

Identification of Novel Protein Biomarkers of Preterm Birth in Human Cervical–Vaginal Fluid

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Spontaneous preterm birth (SPTB) is a major contributor to perinatal morbidity and mortality. However, the diagnosis of preterm labor (PTL) that leads to preterm birth is difficult, and there is a pressing need for improved diagnosis. We utilized multidimensional liquid chromatography–tandem mass spectrometry (LC/LC–MS/MS; MudPIT) and Fluorescence two-dimensional differential in-gel electrophoresis (2D-DIGE) to identify potential biomarkers of PTL and SPTB. MudPIT analysis identified 205 proteins in cervical–vaginal fluid (CVF), 28 of which exhibited significant differences in pairwise and progressive comparisons. Calgranulins, annexins, S100 calcium-binding protein A7, and epidermal fatty acid binding protein were abundant in CVF and differentially present in PTL and SPTB samples, as were the serum proteins α -1-antitrypsin, α 1-acid glycoprotein, haptoglobin, serotransferrin, and vitamin D binding protein. 2D-DIGE identified 17 proteins that were significantly differentially present in PTL and SPTB. Immunoblotting with specific antibodies confirmed the differences and trends of selected markers. Further characterization and quantification of these markers in a larger cohort of subjects may provide the basis for new tests for the early, noninvasive positive prediction of SPTB.

Keywords: Vaginal fluid • Preterm labor • Biomarkers • Prematurity • Pregnancy

Introduction

Spontaneous preterm birth (SPTB) (i.e., before 37 weeks of pregnancy) is a major health concern, accounting for 70–80% of neonatal mortality and increased morbidity in both developed and developing countries.^{2–4} Despite preventive strategies, the rate of SPTB has steadily increased during the past 25 years (National Center for Vital Statistics 2004). Available tocolytic therapies for preterm labor (PTL) delay delivery for 48–72 h,¹ but have had no impact on the rising preterm birth rate in the U.S.

Gestational age (GA) at delivery is the major determinant of neonatal morbidity and mortality. Surviving preterm infants are at increased risk of respiratory distress syndrome, developmental delay, cerebral palsy, and other chronic disorders. The selective use of antepartum steroids as an adjunctive treatment for PTL has been shown to improve neonatal survival, but is efficacious only among patients treated within 7–10 days of delivery.^{2,3} Early diagnosis of SPTB and efficient

diagnostic tools to differentiate between SPTB and PTL may help physicians to determine when to treat and when to avoid treatment. This issue is important given the recommendation to avoid multiple doses of steroids on one hand, and the relatively short-term effect of steroid treatment on the other. Thus, the development of a rapid test that is both sensitive and specific for the diagnosis of PTL would be a major advance in treatment of PTL and prevention of SPTB, and may facilitate the development of effective treatment and prevention strategies to prevent SPTB.

The etiology and pathophysiology of SPTB are multifactorial, and the underlying molecular mechanisms are poorly understood. Previous studies have explored both epidemiologic factors (genetics) and biochemical markers in various body fluids. However, despite a wide range of studies, there is no currently accepted predictive model with adequate utility to justify widespread clinical use.^{4–7} Recent advances in proteomic methods of protein separation, identification, and quantitation enable comprehensive surveys of low- and high-abundance proteins in tissues and various biological fluids. We have applied these technologies to an unbiased examination of cervical–vaginal fluid (CVF) to detect novel biomarkers and potential molecular networks that could play a critical role in SPTB. In this study, we utilized two complementary approaches: fluorescence two-dimensional in-gel electrophoresis

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(2D-DIGE), which is a gel-based separation and quantification approach, and multidimensional liquid chromatography-tandem mass spectrometry (LC/LC-MS/MS; MudPIT), a liquid separation and quantification approach, for in-depth proteome coverage and identification of potential biomarkers of PTL and SPTB in CVF.

Materials and Methods

Sample Collection and Processing. This study was approved by the Oregon Health & Science University Institutional Review Board. All subjects were identified prospectively and gave informed written consent to participate in the study. Asymptomatic control subjects of a targeted GA were enrolled from the outpatient office randomly. Asymptomatic control subjects had to have intact membranes, a GA between 16 and 37 weeks, and no signs of preterm labor or intra-amniotic infection (IAI; i.e., maternal temperature > 38 °C (100.4 °F), fundal uterine tenderness, malodorous vaginal discharge, maternal tachycardia > 100 bpm not otherwise explained, or fetal tachycardia > 160 bpm). PTL was defined as between 16 and 37 weeks GA and the combination of regular uterine contractions (3 in 10 min) with cervical dilation prior to 37 weeks gestation, and preterm birth was defined as a spontaneous delivery occurring prior to 37 weeks gestation. PTL patients had to be between 16 and 37 weeks and have regular uterine contractions (3 in 10 min) and documented cervical dilation. Each symptomatic subject was examined at least twice to determine if they met the definition of PTL. IAI was excluded in all patients by amniocentesis at time of presentation and/or placental histopathology at delivery.

