RESEARCH ARTICLE

A clinically based protein discovery strategy to identify potential biomarkers of response to anti-TNF-α treatment of psoriatic arthritis

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Purpose: Psoriatic arthritis (PsA) can be treated using biologic therapies targeting biomolecules such as tumor necrosis factor alpha, interleukins (IL)-17 and IL-23. Although 70% PsA patients respond well to therapy, 30% patients show no or limited clinical improvement. Biomarkers that predict response to therapy would help to avoid unnecessary use of expensive biologics in nonresponding patients and enable alternative treatments to be explored.

Experimental design: Patient synovial tissue samples from two clinical studies were analysed using difference in-gel electrophoresis-based proteomics to identify protein expression differences in response to anti-TNF-α treatment. Subsequent multiplexed MRM measurements were used to verify potential biomarkers.

Results: A total of 119 proteins were differentially expressed ($p<0.05$) in response to anti-TNF-α treatment and 25 proteins were differentially expressed ($p<0.05$) between "good responders" and "poor responders". From these differentially expressed proteins, MRM assays were developed for four proteins to explore their potential as treatment predictive biomarkers.

Conclusion and clinical relevance: Gel-based proteomics strategy has demonstrated differential protein expression in synovial tissue of PsA patients, in response to anti-TNF-α treatment. Development of multiplex MRM assays to these differentially expressed proteins has the potential to predict response to therapy and allow alternative, more effective treatments to be explored sooner.

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1 Introduction

Psoriatic arthritis (PsA) is a chronic inflammatory arthritis associated with the skin disorder which affects between 0.3 and 1.9% of adults [1–5] and causes considerable morbidity, mortality and substantially affects patient's functionality, psychology, and quality of life [6, 7]. Research has shown that patients with PsA have a high risk of developing major systemic

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Abbreviations: **ACR**, American College of Rheumatology; **AMC**, Academic Medical Centre, Netherlands; **DAS**, disease activity score; **DIGE**, difference in-gel electrophoresis; **EULAR**, European League Against Rheumatism; **OA**, osteoarthritis; **PsA**, psoriatic arthritis; **SVUH**, St Vincent's University Hospital, Dublin, Ireland; **TNF-α**, tumour necrosis factor α

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Clinical Relevance

Psoriatic arthritis (PsA) is a systemic inflammatory and chronic autoimmune disorder causing considerable morbidity, poor quality of life and high rate of mortality. The most effective treatments for PsA are biologic therapies targeting specific biomolecules of the immune system and in particular tumour necrosis factor alpha (TNF-α) and interleukins (IL) IL-17 and IL-23. The precise in vivo mechanism of each different targeted therapy is not fully understood. Although these therapies are highly successful in most patients, a significant proportion of patients show no response or variable clinical improvement. For instance, anti-TNF-α therapy is ineffective in approximately 30% of the patients that receive this treatment. Thus, there is a significant need for the discovery and development of new prognostic and predictive biomarkers that predict disease outcome (without treatment) or response to anti-TNF-α therapy. Additionally, markers that allow disease activity monitoring over time could be very useful. For patients these could limit time on ineffective and expensive treatments and minimise potential side effects. Identification of these predictive biomarkers would enable clinicians to explore alternative targeted treatments sooner in anti-TNF-α nonresponders. This would allow better use of the narrow window to optimise therapeutic interventions before irreversible joint damage occurs.
Furthermore, a more detailed understanding of the molecular events, which occur following treatment, could provide a greater understanding of differences in the therapeutic response and may expose new therapeutic targets. Both are important steps towards the ultimate aim of personalised medicine in which treatment regimes are confidently tailored to individual patients.

comorbidities such as inflammatory bowel disease, cardiovascular disease, diabetes, obesity, other autoimmune disorders, and cancer [8, 9]. In addition to large direct medical costs, PsA results in extensive indirect societal costs for the individual, their family, and affects their work productivity and careers [8, 10, 11].

Despite major advances in therapeutic intervention over the last two decades, the treatment of PsA remains less than satisfactory. Conventional disease-modifying antirheumatic drugs such as methotrexate improve symptoms and reduce disease progression, but rarely result in disease remission. The introduction of cytokine antagonists such as tumour necrosis factor α (TNF-α) inhibitors have dramatically improved the ability to control severe disease but their high costs (£8000-15 000/patient/annum [12]) and uncertain long-term potential toxicity limit their use. In addition, even in combination with methotrexate 30–40% of patients do not respond to these treatments and more than 50% treated in early disease do not achieve remission status [13]. The molecular reasons for this variability are currently unknown.

Predicting a patient's response to treatment intervention is a key challenge. At present, patients who are given expensive biologic drugs such as anti-TNF-α therapies often begin an initial treatment period of a minimum 3 months before treatment response can be assessed adequately using European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR) criteria. In practice this means that about a third of patients (those who do not respond) are exposed to an expensive, ineffective, and potentially toxic drug for at least 3 months before, the treatment is switched to another biologic molecule and the same process repeated. There is therefore a significant need to identify biomarkers, which would predict patient's response to treatment either before initiating therapy or shortly after the treatment starts. Clinically useful biomarkers could either provide information on the likely outcome of disease without treatment (*prognostic*; disease related) or serve to predictively identify a subpopulation of patients who are most likely to respond to therapy (*predictive*; drug related).

As the synovium is the key site of inflammation in patients with PsA, we have used a proteomic strategy to investigate protein expression in synovial tissue biopsies taken from patients before and after anti-TNF-α biologic treatment.

Proteins are attractive biomarker candidates as they can be measured robustly in appropriate biological/clinical samples. Furthermore, changes in protein expression following effective treatment may reveal important information about the mechanism of drug action and/or reveal new potential therapeutic targets. Many studies have been undertaken to attempt to identify prognostic and predictive protein biomarkers for IA [14–16]. However, to-date only C-reactive protein and autoantibody status have been shown as being associated with disease outcome and proven useful in routine clinical practice [17]. A recent advancement has been made with the development of Vectra DA multi-biomarker (12-protein panel) blood test (Crescendo Biosciences), which is used for assessing the disease activity in rheumatoid arthritis patients treated with disease-modifying antirheumatic drugs and biologic therapies [18, 19]. This test along with the C-reactive protein and antibody measurements helps clinicians to make decisions as they go forward with a variety of treatments. However, there are as yet no biomarkers in clinical use, which predict response to therapy in advance. In a recent study, Ademovo et al. [20] have identified and developed a

biomarker panel that simultaneously measures the expression of 57 synovial tissue proteins that could be used to predict the response to anti-TNF-α therapy, help monitor treatment, and support the design of personalised treatment for patients with PsA. However, the translation of this synovial tissue biomarker test to a noninvasive sample such as serum to increase clinical utility is a challenge and further research is underway.

The application of a variety of proteomics techniques including 2DE, LC-MS, SELDI, and protein arrays to analyse diverse protein samples for protein biomarker discovery has generated significant interest in the field of rheumatology over the past few years. Serum and synovial fluid have been used for multiple biomarker discovery studies [21–28] as they represent readily accessible and clinically relevant samples but they are analytically challenging as proteins are present over a wide dynamic range and a small number of highly abundant proteins can mask potential biomarkers. Furthermore, it may be harder to detect changes, which reflect therapeutic response in distal or circulating biofluids, due to synovium biomarker dilution. Proteomic studies of synovial tissue have to date focused on the identification of diagnostic markers, for example to distinguish RA and PsA from each other or from osteoarthritis (OA) [28–35]. For example, Tilleman et al. [34] identified potential diagnostic biomarkers in synovial tissue and Bo et al. [36] did likewise using synovial fibroblasts. Other studies have focussed specifically on modified proteins in RA [37]. Notably, of the proteomics studies, which have sought to identify biomarkers of treatment response, none have used synovial tissue [27, 38, 39]. The current study addresses this issue by using synovial tissue from different patient cohorts and builds on a previous study performed in our lab, whereby PsA patient synovial tissue was used to identify a panel of protein biomarkers that predict response to biologic therapy [20].

To orthogonally verify the observed protein expression changes, usually antibody-based targeted approaches such as western blotting or ELISA are adopted. However, availability of suitable high-quality antibodies to each of the candidate proteins would normally constrain the selection of a small number of proteins for verification. In recent years, alternative quantitative and targeted MS-based approaches have been developed for protein quantification. Selected/multiple reaction monitoring (S/MRM) has emerged as a powerful, robust, sensitive and specific method for routine targeted quantification of proteins in complex biological samples [40, 41]. In S/MRM measurement of the peak areas of selected fragment ions from signature peptides, which are unique to the proteins of interest can be used for quantification [42–44]. This approach has several significant advantages over antibody-based strategies and is particularly suited to the multiplexed measurement of selected proteins [40, 41, 45–47]. For example, Jenkins et al. have reported the simultaneous measurement of 16 cytochrome P450 isoforms [48], de Seny et al. have used the approach to measure the expression of 48 proteins potentially predictive of drug toxicity [29] and Cretu et al. have verified the expression of 12 synovial fluid proteins that were significantly differentially expressed in patients with PsA as compared to healthy controls [23]. These striking characteristics have led to a wide adoption of the S/MRM technique for the targeted quantification of discrete sets of proteins for studies in both model systems [49–51] and clinical samples [20, 23, 45, 52].

