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Mini review

Biomarkers and proteomic analysis of osteoarthritis

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ABSTRACT

Our friend and colleague, Dr. Dick Heinegård, contributed greatly to the understanding of joint tissue biochemistry, the discovery and validation of arthritis-related biomarkers and the establishment of methodology for proteomic studies in osteoarthritis (OA). To date, discovery of OA-related biomarkers has focused on cartilage, synovial fluid and serum. Methods, such as affinity depletion and hyaluronidase treatment have facilitated proteomics discovery research from these sources. Osteoarthritis usually involves multiple joints; this characteristic makes it easier to detect OA with a systemic biomarker but makes it hard to delineate abnormalities of individual affected joints. Although the abundance of cartilage proteins in urine may generally be lower than other tissue/sample sources, the protein composition of urine is much less complex and its collection is non-invasive thereby facilitating the development of patient friendly biomarkers. To date however, relatively few proteomics studies have been conducted in OA urine. Proteomics strategies have identified many proteins that may relate to pathological mechanisms of OA. Further targeted approaches to validate the role of these proteins in OA are needed. Herein we summarize recent proteomic studies related to joint tissues and the cohorts used; a clear understanding of the cohorts is important for this work as we expect that the decisive discoveries of OA-related biomarkers rely on comprehensive phenotyping of healthy non-OA and OA subjects. Besides the common phenotyping criteria that include, gender, age, and body mass index (BMI), it is essential to collect data on symptoms and signs of OA outside the index joints and to bolster this with objective imaging data whenever possible to gain the most precise appreciation of the total burden of disease. Proteomic studies on systemic biospecimens, such as serum and urine, rely on comprehensive phenotyping data to unravel the true meaning of the proteomic results.

Contents

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1. Introduction

The investigator for whom this issue is dedicated, Dr. Dick Heinegård, played a pivotal role in the discovery and validation of biomarkers for osteoarthritis (OA). His seminal work, in characterizing the biochemical composition and interaction of components of cartilage, laid the foundation for all the work that has followed in this field. This paper briefly summarizes his contributions to the field and is followed by an update on the results of proteomic analyses performed since 2009 when a comprehensive review of this topic was last published (De Ceuninck and Berenbaum, 2009). Herein we focus on proteomic studies of four different types of biospecimens that are relevant to the study of joint diseases: cartilage, synovial fluid, serum and urine. We dedicate this work to our friend and colleague, Dick Heinegård.

1.1. Contributions to the field

A PubMed search (April 1, 2014) of papers authored by Dr. Dick Heinegård yielded 325 citations; a total of 16% of these were directly related to molecular markers of joint tissues in health and disease, and an additional 48% of citations involved cartilage biochemistry and chondrocyte biology that greatly inform biomarker work. In addition to cartilage biochemistry, he contributed many fine works related to extracellular matrices of other joint tissues and other tissues in the body, including tendon, bone, skin, sclera, cornea, aorta, eye and kidney (Franzen and Heinegård, 1985; Mörgelin et al., 1989; Oldberg et al., 1989; Saxne and Heinegård, 1989; Reinholt et al., 1990). His musculoskeletal work encompassed OA, rheumatoid arthritis (RA), juvenile inflammatory arthritis, polychondritis, reactive arthritis, psoriatic arthritis, and calcium pyrophosphate deposition disease among others (Saxne et al., 1987; Saxne and Heinegård, 1989). A brief summary of a few of his key works and insights are provided below.

For his entire career, Dr. Heinegård was deeply engaged in understanding disease pathogenesis and elucidating the components of various extracellular matrices, and cartilage and bone in particular, and converting what were enigmas into proteins with known structures and functions. This is nicely exemplified in his early creative determination of the substructures of cartilage proteoglycan and link protein; with Wieslander et al., he created tryptic peptide 'maps' from these proteins based on their cross-reactivity to polyclonal antisera developed to specific epitopes within these proteins (Wieslander and Heinegård, 1979). Beginning as early as 1987, with Inerot et al., he characterized the normal variability in structure and composition of the articular cartilage proteoglycans in the hip (Inerot and Heinegård, 1987). In further work with Wiberg et al. (2003) using molecular electron microscopy in combination with immunogold techniques, he was able to reconstitute and visualize collagen VI microfibril complexes *in vitro*; this work showed that the leucine-rich small proteoglycans (biglycan and decorin) together with matrilins form a link between collagen VI microfibrils and the collagen II and aggrecan networks in the cartilage extracellular matrix. This lifelong interest culminated in a recent paper with Önnerfjord et al., comparing hip and knee cartilage that demonstrated that cartilage constituents differ by joint site (Önnerfjord et al., 2012).

With Petersson et al., he showed that serum cartilage oligomeric matrix protein (COMP) was correlated with knee bone scan abnormalities in individuals with knee pain, suggesting that this marker may be a means of evaluating tissue changes in relation to early stages of OA (Petersson et al., 1998). Further work demonstrated that one function for COMP is to influence the organization of collagen fibrils, thereby contributing to tissue structure. He demonstrated that COMP interacted via its C-terminal globular domain to collagens I and II in the presence of Zn^{2+} and Ni^{2+} but not Ca^{2+}, Mg^{2+}, and Mn^{2+}. Electron microscopy with Rosenberg et al., showed that the interaction occurred at four defined sites on the collagen molecules (Rosenberg et al., 1998).

He also used COMP to gain insights into efficacy of interventions in OA. Working with Sharif et al., he showed that serum COMP increased significantly during the first year of follow-up in patients with progressive OA but not in non-progressors (Sharif et al., 1995). With Joosten et al., he observed synergistic effects of combined treatment with low dose prednisolone, IL-10 and IL-4 on disease activity of collagen induced arthritis reflected in reduced cartilage degradation based on serum COMP concentrations (Joosten et al., 1999a; Joosten et al., 1999c). In further work with Joosten et al., serum COMP also provided insight into the tissue target of biologic treatments of collagen induced arthritis; although both soluble TNF binding protein and anti-IL-1 treatment ameliorated disease activity, only anti-IL-1 treatment normalized COMP levels supporting the histological finding that anti-IL1 decreased cartilage destruction while anti-TNF blocked synovitis (Joosten et al., 1999b).

