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Applications of proteomics in cartilage biology and osteoarthritis research

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1. ABSTRACT

In osteoarthritis (OA) the turnover of extracellular matrix (ECM) macromolecules is disrupted by catabolic changes that lead to the production of a range of inflammatory mediators and the loss and fragmentation of proteoglycans, fibrillar and non-fibrillar collagens. These events result in the degradation and release of ECM fragments, which are potential biomarkers that can be detected in synovial fluid, blood and urine. Proteomics is increasingly applied in cartilage research and has the potential to advance our understanding of the biology of this tissue. It can also provide mechanistic insight into disease pathogenesis and progression and facilitate biomarker discovery. Here we review the area of cartilage and chondrocyte proteomics and published studies relevant to arthritis and OA biomarkers, highlighting areas of current and future research and development. Markers of tissue turnover in joints have the capacity to reflect disease-relevant biological activity potentially enabling a more rational approach to healthcare management. Therefore proteomic studies of cartilage, chondrocytes and their subcellular fractions and other joint cells and tissues may be particularly relevant in diagnostic orthopedics and therapeutic research.

2. INTRODUCTION

A major focus of clinical research in recent years has been the identification of new disease markers that can facilitate early diagnosis and optimize individualized treatments. Such markers can also facilitate the drug discovery process by reducing the high levels of attrition in clinical trials (1). A biomarker is classically defined as a biochemical entity that is used to measure the progress of a disease or the effects of treatment on clinical outcome. Biochemical markers can be measured in blood, serum and urine or a variety of other body fluids and tissues. The National Cancer Institute (NCI) [1] defines a biomarker as 'a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease', and the terms 'molecular markers' or 'signature molecules' have also been used to describe such markers[2]. The term biomarker is all encompassing and can include proteins, protein fragments, metabolites, carbohydrates, nucleic acids (RNA and DNA), cellular features and images.

Osteoarthritis (OA) is a degenerative disease of the entire synovial joint. It involves joint inflammation and destruction of the extracellular matrix (ECM) of articular

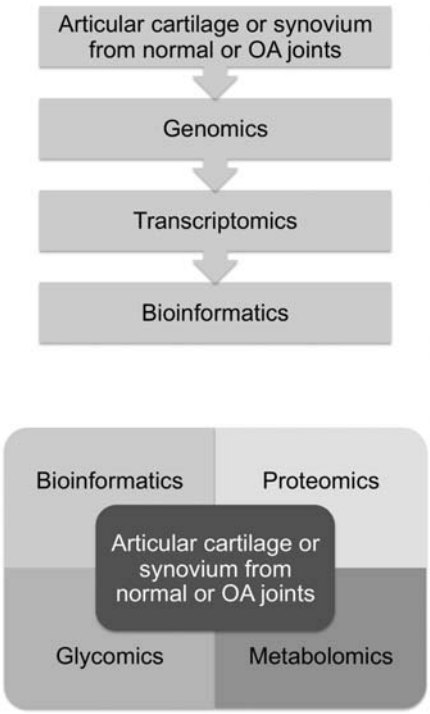

Figure 1. Applications of systems biology in OA research. These -omic techniques can be applied to the study of cartilage and synovium. The same strategies can be applied to synovial fluid, blood (serum) and urine from OA patients.

cartilage (1-3). It is characterized by progressive destruction of articular cartilage, subchondral bone sclerosis, synovial inflammation and osteophyte (bony outgrowth) formation (4). OA can occur in any synovial joint but symptomatic OA in humans is most common in the knee (5). Digits of the hand and the hip are also frequently affected. In general, weight-bearing joints are the worst affected. Risk factors for OA include age, gender, genetics, obesity, poor nutrition and joint injury or instability (6). A biomarker that signifies early OA would be a useful tool to allow screening of individuals with a high risk of developing joint disease so that early detection may facilitate individualized treatment. Cartilage damage in OA is detected radiographically by decreases in joint space width. Radiographic evidence is seen only after significant cartilage degradation has already taken place. Biomarkers have been identified that reliably detect changes to cartilage in radiographically established OA.

Recently, the OARSI[3] /FDA[4] osteoarthritis biomarkers working group has proposed to divide OA biomarkers in two major groups: the so-called soluble or "wet biomarkers", usually measured in a selected body fluid such blood, serum, plasma, urine or synovial fluid and usually representing a modulation of an endogenous substance in these fluids; and the so-called "dry biomarkers" usually consisting of visual analog scales, performed tasks, or imaging (7). The degenerative changes in OA generate collagen and proteoglycan fragments, which are wet biomarkers that can be detected in blood, synovial fluid and urine (8, 9). However, collagen and proteoglycan fragments are only markers of late stage OA; by the time a patient with OA is referred to a specialist, the disease has progressed to the point where intervention does little to alter the course of the disease (9). There are no reliable or established biomarkers for early disease diagnosis and that can be considered as a surrogate for the clinical end point. One reason is the lack of a "good" gold standard that captures the disease in all of its manifestations and that can be used as reference for biomarker qualification. Other factors that hamper biomarker qualification are the absence of a therapy with a strong and rapid structural effect and the lack of prospective cohorts designed for studying the prognostic value of soluble biomarkers. Consequently, new and better biomarker candidates are urgently needed to improve diagnosis, guide molecularly targeted therapy and monitor activity and therapeutic response in OA. There is currently a lack of a coherent pipeline connecting biomarker discovery with well-established methods for validation. Therefore, robust and reliable state-of-the-art diagnostic technologies and coherent multidisciplinary research strategies are needed to fill this gap.

The -omic technologies have resulted in rapid growth and progress in biomarker research (3). The contribution of high throughput technologies such as proteomics and metabolomics to biomarker identification and the diagnostics arena has been disappointing thus far (10). However, approaches (Figure 1) such as proteomics, using mass spectrometry and bioinformatics are making an increasing impact on our understanding of the anatomical structure of cartilage in health and disease. Therefore proteomic techniques hold special promise for the discovery of novel biomarkers that might form the foundation for new tests. Moreover, these technologies are dominating the biomarker research arena and are playing increasingly important roles in laboratories involved in biomarker discovery. The majority of biomarkers are proteins and biochemical metabolites. Proteomics involves large-scale and multi-dimensional studies of protein structure and function (11, 12).

This review provides a brief background to articular cartilage structure and function and the pathogenesis of OA before reviewing the literature that has used proteomic techniques to study cartilage and synovial biology and structural alterations in joints in OA. Proteomic studies of synoviocytes, synovial fluid and subcellular compartments in chondrocytes are also briefly reviewed.

2.1. Articular cartilage

Articular cartilage consists of a single cell type known as the chondrocyte (Figure 2) and unlike the synovium (Figure 3) it is an avascular, aneural and alymphatic connective tissue with unique biological and mechanical properties. Its load-bearing function depends on the structural design of the tissue and the interactions between its unique resident cells, the chondrocytes, and the extracellular matrix (ECM) that makes up the bulk of the

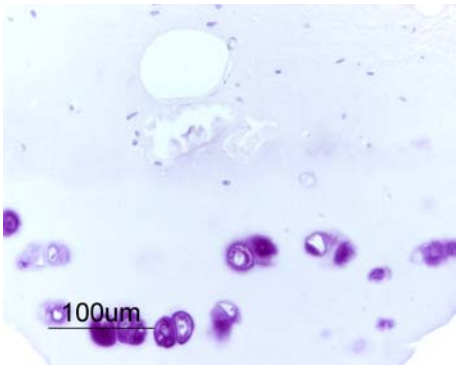

Figure 2. Histology of human articular cartilage. The sample shown in this figure is from a tissue microarray developed by the Cooperative Human Tissue Network (CHTN) of the National Cancer Institute (http://www.chtn.nci.nih.gov/). Cartilage is predominantly an avascular, aneural and alymphatic load-bearing connective tissue consisting of a single cell type known as the chondrocyte. Blood vessels are only present in subchondral bone. The unicellular nature of cartilage and the absence of blood vessels, nerves and lymph vessels highlight its architecturally simple design, making it particularly attractive for tissue engineering and regenerative medicine research.

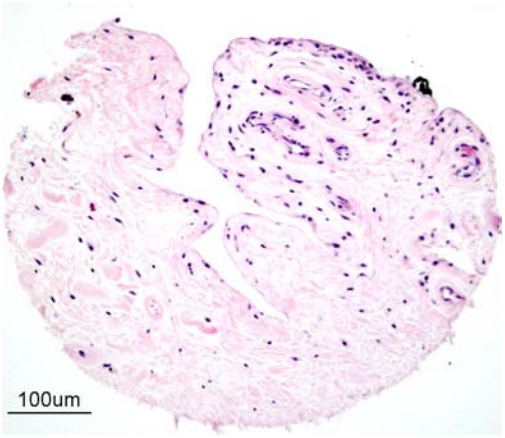

Figure 3. Histology of human synovium. The synovium is a thin layer of connective tissue surrounding the joint capsule. It is only a few cells thick and lines the joint. The composition of the synovium is quite variable but it has two main layers: the outer layer, or subintima, that contains fibrous and adipose tissues and the inner layer, or intima, that consists of a sheet of cells giving rise to the term 'synovial membrane'. The synovium controls the environment within the joint and tendon sheath. It acts as a selectively permeable membrane and produces proteoglycan and glycoproteins that lubricate the joint. Synovial inflammation or synovitis is a frequently observed phenomenon in OA joints and contributes to the pathogenesis of OA through formation of various catabolic and pro-inflammatory mediators altering the balance of cartilage matrix degradation and repair. The sample of synovium shown in this figure is from a tissue microarray developed by the Cooperative Human Tissue Network (CHTN) of the National Cancer Institute (http://www.chtn.nci.nih.gov/).

tissue (13). Chondrocytes are architects of the ECM and build the macromolecular framework of the ECM from three distinct classes of macromolecules: fibrillar and non-fibrillar collagens, proteoglycans, and non-collagenous proteins (14).