Here we have undertaken gel-based proteomic analysis of the synovial tissue to:

(i) identify candidate *predictive* (drug related) proteins present before or shortly after treatment, which distinguish patients at baseline who exhibit a subsequent treatment response;
(ii) identify candidate *prognostic* (disease related) proteins that identify patients at baseline who are at higher risk of an adverse clinical outcome.
(iii) *monitor* changes in protein expression during treatment to (a) establish proteins that might indicate ongoing response to treatment and/or (b) provide mechanistic insight into the cellular and molecular events following therapeutic intervention.

2 Materials and methods

2.1 Sample collection and preparation

Synovial tissue samples were obtained by video-arthroscopy using standard operating procedures in place at St Vincent's University Hospital, Dublin (SVUH) and the Academic Medical Centre, Amsterdam (AMC) [53, 54]. Ethical approval was obtained from the respective hospital's ethics committees and all patients provided written informed consent. Synovial biopsies were frozen in liquid nitrogen and stored at –80°C until use. Samples were prepared by powdering the tissue using a dismembrator (B.Braun, Germany), with all accessories cooled in liquid nitrogen prior to use. At least three and up to six pieces of biopsy tissue were pooled and powdered per patient depending on the size of the individual biopsies. Protein was extracted from the synovial tissue by dissolving powdered biopsies in a 9.5 M urea lysis buffer (9.5 M urea (w/v), 2% CHAPS (w/v) and 20 mM Tris pH 8.8). Insoluble matter was removed by centrifugation and protein concentrations evaluated using a modified Bradford assay with BSA as a protein standard [55].

2.2 Sample cohort details

An initial pilot study was undertaken using a prospective cohort of 12 patients from SVUH with active PsA who received anti-TNF-α therapy (etanercept) [56]. These patients were selected on the basis of strict eligibility criteria, thus each patient had a swollen knee, complied with the British Society for Rheumatology guidelines for prescription of a biologic agent in PsA and had full accompanying clinical activity data

Table 1. Patient data from SVUH pilot patient cohort

Sample ID	Week 0	Week 12	Age (years)	Medication prior to Etanercept therapy	DAS28 (Week 0)	DAS 28 (Week 12)	ACR	Response
EP1	■	■	64	Salazopyrine (1 gm bd), Pregnisolone (7.5 mg od)	5.63	2.92	50	Good
EP2	■		50	.	3.35	1.85	50	Good
EP3	■	■	54	.	–	–	50	Good/moderate
EP4	■	■	63	.	3.28	0.14	70	Good
EP5	■	■	52		5.70	4.48	20	Moderate
EP6	■	■	38	Pregnisolone (5 mg od)	3.08	0.54	70	Good
EP7	■	■	53	.	3.86	1.41	50	Good
EP8		■	34	.	4.67	1.76	70	Good
EP9		■	64	Pregnisolone (5 mg od)	6.31	4.2	20	Moderate
EP10	■	■	31	MTX (22.5 mg/week PO), Pregnisolone (10 mg od)	5.91	5.35	0	Poor/No
EP11	■		56	.	5.58	3.91	0	Moderate
EP12	■		32	.	5.21	2.01	70	Good

Cohort consisted of 12 PsA patients on Etanercept therapy. Shaded squares in columns 2 and 3 indicate which patient synovial tissue samples were available at each time point (shaded = sample available). Week 0 (baseline) $n = 9$, Week 12, $n = 9$. Seven patients had matched week 0 and week 12 samples. Patient response is based on the EULAR criteria as described in the text.

at baseline and at 4 and 12 weeks following commencement of treatment (Table 1). In this cohort of patients, mean age of the patients was 48.6 ± 12.15 (25–64) years. Furthermore, paired synovial tissue samples were obtained from seven of these patients who had arthroscopies both before (week 0, baseline) and 3 months/12 weeks (week 12) following treatment (see Table 1). Three patients had samples available from baseline (week 0) only and two patients had samples from week 12 only. Patient responses were categorised using the EULAR and ACR criteria [57–59]. Patients were classified as good responders if they exhibited a decrease in DAS28 of greater than 1.2 with a week 12 DAS28 of less than or equal to 3.2. Poor response was classified as a decrease in DAS28 of less than 1.2 with a week 12 DAS28 of greater than 5.1. Patients who lay between these two criteria were classified as moderate responders. One patient (EP3) had no DAS28 values recorded but was classified as a moderate responder on the basis of an ACR50 response. Overall in applying these criteria to the 12 patients there, seven were classified as good-responders, one a poor-responder and four as having moderate response (see Table 1).

To consolidate the initial findings a larger cohort of patient samples, taken as part of a prospective clinical trial consisting of 20 patients with active PsA from the Amsterdam Medical Centre, University of Amsterdam (clinical trial number ISRCTN23328456), was subjected to a similar proteomics workflow. Patients were recruited as detailed in van Kujik et al. 2009 and 2010 [60, 61]. Briefly, 20 patients with active PsA were randomized to receive the anti-TNF-α antibody, Adalimumab (40 mg every other week) ($n = 10$) or placebo ($n = 10$) for 4 weeks (see Table 2). In this cohort of patients, mean age of the patients treated with Adalimumab was 42.8 (21–61) years and placebo was 47.2 (25–78) years [60, 61]. Moreover, the mean PsA disease duration was 5.5 (0.4-14.1) years and 8.4 (1.9-18.2) years and the mean methotrexate dose was 18.2 mg/week and 19 mg/week for patients treated with Adalimumab and placebo, respectively [60, 61]. Synovial biopsies were obtained from each patient before treatment and then after 4 weeks. After the second arthroscopy, all patients received Adalimumab in an open label extension study (40 mg every other week). Patients were examined clinically at week 4 and week 12 to determine their overall response to therapy. Again, DAS28 was used to monitor changes in disease activity in response to therapy and patients were classified as previously described for the SVUH cohort.

2.3 Difference in-gel electrophoresis (DIGE)

Apart from a series of studies undertaken to optimise 2DE methods, prior to separation proteins were minimally labelled using CyDyes and DIGE was performed according to the manufacturer's guidelines (GE Healthcare, Bucks, UK). Each sample was minimally labelled with Cy3 fluorescent dye and an internal pooled standard (labelled with Cy5) sample was included. Briefly, 25 μg each of Cy3 labelled sample and 25 μg of Cy5 labelled standard were combined and resuspended in 2× strength sample buffer (8 M Urea, 130 mM DTT, 4% (w/v) CHAPS, 2% (v/v) Pharmalyte 4–7). Immobilized pH gradient (IPG) strips (Immobiline Dry Strip, linear pH 4–7 (or nonlinear pH 3–10 for optimisation experiments)) were rehydrated overnight with the relevant sample mixes. First-dimension separation of proteins by IEF was performed for a total of 75 000 V (2 mA/5W limit per strip) including a final 8000 V step for 1 h to obtain improved resolution. After IEF, the strips were equilibrated, first in 1% (w/v) DTT and then 2.5% (w/v) iodoacetamide, for 15 min each time. IPG strips were each laid into single well 12% PAGE gels and sealed in place with 1% agarose (w/v) in running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v)

Table 2. Details of the AMC patient cohort

Sample ID	Gender	Treatment	Joint	Metho-trexate	Methotrexate dose (mg/week)	DAS28 week 0	DAS28 week 4	DAS28 week 12	Response at week 12
AH1	F	Placebo	Knee	Yes	25	5.40	6.14	4.47	Moderate
AH2	F	Placebo	Knee	No	.	5.05	4.76	2.18	Good
AH3	M	Placebo	Knee	Yes	15	5.58	5.64	3.54	Good
AH4	F	Placebo	Ankle	No	.	6.83	6.94	4.34	Moderate/good
AH5	M	Placebo	Knee	No	.	5.76	6.21	2.84	Good
AH6	M	Placebo	Knee	No	.	4.22	3.92	1.34	Good
AH7	M	Placebo	Knee	Yes	25	4.77	4.72	3.22	Good
AH8	F	Placebo	Ankle	No	.	4.12	4.51	3.15	Good
AH9	M	Placebo	Ankle	No	.	5.97	6.48	4.91	Moderate
AH10	F	Placebo	Knee	Yes	25	4.20	5.09	3.46	Good
AH11	M	Adalimumab	Ankle	No	.	4.76	4.91	3.71	Moderate
AH12	M	Adalimumab	Knee	No	.	5.30	1.67	1.77	Good
AH13	F	Adalimumab	Knee	No	.	5.57	4.51	3.45	Moderate
AH14	M	Adalimumab	Knee	Yes	20	3.06	1.59	1.11	Good
AH15	M	Adalimumab	Knee	Yes	22.5	4.21	3.13	2.56	Good
AH16	M	Adalimumab	Wrist	Yes	20	3.00	0.89	0.53	Good
AH17	F	Adalimumab	Knee	Yes	15	5.11	3.66	2.42	Good
AH18	M	Adalimumab	Knee	No	.	3.66	2.74	2.56	Moderate
AH19	M	Adalimumab	Knee	Yes	10	5.87	2.61	1.81	Good
AH20	M	Adalimumab	Ankle	Yes	25	5.76	1.55	1.46	Good

Cohort consisted of 20 PsA patients on Adalimumab or placebo therapy for 4 weeks before placebo patients were switched to Adalimumab. Patient response was assigned according to the EULAR criteria.