He monitored other matrix molecules as biomarkers to gain further insights into efficacy of interventions. In a study paradigm that was novel and instructive, even today, with Saxne et al. he evaluated cartilage metabolism in arthritis through measurement of proteoglycan in synovial fluid before and after intra-articular injection with a glucocorticoid (Saxne et al., 1986). He established the stability of the proteoglycan measure in samples withdrawn 5 days apart; then the patients were treated with local injections of glucocorticoids that were observed to significantly reduce the proteoglycan concentration in the joint fluid. This was one of the earliest *in vivo* demonstrations that quantification of proteoglycans in synovial fluid appears to have the potential for monitoring the effects of therapy on cartilage metabolism.

With Lorenzo et al. (2004), two key observations were made: that cartilage undergoes metabolic alterations very early in the disease process, even before there is overt fibrillation of the tissue; and notably, in contrast to traditional teaching suggesting minimal or no repair (in the case of collagen II), attempts to repair or replace the extracellular matrix in knee OA were evident based on aggrecan synthesis and increases in cartilage oligomeric matrix protein (COMP), fibronectin, and cartilage intermediate layer protein (CILP) (early events in the process) and collagen synthesis (late event in the process). Importantly, they drew attention to the challenge we have yet to overcome even today, the ability to define and distinguish early from late OA; they defined early OA as the absence of an OA clinical history but the presence of macroscopic lesions in the joint, while acknowledging that "the disease is only recognized in its late stage by clinical and radiological criteria".

In novel work with Sjöberg et al. (2005) he was among the earliest investigators to recognize the ability of components of the extracellular matrix, including the small leucine-rich repeat proteins (SLRPs) fibromodulin, osteoadherin, and chondroadherin, to activate the complement system that forms the core of the innate immune system. With Happonen et al. (2009) he interestingly found that fibromodulin, osteoadherin, and chondroadherin also bound the complement inhibitor C4BP; although not interfering with the ability of C4BP to inhibit complement, this binding apparently sequestered the SLRPs and thereby modulated their pro-inflammatory effects. They confirmed published data (Groeneveld et al., 2005) that decorin and biglycan bound C1-complex recognition protein C1q but did not activate complement (Sjöberg et al., 2009). In the same study, they also showed that lumican had similar properties but with lower affinity for C1q. Moreover, with Happonen et al. (2010) he showed that COMP can activate one complement pathway at the same time as it has the potential to inhibit another. He intuited that the "net outcome of these interactions is most likely determined by the type of released COMP fragments, which may be disease specific". With Kalchishkova et al. (2011), the NC4 domain of collagen IX was shown to inhibit complement by preventing complement C9 polymerization and enhancing cofactor activities of the major soluble complement inhibitors C4BP and Factor H. With Happonen et al. (2012b), compared with healthy controls he found elevated levels of COMP-C3b complexes in the circulation of patients with several rheumatologic diseases, including RA, OA, reactive arthritis, psoriatic arthritis, systemic lupus erythematosus, ankylosing spondylitis, and systemic sclerosis. COMP-C3b correlated with several measures reflecting

disease activity in RA. Because serum COMP did not significantly decrease during TNF-α inhibition, they posited that the intervention caused a reduction in the release of complement activating COMP fragments. The serum COMP-C3b concentrations were significantly higher in patients with arthritis suggesting the importance of joint inflammation for the release of complement-activating COMP. Taken together, these studies demonstrated that ECM factors can both fuel and modulate joint inflammation via the complement cascade. This series of studies was thoroughly summarized in a later review article (Happonen et al., 2012a).

They performed *in vitro* and *in vivo* studies characterizing metabolism of cartilage ECM components in various arthritides. With an *in vitro* system, they demonstrated both enhanced degradation and diminished synthesis of proteoglycans in living cartilage explants in the presence of cell free (10% by volume) inflammatory synovial fluid (Saxne et al., 1988). *In vivo*, in a signal work in 1989, representing the first study of the release of cartilage matrix markers in posttraumatic knee disease in humans, with Lohmander et al. he discovered that proteoglycan fragments in the knee joint fluid were markedly elevated during the initial 3–4 weeks after joint injury (anterior cruciate ligament injury or meniscal trauma) (Lohmander et al., 1989). Remarkably, levels of proteoglycan fragments were still elevated as much as 4 years after injury, which they believed to be due to persistent low-grade synovitis and/or chronic mechanical instability. In subsequent work they established that these pathological elevations even extended for decades after injury (Lohmander et al., 1993). They sagely pointed out that the elevated proteoglycan levels cannot be simply taken to indicate progressive destruction of the joint; rather that this may also reflect increased turnover of matrix components indicating increased synthesis within the joint (Lohmander et al., 1989). They also discovered that COMP and bone sialoprotein (BSP) were released into joint fluid after knee joint injury (Lohmander et al., 1994; Lohmander et al., 1996), and suggested that release of BSP into joint fluid may be associated with active remodeling of the cartilage–bone interface. This work has ultimately led to recent efforts to determine if preventing this cartilage breakdown will improve long term outcomes and decrease the expected 50% incidence of post-traumatic arthritis (Lohmander et al., 2007; Frobell et al., 2008).

He was among the earliest researchers to characterize specific joint tissue component neo-epitopes, products of proteolysis, and propose generating neo-epitope antibodies to detect and monitor ongoing cartilage degeneration and potential therapeutic responses to treatment. With Heathfield et al. (2004) he identified an MMP-13 generated cleavage site neo-epitope in fibromodulin. This work illustrated that he was not averse to disclosing in print what "yielded disappointing results" and to describe their attempts to overcome them. Whereas, full length purified fibromodulin was not cleaved when added to conditioned culture media from IL-1 treated cartilage explants, an N-terminal peptide of fibromodulin was cleaved. They speculated that protein conformation and therefore cleavage site accessibility differed *in vivo* (fibromodulin bound to collagen II) and the fibromodulin peptide from purified full length fibromodulin. With Danfelter et al. (2007) he also identified cleavage events within collagen IX (at both non-triple and triple helical sites) that potentially represent important degradation events that precede the major loss of type II collagen.

