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***In this study researchers examined patients with either erosive rheumatoid arthritis or nonerosive rheumatoid arthritis using synovial fluid protein profiling through a combination of abundant protein immunodepletion, 2-dimensional strong cation exchange/reverse phase liquid chromatography with electrospray ion trap mass spectrometry, and data analysis for relative quantification. This study discovered several candidate biomarkers which were subsequently validated via multiple reaction monitoring MS-MS in patient sera.***

**T**he ability to identify rheumatoid arthritis (RA) patients likely to develop the most severe form of the disease is highly desired. Current markers do not predict subsequent severity (1). To identify a panel of candidate protein biomarkers of RA, immunodepletion, multi-dimensional liquid-phase separations and mass spectrometry was used to characterize the protein profiles of synovial fluid (SF) samples from 10 patients with either the nonerosive or erosive form of the disease with the goal of identifying a panel of candidate protein biomarkers of RA to better predict which patients will develop erosive, disabling disease.

### Methods

A 2-step proteomic approach was used for biomarker discovery and verification. The work reported here focuses on the discovery efforts. For a detailed report of the work, readers should consult reference 2. (Liao, et al). Briefly, in the first step, the hyaluronic acid in SF was digested, the samples were centrifuged, abundant proteins were depleted (IgG and HSA) the samples were size fractionated, concentrated, reduced and alkylated, fractionated by SEC, and digested with trypsin. These digests were then subjected to 2-dimensional strong cation exchange/reverse phase liquid chromatography with electrospray ion trap mass spectrometry to generate protein profiles of SF from patients with either erosive RA (n=5) or nonerosive RA (n=5). A gradient of 4 salt steps was utilized in order to reduce potential carryover of peptides between elution steps. Each of the 10 patient samples produced 27 LC-MS-MS runs. Over 1 million MS-MS spectra were collected from these 10 samples. Data analysis was performed with the Agilent Spectrum Mill software ([www.agilent.com/chem](http://www.agilent.com/chem)). Over 376,000 MS-MS spectra with a sequence tag length >1 were passed through the quality filters in the Spectrum Mill raw data extractor and subsequently searched against a NCBI non-redundant human protein database subset. Spectrum Mill also provided the protein relative abundance by using the ion current measured for each peptide precursor ion in the inter-leaving MS scans of the LC-MS-MS chromatogram. An individual protein's abundance was calculated as the mean ion current measured for all peptide precursor ions derived from that protein.

### Results

By comparing protein profiles of 5 erosive RA SF samples and 5 nonerosive RA SF samples, 33 proteins were selected that were differentially elevated in either erosive or nonerosive RA. A subset of the results is shown in Table 1. 33 candidate biomarkers were chosen from a total of 418 identified proteins. Among

# Biomarker Discovery in Rheumatoid Arthritis Patients by MS-based Synovial Fluid Protein Profiling

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the proteins that were elevated in the SF of patients with erosive RA were C-reactive protein (CRP) and 6 members of the S100 protein family of Calcium binding proteins. For example, CRP was detected in 4 of 5 erosive RA SF samples and 4 of 5 nonerosive samples with the intensity ratio difference between erosive RA and nonerosive RA samples of 0.98:0.02, representing a 49-fold difference in total intensity. An additional example was S100A12 (calgranulin C), detected in all erosive RA SF samples but in only 2 of the nonerosive RA SF samples with an erosive to nonerosive intensity ratio of 0.93:0.07. Additional results may be found in reference 2.

### Conclusions

Several potential protein markers have been identified for the prognosis of the erosive form of RA. The combination of immunodepletion, protein sample fractionation, digestion and 2-dimensional strong cation exchange/reverse phase liquid chromatography MS-MS and Spectrum Mill data analysis provides an effective tool for the discovery of clinically relevant sets of potential disease biomarkers from complex samples.