SDS, and bromophenol blue). Second dimension separations were undertaken at 1 W for 1 h and then at 1W/gel overnight until the dye front reached the end of the gel.

2.4 Gel image analysis

Fluorophore labelled proteins were visualized using a Typhoon 9410 variable imager (GE Healthcare). Excitation/emission wavelengths of 532/580 and 633/670 nm were used for the Cy3- and Cy5-labelled samples and standard, respectively. Gel image analysis was performed with Progenesis Samespots software (version 4.1; Nonlinear Dynamics Ltd. Newcastle upon Tyne, UK). Images were normalised to the Cy5-labelled pooled standard. Analysis of variance (ANOVA) testing was used to examine the significance of changes in protein expression between groups. Either a repeated measure ANOVA (for matched samples at two time points) or a one-way ANOVA (for comparison of grouped baseline samples) was used with a *p*-value of 0.05 used as the cut off for significance. Manual filtering of significant protein spots was performed to eliminate gel artefacts and overlapping peaks.

2.5 Mass spectrometry

For protein identification by MS, preparative gels, each loaded with 450 μg of protein, were silver-stained with an MS compatible stain (PlusOne Silver stain kit, GE Healthcare), omitting the use of glutaraldehyde [62]. Protein spots were excised from the gels and digested using a protocol similar to that of Grassl et al., 2010 but without the use of Ziptips [63]). Briefly, the gel spots were destained, reduced and alkylated, then dehydrated with ACN. The proteins were digested overnight with trypsin (Promega, modified sequencing grade) at 37°C. For LC-MS/MS analysis, dried protein digests were resuspended in buffer A (0.1% formic acid).

Protein identifications were carried out using ion trap and quadrupole-time of flight (TOF) mass spectrometers. Some gel spots were analysed using a nano-LC-ESI-MS/MS coupled to an LTQ-linear ion trap MS (ThermoFisher). After resuspension in buffer A (0.1% formic acid) samples were injected onto an LC Packings PepSep column (75 um id, 150 mm). Nano-HPLC was used to separate peptides using a gradient from 5 to 50% buffer B (0.1% formic acid in 100% MeCN) over 10 min, then up to 90% buffer B over 5 min and introduced into the LTQ ion trap mass spectrometer at 300 nL/min using nanoflow probe. Full MS scans were recorded on the eluting peptides over the 300–2000 m/z range. Tandem MS (MS/MS) spectra were acquired in a data-dependent manner, sequentially on the first to fifth-most intense ion selected from the full MS scan.

All LTQ-MS/MS spectra were searched against the Uniprot/Swissprot (Version 5.0/47.0) sequence database using TurboSEQUEST (v.27; rev.12). Search parameters used were: precursor-ion mass tolerance of 1.4 Da, fragment ion tolerance of 1.0 Da with methionine oxidation and cysteine carboxyamidomethylation specified as differential modifications and a maximum of two missed cleavage sites were allowed. Each peptide used for protein identification

met specific SEQUEST parameters (cross-correlation values of ≥1.9, ≥2.8 and ≥3.2 for single-, double- and triple-charged peptides, respectively) and a peptide probability of <0.001. For all proteins detected with less than three peptides, the spectra were checked manually for satisfactory ion coverage.

Other gel spot samples were analysed using a 6520 Q-TOF (Agilent Technologies) connected via an orthogonal spray Chipcube interface to a 1200 series nanoflow HPLC (Agilent Technologies). Samples were run using a 20-min gradient from 0.1% formic acid, 3% ACN to 0.1% formic acid, 40% ACN using a G4240-62001 HPLC-chip with a 40 nL enrichment column and 75 um × 43 mm separation column both packed with Zorbax 300SB-C18 5 um material. A fragmentor voltage of 175 V was used. Up to eight MS/MS spectra were collected per MS spectrum.

MS/MS spectra acquired on the Q-TOF were searched against human entries in the UniProtKB/Swissprot database (release 15.15/57.15) using Spectrum Mill (rev.A.03.03.084 Service Release 4). Data extraction, MS/MS searching and auto-validation were performed separately. Data were searched with a precursor mass tolerance of ±20 ppm and a product mass tolerance of ±50. Two missed cleavages and a maximum charge of 7 were allowed.

Additionally, some samples were separated using a NanoLC 1D Plus chromatography system (Eksigent, USA) coupled online to an LTQ Orbitrap XL mass spectrometer (ThermoFisher). Each sample was loaded onto a Biobasic C18 PicoFrit column (75 um id, 100 mm) and separated using a 30-min reverse phase gradient at a flow rate of 300 nL/min. The mass spectrometer was operated in positive ion mode at capillary temperature 200°C, capillary voltage 9V, tube lens voltage 100 V and a potential of 1800 V applied to the frit. Data were acquired in automatic data-dependent mode, performing a high-resolution MS scan (300–2000 Da) to select the five most intense ions for MS/MS analysis. Data were searched against Uniprot (Release version 2010_12) using Bioworks Rev 3.3.1 SPI (ThermoElectron). The search parameters used were a peptide tolerance of 20 and a fragment ion tolerance of 1.0 with methionine oxidation and cysteine carboxyamidomethylation specified as differential modifications and a maximum of two missed cleavage sites were allowed. Each peptide used for protein identification met specific SEQUEST parameters (cross-correlation values of ≥1.9, ≥2.8 and ≥3.2 for single-, double- and triple-charged peptides, respectively) and a peptide probability of <0.001.

2.6 MRM verification studies

Skyline v1.1 [64, 65] was used to identify proteotypic peptide sequences from each protein using a database search. The search results were filtered using Peptide Prophet [66] and a minimum probability threshold of 0.9 was given to achieve error rate of 0.022 and sensitivity of 0.642. An MS/MS spectral library of the Q-TOF data was built using skyline containing in-house and public MS/MS libraries including Peptide Atlas, NIST and GPM to help precursor ion and transition selection. The charge states of precursor ions were set to 2 to 3 and the product ions were limited to being singly charged. Both b and y ions were selected. The ion match tolerance was 0.8 Da.

Following reduction with DTT, alkylation with IAA and acetone precipitation, approximately 50 to 70 ug of synovium samples were digested overnight at 37°C with trypsin (Promega, modified sequencing grade), The resulting peptides were evaporated to dryness and resuspended in buffer A (0.1% formic acid) for analysis on a 6450 Triple Quadrupole mass spectrometer connected via an orthogonal spray G4240A Chipcube interface to a 1200 series nanoflow HPLC (Agilent Technologies). Samples were separated using an HPLC-Chip equipped with a 75 μm × 150 mm, 5 μm C-18 300SB-Zorbax analytical column and a 160 nL Zorbax 300SB-C18 5 μm enrichment column using a 38-min gradient of: 0–30% B 0–30 min; 30–95% B 30–35 min; hold 95% B 35–36 min; 95.0% B 36–38 min, followed by column reconditioning for 8 min (where buffer A was 3% ACN, 0.1% formic acid and Buffer B was 90% ACN, 0.1% formic acid). An unscheduled method was used with a cycle time of 3713 ms/cycle (0.27 cycles/s, dwell time of 20 ms per transition and fragmentation voltage was set to 135 V in the positive ion mode.

MS data were imported into Skyline [64, 65] and the coelution of transitions from each peptide and the boundaries for peak integration were checked manually. Peak area values for each parent ion transition were exported into Excel.

3 Results

To investigate the effect of anti-TNF-α therapy on synovial tissue protein expression, we herein followed an unbiased proteomics strategy using a series of synovial tissue samples obtained from PsA patients prior to and after receiving the anti-TNF-α therapy (Etanercept and Adalimumab). Figure 1 illustrates the overall study design and the experimental workflow undertaken for the patient samples used for protein biomarker discovery and verification. The techniques used are detailed in the Materials and methods section.

Study 1 is geared towards monitoring changes in protein expression in response to Etanercept treatment. SVUH cohort patients ($n = 7$) samples at week 0 and week 12 were used.

Study 2 looks at the predictive protein biomarkers in baseline samples, which have the potential to distinguish future responders to Etanercept. SVUH cohort patients who consequently responded to Etanercept treatment ($n = 2$) were compared to nonresponders ($n = 3$).

