

Stratification and Monitoring of Juvenile Idiopathic Arthritis Patients by Synovial Proteome Analysis

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Juvenile idiopathic arthritis (JIA) comprises a poorly understood group of chronic, childhood onset, autoimmune diseases with variable clinical outcomes. We investigated whether profiling of the synovial fluid (SF) proteome by a fluorescent dye based, two-dimensional gel (DIGE) approach could distinguish patients in whom inflammation extends to affect a large number of joints, early in the disease process. SF samples from 22 JIA patients were analyzed: 10 with oligoarticular arthritis, 5 extended oligoarticular and 7 polyarticular disease. SF samples were labeled with Cy dyes and separated by two-dimensional electrophoresis. Multivariate analyses were used to isolate a panel of proteins which distinguish patient subgroups. Proteins were identified using MALDI-TOF mass spectrometry with expression further verified by Western immunoblotting and immunohistochemistry. Hierarchical clustering based on the expression levels of a set of 40 proteins segregated the extended oligoarticular from the oligoarticular patients ($p < 0.05$). Expression patterns of the isolated protein panel have also been observed over time, as disease spreads to multiple joints. The data indicates that synovial fluid proteome profiles could be used to stratify patients based on risk of disease extension. These protein profiles may also assist in monitoring therapeutic responses over time and help predict joint damage.

Keywords: Juvenile • arthritis • proteomics • inflammation • synovial

Introduction

Around one in every thousand children in the U.K. suffer from juvenile idiopathic arthritis (JIA).¹ The worldwide reported incidence varies on a geographical basis, from 0.7 per 1000 in USA to 4.0 per 1000 in Australia.² JIA is a heterogeneous group of inflammatory disorders primarily affecting the musculoskeletal system. Of the seven subsets of JIA identified according to the ILAR classification,³ three, oligoarticular, extended oligoarticular, and polyarticular are the commonest. Adverse outcomes can present to varying degrees regardless of disease subtype, but persistently inflamed joints are a major risk factor.⁴ Typically, the course of JIA is one of exacerbation and remission. In addition, nearly half the patients on disease modifying therapies will have relapses after premature discontinuation

of treatment. The rate of relapses may be influenced by residual synovial inflammation, not clinically apparent.⁵

Current clinical, laboratory or radiological parameters cannot accurately predict disease extension. In approximately 25% of children with oligoarticular JIA, over time the disease will spread to involve many joints, extended oligoarticular disease.⁶ Extended oligo JIA is much more difficult to treat due to its characteristic resistance to first-line therapies.⁷ Predictive tests that forecast disease extension could allow subsequent treatment decisions to be made in a preventative instead of a reactive manner. It is pertinent to define more sensitive markers for determining the risk of unremitting inflammatory arthritis in JIA.

In this study, the synovial fluid (SF) proteome of the persistent oligoarticular patient subgroup is compared to that of patients who show a spread after the first 6 months to involve five or more joints, the extended oligoarticular subgroup. If it was possible to identify these children earlier, one could instigate more effective therapies to prevent joint and periarticular damage. Comparisons are also made between the SF proteome of polyarticular patients who are diagnosed with more than 4 joints involved within 6 months of onset. Comparison between the poly and extended oligoarticular group

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may help us understand why the latter are more resistant to standard therapies than the former. Further differentiation of the oligoarticular group will highlight those proteins which may ultimately contribute to disease extension.

A pilot study uncovered fragments of extracellular matrix and T-cell receptor proteins, products of the degradative proteolytic environs of the recurrently inflamed knee.⁸ The synovial fluid proteome has a dynamic range of protein concentration reflecting its filtration from plasma, but specific additions are made at the site of joint disease.⁹ The current investigation has been designed to reveal SF protein expression patterns to identify reliable predictors of JIA subgroup and track disease course in detail. With further validation, these putative prognostic biomarkers could improve the clinical management of patients.

Methods

Patients. Twenty-two patients with newly diagnosed *untreated* JIA according to ILAR criteria entered this study and were followed for 1 year. At the time of initial sampling, there were 15 children with oligoarticular arthritis and 7 with RF-ve polyarticular arthritis. At 1 year, 5 oligoarticular had been reclassified as having extended oligoarticular JIA.

All patients were examined by a consultant rheumatologist (M.E.R.) who confirmed their diagnosis. For the purposes of this study, only initial synovial fluids from children with disease duration of less than 6 months, and untreated disease, were studied. Arthrocentesis and joint steroid injection was performed according to clinical need. Clinical details recorded included subtype of JIA, age, sex, disease duration, local and erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). Treatments applied after samples were removed are also listed. Local inflammation was defined as both joint swelling and pain on physical examination.

All SFs were aspirated using an aseptic technique and plasma obtained at the same procedure. Samples were immediately centrifuged at 5000g for 15 min at 4 °C, aliquoted and stored at -80 °C until for at least 1 year to allow for subsequent clinical reclassification. Medical Ethics Committee approval was obtained for this study at Green Park Healthcare Trust and patient assent and parent informed consent given (ORECNI 408/03).

Sample Preparation. Samples were dialyzed overnight at 4 °C in distilled water to remove salts, with 3.5 kDa cutoff Slide-A-Lyzer cassettes (Pierce Biotechnology, Inc., Rockford, IL). Each sample was snap frozen in liquid nitrogen and lyophilized overnight on a freeze-dryer (Martin Christ GmbH, Osterode am Harz, Germany). Samples were rehydrated in sample rehydration buffer (8 M Urea, 2% CHAPS and 0.002% bromophenol blue; Invitrogen Ltd., Paisley, U.K.). Protein concentrations were measured using the PlusOne 2-D Quant kit according to manufacturer's guidelines (GE Healthcare, Bucks, U.K.).

Difference In-Gel Electrophoresis (DIGE). DIGE was performed at room temperature with Ettan IEF and vertical gel systems and associated power supply, strips, gels and reagents according to the manufacturer's guidelines (GE Healthcare, Bucks, U.K.). Each synovial fluid and plasma sample was minimally labeled with Cy5 and Cy3 fluorescent dyes and inclusion of an internal pooled standard (Cy2) according to the manufacturer's recommendations. A total of 50 µg of Cy5 and Cy3 samples and Cy2 standard was combined and resuspended in an equal volume of 2× sample buffer (8 M Urea, 130 mM DTT, 4% (w/v) CHAPS, 2% (v/v) Pharmalyte 4-7). A 24 cm Immobiline DryStrip pH 4-7 linear immobilized pH gradient

(IPG) strip was rehydrated overnight with the sample mix. The first-dimension separation of proteins by isoelectric focusing (IEF) was performed for a total of 75 000 Vh (2 mA/5W limit per strip) including a final 8000 V step for 1 h to obtain high quality resolution.

