

REVIEW

The human synovial fluid proteome: A key factor in the pathology of joint disease

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This review aims to summarise our knowledge to date on the protein complement of the synovial fluid (SF). The tissues, structure and pathophysiology of the synovial joint are briefly described. The salient features of the SF proteome, how it is composed and the influence of arthritic disease are highlighted and discussed. The concentrations of proteins that have been detected and quantified in SF are drawn together from the literature on osteoarthritis, rheumatoid arthritis and juvenile idiopathic arthritis. The measurements are plotted to give a perspective on the dynamic range of protein levels within the SF. Approaches to proteomic analysis of SF to date are discussed along with their findings. From the recent literature reviewed within, it is becoming increasingly clear that analysis of the SF proteome as a whole, could deliver the most valuable differential diagnostic fingerprints of a number of arthritic disorders. Further development of proteomic platforms could characterise prognostic profiles to improve the clinician's ability to resolve unremitting disease by existing and novel therapeutics.

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1 The synovial joint triad: SF, synovial membrane, articular cartilage

In order to study the joint disease we need to realise the importance of the interplay between three components: Synovial fluid (SF), synovial membrane (SM) and articular cartilage (see Fig. 1A). In what follows, we use the term 'synovial fluid' to describe the protein components that are soluble in the synovial joint. A number of body fluids are derived from or 'dialysates' of plasma, and thus share some

of its protein content. SF, cerebrospinal fluid and urine feature within this group. SF contains a number of specific additions made from proximal joint tissue, including SM or articular cartilage. SF not only lubricates the bearing surfaces of the joints, but also permits the transport of nutrients from SM to joint cartilage and waste products to the lymph.

The SM, in healthy conditions, is comprised of a layer of cells one to three cells deep (see Fig. 1B). These cells, which are in direct contact with the joint cavity, are mainly macrophage and fibroblast like [1, 2]. The cells are imbedded in a collagen and hyaluronan-rich matrix [3]. The SM is unique compared to the linings of other body cavities as it lacks a true basement membrane; however, this intimal matrix acts as a semipermeable membrane. Furthermore, the superficial capillary network is also fenestrated which may be important in the egression of vascular components beyond the intimal tissue and into the SF [4]. The surface of diarthrodial joints is covered by articular cartilage which is essentially avascular. Hyaline (articular) cartilage is composed of a small number of chondrocytes, which produce, and are imbedded in an extracellular matrix of collagen fibrils and proteoglycans.

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Abbreviations: HA, hyaluronan; MMP, matrix metalloproteinase; OA, osteoarthritis; PMN, polymorphonuclear; RA, rheumatoid arthritis; SF, synovial fluid; SM, synovial membrane; TNF α , tumour necrosis factor alpha

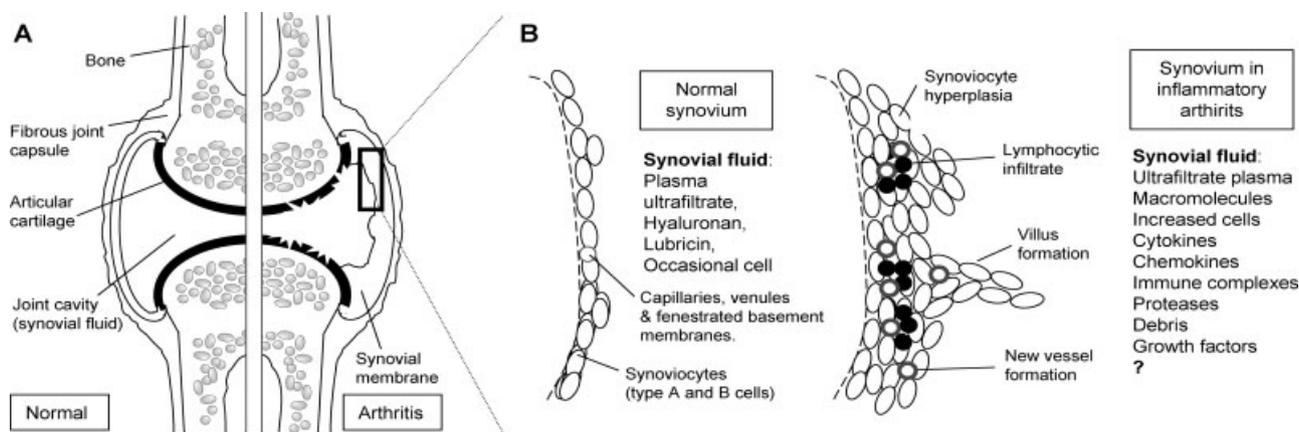


Figure 1. Diagram to represent the structure and pathology of the synovial joint. A comparison is made between the normal and arthritic joint to highlight the main points of protein entry into the SF in disease (A). The arthritic synovial joint is characterised by inflammation and thickening of the SM and a consequent influx of lymphocytes and macrophages through a revascularised SM. In established disease, the proinflammatory environment of SF can stimulate degradation of the articular cartilage, through activated cells. A schematic representation of normal SM in health and the changes that occur in the membrane during inflammation and the subsequent changes observed in the SF are noted (B).

2 Sources of SF components

Normally plasma proteins can enter the SF by passive diffusion. Ropes and Bauer [5, 6] in their classic studies of synovial effusions, equated SF to a dialysate of blood plasma. They found a wide assortment of electrolytes and small molecules present in the effusions at concentrations similar to those of plasma. Concentrations of larger proteins remain substantially less in SF than in plasma (see Table 1). In aspirates from normal knees, the total protein is only 19 mg/mL, a value less than 30% of that in normal plasma [7]. The intrasynovial concentration of any protein represents the net contributions of plasma concentration, synovial blood flow, microvascular permeability and lymphatic removal. However, a number of other properties predict whether a molecule can enter the joint fluid, including; molecular weight, charge, solubility and protein binding [8].

