<td>1031.59</td>
<td>1030.53</td>
<td>97.17</td>
<td>YEKPGSPPR</td>
<td>FN1</td>
<td>Fibronectin</td>
<td>ProteinPilot</td>
</tr>
<tr>
<td>1044.79</td>
<td>GI and GII Intact</td>
<td>1044.48</td>
<td>1044.52</td>
<td>39.39/27</td>
<td>WINLDNNR</td>
<td>PRELP</td>
<td>Prolargin</td>
<td>ProteinPilot, MASCOT</td>
</tr>
<tr>
<td>1060.80</td>
<td>GI Lesion</td>
<td>1061.49</td>
<td>1061.52</td>
<td>99.00</td>
<td>SHNLGGSHPR</td>
<td>CILP2</td>
<td>Cartilage Intermediate Layer Protein-2</td>
<td>ProteinPilot</td>
</tr>
</tbody>
</table>


M/z 735.4 was found to be discriminative between GI and GII intact cartilage regions. The cartilage sections from GII intact region showed a well distributed presence of m/z 735.4 when compared to GI cartilages (Figure 5.10). The spatial distribution of m/z 1031.6 was significantly different among control, GI intact, GII intact and GII lesion groups. Both m/z 735.4 and 1031.6 (Figure 5.11) were identified as fibronectin and were seen to have a higher intensity in the intact regions of moderately degenerated GII patellae.
Figure 5.10 Spatial distribution of discriminative m/z 735.4 for GI intact and GII intact cartilage tissues. Each image shows two consecutive sections obtained from a specific cartilage region. The superficial layer of cartilage is oriented facing the left hand side. The colour intensity bar and scale bar (2 mm) are as shown. The m/z value of 735.4 was identified as fibronectin and is seen to be distributed throughout the GII intact cartilage sections compared to GI intact cartilages. Given alongside is its box plot obtained using SCiLS software showing the distribution intensity.
Figure 5.11 Spatial distribution of discriminative m/z 1031.6 for control, GI intact, GII intact and GII lesion cartilage tissues. Each image shows two consecutive sections obtained from a specific cartilage region. The superficial layer of cartilage is oriented facing the left hand side. The colour intensity bar and scale bar (2 mm) are as shown. M/z 1031.6 was identified as fibronectin and it had higher expression in GII intact cartilage compared to GI intact, control and GII lesion cartilages. The box plot obtained using SCiLS software showing distribution intensity of the four groups are given.
The spatial distribution of m/z 779.4 was found to be differential in control and GII intact cartilages. While this m/z corresponding to fibromodulin was distributed with no particular pattern in GII intact sections, they were confined mostly to superficial and middle layers of cartilage in the control samples (Figure 5.12). M/z 831.4, was seen to be intensely distributed throughout the intact sites of moderately degenerated GII patellae when compared to intact control tissues (Figure 5.13). This m/z was identified as aggrecan and its distribution in each cartilage group (Figure 5.14) was found to correlate well with the proteoglycan loss assessed by toluidine blue staining (Figure 5.4) and results from LC-MS/MS analyses in previous chapters (Figure 3.12 and 4.8).
Figure 5.12 Spatial distribution of discriminative m/z 779.4 for control and GII intact cartilage tissues. Each image shows two consecutive sections obtained from a specific cartilage region. The superficial layer of cartilage is oriented facing the left hand side. The colour intensity bar and scale bar (2 mm) are as shown. The m/z 779.4 was identified as fibromodulin and was seen to be distributed throughout the GII intact sections compared to control cartilage tissues where they were found to be distributed along superficial and middle zone. The box plot shows a higher intensity of signal in GII intact samples.
Figure 5.13 Spatial distribution of discriminative m/z 831.4 for control and GII intact cartilage tissues. Each image shows two consecutive sections obtained from a specific cartilage region. The superficial layer of cartilage is oriented facing the left hand side. The colour intensity bar and scale bar (2 mm) are as shown. M/z 831.4 was identified as aggrecan both by MASCOT and ProteinPilot and was seen to have an intense distribution throughout the GII intact cartilages in comparison to control tissues. The box plot showing the distribution intensity is given.
Figure 5.14 (A) The distribution intensity of m/z 831.4 identified as aggrecan, across all the tissue groups. (B) The peptide levels correlate with the proteoglycan loss seen in histology data (Scale bar ~500 µm).
Comparison of m/z 1044.5, identified as prolargin or PRELP was found be significantly different between intact regions of both GI and GII patellae. The GI intact region had a higher intensity of signal than the GII intact cartilages, as assessed by the box plot (Figure 5.15). On comparing GI and GII lesion site samples, m/z 1061.5 corresponding to CILP2 was found discriminative. It had an intense distribution in GI lesion region, particularly in middle and deep zones of the cartilage (Figure 5.16).
Figure 5.15 Spatial distribution of discriminative m/z 1044.5 in GI intact and GII intact cartilage tissues. Each image shows two consecutive sections obtained from a specific cartilage region. The superficial layer of cartilage is oriented facing the left hand side. The colour intensity bar and scale bar (2 mm) are as shown. M/z 1044.5 was identified as prolargin using both MASCOT and ProteinPilot and it had a lower intensity in GII intact cartilages than the GI intact tissues as seen from the box plot.
Figure 5.16 Spatial distribution of discriminative m/z 1061.5 in GI lesion and GII lesion cartilage tissues. Each image shows two consecutive sections obtained from a specific cartilage region. The superficial layer of cartilage is oriented facing the left hand side. The colour intensity bar and scale bar (2 mm) are as shown. 1061.5 m/z was found to be CILP2 and was seen to have a comparatively higher distribution in middle and deep zone cartilage in GI lesion samples than the GII lesion site cartilage sections. The box plot obtained is shown alongside.
5.4 Discussion