Eighteen subjects ($n = 6$ in each group) were recruited, at a mean GA of 26.9 weeks \pm 7.5 SD (range 15.8–35.9). All subjects in PTL were enrolled sequentially as they presented to the labor and delivery unit of the hospital. The mean maternal parity was 0.8, and 20% of subjects had a prior preterm birth. Human CVF samples were collected prior to digital examination by placing 2 sterile 6 in. Dacron-tipped plastic applicators (Solon, Skowhegan, ME) into the posterior vaginal fornix directly underneath the posterior cervical lip and rotating them for 15 s during a sterile speculum examination. Following collection, protein was extracted into phosphate-buffered saline containing a protease inhibitor cocktail (Roche Diagnostics, Alameda, CA). Samples were spun after extraction to remove any debris and cellular material, and the supernatant was stored at -70 °C. For MudPIT analysis, five maternal CVF samples (100 μ L \times 5) each of control, PTL without preterm delivery, and SPTB without infection were individually pooled and acetone-precipitated. A total of 490 μ g of each pooled sample was dissolved in 10 mM Tris, pH 8.5. For 2D-DIGE experiments, 50 μ g each of GA-matched control, PTL, and SPTB (GA 29–34 weeks) samples were used.

Fluorescence Two-Dimensional Differential in-Gel Electrophoresis (2D-DIGE). GA-matched control/PTL/SPTB (29–34 weeks) sample sets were chosen. For each sample, 50 μ g of CVF protein was labeled with CyDye DIGE Fluor minimal dye (GE Healthcare Bio-Sciences, Piscataway, NJ) at a concentration of 400 pmol of dye/50 μ g of protein. Cy2, Cy3, and Cy5 dyes were used to label control, PTL, and SPTB, respectively, and all three labeled samples were multiplexed and resolved in one gel. Labeled proteins were purified by acetone precipitation, dissolved in IEF buffer, and rehydrated onto a 24-cm IPG strip (pH 4–7) for 12 h at room temperature. The IPG strip was subjected to one-dimensional electrophoresis at 65–70 kVh,

and then equilibrated with dithiothreitol (DTT) and iodoacetamide (IAA) equilibration buffers for 15 min sequentially. Second-dimension 8–16% SDS-PAGE was conducted at 80–90 V for 18 h.

Gels were scanned in a Typhoon 9400 scanner (Amersham Biosciences) using appropriate lasers and filters with PMT voltage set between 550 and 600. Images in different channels were overlaid using pseudo-colors, and differences were visualized using ImageQuant software (Amersham Biosciences). 2D-gel image analysis to identify differentially abundant protein spots was performed using Phoretix 2D evolution, version 2005 (Non-Linear Dynamics, Ltd.). A fixed area was selected from every gel, and a cross-stain analysis protocol was performed. Background subtraction was done using the ‘mode of non-spot’ method, and images were wrapped to maximize the spot matching. A ratiometric normalization algorithm was applied to account for potential concentration differences in protein labeling. Normalized protein spots in the Cy5 and Cy3 channels were compared to the internal standard (Cy2) to generate a ratio of relative amount. The statistical significance of differences in the intensity of protein spots was determined by *t* tests on the averaged gels for each group. Protein spots with a relative ratio >2.0 and a *t*-test value of <0.05 were considered significant.

For the identification of proteins in spots of interest, preparative 2D electrophoresis (2DE) was performed using 700 μ g of CVF protein, and gels were stained with Coomassie Blue R-250 or silver stain. Individual spots were excised from the gel, destained, and subjected to in-gel digestion with trypsin for 16–18 h at 37 °C. Peptides were extracted in ammonium bicarbonate and then filtered with a 0.22-mm MultiScreen filter plate (Millipore, Billerica, MA). Filtered solutions were dried and reconstituted in 5% formic acid (FA) for analysis by mass spectrometry.

Polyclonal Antibodies and Western Immunoblotting. Immunogenic peptides and/or recombinant proteins were used to generate rabbit and goat polyclonal antibodies (DSL Laboratories, Webster, TX). Affinity-purified antibodies were then used for Western blots. Fifty micrograms of CVF protein was resolved on a 4–20% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in Sea Block (Pierce) and incubated with 1 μ g/mL primary antibody (IGFBP-1, calgranulin-A, calgranulin-B, anexin V, or profilin1) overnight at 4 °C. After three washes with TBST, the membranes were incubated with appropriate secondary antibodies tagged with Cy dyes for 1 h in the dark with constant rocking and subsequent washing. Visualization of specific protein bands was done using a Typhoon 9400 variable mode imager (GE Healthcare Bio-Sciences).