The extracellular environment in which chondrocytes exist is highly hypoxic, acidic and hypertonic (15, 16). Chondrocytes are glycolytic cells because the availability of oxygen is significantly lower than synovial fluid and plasma (16). Glucose is an essential source of energy during embryonic growth and fetal development and is vital for mesenchymal cell differentiation, chondrogenesis, and skeletal morphogenesis (17). Cartilage develops in this hypoxic environment and proximity to a blood supply appears to be a determining factor in the formation of bone over cartilage. Hypoxia-dependent up regulation of the hypoxia inducible factor 1 α (HIF-1α) transcriptional activity is critical for differentiation and survival of chondrocytes both during fetal growth and development and after skeletal development is complete (18, 19). The hypoxic conditions also enhance chondrogenic differentiation of mesenchymal stem cells (MSCs) (20). In addition, due to the absence of vasculature, articular cartilage (unlike most tissues) is maintained and functions in a low oxygen environment throughout life. Therefore cartilage is essentially exposed to hypoxia, or, more appropriately, to physiological normoxia, at all times. Therefore, chondrocytes have a specific and adapted response to low oxygen environment, which allows them to survive within the ECM and maintain it (21).

Collagens in the ECM (Figure 4) play important biological and mechanical roles in cartilage. Type II, IX, and XI collagens form a fibrillar meshwork that gives cartilage tensile stiffness and strength (13, 22, 23). Collagen type VI forms part of the matrix immediately surrounding the chondrocytes, enabling them to attach to the macromolecular framework of the ECM and acting as a transducer of biomechanical and biochemical signals in the articular cartilage (24, 25). Embedded in the collagen mesh are large aggregating proteoglycans (aggrecan), which give cartilage its stiffness to compression, its resilience and contribute to its long-term durability (25-28). ECM proteins in cartilage regulate the cell behavior, proliferation, differentiation and morphogenesis (29-37). Small proteoglycans in cartilage include decorin, biglycan, and fibromodulin. Decorin and fibromodulin both interact with the type II collagen fibrils in the matrix and participate in fibrillogenesis and interfibril interactions. Biglycan is mainly found in the immediate surroundings of the chondrocytes, where it may interact with collagen type VI (13, 25). Modulation of the ECM proteins is regulated by an interaction of growth factors with the chondrocytes (38-42). In fact, it has been reported, that IGF-I and TGF-β stimulate the chondrocyte surface expression of integrins, and that this event is accompanied by increasing adhesion of chondrocytes to matrix proteins (43). Other non-collagenous proteins in articular cartilage such as cartilage oligomeric matrix protein (COMP) are markers of ECM turnover and degeneration (44). Tenascin and fibronectin

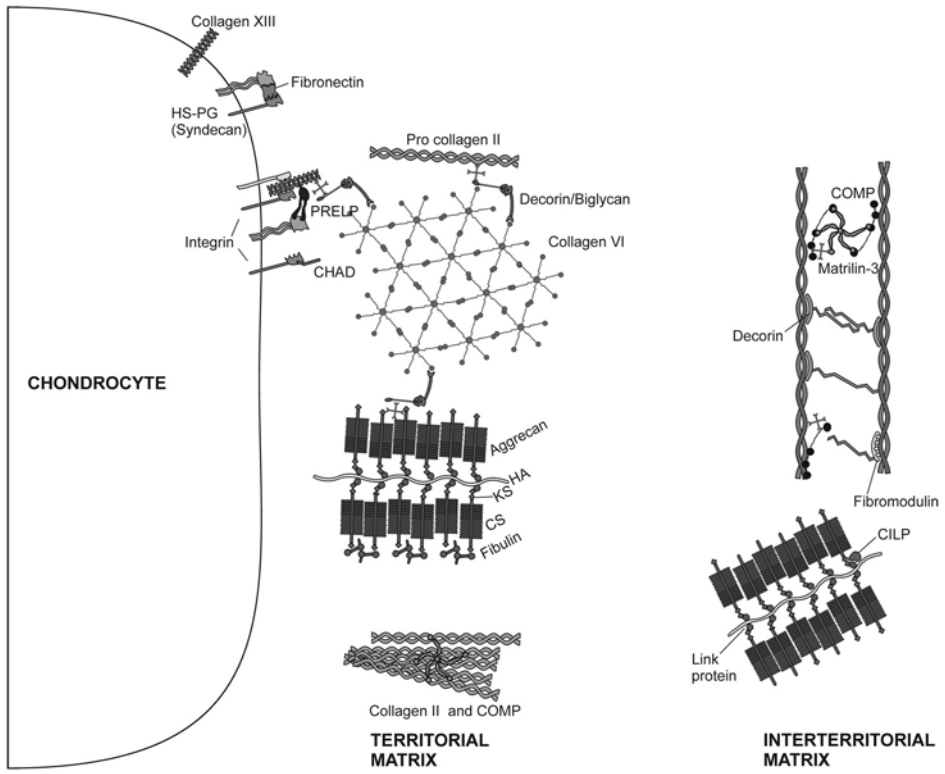

Figure 4. Schematic illustration of the major molecular constituents of the extracellular matrix (ECM) of articular cartilage. This figure illustrates the physical and functional association between the major constituents of cartilage ECM. The molecules are arranged into large multi-molecular assemblies in the territorial and interterritorial ECM of chondrocytes. A number of proteins in the chondrocyte plasma membrane interact with specific ECM molecules, acting as receptors for cell-matrix interactions and mechanoreceptors.

influence interactions between the chondrocytes and the ECM (13, 45). Some of the key fibrillar and non-fibrillar collagens, proteoglycans, and non-collagenous proteins present in the ECM of cartilage are illustrated in Figure 4.

Throughout life, cartilage undergoes continuous internal remodeling as chondrocytes replace matrix macromolecules lost through degradation. Evidence indicates that ECM turnover depends on the ability of chondrocytes to detect alterations in the macromolecular composition and organization of the matrix, including the presence of degraded macromolecules, and to respond by synthesizing appropriate types and amounts of new ECM components (46). It is known that mechanical loading of cartilage creates mechanical, electrical, and physicochemical signals that help to direct the synthesizing and degrading activity of chondrocytes (47). In addition, the ECM acts as a signal transducer for the chondrocytes (48). A prolonged and severe decrease in the use of the joint leads to alterations in the composition of the ECM and eventually to a loss of tissue structure and its specific biomechanical properties, whereas normal physical strain of the joint stimulates the synthesizing activity of chondrocytes and possibly the internal tissue remodeling (49, 50). Articular cartilage can tolerate a tremendous amount of intensive and repetitive physical stress. However, if structural damage occurs to the cartilage, it manifests as an inability to heal even the most minor injury (49, 51-53). Further, aging leads to alterations in the ECM composition and alters the activity of the chondrocytes, including their ability to respond to a variety of stimuli such as growth factors (54-56). All these alterations increase the probability of cartilage degeneration and emphasize the importance of interaction of the chondrocytes with their surrounding ECM since this regulates growth, differentiation, and survival of the chondrocytes in normal and pathophysiological conditions (52, 57-59, 60).

2.2. Osteoarthritis (OA)

Musculoskeletal conditions are leading causes of morbidity and disability, giving rise to enormous healthcare expenditures and loss of work throughout the world (61). Despite its durability, cartilage has a very limited self-maintaining capability and is vulnerable to mechanical injury and prone to structural damage and degradation. Osteoarthritis (OA) is one of the most prevalent and chronic diseases affecting the elderly (62). The symptoms and signs characteristic of OA in the most frequently affected joints are heat, swelling, pain, stiffness and limited mobility. OA is often a progressive and disabling disease, which occurs in the setting of a variety of risk factors, such

as advancing age, obesity, and trauma, that conspire to incite a cascade of pathophysiologic events within joint tissues (63). Other sequelae include osteophyte formation and joint mal-alignment. These manifestations are highly variable, depending on joint location and disease severity. OA is viewed not only as the final common pathway for aging and injuries of the joint, but also as an active joint disease. As medical advances lengthen average life expectancy, OA will become a larger public health problem - not only because it is a manifestation of aging but because it usually takes many years to reach clinical relevance. OA is already one of the ten most disabling diseases in industrialized countries and the most frequent cause of physical disability among older adults globally with most people over 65 years of age show some radiographic evidence of OA in at least one or more joints. Whilst OA is rare in people under 40 it is also becoming more widespread among younger people. This huge financial burden emphasizes the acute need for new and better treatments for articular cartilage defects especially since there are no effective disease-modifying drugs or treatments for OA. Existing pharmaceuticals include analgesics, steroids and NSAIDs, which only treat the symptoms of rheumatoid arthritis (RA) and OA by reducing pain and inflammation. OA is also a major cause of morbidity in animals particularly companion animals such as dogs and horses (64, 65). There is therefore considerable interest in research that could lead to improvements in understanding, diagnosis and treatment of this disease in both humans and animals.