Hyaluronan (HA), a high molecular weight glycosaminoglycan, is secreted into the dialysate from B-type synovium cells. HA is the major macromolecular constituent of SF and it forms a complex with the protein fraction of the dialysate. HA performs a protective role in the SF of articulating joints, the vitreous humour of the eye and in most connective tissues [9].

Until relatively recently it was believed that almost all constituents of SF were obtained solely from plasma. However, with the development of sensitive techniques, it is evident that many of the constituents of SF present in disease states are produced locally. These include: cytokines [10], proteases [11] and antibodies such as rheumatoid factor [12]. These molecules are produced by a variety of cells within the SM including Type A synoviocytes, fibroblasts and infiltrating inflammatory cells. In healthy conditions, the cellular component of SF is low, with the occasional secretion from

synoviocyte, chondrocyte, neutrophil and lymphocyte. In chronic conditions such as osteoarthritis (OA), or chronic inflammatory diseases such as rheumatoid arthritis (RA) and juvenile idiopathic arthritis the primary infiltrates are lymphocytes and plasma cells [13]. The homogeneity of the SM infiltrate in an individual patient enables the use of synovial biopsies to evaluate disease severity and response to treatment [14]. The predominant T cell subtype infiltrate in most chronic arthritides are T helper (CD4+) cells [15], although in the spondyloarthropathies CD8+, suppressor/cytotoxic cell appear to predominate [16].

3 SF proteins quantified by conventional nonglobal proteomic analyses

The following section summarises our knowledge, from the literature to date, of proteins identified within SF by conventional, nonglobal proteomic methods. These methods are limited, by definition, in their ability to study more than one or two proteins at a time within an experiment. In other words they do not possess multiplex or high throughput capabilities characteristic of modern proteomic techniques. Typical examples of antibody-based methods used to study SF include ELISA, Western blotting and immunoprecipitation. Although each of these methods has its limitations for studying SF in a complex multifactorial disease such as arthritis, they have provided invaluable information on individual mechanisms involved in joint development and disease. Indeed, as medical research shifts focus towards the principles of integrated systems biology, the more traditional methods will remain important in validation and can be used in a complementary fashion to further explore large proteomic data sets.

Table 1. Concentrations of the most abundant proteins in SF and blood

	Molecular weight (kDa)	SF			Serum/plasma		
		Normal	OA	RA	Normal	OA	RA
Hyaluronan	2×10^6	3.2	1.5	0.17–2.0	–	–	–
Fibrinogen	340	–	0.15–2.10	0.25–0.55	3.0	–	7.0
Albumin	69	12.0–20.0	11.0–34.0	8.0–35.0	32.7	26.0	29.2–44.0
Haptoglobin	100	0.1	0.2	0.9–4.0	1.8	2.0	2.4–6.0
Ig G	156	–	4.0–30.0	10.3–11.0	–	4.0–12.0	11.0–20.1
TOTAL PROTEIN	–	19.0	40.0–68.0	38.0–84.0	67.7	–	70.0

Concentrations are in units of ng/mL; the approximate molecular weights of each protein are also shown. The data compiled from the literature [4, 40, 93–95] are also plotted alongside the concentration intervals for less abundant proteins in the arthritic SF in Fig. 2.

In the interests of highlighting only those proteins with relevance to clinical outcome, a list was compiled from studies with expression levels correlated to defined pathological processes. Proteins are categorised by their involvement in various established aspects of joint pathology. These categories include inflammation, degradation, cell migration/proliferation, angiogenesis and immunobiology (see Fig. 2). Priority, when compiling the data, was thus given to literature which gave detailed concentrations of proteins in units of mass *per* unit volume and these were standardised to nanogram *per* millilitre for comparative purposes. This approach permits a more authoritative view of the dynamic range of protein levels in the SF. It is important to note that data pertaining to how serum or plasma levels relate to those in the SF were not always present. These data are essential if we are to rely on biological assay of indicators of joint disease in blood in order to make clinically relevant decisions. Though blood is more easily accessed, it would be careless to preclude simultaneous analysis of both fluids at the current juncture in biomarker discovery as it is crucial to have proof of correlation between the two fluids.

It is important to appreciate the stages in the pathology of arthritis which explain in part why these classes of proteins are found at concentrations beyond the normal ranges for SF and even match plasma/serum. The onset of an arthritic disorder is heralded by two fundamental signs of inflammation. In the first of these, SM which encapsulates and nourishes the joint, becomes swollen and thickens (synovitis) due to lymphocytes becoming bound to (lymphostasis) and migrating beyond the synovial blood vessels (Fig. 1B). Hyperplasia, or thickening of the membrane, is promoted by cytokine and growth factor release from migrating cells. During the second component of inflammation, the SM becomes revascularised, making it redder than normal [17–21]. This angiogenic process is initiated by a higher oxygen demand from increases in cell numbers within the SM and hypoxia imposed by lymphocyte engorged arterioles. The revascularisation is altered, however, by the proinflammatory cytokine-enriched environs (IL-1 α , IL-6, tumour necrosis factor alpha (TNF α)) and results in aberrant growth of tortuous, permeable vessels which

propagate beyond the confines of the synovium. This ‘pannus’ impinges on the cartilage and can result in irreparable degradation of the articular surfaces. In describing the early stages of arthritic pathology, points of entry for proteins not normally present in the SF should have become apparent: abnormal blood vessels, the swollen SM and leukocyte cells which penetrate within it.