Multidimensional Liquid Chromatography Tandem Mass Spectrometry (LC-LC-MS/MS; MudPIT) Analysis. A total of 490 μ g each of individually pooled control, PTL, and uninfected SPTB CVF samples were dried and dissolved in 100 μ L of digestion buffer containing 8 M urea, 1 M Tris base, 100 mM methylamine, and 10 mM CaCl₂ (pH 8.5). Samples were reduced and alkylated by first incubating at 50 °C in 12.5 μ L of 0.9 M DTT for 15 min and then in 25 μ L of 1.0 M IAA in dark at room temperature for another 15 min. An additional 12.5 μ L of 0.9 M DTT, along with 210 μ L of water and 1N NaOH, was added to the solution to adjust its pH to 8.5. Samples were digested with 40 μ L of 1 mg/mL trypsin (Promega) stock solution overnight at 37 °C. Digestion was stopped with the addition of 40 μ L of FA and desalted using C18 SepPak Plus

cartridges. Digests (1 mL) were injected onto a polysulfoethyl strong cation-exchange column (2.1-mm i.d. \times 100 mm, 5- μ m particle size and 300-Å pore size (The Nest Group, Southborough, MA)) and fractionated using an HPLC equipped with a UV detector and a fraction collector. Solvent A was 10 mM potassium phosphate (pH 3) with 25% acetonitrile (ACN), and solvent B was 10 mM potassium phosphate (pH 3) and 350 mM KCl with 25% ACN. A 95-min gradient at a flow rate of 200 μ L/min was employed for fractionation of peptides. In total, 80 fractions were collected, evaporated, and resuspended in 100 μ L of 0.1% TFA for desalting using a 96-well Vydac C18 silica spin plate (The Nest Group, Southborough, MA). Fractions were eluted in 80% ACN/0.1% FA, evaporated, and resuspended in 20 μ L of 5% FA, and 5 μ L of each fraction was analyzed on a Q-ToF-2 mass spectrometer connected to a CapLC (Waters, Inc., Milford, MA).

Mass Spectrometry. 2D-LC fractions and gel digests were further separated using a Nanoease C18 75- μ m i.d. \times 15-cm fused-silica capillary column (Waters, Inc.) and a 95-min water/ACN gradient. The mass spectrometer was calibrated using Glu1Fibrinopeptide B. An MS/MSMS survey method was used to acquire spectra. Masses from m/z 400 to 1500 were scanned for MS survey and masses from m/z 50 to 1900 for MS/MS. A total of 10 824 MS/MS spectra was acquired from the 2D-LC fractions. Raw MS/MS spectra were preprocessed with ProteinLynx Global Server v.2.1 software (Waters, Inc.).

On average, 3645 MS/MS spectra from each sample were searched against a combined database containing known contaminants (keratin and albumin), and forward and reverse entries of the Swiss-Prot human database (version 46.6). The peptide identification searches were performed using three independent search engines: TurboSequest (ThermoFinnigan, Waltham, MA), X! Tandem,⁸ and OpenSea.^{9,10} Sequest and X! Tandem are database search engines that match experimental spectrum to theoretical spectrum generated from a theoretical enzymatic digest of the protein database. OpenSea is a *de novo* sequence-based search engine that performs an error-tolerant matching between inexact *de novo* sequences and protein sequences in the database. Peaks software (Bioinformatics Solutions, Ontario, CA) was used to provide *de novo* sequences to the OpenSea search engine. Peptide identifications from individual search engine results were combined into protein identifications using probabilistic protein identification algorithms implemented in Scaffold (v1.3.2, Proteome Software, Portland, OR). Protein identifications that had at least two independent peptide identifications (probability \geq 0.9) were considered to be present in the sample.

Quantitation Using Spectral Counts and Statistical Analysis. Spectral counting, the total number of MS/MS spectra matched to a particular protein, has been used to assess the relative abundance of a protein in a sample.^{11–13} This method has been used to efficiently detect the abundance differences of proteins between two samples without resorting to isotopic labeling.¹⁴ All proteins in a sample with more than two confident peptide identifications were considered for protein quantitation using spectral counts. The protein lists of the samples were further curated by collapsing the spectral counts of similar proteins (e.g., immunoglobulins, α -1-acid glycoproteins 1 and 2, etc.) into a single entry.

Pairwise comparison was performed using χ^2 goodness-of-fit tests to assess whether there were significant differences between the groups (control, PTL, or SPTB) in the spectral counts for each protein. Statistical significance for each protein

was determined after adjusting for multiple comparisons via the false-discovery rate (FDR) method,¹⁵ and the level of significance was set at 0.05 (SAS version 9.1). To reduce the false-positive rate of differentially abundant proteins, only statistically significant proteins that had at least two independent peptides matched to at least four MS/MS spectra (probability \geq 0.8) in at least one of the samples were considered as truly differentially abundant. Fold changes of proteins passing the above criteria were determined using a published formula for calculating spectral count ratios.¹³

Progressive differences in the relative abundance of each protein from the control to PTL to SPTB groups were assessed by fitting generalized linear regression models with a log link function and Poisson distributed errors (i.e., Poisson regression).¹⁶ Orthogonal polynomial contrasts were used to test whether there was an increasing or decreasing trend across the ordinal subject groups. The level of significance was set at 0.05, and as above, adjustments for multiple comparisons were made via the FDR method. Significant trends were confirmed by evaluating regression coefficients of PTL versus control and SPTB versus control groups (i.e., model-based fold changes). These analyses were conducted using the GENMOD procedure in SAS version 9.1.

