TECHNICAL BRIEF

Developing clinically relevant biomarkers in inflammatory arthritis: A multiplatform approach for serum candidate protein discovery

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Received: April 30, 2015
Revised: July 17, 2015
Accepted: August 27, 2015

Purpose: To identify candidate biomarkers that have the potential to distinguish between patients with psoriatic arthritis (PsA) or rheumatoid arthritis (RA) and explore the value of combining different protein discovery platforms for the development of a multiplexed protein biomarker panel.

Experimental design: Serum samples from 32 patients (PsA; $n = 16$ and RA; $n = 16$) defined as active, early onset, and treatment naïve were analyzed using unbiased label-free LC-MS/MS, a microsphere bead-based immunoassay (Luminex xMAP) and an aptamer-based assay (SOMAscan).

Results: LC-MS/MS was used to quantify 324 proteins, while the Luminex xMAP targeted 48 proteins and SOMAscan supported the measurement of 1129 proteins. The combined data from these techniques gave reproducible quantification of 1501 proteins in total. Of these, 42 (LC-MS/MS), 3 (Luminex xMAP), and 127 (SOMAscan) proteins were found to be differentially expressed between PsA and RA ($p < 0.05$).

Conclusion and clinical relevance: Using three different and potentially complementary proteomic platforms we identified a total of 172 proteins that are differentially expressed in patients with PsA compared to RA. These proteins collectively represent candidates for inclusion in a protein signature that could be developed as a diagnostic test to discriminate patients with PsA from RA and therefore be of clinical utility.

Keywords:
LC-MS/MS / Luminex / Multiplexing / Psoriatic and Rhuematoid Arthritis / SOMAscan

A major ambition in clinical proteomics is to develop multiplexed assays that can be used to diagnose disease [1]. Psoriatic arthritis (PsA), a form of inflammatory arthritis that is associated with psoriasis, presents with many clinical features several of which are observed in other diseases making it challenging to diagnose. In particular, with no diagnostic test currently available to distinguish PsA from other arthropathies and most notably rheumatoid arthritis (RA), the diagnosis of PsA is often difficult [2]. At present diagnosis of PsA relies on the experience of the treating clinician. Within rheumatology, it is widely acknowledged that the development of a blood-based test to distinguish PsA from RA would be of significant clinical value [3, 4]. Such a test would assist in early clinical diagnosis and allow more appropriate choices of medication to be made. Thus, while there are some medications that are effective for both PsA and RA (e.g. methotrexate; TNFα inhibitors), there are others that may result in adverse effects in PsA patients (e.g. hydroxychloroquine, corticosteroids). Also,

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Abbreviations: **ACR**, American College of Rheumatology; **PsA**, psoriatic arthritis; **RA**, rheumatoid arthritis; **TFE**, trifluroethanol; **TNF-α**, tumor necrosis factor alpha

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some medications have proven efficacy in RA but not PsA (e.g. rituximab, tocilizumab) and conversely some have proven efficacy in PsA but not RA (e.g. ustekinumab, apremilast, anti-IL17 therapies). Early discrimination of patients with PsA or RA could lead to the introduction of more appropriate and effective medication for individual patients that would result in better long-term outcomes [5, 6].

Ideally, a diagnostic test should be based on an easily accessible biological sample such as blood (serum or plasma). Furthermore, the assay must be suitably robust, specific, and sensitive [1, 7]. Serum represents an ideal biological sample as it is readily available and contains many proteins that are synthesized, secreted, shed, or lost from cells and tissues throughout the body. Fluctuations in the expression levels of these proteins can reflect a pathophysiological state [7]. The most commonly used platforms for the measurement of protein expression include MS, multiplexed ELISAs, and antibody microarrays. Despite the number of technologies available, limitations associated with these techniques have made it challenging to develop multiplexed protein biomarkers [1]. For example, the complexity of the serum proteome and its dynamic range exceeds the sensitivity and analytical capacity of MS. While serum is an easily accessible sample it presents difficulties for protein analysis because less than 20 of the most abundant proteins in serum account for greater than 96% of the total protein content. Moreover the remaining proteins are present at a dynamic concentration range well in excess of 12 orders of magnitude [8]. In MS analysis, the presence of higher abundant proteins interferes with the identification and quantification of lower abundant proteins. To facilitate the detection of these low abundant proteins it is common to remove the high abundant proteins prior to MS analysis. Even after depletion of these proteins, MS can only be used to routinely analyze proteins in ~ the top 5 orders of magnitude of relative abundance (Fig. 2A). The other platforms have limitations too. Drawbacks with multiplexed immunoassay platforms include the lack of availability of suitable high quality protein affinity ligands, cross-reactivity, and the high cost for multiplexed analysis per sample. SOMAscan is a more recently introduced aptamer-based multiplexed proteomic approach that can be used to quantify 1129 proteins in serum spanning over 10 orders of magnitude in abundance (Fig. 2A) [9]. It is apparent that no single technology is suitable for interrogating the entire serum proteome [9]. Individually proteomic techniques provide a modest to good coverage of proteins in complex serum samples, however multiplexing of different proteomic methods has the potential to cover a broader spectrum of serum proteins (Fig. 2A).