The main factors limiting protein entry into the SF from capillaries are the number and size of fenestrations. Increased blood flow and vessel permeability means that proteins which are normally excluded enter now, such that virtually all plasma proteins will reach equivalent concentrations within the joint. Molecular size no longer acts as a limiting factor in what can enter or leave the inflamed joint. This change in filtration is evident from the increased concentrations of higher molecular weight proteins by comparison to the normal joint, such as haptoglobin (85 kDa), Ig G (150 kDa) and fibrinogen (340 kDa, in complex). See Table 1 for the expected ranges of each of the most abundant proteins in both SF and plasma from normal and arthritic patients. In general smaller protein molecules such as albumin are present in normal joints at greater concentrations than larger molecules such as the Igs. The concentrations of cytokines such as IL-1, IL-6 and TNF α albeit on the picogram scale often outstrip plasma levels [22–26]. This clearly indicates the expression localised within or proximal to the joint fluid and thus it is likely that such indicators will be detected in SF at an earlier stage of the disease, than in plasma. Many associated regulatory molecules have also been detected including receptor agonists and soluble receptors shed from leukocytes, macrophages and other invaded peripheral blood cells.

As may be expected, the concentration of a great number of proinflammatory cytokines and acute phase markers derived from synovium and migrating leukocytes increase in SF, in line with the degree of inflammation [24, 25, 27, 28]. Those included within this review include interleukin-1 β , interleukin-6, C reactive protein, serum amyloid A and the recently discovered group of S100 proteins [29, 30]. It is timely to remind at this point that we are only glimpsing a ‘snapshot’ in the timeline of the disease process, which is in

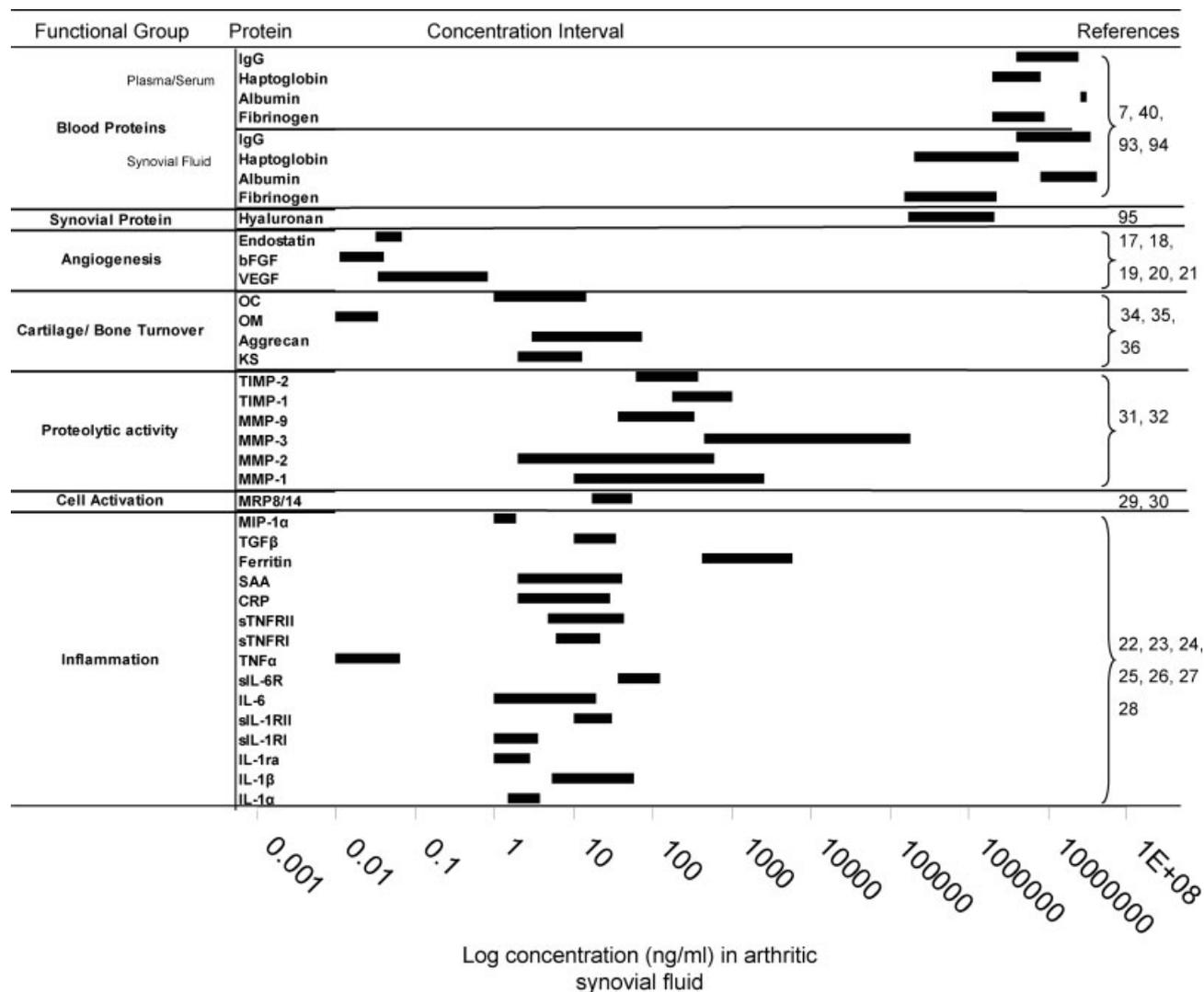


Figure 2. The concentration intervals of synovial proteins in the arthritic joint. Concentrations of several functional classes were compiled from the literature. The data were normalised to nanogram *per* millilitre to allow the comparison across a log scale of protein concentration. The dynamic range extends from mg/mL concentrations for abundant proteins such as albumin and IgG, to pg/mL for scarce proteins, *i.e.* proinflammatory protein TNF α .

constant flux. Therefore samples obtained in a serial fashion, from diagnosis, through early to established disease are invaluable if we wish to detect putative prognostic biomarkers.

