**Proteomic analysis of synovial fluid: current and potential uses to improve clinical outcomes**

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**Abstract**

Introduction:

Synovial fluid (SF) is in close proximity to tissues which are primarily altered during articular disease and has significant potential to better understand the underlying disease pathogeneses of articular pathologies and biomarker discovery. Although development of mass spectrometry-based methods has allowed faster and higher sensitivity techniques, interrogation of the SF proteome has been hindered by its large protein concentration dynamic range, impeding quantification of lower abundant proteins.

Areas covered:

Recent advances have developed methodologies to reduce the large protein concentration dynamic range of SF and subsequently allow deeper exploration of the SF proteome. This review concentrates on methods to overcome biofluid complexity, mass spectrometry proteomics methodologies, extracellular vesicles proteomics and the application of advances within the field in clinical disease, including osteoarthritis, rheumatoid arthritis, spondyloarthritis and juvenile arthritis. A narrative review was conducted with articles searched using PubMed, 1991-2018.

Expert opinion:

The SF proteomics field faces various challenges, including the requirement for rigorous and standardised methods of sample collection/storage, the sensitivity and specificity of proteomic assays, techniques to combat the large protein concentration dynamic range and comprehensive data analysis to reduce falsely identified markers. Additionally, there are challenges in developing multi ‘omic’ integration techniques, with computational integration enhancing analysis.

**Keywords:** mass spectrometry, proteomics, synovial fluid

1. Introduction

Synovial fluid (SF) acts principally as a biological lubricant of the joint, reducing friction between synovial joint articular cartilage surfaces, but also functioning as a nutrient pool for surrounding tissues and allowing movement of cytokines [1,2]. SF is of particular interest for the investigation of orthopaedic pathologies, including osteoarthritis (OA), rheumatoid arthritis (RA), juvenile arthritis, spondyloarthritis, osteochondrosis and synovial sepsis, due to its close proximity to various tissues which are primarily altered during these disease processes, with collection protocols minimally invasive [3-5]. Therefore, SF has significant potential to greater understand the underlying disease pathogeneses of these conditions and biomarker discovery.

The main challenge to SF proteomics is the high dynamic range of protein concentrations. Direct analysis of SF will not enable detection of peptides present at low concentration (which are likely to be the proteins of interest with respect to biomarker discovery) and thus fractionation, depletion and equalisation techniques have been developed.

This article will review recent advances in method development for mass spectrometry (MS)-based SF proteomics, focusing in particular on advances in protein equalisation. We will describe the methods used to identify proteins which may be used clinically either as diagnostic or prognostic biomarkers of arthropathies or potential therapeutic targets. We will focus on recent studies in four major arthropathies osteoarthritis, rheumatoid arthritis, juvenile arthritis and spondyloarthritis. It should be noted that our intention is not to review all novel SF biomarkers, but rather focus on the methodological aspects applicable within the SF proteomics field.

2. Principles

2.1. Biofluid sample complexity

The development of liquid chromatography tandem mass spectrometry (LC-MS/MS) has provided a faster and more sensitive methodology to identify and quantify proteins within complex biological samples [6,7]. However, the complexity and variability of intensity of proteins within biofluids can lead to various challenges associated with proteome analysis [8]. Similarly to serum, the SF proteome is dominated by highly abundant proteins, with an estimated substantial differential expression of 10x compared to the least abundant proteins, with 95% of the serum proteome consisting of approximately only 20 proteins, although representing just 0.1% of the total number of proteins [9-13]. These high abundant proteins can therefore mask and interfere with the detection of low abundant proteins, compromising potential biomarker discovery and understanding of pathogenesis pathways [14]. This sub-optimal identification of low abundant proteins is essentially due to a limited ion capacity restricting the isolation, fragmentation and detection of the least abundant peptide ions within ion trap mass spectrometers. Thus, until improvements within MS technology allow for increased coverage of the native biofluid proteome, at present, focusing on modifying the concentration dynamic range of biofluids to within the mass spectrometer’s detectable dynamic range is the preferred method to quantify low abundant protein biomarkers [15]. However, within the field at this time there is no agreed method to achieve this compression of the protein concentration dynamic range [16]. Numerous techniques have been applied including the use of immuno-affinity depletion through high performance liquid chromatography (LC) columns comprising antibodies to highly abundant proteins [10,17,18]. However, a fundamental flaw to this methodology is the resultant loss of low molecular weight proteins of interest that are closely associated with albumin and other highly abundant proteins [19,20].

2.2 Techniques for reducing protein concentration dynamic range:

2.2.1 Cibacron Blue

Cibacron Blue is a dye-ligand which binds to proteins and enzymes with high affinity [21]. Cibacron Blue is commonly used to remove albumin, with the advantage of a high loading capacity. However, due to broad and nonspecific protein interactions, the dye also binds to NAD, FAD and ATP protein binding sites, eliminating potential proteins of interest [22,23].

2.2.2 Monoclonal Antibodies

Immunoaffinity resins, incorporating monoclonal antibodies against serum albumin, have been found to be effective in the removal of both full-length serum albumin together with serum albumin fragments, with a higher efficiency and specificity than dye-based resins [24,25]. Combination with protein G resin enabled the removal of both immunoglobulins and serum albumin in one step. However, whilst monoclonal antibodies targeting specific epitopes of other proteins have been successful in protein depletion, protein fragments were not bound. The inability to bind to these fragments may be due to protein-protein interactions obscuring the binding site, post translational modifications (PTMs) or binding site disruption due to protein unfolding [23].

2.2.3 Polyclonal Antibodies

Polyclonal antibodies have been used to successfully deplete six highly abundant human serum proteins which corresponded to approximately 85% of the total protein content [22]. These proteins included serum albumin, immunoglobulin G, α1-anti- trypsin, immunoglobulin A, transferrin and haptoglobin.

2.2.4 Centrifugal Ultrafiltration

Centrifugal ultrafiltration of human whole plasma has previously been attempted using cellulose filters to remove proteins with a molecular weight in excess of 30 kDa [26]. This method was however unsuccessful with 2DE revealing retention of some >30 kDa proteins and loss of some <30 kDa proteins. This method has not as yet been adapted for SF.

2.2.5 Precipitation - Albumin Removal

A modified trichloroacetic acid/acetone precipitation provided a simple prefractionation step resulting in the removal of native albumin from human serum [27].

2.2.6 Glycoprotein Enrichment

In order to study glycoproteins, immobilized lectins have been used to enrich these compounds to study glycosylation patterns in human serum via carbohydrate residue binding [28]. Multi-lectin affinity columns were able to capture most glycosylated proteins within serum, allowing analysis of glycosylation patterns.

2.2.7 Combinational Ligand Libraries

An alternative approach to compress the protein concentration dynamic range involves the use of combinational ligand libraries to achieve peptide-based depletion but allowing preservation of the whole proteome [29,30]. This methodology has recently been used in the development of ProteoMinerTM protein enrichment columns (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). This technique was found to generate the largest increase in protein identifications compared to other protein depletion methods when applied to serum [31]. The columns consist of an assortment of porous beads which are bound to combinations of randomised hexapeptides (oligopeptides containing six amino acids) generating 64 million combinations from 20 amino acids to which proteins can selectively bind [32]. Following the introduction and overloading of a complex protein biofluid, ligands selecting for highly abundant proteins will become saturated with excess protein lost in the flow through from the column. Alternatively, low abundant proteins will be completely bound to the column, with binding increasing proportionally to protein content of the biofluid introduced [32,33]. Thus this peptide-based approach produces a compressed protein concentration dynamic range, depleting highly abundant proteins and enriching those less abundant [15]. This technique for quantifying low abundant proteins within a complex biofluid has previously been validated by Hartwig *et al.* (2009) in which human serum was supplemented with *E. coli* lysate. However, the elution solution present within the kit is not compatible with LC-MS/MS analysis, due to the presence of 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) [34]. Although ‘clean-up’ and alternative elution protocols are available, these introduce a further process in sample preparation and subsequently a source of variation. Peffers *et al.* have previously developed an on-bead trypsin digestion protocol for analysis of ProteoMinerTM processed SF [35-37]. However, although the reproducibility of ProteoMinerTM beads when used in conjunction with serum has been examined, reproducibility with SF or on-bead digestions have yet to be investigated [38].

2.3 Proteomic potential of synovial fluid in biomarker discovery- ‘the good and the bad’

Clinical proteomics is the quantitative profiling of proteins within various biofluids for eventual clinical application, aiding diagnosis, clinical monitoring during treatments and assessing prognosis [39,40]. This involves various phases of study including initial biomarker discovery, verification, validation and implementation of clinical trials. These biomarkers have widespread potential application to orthopaedic clinical practice as they offer unbiased parameters to function as a proxy of diseases such as OA. Biofluids such as SF fulfil the practicality recommendations for a biomarker, being relatively easily and economically attainable. Additionally, regarding joint diseases, SF is the primary fluid of choice being in intimate contact with the affected tissues. Whilst blood/serum/plasma provides a more readily accessible biomarker source, this biofluid represents a more complex matrix and although both biofluids suffer from a small number of highly abundant proteins the serum protein profile is more diverse. Furthermore, the serum proteome represents the endogenous processes of many organs and tissues. Therefore SF is a perfect initial surrogate for biomarker discovery but the end-goal ‘holy grail’ remains the provision of a blood/serum assay [41].

Over recent years there have been remarkable advances in MS-based proteomics. These advances now enable almost complete proteome identification of complex biological fluids in hours. Mass spectrometers have become faster and more sensitive generating enormous amounts of data related to not only protein identifications but to protein primary structure, post-translational modifications and splice variants. Even so, higher sensitivity for detecting individual proteins in complex mixtures such as SF is still an issue. New instruments are continually upgraded and novel methodologies for sample preparation are emerging, enabling faster, more sensitive measurements of many proteins in these complex biological mixtures.

In terms of SF proteomic studies, few have led to candidates that have effectively transitioned into the clinic to improve clinical outcome [42]. There are many causes which are well recognised [43,44]. However, the principal difficulties in the translation from proteomic biomarker discovery to clinical use are [45]:

1. A lack of consideration for experimental design before initiating the discovery phase.

2. Deficient biomarker validation strategies, including additional well phenotyped cohort samples.

3. Robustness of the analysis systems used in clinical trials.

Furthermore, a number of additional points need to be addressed within the four phases of a biomarker discovery pipeline; discovery, qualification, verification, and validation.

Firstly though, even before considering these points, other important issues need to be addressed by the community and standardised in order to increase the likelihood of identifying useful clinical SF proteomic markers [46]. These include sample selection; including a lack of standardised operating procedures for this (which can be difficult given the many clinics samples are collected from and availability of SF samples to scientist), insufficient age and sex matched samples, potential interfering patient co-morbidities (again difficult as in OA many of the patients we can access SF from are obese or have other disease issues), a lack of SF from truly ‘normal healthy’ patients for comparison, a lack of longitudinal patient SF samples in the specific orthopaedic condition, sample storage, differing methods used to reduce the high abundance proteins (see section 2.2), protein preparation and digestion techniques, variable MS instrumentation used and diverse data analysis techniques and cut-offs.

On a positive note, the requirement by all good quality journals to use online repository databases to deposit MS data, such as the PRoteomics IDEntifications (PRIDE) Archive database, has increased the capabilities of the field making data accessible to clinical researchers. PRIDE is a centralised, standards compliant, public data repository for MS proteomics data, including protein and peptide identifications (and expression values), post-translational modifications and supporting mass spectra evidence [47]. So long as adequate metadata is included this could be a valuable resource to integrate related studies, increase power and identify future SF biomarkers.

In the discovery phase of a SF proteomic experiment there are potentially 100s-1000s of protein biomarker candidate leads, however the majority of these do not have clinical utility. In order to reduce this gap some laboratories have introduced a verification step that rationalises the process of moving candidate proteins from discovery to validation through the exploitation of the sensitivity and specificity of targeted MS [multiple-reaction monitoring (MRM)]. An emerging trend is to combine immunoassays with MS (also known as “affinity‐MS”). This is a highly reproducible technique which can multiplex up to 150 proteins, overcoming MRM limitations in the reproducible monitoring of low and mid abundant proteins [39,48]. A further method to potentially overcome this limitation is another targeted approach; Parallel Reaction Monitoring (PRM). This does not require a designated triple quadrupole instrument but can utilise hybrid quadrupole-Orbitrap (q-OT) and quadrupole time-of-flight (q-TOF) mass spectrometers. PRM and MRM have demonstrated comparable sensitivity with similar linearity, dynamic range, precision, and repeatability for quantification of proteins. However it is easier to build the data acquisition method with PRM as prior selection of targeted transitions is not required. Increased sensitivity with PRM versus MRM is established because the MS/MS data is acquired in high resolution mode enabling separation of background ions from the target ions and a full MS/MS spectra is attained with all potential product ions and confirming target peptide identification (review-[49]). To the author’s knowledge PRM has not yet been applied to synovial fluid proteomics.

