4 *In Vitro* Models and Proteomics in Osteoarthritis Research

Martin Rydén and Patrik Önnerfjord

Abstract

This review summarizes and exemplifies the current understanding of osteoarthritis *in vitro* models and describes their relevance for new insights in the future of osteoarthritis research. Our friend and highly appreciated colleague, Prof. Alan Grodzinsky has contributed greatly to the understanding of joint tissue biology and cartilage biomechanics. He frequently utilizes *in vitro* models and cartilage explant cultures, and recent work also includes proteomics studies. This review is dedicated to honor his 75-year birthday and will focus on recent proteomic *in vitro* studies related to osteoarthritis, and within this topic highlight some of his contributions to the field.

Keywords

Proteomics · Osteoarthritis · Cartilage

M. Rydén
Orthopaedics, Department of Clinical Sciences Lund, Faculty of Medicine, Lund University, Lund, Sweden

P. Önnerfjord (✉)
Rheumatology and Molecular Skeletal Biology, Department of Clinical Sciences Lund, Faculty of Medicine, Lund University, Lund, Sweden
e-mail: patrik.onnerfjord@med.lu.se

4.1 Introduction

Osteoarthritis (OA), the most common degenerative joint disease, is a major source of pain, disability, and socioeconomic cost worldwide [17]. OA is a complex disease affecting the whole joint and multiple molecular and clinical phenotypes of OA seem to exist [38]. The pathologic changes seen in OA joints include degradation of the articular cartilage, thickening of the subchondral bone, osteophyte formation, variable degrees of synovial inflammation, degeneration of ligaments and, in the knee, the menisci, and hypertrophy of the joint capsule [30]. The degradation of articular cartilage is caused by an increased proteolytic activity of matrix degrading enzymes such as matrix metalloproteinases (MMPs) and aggrecanases. However, as the detailed molecular mechanisms involved in OA initiation and progression remain poorly understood, no effective Disease-Modifying OA Drugs (DMOADs) are currently available.

Human tissue samples for the study of osteoarthritis are often collected at the time of joint replacement when disease progression is in late stage, and limit researchers' ability to study the early development factors that contribute to the disease. The inherent variability of OA disease progression and onset of symptoms between individuals also presents challenges for studying OA pathophysiology. To overcome these limitations, *in vitro* models have been extensively used

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to address queries related to pathological changes, drug-target interactions, molecular pathways and to investigate the roles of pro-inflammatory cytokines in certain conditions.

Proteomics is the analysis of the entire protein complement of a cell, tissue, or organism under a specific, defined set of conditions. Proteomics applications in the field of OA has become rather common [19] as the technology can analyze complex samples in a more discovery-based approach than traditional specific methods such as enzyme-linked immunosorbent assays (ELISA). In this review, we have limited the content and searched the literature for proteomics applications (by mass spectrometry) within OA using in vitro experiments that are further discussed below.

4.2 *In Vitro* Models in Osteoarthritis

One of the main challenges in osteoarthritis research is to find a model that accurately represents the mechanisms of the disease. Different models have been developed, each with its own advantages and disadvantages [12, 22, 49, 50], but there is currently no consensus or gold standard approach. The two most common *in vitro* models are cell cultures and explant cultures.

Cell culture models typically target the chondrocyte as the cell type of interest for OA research but also synoviocytes and other joint cells have been targeted. Monolayer cell culture models are inexpensive, easy to use and allow for many replicates to be made from a single source of tissue. The layout of cells as they are put on a flat surface in a culture flask exposes them to an equal amount of growth factors in the surrounding media. Typical applications of monolayer cultures are the effect of cytokine stimulation, osmotic pressure, or the role of synovium in OA [12]. Like monolayer cell culture models, co-culture models are also used to study the effect of cytokines and osmotic pressure but have the added benefit of allowing cell-cell interactions and crosstalk between cell types to be investigated. However, co-culture experiments can be costly, and are limited because different cell types can require different conditions and cells may de-differentiate depending on the co-culture system. A limitation of cell cultures is the potential loss of chondrogenic phenotype as the chondrocyte is isolated from the extracellular matrix, due to their sensitivity to their molecular environment [11, 22].