As the rate of joint destruction increases proteolytic enzymes (matrix metalloproteinase; MMPs) [26, 31–33] and the peptide products of their degradation, cartilage fragments [26, 34–36], become apparent within the SF. Cytokines such as TNF α and interleukins are released within the synovial blood vessels which induce the expression of intercellular adhesion molecules such as ICAM and E-cadherin. These extracellular proteins are thought to promote the accumulation of activated T cells and soluble forms have been detected in SF [37].

3.1 Limitations of methods of analysis used to date

It is clear from the previous section that many factors are involved in the initiation and the perpetuation of joint inflammation as well as joint destruction and repair. The analysis of single or even groups of mediators is thus unlikely to be as informative as the analysis of a large number of proteins and peptides. Thus to understand what causes joint damage in arthritis we need to look at all the mediators of inflammation, anti-inflammation, destruction and repair.

In the last few decades, theories on the predictors and mechanisms of disease have followed a common trend, whether it be T cells, cytokines, fibroblasts, proteases or osteoclasts. What is clear, however, is that no single agent or

process is wholly responsible rather there is an interaction of these processes leading to a protein footprint that characterises a particular inflammatory insult to the joint. For that reason proteomics is an ideal platform to examine the processes active in arthritis. For a proteomic approach, however, to be successful two conditions are essential:

(i) Expertise in the handling, processing and proteomic analysis of samples.

(ii) Expertise in the classification, and clinical examination of patients to be studied.

4 Considerations and technical challenges of SF proteomics

4.1 Sample characteristics and processing

Paracelsus (1493–1541) introduced the term 'synovia' to name the intra-articular fluid, due to its egg-like appearance and consistency [38]. SF is normally clear, pale and straw coloured in the healthy joint and has a characteristic viscosity attributable to the HA component. SF is only present in small amounts within the normal knee joint (a few drops to a maximum of 4 mL) [39]. The volume of this fluid increases when disease is present to provide an effusion that is clinically apparent and may be easily aspirated for study, hence most knowledge of human SF comes from patients with joint disease. Because of the clinical frequency, volume, and accessibility of knee effusions, our knowledge is largely limited to findings in that joint. Osteoarthritic SF retains the normal colour, clarity and high viscosity but is increased in amount. SF from RA is increased in amount, can be cloudy from suspended cells, less viscous and may clot on standing due to a higher fibrinogen content [40]. The mean molecular weight distribution of HA in RA SF is moderately lower than that of normal fluid [9]. The total amounts of HA actually increase, so pathological fluids are characterised by the dilution of HA and not by the lack of high molecular weight forms. In the context of the physical attributes of SF, this translates into a fluid viscosity which is easier to pipette or aliquot than normal joint fluid. In view of the high viscosity of SF samples, hyaluronidase treatment can be used to facilitate LC techniques such as size fractionation or affinity depletion [41].

Normal SF does not clot as it does not contain sufficient fibrinogen, though inflammation increases the ingress of larger clotting components from the peripheral blood supply. Therefore pathological fluids can clot, such that the speed and size of clot formation relate to the severity of joint inflammation. The recovery of SF does not normally require anticoagulants, though samples can be collected in K-EDTA blood tubes for convenience [42]. Clots can form if samples are left for over 1 h at room temperature, prior to centrifugation, though this will compromise the stability of the SF proteome. Storage and handling of SF should follow similar guidelines agreed by the Plasma Proteome Initiative

at the most recent HUPO congress. Briefly, cellular material should be removed from samples by centrifugation, aliquots made and stored at -80°C . Protease inhibitors can be added to the sample to protect against degradation during purification, prefractionation or IPG strip rehydration for 2-DE [42, 43]. It should be noted, however, that some of the peptide components such as leupeptin in inhibitor cocktails can complicate mass spectrometric identifications.

If a fluid is opaque, yellow-white and of reduced viscosity (low HA concentration), a high total leukocyte count with polymorphonuclear (PMN) cell dominance is the most likely cause. In general, small lymphocytes, monocytes and macrophages (or synovial lining cells) make up the remainder of the leukocytes, and some of each can be found in most of the joint fluids. Normal human SF contains few leukocytes, and PMN cells are difficult or even impossible to find [39]. The total cell count can be used to gauge the activity of the inflammatory disease within the joint. The normal SF has on average a count of 150 cells/mL, whereas OA and RA have average counts of 400 and 10 000, respectively. In the case of OA, clarity may decrease due to the presence of cartilage fragments as the condition progresses.

The SF proteome, as a partial eluent of plasma, is likely to have similar physical attributes which determine the efficacy of a particular analytical approach. SF is slightly alkaline with a pH of approximately 7.3–7.4, which falls during muscular activity. The normal total protein content of SF is approximately 19 mg/mL [7]; normal plasma is 67.7 mg/mL; in arthritic disease the synovial proteome can equate from 38.0 to 84.0 mg/mL [40]. See Table 1 for concentrations of the most prevalent proteins in both fluid types. A number of proteins including albumin, Ig, haptoglobin, fibrinogen and the glycoprotein HA have been detected at high milligram *per* millilitre concentrations within SF. Albumin is the most abundant protein in SF, accounting for ~64% of total protein in normal SF. Immunglobulin levels are normally low in SF, but levels in RA patients become equivalent to those of serum on average 9.5 mg/mL (see Table 1).

The dynamic range between potentially clinically useful proteins can differ by a factor of 10^{10} . For example, $\text{TNF}\alpha$ and haptoglobin have been detected at picogram and milligram *per* millilitre quantities, respectively, close to the detection limits of existing antibody-based affinity methods (see Fig. 2). Further heterogeneity is introduced to various plasma and SF proteins by glycosylation, evident for example among haptoglobins and Igs (see Fig. 3).

To reduce the dynamic range of the SF and enrich lower abundance proteins, serum albumin and Igs can be removed by affinity depletion columns [41]. Although high concentrations of IgGs and albumin may physically mask less abundant proteins with similar *pI* and molecular weight coordinates in 2-DE, depletion columns may also bind proteins in a nonspecific manner [44]. Whether bound directly to a column or indirectly through secondary binding to Igs, and particularly to albumin, depletion should only be used when whole sample integrity is not required.