MALDI IMS has the advantage of speed, sensitivity and molecular specificity to provide visual information about various biological processes occurring in a sample. The spectral intensity in each image it generates correlate with the concentration and distribution of the analyte molecules to that particular region [548]. While this visual representation provides an elementary depiction of the tissue state, advanced software analysis packages, such as SCiLS, facilitate statistical testing and identification of discriminative m/z signals which could be potential disease markers. Like many other major proteomic techniques, MALDI-IMS has the advantage of presenting the global protein profile in a tissue.

Detecting the onset of OA or understanding the pre-OA state of cartilage could provide us with the knowledge to develop appropriate targeted therapies for osteoarthritis. MALDI-IMS is an effective tool to analyze the molecular distribution patterns in tissue sections. This is the first time MALDI-IMS has been utilized to identify and analyze peptide distributions directly on bovine tissues, in order to study the region specific variations occurring in early degenerated cartilages. Using SCiLS, m/z signals were identified, which could discriminate various cartilage-tissue states of the non-healthy patellae.

Using a standard unsupervised segmentation pipeline [567, 568] the classifications of interest were not achieved, perhaps due to the presence of redundant peaks in the spectra or the pixel to pixel variations among the tissues [548] and hence the manual application of ROC plots was performed to determine the significant discrimination between various groups [564].

Since the lesion sample was visually very different from control samples, compared to intact regions, the expectation was to see more discriminative m/z in lesion samples than the intact tissues lying adjacent to it. Instead a lower number of differential m/z was detected between the lesion site cartilages and the control tissues. This could be due to either the higher protein turnover rates in these damaged areas or due to the presence of analytes which could interfere with the ionization process.
The intact regions of the patellae were seen to possess more peptide variations than the lesion site samples, with respect to control tissues. This finding suggests that the intact regions lying adjacent to early cartilage degradation sites could undergo a series of physiological changes, either as a precautionary mechanism to contain the degradation or alternatively these changes in the intact sites could be the glimpse of a pre-OA like state in the tissue, where these changes are the “groundwork” before the actual cartilage degeneration.

While previously greater intra-differential variations were detected among intact and lesion site bovine cartilages using comprehensive LC-MS/MS (Chapter 3 and 4), the present study using IMS was unable to identify any changes between intact and lesion tissues from mildly degenerated (GI) patellae. This could be due to the interference of more abundant proteins in cartilage which can cause suppression of signals from lower abundant ions and result in a less sensitive representation of the tissue health [548]. Also, MALDI-IMS is known to produce fewer m/z signals than an LC-MS approach [569].

On the other hand, the intact site cartilages were discriminatively different when compared to control tissues. The undamaged tissues lying next to GII lesions showed a greater number of peptides whose intensities were significantly different from control samples, whereas the GI intact showed comparatively a fewer number of peptide variations than the control. This finding conflicts with the earlier observation of cartilage tissue analysis using LC-MS/MS (Chapter 3), where it was found that the GI intact site represented a more differential protein profile than the GII intact site. A probable explanation could be the lower abundance levels of the differential proteins in GI patellae, which evidently are detected during comprehensive LC-MS/MS techniques but their signals are lost during IMS due to the interference from the higher abundant proteins’ signals. It is also possible that, while ESI and MALDI ionisation is somewhat complementary, some peptides ionise using both methods but some don’t.

Yet, these observations are significant and suggest that an active alteration occurs in the tissues lying next to the damaged cartilages, where they attempt to surmount the destructive changes. Alternatively, these peptide variations may be indicative of the
beginning of the degeneration process. Either way, the importance of unravelling the pre-to-early OA state could provide us with the early degeneration biomarkers.

In order to identify the parent proteins whose peptide variations were found to be different across various regions in intact and lesion cartilages, on-tissue tandem mass spectrometry was used. The corresponding fragment ion peak-lists were searched using ProteinPilot and MASCOT search engines to establish protein identity. These searches lead to successful identification of m/z 735.4 and 1031.6 as fibronectin, m/z 779.4 as fibromodulin, m/z 831.4 as aggrecan, m/z 1044.5 as prolargin and m/z 1061.5 as CILP2.

Fibronectins are glycoproteins which are found at low concentrations in the extracellular matrix of normal cartilage [139]. Fibronectin was seen to have significantly higher levels in GII intact sites when compared to controls, GI intacts and GII lesions (Figure 5.10 and 5.11). During osteoarthritis, the cartilage is seen to synthesise more fibronectin [570], and this could be what was detected in the GII intact cartilages in the current study. This result is consistent with our previous finding of increased fibronectin in GII intact site cartilages during LC-MS/MS experiment (Figure 3.13).