Acting as a precise and quantitative multiplex filter it confirms only the most credible protein candidates progress to more costly and time-consuming clinical validation studies. The final validation step of a subset of candidate proteins is then undertaken in a larger cohort (often hundreds of samples across many sites) who better represent the heterogeneous population seen in clinical practice. Validated biomarkers could then be commercialised [50].

1. Advance strategies for proteomic analysis of synovial fluid using mass spectrometry
	1. Discovery

The discovery phase in biomarker identification provides an initial list of proteins. The principal tool utilised is MS. As a core MS platform for proteome analysis, matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF-MS) has been used to analyse the SF proteome [4,51,52]. However, its use is becoming less as MS can be employed in combination with front end liquid chromatography separation techniques, i.e. LC-MS/MS, to increase the number of identifications.

In proteomics, protein separation methods are coupled with MS. In the past SF studies were undertaken using 2D gel electrophoresis (2DE) and MS [53,54]. 2DE has the advantage of identifying post translational modifications in combination with MS [55]. Furthermore, 2D gel-based proteomics has the valuable features, of reproducibility and robustness. However, 2D-PAGE based on isoelectric is technically limited as it is restricted to soluble and/or high abundant proteins. The technique often results in a low number of identified membrane proteins or lower identification in complex peptide or protein complexes. Thus, now gel-free proteomics are the principal choice for proteomic studies.

The LC-MS/MS platform supports ‘shotgun proteomics’ or ‘bottom up proteomics’- an approach where complex mixtures of proteins are first enzymatically cleaved into peptides, then separated based on chemical or physical properties and analysed using a mass spectrometer. Thus, it uses the principles of peptide identification and protein inference. The amino acid sequence of each peptide is determined by tandem MS at a predictable elution time [1]. Hybrid instruments principally signify high mass resolution instruments coupled to a front-end component that enables fragmentation (quadrupole time of flight (Q-TOF), Triple-TOF, Q-Orbitrap). These analysers are ubiquitous with biomarker discovery studies because of their unparalleled analytical specificity. The number of proteins identified can be increased by prolonging the LC gradient. However, in SF a major disadvantage associated with LC-MS/MS is that of limited sensitivity when investigations are carried out in highly complex samples, such as SF dominated by a small number of highly abundant proteins (section 2.2).

In data-dependent acquisition methods, the initial ‘MS1’ detection is used to quantify the peptides. Then a subset of precursor ions is isolated and fragmented for a second round of MS; MS2. The MS2 fragments are then utilised for confident peptide identification and peptide quantification [56].

* 1. Protein quantification

There are an increasing number of MS quantification techniques adaptable to clinical proteomics. Stable isotope labelling of amino acids in cell culture (SILAC), an MS1 based method, is a relative quantification metabolic labelling technique incorporating labelled amino acids into proteins through cell culture media supplementation (not applicable to SF). Tandem mass tags (TMT) are a further isobaric labelling reagent similar to iTRAQ [57]. These chemically reactive reagents also impart isotope-based differences to each sample enabling the data to contain the information for up to ten different samples in a single LC-MS/MS run. The quantitative accuracy, which can be distorted using this approach, is overcome when reporter ion quantification occurs in a dedicated MS3 fragmentation event using a technique called synchronous precursor selection [58]. Heavy isotope labelling is another MS1-based quantitation methods utilising Isotope labelling; measuring different protein states in a single MS analysis. This is undertaken through the encoding of the origin of the peptides through independent labelling with for example 2H, 13C, 15N, and 18O. The protein states are then mixed and analysed together [59].

 Of application to biological fluids including SF, chemical labelling through isotope-coded affinity tags (ICAT) or isobaric tags for relative and absolute quantification (iTRAQ) can be used for relative quantification. In ICAT, cysteine residues are labelled by reacting to their sulfhydryl groups [60-62]. However, although this technique has high specificity and reduces sample complexity, improving quantification of low abundance proteins, it limits quantification of high cysteine containing proteins. Currently no published SF studies have used this technique. Tandem mass tags (TMT) or iTRAQ target free amine groups of peptides following trypsin digestion and exist for the concurrent comparison of up to eight or six samples, [63]. This technique has been used in four SF studies to date [64-67].

Label-free (LF) quantification is increasingly used for SF studies due to its simple biochemical workflow, a higher dynamic range of quantification, cost-effectiveness and being non-hypothesis driven. However, there are some disadvantages to LF that are important in relation to SF [68-70]. The greatest disadvantage is reduced sensitivity through peptides from high-abundance proteins such as albumin interfering with peptide identification of lower-abundance proteins.

In LF, unlabelled peptide sample is injected directly onto the LC column and quantified by MS1 ion abundance intensity or spectral counting [71]. As each sample is independently injected, reproducibility is at a premium when injections are consecutive. It is difficult to compare or combine quantification from LF experiments undertaken at different times [72]. Spectral counting is based on the most abundant protein fragments to produce more peptides resulting in more associated MS/MS spectra [73]. The method has been utilised in some studies analysing the SF proteome, where biomarker potential is often believed to lie among the low-abundance proteins with software available to enable simpler analysis of the data [25,74]. Ion abundance uses the peak intensities on the extracted ion chromatogram. Analysis of this challenging data through software tools such as ProegensisQI™ (Waters, Manchester, UK) and PEAKS has become routine in SF proteomics [11,35,36,64,74,75]. However there are many freely available on-line tools to analyse LF data [76].

The recent development of data independent acquisition (DIA) workflows (MS/MS all, SWATH) presents a promising and novel MS quantitation technology [13, 14]. However, DIA requires the latest generation of high-end MS instrumentation and it is yet to be established as a proven and robust MS-based quantitation technology across various biological matrices.

* 1. Verification

Targeted MS or MRM/SRM (Selective Reaction Monitoring) is used to verify discovery findings when the researcher has identified proteins of interests. This enables a sensitive approach using the same or additional samples and a targeted approach. The platform has the capacity for identification and quantification by multiplexing many proteins through the identification of transitions (fragment ions) of quantotypic tryptic peptides of proteins at a specific LC retention time [77]. This method enables improved precision and accurate peptide quantification as the instrument targets fragment ions at a specific time in the LC gradient. Fragment ions are specific to a peptide and peptides need to be unique to the protein of interest. The instrument can potentially identify hundreds of these ions in a single experiment, with improved accuracy compared to data-dependant methods [78]. However, the optimal approach is to use a high mass resolution instrument to identify proteins of interest in a discovery round followed by a targeted approach using a triple quadrupole instrument which can use scheduling (looking for a precursor ion within a specific retention time range) to increase the number of targets.

Whilst there is value in the discovery of differentially expressed proteins in terms of fold change, a more quantitative data set will allow further interpretation and is needed for predictive biology using a systems biology approach [79]. Systems biology studies the complex interactions of biological components through the collection, integration and analysis of complex data sets. Mathematical and mechanistic modelling is then used to discover emergent properties in pathways, cells and tissues. One of the keys to this analysis is the input of real numbers into the models for example as absolute amount of proteins [80]. Additionally, in order to directly compare experiments between laboratory groups in different locations absolute quantification is key.

In absolute quantification some of these methods rely on the principles of surrogacy where, following protease digestion of a protein, the resultant peptides are identified by MS and used to deduce identification of the protein itself. One well described method of absolute quantification is through stable isotope label. There is differential incorporation of stable isotopic nuclei (2H, 13C, 15N, 18O) (which acts as a standard) enabling the ‘light’ and ‘heavy’ form of the same peptide to be resolved in the MS due to their mass difference. A number of methods are available to generate these protein standards [81]. One approach enables the highly accurate parallel absolute quantification of large sets of analyte proteins using an artificial protein QconCAT [82]. Multiple peptides are concatenated into a synthetic gene and expressed as a heterologous QconCAT protein in bacterial cultures allowing large numbers of biological samples to be analysed which is cost effective and reliable. Whilst this latter approach has been applied to cartilage secretomes proteomics it has not yet been applied to SF [83].

1. Extracellular vesicle proteomics

Diseases such as OA, RA, juvenile idiopathic arthritis, spondyloarthritis and other rheumatic diseases affect all the tissues in the joint. Since potential crosstalk between these tissues occurs within SF, it is important to understand the mechanisms of interactions. Extracellular vesicles (EVs) are small cell-derived particles (50 nm-5 µm in diameter) secreted by almost all cell types. These particles include structures such as exosomes, microvesicles and apoptotic bodies. EVs can be released by budding off the cell membrane or secreted as a result of multivesicular endosome fusion with the plasma membrane. EVs function as waste disposal machinery. In absolute quantification, some of these methods rely on the principles of surrogacy where, following protease digestion of a protein, the resultant peptides are identified by MS and used to deduce identification of the protein itself, but also as a transporter of signaling molecules (proteins, RNAs and lipids) enabling cell-to-cell communication [84-86].

In the study of EVs a common EV extraction method is ultracentrifugation and sucrose gradient purification; however, there is no standardised method for EV extraction. One of the main problems in EV extraction is co-precipitation of extracellular matrix proteins and lipids, which can interfere with results or hinder the discovery of potential proteins within the vesicles. Size exclusion chromatography (SEC) was proposed as an alternative way for albumin and high-density lipoproteins (HDL) depletion in EV extraction [87,88]. Western blot analysis demonstrated effective depletion of albumin and HDLs from SF-derived EVs by using SEC. However, matrix proteins such as fibronectin were still present in the EV fraction. In one study extracellular matrix proteins were depleted using Proteinase K and the EV proteome analysed using LC-QTOF/MS in comparison to untreated EVs. MS analysis identified 270 proteins in Proteinase K treated samples, significantly less than 652 proteins identified in untreated samples [88]. However, Proteinase K treated samples showed enrichment in endosomal proteins and depletion of extracellular matrix proteins. Overall, EV extraction from SF using SEC and Proteinase K treatment demonstrated the high recovery of vesicle specific proteins and could be employed for future studies aimed to investigate the proteome of EV in SF.

PTM proteins are of interest in RA diagnosis and prognosis. Citrullinated proteins have been associated with RA and considered as one of the main causes for the disease progression. Citrullinated proteins associated with EVs in the SF of RA, reactive arthritis and OA were identified using MALDI-TOF/MS based proteomics approach. Proteins identified included fibrinogen fragment D, fibrinogen β-chain, fibrin α-chain and Spα. Furthermore, high amounts of non-citrullinated fibronectin/IgG immune complex (IC) were identified in EVs of RA patients alone, indicating the possible activation of immune cells by the representation of the complex in SF during RA [89].

EVs could also be involved in response to drug therapy in RA patients. The effect of disease-modifying anti-rheumatic drugs (DMARDs) on the EV proteome in an experimental model of RA showed the inhibition of changes introduced by IL-1β. Most widely used DMARDs are methotrexate (MTX) and salazosulfapyridine (SASP). These are often found to be more effective when used in combination. Treatment with MTX and SASP suppressed the EV proteome changes induced by IL-1β, suggesting that changes at the EV level can lead to disease mitigation [90].

The abundance of activated macrophages in the joints of OA patients and the ability of EVs to induce immune cells could explain some of the molecular mechanisms involved in disease progression [91,92]. Macrophages extracted from blood and stimulated with SF-derived EVs from OA patient demonstrated upregulation of IL-1β at the mRNA and protein level, however TNFα, IL-10 and IL-6 levels were unchanged. Moreover, EVs stimulated the production of CCL20, CCL15 and CXCL1 chemokines and subsequent release of matrix metalloproteinase 12 (MMP-12) and MMP-7 in macrophages. SF-derived EVs from OA patients have distinct pro-inflammatory properties, which could affect joint homeostasis and initiate cartilage degradation [93].