2.3. The role of chondrocytes and synoviocytes in the pathogenesis of OA

Chondrocytes maintain cartilage by producing and secreting specialized ECM macromolecules responsible for maintaining the structural and functional properties of the tissue (66) (Figure 2). This requires a delicate balance between the synthesis and degradation of the ECM components, which is controlled by the expression of anabolic and catabolic factors and their abundance in the tissue (67). Chondrocytes isolated from OA cartilage show differing patterns of protein expression compared to healthy articular chondrocytes (68, 69). This alteration can be defined in terms of changes in protein expression levels inside the chondrocytes, and also the proteins that are secreted into the surrounding tissue the so-called “secretome”. Alterations in this secretome can further disrupt the homeostasis of the ECM, potentially contributing to further loss of cartilage. Synoviocytes are cells present in the synovial membrane, where they produce components of the synovial fluid and help maintain a barrier between internal joint tissues and the circulatory system (70) (Figure 3). Once the cartilage in the OA affected joint begins to degenerate, ECM degradation products are released into the surrounding synovial fluid (e.g. type II collagen and aggrecan fragments (71, 72)). Both chondrocytes and synoviocytes express receptors that can detect these breakdown products and are stimulated to release a range of proteins, including pro-inflammatory cytokines such as IL-1β and TNF-α, and chemokines (e.g. IL-8). The secretion of IL-8 by chondrocytes into the synovial fluid will induce chemotaxis of inflammatory cells such as neutrophils to the joint. IL-1β and TNF-α bind to their corresponding receptors, (i.e. IL-1R type 1 and TNF-R that are expressed in the plasma membrane of chondrocytes and synoviocytes), thereby initiating inflammatory signaling pathways concluding with translocation of active phosphorylated NF-κB into the nucleus. The active form of NF-κB then induces the expression of a variety of pro-apoptotic and pro-inflammatory genes. These pro-inflammatory cytokines therefore effectively increase the expression and release of a range of catabolic enzymes including matrix metalloproteinases (MMPs) (predominantly MMP-1, MMP-3 and MMP-13), aggrecanases, cathepsins and ADAM-TS (70, 73-75). Studies on the proteome of cartilage, chondrocytes and synoviocytes stimulated by these pro-inflammatory cytokines have the capacity to reveal how protein expression is altered in joint inflammation, providing insights into the signaling processes and may lead to discovery of potential therapeutic targets. These studies can also reveal how new epitopes (neoepitopes) of ECM degradation are formed and how these might be used in novel diagnostic tests.

3. PROTEOMIC APPROACHES IN CARTILAGE BIOLOGY AND OA RESEARCH

Systems biology is increasingly applied in orthopedics and rheumatology to cartilage and synovium in OA and RA (Figure 1). These techniques include genomics, transcriptomics, proteomics, metabolomics, glycomics and bioinformatics and can be applied to the study of cartilage, synovium, synovial fluid and even blood (serum) or urine from OA patients. Proteomics involves the application of specialized analytical techniques that allow the evaluation of the protein composition of tissues, cells and culture supernatants (76-78). One dimensional polyacrylamide gel electrophoresis (1D-PAGE) can be used to separate proteins according to their molecular weight (MW). More complex expression profile maps can be created using two dimensional polyacrylamide gel electrophoresis (2D-PAGE) (79). This method separates the proteins in two stages presenting improved profiling over 1D techniques. In 2D-PAGE, isoelectric focusing is utilized to separate proteins in the first dimension before second dimension separation using traditional SDS-PAGE. Once the gels have been fixed and stained, they are scanned using a densitometer that provides a high-resolution image or map. These images can then be analyzed by image analysis software to establish coordinates for each spot and measure expression levels of proteins and provide evidence of differential expression.

Identification of the proteins that correspond to the stained and visualized spots is achieved by mass spectrometry and bioinformatics (77, 78). The specific spot of interest can be excised from the gel and digested with trypsin, to generate fragments of the protein present. The instrument first ionizes these fragments by creating charged particles. This can be achieved by various methods including electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI) and surface enhanced laser desorption/ionization (SELDI) (80). The charged

fragments are then separated by a mass analyzer depending on the fragments' mass/charge ratio (m/z). Once this separation has occurred, a detector records the ion masses and produces the final mass spectrum of the sample. This provides a peptide mass fingerprint (PMF) that can be used to search databases of known protein PMFs using bioinformatics tools thereby allowing identification of the sample proteins.

Protein antibody microarrays provide another useful approach to determining the types of proteins that are present in a biological sample (81, 82). Antibodies that are directed against a range of inflammatory proteins are attached to a membrane forming an array. When sample media is incubated with this array, proteins that are present in the sample will bind specifically to the antibodies. The bound proteins are then detected and visualized by labeling the attached proteins by fluorescence or chemiluminescence. Cytokine antibody array membranes provide a range of inflammatory related proteins whose altered synthesis could be relevant to the changes that occur in OA affected joints. Studies that employ these protein antibody approaches have the potential to contribute considerably to the OA proteomics research field, especially if the data are supplemented with histology images from the affected joint, *in situ* expression of inflammatory proteins and matrix proteases and measurements of inflammatory mediators such as prostaglandin E_2 (PGE_2) and nitric oxide (NO).

3.1. Proteomic studies of whole extracts of articular cartilage

Proteomic studies on whole cartilage tissue have helped re-examine the molecular composition of articular cartilage and understand that structural changes that are involved in the gradual loss and degradation of articular cartilage in OA. One study employed 1D-SDS-PAGE followed by tandem mass spectrometry to identify over 100 proteins from more than 700 peptide sequences in human OA whole cartilage tissue samples (83). A high proportion of proteins were found to be ECM components like COMP, collagens, CILP and proteoglycans (35%), while many others identified are associated with cellular functioning. The presence of the chemokine CXC12, which is strongly chemotactic for lymphocytes, was highlighted as it has been implicated in OA and RA cartilage degradation. This protein and its receptor CXCR4 can activate lymphocytes and therefore its upregulation in OA cartilage is relevant to the disease.

Proteomic analysis of whole articular cartilage using 1D-SDS-PAGE and NanoLCMS/MS identified 59 proteins that were significantly differentially expressed in OA (84). Expression of HtrA serine protease 11 was noted to be considerably higher in OA cartilage compared to normal tissue. Three proteins from the fibulin family (-1D, -2, -3) had altered expression in OA, which could have implications on the metabolism of cartilage. Another comparison between normal and OA cartilage has shown differential expression of 14 proteins between the two (85). Cartilage extracts were trypsin-digested followed with collagen and proteoglycan removal via centrifugation. Removal and reduction of these ECM components helps to improve separation and diminish interference in protein migration during 2D electrophoresis techniques. Five of the differentially expressed proteins were glycolysis and energy production related enzymes (alcohol dehydrogenase (ADH), adenosine kinase isoenzyme 1 (ADK), flavin reductase (FR), enolase 1 (ENOA) and pyruvate kinase 3 isoform 2 (KPYM)), while 3 signaling proteins were also altered (annexin A1 (ANAX1), tubby protein homolog (TUB) and phosphatidylethanolamine-binding protein (PEBP)). Precursors to collagen type I and VI were identified at higher levels in OA cartilage possibly due to attempts at cartilage repair.

An important contributing factor to cartilage degeneration in OA could be reduced superoxide dismutase 2 mitochondrial (SOD2) levels that were significantly lower in OA samples. The chitinase 3-like protein 1 (YKL-40) protein that is associated with remodeling of the ECM has been shown to be induced by TNF-α and LPS stimulation (86). YKL40 exists as a number of different isoforms that change in several disease states. This protein, which lacks chitinase activity, is secreted by activated macrophages, chondrocytes, neutrophils and synovial cells and is thought to play a role in the process of inflammation and tissue remodeling. It may also play an important role in the capacity of cells to respond to and cope with changes in their environment. Thus far proteomic techniques have not shed any new light on the involvement of these proteins in joint disease.

A proteomic analysis of rat articular cartilage has identified 47 proteins, including latexin, which had not previously been found in the cartilage proteome (87). Latexin is the only known protein inhibitor of zinc-dependent metallocarboxypeptidases. Further studies are required to study its role in chondrocytes and cartilage.

An enhanced procedure to analyze the cartilage proteome was reported using cetylpyridinium chloride (CPC) treatment to selectively precipitate GAGs from cartilage samples (77). CPC has traditionally been used to separate chondroitin sulfate and keratan sulfate in fractions of articular cartilage. This approach improves protein separation by isoelectric focusing resulting in the identification of representative protein spots by MALDI-TOF/TOF-MS. In this study both COMP and fibromodulin were identified. Both molecules have been suggested as potential biomarkers in other studies. The 2D-SDS-PAGE profiles from different studies appear to produce 2D maps that are a similar representation of the cartilage extract proteome. As proteomic techniques are refined, the improved separation and enhanced resolution achieved should lead to the identification of new proteins.

3.2. Proteomic studies of cartilage and chondrocyte secretomes

The degenerative processes that occur as OA progresses are likely to be enhanced by disruption of normal protein and secretion levels by chondrocytes within the tissue. Insights into alterations in the secretome can be gained by culturing cartilage explants for various periods of

time, followed by removal and analysis of secreted proteins in the culture media. Comparisons of healthy and OA cartilage secretions have identified a number of specific proteins that are upregulated in OA. In porcine and human articular cartilage explants the regulatory protein activin A was significantly upregulated in OA (88). This study used medium containing [^{35}S] methionine/cysteine that allowed the identification of newly synthesized proteins after the explants were placed in culture. This approach also showed that levels of connective tissue growth factor (CTGF) and cytokine-like protein C17 were increased in explants from OA cartilage. Another study identified a number of proteins released into media from OA cartilage using 1D-, 2D- and off-gel electrophoresis, with tandem mass spectrometry performed on spots of interest (89). Eleven secreted proteins were also identified by use of antibody microarrays on one of the donor OA samples. Overall, 43 proteins were found to be secreted from cartilage extracts, including chitinase 3-like protein 2 (YKL-39), vitamin D binding protein, tissue inhibitor of metalloproteinase-1 (TIMP-1), pigment epithelium-derived factor (PEDF), osteoprotegerin and tumor necrosis factor receptor 1 (TNF-R1). In another investigation, collagen II neoepitope peptides were identified as by-products of MMP mediated degradation of human OA cartilage stimulated by treatment with IL-1β and oncostatin M (90). There have also been studies on the differential expression of proteins released from mouse cartilage stimulated with IL-1β or all-trans-retinoic acid (Ret A) (91). A number of ECM components and metabolic proteins showed altered levels of expression when the treatment media was compared with control media by 2D-electrophoresis. Using labeling with Cy3 and Cy5, it was possible to provide accurate quantification of the amounts of proteins that were present in the explant culture media. IL-1β treatment resulted in a significant increase in neutrophil gelatinase-associated lipocalin (NGAL), YKL-40, haptoglobin and MMP-3, while Ret A treatment increased COMP, hyaluronan and proteoglycan link protein 1, matrilin-3, serotransferrin and aggrecan G1 domain proteins. Treatment with IL-1β and Ret A both reduced gelsolin secretion into the explant media. The biological significance of these findings is not clear but these studies suggest that IL-1β treatment results in loss of a number of ECM proteins and upregulation of MMP activity.