With longtime colleague Saxne, (Heinegård and Saxne, 2011) he provided a recent review on the role of cartilage matrix in OA. Of pertinence to biomarker studies, they pointed out that the release of fragmented molecules provides opportunities to monitor the disease process and to investigate whether these fragments are involved in propagating OA, for example, by inducing inflammation. They favored the concept that changes in bone are secondary to alterations in articular cartilage, which nevertheless modulate each other's structure.

He contributed to a number of large group efforts to advance biomarkers for use in musculoskeletal diseases including helping to establish a set of criteria for validation of soluble biomarker indicators of radiological damage endpoints in RA and spondyloarthropathies (Maksymowych et al., 2007), helping to establish the BIPEDS (burden of disease, investigative, prognostic, efficacy of intervention, diagnostic and safety) classification system for biomarkers in OA (Bauer et al., 2006) that is applicable to nearly all diseases, and helping to formulate guidance on the application of biomarkers in drug development and clinical trials for OA (Kraus et al., 2011).

2. Proteomic analysis of cartilage in osteoarthritis

Through his lifelong work, Dr. Heinegård has contributed greatly to the cartilage matrix biochemistry field. He contributed not only classical biochemical research techniques, but in recent years he also conducted studies with emerging proteomic tools. Herein we summarize the reports from the last five years of proteomic analyses related to joint tissue, including Dr. Heinegård's work, and focus on four different specimen sources, cartilage tissue, synovial fluid, serum and urine. A list of proteomic techniques, summary of results, as well as phenotyping of sample cohorts tested in these studies are summarized in Table 1.

2.1. Proteomic analysis of cartilage tissue in osteoarthritis

Cartilage consists of scattered chondrocytes and largely extracellular matrix (ECM), which includes predominantly collagens, highly sulfated proteoglycans, hyaluronan, and many other important proteins in minor amounts. Under normal conditions, protein homeostasis is maintained through balanced degradation and synthesis of the components of this highly organized tissue. The balance is disrupted by various pathological conditions including OA. In order to clarify molecular events during OA disease progression and to identify targets for treatment, cartilage proteomic analysis is applied. However, the dynamic range of the amount of protein within cartilage is problematic. Dominant collagen and aggrecan levels overwhelm the signals from other proteins. Anionic macromolecules, including aggrecan and hyaluronan, affect the peptide analysis. Cellular proteins from chondrocytes also hinder the identification of extracellular matrix proteins. Cartilage tissue is a tight physical and chemical network, which makes the protein extraction very difficult. The heterogeneity of cartilage tissue with distance from the cartilage surface further complicates the problem. To solve all these issues, well-established tissue processing methods, sensitive and reliable detection tools, and targeted strategies are imperative. Generally, a combination of physical disruption and chemical extraction is required to extract proteins from highly cross-linked cartilage. There are some modifications added to improve the efficiency of the experimental protocols. Hansen et al. conducted a study comparing the efficiency of surfactant-assisted, ultrasonication-assisted, and surfactant/ultrasonication-assisted digestion combined trypsin for mass spectrometer analysis (Hansen et al., 2009). The results suggested that all three modifications improved the sequence coverage of collagen I, which was the material used for testing. Another study examining ECM of vascular tissue proposed a three-step method to sequentially extract the loosely-linked proteins, cellular proteins, and ECM proteins (Didangelos et al., 2010). The vascular tissue was first pre-washed with PBS, diced and then treated with sodium chloride, which enabled the extraction of loosely associated proteins, including newly synthesized proteins and degradation products. Cellular proteins were extracted subsequently by 0.08% sodium dodecyl sulphate, which is effective in solubilizing cytoplasmic and nuclear membranes and still below the critical micelle concentration. The insoluble ECM-enriched residue was then extracted by guanidine-HCl, a well-developed method to extract strongly bound ECM components. Another group applied a similar strategy to mouse pulmonary and aortic valves wherein tissue was microdissected and decellularized with 2% SDS (Angel et al., 2011). Although they applied this technique to vascular tissue, this concept can be applied to cartilage tissue to separate loosely linked ECM protein, including degraded fragments, cellular components, and ECM of

Table 1
Summary of proteomic studies of cartilage tissue, cartilage explants, and chondrocytes.

Material	Species	Healthy non-OA	OA	Age in years (healthy/OA)	Method	Mass spectrometer used	Number of proteins identified	Note	Reference
Cartilage	Mouse	Post-natal mice	–	–	Sequential extraction	LC-LTQ Orbitrap	703	Sequential extraction of proteins	Wilson et al. (2012)
	Human	Individuals with no joint disease history	Patients who underwent knee replacement	(37–45)/(59–62)	2-DE	LTQ-FT/MS	14	Identified >1400 protein spots; 16 spots were differentially expressed	Guo et al. (2008)
	Human	Individuals with no joint disease history	Patients who underwent knee replacement	NA/(50–82)	1D-SDS PAGE	LC-LTQ Ion Trap	814	59 proteins were differentially expressed	Wu et al. (2007)
	Human	Individuals with no joint disease history	–	Range 36–50	Off-line 2D-LC	Ion Trap/Q-TOF(iTRAQ)	340	Compared different cartilage tissues	Önnerfjord et al. (2012)
Medium of cartilage explant culture	Human	Individuals who underwent trauma/ amputations/autopsies	Patients who underwent joint replacement	(22–86)/(53–83)	Proteoglycan precipitation,2 − DE	LC-Q-TOF	19 labeled proteins	Radio label represented newly synthesized proteins during culture	Hermansson et al. (2004)
	Human	Tissue with no signs of OA	Tissue with pathologic signs of OA	35 & 64	–	LC-triple quadrupole/Ion Trap	16 prominent proteins	Treated with exogenous MMPs and analyzed proteolyzed peptides	Zhen et al. (2008)
	Human	–	patients who underwent knee replacement	range 69–84	1D SDS-PAGE	LC-LTQ/ LC-TQ(QconCAT)	252	9 proteins were differetially expressed by IL-1β; TIMP1 was reduced by IL-1β	Peffers et al., (2013)
	Horse	Marcroscopically healthy tissue	–	–	–	LC–MS/MS (Ion Trap)	–	Explants were treated with IL-1β and anti-inflammatory drug, carprofen	Clutterbuck et al. (2011)
	Horse	–	Tendon with known injury	–	–	LC-Q-TOF/LC-triple quadrupole	–	Explants were treated with IL-1β and PGE_2; COMP fragments were analyzed	Dakin et al. (2014)
Chondrocyte culture	Human	–	Patients who underwent joint replacement	Range 57–80	1D-PAGE	LC-MALDI-TOF/TOF	368 (proteome) & 115 (secretome)	Standardized chondrocyte SILAC protocol. Both cellular proteome and secretome were analyzed	Calamia et al. (2011)
	Human	Marcroscopically healthy tissue with no joint disease history	–	70, 73, & 78	0.2 μm filter & protein precipitation	LC-MALDI-TOF/TOF	75	CS treated chondrocytes. 18 proteins were modulated by CS	Calamia et al. (2012)
Articular cartilage vesicles	Human	Individuals with no joint disease history	Patients who underwent joint replacement	–	Serial centrifugation of collagenase treated cartilage	LC-LTQ	170	6 proteins were only found in healthy and 9 proteins were only in OA	Rosenthal et al. (2011)