SF EV proteomics is an exciting novel field. EVs play an important role in homeostasis of joints by transferring signaling molecules in the SF, and changes in the EV profile could prevent the progression of the disease. The size and relatively low abundance of EVs demands robust extraction techniques for proteomics but with high specificity for vesicular proteins. Furthermore, the employment of MS proteomics techniques could be used to understand the broad range of these particles in SF and the proteins enclosed within them, including the identification and quantification of PTM proteins that may elucidate disease progression and response to therapy.

1. Applications in clinical disease

5.1. Osteoarthritis

OA is the most common form of arthritis, an age-related musculoskeletal disease, characterised by degeneration of articular cartilage, sclerosis of subchondral bone, synovitis and bone proliferation abnormalities [94-96]. Cartilage extracellular matrix (ECM) degradation is primarily driven through increased enzymatic activity of multiple MMPs and a disintegrin and metalloproteinases with thrombospondin motifs (ADAMTSs), although the underlying pathogenesis of OA is yet to be fully understood [97-99]. OA is usually diagnosed through a patient’s history, physical examination and plain radiography [100]. However, due to the insidious nature of OA disease progression, substantial joint pathology is already present at the time of diagnosis [101]. Therefore, there is a need to identify disease biomarkers to accurately identify OA at an earlier stage, allowing timely intervention before the development of significant pathology. Integrating the tissue proteome, including SF, of OA will also allow us to greater understand the underlying pathogenesis of OA as well as potentially identifying novel therapeutic targets.

Various potential OA biomarkers have been identified within human SF, consisting of cartilage, bone and synovium origins, including both markers of synthesis and degradation (Table 1) [102]. Markers of synthesis identified within knee SF include procollagen IIc-peptide, YKL-40 and epitope 846 [103-107]. Synovial degradation product markers identified include TIMP-1, TIMP-2, crosslinked peptides from type II collagen (CTX-II), pentosidine, follistatin-like protein 1, MMP-1, MMP-13, ARGS and C2C [108-116].

Increased activity of MMPs and ADAMTSs during OA leads to cartilage breakdown and the generation of OA-specific peptide degradation products (neopeptides) [117]. During tryptic digestion of biological proteins for MS analysis, arginine and lysine are cleaved on the carboxyl side, unless followed directly by proline [118]. Thus non-tryptic peptide degradation sites may be resultant of this increased enzymatic activity during OA, with peptides with one tryptic and one non-tryptic cleavage site referred to as ‘semi-tryptic’ peptides [117]. Using MS, these neopeptides can be identified and quantified and thus utilised as potential molecular biomarkers of early OA. Additionally, a recent mouse study identified that a 32 amino acid peptide fragment, generated through degradation of aggrecan via increased ADAMTS-4/5 and MMP activity, drives OA pain through Toll-like receptor 2 [119]. Thus, targeting neopeptides may provide analgesia and reduce significant pain associated with joint degeneration. Various studies have identified potential neopeptides associated with OA in equine cartilage and synovial fluid (SF) [11,117,120,121]. Subsequent development of neopeptide antibodies would allow for the detection and monitoring of cartilage degeneration as well as assessing response to therapeutics, in both laboratory and clinical settings [122]. In addition, identification of OA specific neopeptides may provide novel therapeutic targets.

In addition to identifying markers of OA disease, proteomic methodologies have also been applied to SF to investigate novel therapeutics and response to therapies. Davidson *et al.* have previously identified that sulforaphane, an isothiocyanate which is derived from glucosinolate found in broccoli, is chondroprotective within both *in vitro* and *in vivo* OA models [123]. These findings subsequently led to a proof-of-principle trial in which 40 patients undergoing total knee replacement surgery were placed onto a low or high glucosinolate diet for a two week period prior to surgery [35]. Elevations in isothiocyanate levels were identified within the high glucosinolate group which were not present within the low glucosinolate group with SF proteomic analysis identifying discriminant profiles, including 125 differentially expressed proteins. Therefore proteomic methodologies can be applied to assess the effect of novel therapeutic agents as well as the pathways they are involved with. Additionally, SF proteomic analysis has been utilised as a prognostic indicator of procedure outcome. Autologous chondrocyte implantation is a clinical technique applied following traumatic cartilage injuries, consisting of two surgeries to prevent OA onset [36]. However, the procedure currently has a failure rate of 20% with prognostic markers required to predict surgery success and thus allow appropriate management/alternative therapies. Using Isobaric tagging for relative and absolute quantitation (iTRAQ) proteomic analysis and label free quantification of SF, Hulme *et al.* identified numerous biomarker candidates which can separate responders and non-responders, including complement C1s subcomponent and MMP-3 [64].

Table 1 shows studies in which SF proteomics has been used in osteoarthritis investigations

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Authors** | **Tissue type** | **Main techniques** | **Groups** | **Difference in OA** | **References** |
| Liao *et al.,* 2018 | SF | LC-SWATH/MS | OA, RA, traumatic knee arthritis | DKK2, proteins of complement and coagulation cascades ↑ OA;Transaldolase, Profilin‑1, 14‑3‑3 protein zeta/delta, Apolipoprotein A‑IV, Ig delta chain C region ↓ OA | [124] |
| Fernández-Puente *et al.,* 2017 | SF, Serum, Cartilage | MRM LC-MS/MS | SF, cartilage: OA; Serum: OA, control without joint disease | SF: Haptoglobin; Serum: Haptoglobin, VWF ↑ OA | [125] |
| Liao *et al.,* 2015 | SF | 2DE, MALDI-TOF/TOF MS | OA, non-OA articular disease | Haptoglobin ↑ OA | [126] |
| Ritter *et al.,* 2014 | SF, serum | SRM LC-MS/MS | SF: late stage OA; serum: late stage OA, OA progressing group | SF, serum: Clusterin, lubricin ↑ OA progression | [127] |
| Ahrman *et al.,* 2014 | SF | LC-MS/MS | OA, RA, acute pain or trauma | COMP neo-epitopes ↑ OA | [128] |
| Balakrishnan *et al.,* 2014 | SF | PAGE, MRM LC-MS/MS | OA | ADAMDEC1, ANPEP, ASPN, CD84, COLEC10, DKK3, MMRN2, SPARCL1, THY1, VSIG4 ↑ OA | [17] |
| Balakrishnan *et al.,* 2014 | SF | LC-MS/MS | OA, RA | HTRA1, FAP, C4A, C4BPA, CFI, CILP, CRISP3, TTK, NOTCH4, CD5L, COL15A1, SSC5D, RTN4RL2 ↑ OA | [66] |
| Wanner *et al.,* 2013 | SF | LC-MS/MS | early-stage OA, late stage OA, normal control | Complement molecules, coagulation pathway, tenascin, aggrecan ↑ OA Proteins related to the lipid metabolism, molecular transport, small molcule biochemistry network, Compelement factor D ↓ OA | [129] |
| Liao *et al.,* 2013 | SF | 2DE, MALDI-TOF/TOF MS | OA, non-OA articular disease | Apo A-IV, DNM, HLA-B a chain, RNA binding protein homologue 2, PRKA, apo A-I, HLA-DR, HSPA9, RPL27a ↑ OA Secernin, β-catenin-interacting protein 1, Calumenin isoform 5, PTP ↓ OA | [130] |
| Ritter *et al.,* 2013 | SF | 2DE, MALDI-TOF/TOF MS | early-stage OA, late stage OA, non-OA control | Afamin, Clusterin, IGFBP, Lumican, PEDF ↑ OA | [53] |
| Han *et al.,* 2012 | SF | SELDI-TOF/MS | OA, RA | S100A12 ↑ OA | [131] |
| Mateos *et al.,* 2012 | SF | 2DE, LC-MALDI-TOF/TOF MS | OA, RA | COMP, ACAN2, Complement factor D, Tetranectin, Fibronectin, Gelsolin ↑ OA | [4] |
| Sohn *et al.,* 2012 | SF | 2DE, LC-MS/MS | OA, RA, healthy control | Histone-related proteins, NF-kB-related proteins, inflammatory receptors, macrophage-related proteins ↑ OA | [132] |
| Pan *et al.,* 2012 | SF | MB-WCX, MALDI-TOF/TOF MS | OA, healthy control | Peptide peaks of m/z 1864.28 and 5335.63 ↑ OA | [51] |
| de Seny *et al.,* 2011 | SF, serum | SELDI-TOF/MS | SF: OA, RA; serum: OA, RA, healthy control | SF, serum: VTN, C3F ↑ OA Serum: CTAPIII ↓ OA | [133] |
| Kamphorst *et al.,* 2007 | SF | LC-MS/MS | OA, RA, normal control | SF: Collagen II, PRG4, tubulin, matrix Gla protein ↑ OA | [134] |
| Gobezie *et al.,* 2007 | SF | PAGE, LC-MS/MS | early-stage OA, late-stage OA, healthy control | ALB, FGA, FGG, A2M, APOE, APOH, C3, CP, HP, ORM1, DBP, C4b ↑ OA CSTA, ACAN, DCD ↓ OA | [135] |

Abbreviations: SF - synovial fluid; LC - liquid chromatography; MS - mass spectrometry; SWATH -sequential window acquisition of all theoretical mass spectra; OA - osteoarthritis; RA - rheumatoid arthritis; MRM - multiple reaction monitoring; MALDI - matrix-assisted laser desorption/ionization; TOF - time of flight; SRM - single reaction monitoring; 2DE - 2D electrophoresis; PAGE - polyacrylamide gel electrophoresis; SELDI - surface-enhanced laser desorption/ionization.

5.2 Rheumatoid Arthritis

RA a chronic autoimmune disease which leads to joint destruction and synovial inflammation. Patients are diagnosed following physical examination, radiographs and serum analysis. Swollen joints and radiographic erosions are signs of disease progression, whereas factors found in the serum and SF could be used as early diagnostic tools. The presence of rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) in the serum are the main features of RA. It can be identified in serum prior to the onset of disease [136]. Numerous studies have investigated the RA SF proteome (Table 2).

Citrullinated proteins are post-translational modified proteins in which arginine is deaminated to citrulline resulting in autoimmune response through a representation of these proteins as autoantigens, the reaction is catalyzed by peptidylarginine deiminases [137]. However, it is not the only PTM associated with RA; carbamylation and acetylation are also involved in the disease [138,139]. The association with the immune system in RA means immunoglobulins are promising molecules in understanding disease progression, particularly PTMs on IgG in patients with ACPA in serum and SF. The implementation of LC-MS/MS technique on identifying IgG-subtypes with associated Fc-glycans demonstrated that ACPA-IgG4 Fc-glycan-profile differs from control IgG4, with significantly lower amounts of the agalacto and asialylated core-fucosylated biantennary form and higher content of sialylated glycans in ACPA-IgG4 [140].

Identification of the PTMs and proteins that are being modified can be used as robust diagnostic tools and possibly stratify patients for therapy selection. Proteomics analysis of SF during RA is predominantly compared to the SF from OA or other types of arthritis. Several MS studies have shown the increased level of 14-3-3 zeta/delta, 14-3-3 eta proteins and S100A8, S100A12, S100A9 proteins in SF of RA in comparison to OA [4,66,124,141]. Notably, 14-3-3 eta has been verified as a biomarker for RA, increasing the diagnostic capture from 72% when ACPA and RF were used to 78% with the addition of 14-3-3 eta for disease diagnosis [142]. Heterocomplex of the S100A8 and S100A9 proteins (Calprotectin) also correlated to RA and therapeutic responsiveness in patients with RA [143].

Comparison of SF proteome of erosive RA patients and non-erosive RA patients demonstrated higher levels of S100A8, S100A9, S100A11 and S100A12 proteins in erosive RA. Similarly these proteins were significantly abundant in the serum of RA patients when compared to non-erosive RA and healthy patients using MRM quantification methods, indicating that higher levels of these S100 proteins facilitates severe disease progression [144]. Using multiple lectins Bhattacharjee *et al.* enriched glycoproteins from SF of RA and spondyloarthropathy patients then undertook iTRAQ labelling and LC-MS/MS to discover disease-specific proteins. Among the upregulated proteins in RA patients were Lumican, ECM1, S100A8, S100A9, CD44, MPO, VCAM1 and TIMP1 [67].