Explant models are derived directly from *in vivo* tissue and for the study of OA pathophysiology, they have the unmistakable advantage of being a better representation of the *in vivo* tissue compared to cell cultures, as the overall characteristics of the tissue are maintained. An advantage of explant models over *in vivo* models is that they, to a greater extent, allow standardization and controlled variables [56]. Explant models are relatively inexpensive and easy to set up and can be used to study both inflammatory processes and biomechanical loading of the tissue. Despite the benefits over cell culture models, the use of tissue explants also has disadvantages such as chondrocyte death at the edge of the explant and limited number of cells. Explant models are also limited in that few replicates are available from the tissue source [50, 56]. Due to the limitations in sample availability, much of the published work is performed using animals, which is a limitation as not all findings can be directly translated to humans [12].

4.3 Inflammatory Models

The development of inflammatory processes in OA involves the increased expression of catabolic proteins in chondrocytes following cytokine exposure [22]. Cytokines are signaling proteins involved in inflammation response and are produced by nearly all cell types [39]. The balance and interplay of cytokines is increasingly being recognized to have a central role in OA disease progression [60]. To study these effects, models of pro- and anti-inflammatory cytokines are implemented in cell culture or explant models. Some of the most important pro-inflammatory cytokines used for induction of OA-like biological changes in these models are IL-1α, IL-1β, TNF-α, IL-6, leukemia inhibitory factor (LIF), oncostatin-M (OSM), IL-15, IL-17, and IL-18 [18, 40, 60]. In contrast, the major

anti-inflammatory cytokines, which are involved in OA pathogenesis by inhibiting actions of catabolic cytokines, are IL-4, IL-10, IL-11 and IL-13, IL-1 receptor antagonist (IL-1Ra), and interferon (IFN)- γ [18, 60].

Investigations to compare different pro-inflammatory cytokines were made in a cell culture model using RNA-seq [42]. In this study, the authors studied the effect of IFN-γ, IL-1β, IL-4 and IL-17 on gene expression in OA chondrocytes and found that 2800 genes were altered in chondrocytes treated with IL-1β. The mechanisms of IL-1β related to inflammation in OA were also studied in an *in vitro* cell culture model where human articular chondrocytes were cultured with or without recombinant IL-1β [24]. Known proteome changes following IL-1β stimulation, such as activation of the NFKB pathway, and subsequent synthesis of IL-1β, IL-6, IL-8, MMP-13, and ADAMTS-5, were validated by mass spectrometry on articular cartilage from three donors. After the cells had been stimulated with recombinant IL-1β for 20 h, IL-1β, IL-6, MMP-13 and IL-8 were upregulated. The authors also demonstrated that microRNA-140 inhibits the activation of the NFKB pathway, meaning a possible increase in cartilage repair and decrease of cartilage breakdown.

4.4 Mechanical Loading

In addition to biochemical factors, the development of osteoarthritis is affected by the biomechanical homeostasis of the joint. While mechanical loading is essential for the function and maintenance of healthy joints, mechanical *overload* induces molecular events similar to those stimulated by pro-inflammatory cytokines [13, 20]. It is becoming increasingly clear that impact-induced injuries not only cause cell damage, but also initiate progressive tissue damage and are recognized as a risk factor for OA. With this in mind, load-based models using tissue explants are effective systems for simulating the development of post-traumatic OA.

A study of post-traumatic OA has demonstrated a relationship between injury-induced oxidative damage and progressive matrix degradation [34]. Further, *in vitro* models have shown that while moderate intermittent compression have anti-catabolic effects on cartilage homeostasis, the cellular response to high compression involves degradation and decreased biosynthesis of ECM, and upregulation of pro-inflammatory enzymes [27, 29].

Due to variations in experimental protocols defined as injurious compression, direct comparisons of outcomes from models can be difficult. Indeed, mechanical injury can be induced in a multitude of ways; short repetitive loading, cyclic loading over a longer period of time, cartilage can be injured from a weight which drops down from a defined height, and explants can be compressed under confined conditions such that bulging of the explants do not occur [25].