OA has been linked to load-induced injury leading to cartilage damage and degradation. A bovine stifle model of cartilage injury has investigated the secreted protein response to mechanical compression and stimulation with pro-inflammatory cytokines (IL-1β and TNF-α) (92). The proteins found at higher levels in bovine stifle cartilage explant media after mechanical compression injury were mostly intracellular associated proteins. The stress placed upon the ECM and chondrocytes by subjecting them to compressive forces appears to have caused matrix damage and chondrocyte death and cell lysis. The intracellular proteins released included vimentin, pyruvate kinase, glucose-regulated protein 58kDa (GRP58) and glucose-regulated protein 78kDa (GRP78). Proteins associated with stress and immune responses were upregulated when the bovine cartilage explants were exposed to both IL-1β and TNF-α. These cytokines stimulated enhanced release of proteins including YKL-39, YKL-40, MMP-3 and clusterin. Again the enhanced release of these proteins signifies the presence of matrix degrading enzymes and ongoing catabolic reactions in IL-1β and TNF-α treated samples. These studies suggest that activation of the IL-1β and TNF-α signaling pathways leads to the enhanced activity of matrix degrading enzymes and the release of proteins from the ECM.

Proteomic analysis of cartilage explants has distinguished between newly synthesized and existing protein secretion by using stable isotope labeling with amino acids in cell culture (SILAC) (93). This new approach showed that 71% of the proteins released were already present before the SILAC explant culture was started, while 29% were *de novo* synthesized proteins (containing the labeled amino acids). Most of these newly synthesized proteins were related to ECM remodeling (e.g. YKL-40 and TIMP-1). Constituents of the ECM (e.g. COMP, CILP and Aggrecan core protein) made up a large proportion of the non-labeled proteins found in the supernatant, although it is likely that new ECM proteins would have been incorporated into the cartilage tissue as well. The identification of numerous ECM components and proteins involved in metabolic remodeling in the secretome provides evidence for matrix remodeling in this culture model. The high percentage of non-labeled proteins suggests that the majority of released proteins may simply be leaking out from explants rather than being actively synthesized by chondrocytes. However, newly synthesized ECM components may have been incorporated straight into the cartilage to replace the older proteins as ECM turnover occurs.

The previously reported studies have used cartilage explant proteomics where the chondrocytes are maintained within their natural 3-dimentional environment. This can be seen as beneficial because it is a more physiologically relevant model that mimics the natural microenvironment of chondrocytes. Chondrocytes could potentially respond differently once they are placed in monolayer culture. There are also advantages to this approach. As mentioned above, many proteins that are released from the ECM are not newly synthesized. These may interfere with proteomic studies that are aiming to identify newly synthesized proteins in response to inflammatory stimulation. Therefore there are advantages to using both approaches, a strategy that can contribute to a better understanding of chondrocyte biology.

Although the majority of investigations into the proteome of cartilage and chondrocytes have used electrophoresis and mass spectroscopy techniques, an alternative approach is the use of antibody-based microarrays that was used to identify new proteins in the chondrocyte secretome (94). This method revealed a range of cytokines, chemokines, angiogenic and growth factors that were increased when chondrocytes were stimulated with IL-1β and TNF-α. A similar approach but using techniques including CPC precipitation of GAGs, collagenase digestion and a Q sepharose resin batch column

identified eight low molecular weight proteins throughout the 2D map, although the regions corresponding to higher molecular weight proteins were poorly resolved (95). There were increases in MMP-1, MMP-3 and cyclophylin A secretion after stimulation with the cytokines IL-1 and oncostatin M. The proteins YKL40, β_2-microglobin, calgizzarin and cofilin were expressed in both treated and control gels.

The secretome of isolated chondrocytes from rat articular cartilage stimulated with bacterial lipopolysaccharides (LPS) has been examined using proteomics. This study revealed increases in proteins related to immune responses and cartilage degradation (86). LPS stimulation activated toll-like receptors and initiated expression of MMP-3, MMP-1 and YKL-40 along with proteins associated with immune responses. A rat cytokine antibody array was used to confirm that LPS stimulation also increased expression of the chemokines MIP-3α (CCL20) and LIX (CXCL5). These observations suggest that activation of LPS signaling increases chemotactic molecules in chondrocytes. These chemotactic molecules may play an important role in recruiting inflammatory cells to the synovial joint in arthritis thus perpetuating the inflammatory events that occur in the synovial joint.

LPS has been thought to be mainly associated with the inflammatory processes that occur in septic OA (96). However, there is increasing evidence for the presence of LPS receptors, the so-called 'toll-like' receptors 2 and 4 (TLR-2 and TLR-4) in rheumatoid arthritis as well as OA and crystal-induced joint damage (97-99, 100, 101). Research using animal models of arthritis over the last decade suggests that environmental triggering through toll-like receptors may contribute to cartilage and bone degradation (102). Microcrystals of calcium pyrophosphate dihydrate (CPPD) and monosodium urate (MSU) may be deposited in the synovium and in articular cartilage. These crystals are involved in gout and pseudogout and can initiate joint inflammation and cartilage degradation by binding and directly activating resident cells through TLR-2 (101). A study in dogs suggests that increased TLR-4 gene expression in the synovial tissue of OA joints secondary to cruciate ligament damage suggests that activation of innate immunity may play an important role in the pathophysiology of OA (103).

Application of SILAC has enabled the distinction between already present and newly synthesized proteins secreted into media by chondrocytes in monolayer culture (93). This approach allowed 103 newly synthesized secreted proteins to be identified using 1D-SDS-PAGE and LC/MS-MS. Proteins secreted were mostly ECM components along with MMPs and inflammatory mediators. This technique is likely to be refined, improved and adopted in future studies in order to help distinguish between newly synthesized and pre-existing proteins. This is particularly relevant to cartilage because the turnover of many ECM components is very slow and needs to be measured in weeks and months rather than hours and days. This technological advance would be welcome in the area of OA biomarker research because the interesting molecules are likely to be newly synthesized molecules whose expression is transiently switched on or off.

3.3. Proteomic studies of chondrocytes – whole cell lysates

The first proteomic map of the normal human articular chondrocyte was produced by Francisco Blanco and his team using 2D-electrophoresis in 2005 (104). Establishment of this map was an important first step in the characterization of the human chondrocyte proteome. This proteome contained 93 different proteins from 136 spots that were successfully identified by MALDI-TOF-MS. A high proportion of the proteins indentified were involved in cellular organization (26%), while as expected for cells located in a hypoxic environment many of the proteins (16%) related to energy production. The remaining identified proteins had roles in metabolism (12%), transcription, protein synthesis and turnover (12%), signal transduction (8%) and protein fate (14%). Since cytokine-mediated inflammation is a key contributor to OA progression, proteomic maps of normal human chondrocytes stimulated with pro-inflammatory cytokines IL-1β and TNF-α were established to study differentially expressed proteins (105). The IL-1β stimulated chondrocyte map showed 22 proteins with altered regulation when compared to protein expression levels in normal chondrocytes and a further 9 proteins only being expressed following the cytokine stimulation. There were 20 proteins that were found to be modulated by TNF-α and 4 proteins were only identified due to the cytokine treatment compared to un-stimulated cells. A comparison of 2D maps for IL-1β and TNF-α stimulation provided the identification of 18 differentially expressed proteins. There were 76 differentially expressed proteins reported when comparing healthy and OA chondrocytes via a reverse-phase protein array (68). For the first time alterations were found in fibroblast growth factor 23 (FGF23), sex determining region Y box 11 (SOX11), WW domain containing oxidoreductase (WWOX), kruppel-like factor 6 (KLF6) and growth differentiation factor 15 (GDF15) protein levels in OA chondrocytes. Bioinformatic methods were applied to create interaction networks between the protein and microRNA expression providing the potential for improved understanding of OA disease processes.

A study published on the proteome of chondrocytes from healthy donors compared with damaged and intact cartilage from OA patients (106). Chondrocytes showed differences in the protein expression patterns even between intact cartilage from OA patients and healthy cartilage samples. OA chondrocytes (i.e. chondrocytes derived from OA cartilage) showed 17 differentially expressed proteins between damaged and intact regions, of which the protein vimentin was investigated further. Confocal microscopy and western blotting demonstrated disruption in the vimentin network and alterations in vimentin cleavage at OA lesions. The heat shock protein αB-crystallin also showed differential expression in chondrocytes cultured from OA damaged and non-damaged cartilage (69). There was reduced expression of the αB-crystallin in OA chondrocytes, which could have implications for cartilage metabolism. Expression of

chondrocyte specific gene markers (bone morphogenetic protein-2 (BMP-2), Type 2 collagen, aggrecan) was inhibited when siRNA targeted αB-crystallin expression, therefore indicating an important role for this protein in chondrocyte biology. IL-1β and TNF-α produced decreased levels of αB-crystallin in chondrocytes from healthy tissue. Differences in medium osmolarity have also been shown to alter protein expression in human chondrocytes (107). There were significant differences in 20 protein spots when comparing 2D profiles of cells in culture medium of 320 mOsm/kg and 400 mOsm/kg. This suggests that the chondrocytes will actively respond and alter their protein expression depending on the culture conditions they are exposed to. This would need to be taken into account when comparing the results of different studies that have used different culture methods. These culture parameters could have significant impact on protein expression and may lead to variations in proteomic results.