Due to the importance of ACPA in RA and its diagnostic potential, one field of research is the identification of other citrullinated proteins. Tabushi *et al*. used proteomics-based approaches to identify citrullinated proteins in RA SF and identified three: fibrinogen, fibronectin and vimentin. In the study, SF proteins were separated using gel electrophoresis and selected citrullinated proteins using anti-citrulline antibodies. Selected proteins were robustly identified using MALDI-TOF/MS, [145]. Moreover, it was shown that citrullinated SF peptides could be identified without initial selection for citrullinated peptides using antibodies. Raijmakers *et al.* have used ultrafiltration and solid phase extraction to remove peptides from SF. These were then analysed using LC-MS/MS and identified peptides included those with PTMs of methionine oxidation, arginine citrullination, asparagine and glutamine deamidation. Quantification of peptides demonstrated a higher prevalence of citrullinated fibrinopeptide A, a peptide situated in the alpha chain of fibrinogen, in SF of RA patients when compared to SF of other inflammatory diseases [146]. Citrullination of the nuclear proteins, histones, were identified in several studies in SF of RA patients. Citrullinated H2B (cH2B) levels were increased in SF of RA compared to OA and psoriatic arthritis; and in a mouse model co-immunization cH2B together with low dose type II collagen (ldCIA) resulted in a robust arthritis compared to immunization with ldCIA alone [147]. Citrullinated H2A, H4, H3 and H2B histones were identified in ACPA positive SF samples compared to ACPA negative SF. Immune complexes (IC) reactive to the third generation of a cyclic form of the citrullinated peptide (CCP3) in SF were captured and identified using LC-MS/MS approach. Findings demonstrated the presence of citrullinated histones in the majority of ACPA positive samples but not in ACPA negative samples. However, IC of citrullinated histones were not identified in the serum of ACPA-positive samples, indicating that SF microenvironment promotes ACPA to form IC with citrullinated histones [148]. This emphasizes the importance of studying SF along with serum in novel biomarkers discovery, as certain changes in the joint disease could be attributed only to the immediate microenvironment.

Obtaining SF from healthy individuals is one of the obstacles in understanding the whole mechanism of the disease progression at the proteomics level. To overcome such difficulties SF proteomics data obtained from animal models could be a solution. Given the similarity of the porcine immune system to that of human, and the fact that pigs have been used as a model to study other diseases, data generated from healthy SF proteome of the porcine model to some extent may be used as control for disease samples [149]. Furthermore studying the synovial fluid in other animals, such as the horse, has enabled a further understanding of changes in the sf proteome to be deciphered [11]. The horse provides an accepted model for the study of arthropathies as it suffers similar clinical joint diseases, particularly with respect to osteoarthritis, to those seen in man [121,150-153].

Table 2 shows studies in which SF proteomics has been used in rheumatoid arthritis investigations

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Authors** | **Tissue type** | **Groups** | **Main techniques** | **Difference in RA** | **References** |
| Flowers SA. et al., 2017 | SF | RA, OA, spondyloarthritis | LC-MS/MS, Western Blot, ELISA | COMP-lubricin complexes ↑ RA | [154] |
| Hafkenscheid L. et al., 2017 | SF, plasma | RA | LC-MALDI TOF, LC-MS | Fab glycosylation ↑ RA ACPA-positive | [155] |
| Meng X. et al., 2016 | SF, serum | RA, OA, psoriatic arthritis, reactive arthritis, polyarthritis | LC-TOF/MS, ELISA | SF: Citrullinated H4, H2B, H3 and H2A ↑ RA; Serum: Citrullinated H4 ↑ RA | [148] |
| Bhattacharjee M. et al., 2016 | SF | RA | LC-MS/MS | AXL, FLT4, Notch2, CSF/CSFR, NOTCH2, OPN, S100 family, ICAM, VCAM-1, CD300a, PTPN6, PDL2 and TNFR2 | [156] |
| Sohn DH. et al., 2015 | SF, serum | RA, OA, psoriatic arthritis | ELISA, LC-MS | SF, serum: Citrullinated H2B and H2A ↑ RA | [147] |
| Rombouts Y. et al., 2014 | SF, serum | SF: RA; serum: RA, non-RA disease group | LC-MALDI TOF, LC-MS/MS | N-linked glycosylation of Fab region ↑ RA | [157] |
| Lundström SL. et al., 2014 | SF, serum, plasma | RA | LC-MS/MS | Difference in Fc-glycans | [140] |
| Noh R. et al., 2014 | SF, serum | RA, non-RA | LC-MALDI TOF/MS | SF: STEAP4; serum: ZNF658 ↑ RA | [158] |
| Bhattacharjee M. et al., 2013 | SF | RA, spondyloarthritis | LC-MS/MS | VCAM-1, cadherin-5, LUM and ECM1 ↑ RA | [67] |
| Biswas S. et al., 2013 | SF, serum, plasma | RA, OA, trauma patients | LC-TOF/MS/MS | SF: Vimentin, Gelsolin, AHSG, GFAP and A1BG; Serum:GFAP, A1BG ↑ RA | [159] |
| Raijmakers R. et al., 2012 | SF | RA, non-RA inflammatory diseases | LC-MS/MS | Citrullinated fibrinopeptide A ↑ RA | [146] |
| Baillet A. et al., 2010 | SF, serum | RA, OA, inflammatory diseases | SELDI-TOF/MS | SF: S100A12, S100A8, S100A9 ↑ RA | [141] |
| Tabushi Y. et al., 2008 | SF | RA | MALDI-TOF/MS/MS | Citrullinated fibrinogen, fibronectin and vimentin | [145] |
| Liao H. et al., 2004 | SF, serum | SF:erosive-RA, non-erosive-RA; Serum: erosive-RA, non-erosive-RA, healthy control | LC-MS/MS | SF: CRP, 2-plasmin inhibitor, glutathione transferase, triosephosphate isomerase, G3PDH, S100A4, S100A8, S100A9, S100A11, S100A12, S100P, matrix-degrading cysteine proteinase cathepsin B, cystatin B inhibitor, 14-3-3 protein and RhoGDI2 ↑ erosive-RA; Serum: S100A8, S100A9, S100A12 ↑ erosive-RA | [144] |

Abbreviations: SF - synovial fluid; LC - liquid chromatography; MS - mass spectrometry; ELISA - enzyme-linked immunosorbent assay; OA - osteoarthritis; RA - rheumatoid arthritis; MALDI - matrix-assisted laser desorption/ionization; TOF - time of flight; SELDI - surface-enhanced laser desorption/ionization.

5.3 Juvenile Arthritis

Juvenile Idiopathic Arthritis (JIA) is a heterogeneous group of inflammatory diseases, considered the most common cause of disability in children. The disease is defined by several subtypes, including oligoarticular, polyarticular and systemic JIA; differentiated by a distinct clinical manifestation and possible complications. Various studies have used a proteomics approach to interrogate SF of juvenile arthritis (Table 3).

Table 3 shows studies in which SF proteomics has been used in juvenile idiopathic arthritis investigations

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Authors** | **Main Techniques** | **Groups** | **Differences in JA** | **References** |
| Gibson *et al.,* 2006 | 2DE, nESI-MS/MS | Single-event knee joint inflammation, recurrent knee joint inflammation | Collagen X, fibrin beta-chain, and T-cell receptor alpha-region ↑ recurrent group | [160] |
| Agarwal *et al.,* 2008 | ELISA | Polyarticular JIA, RA, OA, ERA | IL-17 ↑ polyarticular JIA | [161] |
| Gibson *et al.,* 2009 | 2DE , MALDI-TOF, Western immunoblotting | Oligoarticular, extended oligoarticular, polyarticular JIA | 40 proteins differentiated between groups | [162] |
| Rosenkranz *et al.,* 2010 | 2DE, MALDI-TOF-MS, Q-TOF-MS | Oligoarticular, polyarticular and systemic JIA | 24 proteins differentiated between JIA subtypes | [163] |
| Gibson *et al.,* 2012 | 2DE, MALDI-TOF/TOF, ELISA | Oligoarticular, extended oligoarticular, polyarticular JIA, psoriatic arthritis, ERA | Truncated isoform of vitamin D binding protein ↓ oligoarticular JIA | [164] |
| Brescia *et al.,* 2017 | Antibody array, ELISA | Oligoarticular JIA, non-arthritic joints | TNFα, sTNFR1, VEGF, IFNγ, IL1α, IL1β, IL-6, IL-4 and IL-17 ↑ oligoarticular JIA. IL-10 ↓ oligoarticular JIA | [165] |

Abbreviations: 2DE - 2D electrophoresis; nESI - nano electrospray ionisation; MS - mass spectrometry; OA - osteoarthritis; RA - rheumatoid arthritis; JIA - juvenile idiopathic arthritis; ERA - MALDI - matrix-assisted laser desorption/ionization; TOF - time of flight; ERA - enthesitis related arthritis.

Gibson *et al.* used 2DE method to identify differentially abundant proteins in SF of JIA patients with recurrent joint inflammation, these proteins were compared to SF of single-event joint inflammation [160]. Six protein spots were differentially abundant in the recurrent joint inflammation group and were identified with nESI-MS/MS as fibrin, fibrinogen, haptoglobin-1 and 2, T-cell receptor type C alpha region (TRAC) and Collagen X (COL10). Furthermore, TRAC and COL10 were identified in the SF of JIA patients with recurrent joint inflammation over time. The authors employed the same approach using MALDI-TOF/TOF to identify differentially abundant proteins between oligoarticular, extended oligoarticular and polyarticular JIA. Among the proteins that were significantly differed between subtypes were six albumin isoforms [5]. Moreover, they have conducted a similar approach to the larger group of JIA patients, where a truncated form of the vitamin D binding protein was significantly decreased in the extended oligoarticular JIA group [164]. Similarly, Rosenkranz et al. demonstrated that JIA subtypes can be differentiated on the SF proteome level. Proteins derived from SF of JIA patients and non-inflammatory disease controls were analysed using 2DE and differentially abundant protein spots subsequently identified by MALDI-TOF/MS or Q-TOF/MS [163]. Noticeably 24 proteins were identified as differentially abundant between JIA subtypes. Given the low sensitivity of 2D gels towards low abundant proteins, it can be assumed that only proteins of high abundance were identified in these studies.

Brescia *et al*. identified increased levels of TNFα, sTNFR1, VEGF, IFNγ, IL1α and IL1β in SF of oligoarticular JIA patients when compared to SF of non-inflammatory joint diseases using a protein array [165]. Furthermore, cytokines such as IL-6, IL-4 and IL-17 were increased in SF of JIA patients, whereas IL-10 was decreased in these patients. Noticeably, in another study IL-17 was also increased in the SF of polyarticular JIA and enthesitis-related arthritis patients when compared to SF of RA and OA patients, making IL-17 a biomarker candidate for JIA [161].

Mor-Vaknin *et al.* utilised LC-MS/MS approach to investigate the nuclear phospholipoprotein DEK in the SF of JIA patients. DEK autoantibodies were previously identified in the JIA patients, mainly in oligoarticular JIA [166]. It was shown that acetylated lysine and phosphorylation in DEK has a unique specificity to the primary macrophages. Unfortunately, there are no current studies in which the SF-derived proteome of JIA patients used high-throughput proteomics with the depletion of highly abundant proteins.

5.4 Spondyloarthropathies

Spondyloarthropathies (SpA) are chronic joint diseases, including ankylosing spondylitis (AS), reactive arthritis, psoriatic arthritis (PsA) and inflammatory bowel disease-associated SpA. There is no robust molecular marker for disease diagnosis, although HLA-B27 has been shown to be associated with SpA development to some extent [167].