Results

2D-LC MS/MS Analysis of the CVF Proteome in SPTB. Multidimensional Protein Identification Technology (MudPIT, 2D-LC MS/MS)¹⁷ has proven to be an effective and robust technique for investigating global changes in protein expression as a function of development and disease.^{18,19} Recently, tandem mass spectra sampling from complex peptide mixtures has been identified as another source for quantitative information besides stable isotope labeling techniques. Several labs have shown that spectral counting linearly correlated with the abundance of the proteins in a complex mixture, was reproducible, and had a wider dynamic range.^{11–13} In this study, we have used offline MudPIT and spectral counting to determine the relative abundance of proteins in control, PTL, and SPTB CVF samples.

1. CVF Proteome. Analysis of a total of 10 824 MS/MS spectra using multiple search engines identified 205 unique proteins in CVF (Supporting Information table). Functional annotation of these proteins revealed that metabolism (25%), immune response (23%), and transport (18%) were the major categories represented in CVF.

2. Control versus PTL without Delivery. Table 1 shows a list of CVF proteins that exhibited significant changes in pairwise comparisons of control, PTL, and SPTB. A comparison of control versus PTL showed 21 proteins with statistically significant ($p < 0.05$) differential abundance. The differential presence of these proteins ranged from +28-fold to –18-fold. Eight proteins, S100 calcium-binding protein A7, mucin-5B precursor, calgizzarin, histone H2B, histone H1.2 (histone H1d), L-lactate dehydrogenase A chain, rho GDP-dissociation inhibitor 2, and 14-3-3 σ were up-regulated by >3 -fold in PTL. S100 calcium-binding protein A7, a development and cell differentiation protein, was the most significantly overexpressed protein (28-fold) in PTL compared to control. Three proteins, desmoplakin (–18-fold), periplakin (–4-fold), and junction plakoglobin (desmoplakin III) (–3-fold) were significantly down-regulated in PTL.

3. Control versus SPTB. A comparison of control versus SPTB showed 30 proteins with statistically significant ($p < 0.05$)

Table 1. CVF Proteins Sharing Significant Changes between Pairwise Comparisons of Control, PTL, and PTB Samples

Swiss-Prot accession number	protein name	spectral count			fold change			p-value		
		control	PTL	PTB	PTL	PTB	PTB	PTL	PTB	PTB
					vs control	vs control	vs PTL	vs control	vs control	vs PTL
P31151	S100 calcium-binding protein A7	1	62	4	28.3	2.1	-13.7	0.000	0.165	0.000
Q9HC84	Mucin-5B precursor	21	85	39	3.9	1.6	-2.4	0.000	0.019	0.000
Q01469	Fatty acid-binding protein, epidermal ^b	209	116	58	-1.9	-4.3	-2.3	0.000	0.000	0.000
P15924	Desmoplakin (DP)	21	0	0	-18.2	-20.3		0.000	0.000	
Q9UBC9	Small proline-rich protein 3	222	146	209	-1.6	-1.2	1.3	0.000	0.531	0.001
P80188	Neutrophil gelatinase-associated lipocalin	17	43	35	2.4	1.8	-1.4	0.001	0.012	0.365
P62328	Thymosin beta-4 (T beta 4)	10	31	35	2.8	2.9	1	0.001	0.000	0.622
P31949	Calgizarin ^b	5	21	14	3.5	2.2	-1.6	0.001	0.035	0.235
P04792	Heat-shock protein beta-1 ^b	30	11	4	-2.6	-6.8	-2.6	0.002	0.000	0.065
P62807	Histone H2B	0	9	0	8.1		-9.2	0.003		0.003
O60437	Periplakin	12	2	2	-4.2	-4.6	-1.1	0.005	0.005	1.000
P01040	Cystatin A (Stefin A) ^b	34	15	18	-2.2	-2.1	1.1	0.006	0.025	0.601
P16403	Histone H1.2 (Histone H1d)	0	8	1	7.3	1.6	-4.6	0.007	0.560	0.028
P04406	Glyceraldehyde-3-phosphate dehydrogenase	16	35	39	2.1	2.1	-1	0.007	0.002	0.642
P13796	L-plastin (Lymphocyte cytosolic protein 1)	8	22	30	2.5	3	1.2	0.009	0.000	0.266
P05109	Calgranulin A ^b	96	134	172	1.4	1.6	1.2	0.012	0.000	0.030
P00338	L-lactate dehydrogenase A chain	0	7	0	6.5		-7.4	0.013		0.013
P14923	Junction plakoglobin (Desmoplakin III)	13	3	0	-3.4	-13	-3.8	0.015	0.000	0.165
P52566	Rho GDP-dissociation inhibitor 2	1	7	12	3.6	5.2	1.4	0.024	0.001	0.249
P31947	14-3-3 protein sigma ^b	2	10	0	3.4	-2.9	-10.1	0.027	0.306	0.002
P04080	Cystatin B (Liver thiol proteinase inhibitor)	50	73	19	1.4	-2.9	-4.2	0.038	0.000	0.000
P12429	Annexin A3 (Annexin III) ^b	14	25	48	1.7	2.9	1.7	0.076	0.000	0.007
P07737	Profilin-1 (Profilin I)	22	34	49	1.5	1.9	1.3	0.107	0.001	0.099
P62805	Histone H4	10	18	4	1.7	-2.4	-4.1	0.128	0.103	0.002
P06702	Calgranulin B ^b	240	272	426	1.1	1.7	1.5	0.157	0.000	0.000
P62937	Peptidyl-prolyl <i>cis-trans</i> isomerase A	14	8	0	-1.7	-13.9	-8.3	0.218	0.000	0.007
P02763	Alpha-1-acid glycoprotein 1 precursor	7	12	38	1.6	4.2	2.7	0.249	0.000	0.000
P01009	Alpha-1-antitrypsin precursor	2	5	57	1.9	16.1	8.5	0.249	0.000	0.000
P80511	Calgranulin C	0	2	21	2.6	15.8	6.2	0.306	0.000	0.000
P01042	Kininogen precursor	3	1	10	-1.9	2.3	4.5	0.306	0.046	0.003
P02774	Vitamin D-binding protein precursor (DBP)	3	6	22	1.7	4.9	2.9	0.313	0.000	0.002
P61626	Lysozyme C precursor	36	30	57	-1.2	1.4	1.7	0.460	0.029	0.004
P08758	Annexin A5 (Annexin V)	0	1	11	1.8	8.7	4.9	0.560	0.001	0.005
P08833 ^a	Insulin-like growth factor binding protein 1	0	1	4	1.8	3.7	2.1	0.560	0.080	0.250
O43707	Alpha-actinin 4	9	11	24	1.2	2.2	1.9	0.654	0.008	0.026
P29508	Squamous cell carcinoma antigen 1	80	83	35	1	-2.6	-2.6	0.814	0.000	0.000
P02787	Serotransferrin precursor (Transferrin)	89	89	194	-1	2	2	0.970	0.000	0.000
P00738	Haptoglobin precursor	16	16	40	-1	2.1	2.2	1.000	0.001	0.001
P02751 ^a	Fibronectin precursor (FN)	0	0	4		3.7	3.7		0.080	0.080