### References

1. Van Riel PL, Wijnands MJ, van de Putte LB. Evaluation and management of active inflammatory disease. In: Klippel JH, Dieppe PA, editors. Rheumatology. 2nd ed. London: Mosby; 1998. p.5.14.11-3.
2. Liao H, Wu J, Kuhn E, Chin W, Chang B, Jones MD, O'Neil S, Clauser, KR, Karl J, Hasler E, Roubenoff R, Zolg W, Guild BC. Use of mass spectrometry to identify protein biomarkers of disease severity in the synovial fluid and serum of patients with rheumatoid arthritis. Arthritis and Rheumatism, Vol. 50, No. 12, December 2004, pp. 3792-3803.

Protein name	Erosive Rheumatoid Arthritis					Non-erosive Rheumatoid Arthritis					Protein Acc. No.	Number unique peptide	Score
	E1	E2	E3	E4	E5	NE1	NE2	NE3	NE4	NE5			
Transferrin	3.87E+08	6.70E+08	7.01E+08	1.48E+09	7.15E+08	1.25E+09	3.10E+08	3.69E+09	4.69E+08	9.13E+08	4557871	74	1203.66
	169	220	387	387	387	387	387	387	387	387			
Serum albumin	5.66E+08	4.87E+08	8.83E+08	1.07E+09	8.07E+08	8.72E+08	3.98E+08	1.65E+09	1.94E+08	3.14E+08	4502027	59	991.85
	519	110	1103	156	54	230	136	391	375	159			
Macroglobulin alpha-A372	1.91E+08	4.22E+08	2.19E+08	9.39E+08	3.18E+08	9.15E+08	3.36E+08	1.50E+09	1.35E+08	2.49E+08	224053	27	416.15
	8	26	21	28	5	6	10	25	14	27			
Kininogen	4.57E+07	0.00E+00	0.00E+00	0.00E+00	0.00E+00	4.01E+08	7.52E+07	1.63E+08	1.08E+08	0.00E+00	125507	19	246.05
	5	0	0	0	0	18	3	1	8	0			
S100 A9	1.81E+09	2.49E+09	9.46E+08	4.17E+09	6.13E+08	5.77E+08	6.81E+08	3.23E+08	5.11E+07	6.31E+07	4506773	14	231.63
	258	142	356	356	89	87	120	5	14	13			
Phosphoglycerate mutase 1	2.87E+08	5.14E+08	3.87E+08	9.02E+08	5.19E+08	4.13E+08	1.45E+08	0.00E+00	0.00E+00	1.73E+08	15301114	13	190.34
	7	13	15	2	3	1	5	0	0	1			
S100 A8	4.80E+08	2.68E+08	3.42E+08	1.49E+09	4.87E+08	1.81E+08	9.35E+08	1.99E+08	3.83E+07	3.80E+07	14729628	11	162.78
	58	28	96	20	36	11	20	1	2	2			
C-reactive protein	0.00E+00	6.39E+08	2.48E+08	1.31E+08	4.98E+08	1.80E+08	3.81E+07	0.00E+00	4.19E+07	7.89E+07	14728083	11	159.23
	0	42	23	136	25	21	2	0	4	12			
RHO GDI 2	6.26E+08	3.18E+08	7.34E+08	1.09E+09	6.34E+08	2.33E+09	3.29E+08	0.00E+00	4.01E+07	2.99E+08	10835002	11	157.73
	8	9	31	4	6	1	1	0	1	1			
14-3-3 Protein beta/alpha	5.80E+07	8.31E+08	3.73E+08	4.56E+08	6.79E+08	0.00E+00	2.76E+07	0.00E+00	4.08E+07	2.79E+08	4507949	9	139.74
	3	4	14	3	1	0	0	0	2	2			
Ig G1 H Nie	0.00E+00	1.16E+08	1.98E+08	2.50E+08	1.09E+08	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	229601	10	134.9
	0	12	10	14	6	0	0	0	0	0			
Osteopontin	9.81E+06	4.64E+08	4.46E+08	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	14724978	9	133.55
	1	10	21	0	0	0	0	0	0	0			