After IEF, the strips were equilibrated first in 1% (w/v) dithiothreitol and then 2.5% (w/v) iodoacetamide. IPG strips were laid into single well 12% PAGE gels and sealed in with 1% agarose (w/v) in running buffer (25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS and bromophenol blue). The second-dimension separation was run at 0.75 W/gel for 19 h. Two preparative pick gels loaded with 500 µg of unlabeled sample were silver-stained for spot picking.

Image and Cluster Analysis. Prelabeled proteins were visualized using a Typhoon 9410 imager (GE Healthcare, Bucks, U.K.). Gel image analysis was performed with Progenesis Samespots software version 2.0 build 2644.18003 (Nonlinear Dynamics Ltd. Newcastle upon Tyne, U.K.), comprising gel warping, DIGE normalization and comparison modules. Briefly, all gel images were aligned to a reference gel and the same spot outlines overlaid onto all images ensuring no data was omitted. The normalized volume (NV) for each spot on each gel was calculated by the software from the Cy3 (or Cy5) to Cy 2 spot volume ratio. The software performs log transformation of the spot volumes to generate normally distributed data. Log normalized volume (LNV) was used in quantifying differential expression.

Replicate normalized volumes were used to create 'master' gels for a series of corresponding SF or plasmas. Quantitative differential spot analysis was performed on 'master' gels of the patient subgroups. Pairwise comparisons were made to establish patient-to-patient and Cy3-to-Cy5 dye variations. Within the Samespots review module, each comparison was filtered to find the spots (a) with a *p*-value ≤ 0.05 for the paired *t* test, and (b) having a greater than 2-fold change in average LNV expression between the groups.

Expression data was analyzed using Epclust, a generic data clustering, visualization, and analysis tool (<http://www.bioinf.ebc.ee/EP/EP/EPCLUST/>). Hierarchical analysis reordered protein expression patterns in an agglomerative fashion, using the unweighted pair-group average (UPGMA) clustering procedure. Pearson ranked correlation was the similarity measure used to group or separate the expression data. A heat map was produced accompanied by a dendrogram depicting the extent of similarity between the different groups in the samples.

Mass Spectrometry Identification and Verification. Protein spots were excised from silver-stained 2D gels and digested by use of the protocol described.¹⁰ Briefly, the gel spots were washed, reduced and alkylated followed by dehydration with acetonitrile. The proteins were digested overnight with trypsin (Promega, modified trypsin) at 37 °C, and the resulting peptides were concentrated on a ZipTip micro purification column and eluted onto an anchor chip target for analysis on a Bruker Autoflex III MALDI TOF/TOF instrument at Alphalyse A/S (Odense, Denmark). The peptide mixture was analyzed in positive reflector mode for accurate peptide mass determination and some of the peptides from each digest were analyzed by MS/MS fragmentation for partial peptide sequencing.

Mass spectra were acquired in the 500–3000 *m/z* scan range. The mass accuracy was calibrated to within 50 ppm using calibration standards (BSA and transferrin) before each run. The peptide masses obtained were used to query the nonredundant sequence database (NRDB-NCBI 2008.04.15) for pro-

Table 1. Patient Demographics^a

patient subgroup	patient subgroup			
	oligo	extended oligo	poly	total
Number	10	5	7	22
Age (years)	7.8 ± 4.15	6.0 ± 3.69	5.1 ± 2.31	6.6 ± 3.60
Male/Female (n)	5/5	0/5	1/6	6/16
Duration of JIA (weeks)	7.0 ± 6.50	11.8 ± 7.6	8.7 ± 6.06	8.7 ± 6.57
No. of Swollen Joints (<i>n</i>)	1.7 ± 0.82	4.0 ± 0.71	4.0 ± 1.63	3.0 ± 1.59
Serum CRP (mg/L)	7.5 ± 1.60	7.1 ± 2.11	13.6 ± 13.14	9.4 ± 7.75
ESR (mm/h)	10.7 ± 7.15	9.8 ± 4.02	50.3 ± 31.63	23.1 ± 25.93
NSAID (yes/no)	8/2	4/1	6/1	18/4
DMARD (yes/no)	0/10	2/3	0/7	2/20
Biologic (yes/no)	0/10	0/5	1/6	1/21

^a Clinical and demographic characteristics of the study subjects. Values are the mean ± standard deviation or the number of subjects. CRP = C-reactive protein; ESR = erythrocyte sedimentation rate. All study patients were rheumatoid factor negative.

tein identification using the Mascot database search program version 2.2.03. The NRDB2 database contains 6 373 249 entries and is maintained and updated by the European Molecular Biology Laboratory. Database search parameters considered: (i) that the trypsin enzyme cleaves on the C-terminal side of KR unless next residue is P, (ii) no fixed modifications, (iii) caramidomethyl (C) and oxidation (M) variable modifications, (iv) up to 1 missed cleavage permitted with no fixed modifications, (v) peptide tolerance set at 60 ppm for the precursor ions, and (vi) 0.7 Da mass tolerance for the fragment ions. The acceptance criteria for PMF based identifications was a minimum Mascot score of 50, using a 95% confidence interval threshold ($p < 0.05$). The peptide ions identified in this study by MALDI-TOF and further CID MS/MS analysis independently matched to single protein entries in the database.