2-DE: two-dimensional gel electrophoresis, LC: liquid chromatography, LTQ: linear trap quadrupole, FT/MS: Fourier transform ion cyclotron resonance mass spectrometer, TOF: Time of fight mass spectrometry, OA: osteoarthritis.

cartilage tissue. In contrast to the Diangelos and Angel groups, who separated cells from matrix, Wilson et al. has developed a method to process whole mouse cartilage for proteomic analysis (Wilson and Bateman, 2008; Wilson et al., 2008; Wilson et al., 2012). They conducted physical disruption by pulverizing liquid nitrogen-frozen tissue to enhance extraction efficiency. Glycan moieties were removed by chondroitinase ABC to improve sequence coverage. Sequential extractions were done by using NaCl to extract soluble proteins and guanidine-HCl to extract NaCl-insoluble proteins. By analyzing these fractions using an LC-LTQ-Orbitrap mass spectrometer, they identified a total of 703 proteins from whole mouse cartilage tissue.

Although it is difficult to establish cartilage proteomic analysis methods, studies in this field are still conducted. Guo et al. performed two-dimensional gel electrophoresis (2-DE) on cartilage extractions from individuals with and without OA respectively and observed over 1400 protein spots in each group (Guo et al., 2008). A total of 16 differentially expressed protein spots were chosen for identification by linear ion trap-Fourier transform ion cyclotron resonance mass spectrometry (LTQ-FT/MS). Another study compared the proteome of cartilage from knee OA to healthy individuals (Wu et al., 2007). The cartilage extractions were fractioned by SDS-PAGE and analyzed by LC–MS/MS. They identified a total of 814 proteins from all samples. Of these proteins, 59 proteins were found to be differentially expressed in OA cartilage compared to non-OA cartilage.

A broad range investigation of different cartilage tissues, including femoral head, humeral head, knee medial tibial condyle, intervertebral disc, nucleus pulposus, meniscus, and tracheal and rib cartilage, has been conducted to provide a background detailed compositional analysis with relative quantification of extracellular matrix proteins (Önnerfjord et al., 2012). This study used isobaric tags for relative and absolute quantification (iTRAQ) to determine the relative quantity of ECM proteins from cartilage tissues, which have been dissected, pulverized, and extracted by guanidine-HCl. Of note, they also collected the extraction residue and performed in situ trypsin digestion and subsequent quantitative analysis. After manually filtering out intracellular proteins, plasma proteins, and proteins quantified in < 50% of all tissues, multivariate analysis of 92 of the 340 identified proteins was performed to detect significantly different protein expression in cartilage from different sites. The results revealed different protein expression patterns between all kinds of cartilages, which may relate to tissue mechanical properties and joint pathology. The investigation of the guanidine extraction residue suggested that several proteins, such as CILP, COMP, and asporin remained partly unextracted, which indicated probable cross-linking with the insoluble residue.

2.2. Proteomic analysis of cartilage explants in osteoarthritis

In order to simulate the behavior of cartilage in a pathophysiological condition and reduce the complexity, some studies have focused on the peptides released to the culture medium when the cartilage explants were treated with cytokines or other chemicals. One study showed the possibility of analyzing released proteins using two-dimensional gel electrophoresis after depleting anionic aggrecan with cetylpyridinium chloride (Hermansson et al., 2004). Nineteen newly synthesized proteins were identified by LC–MS/MS (quadrupole time-of-flight, Q-TOF) after cartilage explants were incubated with radiolabelled culture medium. By comparing the protein pattern from OA to non-OA cartilage, the study concluded that type II collagen synthesis increased in OA and a novel cartilage molecule, activin A, may be an anabolic factor in cartilage. To better simulate cartilage degradation and monitor the released peptides, Zhen et al. digested cartilage tissue with exogenous metalloproteinase enzymes, which are important components of the cartilage degradation cascade in OA (Zhen et al., 2008). A wide variety of released peptides, such as collagen, fibronectin, COMP, CILP, etc., were identified by LC–MS/MS. (Zhen et al., 2008). By monitoring released peptides *in vitro*, this study provided insights into the events and timing of cartilage degradation due to metalloproteinases.

Another study quantified the selected secreted proteins in IL-1β treated OA cartilage explant culture media (Peffers et al., 2013). Knee OA cartilage tissues were diced and incubated with medium supplemented with or without IL-1β. Comparative proteomic analysis of the OA cartilage secretome was performed by label-free LC–MS/MS and a total of 252 proteins were identified. Of these, 9 proteins were differentially expressed in the presence of the IL-1β supplement. Proteins of interest were selected for absolute quantification by QconCAT technology and LC–MS/MS (triple quadrupole) in selected reaction monitoring format (SRM). They found that the TIMP-1 protein was significantly reduced by IL-1β stimulation.