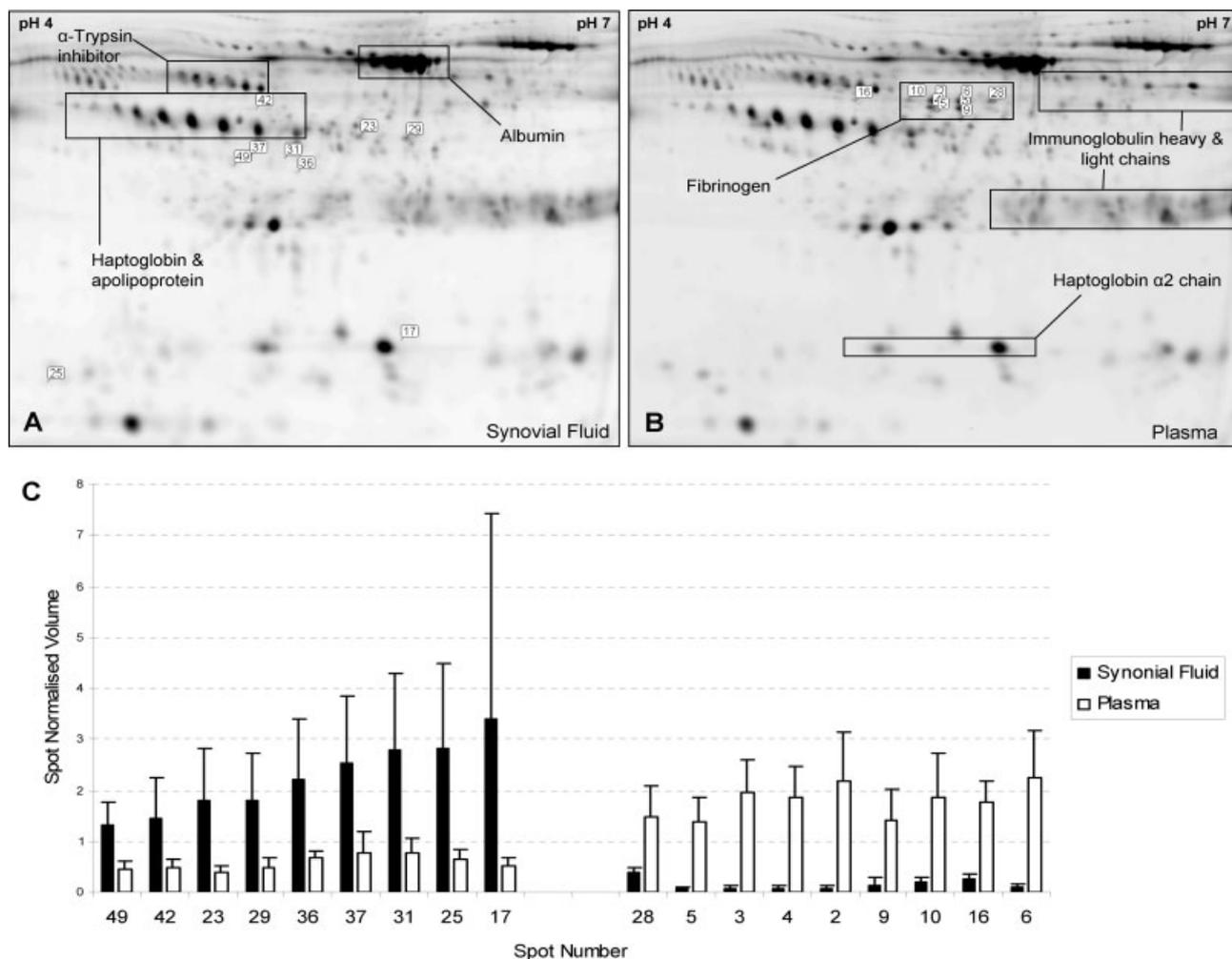


Figure 3. Fluorescence differential in gel electrophoresis (DIGE) of SF and plasma from juvenile idiopathic arthritis (JIA) patients. Briefly, ten paired SF and plasma samples were individually labelled with Cy 5 and Cy 3 fluorophores. An internal standard of all 20 samples was pooled and labelled with Cy 2. 50 µg of each sample pair and internal standard were combined and loaded onto pH 4–7 IPG strips. After IEF in the first dimension, proteins were separated in the second dimension by 12% PAGE. Gels were imaged on a laser scanner with spot matching, normalisation and quantification were processed by a dedicated software package. Nine synovial ‘specific’ (A) and plasma specific (B) proteins are circled and numbered. A number of distinctive protein spot trains are highlighted (boxes), including albumin, Igs and haptoglobin. Identities were made by inference to data published in ExPASy 2-DE protein maps and literature (<http://expasy.org/ch2d/>) [42, 92]. The normalised intensity of each protein across 10 JIA patients is plotted as a bar chart (C) such that black and white bars represent the mean normalised protein spot intensities for SF and plasma, respectively (error bars represent SD).

‘Albuminomics’ itself is a burgeoning offshoot of this very depletion strategy, where research can be conducted on retained depletion columns to investigate the variety of peptides that are bound to this carrier molecule [45]. The wide dynamic range of the synovial proteome precludes the analysis of low abundance, lower molecular weight protein or peptide species by MS unless the sample is prefractionated. Intact and partially degraded proteins or peptide fragments are more likely to leach into the circulating plasma [46]. Low molecular weight components can be enriched by size exclusion or RP chromatography [41, 47]. Characteristics of published and several new proteomic technologies applicable to arthritis research are reviewed in the literature [48–50].