Study 3 monitors the changes in protein expression in response to Adalimumab treatment. AMC cohort patient samples treated with either placebo control ($n = 10$)

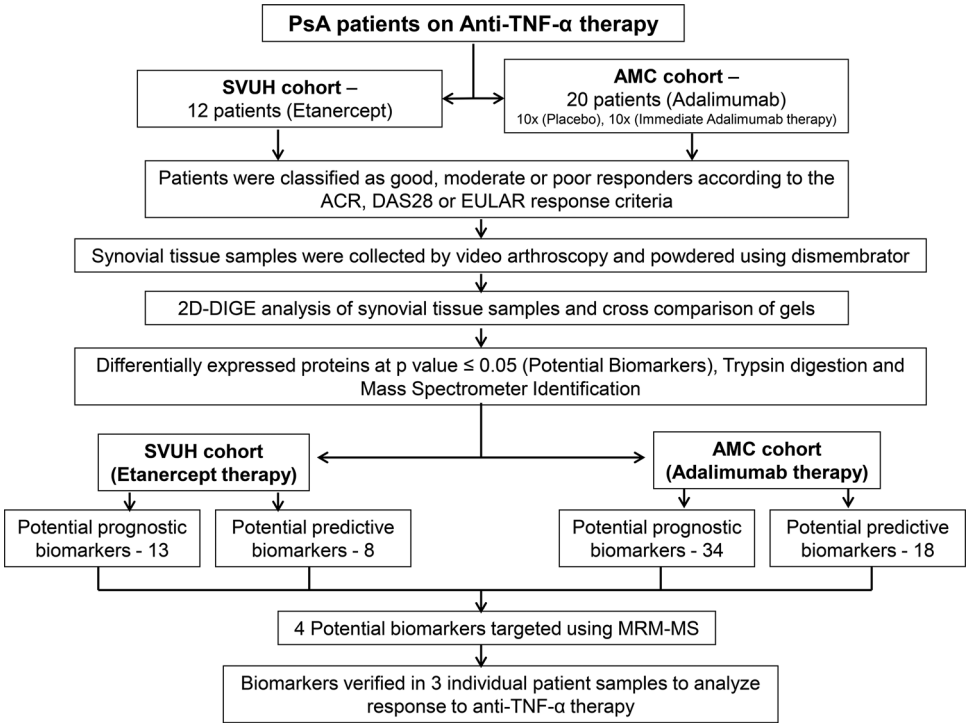

Figure 1. Experimental Workflow. The flowchart depicts the overview of study design illustrating the steps that were undertaken for the patient samples used for protein biomarker discovery and verification.

or Adalimumab ($n = 10$) at week 0 and week 4 were used.

Study 4 looks at the predictive protein biomarkers in baseline samples, which have the potential to distinguish future responders to Adalimumab. The disease-associated protein biomarkers are selected here with more confidence due to the inclusion of placebo control (nontreated) group of patients. AMC cohort patients treated with Adalimumab who consequently responded to the treatment ($n = 8$; good responders) were compared to the patients who poorly responded to the treatment ($n = 2$; nonresponders).

3.1 Study 1. Synovial tissue protein expression changes in response to treatment with anti-TNF-α therapy: SVUH cohort

To identify changes in synovial tissue protein expression in response to treatment with anti-TNF-α (Etanercept) therapy, samples from seven SVUH patients from whom synovial tissue was obtained both pretreatment (week 0) and after 3 months (week 12) (Table 1) were subjected to 2D-DIGE and gel image data were analysed with Progenesis (Fig. 2A). A total of 1176 spots were detected on the 2D-DIGE gels. Figure 2A shows thumbnail images of each individual gel and a large gel image of a pooled reference standard prepared from each patient sample. Annotated on the image are the positions of all the spots that were shown to change significantly in intensity with anti-TNF-α (Etanercept) treatment and which were subjected to identification by MS (Fig. 2A). Out of the 119 protein spots that changed significantly ($p<0.05$), 63 were upregulated in response to anti-TNF-α treatment and 56 were downregulated. Following careful manual selection of the 119 protein spots that changed significantly ($p<0.05$), preparative scale gels were used to isolate a total of 21 of the protein spots (which are annotated on the image in Fig. 2A) were subjected to MS analysis. Manual selection of the spots for MS characterisation was based on the consistency of the change between the gels, the overall intensity of the spot, the extent to which individual spots overlapped with other spots and if there was a clear background in the region of the spot. Out of the 21 spots selected, 20 were identified (see Table 3). Unique IDs were obtained for many spots whilst for others more than one protein was identified in each spot. Some of the differentially regulated proteins upon response to anti-TNF-α (Etanercept) treatment include haptoglobin, annexin A2, serum amyloid P, peroxiredoxin 6, serum albumin, Ig kappa chain C, fibrinogen beta chain.

3.2 Study 2. Analysis of synovial tissue protein expression before anti-TNF-α therapy: Potential predictive biomarkers of response: SVUH cohort

To identify potential predictive biomarkers of response to anti-TNF-α therapy, we sought to determine if there are differences in protein expression at baseline in the synovial tissue of patients who subsequently respond to anti-TNF-α (Etanercept) treatment. To do this, two patients were selected (Table 1, EP4 and EP6) who most clearly showed a good

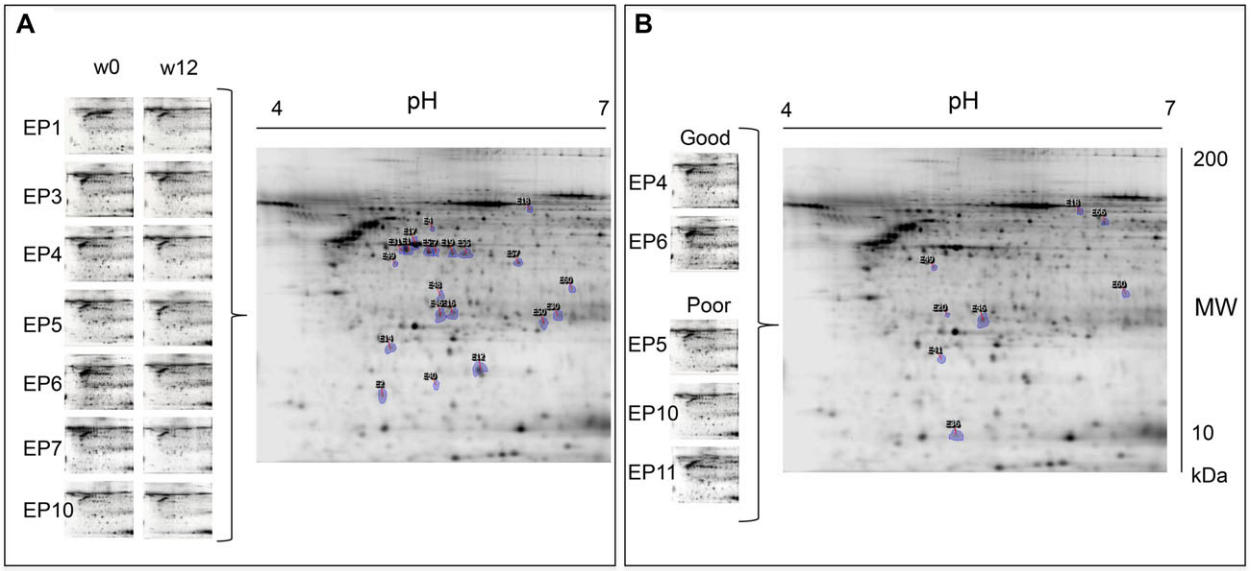

Figure 2. Changes in synovial protein expression in response to treatment with anti-TNF-α therapy – Pilot Cohort. 7 patient synovium samples including pre- (week 0) and post-treatment (w12) with anti-TNF-α therapy were subjected to 2D-DIGE as described in "Materials and methods (A) Depicts protein spots that were found to be significantly altered in expression between week 0 and week 12 of anti-TNF-α treatment and (B) shows differential expressed protein spots in synovium of patients who responded to anti-TNF-α treatment either well (good) or poorly (moderate/poor). Differentially expressed protein spots are annotated on the silver stained 2D-DIGE gel images and the protein identifications are detailed in Tables 3 (A) and 4 (B). Thumbnail gel images show each of the individual gels in each category.

response (defined as EULAR good responders and ACR 70) and three patients (Table 1, EP5, EP10, EP11) who showed a poor response (EULAR moderate or poor response and ACR 20 or less). The five synovial tissue samples taken from the patients before they commenced treatment were analysed by 2D-DIGE (Fig. 2B). Out of the 102 protein spots found to have significantly changed (ANOVA, $p<0.05$), eight protein spots (as annotated in Fig. 2B) were selected according to the criteria described above and were subjected to MS characterisation. Out of these eight protein spots, seven were identified successfully (Table 4). Novel predictive protein candidates include serum albumin, collagen alpha 3, annexin A1 and A2, Ig kappa chain C, BTB/POZ domain-containing protein and tryptase.