Two popular treatments for OA are glucosamine (GS) and chondroitin sulfate (CS). A pharmacoproteomic study investigated articular chondrocytes treated with either/or a combination of these compounds, before IL-1β treatment to initiate an OA related inflammatory state (108). A high proportion of the 18 differentially expressed proteins caused by GS treatment were involved in synthesis and folding processes and signal transduction pathways. The CS treatment caused differential expression of 21 proteins predominantly related to energy production and metabolic pathways. Of particular interest was the expression of the chaperone GRP78, which was only upregulated by GS and not by CS alone. Overall, 31 proteins had modulated expression due to GS and CS treatment alone or in combination, with SOD2 levels reduced by all treatments.

3.4. Proteomic studies of chondrocyte mitochondria

It is now generally accepted that mitochondria are responsible for not only for ATP synthesis from oxidative phosphorylation but also for regulation of apoptotic cell death (109-113). Although chondrocytes mainly use the glycolytic pathway for ATP production (15-17, 114-116), they possess mitochondria and it is increasingly recognized that these organelles may play important roles in disease processes such as OA (117-123). The activity of mitochondrial respiratory chain complexes has been reported to be reduced in OA chondrocytes compared to healthy cells (123). TNF-α and IL-1β have been shown to cause damage to mitochondrial DNA (124). Alterations to mitochondrial function could have serious consequences for any cell type and therefore mitochondrial dysfunction could affect ECM maintenance in chondrocytes and may play a role in the progression of OA.

Mitochondrial fractions have been isolated and purified from healthy chondrocytes before proteomic analysis with 2D-electrophoresis and MALDI-TOF/TOF (119). A 2D reference map was established from which 72 spots were identified, corresponding to 49 distinct proteins. The 2D maps and western blotting of chondrocytes from donors of various ages displayed significant age related reduction in the expression levels of SOD2. SOD2 is involved in the metabolism of the ROS superoxide and thus reduced levels of the enzyme could contribute to increases in ROS and oxidative damage, which can facilitate disease progression. Recently 2D fluorescent differential in-gel electrophoresis (DIGE) has been applied to assess the mitochondrial protein alterations that occur in OA chondrocytes (117). Here an enriched protein fraction revealed differential expression of 73 proteins in OA chondrocytes. Mitochondrial proteins accounted for 23 of the 73 proteins providing further evidence that changes in mitochondria function could influence chondrocyte apoptosis in OA. Differentially expressed mitochondrial proteins included SOD2, TNF receptor-associated protein 1 (TRAP1), inner membrane protein mitofilin (IMMT) and optic atrophy 1 (OPA1). The morphology and remodeling of the cristae in mitochondria are controlled by IMMT and OPA1 and therefore changes in their expression could have implications for chondrocyte energy transduction. Decreases in SOD2 and the chaperone protein TRAP1 could both lead to lack of protection against ROS in mitochondria and such alterations could alter the delicate metabolic equilibrium that is required for chondrocyte function and ECM turnover, or leave chondrocytes susceptible to activation of apoptosis by cell death ligands or the pro-inflammatory cytokines IL-1β and TNF-α. This would decrease the already sparse numbers of these vital cells in OA cartilage.

3.5. Proteomic studies of synovial fluid

The synovium is a specialized soft tissue that lines the non-cartilaginous surfaces within synovial joints. The synovial membrane encapsulates the synovial fluid within joints. It consists of a network of capillaries important for gas and nutrient exchange and for the development of synovial inflammation (synovitis) in OA. The synovium is permeable to water, gases, nutrients, small molecules and proteins, but not to large proteins, proteoglycans, GAGs and oligosaccharides. Biomarkers of OA accumulate in the synovial fluid before they enter circulation. Therefore, proteins secreted from diseased cartilage can be studied and measured in synovial fluid. The majority of proteomic studies of synovial fluid from OA patients have compared protein markers with those found in RA samples. The synovial fluid and plasma of patients with OA, RA and reactive arthritis were analyzed by 2D-electrophoresis and MALDI-TOF-MS (125). All forms of arthritis produced significant levels of fibrinogen β-chain degradation products although these proteins were not present in plasma. This suggests that the synovium creates a barrier preventing these degradation products from entering the circulation. It is also possible that the liver or the kidney (or both) are involved in clearing fibrinogen degradation products.

Another proteomic study identified eighteen protein spots that were significantly higher in synovial samples from OA patients compared to samples from healthy individuals, although poor protein separation in some sections of the 2D gels was a major limitation in this study (126). A number of the differential spots corresponded to haptoglobin α2 chains indicating increased haptoglobin, a protein that is well known for its association

with inflammatory conditions. A proteomic comparison between RA, OA and other inflammatory joint conditions, revealed that S100A8 and S100A9 protein levels were significantly higher in RA than in OA (127). The S100 proteins are a family of calcium binding proteins that are involved in a range of functions. They regulate intracellular processes such as cell growth and motility, cell cycle regulation, transcription and differentiation (128). S100A1 and S100B regulate a diverse group of cellular functions including cell-cell communication, cell growth, cell structure, energy metabolism, contraction and intracellular signal transduction (129). Although some members of the family may function extracellularly and have been shown to be secreted into the secretome, most of them appear to function as intracellular calcium-modulated proteins and couple extracellular stimuli to cellular responses via interaction with other cellular proteins called target proteins (130).

Endogenous peptides present in synovial fluid from OA patients have been profiled by utilizing ultracentrifugation and solid-phase extraction to enrich the sample before identification with LC-MS/MS (131). Here 29 proteins were identified and 6 of these were proposed as possible biomarkers due to their previous association with the disease. In another study, the LC-MS/MS analysis of synovial fluid taken from the knees of OA patients and normal donors led to the identification of dermcidin, aggrecan and cystatin A that have reduced expression in synovial fluid from OA patients (132). Principal component analysis gave distinct clustering of groups for healthy and OA samples although there was not clear separation between early and late OA sufferers. There were 117 proteins recognized while 18 of these showed altered expression between synovial fluids from OA and normal joints.

3.6. Proteomic studies of synoviocyte lysates

Changes in the proteome of synovial cells can be used to monitor alterations in response to a variety of inflammatory and mechanical signals. A 2D-SDS-PAGE protein profile map of synoviocytes has been established with 82 specific proteins being identified (77). This study found that the most abundant proteins in synoviocytes are the filament proteins, vimentin, lamin A and gelsolin. Another study found 25 proteins that showed differential expression between synovial fibroblasts from normal and RA or OA patients (133). Proteins that had significantly higher expression levels in RA and OA were enolase 1, peroxiredoxin 2, SOD2, annexin A1, cathepsin D, S100A4 and S100A10. It was also noted that SOD2 and cathepsin D expression in synovial fibroblasts was upregulated in OA compared to RA cells. The whole cell proteome of chondrocytes and synoviocytes appear to be very similar when compared using 2D gel electrophoresis. Perhaps not surprisingly, the protein expression profiles of these two cells are in fact very similar when compared in this way (77). The major functional role of both these cell types is to maintain the fibrous structures within the synovial joint that surrounds them. While there are obvious differences between the synovium and the ECM cartilage, both are located in a comparable environment and many of the proteins they need to synthesize to survive in the harsh joint microenvironment will be similar.

3.7. Proteomic studies of the synovial membrane

The synovial membrane is effectively a sheet of synovial cells that line the synovium. It is not a real membrane; it is simply a sheet of heterogeneous synovial cells and tissue resident inflammatory cells. Comparisons have been made between synovial tissues from the synovium of OA and RA patients (134). In this study the importance of proteomic approaches was highlighted due to the poor correlation between mRNA and protein levels. A western blot array with 260 immobilized antibodies was used to detect differential protein expression. There were 58 proteins that showed significant changes in expression between the two types of arthritis. These included cathepsin D, Stat1 (signal transducer and activator of transcription 1), p47phox (neutrophil cytosolic factor 1), SOD2 and CD3ε (EGF-like module containing, mucin-like, hormone receptor-like 2). The protein expression profile of OA synovium has also been investigated by 2D gel electrophoresis and MALDI-ESI-MS and compared to the profiles in RA and spondyloarthropathy (135). Although this western blot array is a useful technique, there has to be an appropriate antibody present for a particular protein to be identified. It could be possible that important proteins are missed because an antibody is not included for them, or they may not successfully bind to their corresponding antibody. This approach will only be useful for proteins to which good antibodies have been raised. Having access to high quality monoclonal antibodies capable of recognizing proteins in a variety of different species will facilitate the development of immunoassays for experimental or surrogate biomarkers.

3.8. OA biomarkers in body fluids: proteomic studies of urinary and serum proteins

The ease of obtaining urine samples for clinical analysis is advantageous due to its non-invasive nature. OA biomarkers that can be measured in the urine will therefore be beneficial in early disease and disease monitoring. Discussion of the biomarkers of collagen II neoepitopes is beyond the scope of this review. However, it is important to point out that proteomic techniques were used to identify collagen type II neoepitopes in urine and synovial fluid using an immunoaffinity LC-MS/MS assay (136). A 45 amino-acid peptide (uTIINE) was found to be the most abundant collagen II neoepitope and proved to be a useful biomarker of MMP activity (136-138). Analysis of serum samples from OA patients in a large population-based study has identified an autoantibody to triosephosphate as a novel marker that could be used as a diagnostic biomarker for OA (139). A comparison of 2D protein profiles of serum from healthy individuals, OA patients and OA patients taking soy supplements (a source of anti-inflammatory compounds such as genistein) has been completed to assess the differential proteins (140). The OA serum provided evidence of altered levels of proteins including vitamin D-binding protein precursor and apolipoprotein A-I and A-IV precursors. In samples from patients taking soy protein supplements levels of hemopexin precursor, kininogen, vitamin D-binding protein

precursor and transthyretin were altered. The data presented in Table 1 highlights some of the differentially expressed proteins identified in normal and OA cartilage/chondrocytes and outlines their functional significance.