Given the heterogeneity of individuals and diseases such as inflammatory arthritis, it is widely anticipated that a single protein biomarker is not likely capable of distinguishing PsA from RA. A biomarker panel however, could be potentially more useful. The increased predictive power provided by a multibiomarker panel should compensate for heterogeneity within individual patients. This concept is supported by evidence provided by the recently introduced VECTRA DA test. This is a multibiomarker panel incorporating 12 serum proteins and it has been shown to more accurately reflect disease activity in RA compared to C-reactive protein that was considered the gold standard disease activity marker [10, 11]. To identify a multiplexed biomarker panel that might discriminate PsA and RA, we used three multiplexed proteomic platforms for the analysis of patient serum samples. Using unbiased label-free LC-MS/MS, in combination with the fixed or targeted multiplexed technologies; the Luminex xMAP (a microsphere bead-based immune assay capable of targeting 48 proteins) and SOMAscan (an aptamer-based assay for 1129 proteins) we sought to analyze large numbers of serum proteins spanning 12 orders of magnitude (Fig. 2A).

An aptamer is a short-single strand of nucleic acid, between 20 and 100 base pairs in length. It has been demonstrated that these molecules are capable of folding into a diverse spectrum of intricate structures that can bind to specific proteins. Gold et al. engineered a variation on the aptamer with desirable properties called the SOMAmer. These SOMAmers are modified so that some base pairs are replaced by photo reactive base pairs. Additionally some base pairs are chemically modified with protein like functional groups thereby increasing their affinity for certain target proteins. SOMAmers with properties which make them suitable for use in large multiplexed panels were selected in vitro by a process referred to Systematic Evolution of Ligands by EXponential enrichment (SELEX). A typical selection screens between 10^{14}–10^{15} possible unique nucleic acid sequences complementary to proteins of interest. SELEX is performed in the presence of a polyanionic competitor such as dextrane sulphate that promotes the dissociation of oligonucleotides with fast off-rates, thereby allowing the identification and selection of slow off rate aptamers [9, 12]. It has previously been documented that the SOMAscan is capable of quantifying up to a thousand proteins spanning seven orders of magnitude [9, 12]. This platform has been used to identify biomarkers in various disorders [9, 13, 14].

Serum samples were obtained from a cohort of 32 patients (Table 1) with 16 patients characterized as having PsA according to the classification of psoriatic arthritis criteria and 16 characterized as RA according to the American College of Rheumatology classification criteria (ACR) [15, 16]. Patients whose serum was used for analysis were early stage (symptom duration <12 months) and were treatment naïve. Pregnant patients were excluded from the study. Patient serum was obtained at baseline (onset of enrollment in the study), aliquoted, and stored at –80°C. After inclusion in the study, patients began treatment with either disease modifying antirheumatic drugs; DMARDs (e.g. methotrexate or hydroxychloroquine) or biologic therapy (e.g. anti-TNFα therapy) as selected by their treating clinician. See Table 1 for patient characteristics at baseline. The study was approved by St. Vincent's Healthcare Group Ethics and Medical Research Committee and patients were enrolled only after agreeing to participate in the study and given their informed consent.

Table 1. Patient characteristics at baseline

Baseline characteristics	Total ($n = 32$)	PsA ($n = 16$)	RA ($n = 16$)
Age (years)	44 ± 12	41 ± 11	48 ± 12
Female/male *n* (%)	20 (63%)	7 (44%)	13 (81%)
Symptom duration	< 1 year	< 1 year	< 1 year
ESR (mm/h)	17 ± 21	11 ± 6	26 ± 21
CRP (mg/L)	15 ± 21	6.4 ± 4.2	24 ± 27

CRP, C-reactive protein.

Prior to label-free LC-MS/MS analysis, individual serum samples were used to generate two pools per disease condition (Fig. 1). The 14 most abundant serum proteins (albumin, transferrin, haptoglobin, IgG, IgA, α1-antitrypsin, fibrinogen, α2-macroglobulin, α1-acid glycoprotein, complement C3, IgM, apolipoprotein AI, apolipoprotein AII, and transthyretin) were removed from serum samples using MARS Hu-14 column chromatography (Agilent Technologies, P/N 5188–6557) as previously described [17]. Briefly, 1700 μg of pooled serum samples from each disease group was diluted to 160 μL with commercially available HPLC Buffer A (Agilent Technologies) and centrifuged at 15 000 *g* for 5 min to remove particulate matter and lipids. Samples were then injected onto a MARS Hu-14 column and the low abundant protein fractions were eluted from the column with HPLC Buffer B (Agilent Technologies). Fractions containing the low abundant proteins were concentrated and desalted by centrifugation using 5 kDa spin columns (Agilent Spin Concentrators for Proteins, Agilent Technologies). The column was washed extensively with HPLC Buffer A in between individual pooled sample depletions. The concentrated low abundant proteins were immediately stored at –80°C and subsequently protein concentration in them was determined using a NanoDrop (ND-1000).