Fibronectin fragments are also increased in ECM and synovial fluid during arthritic diseases [135]. These fragments stimulate the degradation of other intact fibronectin molecules [136] and are also found to contribute to proteoglycan loss and in the suppression of new proteoglycan synthesis [137]. Moreover, cytokines and matrix metalloproteinase production are upregulated by the fibronectin fragments and these catabolic molecules then carry on with further degradation of the cartilage matrix [138, 139]. Therefore, our current finding is consistent with the literature, and validates MALDI IMS as a valuable approach to discriminate the changes in OA cartilage.

Fibromodulin was also observed to have a discriminatively higher level in intact tissues from GII patellae compared to control cartilages (Figure 5.12). Fibromodulin is a small leucine rich proteoglycan, which is known to be involved in fibrillogenesis by binding onto the collagen fibril and also influences collagen cross-linking [444].
Fibromodulin was found during MALDI-IMS in human OA cartilage tissues [550] but not in control samples. Interestingly, the same peptide (m/z 1361.7) was found to be lowered in equine OA cartilages by another study[551]. Earlier work done in canine spontaneous OA cartilage showed an increase in this protein and they concluded it acts as the repair mechanism which the cartilage takes on to limit the destruction [571]. Therefore, the increase in fibromodulin seen in intact GII cartilage could be the anabolic response to the adjacent lying damaged tissue.

Aggrecan (m/z 831.4) was seen to have an intense distribution in the GII intact cartilages in comparison to the control tissues (Figure 5.13). Among various proteoglycans, aggrecan is the largest and most prevalent in ECM. It is composed of chondroitin sulphate and keratin sulphate glycosaminoglycan chains, which are attached to a hyaluronic acid molecule with the help of link proteins [55]. This structure is called a proteoglycan aggregate. The proteoglycan aggregates with their negative charges, are immobilized within the collagen inter-fibrillar space and maintain a high osmotic pressure environment in the cartilage. This arrangement results in high pressurization in cartilage’s fluid phase, providing stiffness and viscoelastic properties to the tissue [56].

Aggrecan is known to have an increased synthesis during early stages of cartilage degeneration [285, 572]. Matyas et al. found an increased aggrecan mRNA expression in experimental canine OA [573]. This early increase in aggrecan synthesis is lost when the degeneration advances, softening the cartilage tissue further. Toluidine blue staining was used to estimate the level of proteoglycan loss [483] from the cartilage (Figure 5.14B) and the corresponding IMS analysis of aggrecan peptide m/z 831.4 across the cartilage groups showed a similar profile to that of the proteoglycan level found during histological analysis (Figure 5.14A). Moreover, the LC-MS/MS analysis of bovine cartilages in the earlier chapter, showed similar abundance level of aggrecan (Figure 3.12).

Prolargin is a proline- and arginine-rich glycoprotein [574], which is found in the cartilages and accumulates in tissue with age [575]. It interacts with collagen type I and II [576] and is believed to be involved in matrix organization [577].
experimental model of OA in mice, the expression of PRELP was upregulated compared to normal cartilage suggesting an active repair mechanism to “heal” the degenerating cartilage [578]. In the present study, m/z 1044.5 was identified as PRELP and was seen to be discriminately higher at the intact site cartilage from patellae showing mild degeneration (GI) compared to intact cartilages lying next to GII lesion cartilages (Figure 5.15). This suggests that PRELP has a stronger spatial distribution in undamaged tissues when the cartilage structural degeneration is at its earliest stage and this underscores its potential as an investigative or diagnostic OA biomarker.

CILP2 also was found to be significantly discriminative in the present work. It was found to have more intense level in mildly degenerate cartilage (GI lesion) compared to GII lesion cartilage tissues (Figure 5.16). As the level of degeneration advances from mild to moderate, the cartilage spatial distribution of CILP2 was found to decrease, emphasizing its potential as a marker for progression of the diseased state. This observation is similar to the downregulation of CILP2 gene expression seen in degenerated cartilage from the osteoarthritic mouse model [388] and could be the direct result of degeneration occurring in the tissue.

Although MALDI-IMS is widely used as a visualisation technology, the present study combined the use of advanced software analysis package called SCiLS to identify statistically tested discriminative m/z channels. After performing on-tissue mass profiling, a wide array of discriminating molecules were identified. The attempt to identify all of the discriminative m/z with on-tissue MS/MS approach was unsuccessful, due to a lack of adequate signal in the samples. Further effort in studying the regional variations in a degenerating cartilage, can contribute to unravelling the elusive pre-to-early OA state. The major limitation of MALDI-IMS in cartilage proteomics which needs to be overcome, is bypassing the highly abundant cartilage proteins, to detect the differential expression of the lower abundant molecules. Once this is achieved, MALDI-IMS may prove a powerful method representing the global protein profile of the tissue, while retaining its integrity.
5.5 Conclusions

- The region-to-region variation in intact and lesion cartilage tissues from bovine patellae showing mild-to-moderate level of degeneration, was determined using MALDI-IMS.
- The analysis showed that MALDI-IMS can be successfully used to discriminate the regional variations occurring in cartilage tissues.
- The healthy control patellae and GI patellae did not reveal much inherent peptide variation, when analysed by MALDI-IMS.
- Six m/z signals were found to be discriminative between the intact and lesion sites of GII patellae.
- Intact regions of non-healthy patellae showed a higher number of discriminative m/z signals when compared to healthy controls, than the lesion vs. healthy comparison. Therefore, the differential m/z signal variation observed at two different sites in a non-healthy patella gave the basis for speculation that these peptide/protein changes could be the tissue’s active reaction towards the cartilage disintegration.
- M/z signals corresponding to fibronectin, fibromodulin, aggrecan, prolargin and CILP2 were effectively identified by on-tissue tandem mass spectrometry.
- Fibronectin, fibromodulin, aggrecan and prolargin were found to have increased expression in intact regions of the non-healthy patellae and can be used as potential biomarkers to discriminate the pathophysiological state of the cartilage. Further study in this area can provide more insight to the early cartilage degeneration changes.
CHAPTER 6