4.5 Proteomics

The advances in mass spectrometry (MS) over the last decade has enabled this powerful technology to be frequently used in proteomics applications [1, 33]. The most common application "bottom-up proteomics" includes an enzymatic digestion of proteins e.g. extracted from cells, tissue or released into cell/tissue culture media. Proteins are digested with a sequence-specific enzyme like trypsin to generate a complex mixture of peptides. Peptides are usually separated by liquid chromatography, after which they enter a mass spectrometer where peptide ions are measured, to enable their identification and quantification. The data acquisition methods can be either discovery based or targeted against a predetermined list of peptides. Both approaches have been utilized for *in vitro* studies in osteoarthritis research, see Table 4.1. The discovery approach has the advantage of not being biased against any particular proteins, although in practice there is some bias towards medium and high abundant proteins as the precursor selection for MSMS experiments are based on top N most intense peptides i.e. data-dependent acquisition (DDA), and thereby low abundant proteins can be missed out. There is another discovery approach using data-independent acquisition (DIA) where all precursors within a certain m/z

range, sequential isolation windows of typical 20–25 Da, are selected for fragmentation to cover a typical mass range for proteolytic digests. This results in highly complex and data information-rich datasets that typically are matched against spectral libraries using a targeted analysis [61]. This latter approach combines an untargeted data collection with a targeted data analysis, reducing missing values and in addition enables data to be re-analyzed against more comprehensive libraries.

The traditional targeted approach, multiple reaction monitoring (MRM) is usually performed on triple quadrupole instruments using preset precursor mass filter (Q1), collision cell for fragmentation (Q2) and precursor fragment mass filter (Q3) with combined settings called peptide transitions which can be identified and optimized for highest sensitivity in pilot experiment [16]. The targeted approach has the advantage of high sensitivity, better reproducibility and fewer missing values and it is suitable for absolute quantification using heavy-isotope labeled standards while having limitations in a number of precursors as well as being limited to the pre-selection of targets missing out on novel findings (non-discovery). The development of parallel reaction monitoring (PRM) does increase the number of targets available and also results in full MSMS scans as the third quadrupole is replaced with a high-resolution mass analyzer (orbitrap) [43].

4.6 Proteomics Applications Using *In Vitro* Models

Mass spectrometry-based proteomics is a powerful technique that has increasingly enabled insights generated from OA *in vitro* experiments and has been used for characterizing biological elements relevant to OA pathophysiology, such as extracellular matrix components and structure. Cartilage is the most used tissue in OA *in vitro* studies and in proteomics application special considerations must be taken into account due to its physical characteristics. Articular cartilage is an avascular tissue with few cells and an extensive extracellular matrix (ECM) with major components being collagen (mainly type II) and proteoglycans (mainly aggrecan). The crossed-linked collagen network results in poor extractability with only a minor part (soluble fraction) being measured [41]. The high level of aggrecan can cause problems as the highly negatively charged glycosaminoglycan chains can interfere in the sample preparation steps ultimately affecting the chromatography performance [21]. However, despite these difficulties, more than 1000 additional proteins can be identified using current proteomics technologies [8].

Earlier in this review, we described a typical bottom-up proteomics workflow and here, we will demonstrate some of its applications using *in vitro* experiments related to OA. An experimental workflow for an in vitro model using proteomics is shown in Fig. 4.1.

In early proteomics studies, a commonly applied technique was two-dimensional gel electrophoresis (2D SDS-PAGE), which separated complex mixtures of a sample by isoelectric point (pI) and molecular weight. Following this, spots of interest and subsequent differentially expressed proteins could be identified by MS while quantification was usually performed using image analysis and intensities of matching protein spots [23]. Some limitations of this technique include low sensitivity, labor intensive work and poor ability for automation [48]. A simpler alternative was the more straightforward "salami" approach where sample lanes from 1D gels were cut into multiple bands followed by LC-MS analysis. However, due to these limitations and overall technological developments in the field, separation by liquid chromatography coupled with MS (gel-free proteomics) has been the preferred method in recent studies.