Inflammatory cytokines, including Inteleukin-17 and TNFα, have been found to be involved in PsA progression and anti-TNFα therapy demonstrated improvement in PsA patients [168-170]. Ademowo *et al.* investigated synovial tissue of PsA patients using LC-MS/MS and label-free quantification to compare the proteome of anti-TNFα treatment responders to the non-responders. Furthermore, 107 proteins were selected for the biomarker panel from which an MRM assay was developed for 57 proteins. The biomarker panel was tested on the larger group to predict treatment responders. Predictive proteins included S100A8, S100A10, Ig kappa chain C, fibrinogen-α and γ, haptoglobin, COL2A1, vitronectin, cofilin, prolargin and 14-3-3 protein epsilon [171]. A follow up study from this group compared paired synovial tissue of PsA patients before the anti-TNFα treatment and after 12 weeks of treatment; 115 proteins demonstrated a change in abundance as a response to the treatment. These patients were also classified as responders and non-responders based on the European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR) criteria, with 25 proteins differentially abundant between the groups. Moreover, synovial tissue from the clinical trial using anti-TNFα was used to identify biomarkers for treatment effectiveness. From these two studies, authors selected albumin, haptoglobin, serum amyloid P and apolipoprotein AI for MRM assay, which could predict the response to treatment in SpA patients [172]. In the above-mentioned studies, a proteomics approach demonstrated the diagnostic ability in selecting effective treatment of PsA. Chang *et al.* used a similar approach by studying synovial tissue of RA, OA and AS patients, however, only proteins that were higher abundance in the RA group in comparison to others were analysed in depth [173]. Thus, AS synovial tissue was used as a control, similar to other studies involving SpA patients [147,148,154].

There are only a few studies which have used a proteomics approach to investigate the SF of SpA. Given the complexity of the disease, development of novel diagnostic and therapeutic tools could benefit from suchlike studies. Cretu *et al.* used an LC-MS/MS approach to study the proteome of SF in PsA patients in comparison to early-OA patients using label-free quantification. 137 proteins were found to be differentially abundant between the groups, including 44 upregulated proteins in PsA with a ratio > 2. Pathway analysis demonstrated the involvement of these proteins in acute phase response signalling, inflammatory response, production of nitric oxide and reactive oxygen species in macrophages. Furthermore, 17 proteins were selected for SRM assay of which 13 proteins were positively validated. Additionally, these 17 proteins were analysed in an independent cohort of PsA and early-OA SF using SRM. 12 proteins were confirmed to be abundant in PsA, including MMP3, EPO, M2BP, H4, H2AFX, ORM1, CD5L, PFN1 and C4BP proteins [174]. Bhattacharjee *et al.* analysed SF of RA and SpA patients using LC-MS/MS with iTRAQ labelling [67]. Notably MRM validated proteins myeloperoxidase, ORM1 and S100A9 from the Cretu *et al.* study were significantly less abundant in SpA SF when compared to RA in this study. Whereas proteins M2BP, C4BP and MMP-3 and CD5L have been confirmed to be highly abundant in SpA patients in comparison to RA, similarly to the study involving early-OA SF. Hence, the comparison of multiple inflammatory joint arthritis diseases could provide more accurate proteome profiles for the diseases and potentially more specific biomarkers.

1. Conclusion

The greatest unmet needs to improve clinical outcomes include biomarker discovery enabling tests that provide early intervention when a patient would present otherwise healthy and before the disease has progressed to a point which is irreversible. The field of MS-based biomarker discovery in SF has been split into factions of pessimism and cautious optimism. The field has developed considerably with regards to emphasis on good experimental design and the need to reduce false discovery. The limited success of shotgun proteomics in the development of clinical biomarkers and therapeutic targets should not dissuade scientists involved in biomarker discovery from adopting MS technology as a biomarker discovery tool.

1. Expert Commentary

One important application of SF proteomics is in the identification of early disease markers for arthropathies, particularly in the case for OA, allowing timely intervention before the development of significant pathology. However, there are many challenges within the SF proteomics field including the requirement for rigorous and standardised methods of sample collection and storage, the sensitivity and specificity of proteomic assays, the availability of high-quality samples (including relevant controls), and a comprehensive analysis of the data to reduce falsely identified markers. However, with increasingly sensitive instrumentation and other ‘omics’ technologies some recent challenges have emerged.

a. Multi ‘omics’

In recent years there has been a huge increase in the number of publications reporting results using ‘omics’ techniques, principally transcriptomics, genomics, metabolomics and proteomics [175]. One of the challenges this poses is the need to develop multi ‘omic’ integration techniques. Computational integration of these separate datasets can work synergistically, greatly enhancing the information that can be obtained as opposed to analysing them separately. Considering this, various software programs are now available which undertake pathway analysis and multi ‘omics’ data visualisation, generating joint pathway p- values [176-179]. In most cases, an optimal experimental design involves the splitting of samples which are subsequently used for appropriate ‘omics’ studies, termed a ‘split sample study’ design [175].

Recently, a study has used a multi ‘omics’ integration approach, utilising genomics, proteomics and miRNomics of human plasma for biomarker discovery in the study of the inherited cardiac disorder Brugada syndrome, providing further information on disease pathogenesis [180]. Within musculoskeletal biology, Ritter *et al.* carried out MS proteomic analysis of early and late stage human OA SF and combined these results with transcriptomic results of articular tissues to inform on the source of differentially abundant synovial proteins [53]. Thus, a multi ‘omics’ approach can yield even more information and further support/validate a study even if the complementary omics approaches are not performed on the same source material or data is drawn from publicly available data repositories. However, despite the growing field of multi ‘omics’ approaches, to date no studies have combined NMR metabolomics and MS proteomic datasets. This is an area which we are currently developing within our group at The University of Liverpool, undertaking computational integration of these ‘omics’ datasets for SF analysis.

b. Removal or equalisation of high abundance proteins

Whilst a vast array of proteomic data present exciting opportunities for biomarker discovery among arthritic diseases to improve clinical outcome through improved early diagnostics, stratification and, the identification of therapeutic targets, it is critical that studies are extensively optimized to allow for the success of novel candidate markers. One of the major problems with SF is the small number of highly abundant proteins rendering the identification of low abundance proteins challenging. However, as technologies improve, various methodologies have developed to reduce the protein concentration dynamic range, enriching less abundant proteins and maximising the total number of identified proteins, maximising the potential for biomarker discovery. Maximising the number of quantifiable proteins within SF samples is also improving pathway analysis and strengthening the conclusions which can be drawn.

1. Five-year View

Undertaking MS-based analysis has been cost prohibitive for many researchers and therefore a major limitation of advancing knowledge within the field. Subsequently this has resulted in numerous underpowered studies for which firm conclusions have been difficult to draw. However, a combination of developing MS methods, allowing a faster and higher sensitivity methodology, and advancing techniques to allow deeper SF proteome interrogation, is maximising the data that can be obtained within studies as well as allowing this technique to be more accessible to research groups. As technology continues to advance, improved methodologies to reduce the protein concentration dynamic range of SF are likely to develop further over the next five years, allowing even deeper analysis of the SF proteome and quantification of the lowest abundant proteins.

Within the coming years, as the various ‘omics’ fields have developed, the next important step for the field will be within the synergistic computational integration of these areas. Combining these datasets will inevitably greatly improve the information that can be gleaned from separate analyses, as well as providing key information for improving pathway analysis. The number of software platforms available to undertake this approach are ever growing, developing and improving, thus making this analysis easier to undertake.

Within SF proteomics, the identification of early biomarkers for orthopaedic pathologies has been a key objective. However, as mentioned previously, markers of clinical prognosis and therapeutic effects are growing areas of interest. In addition, an exciting potential area of research is the identification of protein pain biomarkers of musculoskeletal pain within SF. Factors of interest include nerve growth factor, a neuromediator, which is likely to be a key regulator involved in inflammatory and neuropathic pain and the neurotransmitter substance P which has been suggested as a potential biomarker for pain in chronic equine OA joints [181,182]. This is an exciting area of research, which will undoubtedly develop over the coming years, potentially providing a panel of pain markers which may have potential to deliver a more bespoke clinical analgesic therapeutic plan, identifying pain pathways implicated for individuals, generating novel therapeutic targets for the next generation of analgesics, and a potential pain panel to quantifiably monitor the effectiveness of future analgesics.

Review Criteria

Studies included in this article were identified by searching PubMed using the terms “osteoarthritis”, “rheumatoid arthritis”, “spondyloarthritis”, “juvenile arthritis”, “extracellular vesicles” AND “synovial fluid” as well as “biofluid sample complexity”. The literature search was limited to full-text original research articles and reviews written in English and published between 1991 and 2018. In addition, further relevant articles were identified from the authors’ personal archives.

Declaration of Interest

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**References**

1. Blewis ME, Nugent-Derfus GE, Schmidt TA, Schumacher BL, Sah RL. A model of synovial fluid lubricant composition in normal and injured joints. *Eur Cell Mater*, 13, 26-39 (2007).

2. Tamer TM. Hyaluronan and synovial joint: function, distribution and healing. *Interdiscip Toxicol*, 6(3), 111-125 (2013).

3. Anderson JR, Phelan MM, Clegg PD, Peffers MJ, Rubio-Martinez LM. Synovial Fluid Metabolites Differentiate between Septic and Nonseptic Joint Pathologies. *Journal of proteome research*, 17(8), 2735-2743 (2018).

4. Mateos J, Lourido L, Fernandez-Puente P *et al.* Differential protein profiling of synovial fluid from rheumatoid arthritis and osteoarthritis patients using LC-MALDI TOF/TOF. *Journal of proteomics*, 75(10), 2869-2878 (2012).

5. Gibson DS, Finnegan S, Jordan G *et al.* Stratification and monitoring of juvenile idiopathic arthritis patients by synovial proteome analysis. *Journal of proteome research*, 8(12), 5601-5609 (2009).

6. Hsueh M-F, Önnerfjord P, Kraus VB. Biomarkers and proteomic analysis of osteoarthritis. *Matrix Biology*, 39, 56-66 (2014).

7. Mahendran SM, Oikonomopoulou K, Diamandis EP, Chandran V. Synovial fluid proteomics in the pursuit of arthritis mediators: An evolving field of novel biomarker discovery. *Critical Reviews in Clinical Laboratory Sciences*, 54, 495-505 (2017).

8. Puangpila C, Mayadunne E, El Rassi Z. Liquid phase based separation systems for depletion, prefractionation, and enrichment of proteins in biological fluids and matrices for in-depth proteomics analysis-An update covering the period 2011-2014. *Electrophoresis*, 36(1), 238-252 (2015).

9. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics*, 1, 845-867 (2002).

10. Fernandez-Costa C, Calamia V, Fernandez-Puente P, Capelo-Martinez JL, Ruiz-Romero C, Blanco FJ. Sequential depletion of human serum for the search of osteoarthritis biomarkers. *Proteome Sci*, 10(1), 55 (2012).

11. Peffers MJ, McDermott B, Clegg PD, Riggs CM. Comprehensive protein profiling of synovial fluid in osteoarthritis following protein equalization. *Osteoarthritis Cartilage*, 23(7), 1204-1213 (2015).

12. Roche S, Tiers L, Provansal M, Piva MT, Lehmann S. Interest of major serum protein removal for Surface-Enhanced Laser Desorption/Ionization - Time Of Flight (SELDI-TOF) proteomic blood profiling. *Proteome Sci*, 4, 20 (2006).

13. Seligner B, Kellner R. Biomarker Discovery in Renal Cell Carcinoma Applying Proteome-Based Studies in Combination with Serology. In: *Proteomics in Drug Research.* Hamacher, M, Marcus, K, Stuhler, K, Van Hall, A, Warscheid, B, Meyer, HE (Ed.^(Eds) (Wiley, Weinheim, 2006) 227.

14. Shen Y, Jacobs JM, Camp 2nd DG *et al.* Ultra-high-efficiency strong cation exchange LC/RPLC/MS/MS for high dynamic range characterization of the human plasma proteome. *Anal Chem*, 76, 1134-1144 (2004).

15. Fonslow BR, Carvalho PC, Academia K *et al.* Improvements in proteomic metrics of low abundance proteins through proteome equalization using ProteoMiner prior to MudPIT. *Journal of proteome research*, 10(8), 3690-3700 (2011).

16. Fernandez-Costa C, Calamia V, Fernandez-Puente P, Capelo-Martinez JL, Ruiz-Romero C, Blanco FJ. Sequential depletion of human serum for the search of osteoarthritis biomarkers. *Proteome Sci*, 10, 55 (2012).

17. Balakrishnan L, Nirujogi R, Ahmad S *et al.* Proteomic analysis of human osteoarthritis synovial fluid. *Clinical proteomics*, 11, 6 (2014).

18. Garner BC, Kuroki K, Stoker AM, Cook CR, Cook JL. Expression of proteins in serum, synovial fluid, synovial membrane, and articular cartilage samples obtained from dogs with stifle joint osteoarthritis secondary to cranial cruciate ligament disease and dogs without stifle joint arthritis. *American journal of veterinary research*, 74, 386-394 (2013).