GENERAL DISCUSSION

6.1 General Summary

In this study, the early degeneration occurring in cartilage tissue was studied in detail with the aim of understanding the pre-OA state better. An overall schematic representation of this study is shown in Figure 6.1. It is found to support the hypothesis that the proteomic data obtained from the cartilage tissues showing signs of early degeneration, correlates well with its microstructural changes.

The cartilages for this study, were obtained from bovine patellae showing no signs of degeneration (G0) as well as mild degeneration at the distal lateral surface of the patellae (GI) and finally, from patellae with moderate level of degeneration at the distal lateral surface (GII). The GI and GII cartilage samples represented the early degeneration seen during OA pathology [266]. Additionally, from each patella used in this study, two samples were obtained from the two different regions in the patellae such that if the patella have a degenerated region, the sample was obtained from the damaged region (referred to as ‘lesion’ in this work, for convenience and not to be confused with clinical manifestation of a lesion) and from its adjacent intact site with no surface disruption (termed as ‘intact’).

At first, the microstructural changes in various cartilage groups were studied using DIC optical imaging. It was confirmed that, two intact regions from the healthy-appearing patellae did not display any structural variations. However, the intact and lesion site osteochondral tissues showed significant microstructural changes involving surface layer appearance, zonal arrangement, chondrocyte morphology, matrix texture, changes in ZCC and subchondral bone and also in their histological staining. These changes were more pronounced in the GII patellae samples, than the GI patellae. Also, of much importance is that intact tissue (from both GI and GII) were
structurally similar to the healthy tissues, except for an increased ZCC thickness and bone changes seen in some samples. These microstructural features could be analogous to the pre-OA state.

For determining the protein variations associated with various cartilage groups analysed in this study, comprehensive iTRAQ labelled LC-MS/MS was performed. Initial experiments involved using whole cartilage tissue as samples for the proteomic analysis. It was found that the protein profiles of cartilage from adjacent sites in a healthy patella, showed little variations. Interestingly, the comparison of protein profiles from the lesion site to its adjacent intact site, was found to be significantly different. Also, it was seen that the intact region cartilage from the patellae with mild degeneration (GI), have more profound region specific differences to its corresponding lesion site, than the GII patellae, where fewer number of proteins with differential level were detected between its intact and lesion site. This observation strongly suggests that the cartilage undergoes active metabolic alteration at the very early stage of degeneration, but with time, its anabolic efforts are overpowered by the catabolic activities in the cartilage, as seen in the GII patellae samples. This study brings forth the hypothesis that intact region in a tissue behaves as a relatively early stage of the disease (pre-OA) whereas the lesion tissue region is the glimpse of aftermath of the disease.

Consistent with previous literature, this study too saw significant protein variations between the healthy and non-healthy (GI and GII patellae samples) cartilages. The proteins, which were differentially present in the intact regions of GI patellae compared to healthy cartilage tissues, were found important in this study as they could play vital roles in the pre-to-early OA state. Furthermore, by correlating the microstructural changes observed in cartilage and subchondral bone to the respective protein profiles, a set of proteins were identified and validated which could potentially be used as biomarkers for OA disease progression and pathophysiology.
Figure 6.1 Schematic showing overall summary of this work. For each study, samples were obtained from bovine patellae as shown in the block, followed by microstructural study (Chapter 2), proteomic study of intact and ‘lesion’ whole cartilage (Chapter 3), proteomic analysis of cartilage secretome from intact and ‘lesion’ site explants (Chapter 4) and finally on-tissue IMS and protein mass fingerprinting on cartilage tissues from intact and ‘lesion’ sites (Chapter 5).
In addition, cartilage explants from different examined groups were grown in culture medium over a time period of 96 hours to study the secretome profile of those tissues. The medium was unstimulated, hence the variations observed in the secretome were spontaneous. The two control samples from healthy tissues had a minimal number of proteins with differential expression. However, the intact and lesion site samples from non-healthy patellae had a significant number of proteins which had differential expression between these two cartilage groups. It was found that the GII patellae had a higher number of proteins with differential expression than the GI patellae. This enhanced secretion/loss of proteins from the GII cartilage explants could be the explanation for the detection of lower number of proteins with differential levels when the whole cartilage tissue from GII patellae were analysed. This theory is further supported by the microstructural analysis of GII samples, which revealed that they possess a ‘destructured’ fibrillar network which could further facilitate the movement of molecules out of the tissue.

The secretome from healthy and non-healthy tissues also showed significant differences in the protein profiles. More number of proteins with differential expression was identified in the intact cartilage secretome samples than the lesion samples when compared with the healthy cartilage secretome. These proteins which are secreted from the intact cartilage explants could have similar secretory pattern during pre-to-early OA in vivo.