4. OA biomarkers: discovery, validation and commercialization

Biomarker discovery and validation for OA has accelerated significantly as we have increased our understanding of the anatomy, physiology, biochemistry and cell biology of joint tissues and their molecular complexity (141). One of the main issues responsible for driving this agenda has been the acute need for improved OA outcome measures in clinical trials (141, 142). There is an acute need for new biomarkers that provide information about early responses in cartilage. These biomarkers will be potentially useful in a clinical setting for detecting early, pre-radiographic changes in joints. We also need to identify biomarkers that may be useful for characterizing the status, prognosis and measurement of treatment response in OA. Current research in this area is aimed at developing an analytical toolbox with the potential to improve the clinical development process (143, 144). Many OA biomarker patents have been published and an increasing number of these include post-genomic techniques. It remains to be seen how many of the filed patents turn into products, tests and assays that can diagnose early OA and predict chronic disease.

5. Areas for future research

It is clear that combining existing biomarkers in new tests may improve their prognostic accuracy and help identify at-risk patients (145). However, there is still a great deal of work that needs to be done. The challenge now is to identify sensitive and reliable OA biomarkers that can be accurately and reproducibly measured in blood or urine. The assays for these biomarkers should be robust and easy to establish in any laboratory without the need for expensive and complicated equipment. Identification of such biomarkers is especially critical in the early phases of OA so that any treatments (i.e. NSAIDs), moderate exercise and weight loss can be started as soon as possible to slow down progression of the disease. There will be many challenges to using proteomics in biomarker discovery, validation and qualification in the coming years. The main challenge is the high cost of proteomic techniques and their reliance on state-of-the-art equipment. Future research in this area will benefit from advances and refinements in proteomic technology and advanced imaging and image analysis research. Research effort will also need to focus on identification and validation of panels of biochemical markers that may be correlated with joint imaging modalities (i.e. radiography, ultrasound, MRI) and used as non-invasive and reliable diagnostic and prognostic indicator of disease severity and response to pharmacotherapy and physiotherapy.

5.1. Glycomics

Many proteins are unable to fold properly if they are incorrectly glycosylated and may be directed to the wrong part of the cell. Defects in the assembly of sugar molecules or the sugar-protein hybrids are the basis for a growing list of human diseases (146). Glycomics is the comprehensive study of all glycans expressed in biological systems (146). Therefore, it is also important to apply 'glycomic' approaches to joint tissues in order to understand disease related changes in the 'glycome' (147). The glycome exceeds the complexity of the proteome due to the greater diversity of the glycome's constituent carbohydrates. The 'glycome' is further complicated by the sheer number of possibilities for combinations and interactions between carbohydrates and proteins. Such approaches will help identify the sugars (glycans) as well as the sugar-protein and sugar-fat hybrid molecules that are produced by joint tissues and changes in these molecules in ageing and disease. The biosynthesis of glycan relies on a number of highly competitive processes involving glycosyl transferases.

There are a small number of publications on these enzymes in normal and OA cartilage (148, 149). However, these are older studies that did not have access to currently available proteomic and glycomic approaches. We predict that this will be a fruitful area for future research. Molecular imaging of the glycome with chemical reporters has been proposed and offers a new avenue for probing changes in the glycome that accompany disease processes in joints (150). This work will also be facilitated by the development and expansion of glycome databases such as GlycomeDB[5] and establishment of new tissue and/or disease specific databases.

5.2. Plasma membrane proteomics

Membrane proteins present a major challenge to the comprehensive analysis of the proteome (151, 152). The large-scale analysis of membrane proteins is difficult due to the fact that membrane proteins require a carefully balanced hydrophilic and lipophilic environment. Furthermore, membrane proteins are rarely detectable using 2-D PAGE. There are thousands of published papers that have presented evidence for altered expression of membrane antigens in cancer. New methods for 2-D PAGE and protein identification are being developed for membrane proteomics in cancer research and immunology. Membrane proteins are also important markers of chondrocytes and other joint cells. The membrane proteome includes many diverse classes of protein molecules. Some of these are ion channels (see below) whereas others are receptors for growth factors, cytokines and extracellular matrix molecules (Figure 4). Many of these are multi-functional proteins. Difference gel electrophoresis (DIGE), in which two protein samples are separately labeled with different fluorescent dyes and other methods of cell surface labeling have been used to study membrane proteins and cell surface antigens (153). However, the poor solubility of membrane proteins remains a major obstacle to research progress in this area.

5.3. The chondrocyte channelome

Ion channels are the essential components that control ion movement in and out of the cell (154). They are embedded within the plasma membrane and usually

Table 1. Highlighted proteins identified in osteoarthritis related proteomic studies

Protein Name	Function	Reference	Differentially Expressed	Source
COMP	ECM component protein that interacts with collagens and fibronectin.	(83)		Articular Cartilage - Human
Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1) (CXCL12)	Chemokine	(83)		Human
Cartilage intermediate layer protein (CILP)	ECM constituent	(83)		Human
HtrA serine protease 11	Intercellular Protease	(84)	✓	Human
Fibulins (-1D, -2 and -3)	Secreted glycoprotein incorporated ECM.	(84)	✓	Human
Alcohol dehydrogenase (ADH)	Intracellular metabolic enzyme.	(85)	✓	Human
Adenosine kinase isoenzyme 1 (ADK)	Intracellular metabolic enzyme.	(85)	✓	Human
Flavin Reductase (FR)	Intracellular Metabolic enzyme.	(85)	✓	Human
Enolase 1 (ENO1)	Intracellular Metabolic enzyme.	(85)	✓	Human
Pyruvate Kinase 3 isoform 2 (KPYM)	Intracellular Metabolic enzyme.	(85)	✓	Human
Annexin A1 (ANXA1)	Intracellular Protein – involved in functions at the inner cellular membrane and exocytosis.	(85)	✓	Human
Tubby protein homolog (TUB)	Intracellular signaling protein.	(85)	✓	Human
Phosphatidylethanolamine-binding protein (PEBP)	Intracellular binding protein.	(85)	✓	Human
Superoxide dismutase 2 mitochondrial (SOD2)	Mitochondrial enzyme that protects from oxidative stress.	(85)	✓	Human
YKL-40	Secreted protein that may have a role in tissue remodeling.	(86)	✓	Rat (distal femoral condyles)
Latexin	Specific inhibitor of zinc-dependent metallocarboxypeptidases	(87)		Rat
Activin A	Secreted protein for endocrine signaling.	(88)	✓	Cartilage Secretome – Human/Pig
Connective tissue growth factor (CTGF)	Secreted protein involved in regulation of ECM metabolism.	(88)	✓	Human/Pig
Cytokine-like protein C17	Cytokine	(88)	✓	Human/Pig
YKL-39	Secreted protein signaling.	(89)		Human
Vitamin D binding protein	Binds vitamin D in plasma.	(89)		Human
TIMP-1	Inhibitor of matrix metalloproteinases.	(89) (93)	 ✓	Human Human
Pigment epithelium-derived factor (PEDF)	Neurotrophic protein.	(89)		Human
Osteoprotegerin (OPG)	A TNF receptor superfamily member.	(89)		Human
Tumor necrosis factor receptor 1 (TNF-R1)	Cytokine receptor – transmembrane protein.	(89)		Human
Collagen II neoepitope peptides	ECM constituents	(136)	✓	Human
Neutrophil gelatinase-associated lipocalin (NGAL)	Transport of small lipophilic substances (Potential)	(91)	✓	Mouse
YKL-40	Secreted protein that may have a role in tissue remodeling.	(91) (92) (93)	✓ ✓ ✓	Mouse Bovine (Stifle) Human
Haptoglobin	Binds hemoglobin in the plasma.	(91)	✓	Mouse
MMP-3	ECM degradation.	(91) (92)	✓ ✓	Mouse Bovine (Stifle)
COMP	ECM component protein that interacts with collagens and fibronectin.	(91)	✓	Mouse
Hyaluronan and proteoglycan link protein 1	Stabilizes the aggregates of proteoglycan monomers with hyaluronic acid in the extracellular cartilage matrix.	(91)	✓	Mouse
Matrilin-3	ECM component.	(91)	✓	Mouse
Serotransferrin	Iron binding transport protein.	(91)	✓	Mouse
Aggrecan G1 domain proteins	A proteoglycan – ECM component.	(91)	✓	Mouse
Gelsolin	Actin-modulating protein	(91)	✓	Mouse
GRP58	Chaperone associated protein	(83)	✓	Bovine (Stifle)
GRP78 (HSPA5)	Chaperone protein	(83)	✓	Bovine (Stifle)
MMP-1	ECM degradation	(95) (86)	✓ ✓	Chondrocyte Secretome – Human/Bovine (Nasal) Rat
MMP-3	ECM degradation	(95) (86)	✓ ✓	Human/Bovine (Nasal)