In-solution tryptic digestion was carried out on the pooled depleted serum samples proteins using sequencing grade modified porcine trypsin (Promega) as previously described [17]. Samples (containing 50 μg protein) were subjected to reduction (10 mM DTT, 50 mM NH_4HCO_3, and 50% trifluoroethanol (TFE) for 30 min at room temperature) and

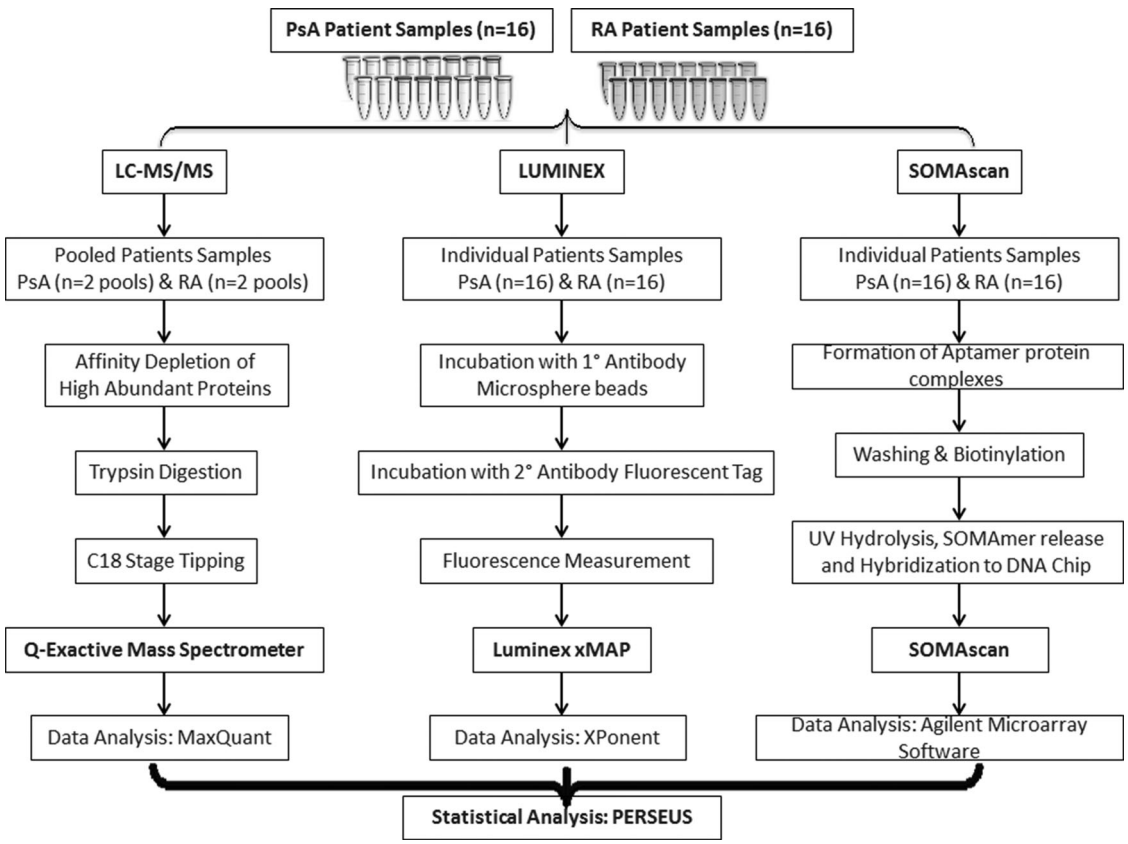

Figure 1. A multi-platform proteomic workflow. (A) LC-MS/MS, (B) Luminex, and (C) SOMAscan analysis of PsA and RA serum samples.

alkylation (20 mM iodoacetamide for 30 min at room temperature in dark). DTT was added to a final concentration of 10 mM. Buffer exchange was carried out using 5 kDA spin concentrators (Agilent) and washed three times with 3 mL of 5% v/v TFE, 50 mM NH_4HCO_3. Samples were recovered and the concentrator then washed twice with 50 μL of 5% TFE in 50 mM NH_4HCO_3. 20 μg trypsin was resuspended in 20 μL of 50 mM NH_4HCO_3 and appropriate amount of trypsin was added to the samples (trypsin to substrate ratio = 1:100) that were incubated at 37°C for 20 h at 500 rpm in a thermomixer The digested samples were evaporated to dryness, resuspended in 0.5% TFA and then desalted and purified using C18 resin pipette stage tips. Purified samples were dried under vacuum and resuspended in mass spectrometer compatible buffer A (3% ACN, 0.1% formic acid) and stored in aliquots of 50 μL at an estimated concentration of 1 μg/μL at –80°C. The equivalent of 6 μg of sample was then washed through a C18 stage tip as described previously [18, 19]. A technical reference pool sample was prepared by pooling equal amount of protein derived from all the stage tipped samples. Label-free nano-flow LC-MS/MS analysis was performed on a Q-Exactive mass spectrometer equipped with a Dionex Ultimate 3000 (nano-RSLC) chromatography system (Thermo Fisher Scientific). Two microliters (equivalent to 2 μg of digested protein) of each sample was injected onto a fused silica emitter (75 μm id, pulled using a laser puller (Sutter Instruments P2000)), packed with Reprocil Pur C18 (1.9 μm) reverse phase media and was separated by an increasing acetonitrile gradient over 101.5 min (flow rate of 250 nL/min). The mass spectrometer was operated in positive ion mode with a capillary temperature of 320°C, and with a potential of 2300 V applied to the frit. All data were acquired in automatic data-dependent switching mode. A high resolution (70 000) MS scan (300–1600 m/z) was performed using the Q Exactive to select the 12 most intense ions prior to MS/MS analysis using HCD.