Clutterbuck et al. adopted a different strategy to investigate the role of inflammatory mediators in a cartilage model system (Clutterbuck et al., 2011). They compared the proteomic profile of cartilage explant culture media when treated under three conditions, medium alone, presence of the inflammatory mediator IL-1β, and IL-1β with the anti-inflammatory drug, carprofen. The peptides within the culture media were analyzed by qualitative LC–MS/MS and lists of proteins within the media were generated. The selected proteins were quantified by Western blotting. In discovery assay many proteins were identified, including aggrecan core protein, COMP, thrombospondin-1 (TSP-1), clusterin (CLU), matrix metalloproteinases MMP-1 and MMP-3, to name a few. By quantitative assay, they found that IL-1β increased MMP-1, MMP-3 and TSP-1 and decreased the CLU precursor released into the culture media. In a follow-up study, Williams et al. demonstrated that the addition of carprofen inhibited the release of MMP-1, -3 and -13 in the IL-1β treated equine cartilage explants (Williams et al., 2013). Differences in the secretome from cartilage explants were also investigated by Polacek et al. and included stable isotope labeling with amino acids in tissue culture media (SILAC) allowing them to target newly synthesized proteins in the culture media where only 25–30% of the proteins were labeled, indicative of matrix turnover (Polacek et al., 2010).

Dakin et al. have investigated the proteomic profiling of injured tendon during all stages of disease and focused on identifying specific cleavage patterns of COMP (Dakin et al., 2014). They simulated tendon injury by stimulating tendon explants with inflammatory mediators, IL-1β and prostaglandin E_2, to induce COMP protein degradation and characterized the effect of this stimulation on the cleavage patterns of COMP by LC–MS/MS (Q-TOF). They also quantified the COMP cleavage fragments by quantitative proteomic analysis in multiple reaction monitoring format (MRM). IL-1β was found to enhance the proteolytic cleavage and release of COMP fragments from explants. However, PGE_2 showed no catabolic effect. They also identified two cleavage fragments released in early stages of simulated tendon injury (presence of IL-1β) that could be used to develop a neo-epitope based assay for tendon injury.

2.3. Proteomic analysis of chondrocyte proteins in osteoarthritis

Besides cartilage tissue based and cartilage explant based studies, there are also chondrocyte-based studies, which reduce the complexity of the sample analysis. Calamia et al. developed a quantitative proteomic method applying stable isotope labeling with amino acids in cell culture (SILAC) on human articular chondrocytes (HACs) (Calamia et al., 2011). They used this technique to study the effect of the proinflammatory mediator, interlukin-1β, on *in vitro* cultured chondrocytes, and analyzed the changes of the cellular proteome and secretome. In the HAC proteome, they revealed a decreased expression of proteins involved in the actin cytoskeleton structures. Meanwhile, a global increase of protein chaperones, including GRP78 and HSP71, was also identified. In the secretome, they revealed the increase of three protein clusters, including proinflammarory mediators and proteases, type VI collagen and related molecules, and TGF-β pathway related

proteins. Another related study published a year later by the same group investigated the effect of chondroitin-sulfate on IL-β modulated HACs (Calamia et al., 2012). They identified 75 proteins from the secretome and, of these, 18 proteins that were modulated by chondroitin-sulfate. Chondroitin-sulfate intervention decreased the expression of several complement molecules and led to overexpression of TNF-α induced protein (Tumor necrosis factor-inducible gene 6 protein, TSG6). They also revealed the decrease of MMP1 and MMP-3 activation related to TSG6 overexpression. Chondroitin-sulfate also increased the expression of an anti-angiogenic molecule, thrombospondin-1.

A different research topic related to cartilage is articular cartilage vesicles (ACVs). ACVs are extracellular small organelles secreted by chondrocytes that have the ability to generate calcium pyrophosphate dihydrate-like crystal (Derfus et al., 1992). ACVs also carry RNA for type II collagen, aggrecan and other molecules (Mitton et al., 2009). Such vesicles might be one method for chondrocytes to communicate with each other. The latest study in this field conducted proteomic analysis on healthy non-OA and OA cartilage (Rosenthal et al., 2011). Articular cartilage vesicles were isolated by serial centrifugation of collagenase treated cartilage. Proteomic analyses of isolated ACVs identified approximately 170 proteins having >1 representative peptide per protein and a false discovery rate ≤5%. Of these, 6 proteins were exclusively found in healthy cartilage while 9 proteins were only found in OA cartilage. They also observed a decrease of matrix proteoglycans and an increase of TGF-β related protein, βig-H3, vitronectin, and serine protease HtrA1.

2.4. Summary of proteomic studies of cartilage

In summary, current studies have focused on solving the difficulties involved with performing proteomic analysis of cartilage tissue. A comprehensive and strategic tactic should be considered for planning the study. Currently, several studies already reported the results of differential proteomic analysis of cartilage tissue in healthy and OA patients. Tissue-wide studies suggest that protein components are not always the same. Tissue resources, different joints or different regions from the same joints, should be considered for further studies. Explant model have reduced the complexity of cartilage tissue studies. This system allows for manipulation of the cartilage explants with inflammatory mediators and/or anti-inflammatory drugs to demonstrate what may happen during cartilage degradation. Chondrocyte-based studies reduce the complexity of sample analysis and can reveal the changes of the cellular proteome and secretome in response to various interventions. However, a more comprehensive study design should be considered to simultaneously evaluate concurrent events, not only in culture media, but also the cartilage explant extractable and non-extractable components.

3. Proteomic analysis of body fluid in osteoarthritis

3.1. Proteomic analysis of synovial fluid in OA

In 1991, Dr. Heinegård and Saxne noted that levels of fragments of select macromolecules in synovial fluid correlated with processes in the joint cartilage (Heinegård and Saxne, 1991). They also noted that fragments lost from the articular cartilage were released into the synovial fluid in proportion to the activity of the disease process (Heinegård et al., 1987). For these reasons, synovial fluid analyses are of the utmost value for assessing, most directly, joint tissue metabolism. Although more difficult to obtain than a blood sample, synovial fluid is much more readily obtained than joint tissue such as by synovial or cartilage biopsies. An overview of the proteomic techniques that have been used in analyses of OA body fluid samples is listed in Table 2.