Western Immunoblotting and Immunohistochemistry. Western immunoblot was used to confirm the mass spectrometry

findings of selected proteins and validate DIGE expression levels across subgroups. The proteins within 10 μ L denatured synovial fluid samples were separated using NuPAGE 4–12% Bis-Tris gels and transferred to 0.45 μ m PVDF membrane (Millipore Corporation, MA) with an XCell II mini gel tank and blot module, associated buffers and power supply, according to the manufacturer's guidelines (Invitrogen Ltd., Paisley, U.K.). Nonspecific binding was blocked overnight at 4 °C using 5% nonfat milk in PBS with 0.1% Tween 20 (PBST) (Sigma-Aldrich Company Ltd., Gillingham, U.K.). Antihuman primary antibodies to Complement C3c (Rabbit polyclonal ab15980; Abcam PLC, Cambridge, U.K.), Vitamin D Binding Protein and Apolipoprotein II (Monoclonal clones 359501 and 395906, respectively; R&D Systems, Minneapolis, MN) were incubated with blotted membranes at a dilution of 1:200 in PBST, for 1 h at room temperature. After the membranes were washed 3 times (5 min each) with PBST, they were incubated in horseradish

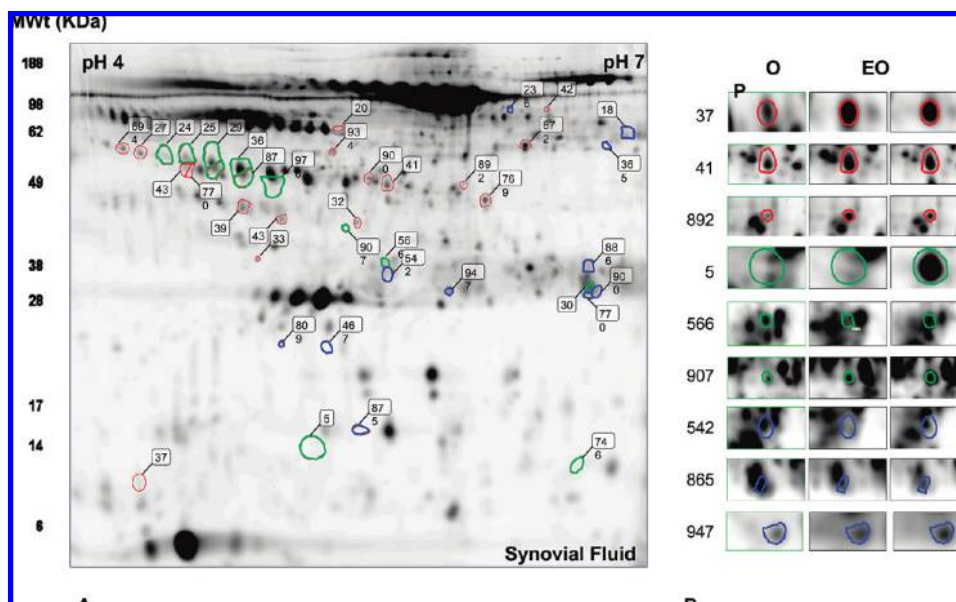


Figure 1. Fluorescence difference in-gel electrophoresis (DIGE) of synovial fluid. DIGE reveals ~1300 spots per gel within the pH 4–7 range for synovial fluid (A). Spot filtering on ‘master’ gels reveals 426 protein spots which are consistently expressed in synovial fluid across patient subgroups. A series of 40 synovial fluid proteins, encircled and numbered above, were expressed at a 2-fold or higher level than could be detected in the other two patient subgroups with statistical significance by ANOVA ($p < 0.05$). Representative protein expression patterns in synovial fluid are shown in selected areas of ‘master’ gels (B) (37, 41, 892, 5, 566, 907, 542, 865, 947). The colors (red, green, blue) used to encircle spots and spot numbers correlate to the clusters observed in the data after hierarchical cluster analysis, shown in Figure 2B. Forty proteins spots were cut from replicate preparative gels and MALDI-TOF and nESI-MS/MS were used to ascertain their identity.

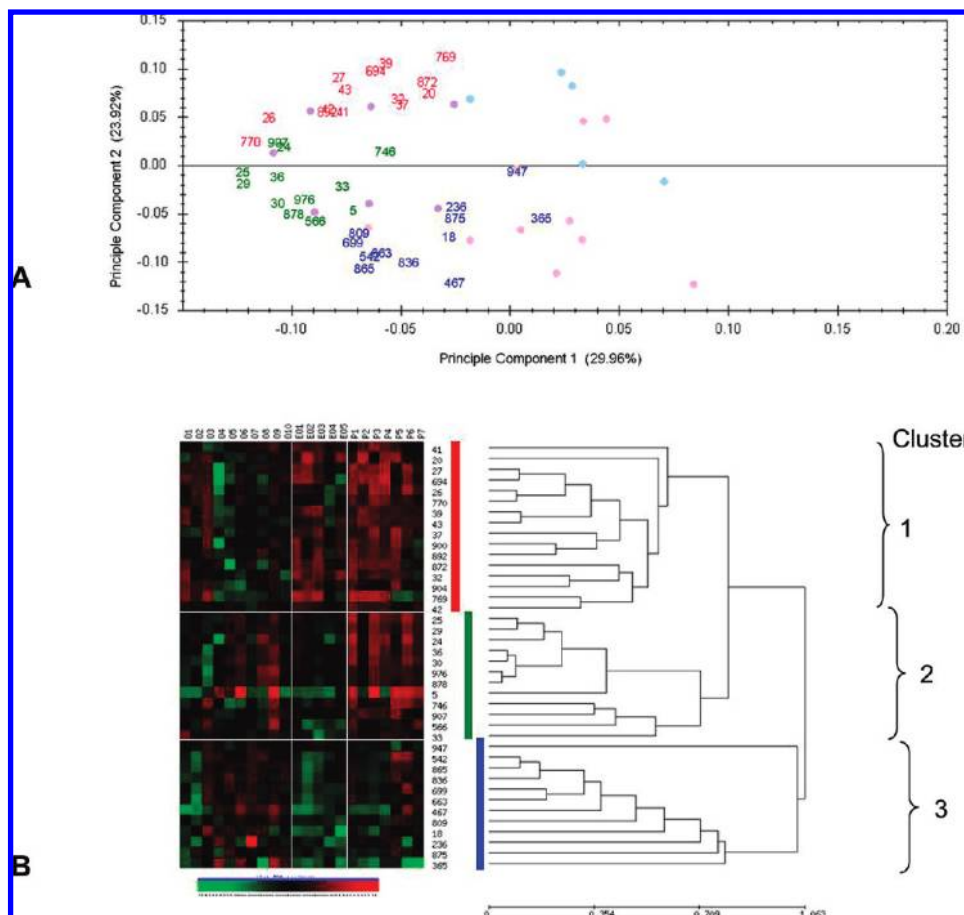


Figure 2. Multivariate analysis of proteins differentially expressed across patient subgroups. Principle component analysis of proteins differentially expressed across patient subgroups (A). Synovial fluid proteome data from each patient is represented by single dots; oligoarticular patients in pink, extended oligoarticular in blue and polyarticular in purple. The variance in the 40 selected proteins is also represented by the numbers which are color coded with respect to the subsequent cluster analysis. Hierarchical cluster analysis of proteins expressed with statistically significant differences between patient subgroups (B). The interindividual and intergroup variation in 40 preselected proteins is represented in heat map form. The protein expression data was reordered by hierarchical cluster analysis (HCA) using Pearson centered correlation (UPGMA), revealing distinguishing expression patterns. Three main clusters of the proteins are highlighted by the colored bars (red, green blue). Each patient sample is represented by a single numbered column (oligoarticular O1–O9, extended oligoarticular EO1–EO5 and polyarticular P1–P7). The location of these spots from the comparative heat map is shown in Figure 1A.