This work also achieved to identify a novel biomarker protein, WFDC18-like protein which have differential levels, in both whole cartilage tissue and secretome analysis. With a potential anti-protease activity in cartilage, the early upregulation of this protein in the GI intact samples is noteworthy. A detailed investigation of this protein in relation to early cartilage degeneration can lead to further exploration of OA pathophysiology.

Finally, molecular imaging was performed using MALDI-IMS to compare region-to-region variation in intact and lesion cartilages. Peptide/protein variations in control sections were negligible. But it was also found that the IMS was not sensitive enough
to identify the peptide variations in intact and lesion samples of GI patellae and a probable reason could be the lower abundance levels of these proteins in the tissue which is not detected effectively during MALDI imaging. Yet, discriminative m/z signals were found between the intact and lesion sites of GII patellae. Similar to the previous findings in this study, the intact region of the non-healthy patellae showed more number of discriminative m/z signals than the corresponding lesion site cartilage, when compared against the healthy tissues. This strengthens the speculation that the intact cartilage in a non-healthy patella actively respond to the adjacent degeneration. Using on-tissue tandem MS, m/z signals corresponding to fibronectin, fibromodulin, aggrecan, prolargin and CILP2 were identified. This study reports that MALDI-IMS could be successfully employed in gaining insight to early cartilage degeneration.

6.2 Correlating Structural and Biochemical Data

Through this work, the microstructural changes seen during early cartilage degeneration occurring in bovine patellae were studied in detail. Following this, the individual cartilage group’s protein profile was assessed by analysing the tissue and its secretome. The proteins with significant differential expression among various cartilage groups were correlated to various microstructural features such as cartilage surface layer disruption, chondrocyte clustering, cartilage texture, proteoglycan loss, bone spicule formation and angiogenesis. The protein variations detected in the samples were mostly found to complement their tissue state.

6.3 Biomarker Panel

A set of proteins were identified following comprehensive liquid chromatography-tandem mass spectrometry based proteomic analysis of cartilage and its secretome. These proteins which were differentially identified in the intact region of patellae with mild degeneration, could play a vital role in early cartilage degeneration. WFDC18-like, COMP, COL2A1, COL12A1, THBS1, PCOLCE and TGFBI proteins were consistently found to be differential at the sites of pre-to-early cartilage degeneration in both cartilage and secretome analysis. Further investigation into these ensemble of
proteins can provide explanation of how they influence the structural changes associated with pre-to-early OA.

6.4 Limitations

Like any other work, this study too is not without limitations. Cartilage derived proteins are found in biological fluids like synovial fluid, serum and urine which directly or indirectly are in contact with the cartilage. For most biomarker discovery based research, samples are these biological fluids. In the present work, whole cartilage samples were analyzed with the aim of understanding the cartilage ECM homeostasis in early cartilage degeneration. This lead to identification of a set of proteins which can be potential candidates for further biomarker study, involving biological fluids.

The age and clinical status of the bovine animals used were uncertain, as they were collected from the abattoir. So it is probable that a few changes seen in this work, could be due the age of animal or previous injury to the animal.

Allusion to words like ‘healthy’ and ‘non-healthy’ in this work is used as the terms of convenience and does not refer to the clinical status of the animal as all the evaluations were based on visual, tactile and histological inspection.

For whole cartilage tissue proteomic analysis, the supernatant from homogenized cartilages were used without removing the highly abundant proteins. This could have affected the sensitivity of the experiment resulting in loss of the lower abundant proteins like MMPs in the final proteome profile. Another limitation was the difficulty in pin-pointing the non-tryptic cleavages in peptide summary, which could be present in the sample before trypsin digestion due to activity by other proteases in the tissues. Trypsin might also produce these cleavages during digestion and especially in the overnight incubation. Therefore, it is difficult to pinpoint the origin of these peptides unambiguously. iTRAQ labels 115 and 116 are known to interfere with the peptides ending with proline or contained a proline (P) immediately followed by glutamine (Q). To avoid the tampered data, those peptides were omitted during the data trimming stage.
The secretome samples in this study had relatively lower levels of total protein content. The in vitro nature of the experiment also could have triggered some aberrant reactions in the cartilage explants which could have affected the final secretome.

Another limitation was the use of two different instruments for LC-MS/MS experiments- QSTAR XL hybrid and TripleTOF 6600 Quadrupole-Time-of-Flight mass spectrometers. The former was used for cartilage proteome analysis and the latter for MRM experiments and cartilage secretome analyses. It was seen that TripleTOF instruments had a higher dynamic range and sensitivity than the QSTAR XL and was able to detect proteins like MMPs, which were not detected in the cartilage proteome analyses using QSTAR XL.

The highly abundant cartilage proteins again posed an obstacle in detecting the differential expression of the lower abundant molecules in MALDI-IMS, hence resulting in a lesser sensitive protein profile. Protein identification using on-tissue tandem mass spectrometry, based on the discriminative m/z signals identified by IMS, was found onerous due to lack of adequate signals in the sample.

Another major limitation for this study was the overall high cost of the iTRAQ label-based proteomics experiments, which limited the number of samples analysed in each experiment.

6.5 Future Works

The proteins found to have differential expression in the secretome analysis, need to be validated further with an MRM assay. Proteins such as WFDC18-like identified in this work, which is not characterised yet at the protein-level should be investigated to understand its role and implications of its increased level at the early cartilage degeneration sites.