^a Proteins that showed significant fold change but did not reach statistical significance due to small number of spectral counts. ^b Proteins that also showed differential expression from 2D-DIGE analysis. Bold values in p-value columns are < 0.05.

differential abundance. Seven proteins, α-1-antitrypsin precursor, calgranulin C, annexin A5 (annexin V), rho GDP-dissociation inhibitor 2, vitamin D-binding protein precursor (DBP), α-1-acid glycoprotein 1 precursor, and L-plastin (lymphocyte cytosolic protein 1), were up-regulated by >3-fold in SPTB. Alpha-1-antitrypsin, a protease inhibitor, was the most significantly overexpressed protein (16-fold), followed by calgranulin C (~16-fold) and annexin A5 (8.5-fold) in SPTB. Six proteins, desmoplakin (DP), peptidyl-prolyl *cis-trans* isomerase A, junction plakoglobin (desmoplakin III), heat-shock protein β-1, periplakin, and epidermal fatty acid-binding protein, were down-regulated by >3-fold in SPTB.

4. PTL without Delivery versus SPTB. A comparison of PTL versus PTB showed 25 proteins with statistically significant (*p* < 0.05) differential abundance. Four proteins, α-1-antitrypsin precursor (8.5-fold), calgranulin C (6.2-fold), annexin A5 (annexin V) (4.9-fold), and kinninogen (4.5-fold), were up regulated by >3-fold in PTB CVF. Eight proteins, S100 calcium-binding protein A7 (-13-fold), 14-3-3 σ (-10.1-fold), histone H2B (-9.2-fold), peptidyl-prolyl *cis-trans* isomerase A (-8.3-fold), L-lactate dehydrogenase A chain (-7.4-fold), histone H1.2 (-4.6-fold),

cystatin B (-4.2-fold), and histone H4 (-4.1-fold), were down-regulated by >3-fold in SPTB compared to PTL.

5. Trend Analysis. To estimate the trend and linearity of the relative abundance of common proteins found in control, PTL, and SPTB, a GENMOD linear regression model (SAS version 9.1) was used by fitting generalized linear regression models with a log link function and Poisson distributed errors (i.e., Poisson regression).¹⁶ The level of significance was set at 0.05, and adjustments for multiple comparisons were made via the FDR method. Sixteen proteins were found to be differentially (*p* < 0.003) present in all three samples (Table 2). Thirteen proteins consistently showed a statistically significant increase in SPTB > PTL without delivery > control. Only three proteins, epidermal fatty acid-binding protein, heat-shock protein beta-1, and desmoplakin showed a decrease in SPTB < PTL without delivery < control.