19. Peffers MJ, Riggs CM, Phelan MP, Clegg PD. Identification of Disease Specific Metabolic Fingerprints in Early Osteoarthritis. *Equine Veterinary Journal*, 47, 13 (2015).

20. Zhou M, Lucas DA, Chan KC *et al.* An investigation into the human serum "interactome". *Electrophoresis*, 25, 1289-1298 (2004).

21. Subramanian S. Dye-ligand affinity chromatography: the interaction of Cibacron Blue F3GA with proteins and enzymes. *CRC critical reviews in biochemistry*, 16, 169-205 (1984).

22. Björhall K, Miliotis T, Davidsson P. Comparison of different depletion strategies for improved resolution in proteomic analysis of human serum samples. *PROTEOMICS*, 5, 307-317 (2005).

23. Zolotarjova N, Martosella J, Nicol G, Bailey J, Boyes BE, Barrett WC. Differences among techniques for high-abundant protein depletion. *PROTEOMICS*, 5, 3304-3313 (2005).

24. Steel LF, Trotter MG, Nakajima PB, Mattu TS, Gonye G, Block T. Efficient and specific removal of albumin from human serum samples. *Molecular & cellular proteomics : MCP*, 2, 262-270 (2003).

25. Wang YY, Cheng PCP, Chan DW. A simple affinity spin tube filter method for removing high-abundant common proteins or enriching low-abundant biomarkers for serum proteomic analysis. *PROTEOMICS*, 3, 243-248 (2003).

26. Georgiou HM, Rice GE, Baker MS. Proteomic analysis of human plasma: Failure of centrifugal ultrafiltration to remove albumin and other high molecular weight proteins. *PROTEOMICS*, 1, 1503 (2001).

27. Chen Y-Y, Lin S-Y, Yeh Y-Y *et al.* A modified protein precipitation procedure for efficient removal of albumin from serum. *ELECTROPHORESIS*, 26, 2117-2127 (2005).

28. Yang Z, Hancock WS. Approach to the comprehensive analysis of glycoproteins isolated from human serum using a multi-lectin affinity column. *Journal of Chromatography A*, 1053, 79-88 (2004).

29. Furka A, Sebestyen F, Asgedom M, Dibo G. General method for rapid synthesis of multicomponent peptide mixtures. *Int J Pept Protein Res*, 37, 487-493 (1991).

30. Lam KS, Salmon SE, Hersh EM, Hruby VJ, Kazmierski WM, Knapp RJ. A new type of synthetic peptide library for identifying ligand-binding activity. *Nature*, 354, 82-84 (1991).

31. Pisanu S, Biosa G, Carcangiu L, Uzzau S, Pagnozzi D. Comparative evaluation of seven commercial products for human serum enrichment/depletion by shotgun proteomics. *Talanta*, 185, 213-220 (2018).

32. Righetti PG, Boschetti E. The ProteoMiner and the FortyNiners: searching for gold nuggets in the proteomic arena. *Mass Spectrom Rev*, 27, 596-608 (2008).

33. Hartwig S, Czibere A, Kotzka J *et al.* Combinatorial hexapeptide ligand libraries (ProteoMiner): an innovative fractionation tool for differential quantitative clinical proteomics. *Arch Physiol Biochem*, 115, 155-160 (2009).

34. Williams A. Proteomic studies of an explant model of equine articular cartilage in response to proinflammatory and anti-inflammatory stimuli. (Ed.^(Eds) (2014) 1-237.

35. Davidson R, Gardner S, Jupp O *et al.* Isothiocyanates are detected in human synovial fluid following broccoli consumption and can affect the tissues of the knee joint. *Sci Rep*, 7(1), 3398 (2017).

36. Hulme CH, Wilson EL, Peffers MJ *et al.* Autologous chondrocyte implantation-derived synovial fluids display distinct responder and non-responder proteomic profiles. *Arthritis Res Ther*, 19(1), 150 (2017).

37. Peffers MJ, McDermott B, Clegg PD, Riggs CM. Comprehensive protein profiling of synovial fluid in osteoarthritis following protein equalization. *Osteoarthritis Cartilage*, 23, 1204-1213 (2015).

38. Li L, Sun C, Freeby S *et al.* Protein sample treatment with peptide ligand library: coverage and consistency. *Journal of Proteomics & Bioinformatice*, 2, 485-494 (2009).

39. Di Meo A, Diamandis EP, Rodriguez H, Hoofnagle AN, Ioannidis J, Lopez M. What Is Wrong with Clinical Proteomics? *Clinical Chemistry*, 60(10), 1258 (2014).

40. Chiou SH, Wu CY. Clinical proteomics: current status, challenges, and future perspectives. *The Kaohsiung journal of medical sciences*, 27(1), 1-14 (2011).

41. Ruiz-Romero C, Blanco FJ. Proteomics role in the search for improved diagnosis, prognosis and treatment of osteoarthritis. *Osteoarthritis Cartilage*, 18(4), 500-509 (2010).

42. Poste G. Bring on the biomarkers. *Nature*, 469(7329), 156-157 (2011).

43. Poste G. Biospecimens, biomarkers, and burgeoning data: the imperative for more rigorous research standards. *Trends Mol Med*, 18(12), 717-722 (2012).

44. Horvath AR, Bossuyt PM, Sandberg S *et al.* Setting analytical performance specifications based on outcome studies - is it possible? *Clin Chem Lab Med*, 53(6), 841-848 (2015).

45. Drucker E, Krapfenbauer K. Pitfalls and limitations in translation from biomarker discovery to clinical utility in predictive and personalised medicine. *EPMA J*, 4(1), 7 (2013).

46. Issaq HJ, Waybright TJ, Veenstra TD. Cancer biomarker discovery: Opportunities and pitfalls in analytical methods. *Electrophoresis*, 32(9), 967-975 (2011).

47. Vizcaino JA, Csordas A, del-Toro N *et al.* 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res*, 44(D1), D447-456 (2016).

48. Li H, Popp R, Borchers CH. Affinity-mass spectrometric technologies for quantitative proteomics in biological fluids. *bioRxiv*, 114751 (2017).

49. Rauniyar N. Parallel Reaction Monitoring: A Targeted Experiment Performed Using High Resolution and High Mass Accuracy Mass Spectrometry. *International journal of molecular sciences*, 16(12), 28566-28581 (2015).

50. Drabovich AP, Martinez-Morillo E, Diamandis EP. Toward an integrated pipeline for protein biomarker development. *Biochim Biophys Acta*, 1854(6), 677-686 (2015).

51. Pan X, Huang L, Chen J, Dai Y, Chen X. Analysis of synovial fluid in knee joint of osteoarthritis:5 proteome patterns of joint inflammation based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *International orthopaedics*, 36(1), 57-64 (2012).

52. Wang F, Chen FF, Gao WB *et al.* Identification of citrullinated peptides in the synovial fluid of patients with rheumatoid arthritis using LC-MALDI-TOF/TOF. *Clin Rheumatol*, 35(9), 2185-2194 (2016).

53. Ritter SY, Subbaiah R, Bebek G *et al.* Proteomic analysis of synovial fluid from the osteoarthritic knee: comparison with transcriptome analyses of joint tissues. *Arthritis and rheumatism*, 65(4), 981-992 (2013).

54. Yamagiwa H, Sarkar G, Charlesworth MC, McCormick DJ, Bolander ME. Two-dimensional gel electrophoresis of synovial fluid: method for detecting candidate protein markers for osteoarthritis. *J Orthop Sci*, 8(4), 482-490 (2003).

55. Larsen MR, Trelle MB, Thingholm TE, Jensen ON. Analysis of posttranslational modifications of proteins by tandem mass spectrometry. *BioTechniques*, 40(6), 790-798 (2006).

56. Karpievitch YV, Polpitiya AD, Anderson GA, Smith RD, Dabney AR. Liquid Chromatography Mass Spectrometry-Based Proteomics: Biological and Technological Aspects. *Ann Appl Stat*, 4(4), 1797-1823 (2010).

57. Zhang L, Elias JE. Relative Protein Quantification Using Tandem Mass Tag Mass Spectrometry. In: *Proteomics: Methods and Protocols.* Comai, L, Katz, JE, Mallick, P (Eds.) (Springer New York, New York, NY, 2017) 185-198.

58. Navarrete-Perea J, Yu Q, Gygi SP, Paulo JA. Streamlined Tandem Mass Tag (SL-TMT) Protocol: An Efficient Strategy for Quantitative (Phospho)proteome Profiling Using Tandem Mass Tag-Synchronous Precursor Selection-MS3. *Journal of proteome research*, 17(6), 2226-2236 (2018).

59. Chahrour O, Cobice D, Malone J. Stable isotope labelling methods in mass spectrometry-based quantitative proteomics. *Journal of pharmaceutical and biomedical analysis*, 113, 2-20 (2015).

60. Mann M. Functional and quantitative proteomics using SILAC. *Nat Rev Mol Cell Biol*, 7(12), 952-958 (2006).

61. Shiio Y, Aebersold R. Quantitative proteome analysis using isotope-coded affinity tags and mass spectrometry. *Nat Protoc*, 1(1), 139-145 (2006).

62. Wiese S, Reidegeld KA, Meyer HE, Warscheid B. Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research. *Proteomics*, 7(3), 340-350 (2007).

63. Megger DA, Bracht T, Meyer HE, Sitek B. Label-free quantification in clinical proteomics. *Biochim Biophys Acta*, 1834(8), 1581-1590 (2013).

64. Hulme CH, Wilson EL, Fuller HR *et al.* Two independent proteomic approaches provide a comprehensive analysis of the synovial fluid proteome response to Autologous Chondrocyte Implantation. *Arthritis research & therapy*, 20(1), 87 (2018).

65. Balakrishnan L, Nirujogi RS, Ahmad S *et al.* Proteomic analysis of human osteoarthritis synovial fluid. *Clinical proteomics*, 11(1), 6 (2014).

66. Balakrishnan L, Bhattacharjee M, Ahmad S *et al.* Differential proteomic analysis of synovial fluid from rheumatoid arthritis and osteoarthritis patients. *Clinical proteomics*, 11(1), 1 (2014).

67. Bhattacharjee M, Sharma R, Goel R *et al.* A multilectin affinity approach for comparative glycoprotein profiling of rheumatoid arthritis and spondyloarthropathy. *Clinical proteomics*, 10(1), 11 (2013).

68. Bantscheff M, Eberhard D, Abraham Y *et al.* Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. *Nat Biotechnol*, 25(9), 1035-1044 (2007).

69. Mann M. Comparative analysis to guide quality improvements in proteomics. *Nat Methods*, 6(10), 717-719 (2009).

70. Patel VJ, Thalassinos K, Slade SE *et al.* A comparison of labeling and label-free mass spectrometry-based proteomics approaches. *Journal of proteome research*, 8(7), 3752-3759 (2009).

71. Bubis JA, Levitsky LI, Ivanov MV, Tarasova IA, Gorshkov MV. Comparative evaluation of label-free quantification methods for shotgun proteomics. *Rapid Commun Mass Spectrom*, 31(7), 606-612 (2017).

72. Tabb DL, Vega-Montoto L, Rudnick PA *et al.* Repeatability and reproducibility in proteomic identifications by liquid chromatography-tandem mass spectrometry. *Journal of proteome research*, 9(2), 761-776 (2010).

73. Arike L, Peil L. Spectral counting label-free proteomics. *Methods Mol Biol*, 1156, 213-222 (2014).

74. Zhang J, Xin L, Shan B *et al.* PEAKS DB: de novo sequencing assisted database search for sensitive and accurate peptide identification. *Mol Cell Proteomics*, 11(4), M111 010587 (2012).

75. McIlwraith CW, Kawcak CE, Frisbie DD *et al.* Biomarkers for equine joint injury and osteoarthritis. *J Orthop Res*, 36(3), 823-831 (2018).

76. Gonzalez-Galarza FF, Lawless C, Hubbard SJ *et al.* A critical appraisal of techniques, software packages, and standards for quantitative proteomic analysis. *OMICS*, 16(9), 431-442 (2012).

77. Parker CE, Domanski D, Percy AJ *et al.* Mass spectrometry in high-throughput clinical biomarker assays: multiple reaction monitoring. *Top Curr Chem*, 336, 117-137 (2014).