4.6.1 Targeted Proteomics Applications

4.6.1.1 Explant Cultures

A cartilage explant study by Melin-Fürst et al. [14] used MRM, a targeted approach, to

Table 4.1 In vitro studies in osteoarthritis using proteomics applications

Culture model type	OA model type	Treatments	Species	Analytical methods	MS method	Reference
Cartilage explant	Inflammation	± (IL-1β/TNF-α)	Equine	SDS-PAGE, LC-MS, NMR metabolomics	Discovery (DDA)	[2]
Cartilage explant	Inflammation	± (OSM/TNF-α, IL-17A)	Bovine	ELISA, LC-MS	Discovery (DDA)	[51]
Cell culture	Inflammation	± IL-1β	Human	SILAC, SDS-PAGE, LC-MS (MALDI)	MALDI MSMS	[10]
Cell culture	Inflammation	± IL-1β, ± nicotine	Human	SILAC, 2D LC-MS (MALDI)	MALDI MSMS	[31]
Cell culture, cartilage explant	OA secretome	Macrosopically normal	Human	SILAC, SDS-PAGE, LC-MS	Discovery (DDA)	[44]
Cartilage-synovium coculture	Tissue crosstalk, inflammation	± IL-1α, ± IL-1Ra	Bovine	LC-MS	Discovery (DDA)	[37]
Cell culture	OA secretome	Chondrogenesis BMSCs	Human	SILAC, SDS-PAGE, LC-MS	Discovery (DDA)	[46]
Cartilage, meniscus explant	Inflammation	± IL-1α, ± IL-1β	Porcine	MMP activity, NO, sGAG release, aggregate modulus, permeability	No MS	[36]
Cartilage explant	Inflammation	± IL-1β, ± carprofen	Canine	SDS PAGE, LC-MS, machine learning	Discovery (DDA)	[54]
Cartilage explant	PTOA, inflammation	± IL-1α	Bovine	MMP activity assay, SDS PAGE, LC-MS	Discovery (DDA)	[45]
Cartilage explant	Inflammation	± IL-1α, wt, Adamts5Δcat	Mouse	SDS-PAGE, LC-MS, microarray	Discovery (DDA)	[59]
Cell culture	Inflammation	± IL-1β	Human	2D SDS-PAGE, MALDI	MALDI MSMS	[47]
Cell culture	Inflammation	± IL-1β, ± miRNA-140	Human	RT-qPCR, LC-MS, Western blot	Discovery (DDA)	[24]
Osteochondral explants	Inflammation	±LPS, ± TGF-β RI inhibitor	Human	ELISA	No MS	[15]
Cartilage explant	Inflammation	± (IL-1β/TNF-α)	Equine	sGAG, RT-qPCR, Western blot	No MS	[35]
Cell culture	Inflammation, drug response	IL-1β + chondroitin sulfate	Human	LC, MALDI	MALDI MSMS	[9]
Cell culture	OA secretome	Different zones (N, UOA, WOA)	Human	2D SDS-PAGE, LC-MS	Discovery (DDA)	[26]
Cell culture	OA proteome	N vs OA chondrocytes	Human	SDS-PAGE, LC-MS	Discovery (DDA)	[57]
Cartilage explant	Dynamic loading	Loading ± IGF-1 or ± TGF-β	Bovine	ELISA	No MS	[13]
Cartilage explant	OA secretome	Different zones (N, UOA, WOA)	Human	iTRAQ, 2D LC-MS	MALDI MSMS	[32]
Cartilage explant	PTOA, inflammation	± (injury, TNF-α, IL-1α)	Bovine	SDS PAGE, LC-MS	Discovery (DDA)	[52]
Cartilage explant	PTOA, inflammation	± injury, ± (IL-6/TNF/sIL6R), ± Dex	Bovine	LC-MS	Discovery (DDA)	[7]
Cell culture	Inflammation	± IL-1β (N vs OA synoviocytes)	Human	IHC, LC-MS	Discovery (DIA)	[55]
Cartilage explant	PTOA, inflammation	± (injury, TNF-α, IL-1α)	Bovine	2D LC-MS	ITRAQ	[53]
Cartilage explant	PTOA, inflammation	± injury, ± (IL-6/TNF/sIL6R)	Human	LC-MS	Targeted (MRM)	[58]
Cartilage explant	PTOA, inflammation	± injury, ± (IL-6/TNF/sIL6R), ± Dex	Bovine, Human	LC-MS	Discovery (DDA)	[6]
Cartilage explant	Inflammation	± IL-1α	Bovine	ELISA, LC-MS	Targeted (MRM)	[14]