2D-DIGE Analyses of Control, PTL, and SPTB CVF. Two-dimensional gel electrophoresis has been widely used to characterize the serum proteome²⁰ to identify biomarkers for cancer and other diseases. To enhance sensitivity, reproducibility, and detection over a wide dynamic range, we utilized a

Table 2. CVF Proteins Showing Progressive Differences in Relative Abundance, Control < PTL < PTB

Swiss-Prot accession number	protein name	spectral count			p-value ^a
		control	PTL	PTB	
P06702	Calgranulin B	240	272	426	0.000
P02787	Serotransferrin precursor	88.5	89	194	0.000
P05109	Calgranulin A	96	134	172	0.000
Q01469	Fatty acid-binding protein, epidermal	209	116	58	0.000
P01009	Alpha-1-antitrypsin precursor	2	5	57	0.000
P07737	Profilin-1 (Profilin I)	22	34	49	0.002
P12429	Annexin A3 (Annexin III)	14	25	48	0.000
P00738	Haptoglobin precursor	16	16	40	0.002
P04406	Glyceraldehyde-3-phosphate dehydrogenase, liver	16	35	39	0.003
P02763	Alpha-1-acid glycoprotein 1 precursor (AGP 1)	7	12	38	0.000
P62328	Thymosin beta-4 (T beta 4)	10	31	35	0.000
P13796	L-plastin (Lymphocyte cytosolic protein 1)	8	22	30	0.001
P80511	Calgranulin C	1	3	22	0.003
P02774	Vitamin D-binding protein precursor	3	6	22	0.001
P04792	Heat-shock protein beta-1 (HspB1)	30	11	4	0.000
P15924	Desmoplakin (DP)	22	1	1	0.003

^a Statistical significance was defined at FDR of 0.05 after adjustment for multiple comparisons.

multiplex proteomic analysis approach to label proteins with fluorescent cyanine dyes. Three GA-matched controls, PTL, and SPTB CVF (GA 29–34 weeks) were labeled with Cy3, Cy5, and Cy2, respectively. Each labeled GA-matched sample pair was resolved in the same gel. Intensity of the green or red colors indicates the differentially abundant protein levels, and yellow represents comparatively similar abundance. Pseudo-color visualization of control/PTL/SPTB gel maps (ImageQuant; GE Healthcare) showed a distinct pattern of up-regulation of proteins in PTL/SPTB (Figure 1A–C).

A quantitative analysis of the CVF proteome resolved by 2D-DIGE was done using Phoretix 2D evolution software. The use of an internal standard (Cy2) as the third channel increased the quality of the analysis by providing references for spot normalization and matching. The spot-quantification protocol matched, on average, 590 spots in pairwise comparisons. Seventeen proteins were differentially present by more than 2-fold in PTL without delivery and SPTB CVF samples (Table 1).

Eleven proteins were up-regulated, and six proteins were down-regulated in SPTB when compared with PTL without delivery. Protein identification from the gel spots revealed that 14-3-3 σ , or stratifin, showed the highest overexpression (11-fold), followed by annexin A2 (7.5-fold), cystatin A (5.7-fold), calgranulin B (4-fold), and cellular retinoic acid-binding protein 2 (3.9-fold). Involucrin was highly down-regulated (15-fold), followed by epidermal fatty acid binding protein (6.3-fold) and cytoplasmic actin 2 (2.9-fold) in the SPTB sample.

MudPIT experiments revealed many differentially abundant SPTB biomarkers with high confidence (>90%), some of them complementary to the 2D-DIGE experiments. The differentially abundant proteins common between 2D-DIGE and MudPIT were 14-3-3 σ , calgranulin B, S100 calcium binding protein A7, α -1-antitrypsin, and cystatin A. MudPIT additionally identified many smaller differentially abundant proteins that were difficult to separate and visualize using 2D gel conditions. Furthermore, as a result of the all-liquid fractionation, better sequence coverage was obtained for many of the identified proteins in MudPIT versus the 2D gel experiments. Immunoblotting with specific antibodies for calgranulins, annexin V, and profilin 1 confirmed the consistent trends (Figure 2) observed in MudPIT analysis. IGFBP-1 exhibited a distinct proteolytic pattern and higher levels of expression in SPTB. The

majority of the IGFBP-1 detected corresponded to a low-molecular-weight, 11-kDa proteolytic fragment previously described as an amniotic fluid biomarker for intra-amniotic infection.²¹

Discussion

SPTB is a major problem in perinatal medicine worldwide and is the leading cause of perinatal deaths not attributable to congenital malformations. An estimated 8 million perinatal deaths occur annually, primarily due to prematurity and neonatal sepsis.^{22,23} The rate of SPTB has not decreased over the last three decades, in spite of improved healthcare,²⁴ and in the U.S., the rate of SPTB has continuously increased over the past 25 years to a rate of 12.5% in 2004 (National Vital Statistics 2004). Numerous studies have attempted to identify markers of SPTB. Associations between epidemiologic risk factors, cervical length, cervical–vaginal fetal fibronectin (fFN) single nucleotide polymorphisms, maternal medical conditions, vaginal infections, and protein biomarkers in amniotic fluid and other biologic fluids have been analyzed in the hope of developing a useful model for the positive prediction of SPTB. To date, however, no robust marker has been validated for general clinical use.