78. Liebler DC, Zimmerman LJ. Targeted quantitation of proteins by mass spectrometry. *Biochemistry*, 52(22), 3797-3806 (2013).

79. Otto A, Bernhardt J, Hecker M, Becher D. Global relative and absolute quantitation in microbial proteomics. *Curr Opin Microbiol*, 15(3), 364-372 (2012).

80. Brownridge P, Holman SW, Gaskell SJ *et al.* Global absolute quantification of a proteome: Challenges in the deployment of a QconCAT strategy. *Proteomics*, 11(15), 2957-2970 (2011).

81. Dupuis A, Hennekinne JA, Garin J, Brun V. Protein Standard Absolute Quantification (PSAQ) for improved investigation of staphylococcal food poisoning outbreaks. *Proteomics*, 8(22), 4633-4636 (2008).

82. Beynon RJ, Doherty MK, Pratt JM, Gaskell SJ. Multiplexed absolute quantification in proteomics using artificial QCAT proteins of concatenated signature peptides. *Nat Methods*, 2(8), 587-589 (2005).

83. Peffers MJ, Beynon RJ, Clegg PD. Absolute quantification of selected proteins in the human osteoarthritic secretome. *Int J Mol Sci*, 14(10), 20658-20681 (2013).

84. Nagarajah S. Exosome Secretion - More Than Simple Waste Disposal? Implications for Physiology, Diagnostics and Therapeutics. *J Circ Biomark*, 5, 7 (2016).

85. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol*, 200(4), 373-383 (2013).

86. Fu H, Hu D, Zhang L, Tang P. Role of extracellular vesicles in rheumatoid arthritis. *Mol Immunol*, 93, 125-132 (2018).

87. Baranyai T, Herczeg K, Onodi Z *et al.* Isolation of Exosomes from Blood Plasma: Qualitative and Quantitative Comparison of Ultracentrifugation and Size Exclusion Chromatography Methods. *PloS one*, 10(12), e0145686 (2015).

88. Foers AD, Chatfield S, Dagley LF *et al.* Enrichment of extracellular vesicles from human synovial fluid using size exclusion chromatography. *Journal of extracellular vesicles*, 7(1), 1490145 (2018).

89. Skriner K, Adolph K, Jungblut PR, Burmester GR. Association of citrullinated proteins with synovial exosomes. *Arthritis Rheum*, 54(12), 3809-3814 (2006).

90. Tsuno H, Suematsu N, Sato T *et al.* Effects of methotrexate and salazosulfapyridine on protein profiles of exosomes derived from a human synovial sarcoma cell line of SW982. *Proteomics Clin Appl*, 10(2), 164-171 (2016).

91. Malda J, Boere J, van de Lest CH, van Weeren P, Wauben MH. Extracellular vesicles - new tool for joint repair and regeneration. *Nat Rev Rheumatol*, 12(4), 243-249 (2016).

92. Kraus VB, McDaniel G, Huebner JL *et al.* Direct in vivo evidence of activated macrophages in human osteoarthritis. *Osteoarthritis and cartilage*, 24(9), 1613-1621 (2016).

93. Domenis R, Zanutel R, Caponnetto F *et al.* Characterization of the Proinflammatory Profile of Synovial Fluid-Derived Exosomes of Patients with Osteoarthritis. *Mediators Inflamm*, 2017, 4814987 (2017).

94. Kramer CM, Tsang AS, Koenig T, Jeffcott LB, Dart CM, Dart AJ. Survey of the therapeutic approach and efficacy of pentosan polysulfate for the prevention and treatment of equine osteoarthritis in veterinary practice in Australia. *Aust Vet J*, 92, 482-487 (2014).

95. Mobasheri A, Bay-Jensen A-C, van Spil WE, Larkin J, Levesque MC. Osteoarthritis Year in Review 2016: biomarkers (biochemical markers). *Osteoarthritis and cartilage*, 25, 199-208 (2017).

96. Truong LH, Kuliwaba JS, Tsangari H, Fazzalari NL. Differential gene expression of bone anabolic factors and trabecular bone architectural changes in the proximal femoral shaft of primary hip osteoarthritis patients. *Arthritis research & therapy*, 8, R188 (2006).

97. Li Y, Xu L, Olsen BR. Lessons from genetic forms of osteoarthritis for the pathogenesis of the disease. *Osteoarthritis and cartilage*, 15, 1101-1105 (2007).

98. Marhardt K, Muurahainen N. Development of a Disease-Modifying OA Drug (DMOAD) in Knee Osteoarthritis: The Example of Sprifermin. *Drug Research*, 65, S13-S13 (2015).

99. Struglics A, Larsson S, Pratta MA, Kumar S, Lark MW, Lohmander LS. Human osteoarthritis synovial fluid and joint cartilage contain both aggrecanase- and matrix metalloproteinase-generated aggrecan fragments. *Osteoarthritis and Cartilage*, 14, 101-113 (2006).

100. Sinusas K. Osteoarthritis: Diagnosis and Treatment. *American Family Physician*, 85, 49-56 (2012).

101. Brommer H, van Weeren PR, Brama PA. New approach for quantitative assessment of articular cartilage degeneration in horses with osteoarthritis. *American journal of veterinary research*, 64, 83-87 (2003).

102. Nguyen LT, Sharma AR, Chakraborty C, Saibaba B, Ahn M-E, Lee S-S. Review of Prospects of Biological Fluid Biomarkers in Osteoarthritis. *International journal of molecular sciences*, 18 (2017).

103. Conrozier T, Carlier MC, Mathieu P *et al.* Serum levels of YKL-40 and C reactive protein in patients with hip osteoarthritis and healthy subjects: a cross sectional study. *Annals of the rheumatic diseases*, 59, 828-831 (2000).

104. Guan J, Liu Z, Li F *et al.* Increased Synovial Fluid YKL-40 Levels are Linked with Symptomatic Severity in Knee Osteoarthritis Patients. *Clinical laboratory*, 61, 991-997 (2015).

105. Kobayashi T, Yoshihara Y, Yamada H, Fujikawa K. Procollagen IIC-peptide as a marker for assessing mechanical risk factors of knee osteoarthritis: effect of obesity and varus alignment. *Annals of the rheumatic diseases*, 59, 982-984 (2000).

106. Lohmander LS, Ionescu M, Jugessur H, Poole AR. Changes in joint cartilage aggrecan after knee injury and in osteoarthritis. *Arthritis and rheumatism*, 42, 534-544 (1999).

107. Väänänen T, Koskinen A, Paukkeri E-L *et al.* YKL-40 as a novel factor associated with inflammation and catabolic mechanisms in osteoarthritic joints. *Mediators of inflammation*, 2014, 215140 (2014).

108. Germaschewski FM, Matheny CJ, Larkin J *et al.* Quantitation OF ARGS aggrecan fragments in synovial fluid, serum and urine from osteoarthritis patients. *Osteoarthritis and cartilage*, 22, 690-697 (2014).

109. Ishiguro N, Ito T, Ito H *et al.* Relationship of matrix metalloproteinases and their inhibitors to cartilage proteoglycan and collagen turnover: Analyses of synovial fluid from patients with osteoarthritis. *Arthritis & Rheumatism*, 42, 129-136 (1999).

110. Kumahashi N, Swärd P, Larsson S, Lohmander LS, Frobell R, Struglics A. Type II collagen C2C epitope in human synovial fluid and serum after knee injury--associations with molecular and structural markers of injury. *Osteoarthritis and cartilage*, 23, 1506-1512 (2015).

111. Larsson S, Lohmander LS, Struglics A. Synovial fluid level of aggrecan ARGS fragments is a more sensitive marker of joint disease than glycosaminoglycan or aggrecan levels: a cross-sectional study. *Arthritis research & therapy*, 11, R92 (2009).

112. Lohmander LS, Atley LM, Pietka TA, Eyre DR. The release of crosslinked peptides from type II collagen into human synovial fluid is increased soon after joint injury and in osteoarthritis. *Arthritis & Rheumatism*, 48, 3130-3139 (2003).

113. Özler K, Aktaş E, Atay Ç, Yılmaz B, Arıkan M, Güngör Ş. Serum and knee synovial fluid matrixmetalloproteinase-13 and tumor necrosis factor-alpha levels in patients with late stage osteoarthritis. *Acta orthopaedica et traumatologica turcica*, 50, 670-673 (2016).

114. Rübenhagen R, Schüttrumpf JP, Stürmer KM, Frosch K-H. Interleukin-7 levels in synovial fluid increase with age and MMP-1 levels decrease with progression of osteoarthritis. *Acta orthopaedica*, 83, 59-64 (2012).

115. Senolt L, Braun M, Olejárová M, Forejtová S, Gatterová J, Pavelka K. Increased pentosidine, an advanced glycation end product, in serum and synovial fluid from patients with knee osteoarthritis and its relation with cartilage oligomeric matrix protein. *Annals of the rheumatic diseases*, 64, 886-890 (2005).

116. Wang Y, Li D, Xu N *et al.* Follistatin-like protein 1: a serum biochemical marker reflecting the severity of joint damage in patients with osteoarthritis. *Arthritis research & therapy*, 13, R193 (2011).

117. Peffers MJ, Thornton DJ, Clegg PD. Characterization of neopeptides in equine articular cartilage degradation. *J Orthop Res*, 34(1), 106-120 (2016).

118. Siepen JA, Keevil E-J, Knight D, Hubbard SJ. Prediction of missed cleavage sites in tryptic peptides aids protein identification in proteomics. *Journal of proteome research*, 6, 399-408 (2007).

119. Miller RE, Ishihara S, Tran PB *et al.* An aggrecan fragment drives osteoarthritis pain through Toll-like receptor 2. *JCI Insight*, 3 (2018).

120. Skiöldebrand E, Ekman S, Mattsson Hultén L *et al.* Cartilage oligomeric matrix protein neoepitope in the synovial fluid of horses with acute lameness: A new biomarker for the early stages of osteoarthritis. *Equine Veterinary Journal*, 49, 662-667 (2017).

121. Peffers MJ, Cillero-Pastor B, Eijkel GB, Clegg PD, Heeren RM. Matrix assisted laser desorption ionization mass spectrometry imaging identifies markers of ageing and osteoarthritic cartilage. *Arthritis Res Ther*, 16(3), R110 (2014).

122. Peffers M, Jones AR, McCabe A, Anderson J. Neopeptide Analyser: A software tool for neopeptide discovery in proteomics data. *Wellcome Open Res*, 2, 24 (2017).

123. Davidson RK, Jupp O, de Ferrars R *et al.* Sulforaphane Represses Matrix-Degrading Proteases and Protects Cartilage From Destruction In Vitro and In Vivo. *Arthritis & Rheumatism*, 65, 3130-3140 (2013).

124. Liao W, Li Z, Li T, Zhang Q, Zhang H, Wang X. Proteomic analysis of synovial fluid in osteoarthritis using SWATHmass spectrometry. *Molecular medicine reports*, 17(2), 2827-2836 (2018).

125. Fernandez-Puente P, Calamia V, Gonzalez-Rodriguez L *et al.* Multiplexed mass spectrometry monitoring of biomarker candidates for osteoarthritis. *Journal of proteomics*, 152, 216-225 (2017).

126. Liao W, Li Z, Zhang H, Li J, Wang K, Yang Y. Proteomic analysis of synovial fluid as an analytical tool to detect candidate biomarkers for knee osteoarthritis. *International journal of clinical and experimental pathology*, 8(9), 9975-9989 (2015).

127. Ritter SY, Collins J, Krastins B *et al.* Mass spectrometry assays of plasma biomarkers to predict radiographic progression of knee osteoarthritis. *Arthritis research & therapy*, 16, 456 (2014).

128. Ahrman E, Lorenzo P, Holmgren K *et al.* Novel cartilage oligomeric matrix protein (COMP) neoepitopes identified in synovial fluids from patients with joint diseases using affinity chromatography and mass spectrometry. *The Journal of biological chemistry*, 289(30), 20908-20916 (2014).

129. Wanner J, Subbaiah R, Skomorovska-Prokvolit Y *et al.* Proteomic profiling and functional characterization of early and late shoulder osteoarthritis. *Arthritis research & therapy*, 15(6), R180 (2013).

130. Liao W, Li Z, Wang H, Wang J, Fu Y, Bai X. Proteomic analysis of synovial fluid: insight into the pathogenesis of knee osteoarthritis. *International orthopaedics*, 37(6), 1045-1053 (2013).