Triosephosphate isomerase	5.42E+08	1.03E+09	5.30E+08	0.00E+00	7.20E+08	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.10E+08	4507645	9	130.03
	3	4	13	0	3	0	0	0	0	1			
Prosaposin	0.00E+00	3.76E+08	1.06E+08	3.19E+08	0.00E+00	1.67E+08	0.00E+00	0.00E+00	0.00E+00	0.00E+00	15298143	10	123.62
	0	15	5	3	0	1	0	0	0	0			
S100 A12	1.91E+08	2.90E+08	2.57E+08	3.78E+08	1.76E+08	1.61E+08	3.83E+08	0.00E+00	0.00E+00	0.00E+00	2146972	7	116.15
	25	68	28	16	46	8	6	0	0	0			
Cathepsin B	2.08E+08	2.93E+08	3.49E+08	3.94E+08	4.15E+08	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	4503139	8	113.29
	1	10	1	1	1	0	0	0	0	0			
Apolipoprotein C-II	5.01E+07	1.71E+07	0.00E+00	0.00E+00	0.00E+00	5.65E+08	3.10E+09	7.45E+08	3.06E+08	0.00E+00	2134777	8	106.13
	8	1	0	0	0	7	7	4	19	0			
Thymosin beta-4	5.44E+07	2.57E+07	1.61E+09	7.51E+08	4.83E+07	0.00E+00	0.00E+00	0.00E+00	0.00E+00	9.17E+07	11056061	7	103.65
	8	3	1	3	10	0	0	0	0	7			
Glyceraldehyde-3-phosphate dehydrogenase	6.12E+08	7.08E+08	3.29E+08	5.13E+08	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	35053	6	100.01
	14	5	10	1	0	0	0	0	0	0			
Neutrophil defensin alpha-3	4.28E+08	3.12E+09	5.36E+09	2.71E+09	1.36E+08	0.00E+00	2.12E+07	0.00E+00	2.47E+07	2.62E+08	4885179	7	95.69
	43	16	20	10	9	0	5	0	5	3			
CD14 antigen	0.00E+00	0.00E+00	3.41E+08	6.84E+08	8.50E+08	0.00E+00	1.89E+08	0.00E+00	0.00E+00	0.00E+00	4557417	6	94.14
	0	0	2	3	7	0	1	0	0	0			
Peptidylprolyl isomerase A	3.09E+08	2.57E+08	3.32E+08	2.16E+08	1.37E+08	0.00E+00	0.00E+00	0.00E+00	1.24E+08	6.77E+08	10863927	6	93.77
	11	3	11	2	2	0	0	0	0	0			
Transgelin 2	3.83E+08	3.25E+08	3.53E+08	0.00E+00	1.94E+08	3.55E+07	0.00E+00	0.00E+00	0.00E+00	0.00E+00	14728128	6	91.47
	1	11	13	0	1	1	0	0	0	0			
alpha-A702 plasmin inhibitor	1.05E+08	3.81E+08	5.04E+08	9.83E+08	3.71E+08	8.35E+07	6.49E+07	0.00E+00	0.00E+00	0.00E+00	11386143	5	82.8
	1	2	3	7	2	1	3	0	0	0			
Glutathione transferase	2.71E+08	9.72E+08	7.05E+08	0.00E+00	0.00E+00	7.82E+08	0.00E+00	0.00E+00	0.00E+00	0.00E+00	14766346	5	79.03
	3	3	9	0	0	1	0	0	0	0			
S100C Protein	2.59E+08	2.42E+08	6.75E+07	1.19E+08	1.30E+08	0.00E+00	0.00E+00	0.00E+00	1.27E+07	0.00E+00	5032057	5	73.04
	2	18	2	3	1	0	0	0	2	0			

**Table 1.** Subset of Spectrum Mill Protein Summary for erosive and nonerosive rheumatoid arthritis synovial fluid samples. A dark red color indicates high abundance. An orange to yellow to white color indicates progressively less abundance. The numbers in the colored boxes represent the protein's mean intensity value and the number below list the number of peptide spectra that match to that protein. Additional details may be found in reference 2.



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