3.3 Study 3. Synovial tissue protein expression changes in response to anti-TNF-α therapy: placebo controlled, randomised clinical trial patients from AMC cohort

Given that differences in synovial tissue protein expression exist in response to Etanercept-based anti-TNF-α treatment, we sought to explore and identify the changes in synovial tissue protein expression in response to treatment with Adalimumab-based anti-TNF-α therapy. To do this, 2D-DIGE-based proteomic analysis was performed on synovial tissue samples obtained from the 20 AMC patients at two time-points (baseline (week 0) and 4 weeks after starting anti-TNF-α therapy (Adalimumab) or placebo treatment (week 4) (Table 2). Overall, 40 2D-DIGE gels were analysed to determine changes in protein expression before and after treatment (Fig. 3A). A total of 1120 spots were detected on the 2D gels. Only spots that changed exclusively in the gels of patients on anti-TNF-α treatment (and were not changed over the 4-week period in the placebo group) were regarded as changing with treatment. Using this criterion, 91 spots were significantly different ($p<0.05$) between week 0 and week 4. Out of these, 50 were upregulated and 41 downregulated in response to anti-TNF-α (Adalimumab) therapy. After manual selection, 26 of these protein spots were excised, digested and subjected to MS for protein identification (Fig. 3A; Table 5). Figure 3A shows thumbnail images of selected individual gels and a large gel image of the pooled reference standard prepared from all patient samples. Annotated on the image are the positions of the 26 spots that were shown to change significantly in intensity. Protein identifications were obtained for 24 of the selected spots (Table 5). Unique IDs were obtained for many spots whilst for others more than one protein was identified in each spot. Some of the interesting differentially expressed proteins upon response to anti-TNF-α (Adalimumab) treatment include haptoglobin, serum albumin, ubiquitin conjugating enzyme E2, annexin A1, A2 and A6, serum amyloid P, heat shock cognate 71 kDa protein, fibrinogen beta chain, pyruvate kinase isozymes M1/M2, collagen alpha 3 and cathepsin B. Interestingly, some of these proteins such as haptoglobin, actin, serum albumin, annexin A2, serum amyloid P, collagen alpha 3 and fibrinogen were

Table 3. The top ranking proteins identified in gel spots exhibiting significant changes ($p<0.05$) in spot volumes in matched samples at week 0 and week 12 ($n = 7$) during anti-TNF-α treatment in the SVUH study

Spot number	Protein	Accession number	Fold change	ANOVA/ *t*-test value	Number of peptides matched (% coverage)	Protein score	pI/mass kDa
E1	Haptoglobin	P00738	0.47	0.004	8 (21.2)	80.22	6.1/45.2
E2	Actin (cytoplasmic)	P63261	0.49	0.04	1 (4.8)	10.22	5.3/41.8
E4	Serum albumin	P02768	0.28	0.003	1 (1.6)	10.2	5.9/66.5
E5	Haptoglobin	P00738	0.36	0.0003	5 (14.0)	50.24	6.1/45.2
E7	Serum albumin	P02768	0.59	0.01	7 (12.8)	70.20	5.9/66.5
	Haptoglobin	P00738			4 (9.6)	40.22	6.1/45.2
E12	Haptoglobin	P00738	0.55	0.047	2 (3.7)	18.2	6.1/45.2
E14	Annexin A2	P07355	1.62	0.001	2 (5.6)	20.15	7.6/38.6
E16	Serum amyloid P	P02743	0.73	0.03	4 (20.2)	40.23	6.1/25.4
E17	Actin (cytoplasmic)	P63261	1.23	0.007	9 (25.9)	90.29	5.3/41.8
	Actin (alpha)	P68133			6 (17.0)	60.24	5.2/42.1
E18	Serum albumin	P02768	1.43	0.01	2 (3.78)	20.18	5.9/66.5
E19	Haptoglobin	P00738	0.58	0.01	7 (19.0)	70.23	6.1/45.2
	Protein disulfide-isomerase A3	P30101			4 (5.90)	40.21	6.0/56.8
E30	Peroxiredoxin 6	P30041	0.86	0.023	2 (10.3)	20.18	6.0/25.0
E31	No ID		0.65	0.010			
E40	Annexin A2	P07355	0.53	0.044	2 (6.2)	20.20	7.6/38.6
E46	Ig kappa chain C	P01834	0.49	0.018	1 (18.90)	10.24	5.6/11.6
E48	Annexin A2	P07355	0.65	0.020	4 (12.40)	40.19	7.6/38.6
E49	Collagen alpha-3 (VI)	P12111	0.62	0.056	6 (2.3)	60.23	6.3/343.7
	BTB/POZ domain-containing protein	Q96CX2			4 (14.50)	40.25	5.5/35.7
	Annexin A2	P07355			3 (9.40)	30.20	7.6/38.6
E50	Annexin A2	P07355	0.43	0.007	3 (9.40)	30.20	7.6/38.6
E55	Fibrinogen beta chain	P02675	0.57	0.037	8 (19.90)	80.26	8.5/55.9
E57	Fibrinogen beta chain	P02675	0.59	0.051	8 (21.40)	80.25	8.5/55.9
E60	Tryptase alpha-1	P15157	1.65	0.054	1 (4.00)	10.19	6.6/30.5

All spots were analysed using an LTQ ion-trap MS and where more than one protein was identified for a single spot, only those identifications made on the basis of at least two peptides and a protein score of 25 or above are listed. A full list of proteins identified is shown in Table 1-Supporting Information Material. pI and molecular weights obtained from SwissProt/Uniprot are shown. Spots E49, E57 and E60 have slightly borderline nonsignificant ANOVA values (highlighted in grey).

found to change upon both the Etanercept (SVUH patient group) and Adalimumab (AMC patient group) therapies but others were found to be altered with treatment in one patient group and not the other.

3.4 Study 4. Differences in synovial tissue protein expression before anti-TNF-α therapy: Potential predictive biomarkers of response: AMC cohort

Upon observing, that differences in synovial tissue protein expression subsist in response to different forms of anti-TNF-α therapies, we further sought to investigate and identify the potential proteins that could predict response to Adalimumab-based anti-TNF-α therapy and to explore if they were different than Etanercept-based anti-TNF-α therapy. In order to do this, 2D-DIGE gels from patient samples at baseline (week 0) from both the treatment and placebo sets of the study, were grouped according to subsequent response status. The "good responders" group comprised patients AH2, AH5, AH6, AH12, AH14, AH15, AH16, AH17, AH19 and AH20 ($n = 10$) who all met EULAR criteria for definition of a good responder (see Table 2). Patients AH1, AH9, AH11 and AH13 were grouped together as "poor/moderate responders" for whilst they demonstrated some response to treatment they still had moderate disease activity (DAS28>3.2) after 3 months of anti-TNF-α (Adalimumab) therapy (see Table 2). The remaining six patients (AH3, AH4, AH7, AH8, AH10 and AH18) who did not fall into either category on the basis of DAS28 score (detailed in the sample cohort details section in Materials and methods) for good or poor/moderate responders were excluded from this analysis. A total of 25 protein spots were found to have significantly ($p<0.05$) different levels between the two groups with eight protein spots of higher intensity in "good responders" and 17 higher in "poorer responders". Out of these protein spots, 14 were selected and excised, digested and subjected to MS from which identifications were achieved for all 14 (Table 6). Figure 3B shows thumbnail images of the gels used for analysis and a larger image of a pooled reference standard image with these 14

Table 4. SVUH Study. Protein identifications obtained for gel spots with significantly different volumes before commencement of treatment in patients who subsequently responded well to anti-TNF-α therapy (Etanercept) (ACR 70) compared to those who responded poorly (ACR 20 or less)

Spot number	Protein	Accession number	Fold change	ANOVA/ *t*-test value	Number of peptides matched (% coverage)	Protein score	p*I*/mass kDa
E18	Serum albumin	P02768	0.58	0.053	2 (3.78)	20.18	5.9/66.5
E20	Collagen alpha 3(VI)	P12111	3.32	0.037	6 (1.9)	60.23	6.3/343.7
E36	Serum albumin	P02768	1.21	0.011	2 (3.60)	20.21	5.9/66.5
E41	Annexin A1	P04083	2.08	0.047	2 (6.4)	20.20	6.6/38.7
E46	Ig kappa chain C	P01834	2.20	0.021	1 (18.90)	10.24	5.6/11.6
E49	Collagen alpha-3 (VI)	P12111	2.06	0.054	6 (2.3)	60.23	6.3/343.7
	BTB/POZ domain-containing protein	Q96CX2			4 (14.50)	40.25	5.5/35.7
	Annexin A2	P07355			3 (9.40)	30.20	7.6/38.6
E60	Tryptase	P15157	2.71	0.019	1 (4.00)	10.19	6.6/30.5
E66	No ID		0.583	0.017			

Spot numbers correlate to those on Fig. 2B. The fold change given is for the group of good responders relative to the group of poor responders. All spots were analysed using an LTQ ion-trap MS and for spot E49, where more than one protein was identified for a single spot, only those identifications made on the basis of at least two peptides and a protein score of 25 or above are listed. A full list of proteins identified for E49 is shown in Table 1-Supporting Information Material. pI and molecular weights obtained from SwissProt/Uniprot. Spots E18 and E49, has a slightly borderline nonsignificant ANOVA value (highlighted in grey).