131. Han MY, Dai JJ, Zhang Y *et al.* Identification of osteoarthritis biomarkers by proteomic analysis of synovial fluid. *The Journal of international medical research*, 40(6), 2243-2250 (2012).

132. Sohn DH, Sokolove J, Sharpe O *et al.* Plasma proteins present in osteoarthritic synovial fluid can stimulate cytokine production via Toll-like receptor 4. *Arthritis research & therapy*, 14(1), R7 (2012).

133. de Seny D, Sharif M, Fillet M *et al.* Discovery and biochemical characterisation of four novel biomarkers for osteoarthritis. *Annals of the rheumatic diseases*, 70(6), 1144-1152 (2011).

134. Kamphorst JJ, van der Heijden R, DeGroot J *et al.* Profiling of endogenous peptides in human synovial fluid by NanoLC-MS: method validation and peptide identification. *Journal of proteome research*, 6(11), 4388-4396 (2007).

135. Gobezie R, Kho A, Krastins B *et al.* High abundance synovial fluid proteome: distinct profiles in health and osteoarthritis. *Arthritis research & therapy*, 9, R36 (2007).

136. Nielen MM, van Schaardenburg D, Reesink HW *et al.* Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. *Arthritis Rheum*, 50(2), 380-386 (2004).

137. Anzilotti C, Pratesi F, Tommasi C, Migliorini P. Peptidylarginine deiminase 4 and citrullination in health and disease. *Autoimmun Rev*, 9(3), 158-160 (2010).

138. Juarez M, Bang H, Hammar F *et al.* Identification of novel antiacetylated vimentin antibodies in patients with early inflammatory arthritis. *Ann Rheum Dis*, 75(6), 1099-1107 (2016).

139. Shi J, Knevel R, Suwannalai P *et al.* Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. *Proc Natl Acad Sci U S A*, 108(42), 17372-17377 (2011).

140. Lundstrom SL, Fernandes-Cerqueira C, Ytterberg AJ *et al.* IgG antibodies to cyclic citrullinated peptides exhibit profiles specific in terms of IgG subclasses, Fc-glycans and a fab-Peptide sequence. *PLoS One*, 9(11), e113924 (2014).

141. Baillet A, Trocme C, Berthier S *et al.* Synovial fluid proteomic fingerprint: S100A8, S100A9 and S100A12 proteins discriminate rheumatoid arthritis from other inflammatory joint diseases. *Rheumatology (Oxford)*, 49(4), 671-682 (2010).

142. Maksymowych WP, Marotta A. 14-3-3eta: a novel biomarker platform for rheumatoid arthritis. *Clinical and experimental rheumatology*, 32(5 Suppl 85), S-35-39 (2014).

143. Nordal HH, Brokstad KA, Solheim M, Halse AK, Kvien TK, Hammer HB. Calprotectin (S100A8/A9) has the strongest association with ultrasound-detected synovitis and predicts response to biologic treatment: results from a longitudinal study of patients with established rheumatoid arthritis. *Arthritis Res Ther*, 19(1), 3 (2017).

144. Liao H, Wu J, Kuhn E *et al.* Use of mass spectrometry to identify protein biomarkers of disease severity in the synovial fluid and serum of patients with rheumatoid arthritis. *Arthritis and rheumatism*, 50(12), 3792-3803 (2004).

145. Tabushi Y, Nakanishi T, Takeuchi T *et al.* Detection of citrullinated proteins in synovial fluids derived from patients with rheumatoid arthritis by proteomics-based analysis. *Ann Clin Biochem*, 45(Pt 4), 413-417 (2008).

146. Raijmakers R, van Beers JJ, El-Azzouny M *et al.* Elevated levels of fibrinogen-derived endogenous citrullinated peptides in synovial fluid of rheumatoid arthritis patients. *Arthritis research & therapy*, 14(3), R114 (2012).

147. Sohn DH, Rhodes C, Onuma K *et al.* Local Joint inflammation and histone citrullination in a murine model of the transition from preclinical autoimmunity to inflammatory arthritis. *Arthritis Rheumatol*, 67(11), 2877-2887 (2015).

148. Meng X, Ezzati P, Smolik I, Bernstein CN, Hitchon CA, El-Gabalawy HS. Characterization of Autoantigens Targeted by Anti-Citrullinated Protein Antibodies In Vivo: Prominent Role for Epitopes Derived from Histone 4 Proteins. *PLoS One*, 11(10), e0165501 (2016).

149. Bennike TB, Barnaby O, Steen H, Stensballe A. Characterization of the porcine synovial fluid proteome and a comparison to the plasma proteome. *Data Brief*, 5, 241-247 (2015).

150. Peffers M, Liu X, Clegg P. Transcriptomic signatures in cartilage ageing. *Arthritis research & therapy*, 15(4), R98 (2013).

151. Goodrich LR, Nixon AJ. Medical treatment of osteoarthritis in the horse - a review. *Veterinary journal (London, England : 1997)*, 171(1), 51-69 (2006).

152. Innes JF, Clegg P. Comparative rheumatology: what can be learnt from naturally occurring musculoskeletal disorders in domestic animals? *Rheumatology (Oxford)*, 49(6), 1030-1039 (2010).

153. McIlwraith CW, Frisbie DD, Kawcak CE. The horse as a model of naturally occurring osteoarthritis. *Bone & joint research*, 1(11), 297-309 (2012).

154. Flowers SA, Kalamajski S, Ali L *et al.* Cartilage oligomeric matrix protein forms protein complexes with synovial lubricin via non-covalent and covalent interactions. *Osteoarthritis Cartilage*, 25(9), 1496-1504 (2017).

155. Hafkenscheid L, Bondt A, Scherer HU *et al.* Structural Analysis of Variable Domain Glycosylation of Anti-Citrullinated Protein Antibodies in Rheumatoid Arthritis Reveals the Presence of Highly Sialylated Glycans. *Mol Cell Proteomics*, 16(2), 278-287 (2017).

156. Bhattacharjee M, Balakrishnan L, Renuse S *et al.* Synovial fluid proteome in rheumatoid arthritis. *Clinical proteomics*, 13, 12 (2016).

157. Rombouts Y, Willemze A, van Beers JJ *et al.* Extensive glycosylation of ACPA-IgG variable domains modulates binding to citrullinated antigens in rheumatoid arthritis. *Annals of the rheumatic diseases*, 75(3), 578-585 (2016).

158. Noh R, Park SG, Ju JH *et al.* Comparative proteomic analyses of synovial fluids and serums from rheumatoid arthritis patients. *J Microbiol Biotechnol*, 24(1), 119-126 (2014).

159. Biswas S, Sharma S, Saroha A *et al.* Identification of novel autoantigen in the synovial fluid of rheumatoid arthritis patients using an immunoproteomics approach. *PLoS One*, 8(2), e56246 (2013).

160. Gibson DS, Blelock S, Brockbank S *et al.* Proteomic analysis of recurrent joint inflammation in juvenile idiopathic arthritis. *Journal of proteome research*, 5(8), 1988-1995 (2006).

161. Agarwal S, Misra R, Aggarwal A. Interleukin 17 levels are increased in juvenile idiopathic arthritis synovial fluid and induce synovial fibroblasts to produce proinflammatory cytokines and matrix metalloproteinases. *The Journal of rheumatology*, 35(3), 515-519 (2008).

162. Gibson DS, Finnegan S, Jordan G *et al.* Stratification and Monitoring of Juvenile Idiopathic Arthritis Patients by Synovial Proteome Analysis. *Journal of proteome research*, 8, 5601-5609 (2009).

163. Rosenkranz ME, Wilson DC, Marinov AD *et al.* Synovial fluid proteins differentiate between the subtypes of juvenile idiopathic arthritis. *Arthritis and rheumatism*, 62(6), 1813-1823 (2010).

164. Gibson DS, Newell K, Evans AN *et al.* Vitamin D binding protein isoforms as candidate predictors of disease extension in childhood arthritis. *Journal of proteomics*, 75(17), 5479-5492 (2012).

165. Brescia AC, Simonds MM, Sullivan KE, Rose CD. Secretion of pro-inflammatory cytokines and chemokines and loss of regulatory signals by fibroblast-like synoviocytes in juvenile idiopathic arthritis. *Proteomics. Clinical applications*, 11(5-6) (2017).

166. Mor-Vaknin N, Kappes F, Dick AE *et al.* DEK in the synovium of patients with juvenile idiopathic arthritis: characterization of DEK antibodies and posttranslational modification of the DEK autoantigen. *Arthritis and rheumatism*, 63(2), 556-567 (2011).

167. Stolwijk C, Boonen A, van Tubergen A, Reveille JD. Epidemiology of spondyloarthritis. *Rheum Dis Clin North Am*, 38(3), 441-476 (2012).

168. Menon B, Gullick NJ, Walter GJ *et al.* Interleukin-17+CD8+ T cells are enriched in the joints of patients with psoriatic arthritis and correlate with disease activity and joint damage progression. *Arthritis Rheumatol*, 66(5), 1272-1281 (2014).

169. Myers WA, Gottlieb AB, Mease P. Psoriasis and psoriatic arthritis: clinical features and disease mechanisms. *Clin Dermatol*, 24(5), 438-447 (2006).

170. Lemos LL, de Oliveira Costa J, Almeida AM *et al.* Treatment of psoriatic arthritis with anti-TNF agents: a systematic review and meta-analysis of efficacy, effectiveness and safety. *Rheumatol Int*, 34(10), 1345-1360 (2014).

171. Ademowo OS, Hernandez B, Collins E *et al.* Discovery and confirmation of a protein biomarker panel with potential to predict response to biological therapy in psoriatic arthritis. *Annals of the rheumatic diseases*, 75(1), 234-241 (2016).

172. Collins ES, Butt AQ, Gibson DS *et al.* A clinically based protein discovery strategy to identify potential biomarkers of response to anti-TNF-alpha treatment of psoriatic arthritis. *Proteomics. Clinical applications*, 10(6), 645-662 (2016).

173. Chang X, Cui Y, Zong M *et al.* Identification of proteins with increased expression in rheumatoid arthritis synovial tissues. *The Journal of rheumatology*, 36(5), 872-880 (2009).

174. Cretu D, Prassas I, Saraon P *et al.* Identification of psoriatic arthritis mediators in synovial fluid by quantitative mass spectrometry. *Clinical proteomics*, 11(1), 27 (2014).

175. Cavill R, Jennen D, Kleinjans J, Briede JJ. Transcriptomic and metabolomic data integration. *Brief Bioinform*, 17(5), 891-901 (2016).

176. Eichner J, Rosenbaum L, Wrzodek C, Häring H-U, Zell A, Lehmann R. Integrated enrichment analysis and pathway-centered visualization of metabolomics, proteomics, transcriptomics, and genomics data by using the InCroMAP software. *Journal of Chromatography B*, 966, 77-82 (2014).

177. García-Alcalde F, García-López F, Dopazo J, Conesa A. Paintomics: a web based tool for the joint visualization of transcriptomics and metabolomics data. *Bioinformatics (Oxford, England)*, 27, 137-139 (2011).

178. van Iersel MP, Kelder T, Pico AR *et al.* Presenting and exploring biological pathways with PathVisio. *BMC bioinformatics*, 9, 399 (2008).

179. Xia J, Fjell CD, Mayer ML, Pena OM, Wishart DS, Hancock REW. INMEX--a web-based tool for integrative meta-analysis of expression data. *Nucleic acids research*, 41, W63-70 (2013).

180. Scumaci D, Oliva A, Concolino A *et al.* Integration of “Omics” Strategies for Biomarkers Discovery and for the Elucidation of Molecular Mechanisms Underlying Brugada Syndrome. *PROTEOMICS - Clinical Applications*, 1800065 (2018).

181. Grauw JC, Lest CHA, Weeren R, Brommer H, Brama PAJ. Arthrogenic lameness of the fetlock: synovial fluid markers of inflammation and cartilage turnover in relation to clinical joint pain. *Equine Veterinary Journal*, 38, 305-311 (2010).

182. Isola M, Ferrari V, Miolo A *et al.* Nerve growth factor concentrations in the synovial fluid from healthy dogs and dogs with secondary osteoarthritis. *Veterinary and Comparative Orthopaedics and Traumatology*, 24, 279-284 (2011).