				Rat
YKL-40	Secreted protein that may have a role in tissue remodeling.	(95) (86)	✓	Human/Bovine (Nasal) Rat
Cyclophilin A (peptidylprolyl isomerase A)	Accelerates the folding of proteins and catalyses cis-trans transformations.	(95)	✓	Human/Bovine (Nasal)
β_2-microglobin	Serum protein.	(95)		Human/Bovine (Nasal)
S100A11 (Calgizzarin)	Range of intercellular signaling functions and can be secreted.	(95)		Human/Bovine (Nasal)
Cofilin	Actin-modulating.	(95)		Human/Bovine (Nasal)
MIP-3α (CCL20)	Chemokine	(86)	✓	Rat
LIX (CXCL5)	Chemokine	(86)	✓	Rat
Fibroblast growth factor 23 (FGF23)	Regulator of phosphate homeostasis.	(68)	✓	Chondrocyte Whole Cell Lysate - Human
SRY (sex determining region Y)-box 11 (SOX11)	Transcription regulator protein.	(68)	✓	Human
WW domain containing oxidoreductase (WWOX)	Oxidoreductase enzyme with a range of roles.	(68)	✓	Human
Kruppel-like factor 6 (KLF6)	Transcription activator protein.	(68)	✓	Human
Growth differentiation factor 15 (GDF15)	Regulates tissue differentiation.	(68)	✓	Human
Vimentin	Cytoskeletal protein.	(106)	✓	Human
αB-crystallin	Small heat shock protein	(69)	✓	Human
Lamin A	Component of the nuclear lamina.	(77)		Synoviocytes - Human
Vimentin	Cytoskeletal protein.	(77)		Human
Gelsolin	Actin-modulating protein	(77)		Human
Enolase 1 (ENO1)	Intracellular metabolic enzyme.	(133)	✓	Human
Peroxiredoxin 2	Role in redox regulation of the cell	(133)	✓	Human
Superoxide dismutase 2 mitochondrial (SOD2)	Mitochondrial enzyme that protects from oxidative stress.	(133)	✓	Human
Annexin A1 (ANXA1)	Intracellular Protein – involved in functions at the inner cellular membrane and exocytosis.	(133)	✓	Human
Cathepsin D	Protease responsible for intracellular protein breakdown.	(133)	✓	Human
S100A4	Range of intercellular signaling functions and can be secreted.	(133)	✓	Human
S100A10	Range of intercellular signaling functions and can be secreted.	(133)	✓	Human
Stat1	Signaling protein.	(134)	✓	Whole Synovial Membrane - Human
Cathepsin D	Protease responsible for intracellular protein breakdown.	(134)	✓	Human
Neutrophil cytosolic factor 1 (p47phox)	Subunit of neutrophil NADPH oxidase.	(134)	✓	Human
Superoxide dismutase 2 mitochondrial (SOD2)	Mitochondrial enzyme that protects from oxidative stress.	(134)	✓	Human
Egf-like module containing, mucin-like, hormone receptor-like 2 (CD3-12 or CD3ε)	A transmembrane receptor, associated with cells of the immune system.	(134)	✓	Human
Superoxide dismutase 2 mitochondrial (SOD2)	Mitochondrial enzyme that protects from oxidative stress.	(119) (117)	✓	Chondrocyte Mitochondria – Human Human
TNF receptor-associated protein 1 (TRAP1)	Mitochondrial heat shock protein.	(117)	✓	Human
Inner membrane protein mitofilin (IMMT)	Protein involved in binding and organizing localization of cellular structures.	(117)	✓	Human
OPA1	Mitochondrial protein similar to dynamin-related GTPases.	(117)	✓	Human
Haptoglobin α2	Binds hemoglobin in the plasma.	(126)	✓	Synovial Fluid - Human
S100A8	Calcium binding proteins	(127)	✓	Human
S100A9	Calcium binding proteins	(127)	✓	Human
Dermcidin	A secreted protein related to range of biological activities.	(132)	✓	Human
Aggrecan	A proteoglycan – ECM component.	(132)	✓	Human
Cystatin A	Intracellular thiol proteinase inhibitor.	(132)	✓	Human
Collagen II neoepitope – uTIINE	Collagen fragments released from ECM.	(136) (138) (137)	✓ ✓ ✓	Urine - Human
Triosephosphate auto-antibody	Antibody to triosephosphate compounds	(139)	✓	Serum - Human
Vitamin D-binding protein precursor	Binds vitamin D in plasma.	(140)	✓	Human
Apolipoprotein A-I and A-IV	Protein components of high density lipoprotein (HDL) in plasma.	(140)	✓	Human

Hemopexin precursor (OQHU)	Hemopexin is a plasma glycoprotein that transports heme from the plasma to the liver.	(140)	✓	Human
Kininogen (specific subtype not yet determined)	Two types: kininogen (HMWK) and low molecular weight kininogen (LMWK). HMWK is essential for blood coagulation and assembly of the kallikrein-kinin system. Also, bradykinin, a peptide causing numerous physiological effects, is released from HMWK. In contrast to HMWK, LMWK is not involved in blood coagulation.	(140)	✓	Human
Transthyretin	Thyroid hormone-binding protein.	(140)	✓	Human

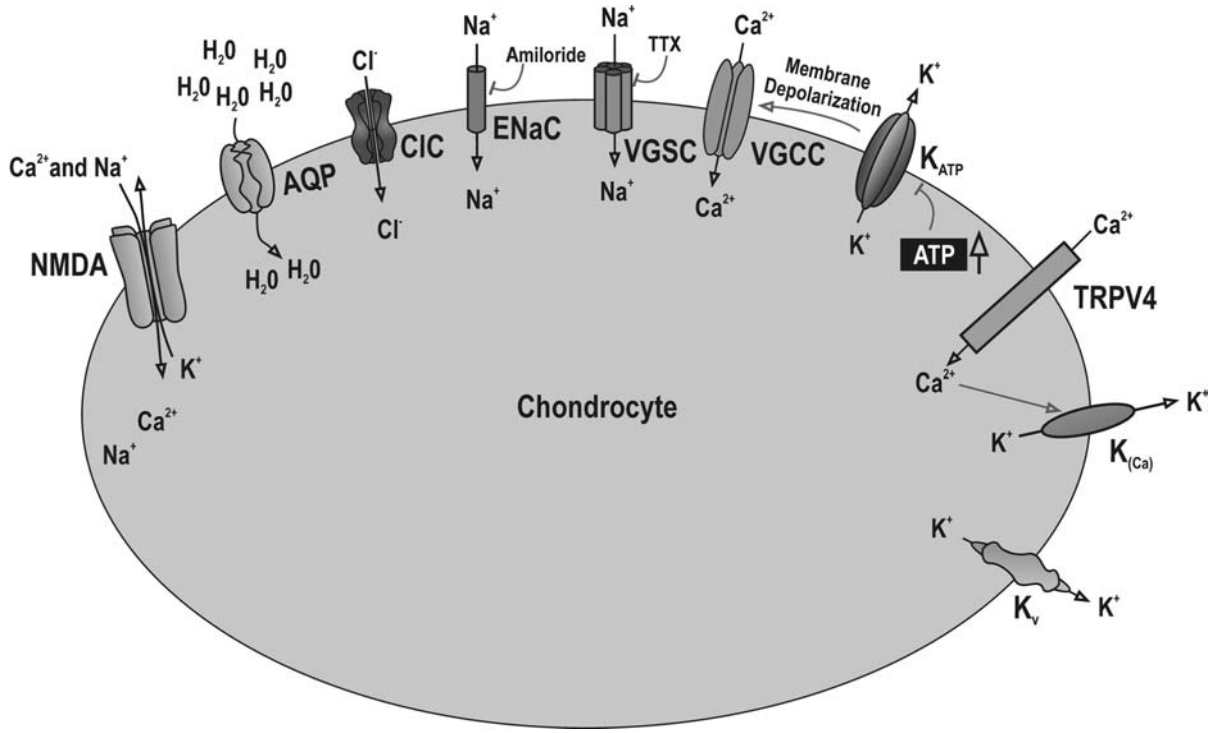

Figure 5. The chondrocyte channelome. This figure illustrates some of the major channel proteins identified to date, either by electrophysiological, immunological or molecular biological techniques. Proteomic studies are needed to study the detailed molecular composition of the chondrocyte membrane, including its channelome. Note that in this figure, $K_{(Ca)}$ is taken to be equivalent to any calcium activated potassium channel including BK and SK. AQP, Aquaporin channel; BK, Calcium-activated potassium cannel, high conductance; ClC, Chloride channel; ENaC, Epithelial sodium channels; KATP, ATP dependent potassium channel; Kv, Voltage-gated potassium channel; NMDA, N-methyl D-aspartate; SK, Calcium-activated potassium channel, low conductance; TRP, Transient receptor potential channel; VGCC, Voltage-gated calcium channels; VGSC, Voltage-gated sodium channel. Adapted with permission from (159).

consist of one or more proteins with a central aqueous pore, which opens by conformational change (155). The stimulus for opening (gating) is specific to each ion channel, and may be voltage, chemically or mechanically induced (156). There is growing interest in the expression and function of ion channels in chondrocytes and other joint cells. Part of this interest stems from the fact that many ion channels are involved in mechanotransduction, chemotransduction and osmoregulation. A number of studies have now shown the presence of an ever-expanding list of ion channels in chondrocytes. We have recently reviewed the literature on the expression and proposed roles of these channels in the chondrocyte channelome (Figure 5). It is important to bear in mind that ion channels are also important drug targets because of their localization in the chondrocyte plasma membrane. A number of research groups including ours have used electrophysiology, molecular biology and immunohistochemistry to study ion channels in articular chondrocytes. It is likely that some ion channels in chondrocytes are multifunctional, serving a number of different physiological purposes. The processes of mechanical and chemical sensing and metabolic regulation in the joint may well be intricately linked and make use of a number of ion channels as common denominators. Ion channels are important for cell function and further investigations are required to explore the full complement of channels present in the chondrocyte channelome. This knowledge will help us understand the unique biology of chondrocytes and may lead to the development and formulation of therapeutic strategies to treat arthritis. Proteomic studies on the chondrocyte channelome have not been carried out. This should be a high priority for future studies.