identified differentially expressed spots annotated. Potential predictive proteins for response to Adalimumab-based anti-TNF-α therapy include annexin A1 and A2, serum albumin, haptoglobin, apolipoprotein A1, collagen alpha 3, actin, rho-GDP-dissociation inhibitor 2, alpha-1B-glycoprotein, 78kDa glucose-related protein, replication protein A, pyruvate kinase M1/M2, heat shock protein 70kDa and 71kDa, vimentin and lamin-B2. As seen before, some of the potential predictive proteins for response to Etanercept (SVUH patient group) and Adalimumab (AMC patient group) based anti-TNF-α therapy

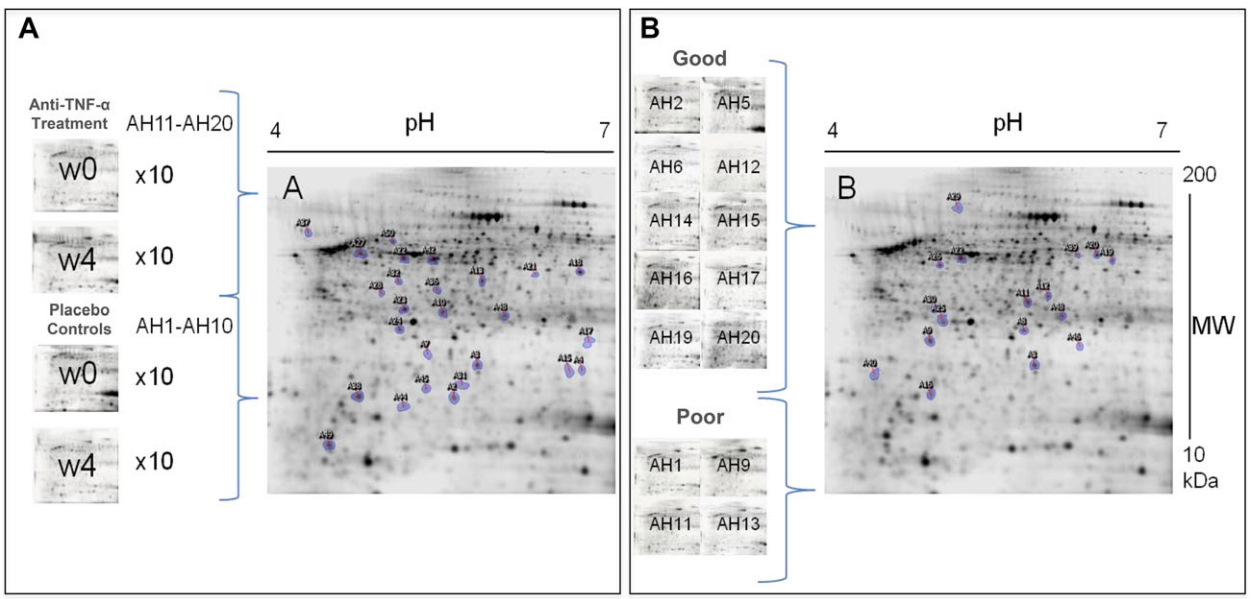

Figure 3. Changes in synovial protein expression in response to treatment with anti-TNF-α therapy – Study Cohort. 20 patient synovium samples including pre- (week 0) and post-treatment (week 4) with anti-TNF-α therapy or placebo treatment controls from the Amsterdam Medical Centre were subjected to 2D-DIGE analysis (i) illustrates the samples used for 2D gel analysis and shows a composite reference gel image (A) annotated with differentially expressed protein spots that changed significantly in expression between week 0 and week 4 of anti-TNF-α treatment. (ii) Shows individual thumbnail gel images for good and poor responders and a composite reference gel image annotated with spots that showed differential protein expression (at baseline) between good and poor responders. The corresponding protein identifications are detailed in Tables 5 (A) and 6 (B).

Table 5. AMC Study. Protein identifications obtained from gel spots identified as having siginificant ($p<0.05$) changes in spot volume after 4 weeks treatment with anti-TNF-α therapy (Adalimumab)

Spot number	Protein	Accession number	Fold change (post relative to pre)	ANOVA value	Number of peptides matched (% coverage)	Protein score	pI/mass kDa
A2	Transthyretin	P02766	1.37	0.041	2 (9)	27.69	5.52/15.9
	Ubiquitin-conjugating enzyme E2	P61088			2 (13)	25.32	6.13/17.1
A3	Haptoglobin	P00738	0.44	0.023	6 (10)	106.81	6.13/45.2
A4	Serum albumin	P02768	0.39	0.009	1 (1)	12.90	5.92/69.4
A7	Serum albumin	P02768	1.31	0.043	4 (6)	68.85	5.92/69.4
A10	Actin	P63261	0.64	0.025	10 (30)	162.57	5.31/41.8
	Annexin A1	P04083			3 (11)	58.24	6.57/38.7
	Ig Kappa Chain C	P01834			2 (34)	51.14	5.58/11.6
	Serum amyloid P	P02743			3 (15)	43.68	6.1/25.4
A13	Pyruvate kinase isozymes M1/M2	P14618	0.51	0.016	8 (17)	163.64	7.95/57.9
A15	Heat shock cognate 71 kDa protein	P11142	0.61	0.023	7 (12)	133.98	5.38/70.9
	Lamin A/C	P02545			4 (5)	59.95	6.57/74.1
A17	Serum Albumin	P02768	0.70	0.007	2 (3)	33.52	5.92/69.4
	Mn superoxide dismutase	P04179			2 (9)	31.12	8.34/24.7
A18	Fibrinogen beta chain	P02675	0.51	0.019	5 (12)	79.07	8.54/55.9
	Pyruvate kinase isozymes M1/M2	P14618			4 (9)	74.68	7.95/57.9
	Serum albumin	P02768			3 (4)	41.83	5.92/69.4
A21	No ID		0.51	0.007			
A22	Haptoglobin	P00738	0.70	0.034	9 (25)	182.31	6.28/46.7
	Collagen alpha 3 (VI)	P12111			7 (2)	99.98	6.27/344
	Serum albumin	P02768			4 (7)	74.42	5.92/69.4
	Actin	P63261			4 (12)	66.68	5.35/42
	Lamin-B2	Q03252			4 (7)	64.08	5.28/67.7
A23	Actin	P63261	1.31	0.003	6 (15)	98.04	5.31/41.8
	ATP synthase	P06576			3 (5)	54.54	5.26/56.5
A24	Cathepsin B	P07858	0.76	0.038	3 (9)	45.01	5.88/37.8
A28	EF-hand domain-containing protein	Q96C19	0.68	0.003	5 (27)	90.39	5.15/26.7
	Protein disulfide-isomerase	P07237			5 (9)	86.17	4.76/57.1
	Protein AMBP	P02760			2 (5)	32.78	5.95/39.0
A31	Actin-related protein	O15511	0.74	0.008	6 (33)	93.03	5.47/16.3
	Actin	P63261			3 (8)	47.88	5.31/41.8
A32	Tubulin	P07437	1.64	0.004	5 (13)	70.6	4.78/49.7
	Serum albumin	P02768			3 (5)	46.49	5.92/69.4
	Collagen alpha 1 (I)	P02452			3 (2)	44.22	5.61/139
	Thioredoxin domain containing protein	Q8NBS9			2 (5)	35.21	5.63/47.6
	Ceruloplasmin	P00450			2 (2)	29.13	5.44/122
A34	Haptoglobin	P00738	0.71	0.006	9 (25)	180.66	6.13/45.2
	Vimentin	P08670			3 (6)	35.02	5.06/53.6
A36	Collagen alpha 3 (VI)	P12111	0.73	0.028	3 (1)	46.08	6.27/343.7
	Glyoxalase domain containing protein	Q9HC38			2 (7)	27.54	5.4/34.8
A37	Lactotransferrin	P02788	1.53	0.002	14 (25)	242.76	8.5/78.2
	Ig alpha-1 chain	P01876			4 (15)	84.29	6.08/37.6
	Endoplasmin	P14625			5 (7)	81.95	4.76/92.4
	Serum albumin	P02768			4 (5)	65.24	5.92/69.4
A38	Dermcidin	P81605	1.44	0.013	1 (10)	14.69	6.09/11.3
A42	Serum albumin	P02768	0.67	0.028	12 (26.9)	120.3	5.92/69.4
A44	Annexin A2	P07355	0.65	0.024	1 (3.2)	10.20	8.53/40.4
A45	Annexin A6	P08133	0.72	0.0003	5 (10.5)	50.30	4.9/54
A48	Annexin A2	P07355	1.55	0.024	5 (19.5)	50.30	8.53/40.4
A49	No ID		1.21	0.002			
A50	Collagen alpha 3	P12111	1.22	0.009	1 (0.3)	10.10	6.27/344

Spots A2–A38 were identified using a Q-TOF MS and spots A39-A48 using an Orbitrap MS. Spot numbers correlate to those on Fig. 3A. In the case of more than one protein being identified for a particular spot only proteins with a score greater than 25 and more than two peptides are listed. The full list of proteins identified is in the Supporting Information Material. pI and molecular weights were obtained from Uniprot. For all spots, the values given for fold change and ANOVA are those for the entire group pre and post.