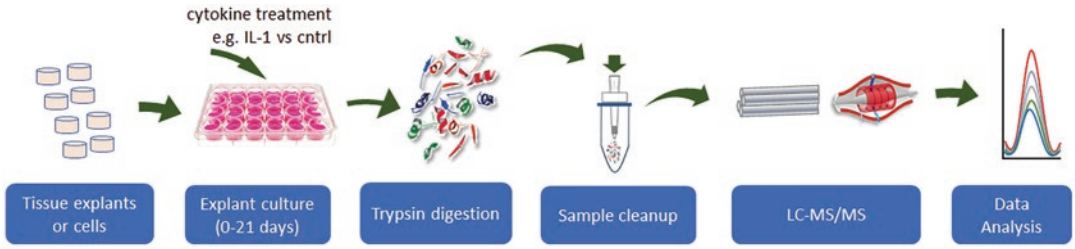

Fig. 4.1 Schematic workflow of an *in vitro* model using proteomics. Tissue explants or cells are kept in culture and exposed to different treatment conditions e.g. cytokines vs control. Cell culture media is replaced every 2–3 days and various time points are collected for further MS sample preparation (reduction, alkylation, ethanol precipitation, trypsin digestion, excess GAG removal, desalting) before peptides are separated using reversed phase liquid chromatography (nano-LC) and finally analyzed by mass spectrometry. The data generated is processed using specific software and searched against a protein sequence database or spectral library for identification and quantification

characterize the inflammatory processes involved cartilage degradation, induced by IL-1α in bovine cartilage explants, and monitor interactions with the complement system. Following cytokine stimulation, the authors found a decrease in proteoglycan and collagen content in the cartilage, and activation of the complement.

MRM was also used in a study to characterize cartilage response to mechanical injury and cytokine treatment [58]. The targeted approach allowed the authors to monitor a predefined set of potential molecular biomarkers including cleavage neoepitopes, see also Fig. 4.2. In this work, both the explant culture media (individual time points) and the final explant, representing the sum of events during the entire culture period, were measured. The neo-epitope measurements give extra biological value as it represents an active proteolytic event being measured in addition to the overall protein release.

4.6.2 Discovery Proteomics Applications

4.6.2.1 Cell Cultures

Several comparative proteomic analyses that aim to describe the proteome of OA have been conducted as cell culture models analyzed using discovery-based acquisition methods. One such study used 2D SDS-PAGE and subsequent discovery MS to analyze the proteome of human articular chondrocytes and described the phosphorylation status of differentially expressed proteins in OA progression [26]. Another study reported on the role of hypertrophy-like alterations in chondrocytes in OA using high resolution MS [57].

Secretome analysis can provide information on the mechanisms behind remodeling of ECM in response to drug treatment or mechanical load and thereby provide insights into the pathogenesis of OA. The effect of chondroitin sulfate in the presence of IL-1β on proteins in chondrocyte secretome was examined in a study from 2012 by 2D SDS-PAGE and SILAC in a discovery workflow [9]. The study found 75 proteins in the secretome, 18 of which were modulated by chondroitin sulfate, and provided evidence of its anti-angiogenic, anti-inflammatory, and anti-catabolic properties.

Using bovine cartilage explant monoculture and cartilage-synovium co-culture, a recent study investigated the role of cartilage-synovium crosstalk in a discovery-based mass spectrometry experiment [37]. Sustained doses of IL-1Ra were shown to suppress cytokine-induced catabolism in cartilage more effectively in the presence of synovium, which was associated with endogenous production of anti-catabolic factors.