5 Approaches and findings from global proteomic analyses

5.1 SF analysis

A number of the recent technological advances in high throughput protein separation and identification have already been applied to the analysis of the synovial proteome. This section tracks the progress made to date, starting with classic 2-DE. Early on in the history of body fluid proteomics, Felgenhauer and Hagedorn suggested the 2-DE technique to screen complex soluble protein mixtures for pathological components. Despite the technological restrictions of the

time, they were able to identify the SF as a filtrate of serum due to the resemblance of separated protein patterns between the two body fluids [51]. The authors propose that the serum-derived fraction of a body fluid, such as SF, depends on the selectivity of the respective blood–body fluid barrier. The qualitative feature of the protein ‘maps’ derived by 2-DE remains one of its unique strengths, allowing the identification of unique or differentially expressed proteins and their PTMs. Scott *et al.* some years later detected a high molecular weight fibrin-complex species in RA SF by 2-DE immunoblots. The fibronectin complexes were able to remove Ig complexes from SF *in vitro* and thought to assist in phagocytosis [52]. Further study by 2-DE revealed that a number of fibronectin complexes were present in SF, whereas only a single fibronectin component was present in paired serum from RA patients [53]. SF from osteoarthritic patients, analysed in a later series of 2-DE immunoblot-based investigations, was found to contain EIIIA sequences which can promote catabolic responses in chondrocytes [54, 55].

The reproducibility of 2-DE as an analysis technique was first tested extensively on SF and paired serum and synovial tissue by Fritz *et al.* [56], with repeat runs giving consistent protein spot patterns. They found though, that the number of proteins in SF (45) was always lower than in matched serum (50) or tissue (69). Spot numbers were so low due to the poor resolution and dynamic range attributes of the custom micro 2-DE system employed, but the group were the first to successfully separate soluble protein from the synovial tissue and developed their ideas further to try and characterise the histology of cryostat sections [57]. The only difference in spot pattern noted between RA and non-RA patients was the increased density of the IgG spots [56]. In spite of limited sensitivity, these investigations demonstrated that an additional validity and clarity could be added by the introduction of adjacent tissues such as SM, cartilage and bone, such that the origin of protein expression can be established. The ability to localise protein expression could help in the development of more effective therapeutics, directed to specific areas of the joint depending on the individual patient’s state of disease. Sera and SF from RA patients have also been evaluated for anti-HLA class II beta-chain antibodies using single and 2-D immunoblots [58]. The antibodies from RA sera and SFs which reacted with class II beta-chain determinants were predominantly IgM and IgA with minimal IgG. Thus determinants of autoimmunity, a characteristic of established arthritic disease, can also be investigated in the SF of inflamed joints.

Since these preliminary proteomic studies, vast improvements have been made in the various components required for sensitive and high resolution 2-DE. Advances include the manufacture of IPGs for more precise IEF, a new generation of fluorescent stains and software-based spot recognition and analysis, all of which have increased the sensitivity and reliability of the 2-DE technique. The advent of soft ionisation of peptides from protein digests also justified the application of MS to identify spots cut from gels.

Sinz *et al.* [42] used an adaptation of this ionisation known as MALDI PMF in conjunction with high capacity 2-DE, where up to 900 µg of the SF protein was loaded *per* IPG strip. Paired SF and plasma samples were separated over a pH 4–7 range, with approximately 300 spots *per* gel visualised by coomassie staining. Among the 150 proteins identified, a number of differentially expressed proteins were detected in the plasma and SF of RA patients, but not in OA patients. Calgranulin B, or migration inhibitory factor-related protein-14 (MRP14), was identified exclusively in five out of six SF samples from RA [42]. This molecule could act as a marker of activated phagocytes recruited to the inflamed joint as these initial findings have been validated through a number of other studies [41, 59]. Interestingly, calgranulin A (MRP8), which forms complexes with and is coexpressed with MRP14, could not be identified in any of the gels. Calgranulin C (MRP12) detected using gels with a higher resolving power may contribute to leukocyte migration into the chronically inflamed joint and has been detected in SF by other groups [60]. Plasmin derived, fibrinogen beta chain degradation products detected in the joint are thought to result from an imbalance between fibrin formation and degradation and are indicative of a proteolytic environment. Serum amyloid A, which may have detrimental effects in chronic inflammation evident in established rheumatic disease, was found in the low molecular mass region of gels in agreement with Doherty *et al.* [61]. The calcium binding protein MRP14 (S100A9) was further characterised in SF as a disease-associated discriminatory marker by Drynda *et al.* [59]. Results were confirmed by ELISA measurements of the MRP8/14 complex and demonstrated that plasma levels correlate well with those in SF. Therefore, a blood test may be able to distinguish RA patients from a range of other inflammatory disorders including OA. Furthermore, hetero-complex levels are reduced in response to anti-TNF α treatment, showing promise as a molecular monitor on therapeutic status [59].

Evidence concerning the reliability of modern 2-D gel techniques to analyse SF has been recently published, which should encourage continued use of this method. In an investigation of intrarun variability, correlation coefficients of 0.89 or higher were achieved for triplicate runs of two samples. Variables such as protein loading, pH range for IPG strips, IEF conditions and albumin or globulin depletion were tested and optimised [44]. An 8–16% gradient gel provides a better separation than linear gels and resolution greatest with 50 µg of SF protein. Most of the proteins had a *pI* between 4 and 7 and apparent molecular weight less than 70 kDa. One hundred and thirty-nine valid protein spots were visualised, 18 of which differed five-fold and nine spots differed more than 100-fold among themselves, which were differentially expressed across individual OA patients. MS was not used in this instance, but instead identities were inferred from Expasy 2-D plasma maps (<http://expasy.org/ch2d/>). The authors claim a sensitivity of 1–2 ng, increased by SYPRO ruby fluorescent post-run gel stain. A further