peroxidase conjugated secondary antibodies diluted to 1:2500–1:5000 in PBST (Sigma-Aldrich Company Ltd., Gillingham, U.K.), for 1 h at room temperature. After a further 6 washes, bands were visualized using Supersignal West Pico chemiluminescent substrate (Pierce Biotechnology, Inc., Rockford, IL), imaged with a Autochemi CCD camera and analyzed with Labworks software version 4.0.0.8 (UVP Ltd., Cambridge, U.K.).

Immunohistochemistry was performed on synovial membrane biopsies to ascertain the localization and level of tissue expression for selected proteins as well as providing further validation. Synovial membrane tissues were obtained from each patient by needle biopsy, coated in OCT compound, snap frozen in liquid nitrogen and stored at -80°C . Cryostat sections of $7\ \mu\text{m}$ were cut (Leica CM 1900; Meyer Instruments, Inc.), fixed in acetone at 4°C for 10 min, air-dried and then rehydrated in PBS for 10 min at room temperature. Endogenous peroxidase was blocked by incubation with a 3% hydrogen peroxide solution for 10 min. Sections were rinsed in PBS and probed overnight at 4°C with primary antibodies, as above, diluted from 1/25 to 1/50 in PBS. Sections were incubated with the Envision+ Dual link system –HRP (DAKO, Denmark)

according to the manufacturers guidelines for 30 min at RT. Again sections were washed twice in PBS for five minutes, stained with DAB solution, rinsed in water for 5 min and counterstained in Mayers hematoxylin for 30 s. Sections were washed then dehydrated through graded alcohols and xylene, and allowed to air-dry for 5 min. Coverslips were mounted with DPX medium (HD Supplies, Ayelsbury, UK) and sections imaged with an Olympus BX41 light microscope and JVC 3CCD camera (Olympus Ltd., Essex, UK).

Results

Proteome Description and Significant Expression Patterns. The simultaneous analysis of individual synovial fluids from 22 JIA patients (Table 1) was used to initially isolate joint-specific protein expression profiles. A characteristic pattern of high-abundance proteins form a series of typical charge trains (see Figure 1A), consistent with previous work by other laboratories and our own.^{8,11}

Approximately 1300 spots per synovial fluid gel image were detected and matched across patients. Spot filtering on ‘master’

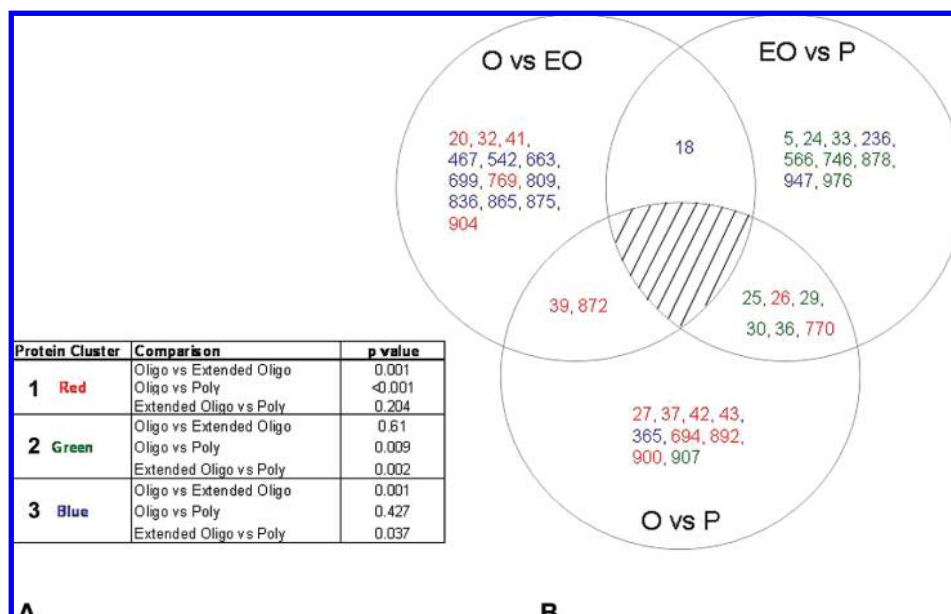


Figure 3. Statistical analysis of subgroup comparisons by protein clusters. The significance of these clusters to differentiate patient subgroups was tested by Tukey-Kramer HSD post hoc test of multiple comparisons (A). The unpaired *t* test revealed proteins with significant intersubgroup variations (B). The *p*-values are summarized in Table 2 and overlaps of protein expression comparisons which reached statistical significance between subgroups are represented within the Venn diagram.

gels reveals 426 protein spots which are consistently expressed in synovial fluid across patient subgroups. Attention was focused on a series of 40 synovial fluid proteins (circled and labeled in Figure 1A) as these spots were expressed at a 2-fold or higher level than could be detected in the other two patient subgroups ($p < 0.05$). Certain proteins predominate in two patient subgroups, for example, proteins 37 and 892 abundant in both the polyarticular and extended oligoarticular patient subgroups, whereas others apparently predominate in a single subgroup, for example, proteins 5 and 907 in polyarticular or protein 865 in oligoarticular patients (highlighted in Figure 1B). Multivariate analysis was used to visualize inter-subgroup expression patterns of the 40 selected protein spots.