Label-free LC-MS/MS data analysis was undertaken using Maxquant (V1.4.1.3) to automatically extract peptide isotopic patterns, align, and normalize total peptide signals across multiple samples [20]. Protein identifications were generated using Maxquant's inbuilt Andromeda search engine and searching against the Uniprot Human database (release 07–2014). Search parameters consisted of enzyme: trypsin; allowing up to two missed cleavages; species: Homo sapiens; fixed modification: carbamidomethylated cysteine; variable modification: oxidation methionine; and minimum peptide length of 7 amino acids [21]. The precursor mass tolerance window was set to 6 ppm and the product mass tolerance was 20 ppm. Peptide and protein FDR were set to 0.01%. The label-free quantification values were generated with a minimum of two peptides required per protein and with the maximum number of peptides compared across all samples. Protein ratios were calculated as the median of all ratios of common peptides identified across different samples [21].

Using the same 32 patient samples, individual samples were subjected to in-house developed and validated multiplexed immunoassays to measure 48 analytes using Luminex xMAP proteomics technology (Austin TX, USA) (Fig. 1). The assays were performed as previously described [22–24]. Forty-eight different carboxylated magnetic beads, each with a distinct emitting fluorescence pattern, were purchased from Luminex Corporation (Austin, TX, USA). Capture antibodies (commercially purchased) for 48 analytes were covalently coupled to the microspheres as described previously [22–24]. Briefly, specific heterophilic immunoglobulins were preabsorbed from all samples with heteroblock (Omega Biologicals, Bozeman, MT, USA). Patient samples were run undiluted and diluted 1:50 in high performance ELISA buffer (Sanquin, Amsterdam, The Netherlands). Calibration curves from recombinant protein standards were prepared using two fold dilution steps in serum diluent (R&D Systems, Abingdon, UK) as described previously [22–24]. A mixture containing 1000 microsphere beads per mediator (total volume 10 μL/well) was incubated together with a standard, sample, or blank for 1 h at room temperature. After washing, biotinylated antibodies (250 ng) were added to each sample and incubation continued for an additional 60 min. Beads were then washed and incubated for 10 min with 50 ng streptavidin rphycoerythrin (BD Biosciences, San Diego, California, USA). The fluorescence intensity of the beads was measured in a final volume of 100 μl high performance ELISA buffer. Acquisition was performed with a BioRad FlexMAP3D (Bio-Rad laboratories, Hercules, USA) in combination with xPONENT software, version 4.2 (Luminex). Data were analyzed by 5-parametric curve fitting using Bio-Plex Manager software, version 6.1.1 (BioRad).

In parallel, the 32 individual patient serum samples were subjected to a multiplexed aptamer-based assay (SOMAscan) that has been developed by Gold et al. to measure the levels of 1129 proteins as described before (Fig. 1) [9]. Briefly, in the first step of the assay 150 μL of serum was incubated with Slow Off Rate Modified Aptamers (SOMAmers, labelled with photocleavable biotin and a fluorescent dye) in-solution and protein-SOMAmer complexes formed. Following this, the complexes were applied to streptavidin beads, then washed extensively to remove no specifically bound proteins. Following this the remaining bound proteins were biotinylated. The protein-SOMAmer complexes were released into solution by photo-activated cleavage and bound to avidin beads by virtue of the biotin-labeled proteins. Following washing to remove free SOMAmers those covalently attached to protein were released by increasing the pH and hybridized to a DNA microarray. The identity and concentration of proteins in the original sample were revealed by spot localization and fluorescence intensity on the microarray. Microarray slides were imaged (Agilent G265, MicroArray Scanner System, Agilent technologies) and resulting tiff images were processed (using Agilent feature extraction software version 10.5.1.1) [9]. SOMAscan analysis was carried out by Somalogic, Inc. Boulder, CO, USA.