study by the same group used two software packages and performed trail analyses, focussing on the efficiency of attributes such as spot detection in SF [62]. We have used the 2-DE technique to characterise the SF specific proteins which distinguish juvenile arthritis patients with recurrent inflammation of the knee joint [43]. Fragments of the T-cell receptor and collagen X were identified in SFs of children with persistent inflammatory episodes. Further studies using more sensitive fluorescence difference in gel electrophoresis (DIGE) have revealed interesting distinctions between the local and systemic molecular signatures of juvenile arthritis subgroups (Fig. 3, manuscripts in preparation). The most predominant proteins can be easily distinguished in distinct spot trains and it is clear that the majority of fibrinogen remains exclusively in the blood (Figs. 3A and B). A number of proteins with synovial 'specific' expression are highlighted and quantified to demonstrate the ability to reliably differentiate molecular fingerprints of local and systemic disease across patient groups by this gel-based approach (Fig. 3A and C).

Aside from gel electrophoresis, several other separation methods have been used to process SF in the liquid phase prior to MS. A number of small molecules including oligosaccharides from glycosaminoglycan digests, estrogens and androgens, and bacteria-derived muramic acid have been detected in SF pre-MS by liquid and GC separations [63–65]. A recent study adopted a two dimensional liquid chromatographic approach (LC/LC) coupled with MS/MS to generate protein profiles from prefractionated SF [66]. Selected reaction monitoring (previously known as MRM) acquires data from specific product ions which correspond to mass/charge selected precursor ions. These product ions can be recorded *via* two or more stages of MS in a temporal or spatial manner so the relative abundance of a protein can be determined across several different samples. Thirty SF proteins were selected due to their raised levels in erosive RA than non-erosive RA, including C reactive protein (detected at 49-fold higher levels in erosive patients) and the metabolic enzyme triosephosphate isomerase (TPI) [41]. Attention was focused on the S100 family of calcium binding proteins (A4, A8, A9, A11, A12 and P) and their ability to discriminate erosive from nonerosive patients was tested on pooled sera. Only S100A8, A9 and A12 were increased in erosive versus non-erosive RA patients with fold difference ranges of 3.0–6.0-fold, 9.4–14.1-fold and 4.4–11.1-fold, respectively [66].

Advances in biochip and affinity based proteomic technologies have been applied to SF in a drive towards high throughput, real time devices with near clinical application. Uchida *et al.* [67] used the ProteinChip platform to detect biomarker candidates which can distinguish between RA and OA. Surface enhanced laser desorption/ionisation MS identified myeloid related protein 8 (MRP8) among these candidates, in agreement with previously mentioned studies of RA. Results from peptide fingerprint mapping and PSD analysis were verified by enzyme immunoassay. The authors propose that the ProteinChip platform could provide clinically

useful markers, though further study is required to characterise temporal expression patterns as disease progresses. Surface plasmon resonance detection of antibody–antigen interactions in biochip format has also been applied to the study of SF autoantibodies [68]. This detection has real-time measurement qualities, can be used in a nonredundant manner and is not reliant on labelled antibodies. An inhibitor affinity extraction method has been used to extract and enrich individual MMPs from SF onto a sepharose cartridge [69]. This process was automated on an SPE workstation and the identity of each extracted MMP was confirmed by gelatin zymography. What is really significant about this approach is that the function of active molecules (*i.e.* the active enzyme only in this case) can be probed and assayed in the diseased joint. The authors claim that MMP extraction yields of approximately 98% could be achieved with as little as 13 s sample contact time with the affinity matrix [69]. In theory if the automated extraction techniques applied in this study were coupled with modern MS identification, extremely rapid sample turn around times could be achieved.

5.2 Serum/plasma analysis

A similar range of proteomic technologies have been used to examine and validate SF findings in a parallel approach to find serum/plasma based indicators of joint disease. Autoantibodies have been detected binding to chondrocyte antigenic proteins in sera separated by 2-D gel from arthritic patients [70]. Nineteen protein spots were recognised only by antibodies present within OA era. Of these, antitriosephosphate isomerase antibodies were detected in ~24% of OA patient's serum and SF samples, whereas <6% of RA and lupus patient samples were positive.

Hueber *et al.* used a custom autoantigen microarray to detect B cell derived autoantibodies which target citrullinated proteins and peptides in serum. RA patients could be stratified by cluster analysis of autoantibody levels to citrullinated or native proteins, into groups predictive of disease severity [71]. Commercially available BioChips (Ciphergen) have also been used recently to differentiate serum protein profiles indicative of response to conventional therapeutics in juvenile idiopathic arthritis patients with severe systemic onset disease [72].

The same principles of sample separation and analysis can be used to focus on molecules other than proteins in serum and SF. Phospholipids have been identified by MALDI and characterised as indicators of disease severity in the early stages of RA [73]. Furthermore, diminished disease activity in patients who display a response to anti-TNF α therapy is accompanied by corresponding increases in the ratio of phosphatidylcholine/lysophosphatidylcholine in plasma. The peptidome of SF has also been analysed by MALDI after RP HPLC fractionation to enrich the low molecular weight protein [47]. Over 500 peptide signals have been recorded in SF and over 5000 in plasma [74]. Once cleaved into low molecular weight peptides, many proteins

may have functions beyond our original perceptions. Peptide profiles could be used to differentiate disease subgroups to predict patients more likely to suffer severe disease [75].

5.3 Synovial cell and tissue proteomics

It is likely that markers of bone and cartilage destruction and synovial specific cell migration would also be useful in the clinical setting. Of course several specific subproteomes are secreted from and are contained within the proximal tissue and cells suspended in SF. The current state of proteomic research on chondrocytes, fibroblasts and cells which have migrated into the inflamed joint are therefore also briefly described.