Cluster and Statistical Analysis Differentiates Proteins Which Segregate Patient Subgroups. Principle component analysis of all data indicated that the synovial fluid proteome forms distinct groups of patients in an unsupervised fashion (Figure 2A). Overlaps between subgroups are apparent, but the variance is reduced in the panel of the 40 selected proteins. These proteome patterns were further explored by hierarchical cluster analysis and visualization in heat map form (Figure 2B). Pearson ranked correlation revealed three distinctive clusters. Cluster 1 contains proteins consistently overexpressed in both extended oligoarticular and polyarticular patients, whereas proteins in cluster 2 predominate only in the polyarticular subgroup. Intriguingly, proteins in cluster 3 are consistently overexpressed in the oligoarticular and polyarticular subgroups relative to those patients with disease extension. It seemed conceivable that these three distinct clusters could be used in combination to differentiate disease subgroups. Further statistical analysis was used to test the significance of inter-subgroup patterns of the identified proteome clusters.

The Tukey-Kramer test reveals that patients with extended oligoarticular and polyarticular disease have significant differences in protein clusters 1 and 3 ($p = 0.001$ for both) and clusters 1 and 2 ($p = 0.001$, $p = 0.009$, respectively), when compared to the oligoarticular subgroup. The unpaired *t* test

revealed proteins with significant fluctuations between patient subgroups. The *p*-values are summarized in Table 2 and overlaps between subgroup comparisons are represented by Venn diagram in Figure 3B. Having established the significance of protein expression patterns, protein identities were investigated using mass spectrometry.

Discriminatory SF Proteome Identification, Inter-Subgroup Variation and Patient Monitoring over Disease Course. The identities and relative expression levels of the 40 selected proteins are listed in Table 2. Six albumin isoforms (*a*, *g*, *h*, *k*, *p* and *t*) were identified from 11 distinct protein spots, predominating in cluster 1. Within cluster 3, albumin isoforms *t* and *k* were overexpressed 4.8- ($p = 0.049$), 2.9- ($p = 0.018$) and 3.0- ($p = 0.038$) fold in extended oligoarticular patients when compared to the oligoarticular subgroup. Conversely, albumin isoforms *a* and *g* are under expressed 2.7- ($p = 0.021$) and 6.9- ($p = 0.021$) fold, respectively, in patients with disease extension. Intriguingly, albumin complexed with the nonsteroidal anti-inflammatory drug (NSAID) S-naproxen (spot numbers 41 and 904) was detected at significantly raised levels in extended oligoarticular contrasted with oligoarticular patients.

Apolipoproteins AI and AII were overexpressed 2.9- ($p = 0.008$) and 2.6- ($p = 0.046$) fold, respectively, in polyarticular patients when contrasted against oligoarticular and extended oligoarticular subgroups. In clusters 1 and 3, transferrin was identified as two independent protein spots (42,33) representing nonglycosylated and N-lobe forms. Levels of the nonglycosylated transferrin were raised 2.0- ($p = 0.014$) fold in patients who showed disease extension, whereas the transferrin N-lobe displayed the inverse with 2.6- ($p = 0.045$) fold less, relative to oligoarticular patients. Complement component C3c and hemopexin display analogous overexpression in polyarticular patients when balanced alongside oligoarticular and extended oligoarticular subgroups. Interestingly, from the point of view of JIA pathology, vitamin D binding protein was detected in polyarticular patients at significantly higher levels, 2.2- ($p = 0.019$) fold, relative to oligoarticular patients in cluster 1,