The structure based proteomic analysis of cartilage has proven to be an ideal direction to capture the elusive ‘pre-to-early’ OA state. Future experiments with age matched bovine samples including cartilage, secretome, synovial fluid and serum or with other large animal models where OA is surgically induced (ACL transection or meniscectomy) could provide more information on the initiation of the disease. Also,
more multi-scalar microstructural studies including more SEM based studies could be correlated to the proteomics findings in the sample.

Furthermore, the findings from the bovine animal model of the study need to be translated into human samples. Currently, this work is progressing towards the studies using human samples from donors with no joint history.
APPENDICES

Appendix A

iTRAQ Labelling Scheme

Proteomic Study I

Proteomic Study II
Appendix B

Detailed iTRAQ Data Processing

ALIGNMENT OF INDIVIDUAL RUNS to the COMBINED RUN

1) The combined search was assessed in terms of False Discovery Rate (FDR) score and a total of 191 unique proteins were found above the threshold and were accepted into the analysis. Any proteins below the FDR score were pruned off and not used in the steps described below.

The matched peptides from the combined search was used as the backbone for alignment of the individual runs, in order to avoid erroneous allocation of the same peptides to different entries of identical/near identical proteins in the searched database, as shown below.

2) In practice, this meant that firstly the combined search was trimmed down to accepted proteins only (i.e. above the FDR cut-off), then any duplicate peptide matches to the same protein was deleted so that each peptide was present only once in the document. Note that the only information taken from the combined search was which protein names and corresponding accession numbers should be used for each peptide found in the individual searches.

3) For each individual search, any information of FDR score, protein names and accession was disregarded. Instead, any peptide also found above the FDR threshold in the combined search were allowed and assigned the protein name linked to it in the combined search. Excel formulas were used to search and insert the correct names and accession numbers, as determined in the combined search, next to each matched peptide in the individual search. The above process resulted in six separate individual runs that were perfectly aligned in terms of protein IDs.

NORMALIZATION of iTRAQ areas

1) Each individual run was then processed to ensure equal iTRAQ signal from each of the eight iTRAQ labels, essentially correcting for any unequal loading. The first step was to add 8 columns where the value 1 was added to each area. (This was done to enable log transformation of values found to be 0 in certain labels.)

2) Then 8 more columns were added, where the log (LN) transformed values for each measured area +1 was entered.

3) Any instances of matched peptides ending with a proline or containing a proline immediately followed by a glutamine was identified and highlighted, since these have both been found to interfere with the 115 (PQ) and 116 (N-terminal P) labels. Any such peptides were placed at the very top of the document. Any matched peptides found to contain a missed cleavage was also highlighted. These were combined and placed at the top, just below the peptides compromised in terms of PQ and N-terminal P.

4) The average log transformed value for each label was obtained separately of 1) correctly cleaved peptides not containing a PQ or an N-terminal P, and 2) miscleaved peptides not containing a PQ or an N-terminal P. These values were used to obtain a dual set of correction

<table>
<thead>
<tr>
<th>Combined run (protein name and accession)</th>
<th>Individual runs (iTRAQ info)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein name</td>
<td>Accession number</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
factors for each label, adjusting each label to the one showing the highest average overall (in order not to introduce any negative numbers).

5) The correction factors were used to correct all log transformed values, including those linked to a matched peptide with a PQ or an N-terminal P in a further 8 columns. Note that separate correction factors were used for miscleaved and correctly cleaved peptides. The calculations were double-checked by ensuring that the average values for all labels were identical.

6) Finally, all corrected values were back-transformed to normal space and 1 subtracted from each value.

SUMMING of iTRAQ LABELS for each protein and each label

The pivot table function in Excel was used to generate for each iTRAQ label, the sum of all areas for each protein separately. For all spectra matched to a peptide containing a PQ or ending with a P, the numbers were adjusted manually by either deleting the compromised spectrum across all eight labels (if there were also non-compromised spectra available for the protein in question) or, if only P/PQ spectra were available, the final sums would be deleted for 115 only (in the case of PQ) or 116 only (in the case of a matched peptide ending with a P).

MASTER FILE, INTER-RUN COMPARISONS and STATS

1) A master file was constructed using the protein names, accession numbers and normalised iTRAQ signal data (summed areas) from each run. The protein names and accessions were those obtained in the combined search and hence these were identical across runs when identical proteins were identified. The iTRAQ data inserted in the master file were as processed above, i.e. the sums of each protein and label for each LC-MS/MS run.

2) For statistical analysis, the sums were first log transformed and then, in a separate set of columns, ratios of these logs were obtained for each protein between each individual sample and the sample pool, which was repeated between runs. These ratios are the only numbers that can be compared reliably between runs. (Since the numbers are in log space, ratios were obtained by subtracting the log value of the sample pool from that of the sample in question.) The result of these calculations is what is referred to as the “log ratios”, which give the relative abundance of each quantified protein in each analysed sample.

3) All ratios were then organised in groups of healthy control site A (n=3), healthy control site B (n=3), bull site A (n=3), bull site B (n=3), Grade I intact (n=7), Grade I lesion (n=7), Grade II intact (n=5), Grade II lesion (n=5) to simplify further calculations.
Appendix C

For the comparison of healthy vs. non-healthy cartilages

The two separate cartilage iTRAQ based proteomic studies (labelling schemes as shown in Appendix A) were linked, by two separate samples (BJ13 (Grade I intact) and BJ31 (Grade II intact) which were present in run 3 and 4 of the initial study, respectively. Both samples were also analysed in both runs performed for the second study.