Table 2. A summary of proteomic studies relating to cartilage, other joint tissues and osteoarthritis

Tissue/Cell/Fluid Investigated	Proteomic Approaches	Main Findings	Reference
Articular Cartilage - Human	1D-SDS-PAGE, (LC)/MS/MS	Over 100 proteins identified, high proportion were ECM components	(83)
Human	1D-SDS-PAGE, nanoLC/MS/MS on normal and OA tissue.	59 differentially expressed proteins, including HtrA serine protease 11 and fibulin proteins	(84)
Human	2D-SDS-PAGE, LTQ-FS/MS comparing OA and normal tissue.	Over 1400 protein spots resolved in both OA and normal tissue. There were 14 differentially expressed proteins identified.	(85)
Rat	2D-SDS-PAGE, MALDI TOF/TOF MS	147 protein spots visualized on 2D Gels, with 47 identified. Latexin identified in this cartilage proteome.	(87)
Human	2D-SDS-PAGE, MALDI TOF/TOF MS	Improved resolution and focusing with techniques used. 16 representative proteins extracted and identified.	(77)
Articular Cartilage Secretome - Human and Pig	[35S] Methionine/Cysteine radiolabeling, 2D-SDS-PAGE, HPLC MS/MS, comparing OA and normal cartilage.	170 proteins identified in the proteome. 19 were newly synthesized due to radiolabeling. Activin A significantly up-regulated in OA.	(88)
Human	1D, 2D and off gel electrophoresis, Tandem MS, Antibody microarray.	43 proteins identified in the secretome, including YKL-39, TNFR1 and TIMP-1.	(89)
Human	Q-TOF/MS	Collagen II neoepitope peptides identified after IL-1β and oncostatin M treatment.	(90)
Mouse (Proximal femoral head cartilage)	2D-SDS-PAGE, Tandem MS.	IL-1β and Ret A treatment caused a number of proteins to be differentially expressed.	(91)
Bovine (Stifle cartilage)	SDS-PAGE-LC-MS/MS	Proteins released due to mechanical compression injury were mostly intercellular, while IL-1β and TNF-α treatments caused up-regulation of stress response proteins.	(92)
Human	SILAC, 1D-SDS-PAGE, LC Q-TOF/MS	25-30% of proteins were labeled and therefore newly synthesized. These new proteins were related to ECM metabolism.	(93)
Chondrocyte Secretome - Human (Articular cartilage chondrocytes)	Cytokine protein microarray comparing non-stimulation with IL-1β and TNF-α stimulation.	Cytokines, chemokines and growth or angiogenesis factors were upregulated by the stimulating inflammatory treatments.	(94)
Human (Articular cartilage chondrocytes) + Bovine (Nasal chondrocytes)	ESI-MS, 2D-SDS-PAGE.	Chondrocytes from OA cartilage stimulation with IL-1β and oncostatin M were compared with non-stimulated chondrocytes. Stimulation increased several proteins including MMP-1 and MMP-3.	(95)
Rat Chondrocytes (distal femoral condyles)	1D-SDS-PAGE, LC-MS investigating responses to LPS treatment.	LPS treatment induced stress-response proteins including MMP-1, MMP-3 and YKL-40.	(86)
Human articular chondrocytes	SILAC, 1D-SDS-PAGE, LC Q-TOF/MS	90% of proteins contained the labeled amino acids, and were therefore newly synthesized.	(93)
Chondrocyte Lysate - Human	2D-SDS-PAGE, MALDI-TOF-MS	136 protein spots visualized, with 93 identified proteins.	(104)
Human	2D-SDS-PAGE, MALDI-TOF-MS, comparing inflammatory stimulation by IL-1β and TNF-α with non-stimulated chondrocytes.	IL-1β treatment caused 22 differentially expressed proteins. TNF-α treatment caused 20 differentially expressed proteins.	(105)
Human	Reverse-phase protein array comparing normal and OA chondrocytes.	76 differentially expressed proteins, including FGF23, SOX11, WWOX, KLF6 and DGF15.	(68)
Human	2D-SDS-PAGE, Tandem MS, comparing damaged and intact OA, and normal chondrocytes.	17 differentially expressed proteins between chondrocytes from intact and damaged OA cartilage regions.	(106)
Human	2D-SDS-PAGE, Western blotting, comparing OA and normal cartilage.	αB-Crystallin levels reported as reduced in OA chondrocytes.	(69)
Human	2D DIGE, MALDI-TOF/TOF-MS comparing varying osmolarity.	20 differential spots between different osmolarities, with 18 of these proteins identified.	(107)
Human	2D-SDS-PAGE, MALDI-TOF/TOF-MS evaluating proteomic effects of chondroitin (CS) and glucosamine (GS) sulfate after IL-1β stimulation.	The CS and/or GS sulfate treatment differentially regulated 31 proteins, when compared to control non-treated cells (All stimulated with IL-1β). Both treatments reduced SOD2 levels. GS upregulated the chaperone GRP78.	(108)
Synoviocytes - Human	2D-SDS-PAGE, MALDI-TOF/TOF-MS.	82 proteins identified, the most abundant being vimentin, gelsolin and lamin A.	(77)
Human	2D-SDS-PAGE, MALDI-TOF-MS, comparing OA, RA and healthy synovial fibroblasts.	The comparison of healthy and RA or OA cells gave 25 differentially expressed proteins.	(133)
Whole Synovial Membrane - Human	Western blot array comparing proteins from RA and OA tissue.	260 proteins detected between the samples, with 58 differentially expressed between the two arthritis types.	(134)
Human	2D-SDS-PAGE, Tandem Mass Spectroscopy, Cluster Analysis, comparing RA, OA and spondyloarthropathy.	It was demonstrated how the cluster analysis could separate the three diseases in separate distinct groups.	(135)
Chondrocyte Mitochondria - Human	2D-SDS-PAGE, MALDI-TOF/TOF.	72 protein spots visualized with 49 proteins identified from the 2D reference map.	(119)
Human (enriched protein fraction)	2D fluorescent DIGE, MALDI-TOF/TOF-MS.	73 proteins differentially expressed, including SOD, TRAP1, IMMT and OPA1.	(117)
Synovial Fluid - Human	2D-SDS-PAGE, MALDI-TOF-MS on RA, OA and reactive arthritis samples.	All arthritis forms gave fibrinogen β-chain degradation products.	(125)

Human	2D-SDS-PAGE comparison of OA and healthy patients.	18 protein spots significantly higher in OA patients.	(126)
Human	2D-SDS-PAGE, Mass Spectrometry comparing various inflammatory joint conditions including RA and OA.	S100A8 and S100A9 protein levels were significantly higher in RA than in OA.	(127)
Human	Ultracentrifugation, NanoLC/MS on OA patient samples.	29 proteins identified in synovial fluid, with 6 being possible biomarkers.	(131)
Human	LC-MS/MS on normal and OA patient samples.	117 proteins recognized in the synovial fluid, with 18 of these differentially expressed.	(132)
Urine - Human	Immunoaffinity LC-MS/MS	Collagen II neoepitopes analyzed with uTIINE the most abundant neoepitope.	(138)
Human	Immunoaffinity LC-MS/MS	Further validation of the uTIINE neoepitope for use in immunoaffinity LC-MS/MS assay, demonstrating MMP's collagen II degradation.	(90)
Human	NMR, Principal component analysis on OA and normal samples.	Urinary metabolite profile created. OA and healthy sample profiles can help separate samples into distinct groups.	(8)
Serum - Human	2D-SDS-PAGE, MALDI-TOF-MS, on RA, OA and healthy samples.	An auto-antibody to triosephosphate identified and investigated as a diagnostic biomarker.	(139)
Human	2D-SDS-PAGE, MALDI-TOF, comparing healthy, OA and OA with soy treatment.	Several proteins altered in OA, and also when OA patients received soy treatment.	(140)

6. CONCLUDING REMARKS

Proteomic approaches continue to facilitate progress in basic cartilage biology and OA research creating an increasingly detailed interactive picture of the proteins involved in this disease at the molecular, cellular and tissue levels. By 'interactive' we mean a dynamic model that clarifies the roles of distinct classes of proteins in cartilage turnover. The synovial joint consists of several different components including articular cartilage, chondrocytes, synovial membrane, synoviocytes and synovial fluid and all of these can be studied in health and disease. Table 2 summarizes the proteomic studies that have focused on these tissues.

Studying each of the different components of the synovial joint will require different strategies and techniques. For example, studying extracts of synovial fluid and whole cartilage from normal and OA joints will require pre-treatment with chemicals that precipitate GAGs. This is especially important if 2D gels are used for protein separation. Although the removal of GAGs with CPC may be a technical pre-requisite for 2D-PAGE, it may result in the loss of potential biomarkers (proteins, glycoproteins or complex sugars) that are closely associated with GAGs. High-throughput techniques are also likely to have an impact in this field.

The benefits of advances in OA proteomics include potential improvements in therapeutic treatments and biomarker discovery for early disease diagnosis and monitoring of disease severity. Many differentially expressed proteins have been identified in OA tissues/cells compared to healthy samples. The increase or decrease in levels of these proteins could have an important contributing role in to the development of OA. Some considerations need to be taken into account when drawing conclusions from studies reporting on proteins that have shown altered levels of expression or release. Proteomic studies of whole cartilage have identified many proteins that were already known to be present in the ECM (Figure 4) before many of these proteomic approaches were even developed. ECM constituents such as collagen fragments and COMP often appear to have raised release or expression in OA conditions. Future proteomic studies will need to reveal more than just evidence of ECM degradation. Such information may include altered post-translational modifications and other structural alterations. There are also some proteins that are differentially expressed when comparing normal and affected tissues from completely unrelated diseases (157). Heat-shock proteins and metabolic enzymes such as enolase-1 are associated with cellular stress responses and therefore will show significant up-regulation in a spectrum of other diseases (158). Further research into the proteome of joint tissues should provide evidence of proteins specifically altered in OA, proteins that play key roles in disease pathogenesis. The low abundant proteins may prove to be the most interesting ones rather than the major components of cartilage ECM. Once potential candidates have been identified using proteomic approaches, there is still a considerable amount of work that is required before these candidates become true biomarkers. Validation and qualification studies require in depth quantitative analysis. Future research using proteomics will no doubt provide a more robust set of biomarkers and a better understanding of the basic biology of cartilage and the molecular pathogenesis of this disease.

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Footnotes: [1]http://www.cancer.gov/, [2]http://www.cancer.gov/dictionary/?CdrID=45618, [3]Osteoarthritis Research Society International - http://www.oarsi.org/, [4]U.S. Food and Drug Administration - http://www.fda.gov/, [5]http://www.glycome-db.org/

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