Several biological fluids, including CVF, saliva, and plasma, have all been used as a source to detect markers for SPTB. However, none of these markers were found to be good predictors of preterm delivery. Various hormones in saliva have been evaluated as potential biomarkers of SPTB. Of these, only salivary estriol has been shown to be a marker for SPTB, usually beyond 32 weeks of gestation.^{25–27} Since infants delivered beyond 32 weeks of gestation are at low risk for neonatal morbidity and death compared to infants delivered at earlier GAs, the clinical usefulness of this marker is limited. Serum or plasma components have been evaluated extensively for markers of SPTB. Goldenberg et al.^{28,29} have shown that a serum granulocyte colony-stimulating factor (G-CSF) level above the 75th percentile and serum ferritin level above the 90th percentile are among the strongest predictors of SPTB. High α -fetoprotein, alkaline phosphatase, and corticotrophin-releasing hormone levels are also potential serum markers of SPTB.^{30,31} Several substances in CVF have been previously evaluated as possible biomarkers for SPTB. Of all the markers

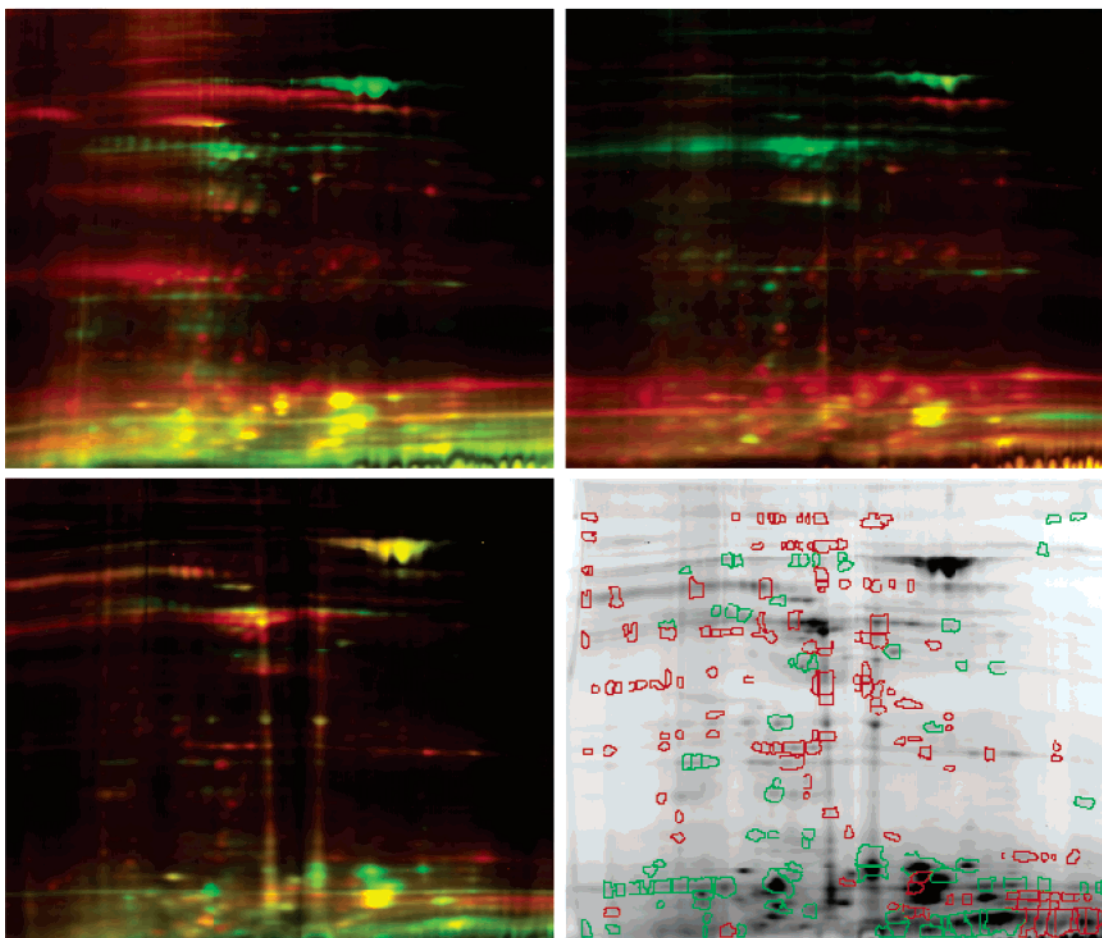
A. Control vs PTB**B. Control vs PTL****C. PTB vs PTL****D. PTB vs PTL**

Figure 1. 2D-DIGE analysis of PTL, PTB, and control CVF samples. (A) Overlay of PTB (green) and control (red), (B) PTL (green) and control (red), and (C) PTB (green) and PTL (red); (D) map of the differentially abundant proteins between PTB and PTL. (D) Differential spot map of panel C. Spots outlined in green represent >2-fold higher in PTB, and those in red represent >2-fold lower in PTB with respect to PTL. The spot map was generated by Phoretix Evolution. Proteins identified are numbered and shown in Table 1.

to date, only fFN in the cervix or vagina has been shown to be a reliable negative predictor for SPTB at approximately 24–26 weeks of gestation³² in women with symptoms of preterm PTL. However, at other gestational ages, particularly prior to 24 weeks, fFN has a low sensitivity for SPTB (<20%).³³

The multiple proteomic approaches employed in this study identified distinct sets of proteins that were differentially abundant in CVF of women delivering preterm compared to those found in the CVF of women with SPTL who delivered at term (Tables 1 and 2). Pairwise comparisons of asymptomatic controls with samples of patients with PTL and SPTB for the first time revealed the presence of a unique set of markers for PTL that are distinct from SPTB (Table 1). It is likely that further studies of these potential PTL markers will facilitate a better understanding of the mechanism of PTL and provide new avenues for therapy.