Table 6. List of protein identifications obtained from gel spots identified as having siginificantly ($p<0.05$) different volumes in baseline (pretreatment) samples from patients who responded well to anti-TNF-α therapy (Adalimumab) compared to those who responded poorly

Spot number	Protein	Accession number	Fold change	ANOVA	Number of peptides matched (% coverage)	Protein score	p*I*/mass kDa
A8	78kDa glucose-related protein	P11021	0.57	0.027	5 (9)	81	5.07/72.3
	Collagen alpha 3 (VI)	P12111			4 (1)	69.79	6.27/343.7
A9	Annexin A2	P07355	0.43	0.048	9 (27)	172.15	7.58/38.6
A12	Annexin A1	P04083	0.52	0.035	7 (22)	129.29	6.57/38.7
	Serum albumin	P02768			4 (6)	51.72	5.92/69.4
	Replication protein A	P15927			2 (6)	30.85	5.75/29.2
A19	Pyruvate kinase isozymes M1/M2	P14618	0.44	0.010	6 (15)	102.87	7.95/57.9
A20	Pyruvate kinase isozymes M1/M2	P14618	0.35	0.005	6 (14)	97.39	7.95/57.9
A22	Haptoglobin	P00738	1.72	0.044	9 (25)	182.31	6.13/45.2
	Collagen alpha 3	P12111			7 (2)	99.98	6.27/343.7
	Serum albumin	P02768			4 (7)	74.42	5.92/69.4
	Actin	P63261			4 (12)	66.68	5.31/41.8
	Lamin-B2	Q03252			4 (7)	64.08	5.28/67.7
A25	Collagen alpha 3 (VI)	P12111	0.74	0.023	6 (2)	88.46	6.27/343.7
	Rho GDP-dissociation inhibitor 2	P52566			6 (33)	100.53	5.1/23
	Heat shock 70 kDa protein	P08107			4 (8)	67.84	5.48/70.1
	Apolipoprotein A-I	P02647			3 (14)	46.57	5.56/30.8
A26	Heat shock 70 kDa protein	Q5SP16	0.42	0.004	5 (8)	72.56	5.48/70.1
A29	Alpha-1B-glycoprotein	P04217	1.90	0.029	5 (10)	63.00	5.58/54.3
A30	Heat shock cognate 71 kDa protein	P11142	1.61	0.009	11 (20)	197.51	5.38/71
	Collagen alpha 3 (VI)	P12111			3 (1)	47.32	6.27/343.7
	Actin	P63261			3 (6)	41.11	5.3/42
	Serum albumin	P02768			3 (5)	40.78	5.92/69.4
A39	Isoform M1 of pyruvate kinase isozymes M1/M2	P14618	0.49	0.007	4	40.18	7.96/66
A40	Vimentin	P08670	1.90	0.004	2	20.24	5.06/54
A46	Vimentin	P08670	0.59	0.021	1 (4.1)	10.30	5.06/54
A48	Annexin A2	P07355	0.58	0.019	5 (19.5)	50.30	8.53/40.4

Spots A8–A30 were identified using a Q-TOF MS and spots A39–A48 using an Orbitrap MS. The spot numberings correlate to those on Fig. 3B. In the case of more than one protein being identified for a particular spot only proteins with a score greater than 25 and more than two peptides are listed–the full list of proteins identified is in the Supporting Information Material. pI and molecular weights obtained from Swissprot/Uniprot.

were same but some were found to be different and unique to individual patient and treatment group.

3.5 Towards verification of potential biomarkers

Our results have demonstrated that many proteins change in the synovial tissue during the anti-TNF-α treatment and differences in protein expressions also exist with the use of different anti-TNF-α therapies involving specific TNF-α inhibitors. To confirm the observed synovial tissue protein changes we are developing MRM assays to each of the candidate proteins. MRM assays to some of the proteins have been reported in another recently published study from our group [20]. For the current study, to illustrate the MRM approach, we developed MRMs to four proteins. Following MRM assay validation, two proteotypic peptides were chosen for measurement of serum albumin and Apolipoprotein AI and 1 suitable proteotypic peptides was found suitable for each of serum amyloid P and haptoglobin proteins. Figure 4 shows the results from initial verification in which the proteins were measured in three patients from the AMC Amsterdam cohort at baseline (week 0) and week 4 of anti-TNF-α (Adalimumab) treatment. As can be seen in Figure 4, there was good agreement between the 2D-DIGE gel and MRM data as to whether each of the proteins was up or downregulated with anti-TNF-α treatment. For example, for haptoglobin, the MRM average fold change (in the three verification patient samples) over the first 4 weeks of treatment was 3.12 compared with an average fold change of 1.70 for gel spots identified as haptoglobin in all samples in the main cohort. The corresponding values (MRM vs. 2D gel spots) for serum amyloid P were 3.68 and 1.56. The two peptides measured for albumin appear to respond differently to anti-TNF-α treatment, with one being upregulated (average fold change = 0.30) and the other downregulated (fold change = 3.22). Albumin also

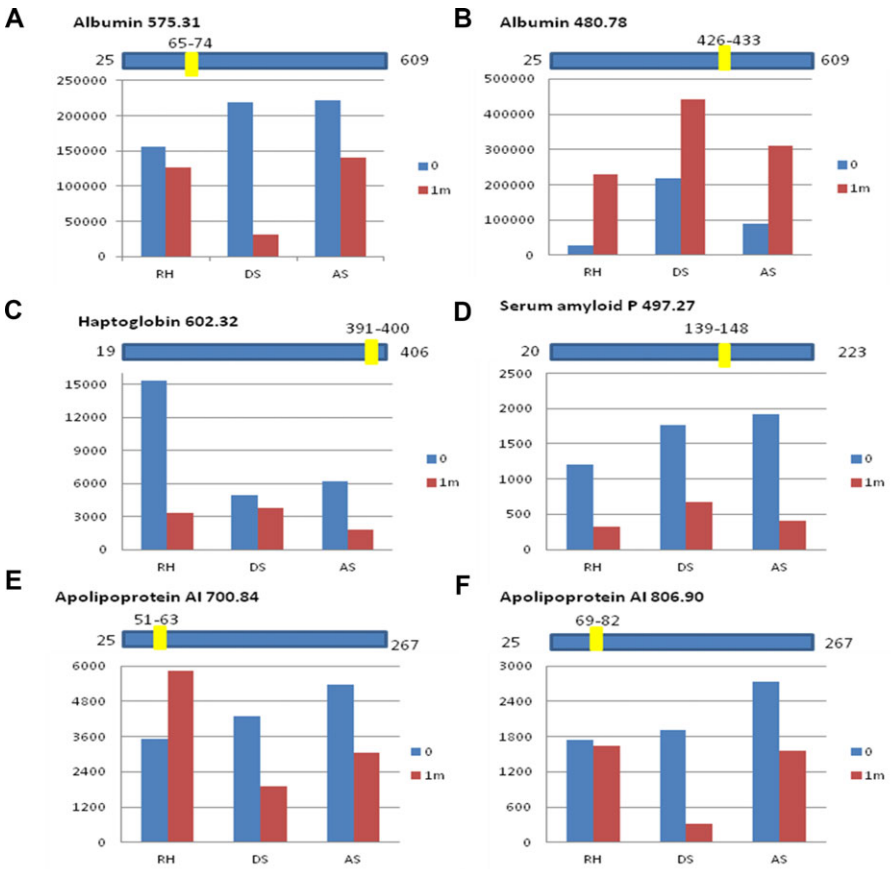

Figure 4. MRM measurement of potential biomarkers. Peak areas for the selected transitions of peptides from selected proteins **(A, B)** Albumin, **(C)** Haptaglobin **(D)** Serum amyloid, and **(E, F)** Apolipoprotein, were measured in patients from the AMC Amsterdam cohort at baseline (week 0) and week 4(1month; 1 m) of Adalimumab treatment.

exhibited similar behaviour in the gel study with some albumin containing gel spots upregulated and some downregulated by treatment. Apolipoprotein AI appeared to be very slightly upregulated with anti-TNF-α treatment in 2D gel experiments but in the MRM experiments both peptides were downregulated by anti-TNF-α treatment with average fold changes of 1.53 and 2.97.

4 Discussion

A wide range of different proteomic approaches, including 2DE, LC-MS, SELDI and protein arrays have been used with diverse protein samples (tissues, fluids, cell lines, etc.) for protein biomarker discovery. In practice, each of these different proteomic techniques has relative advantages and disadvantages [67–69]. Here, we have used a 2D gel-based strategy, including the use of DIGE [70], for synovial tissue biomarker discovery. A major limitation with the translation of biomarkers to clinical utility is the quality and the type of samples used in the discovery phase. Biological fluids such as serum, plasma, urine and saliva represent easily accessible bodily fluids, thereby making them an optimal sample type for a clinical assay but not necessarily for discovery experiments. Notably, proteins secreted at the site of disease are shed into the bloodstream, where they are subjected to an enormous dilution factor, thus making it difficult using existing proteomic approaches to identify with appropriate sensitivity changes between the disease and respective controls [71, 72]. Therefore, biological samples obtained directly from the site of inflammation, such as synovial fluid or synovial tissue in the case of articular disease, more likely are truly representative of the disease-related proteome. The challenge though is that the collection of these samples is invasive, is associated potentially with adverse effects and not all PsA patients present with swollen joints which can be biopsied or which provide sufficient synovial fluid sample for collection. This makes the acquisition of tissue samples for discovery challenging and their routine use for diagnostic purposes unlikely. We recognise therefore that the protein changes identified (using synovial tissue) here will require validation on both tissues and biological fluids. The latter, ideally in blood, will be essential if these proteins are to form a test that will have clinical utility.