Table 2. Mass Spectrometry of Selected Proteins^a

Spot ID	Log Normalised Spot Volume-Mean (SD)			Inter-group Comparison-Fold difference, unpaired T-test (p<0.05)						Protein Name	Mass Spectrometry Data-MALDI-TOF/TOF						
	Oligo	Ext Oligo	Poly	OE/O	p	P/O	p	P/OE	p		Accession Number	pl	MW (KDa)	Mascot Score	Sequence Coverage (%)	Matched Peptides	Verification CID ions
26	1.481 (0.370)	2.193 (0.357)	1.993 (0.422)	0.612	0.049	1.245	0.086	2.036	0.547	Albumin, isoform CRA-t	119626083	6.66	58614	53	5	3	2
20	2.222 (0.475)	2.282 (0.436)	2.587 (0.360)	1.151	0.953	0.543	0.017	0.471	0.019	Leucine-rich alpha-2-glycoprotein 1	16418467	6.45	38154	189	23	7	2
27	2.085 (0.623)	2.176 (0.309)	2.176 (0.309)	0.985	0.115	2.265	0.011	2.275	0.311	HP protein	47124562	8.48	31362	80	28	8	3
37	1.250 (0.390)	1.780 (0.343)	1.616 (0.399)	2.284	0.018	1.378	0.080	0.603	0.364	Albumin, isoform CRA-k	119626074	5.97	47257	130	24	11	3
32	2.300 (0.440)	2.609 (0.249)	2.832 (0.285)	1.824	0.206	2.296	0.046	1.259	0.359	Apolipoprotein A-II isoform CRA_c	119573006	6.15	8958	108	32	4	1
39	2.079 (0.409)	2.493 (0.437)	2.539 (0.196)	2.306	0.047	2.337	0.032	1.013	0.721	No Identification	-	-	-	-	-	-	
41	2.723 (0.382)	3.203 (0.175)	3.108 (0.323)	0.567	0.006	1.367	0.084	2.413	0.918	Serum Albumin with S-Naproxen, Chain A	168988718	5.63	65778	322	32	25	8
42	1.193 (0.227)	1.488 (0.231)	1.504 (0.336)	0.410	0.014	0.767	0.042	1.870	0.655	Apo-serum transferrin (non-glycosylated), Chain A	110590597	6.58	74643	377	38	26	8
43	1.681 (0.375)	1.899 (0.366)	2.136 (0.252)	2.409	0.417	2.449	0.019	1.681	0.108	Vitamin D binding protein/ group specific component	455970	5.34	52916	205	9	7	5
694	1.900 (0.637)	2.372 (0.705)	2.551 (0.446)	0.408	0.120	1.007	0.036	2.467	0.811	Albumin, isoform CRA_h	119626071	5.92	68568	75	17	9	3
769	1.927 (0.555)	2.484 (0.630)	2.269 (0.815)	0.383	0.038	0.881	0.116	2.238	0.941	Albumin, isoform CRA_k	119626074	5.97	47257	255	32	15	4
770	2.287 (0.441)	2.266 (0.312)	2.892 (0.396)	0.369	0.526	0.891	0.043	2.415	0.033	No Identification	-	-	-	-	-	-	
872	2.222 (0.536)	2.704 (0.354)	2.676 (0.420)	0.748	0.020	2.254	0.040	3.015	0.962	HP Protein	47124562	8.48	31362	218	40	13	3
892	1.698 (0.326)	2.040 (0.425)	2.006 (0.505)	3.020	0.054	2.910	0.035	0.963	0.974	Albumin, isoform CRA_h	119626071	5.92	68568	225	19	12	5
900	2.273 (0.479)	2.596 (0.338)	2.621 (0.520)	0.699	0.167	2.224	0.042	3.181	0.590	Haptoglobin	3337390	6.14	38209	232	31	14	3
904	1.486 (0.471)	1.911 (0.304)	1.657 (0.315)	0.345	0.045	1.233	0.063	3.578	0.245	Serum Albumin with S-Naproxen, Chain A	168988718	5.63	65778	340	23	16	7
5	2.613 (0.901)	2.213 (0.523)	3.338 (0.870)	3.249	0.150	3.624	0.196	1.115	0.044	Albumin, isoform CRA_h	119626071	5.92	68568	168	22	17	4
24	2.463 (0.520)	2.440 (0.455)	3.073 (0.428)	0.256	0.725	0.706	0.054	2.756	0.040	Haptoglobin	3337390	6.14	38209	191	33	14	3
25	2.864 (0.360)	2.989 (0.264)	3.486 (0.417)	2.206	0.788	1.008	0.028	4.891	0.021	Leucine-rich alpha-2-glycoprotein 1	16418467	6.45	38154	124	16	5	3
29	3.148 (0.359)	3.166 (0.237)	3.604 (0.352)	0.336	0.781	1.774	0.029	5.274	0.023	Zinc-Alpha-2 Glycoprotein, Chain B	4699583	5.7	31626	266	51	17	4
30	3.250 (0.487)	3.354 (0.385)	3.737 (0.387)	0.144	0.599	0.538	0.026	3.725	0.033	Complement Component C3c, Chain C	78101271	4.79	39463	310	36	13	4
33	0.506 (0.373)	0.152 (0.382)	0.703 (0.186)	0.240	0.116	0.099	0.620	0.410	0.008	Lipid-free Apolipoprotein A-I	90108664	5.27	28061	91	26	6	3
36	3.557 (0.508)	3.721 (0.199)	4.072 (0.331)	0.063	0.929	0.160	0.028	2.558	0.043	Hemopexin	11821561	6.55	51643	159	22	12	4
566	1.897 (0.507)	1.458 (0.247)	1.996 (0.555)	1.388	0.121	2.205	0.989	1.589	0.035	Albumin, isoform CRA_k	119626074	5.97	47257	326	23	11	5
746	1.965 (0.550)	2.053 (0.274)	2.488 (0.366)	1.953	0.480	2.259	0.092	1.157	0.044	Albumin, isoform CRA_p	119626064	8.42	25276	169	13	4	3
878	3.311 (0.173)	3.073 (0.370)	3.175 (0.451)	2.351	0.015	3.757	0.408	1.473	0.078	No Identification	119579601	6.31	39944	308	17	5	2
907	1.076 (0.311)	1.171 (0.136)	1.427 (0.384)	2.391	0.988	2.122	0.038	0.887	0.059	Leucine-rich alpha-2-glycoprotein 1	16418467	6.45	38154	90	21	7	2
978	3.527 (0.592)	3.599 (0.160)	3.879 (0.243)	1.718	0.336	2.552	0.616	1.486	0.040	Albumin, isoform CRA_g	119626070	5.66	22001	160	35	8	3
18	2.714 (0.514)	2.061 (0.453)	2.590 (0.413)	0.961	0.030	2.502	0.284	2.603	0.032	Immunoglobulin heavy chain variable region	39937967	5.13	16641	86	38	7	1
236	1.872 (0.760)	1.487 (0.308)	1.809 (0.485)	0.421	0.301	1.199	0.352	2.850	0.030	No Identification	-	-	-	-	-	-	
365	1.855 (0.723)	1.442 (0.501)	1.150 (0.411)	2.868	0.093	2.075	0.048	0.724	0.262	Leucine-rich alpha-2-glycoprotein 1	16418467	6.45	38154	280	21	6	3
467	1.984 (0.751)	1.318 (0.466)	1.732 (0.563)	0.832	0.021	2.498	0.311	3.002	0.244	Albumin, isoform CRA_g	119626070	5.66	22001	376	35	9	6
542	2.402 (0.530)	2.023 (0.317)	2.439 (0.708)	0.911	0.023	2.828	0.400	3.104	0.133	Immunoglobulin kappa 1 light chain	170684606	5.72	23238	177	40	6	3
663	2.286 (0.535)	1.872 (0.195)	2.175 (0.451)	2.351	0.015	3.757	0.408	1.473	0.078	No Identification	-	-	-	-	-	-	
699	2.657 (0.420)	2.324 (0.161)	2.676 (0.481)	1.026	0.020	3.850	0.666	3.753	0.145	Immunoglobulin light chain	149673887	6.97	23380	237	54	8	4
800	0.661 (0.370)	0.333 (0.143)	0.624 (0.328)	1.114	0.021	4.229	0.750	3.796	0.074	Albumin, isoform CRA_a	119626064	6.45	25276	17	13	4	1
836	2.843 (0.528)	2.522 (0.355)	2.782 (0.541)	0.819	0.045	3.500	0.754	4.276	0.167	Chain A, Human Serum Transferrin, N-iso	29726565	6.79	36533	649	41	16	9
865	2.909 (0.508)	2.576 (0.347)	2.897 (0.479)	4.813	0.040	3.611	0.983	0.750	0.090	No Identification	-	-	-	-	-	-	
875	1.828 (0.372)	1.536 (0.167)	1.702 (0.324)	0.184	0.023	0.606	0.510	3.302	0.256	No Identification	-	-	-	-	-	-	
947	1.030 (0.357)	1.185 (0.138)	1.833 (0.234)	0.134	0.616	2.334	0.055	17.674	0.001	Chain A, Human Vitamin D Binding Protein	18655434	5.66	51183	361	20	8	1

^a Protein name, mass spectrometry data, fold differences between subgroups and their statistical significance are compiled for the 40 synovial fluid associated proteins. Spot tryptic digests were identified using matrix assisted laser desorption ionisation (MALDI-TOF/TOF), correlated to compiled peptide data (Matrixscience). P-values in bold highlight inter-subgroup comparisons which reached statistical significance ($p < 0.05$) by unpaired *t* test. Peptide ion sequence and peak lists can be found in Supplementary Tables 3A–C.

whereas the protein was under expressed 2.1- ($p = 0.001$) fold relative to the extended oligoarticular subgroup in cluster 3.