Synovial fluid is a dialysate of plasma to which are added components produced locally by joint tissues, including hyaluronan and lubricin. Thus synovial fluid contains proteins originally from serum and surrounding tissues such as synovial tissue, meniscus and articular cartilage. Higher concentrations of cartilage degradation products in the synovial fluid compared to serum or other body fluids, are usually taken to indicate a joint origin of the protein. Although harvest of synovial fluid is more practical than collection of affected cartilage by biopsy, collection of synovial fluid in large-scale cohort studies would be challenging. The high concentrations of anionic hyaluronan and abundant serum proteins in synovial fluid make the detection even more complicated. The preparation of synovial fluid for proteomic studies is a critical step for success. Generally, synovial fluid samples need to be treated with hyaluronidase to remove the macromolecular hyaluronan. Depletion of highly abundant proteins may be needed in addition to removal of contaminating red blood cells if the arthrocentesis was traumatic. Kong et al. have optimized a pretreatment method of synovial fluid for proteomic analysis (Kong et al., 2012). By removing hyaluronan, albumin, and immunoglobulin simultaneously, they observed much better proteome resolution and higher protein spot intensity when evaluating by two-dimensional gel electrophoresis (2-DE) separation.

In another study employing 2-D gel electrophoresis, Yamagiwa et al. that achieved low intrasample variability (Yamagiwa et al., 2003), a high level of heterogeneity was observed between different OA synovial fluid samples. They identified 18 protein spots with more than 5-fold differences and 9 protein spots with more than 100-fold differences between subjects. A total of 342 protein spots were examined and total of 135 unique proteins identified in a study employing one-dimensional gel electrophoresis to reduce the complexity of synovial fluid followed by analysis of in-gel processed samples with iontrap LC–MS/MS (Gobezie et al., 2007). After 18 proteins representing keratin species were removed, a total of 117 synovial fluid proteins remained. Of these, 18 proteins were identified with significant differences between OA patients and healthy controls. Ritter and Gobezie further improved the sensitivity of their method by using two-dimensional gel electrophoresis and a more sensitive LC–MS/MS system. They identified 66 proteins that were differentially present in healthy and OA synovial fluid (Ritter et al., 2013). Another study modified the immunodepletion step to yield additional insights into the synovial fluid proteome (Mateos et al., 2012); employing nano-LC-MALDI-TOF/TOF analysis, and after excluding the abundant proteins reported in the previous study, they identified a total of 136 proteins involved in the formation and remodeling of the extracellular matrix, including fibronectin, kininogen-1, cartilage acidic protein 1, and COMP. These discoveries were generated by mass spectrometry and verified by immunoblotting on individual samples. A different strategy utilized the surface enhanced laser desorption/ionization-time-of-flight (SELDI-TOF) technique for analysis of synovial fluid samples from OA and RA patients (Han et al., 2012). The advantages of SELDI are high throughput capacity and chromatographic chemistry to provide accuracy and reproducibility. The results were interrogated by artificial neural network (ANN) and three mass spectrometry peaks were identified as potential markers to differentiate OA from RA with a sensitivity of 89.4% and a specificity of 91.2%. Of these three peaks, one of them was identified by Western blot as S100 calcium binding protein A12 (S100A12). The latest report brought discovery proteomic analysis to the next level (Balakrishnan et al., 2014b). In this study, abundant proteins were depleted from pooled OA synovial fluid then subjected to SDS-PAGE separation at the protein level, and strong cation exchange (SCX) and isoelectric focusing fractionation (OFFGEL fractionators) at the peptide level to reduce the complexity of the synovial fluid. The processed peptides were then analyzed on a Fourier transform LTQ-Orbitrap Velos mass spectrometer; 5544 peptides corresponding to 677 proteins were identified. Of these, 545 proteins have not been previously reported including: ADAM-like decysin 1 (ADAMDEC1), alanyl (membrane) aminopeptidase (ANPEP), CD84, fibulin 1 (FBLN1), matrix remodeling associated 5 (MXRA5), secreted phosphoprotein 2 (SPP2) and spondin 2 (SPON2). Other than screening for novel proteins in synovial fluid, there are also studies that looked for neo-epitopes. Åhrman et al. used antibody-affinity

Table 2
Summary of proteomic studies of synovial fluid and serum.

Material	Species	Healthy non-OA	OA	Age in years (mean ± std or range)	Method	Mass spectrometer used	Number of proteins identified	Note	Reference
Synovial Fluid	Human	Radiographic healthy and no joint disease history	Inner-third meniscal tear (early); knee replacement (late)	NA/above 45/NA	1D-PAGE	LC-LCQ Iontrap	135	18 proteins were differentially expressed between OA and non-OA individuals	Gobezie et al. (2007)
	Human	Individuals with no inflammatory arthritis	Meniscal tear/MRI validated (early); knee replacement (late)	Range 45–65	2D-DIGE	MALDI-TOF/LC-triple quadrupole	–	66 proteins were differentially expressed; 5 proteins were confirmed by SRM	Ritter et al. (2013)
	Human	–	Diagnosed OA/RA per ACR criteria	(73 ± 8)/(53 ± 10)	Hyaluronidase, immunodepletion. 2-DE	LC-MALDI-TOF/TOF	136	Comparing RA to OA they identified novel RA and OA proteomic signatures	Mateos et al. (2012)
	Human	–	Diagnosed OA/RA per ACR criteria	(70.2 ± 5.4)/(68.4 ± 4.9)	ProteinChip array	SELDI-TOF	–	37 peaks were different comparing OA to RA. 3 peaks were potential markers by ANN analysis for differentiating OA/RA	Han et al. (2012)
	Human	–	Diagnosed OA per ACR criteria	65	Depletion, 1D-PAGE/SCX/Offgel	LC-LTQ-FT Orbitrap/ LC-triple quadrupole	677	Highest identified numbers of proteins to date; among these, 545 proteins have not been previously reported	Balakrishnan et al. (2014b)
Serum	Human	Individuals with no inflammatory/joint disease history	Diagnosed OA per ACR criteria	(47–64)/(30–92)	ProteinChip array	SELDI-TOF	4	345 serum samples were analyzed due to the high capacity of SELDI technique	de Seny et al. (2011)
	Human	Radiographic healthy and no joint disease history	>50 yr and diagnosed OA per ACR criteria. (progressor & non-progressor)	(61.9 ± 11.7)/ (71.5 ± 4.5)/(67.5 ± 6.4)	Depletion, desalt, isoelectric fractionation,ProteinChip array	SELDI-TOF	3	69 OA plasma samples, including progression, nonprogression, and non-OA were analyzed	Takinami et al. (2013)
	Human	Individuals with no joint disease history	Diagnosed OA per ACR criteria with radiograph validation	Range 58–90	Depletion	LC-MALDI TOF/TOF (iTRAQ)	262	6 proteins were modulated in moderate OA, 13 proteins in severe OA, and 7 proteins in both severity grades	Fernandez-Puente et al. (2011)

2-DE: two-dimensional gel electrophoresis, 2D-DIGE: two-dimensional difference gel electrophoresis, MALDI: matrix assited laser desorption/ionization, SELDI: surface enhanced laser desorption/ionization, LC: liquid chromatography, TOF: Time of fight mass spectrometry, OA: osteoarthritis, ACR: American College of Rheumatology, ANN: artificial neural network.