Another major limitation with the translation of biomarkers to clinical utility is the identification of a relevant clinical question that addresses an area of unmet need [73]. In this work, we attempted to attend to both of these issues by developing a robust methodology for the discovery of potential protein biomarkers samples to predict and measure response to anti-TNF-α therapy in PsA, for which there is yet to date a great clinical unmet need. We have applied this to high-quality synovial tissue samples collected and processed

under SOP-like conditions from well-characterised patients including those enrolled in a controlled clinical study. To ensure high-quality 2DE separation, our experience of 2DE [74] has shown us that each sample type requires careful optimisation. We therefore undertook a number of experiments to ensure reproducible resolution of synovial proteins. An initial SVUH pilot study showed that significant changes in protein expression between patients during treatment and before the commencement of treatment can be measured and suggested that the approach could successfully (i) provide mechanistic information of the action of anti-TNF-α therapy and (ii) identify proteins predicting response to anti-TNF-α therapy.

In an initial SVUH pilot study, we selected proteins that were changing significantly and also of relatively high intensity and as a consequence some of these proteins were identified are abundant tissue and serum proteins including haptoglobin, albumin, collagen and fibrinogen (Tables 3 and 4). We are of the opinion that these proteins cannot be excluded as potential members of a multiplexed biomarker panel. From a larger, placebo-controlled AMC cohort we identified a more comprehensive list of potential biomarkers (Tables 5 and 6). As might be anticipated there was overlap between the proteins identified in the SVUH study and the AMC study although the increased number of samples in the latter afforded the opportunity to identify additional potential biomarkers. There were also differences in the proteins identified and this may be a consequence of the different time points used in the two studies or subtle differences in the mechanism of action of Etanercept and Adalimumab [75]. For example, Adalimumab (i) fully human monoclonal IgG antibody), but not Etanercept (a construct comprising soluble TNF-α receptors coupled to a monoclonal antibody) can lyse cells that express TNF-α on their surface and thus suppresses the inflammatory response more efficiently [76]. However, Etanercept (but not Adalimumab) is known to bind both TNF-α and lymphotoxin (TNF-β) and thus suppresses inflammatory responses efficiently employing two routes [77]. There was a good agreement in the potential baseline protein predictors of response between the two cohorts. Some of the proteins identified were found in more than one gel spot and this is likely to arise from variable post-translational modifications and/or protein proteolysis. Some PTMs can be seen as changes in the pattern of 'trains' of protein spots on a 2D gel and the appearance of lower molecular weight forms of proteins on 2DE gels may be indicative of proteolytic cleavage events and/or protein processing. This is likely to be of significant advantage and is particularly important in the analysis of samples from patients with PsA–a significant inflammatory disease, where it is known that several PTMs and proteolysis processes are important in the pathophysiology. For example, TNF-α is known to activate the release of proteolytic enzymes by chondrocytes [78]. These observations highlight the value of gel-based proteomics studies, which retain information of intact and degraded protein moieties.

From the analysis of the two cohorts, we have obtained a list of 43 proteins in synovium that change in expression with anti-TNF-α treatment. The proteins identified can be mostly classified into broad functional groups. Unsurprisingly several of the proteins detected were acute-phase proteins including haptoglobin, ceruloplasmin, transthyretin, fibrinogen, albumin and manganese superoxide dismutase. Three different annexins were found: annexin A1, A2 and A6, as were several cytoskeletal proteins: actin, lamin, vimentin and tubulin. Others proteins included collagens, immunoglobulins and transferases. Some of these proteins- haptoglobin, actin, serum albumin, annexin A2, serum amyloid P, Collagen alpha 3 and fibrinogen–were found to change in both the SVUH and AMC patients, but others were found to be altered with treatment in one patient group but not the other. Clearly, this may have been due to many reasons: the different time-points of sampling, lack of control samples in the SVUH cohort and perhaps the different types of anti-TNF-α therapy used. Several of the proteins identified have been shown in other studies to be disease-related, and specifically to be involved in inflammation/TNF-α pathways. For example, haptoglobin is locally induced in inflamed joints in arthritis [79, 80] and annexins are implicated in the down-regulation of inflammation [81]. Furthermore, annexin A1 has been identified as a secreted protein that mediates TNF-α-stimulated MMP-1 secretion [82]. Ubiquitin-conjugating enzyme Ubc13 E2 which is found to be upregulated in response to Adalimumab treatment in the AMC patient group is known to be a critical component of TNF receptor-associated factor (TRAF) and nuclear factor-kappa-B (NF-κB) mediated inflammatory response [83]. This indicates that a number of the changes observed here are in proteins related to TNF-α function and inflammation. The response of the acute-phase proteins to anti-TNF-α therapy is generally as would be expected following a reduction of inflammation. However it is noteworthy that ceruloplasmin, a positive acute-phase protein, appeared to increase with anti-TNF-α treatment.

Analysis of synovial tissue protein expression before treatment showed that in general there were significant differences between individual patients. However, linking the clinical outcome data to the protein expression profiles revealed consistent changes in protein expression between patients who either responded well to anti-TNF-α therapy or those who responded poorly. These consistently altered proteins included albumin, collagen alpha 3 and annexins A1 and A2. Interestingly, these proteins were identified in both the SVUH and AMC cohorts.

Amongst the proteins identified in the AMC cohort as potential predictive biomarkers of response there was a much lower occurrence of acute phase proteins with just one form of haptoglobin and apolipoprotein AI found to be present at significantly different levels between good and poorer responders. Several heat shock proteins (HSPA1A/B, HSPA8, HSPA5) appear in the list of proteins that may discriminate between good and poor responders. Other proteins, which

seem to show altered expression according to patient response, were three forms of pyruvate kinase, two forms of vimentin and two forms of annexin A2, as well as annexin A1. Obviously, it is clear from this 2D-DIGE-based study that mostly only strongly expressed proteins were detected, which shows that 2D-DIGE proteomic technology is not really suitable for the identification of low-abundant protein biomarkers, unless sample depletions (to remove high-abundant proteins) is undertaken prior to 2D-DIGE analysis.

In this 2D-DIGE-based study, we have undertaken the development of MRM's to four proteins: albumin, haptoglobin, apolipoprotein AI and serum amyloid P and have developed a robust MRM assay to each protein based on either 2 (albumin and apolipoprotein AI) or 1 (serum amyloid P and haptoglobin) peptide. MRM assays to some of the other proteins have been reported in another recently published LC-MS/MS-based study from our group [20]. Our study builds on this recently published LC-MS/MS study and utilizes a different proteomics approach (2D-DIGE) for the identification of novel predictive and prognostic biomarkers in response to Etanercept- and Adalimumab-based anti-TNF-α therapy in patients with PsA. The experiments illustrate the applicability of MRM methodology for the validation of potential biomarkers. For the four selected proteins of interest, it was possible to measure peak areas for the selected transitions and the response of these proteins to anti-TNF-α therapy was generally similar to that seen in the 2D-DIGE experiments identifying them as potential biomarkers. The authors of this study are aware that the scale of these preliminary exploratory validation experiments is too small for conclusive validation and further experiments are underway to develop MRM transitions for all proteins of interest, for full validation. Furthermore, to aid the translation of biomarker discovery into verification model, the proteomic changes observed in the synovial tissue samples are being investigated in accessible bodily fluids (serum samples from PsA patients) for clinical utility.

In conclusion, a DIGE-based strategy has demonstrated that anti-TNF-α therapy caused detectable changes in protein expression levels in PsA synovial tissue and that it may be possible to predict subsequent response to anti-TNF-α therapy by the measurement of protein expression levels before treatment commencement. Preliminary MRM measurements have illustrated the suitability of this methodology to validate the list of potential biomarkers generated in the DIGE-based studies in a potentially high-throughput and multiplexed manner.

Author contributions: E.S.C. performed and interpreted the experimental work, and drafted the manuscript. A.Q.B. helped with interpretation of data and final drafting of the manuscript. D.S.G., U.F., E.P. and M.J.D. were involved with study concept and design, and interpretation of data and critical analyses of the manuscript. A.W.V.K., D.M.G., D.J.V. and P.P.T. undertook the arthroscopic biopsies, and were involved with study concept and design as well as interpretation of data and critical analyses of the manuscript. O.F. and S.R.P. were involved in obtaining financial support, study concept and design, interpretation of data, critical comments on drafting of the manuscript and they assisted in the preparation of the final manuscript. All authors read and approved the final version of the manuscript.

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The remaining authors have declared no conflict of interest.

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