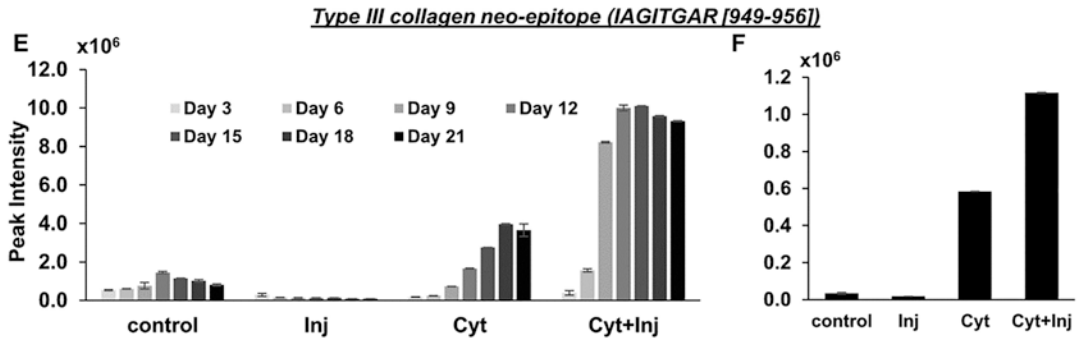

Fig. 4.2 Collagen type III cleavage neo-epitope release into explant culture media over 21 days (E) and the remaining peptide left in explant after the complete culture period (F). Values are average peak intensities of two technical replicates using targeted proteomics (MRM). Modified figure reprinted with permission from [58]

Though most of the discovery workflows relied on data dependent acquisition (DDA), one study, focusing on the difference in the phosphoproteome of OA and acute joint fracture in synovial tissue, used data independent acquisition (DIA) in an IL-1β–treated human synoviocyte (HS) *in vitro* model to verify their results [55]. The study found that IL-1β could induce HS to secrete proteins associated with the endosomal/vacuolar pathway, endoplasmic reticulum/Golgi secretion, complement activation, and collagen degradation.

4.6.2.2 Explant Cultures

Cartilage degradation is a well-established process in OA pathogenesis, and as such, a commonly studied model is the degradation of cartilage upon cytokine stimulation in explant culture. Several proteomic studies have employed this model to measure the effects of pro-inflammatory cytokines on articular cartilage. One study from 2016 investigated the chondrocyte response to IL-1α within native cartilage tissue and its secretome using discovery LC-MS and whole-genome expression profiling using microarray [59]. The study was the first to report on the effects of IL-1α in native cartilage and cartilage lacking the catalytic domain of ADAMTS5 (aggrecanase) and identified more than 150 proteins modulated by IL-1α. Additionally, the combined LC-MS and microarray analysis permitted the authors to differentiate between proteins modulated by IL-1α on gene expression level and those which were a product of ECM degradation.

Discovery LC-MS using DDA was used in a metabolomics and proteomics study published in 2020, where Anderson et al. studied OA pathogenesis using an equine cartilage explant model [2]. In the study, nine potential novel OA neoepitope peptides were discovered.

Unbiased labeled approaches such as Isobaric Tag for Relative and Absolute Quantitation (ITRAQ) were used in two studies. Stevens et al. used traumatic injury, IL-1β or TNF-α compared to control showing increased levels of MMPs and proteins of the innate immunity while the mechanical injury mainly led to an increased release of intracellular proteins [53]. Lourido et al. conducted a comparative profiling of proteins in early- versus late-stage OA, and subsequent clustering analysis and found upregulation of periostin and downregulation of osteoprotegerin in OA [32]. In a recent study a cartilage injury model was described using articular cartilage explants treated with cytokines and mechanical injury [7]. The addition of dexamethasone was shown to rescue the catabolic response but not the anabolic dysregulation. The treatment effects in this model are illustrated in a heatmap (Fig. 4.3).

4.7 Concluding Remarks and Future Perspectives

In this review, we have shown an increased use of proteomics applications within *in vitro* models during the last decade and this trend will