Statistical analysis (Univariate Student's *t*-tests and Principal Component Analysis) of the LC-MS/MS, Luminex, and

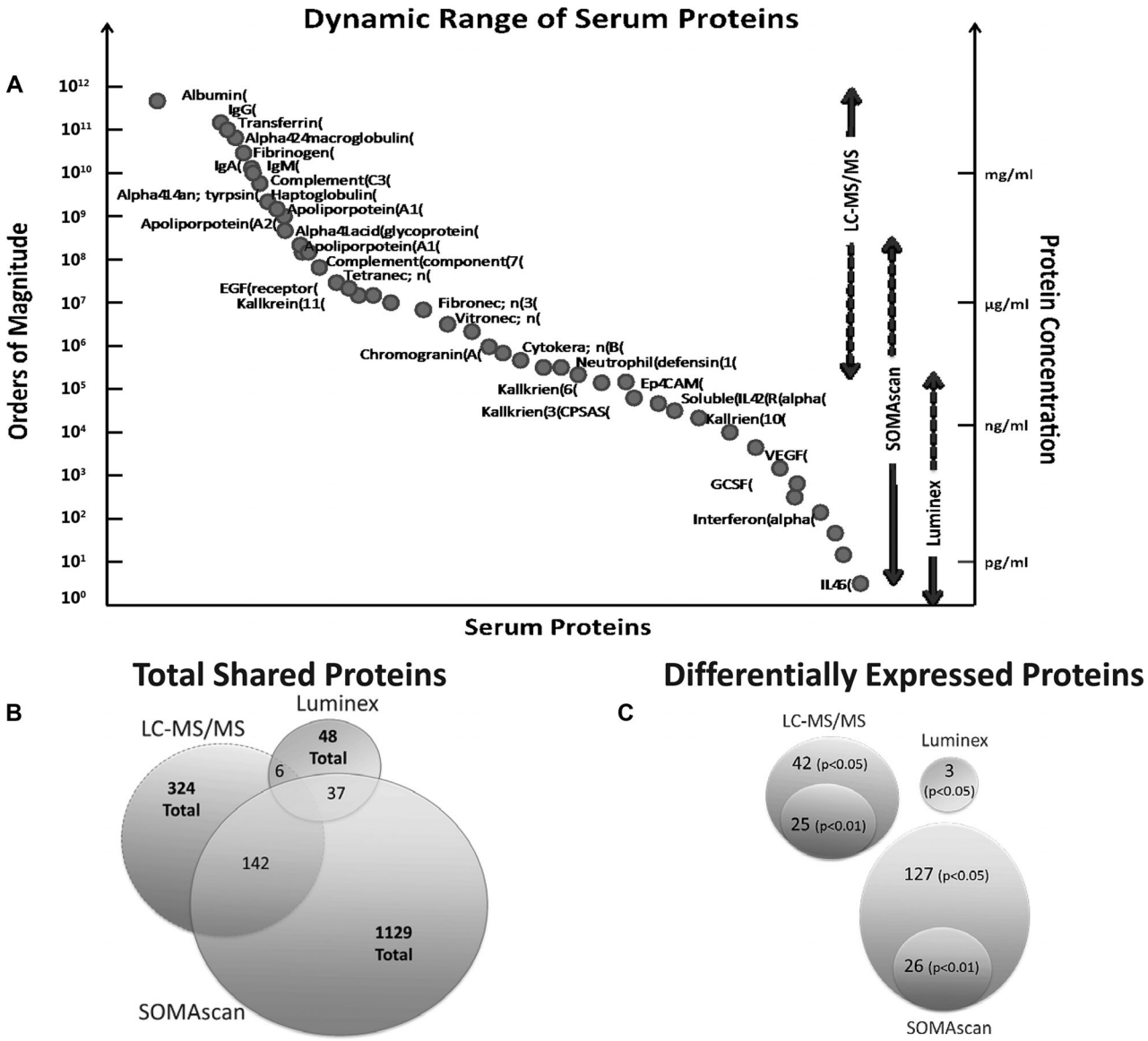

Figure 2. Comprehensive proteomic analysis of PsA and RA serum reveals differentially expressed proteins. (A) LC-MS/MS, Luminex, and SOMAscan platforms were used to perform a comprehensive proteomic analysis of PsA and RA serum proteins. (B) Venn diagram of proteins differentially expressed in PsA and RA ($p \leq 0.05$).

SOMAscan data were performed using the statistical software package Perseus (version 1.4.1.3) provided as part of Maxquant.

Applying these three proteomic technologies—label-free LC-MS/MS, Luminex xMAP, and SOMAscan—we successfully identified and quantified 324 proteins, 48 cytokines & chemokines and 1129 proteins, respectively (Fig. 2B). Of these, 42, 3, and 127 proteins were differentially expressed between PsA and RA (at p value < 0.05). The corresponding figures were 25, 0, and 26 proteins when a higher p value of < 0.01) (Fig. 2C). Some overlap in the proteins measured by the three platforms was observed. Principal component analysis of differentially expressed proteins from all three platforms allowed the separation of patients with PsA form RA (Fig. 3A–C). Principal component analyses of the LC-MS/MS and SOMAscan data revealed good separation between the PsA and RA patient groups, however the separation for the Luminex data were not clear (Fig. 3B). Interestingly there was no overlap in the proteins shown to be differentially expressed by the three platforms. This was somewhat unexpected because 37 proteins of the 48 measured on the Luminex platform were also targeted by the SOMAscan assay and 142 of the proteins identified by LC-MS/MS were present on the SOMAscan panel of 1129 proteins (Fig. 2B).