Ratios were aligned through a series of calculations for both studies, aligning separate runs within each study to a common denominator.

For the Proteomic study I, ratios were first obtained against a pooled reference sample which was repeated between all four runs. This procedure aligned the values between runs within the initial study. Each aligned sample/pool ratio was then aligned to the Grade I intact sample 13 (present in Run 3), in order to link the initial study to the Proteomic study II (where this was present in each run). An alternative alignment was also performed against the Grade II intact sample 31, in order to compare the use of the two reference points to each other.

The same two sets of ratios (against Grade I intact sample 13 and Grade II intact sample 31) were also obtained from the Proteomic study II, where both Grade I intact 13 and Grade II intact 31 samples were present in each of the two performed runs. As mentioned above, this was done to control for differing results using different reference points and the expectation was that similar or identical results would result from using Grade I intact 13 and Grade II intact 31 to link the two studies. Finally, all resulting ratios (for both studies) were then aligned to the average value of the six samples analysed from healthy cow, which constitutes the baseline for the linked study (as well as the second study).

Double checking using Grade I intact Vs Grade II intact samples from the two studies showed that the results were identical only for the Proteomic study II (i.e. whichever samples was chosen as the reference sample, the results would not change). For the Proteomic study I, where the Grade I and Grade II reference samples were not present in the same run, the results would however vary to some extent but only in regards to the distribution of the set of results from the Proteomic study I, in relation to the gender samples (bull vs. cow). Hence, this comparison was excluded during final analysis. The internal relationship between the samples from the Proteomics study I remained unaffected by the choice of reference point.

In order to eliminate any bias stemming from the choice of linking reference, an average of the ratios obtained by the two references was used for each protein and each sample. For proteins only detected in one of the two reference samples, values were calculated based on the available reference sample only.
**Appendix D**

Proteins found significantly different (p<0.05) when non-healthy cartilage samples were compared to healthy cartilages.

- ALDOA Fructose-bisphosphate aldolase
- GAPDH Glyceraldehyde-3-phosphate dehydrogenase
- PGAM1 Phosphoglycerate mutase 1
- CKM Creatine kinase M-type
- COL2A1 Collagen alpha-1(II) chain
- COL6A1 Uncharacterized protein
- COL6A2 Uncharacterized protein
- COL6A3 Uncharacterized protein
- COL9A1 collagen, type IX, alpha 1
- COL9A2 collagen, type IX, alpha 2
- COL9A3 collagen, type IX, alpha 3
- COL11A1 collagen alpha-1(XI) chain
- COL12A1 Uncharacterized protein
- COL15A1 collagen, type XV, alpha 1; COL15A1 collagen alpha-1(XV) chain
- PCOLCE Procollagen C-endopeptidase enhancer
- THBS1 Thrombospondin-1
- THBS4 Thrombospondin-4
- TNC Uncharacterized protein
- FN1 Embryospecific fibronectin 1 transcript variant
- CHAD Chondroadherin
- EDIL3 EGF-like repeat and discoidin I-like domain-containing protein 3
- MATN3 matrilin 3
- COMP Cartilage oligomeric matrix protein
- CILP Cartilage intermediate layer protein 1
- CILP2 Cartilage intermediate layer protein 2
- VIT Vitrin
- HSPG2 heparan sulfate proteoglycan 2
- DCN Decorin
- FMOD Fibromodulin
- LUM Lumican
- OGN Mimecan
- ACAN Isoform 2 of Aggrecan core protein
- TGFBI Transforming growth factor-beta-induced protein ig-h3 precursor
- PRELP Prolargin
- C1QB Complement C1q subcomponent subunit B
- HBA1;HBA Hemoglobin subunit alpha
- HBB Hemoglobin subunit beta
- ALB Serum albumin
- LYZ1 Lysozyme C, milk isozyme
- CLU Clusterin
- ACTB Actin, cytoplasmic 1; ACTG1 Actin, cytoplasmic 2
- MSN Moesin
- GSN Gelsolin A
- VIM Vimentin
- HTRA1 HtrA serine peptidase 1
- SERPINF1 Pigment epithelium-derived factor
WFDC18 WAP four-disulfide core domain protein 18-like [Bos taurus]
LMNA Uncharacterized protein
RNASE4;ANG Ribonuclease, RNase A family, 4
EEF1A1 Elongation factor 1-alpha 1
ANXA1 Annexin A1
ANXA8L1 Annexin A8
PPIA Peptidyl-prolyl cis-trans isomerase A
PREDICTED: target of Nesh-SH3 [Bos mutus] - 99% identical
SRPX2 Sushi repeat-containing protein SRPX2
ANG Angiogenin-1
Appendix E

Proteins found significantly different (p<0.05) when intact cartilage samples were compared to lesion site cartilages.