Hulkower *et al.* utilised radioactive isotope labelling (^{32}P) to study rabbit synovial chondrocyte response to interleukin-1 alpha treatment. Phosphoproteins formed by the incorporation of the ^{32}P isotope for 30 min could be visualised by making autoradiographs of cell extracts separated by 2-DE. Interleukin-1 alpha produced marked changes in the pattern of protein phosphorylation [76], which were similar to those introduced by synovial cytokines. Activated chondrocytes have been shown to produce proteins which participate in cartilage degradation of joints [77]. A number of studies focus on the proteome of immune system cells which enter the joint *via* the inflamed synovium [78–80]. Human peripheral monocytes, sourced from SF, labelled overnight *in vitro* using ^{35}S -methionine metabolic labelling to quantify protein secretion. Culture medium conditioned by labelled PMN cells was separated by 2-DE and visualised by autoradiography. Thrombospondin secretion was confirmed by immunoprecipitation and the authors propose that abnormally high levels may indirectly induce destructive changes within the SM [79]. Serum amyloid A derived fragments (98–104) detected in rheumatic SFs by HPLC-MS are capable of stimulating interferon gamma production by CD4 positive T-lymphocytes [80].

A number of proteomic studies have focused on fibroblast-like cells, also known as synovial lining cells or synoviocytes, as they also participate in the propagation of arthritic disorders [81–85]. In the most recent study, Dasuri *et al.* [83] investigated the synovial fibroblast proteome by 2-DE MALDI-MS, identifying a number of potential autoantigens and proteins previously implicated in RA disease pathology. Other groups also used a gel-based separation and MS identification approach to investigate the mode of action of non-steroidal anti-inflammatory drugs, and to detect serine protease secretion from synovial fibroblasts isolated from arthritis patients [84].

These studies collectively demonstrate the power of investigating the cells suspended in SF as they can make specific additions to the SF proteome in joint health or disease. Synovial cell pellets should therefore be retained as a valuable resource, which contribute additional levels of perception concerning the complexity of the SF proteome and how it may be used in a clinical situation for diagnostic or prognostic measurements. Furthermore, they may present

putative targets to direct more specific antiinflammatory and antidegradative therapeutics, which could resolve disease persistence and prevent joint damage.

Proteome analysis of SM tissue could lead to further understanding of the causes of joint pathology. As the initiators of synovium inflammation and hyperplasia remain elusive, it is important to gain samples from the earliest stages of arthritic disease. Early gel based experiments were able to differentiate the histomorphology of cryostat sections as the characteristic of either exudative or proliferative synovitis and probe for autoantigens in RA [86, 87]. Modern gene array and multi-Western blot platforms have been used to simultaneously characterise transcript and protein levels in RA compared to OA in a patient's synovial tissue [88]. The authors report that changes in transcript levels did not always translate into changes in the corresponding protein levels. Protein localisation and expression levels confirmed by immunohistochemistry demonstrated that stat 1, involved in interferon signal transduction, was overexpressed in perivascular macrophages and CD3 positive T-lymphocytes in RA patients [88]. More recently, Tilleman *et al.* [89] demonstrated the ability to segregate spondyloarthropathy patients from RA and OA patient groups by hierarchical cluster analysis of differentially expression patterns derived from a 2-DE-MS investigation of inflamed synovium. Considered together, synovial tissue analysed in combination with fluids could reveal biomarkers secreted by various types of SM cells as disease progresses from early clinically unapparent disease to established chronic inflammation. The study of synovium by proteomic platforms should be considered as a key to our understanding of the stages of cellular pathology involved in the development of joint diseases.

6 Clinical application of synovial proteomics

The application of global proteomic rationales to the study of SF, plasma and synovial cells and tissue provides the opportunity to tackle a wide variety of clinical challenges encountered with a multifaceted disease such as arthritis. These challenges are clearly defined in a recent review of proteomics to study autoimmunity by Hueber and Robinson [90] and include the ability to identify patients more likely to develop severe disease or screening therapeutic response in a given individual. Profiling disease state-specific changes in the SF proteome represents a powerful approach to provide personalised treatment tailored to individual patients.

The concept of individualised medicine through the characterisation of changes in proteome response has been proposed for both plasma and SF [47, 50, 91]. Long-term patient 'proteome records' showing gradual disease associated changes over time, may allow tracking and refinement of therapeutic responses in a given individual [91]. Several studies demonstrate the potential of proteomics to monitor the changes in serial SF samples, collected during clinical trials of

novel biological drugs [59, 92]. What is more, proteomic analysis offers a suitable alternative to conventional molecular measures of disease status and progression, particularly where limited sample volume may restrict the number of singular assays which can be processed *per* patient.

Existing biomarkers which relate to the extent of inflammation do not act as sentinels of joint damage, which can occur in patients with clinically unapparent disease, nor do they portray the efficacy of treatment. Classic measures of acute phase response, erythrocyte sedimentation rate (ESR) and C reactive protein correlate well with concurrent inflammatory activity and correlate with clinical improvement but are poor predictors of future severity [41, 92]. Large-scale proteomic analysis of SF is therefore of particular importance not only in monitoring therapeutic responses, but also holds a great potential to diagnose arthritic disorders [50].

7 Conclusions

Many technologies have been used in the field of SF proteomics, including MS, 2-DE, ELISA, and protein microarray. The data derived by such techniques are thus inevitably composed of heterogeneous measurements that require integration to allow comparison. Data normalisation and scaling techniques will be critical in building predictive models of arthritic disease. The clinical challenges in arthritis are to develop robust biomarkers for predictors of outcome and disease progression. To this end, clinicians and scientists need to work in tandem. Clinicians must ensure that patient's disease and subtype are rigorously categorised according to internationally recognised criteria. Detailed clinical assessments are undertaken and that samples are obtained and stored according to protocols. Scientists must ensure that their methodologies are undertaken in such a way that their techniques are sensitive, findings reproducible and pertinent to the clinical questions asked.

8 References

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