The acute phase reactant, haptoglobin, was positively identified in extracts from independent spots (900, 24 and 878) and significantly overexpressed in polyarticular patients 2.3- to 4.3-fold when contrasted with all oligoarticular patients. Two glycoprotein species of alpha-2-glycoprotein 1 were identified in four discrete protein spots (26, 25, 29 and 365), a leucine-rich and a zinc-complexed species. In addition, several spots (18, 542 and 699) were identified as Immunoglobulin (Ig) fragments within cluster 3 at significantly reduced levels in the patients with disease extension to oligoarticular patients. Ig heavy chain variable region, IgK1 and Ig light chains were underexpressed 5.4- ($p = 0.030$), 3.0- ($p = 0.023$) and 2.9- ($p = 0.020$) fold in the extended oligoarticular subgroup relative to the oligoarticular subgroup. A number of protein spots remained unidentified, attributable to low protein yield from the gel core extraction process.

Western immunoblotting of synovial fluids and immunohistochemistry of synovial membranes from a representative set of patients validates the mass spectrometry identifications of selected proteins, in Figure 4. Western blot apparent band densities are consistent with levels ascertained from DIGE spot intensities. Immunohistochemistry reveals perivascular expression of apolipoprotein II and vitamin D binding protein with synovial membrane, indicative of macrophage and T-lymphocyte activation; complement C3c is not detected.

Synovial fluid samples from patients with recurrent joint aspirations were used to monitor the panel of proteins as disease evolves. Data representative of five patients monitored in this fashion are shown for a single polyarticular individual in Figure 5. Haptoglobin, transferrin, albumin complexed with S-naproxen and alpha-2-glycoprotein 1 all follow similar trends within the affected joints over the 2 year period. It was evident that peaks in levels of these proteins coincided with higher numbers of inflamed joints across the illustrated individual.

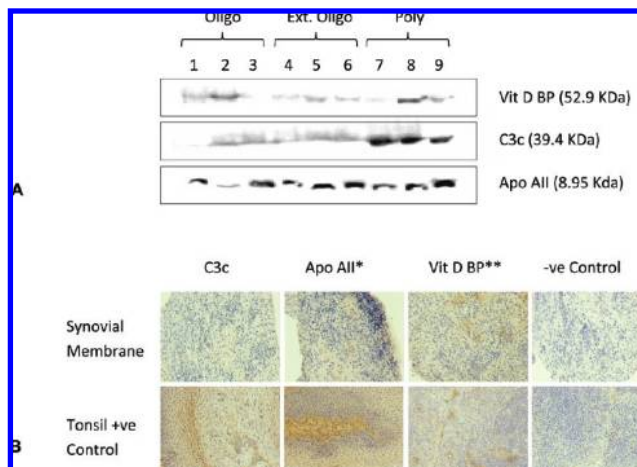


Figure 4. Verification of protein expression and localization in synovial fluid and membrane. Western immunoblotting of synovial fluids from representative oligoarticular (1–3), extended oligoarticular (4–6) and polyarticular (7–9) patients (A). Band densities of vitamin D binding protein, complement C3c and apolipoprotein II concur with protein expression levels measured by DIGE. Representative immunohistochemistry of synovial membrane from a polyarticular patient (B), all captured at 10× magnification. Apolipoprotein AII and vitamin D binding protein expression is perivascular in nature, whereas complement C3c is not detected.

In contrast, the nonglycosylated form of transferrin displayed a gradual decline across the sampled joints, whereas complement component C3c showed a similar trend up to the 73 week point before rising to levels detected in the initial sample.

Discussion

This is the first study to analyze the synovial proteome in JIA and relate it to subsequent disease subtypes. Many pro-

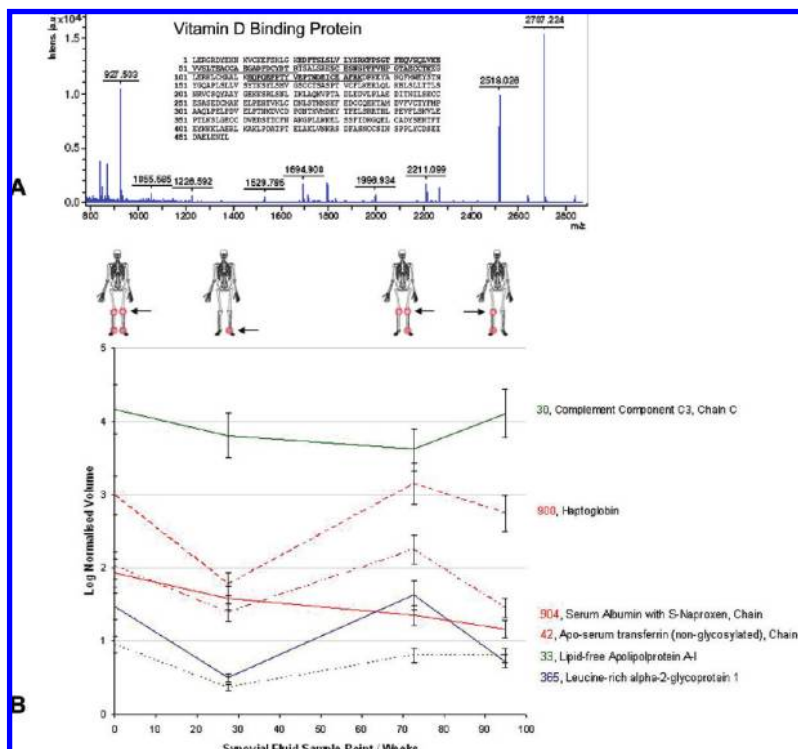


Figure 5. Intra-individual proteome observation during disease course. A representative MALDI-TOF mass spectra of spot 947 identified as vitamin D-binding protein (A). The matched peptide sequences are underlined within the sequence of vitamin D-binding protein. The expression levels of 6 representative proteins (42, 900, 904, 30, 33, 365) are shown for a series of synovial fluid samples from a single patient over approximately 2 years (B). The female patient was diagnosed with polyarticular JIA at the age of 3 years old, 3 months after disease onset and was rheumatoid factor negative. The log normalized spot volumes from DIGE images of samples are plotted against time in weeks. The body above each data point indicates the number of swollen joints at the time of aspiration (encircled red) and the joint on which DIGE proteome analysis was conducted (black arrow). Samples were taken from the left knee 1 month after diagnosis (0 weeks) and on subsequent appointments from the left ankle (28 weeks), from the left knee again (73 weeks) and from the right knee (95 weeks).