The full list of differentially expressed proteins will be reported as part of a future study once they have been subjected

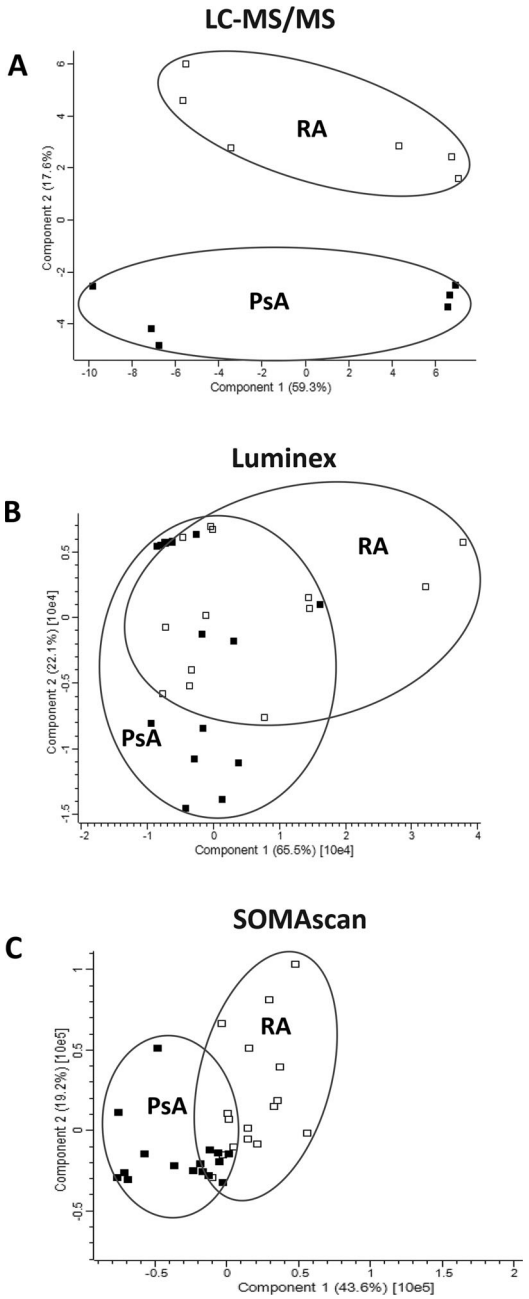

Figure 3. Principal component analysis of differentially expressed proteins in PsA and RA serum. Principal component analysis of (A) 42 proteins identified by LC-MS/MS, (B) three proteins identified by Luminex and (C) 127 identified by SOMAscan.

to clinical evaluation using additional patient samples from separate patient cohorts. However, it is of interest to note that of the three proteins identified as differentially expressed between PsA and RA from the Luminex data, two (interleukin-6 and leptin) are part of the Vectra DA multiprotein biomarker test that provides a molecular measure of disease activity in RA patients [10, 25].

The discovery and development of biomarkers is a multistep process involving an initial biomarker discovery, subsequent biomarker clinical evaluation, and validation [26–28]. In a clinical proteomics study, label-free LC-MS/MS is suitable for analyzing large number of clinical samples [27, 28]. However, LC-MS/MS alone does not cover the large dynamic range of proteins that are spread across 12 orders of magnitude in complex biological samples such as serum or plasma. Evidently, much deeper comprehensive analysis could be performed via individual sample fractionation prior to LC-MS/MS analysis, which allows for the identification of several thousand proteins. However, this considerably increases the cost and time of such experiments and combining data across the fractionated samples is challenging not least in maintaining analytical reproducibility [29, 30]. Thus, it is more practical and economical and common to use fractionation-free approaches for proteomic analysis. Here, we have investigated the potential of combining proteomic techniques to improve protein coverage while retaining quantitative reproducibility.

In this study, we were able to identify and quantify 324 proteins using label-free LC MS, while a multiplexed immunoassay Luminex xMAP targeted 48 proteins and SOMAscan supported the measurement of 1129 proteins (Fig. 2B). By combining the data from these techniques we were able to reproducibly quantify a total of 1501 proteins. Furthermore, these techniques allowed us to analyze serum proteins that spanned about 12 orders of magnitude in relative abundance (Fig. 2A). Statistical analysis of all three datasets revealed lists of proteins that were significantly differentially expressed in serum samples of patients with PsA and RA (Fig. 2C). While there was some overlap in the proteins measured by the three platforms, there was no overlap in the proteins that were shown to be differentially expressed proteins between PsA and RA by all three platforms. This was not surprising to find, given that each of the three proteomic platforms utilize different methodologies and have different capabilities [31]. Principal component analysis of differentially expressed proteins from all three platforms allowed the separation of patients with PsA form RA (Fig. 3A–C). Principal component analyses for the LC-MS/MS and SOMAscan data revealed good separation between the PsA and RA patient groups, however the separation for the luminex data were not clear (Fig. 3B). This may be because principal component analysis searches for linear combinations of variables and the relationships in the Luminex data may be nonlinear in nature, as is often the case with complex biological data [32].

Many candidate biomarkers for PsA and RA have already been reported in the literature, however relatively few have reached clinical use. It has become evident that a significant challenge in the transition of biomarker candidates to clinical diagnostic assays lies in the development of suitably robust biomarker assays, especially multiplexed assays, and their clinical validation in appropriate patient sample cohorts [33]. Thus, translating findings made during this discovery phase into the clinic presents as an onerous task that will require a rigorous validation strategy and systematic study design.