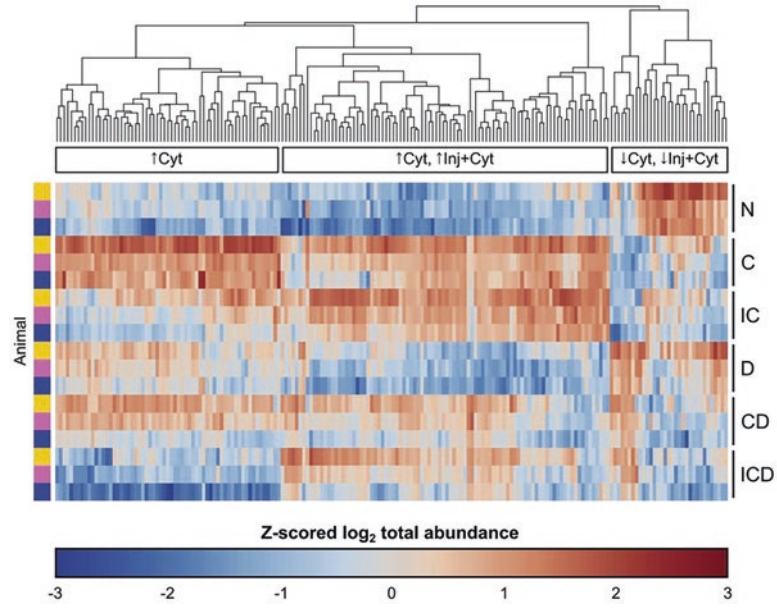

Fig. 4.3 Heatmap of proteins significantly affected by disease treatment. Treatment effects were evaluated by pairwise comparisons of MS abundance data of different disease treatments within each animal replicate. Proteins were selected that had a differential effect of C or IC treatments and that were present in at least three time points across at least one consistent treatment condition between all three biological replicates, resulting in 188 selected proteins. The raw abundance value for each filtered protein was summed over all time points and $\log_2$-transformed. For visualization, the $\log_2$-transformed values were normalized via z-scoring across all treatment conditions, excluding injury alone and injury with Dex: control (N), cytokine (C), injury + cytokines (IC), Dex (D), cytokines + Dex (CD), and injury + cytokines + Dex (ICD). Proteins are plotted on the horizontal axis and ordered based on their hierarchical clustering (Euclidian distance) across all six selected treatment conditions. Each individual replicate is plotted on the vertical axis, ordered by treatment condition and then by animal. The clustering reveals three major patterns of protein release: increased release by cytokines alone (↑Cyt), an increase by both cytokines and injury + cytokines (↑Cyt, ↑Inj + Cyt), and decreased release by cytokines and injury + cytokines (↓Cyt, ↓Inj + Cyt). (Figure reproduced from [7] under a Creative Common license)

most likely continue. The high sensitivity and selectivity offered by current and future MS instruments allow identification and quantification of a large number of proteins in highly complex samples. These information-rich data sets further allow much more detailed results to be obtained including individual peptide levels (relative abundances), post-translational modifications (e.g. measuring catabolic proteolytic events, phosphorylation states and protein synthesis), hence making this a very attractive technology. Novel developments in MS instrumentation, data acquisition methods as well as improved tools for data processing and evaluation tools will enable an increased depth of the proteome improving the bioinformatic evaluation of the data to identify proteins involved in specific functional pathways and interpretation of protein-protein association networks. Clustering analysis can also identify proteins following similar patterns e.g. grouping proteins according to their kinetic release profile [6].

In comparison to cell culture models, explant models better represent the extracellular matrix environment *in vivo* and also possess the ability to perform mechanical loading experiments. The kinetics of specific proteins release can easily be addressed as the culture medium needs to be replaced every 2–3 days creating a longitudinal experiment that can reflect both early-, mid- and late stages in the model. This is a great potential

when healthy donors are available as early changes can be investigated. *In vitro* models are also well suited for molecular therapeutic interventions e.g. applying potential drug candidates for treatment purposes. One example is the glucorticoid dexamethasone that has been extensively studied with somewhat conflicting results in the past [5] but in explant culture, the combination of dexamethasone with insulin-like growth factor-1 inhibited both the loss of soluble proteoglycan (sGAG) and collagen, rescued the suppression of matrix biosynthesis and inhibited loss of chondrocyte viability induced by IL-1α treatment [28]. Drug delivery is an important aspect to this as without a carrier, intra-articular injections of the drug is rapidly cleared from the joint cavity. The Grodzinsky group has circumvented this drawback by linking the drug to a basic carrier enabling rapid uptake and sustained delivery both in vitro [3] and in vivo [4]. The Dex treatment effect was further investigated using proteomics in a post-traumatic *in vitro* model where it suppressed most of the proteins affected by cytokine+injury treatment versus control [6, 7].

The ground-breaking work of Prof. Grodzinsky and his contributions to the research community has been widely recognized by numerous awards. He is considered as a world leader in his field and in 2021, he received the most prestigious Lifetime Achievement Award by the Osteoarthritis Research Society International.

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