inflammatory, anti-inflammatory and mediators of tissue repair and destruction have been identified in JIA.^{12–15} Conventional measures of the acute phase response CRP and ESR were recorded within this study at similar levels in oligo and extended oligoarticular groups, potentially confounding clinical differentiation of patients who will likely develop more aggressive disease. Significantly higher levels were observed in the polyarticular patients signifying the increased disease activity experienced at an early stage by polyarticular patients. Proteomic profiles offer us a powerful tool to identify those biomarker profiles present not only in disease subsets, but which predict evolution into a particular disease subset. Our results suggest that for each disease subset a proteomic profile exists and that this profile has the potential to predict disease evolution. Furthermore, the SF proteomic pattern observed in an individual patient was consistent between joints of that patient, possibly reflecting the degree of joint inflammation. The consistency of protein pattern across joints suggests that the biomarker profile truly represents a disease subtype.

Synovial fluid acts as a pool for proteins and peptides secreted and shed locally from suspended cells, synovial membrane and cartilage. Potential biomarkers which have relevance to joint inflammatory status and disease progression can be found at enriched levels within the synovial fluid proteome.⁸ The current study has provided evidence that JIA patient subgroups can be segregated in early disease based on the clustering patterns of 40 synovial fluid proteins. By extension of this of this finding, it is possible to differentiate JIA

patients who suffer disease extension within the first year of diagnosis. Several acute-phase proteins, albumins, glycoproteins and immunoglobulins comprise the bulk of the discriminatory subproteome isolated in this study. Though many of these are abundant in plasma, substantial evidence suggests these molecules may govern a wide range of inflammatory pathways with bearing on the pathology of JIA, in particular disease extension.

A number of these proteins belong to the negative acute phase proteins (APPs) and previous studies suggest that plasma concentrations decrease in response to inflammation because of increased rates of transcapillary escape degradation.¹⁶ It has been suggested that transient infiltration of negative APP plasma proteins may partially explain the relapse-remission cycles characteristic of JIA and other forms of inflammatory arthritis.¹⁷

Inhibitory acute phase proteins were associated with significant differences in JIA subgroup expression patterns. When compared to the synovial fluid proteome levels of the oligoarticular patient subgroup, apolipoprotein A-I was reduced in patients with extended oligoarticular disease, whereas apolipoprotein A-II was conversely higher. Both apolipoprotein species were significantly raised in the polyarticular patients. In rheumatoid arthritis patients, apolipoprotein A-I plasma levels are lower than normal controls,^{18,19} whereas the levels are increased in synovial fluid.^{20,21} Apo A-I immunohistological staining is confined to perivascular areas within the synovium of RA patients.²² It has been suggested that Apo A-I could

inhibit the production of proinflammatory cytokine production²³ and may limit disease recurrence by inhibition of interactions between T lymphocytes and monocytes.²⁴

In line with apolipoprotein A-I levels, extended oligoarticular patients display a general trend toward reduced transferrin, haptoglobin and complement C3c relative to the other two patient subgroups. These host-response proteins could be involved in the spread of inflammation to previously unaffected joints. Haptoglobin has been shown to be involved in angiogenesis, tissue remodeling and cell migration.²⁵ In addition, transferrin has also been shown to promote angiogenesis, whereas decreased levels of complement components may protect breast cancer cells from complement mediated immune surveillance.^{26,27}

Complement component C3c is increased in SF in RA patients versus traumatic arthritis, which correlates to levels of PMN cells in the SF. Therefore, C3c in SF may elucidate the inflammatory activity in the SM.²⁸ In synovial tissue, distinct deposits of C3c (and C9) are localized in the synovial vasculature and the synovial lining in adult RA.²⁹ Evidence of complement activation in synovial fluid is available, but their role in joint inflammation remains unclear.^{30,31} In addition, previous studies have identified vitamin D binding protein as the costimulatory molecule for a metabolite of C5a resulting in enhanced chemotactic activity.³²

A number of proteins associated with tumor spread were also identified in the panel of discriminatory markers. Increased serum leucine rich alpha-2-glycoprotein (LRG) has been observed in patients with several types of cancer including lung and liver cancers by 2-DE.^{33,34} Zinc alpha-2-glycoprotein (ZAG) is overexpressed in prostate cancer and may be a serum marker of early tumor development with levels predictive of metastatic spread.^{35,36}

Conclusions

The samples used in this study were only taken at the time of initial knee inflammation, therefore, are most relevant to the disease pathology in early disease. The authors consider this prerequisite vital in the discovery of biomarkers which not only 'describe' the initial disease processes, but may also act as sentinels to predict subsequent disease outcome. The data provided indicates that synovial fluid proteome profiles could be used to classify patients based on existing clinical definitions and predict disease evolution. By cataloguing the protein profiles of joint status as disease progresses, it may also be possible to monitor therapeutic response over time. Considering the heterogeneity of patient outcome in JIA, therapeutic suppression of synovial joint inflammation, while neglecting the systemic components of chronic disease, may not represent the best approach to management of nonresponsive disease. Longer lasting remission could be possible by taking systemic sentinels into account.

It is envisioned that a simple synovial fluid or plasma sample will predict the subsequent course of disease for that patient, thus, enabling early appropriate intervention. This course of action could avoid months of fruitless treatment, commencing biologics earlier, and thus reducing pain, disability and joint damage. As with all translational research, a final properly powered prospective study to assess the robustness of such biomarkers to predict outcome is required. Cross validation of putative biomarkers is a vital feature of the study design, in agreement with published discovery strategies.³⁷

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Supporting Information Available: Peptide ion sequence and peak lists can be found in Supplementary Tables 3A–3C. This data corresponds with Table 2 in main manuscript. Peptide position in the matched protein, observed mass (*m/z*) and amino acid sequence are listed for each protein match listed in the manuscript. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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