Table 3
Summary of proteomic studies of urine.

Material	Species	Healthy non-OA	OA	Age in years	Method	Mass spectrometer used	Target	Note	Reference
Urine	Human	Individuals with no radiographic signs of OA	Patients diagnosed with scored radiographic OA		Immunoaffinity enrichment	LC-Q-TOF	TIINE	Several urine OA markers were identified and a specific one was selected; TIINE was elevated in symptomatic OA compared to non-OA	Nemirovskiy et al. (2007). Nemirovskiy et al. (2010)
	Human	Individuals with no radiographic signs of OA	Patients with joint pain and scored radiographic OA	Above 55	Immunoaffinity enrichment	LC-Triple quadrupole (MRM)	Aggrecan ^{374}ARGS. ^{1820}AGEG	The aggrecan ARGS aggrecanase-generated neo-epitope was elevated in radiographic OA	Dufield et al. (2010)
	Human	Individuals with no joint disease history	Patients who underwent knee replacement with scored radiographic OA	(25.6 ± 2.6)/ (76.0 ± 5)	2D-DIGE	LC–MS/MS (Ion Trap)	Fib3-1, Fib3-2	ELISA assay results suggested these two markers were elevated in knee OA patients	Henrotin et al. (2012)

2D-DIGE: two-dimensional difference gel electrophoresis, LC: liquid chromatography, TOF: Time of fight mass spectrometry, OA: osteoarthritis.

enrichment of COMP fragments in synovial fluid samples to identify protein cleavage sites (Åhrman et al., 2014). They enriched proteins from synovial fluid of joint disease patients, including those with acute trauma, OA and RA, and the enriched proteins were fractioned by SDS-PAGE. Bands of interest were excised and prepared for identification by IonTrap Mass spectrometry. They identified a total of twelve novel neo-epitopes in COMP; two neo-epitopes were identified in acute trauma patients, six neo-epitopes were from OA patients, and five neo-epitopes were from RA patients. These neo-epitopes are candidate biomarkers that potentially could depict early events in disease.

Besides discovery proteomics, targeted proteomic analysis has been applied to the study of synovial fluid. Selected Reaction Monitoring (SRM) was applied in a study to validate differential protein expression among OA and control synovial fluids (Ritter et al., 2013). Balakrishnan et al. employed Multiple Reaction Monitoring (MRM), the advanced format of SRM, to validate the expression of ANPEP, osteoglycin (OGN), and dickkopf WNT signaling pathway inhibitor 3 (DKK3). Another important technique applied in modern proteomics is isotope labeling, which uses different weight isotopes to modify peptides. Water containing ^{16}O or ^{18}O have been employed to label OA and control synovial fluid with relative quantification determined by calculating the ratio of ^{16}O/^{18}O (Wanner et al., 2013). Isobaric Tag for Relative and Absolute Quantitation (iTRAQ) is another technique using heavy isotope labels for peptide quantification. This method is based on placing isobaric mass tags at the N-terminals of peptides. Labeled by two different iTRAQ tags and analyzed by mass spectrometry, depleted synovial fluid from RA and OA yielded a total of 575 proteins out of which 135 proteins were differentially expressed by ≧3-fold (Balakrishnan et al., 2014a).

3.2. Proteomic analysis of serum in OA

Serum samples are some of the most commonly accessible biospecimens, although more remote than synovial fluid from the individual joint organ. Cartilage degradation markers, released from cartilage into synovial fluid, are diluted in serum and mixed with the highly abundant proteins, such as albumin present in serum, creating a complex mixture of proteins. Moreover, the peptide of interest may originate from multiple joints and non-joint tissues, further complicating the ability to discern a contribution of a specific index joint to the serum concentration. Therefore, highly reliable sample processing and detection techniques are necessary for proteomic analysis of serum in OA. Widely used methods, such as immunodepletion for reducing the highly abundant proteins, have been applied; novel strategies are still developing. A recent study reported a method for pre-processing human serum samples based on a chemical depletion method involving two sequential protein precipitation steps with acetonitrile and DTT, with a subsequent two-dimensional difference gel electrophoresis (2D-DIGE) analysis and MALDI-TOF/TOF analysis to identify OA biomarkers (Fernandez-Costa et al., 2012). Reproducibility was examined by technical and biological replicates. Gel-based quantitative differential profiling yielded 16 protein forms that were significantly changed in OA samples compared to samples from healthy individuals without OA. ProteinChip array in surface enhanced laser desorption/ionization-time-of-flight (SELDI-TOF) technique was also developed and applied to the study of OA serum (de Seny et al., 2005; de Seny et al., 2011). By SELDI-MS, they compared serum from a large OA cohort to those of healthy individuals and OA patients and they identified four novel markers for OA, including V65 vitronectin fragment, C3f peptide, CTAP-III, and m/z 3762 protein. Another OA proteomic study in plasma was reported by Takinami et al. (2013). This group applied SELDI-MS to plasma samples from progressive OA, non-progressive OA, and healthy individuals without OA. They also used an affinity column to deplete the highly abundant proteins to raise the sensitivity of the assay. Three differentially expressed peaks between OA and healthy groups were selected from SELDI-MS analysis and identified by MALDI-MS/MS, including apolipoprotein C-I, C-III and N-terminal truncated transthyretin. The results were validated by the loss of ion peaks in SELDI-MS after targeted immunoprecipitation of these proteins from plasma.