At the outset, access to validation cohorts and knowledge of the number of patient samples required for the clinical evaluation and validation is key. Additionally, a good understanding of the analytical and regulatory requirements for a clinical assay is essential [31, 34, 35]. Confounding factors have a huge impact on the validation of biomarkers, which range from genetic heterogeneity in individual patients to the different methods used for measuring proteins. Indeed, the identification of appropriate bioinformatic and statistical tools capable of integrating data derived from multiple proteomic platforms present a significant challenge as we move forward [31, 36, 37]. Thus, although multiplexing of different proteomic techniques provides numerous advantages such as broad range coverage of complex biological samples, it adds to the complexity of the validation strategy.

In conclusion, we anticipate that the proteins identified here may support the development of a biomarker panel that could distinguish patients with early PsA from those with RA. Studies to evaluate the discriminatory potential of this panel are currently underway. Furthermore, continued proteomic discovery on a larger initial cohort of patients is being undertaken to support the robust identification of additional biomarkers. We believe these have the potential to result in the development of a biomarker signature that could be used to distinguish patients with PsA from those with RA with high sensitivity and specificity and as such would provide a much needed tool to support clinical decision making. It is of note here that this study did not include a psoriasis (Ps) cohort. A future interesting study might include Ps patients. Investigating the differences in proteins expressed by Ps patients from those with PsA and RA might prove very fruitful and could contribute to our understanding of the pathogenic mechanisms that underpin IA.

The authors would like to acknowledge financial support from the European Commission under the EU FP7 Programme "MI-AMI" – Monitoring Innate immunity in Arthritis and Mucosal Inflammation. The authors would also like to thank Tony Bartlett, David MacAllan, Kirsten Wall, and Evan King for helpful discussions and access to SOMAscan technology. The UCD Conway Institute and Proteome Research Centre is funded by the Programme for Research in Third level Institutions, as administered by Higher Education Authority of Ireland.

Angela Mc Ardle, Aisha Qasim Butt, Agnes Szentpetery, Wilco de Jager, Sytze de Roock, and Stephen R. Pennington declare no conflicts of interest. Oliver Fitzgerald declares the receipt of research grant support from Pfizer, Abbott, Roche, MSD, Amgen, and UCB.

References

[1] Wilson, R., Sensitivity and specificity: twin goals of proteomics assays. Can they be combined? *Expert Rev. Proteomics* 2013, *10*, 135–149.

[2] Butt, A. Q., Mc Ardle, A., Gibson, D. S., FitzGerald, O., Pennington, S. R. Psoriatic arthritis under a proteomic spotlight: application of novel technologies to advance diagnosis and management. *Curr Rheumatol Rep.* 2015, *17*(5); 35.

[3] Gladman, D. D., Clinical, radiological, and functional assessment in psoriatic arthritis: is it different from other inflammatory joint diseases? *Annal. Rheumatic Dis.* 2006, *65*, iii22–24.

[4] Taylor, W., Gladman, D., Helliwell, P., Marchesoni, A., Mease, P., Mielants, H., Group, C. S., Classification criteria for psoriatic arthritis: development of new criteria from a large international study. *Arthritis Rheumatism* 2006, *54*, 2665–2673.

[5] Leung, Y. Y., Tam, L. S., Kun, E. W., Li, E. K., Psoriatic arthritis as a distinct disease entity. *J. Postgraduate Med.* 2007, *53*, 63–71.

[6] Ritchlin, C. T., Kavanaugh, A., Gladman, D. D., Mease, P. J. et al., Treatment recommendations for psoriatic arthritis. *Anal. Rheumatic Dis.* 2009, *68*, 1387–1394.

[7] Anderson, N. L., Anderson, N. G., The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell. Proteomics* 2002, *1*, 845–867.

[8] Millioni, R., Tolin, S., Puricelli, L., Sbrignadello, S. et al. High abundance proteins depletion vs low abundance proteins enrichment: comparison of methods to reduce the plasma proteome complexity. *PloS One* 2011, *6*, e19603.

[9] Gold, L., Ayers, D., Bertino, J., Bock, C. et al., Aptamer-based multiplexed proteomic technology for biomarker discovery. *PloS One* 2010, *5*, e15004.

[10] Eastman, P. S., Manning, W. C., Qureshi, F., Haney, D. et al., Characterization of a multiplex, 12-biomarker test for rheumatoid arthritis. *J. Pharm. Biomed. Anal.* 2012, *70*, 415–424.

[11] Mc Ardle, A., Flatley, B., Pennington, S. R., FitzGerald, O., Early biomarkers of joint damage in rheumatoid and psoriatic arthritis. *Arthritis Res. Ther.* 2015, *17*, 141.

[12] Wilson, R., High-content aptamer-based proteomics. *J. Proteomics* 2011, *74*, 1852–1854.

[13] Ostroff, R. M., Bigbee, W. L., Franklin, W., Gold, L. et al., Unlocking biomarker discovery: large scale application of aptamer proteomic technology for early detection of lung cancer. *PloS One* 2010, *5*, e15003.