COMP Cartilage oligomeric matrix protein
ACAN Isoform 2 of Aggrecan core protein
ALB Serum albumin
COL6A3 Uncharacterized protein
LYZ1 Lysozyme C, milk isoyme
C4A Uncharacterized protein
IGL Immunoglobulin light chain, lambda gene cluster [Bos taurus]
THBS1 Thrombospondin-1
MB Myoglobin
COL6A2 Uncharacterized protein
TNC Uncharacterized protein
WFDC18 WAP four-disulfide core domain protein 18-like [Bos Taurus]
HBB Hemoglobin subunit beta
APOA1 Apolipoprotein A-I
EDIL3 EGF-like repeat and discoidin I-like domain-containing protein 3
RNASE4 Ribonuclease, RNase A family, 4
COL6A1 Uncharacterized protein
ANXA2 Annexin A2
GSTP1 Glutathione S-transferase P
OGN Osteoglycin
FMOD Fibromodulin
CLEC3A C-type lectin domain family 3 member A
GSN Gelsolin a
ANG Angiogenin-1
HBA1 Hemoglobin subunit alpha
CCL16 Uncharacterized protein
TIMP3 Uncharacterized protein
CILP Cartilage intermediate layer protein 1 [Bos Taurus]
ENO1 Alpha-enolase
TGFBI LOC539596 protein
LUM Lumican
PGLYRP1 Peptidoglycan recognition protein 1
IGFBP6 Insulin-like growth factor-binding protein 6
PPIA Peptidyl-prolyl cis-trans isomerase A
FIBIN Fin bud initiation factor homolog
FGA Fibrinogen alpha chain
LMNA Lamin A
APOD Uncharacterized protein
CLEC3B Tetranectin
VIT Vitrin
PCOLCE Procollagen C-endopeptidase enhancer
LO504773 Regakine-1
HHIPL2 Hedgehog interacting protein-like 2-like
C1QB Complement C1q subcomponent subunit B
TNFRSF11B Tumor necrosis factor receptor superfamily member 11B
SMOC2 MGC148871 protein
**Appendix F**

Proteins found significantly different (p<0.05) when non-healthy cartilage secretome were compared to healthy cartilage secretome.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPI</td>
<td>Glucose-6-phosphate isomerase</td>
</tr>
<tr>
<td>ENO1</td>
<td>Alpha-enolase</td>
</tr>
<tr>
<td>COL5A1</td>
<td>Uncharacterized protein</td>
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<td>COL2A1</td>
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<td>COL12A1</td>
<td>Collagen alpha-1(XII) chain</td>
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<td>Lysyl oxidase homolog 2</td>
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<td>PCOLCE</td>
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<td>Thrombospondin-1</td>
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<td>TNC</td>
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<td>Osteopontin</td>
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<td>Cartilage oligomeric matrix protein</td>
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<td>Complement component C9</td>
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<td>C1QB</td>
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<td>Clusterin</td>
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<td>Vimentin</td>
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</table>
YWHAQ 14-3-3 protein theta
PDIA3 Protein disulfide-isomerase
PGLYRP1 Peptidoglycan-recognition protein
LAP LAP-like antimicrobial peptide (Fragment)
BRN Brain ribonuclease
CHADL Uncharacterized protein (Fragment)
Appendix G

Proteins found significantly different (p<0.05) when intact site cartilage secretome were compared to lesion site cartilage secretome.

ABI3BP ABI family, member 3 (NESH) binding protein
ACAN Aggrecan core protein
AEBP1 Uncharacterized protein
ALB Serum albumin
AMBP Protein AMBP
ANG1 Angiogenin, ribonuclease, RNase A family, 5
ANG2 ANG2 protein
APOH Beta-2-glycoprotein 1
BGN Biglycan
C4 Complement C4 (Fragments)
C9 Complement component C9
CCL16 C-C motif chemokine
CILP Uncharacterized protein
CILP2 Uncharacterized protein
CLEC3B Tetranectin
CLU Clusterin
COL12A1 Collagen alpha-1(XII) chain
COL15A1 Uncharacterized protein
COMP Cartilage oligomeric matrix protein
CTGF Connective tissue growth factor
ECSOD Superoxide dismutase [Cu-Zn]
ENO1 Alpha-enolase
EPYC Epiphycan
F13A1 Coagulation factor XIII A chain
FBLN1 Fibulin-1
Uncharacterized protein
FGA Fibrinogen alpha chain
FN1 Embryo-specific fibronectin 1 transcript variant
GPI Glucose-6-phosphate isomerase
HIST1H1D Uncharacterized protein
LAP LAP-like antimicrobial peptide (Fragment)
LECT2 Leukocyte cell-derived chemotaxin-2
LOC100847724 (WFDC18) Uncharacterized protein/WFDC18
LOC100852118 (C4) Uncharacterized protein
TGFBI LOC539596 protein
LUM Lumican
MFGE8 MFGE8 protein
NOV Nephroblastoma overexpressed
PCOLCE2 Uncharacterized protein
PDIA3 Protein disulfide-isomerase
PGLYRP1 Peptidoglycan-recognition protein
PRELP Proline/arginine-rich end leucine-rich repeat protein
PRG4 Lipid phosphate phosphatase-related protein type 2
RBP4 RBP4 protein
SAA3 Serum amyloid A protein
SERPINF1 Pigment epithelium-derived factor
SPARC
SPP1 Osteopontin
SRPX2 Sushi repeat-containing protein SRPX2
SSC5D Uncharacterized protein
THBS1 Thrombospondin-1
TIMP1 Metalloproteinase inhibitor 1
TNC Tenascin C
Uncharacterized protein
VIM Vimentin
YWHAQ 14-3-3 protein theta
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