iTRAQ has also been applied in proteomic research of OA serum. Fernandez-Puente et al. (2011), aiming to identify protein biomarkers of OA progression, performed differential quantitative proteomic analysis of serum samples from healthy individuals without OA, or moderate or severe OA (Kellgren–Lawrence (Kellgren and Lawrence, 1957) grades 2 and 4 respectively) and identified 262 quantifiable proteins by the calculated iTRAQ ratio. Of these, they identified three groups of proteins with differential expression comparing OA and non-OA healthy groups; six proteins were modulated only in moderate OA, 13 proteins only in severe OA, and 7 proteins in both. These proteins were from various categories, including complement components, lipoproteins, von Willebrand factor, tetranectin, and lumican. This was consistent with a previous report in which proteins, such as COMP, were modulated in OA samples (Morozzi et al., 2007). Further validation is needed to evaluate the potential capability of using these protein markers for OA prognosis.

3.3. Proteomic analysis of urine in OA

Urine is an easily accessible biofluid and can be obtained non-invasively. The simplicity of urine, compared to serum or synovial fluid, makes it attractive for discovery of biomarkers of disease; for this reason, urine proteomics in OA is an emerging field. However, the potential biomarkers released into synovial fluid from cartilage during disease progress have been highly diluted. Moreover, although urine is much less complex than serum or synovial fluid, a high percentage of albumin and abundance of small molecular weight proteins in urine present challenges for urinary proteomics. Modern proteomic

techniques, with high sensitivity and accuracy, improve the feasibility of identifying biomarkers in urine. We summarize the proteomic techniques and peptides targeted in the few existing urinary proteomic studies of OA. We also describe the phenotyping of sample cohorts used in these studies (Table 3). In a study by Nemirovskiy and colleagues, several MMP-driven Type II collagen neo-epitopes (TIINE) were identified by liquid chromatography coupled with a quadrupole time-of-flight (Q-TOF) mass spectrometer (Nemirovskiy et al., 2007). They raised antibodies against conserved sequences among these peptides and used these antibodies to prepare affinity column coupled to LC–MS/MS. A 45-mer peptide was observed in urine and synovial fluid samples. By triple quadrupole mass spectrometer with MRM, the five hydroxylated (5OH) form of this 45-mer peptide was found to be the most abundant peptide in urine and synovial fluid samples. A subsequent study that applied this assay to the analysis of human urine samples, including symptomatic OA, radiographic OA and non-OA subjects, showed elevated uTIINE 45-mer 5OH peptide in symptomatic OA compared to non-OA controls (Nemirovskiy et al., 2010).

Another potential biomarker in urine is aggrecan fragments, an indicator of the aggrecanase and metalloproteinase-activity involved in cartilage degradation. Aggrecan is one of the most abundant proteins within cartilage tissue. Therefore, monitoring the activity of aggrecanases (ADAMTS-4 and ADAMTS-5) and metalloproteinases, through monitoring of fragments generated by these proteases, is considered important for understanding OA. Dufield and colleagues have developed an immunoaffinity LC–MS/MS assay to quantify the specific ^{374}ARGS aggrecanase generated fragment within human urine (Dufield et al., 2010). They demonstrated the ability of the immunoaffinity column to enrich this peptide signal from urine. Although there was overlap in the groups in a human proof-of-concept study, urinary ARGS was elevated in subjects with radiographic OA (hand/spine/knee/hip OA) compared with symptomatic OA or subjects with no radiographic OA in these joints. They confirmed the significant elevation of urinary ARGS fragment from another human OA cohort (0.56 ± 0.15 ng/mg creatinine) compared to healthy controls (0.36 ± 0.15 ng/mg creatinine). Differential proteomic evaluation of urine samples has also been conducted by Henrotin and co-workers who analyzed urine samples from women with severe OA undergoing knee replacement and non-OA healthy individuals (Henrotin et al., 2012). Two-dimensional differential gel electrophoresis (2D-DIGE) was employed with identification by LC–MS/MS of protein spots with differential expression ≧1.5-fold (OA:healthy). Thirteen differentially expressed proteins were identified; of these, 2 peptides from fibulin 3 attracted their attention. Two enzyme-linked immunosorbent assays, targeting these two peptides, were developed and used to analyze serum from a large human cohort. These two peptides were elevated in OA patients with area under the receiver operating characteristic curve of 0.75 and 0.83 for Fib3-1 and Fib3-2 respectively for diagnosing OA. Overall, this study suggested that Fib3-1 and Fib3-2 are both potential knee OA biomarkers that could be used to discriminate individuals with knee OA from non-OA individuals.

3.4. Summary of proteomic studies of body fluid

In summary, to date the differential expression patterns of proteins in OA have been evaluated primarily by proteomic analyses of synovial fluid or serum. Synovial fluid contains the high abundant serum proteins and highly anionic hyaluronan that contributes to the difficulty of analyzing synovial fluid samples. Different kinds of affinity depletion have been conducted to remove abundant proteins that mask the signals of other less abundant proteins. Pre-treatment of samples with hyaluronidase can reduce the viscosity of synovial fluid and the negative influences of anionic charge. Similar to synovial fluid, serum contains some highly abundant proteins that overwhelm the detection of other proteins. Thus, both synovial fluid and serum proteomic analyses can benefit from affinity depletion of highly abundant proteins. Although the cartilage proteins present in urine are, in most cases, more dilute than in serum or synovial fluid, the protein composition of urine is much less complex than serum or synovial fluid. In addition to this, urine collection is non-invasive. These benefits make urine an attractive source of potential biomarkers. However, making the link from urine samples to joint disease or even a specific joint is a very challenging task. Therefore, in contrast to serum and synovial fluid, to date, few proteomic studies in OA have focused on urine. Only a few urine peptide fragments with the potential to be biomarkers of OA have been reported and validated. A comprehensive discovery proteomic evaluation to identify the peptides related to cartilage degradation is provided by cartilage proteomics. Taken all together, proteomics methods have identified many proteins that may relate to pathological mechanisms of OA. Further targeted approaches to validate the role of these proteins in OA are needed.

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