[14] Sattlecker, M., Kiddle, S. J., Newhouse, S., Proitsi, P. et al., Alzheimer's disease biomarker discovery using SOMAscan multiplexed protein technology. *Alzheimer's Dementia* 2014, *10*, 724–734.

[15] Aletaha, D., Neogi, T., Silman, A. J., Funovits, J. et al., 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheumatism* 2010, *62*, 2569–2581.

[16] Kay, J., Upchurch, K. S., ACR/EULAR 2010 rheumatoid arthritis classification criteria. *Rheumatology* 2012, *51*, vi5–9.

[17] Morrissey, B., O'Shea, C., Armstrong, J., Rooney, C. et al., Development of a label-free LC-MS/MS strategy to approach the identification of candidate protein biomarkers of disease recurrence in prostate cancer patients in a clinical trial of combined hormone and radiation therapy. *Proteomics Clin. Appl.* 2013, *7*, 316–326.

[18] Rappsilber, J., Ishihama, Y., Mann, M., Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* 2003, *75*, 663–670.

[19] Turriziani, B., Garcia-Munoz, A., Pilkington, R., Raso, C. et al., On-beads digestion in conjunction with data-dependent mass spectrometry: a shortcut to quantitative and dynamic interaction proteomics. *Biology* 2014, *3*, 320–332.

[20] Cox, J., Mann, M., MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 2008, *26*, 1367–1372.

[21] Cox, J., Neuhauser, N., Michalski, A., Scheltema, R. A., Olsen, J. V., Mann, M., Andromeda: a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res.* 2011, *10*, 1794–1805.

[22] de Jager, W., Hoppenreijs, E. P., Wulffraat, N. M., Wedderburn, L. R. et al., Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. *Annal. Rheumatic Dis.* 2007, *66*, 589–598.

[23] de Jager, W., Prakken, B. J., Bijlsma, J. W., Kuis, W., Rijkers, G. T., Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. *J. Immunol. Methods* 2005, *300*, 124–135.

[24] de Jager, W., te Velthuis, H., Prakken, B. J., Kuis, W., Rijkers, G. T., Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. *Clin. Diagnostic Lab. Immunol.* 2003, *10*, 133–139.

[25] Curtis, J. R., van der Helm-van Mil, A. H., Knevel, R., Huizinga, T. W. et al., Validation of a novel multibiomarker test to assess rheumatoid arthritis disease activity. *Arthritis Care Res.* 2012, *64*, 1794–1803.

[26] Ademowo, O. S., Hernandez, B., Collins, E., Rooney, C. et al., Discovery and confirmation of a protein biomarker panel with potential to predict response to biological therapy in psoriatic arthritis. *Annal. Rheumatic Dis* 2014, *0*, 1–8.

[27] Collins, B. C., Miller, C. A., Sposny, A., Hewitt, P. et al., Development of a pharmaceutical hepatotoxicity biomarker panel using a discovery to targeted proteomics approach. *Mol. Cell. Proteomics* 2012, *11*, 394–410.

[28] Staunton, L. C., Tonry, C., Ademovo, S., Dharsee, M. et al., in: C. EaS. Gaskell (Eds), *Quantitative Proteomics*, RCS- royal society of chemistry, 2014. DOI:10.1039/9781782626985.

[29] Court, M. G. J., Masselon, C. D., Urine sample preperation and fractionation for global proteome profiling by LC-MS. *Methods Mol. Biol.* 2015, *1243*, 175–176.

[30] Prieto, J. H. F. E., Koncarevic, S., Yates, J., Becker, K., Large-scale differential proteome analysis in *Plasmodium falciparum* under drug treatment. *Methods Mol. Biol.* 2015, *1201*, 268–279.

[31] Omenn, G. S., Strategies for plasma proteomic profiling of cancers. *Proteomics* 2006, *6*, 5662–5673.

[32] IJ, Principal component analysis. *Encylopedia Stat. Behav. Sci.* 2014, q6. DOI: 10.1002/9781118445112.stat06472.

[33] Gibson, D. S., Rooney, M. E., Finnegan, S., Qiu, J. et al., Biomarkers in rheumatology, now and in the future. *Rheumatology* 2012, *51*, 423–433.

[34] Hernandez, B., Parnell, A., Pennington, S. R., Why have so few proteomic biomarkers "survived" validation? (Sample size and independent validation considerations). *Proteomics* 2014, *14*, 1587–1592.

[35] Vidal, M., Chan, D. W., Gerstein, M., Mann, M. et al., The human proteome—a scientific opportunity for transforming diagnostics, therapeutics, and healthcare. *Clin. Proteomics* 2012, *9*, 6.

[36] Chandra, P. E., Sokolove, J., Hipp, B. G., Lindstrom, T. M. et al., Novel multiplex technology for diagnostic characterization of rheumatoid arthritis. *Arthritis Res. Therapy* 2011, *13*, R102.

[37] Wener, M. H., Multiplex, megaplex, index, and complex: the present and future of laboratory diagnostics in rheumatology. *Arthritis Res. Therapy* 2011, *13*, 134.