Progressive analysis (trend analysis) revealed a potential list of markers that exhibited a gradual increase from asymptomatic controls to SPTB. These markers could be beneficial to monitor

the risk of SPTB through serial measurement. While this study did not include serial evaluation of markers, the presence of protein biomarkers in PTL patients before SPTB suggests that serial sampling of patients with PTL symptoms may prove to be a useful way to follow subjects with preterm contractions to determine when interventions such as antenatal steroids should be administered. Trend analysis identified the S100 proteins as one group of molecules showing significant statistical differences. The S100 proteins calgranulins A, B, and C have been previously described as differentially present in maternal serum and amniotic fluid of women with SPTB, and are generally up-regulated in the setting of infection and inflammation.²¹ S100 proteins are thought to modulate biological activity via calcium binding,³⁴ and increased levels of S100 proteins in neonatal CSF, blood, and urine have been associated with neonatal brain damage.^{35,1} The differential abundance of S100 proteins in our study may reflect the increased prevalence of subclinical IAI and inflammation in women with PTL resulting in SPTB compared to those who go to term.

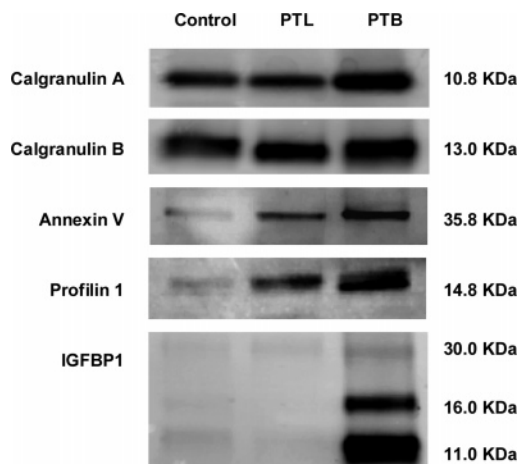


Figure 2. Immunodetection of potential biomarkers of spontaneous preterm birth in human CVF. A total of 50 μ g of CVF protein representing each sample group was blotted and probed with specific antibodies. IGFBP-1 bands represent intact protein (~30 kDa) and proteolytic fragments (~16 and ~11 kDa).

Similarly, trend analysis also showed the presence of negative predictors of SPTB, including epidermal fatty acid binding protein, heat shock protein beta-1, and desmoplakin (Table 2). It is possible that immunoassays assessing both up- and down-regulated proteins will convey even greater sensitivity and specificity to the diagnosis of SPTB.

Important acute-phase reactants exhibited increasing abundance in our pairwise comparisons and trend analysis (Tables 1 and 2). These included α -1-acid glycoprotein (A1AG), α -1-antitrypsin precursor, and annexins A3 (annexin III) and A5 (annexin V). Elevated levels of A1AG have been previously reported prior to delivery in rhesus macaques,³⁶ while α -1-antitrypsin, a glycoprotein protease inhibitor released by leukocytes in response to inflammatory stimuli, appears to play an important role in maintenance of the uterine surface and placental attachment.³⁷ The production of α -1-antitrypsin by human trophoblastic tissue has been demonstrated.³⁸ The physiologic role of annexin III has not been fully determined; however, its proposed function as a mediator of intracellular calcium signaling and transmembrane calcium transportation, as well as its presence in the placenta and in neutrophils,³⁹ supports a possible role for this protein in the pathophysiology of SPTB. Annexin V has been implicated in pregnancy loss,⁴⁰ preeclampsia, and intrauterine growth restriction,⁴¹ suggesting a possible mechanistic role for annexin 5 in decidual infarction/placental-mediated PTL.

Several CVF proteins that were differentially abundant in PTL and SPTB are integral to cytoskeletal structure, arrangement, and motility. Profilin-1, rho GDI 2, and thymosin β -4 are all involved in the organization and biogenesis of the actin cytoskeleton and were up-regulated in SPTB. Profilin-1, rho GDI 2, and thymosin have also been implicated in either the inhibition of actin polymerization or disruption of the actin cytoskeleton.⁴² Profilin-1 is also known to bind to poly-L-proline motifs⁴³ and has been implicated in host-pathogen interactions. *Listeria* and *Shigella* bacteria produce profilin-1-binding proteins that enable them to use the host-cell cytoskeleton for invasion of neighboring cells.⁴³ Involucrin is an epithelial structural protein and is a marker of early differentiation of epidermal cells. It has been used as a biomarker of early differentiation in the cervix in chemoprevention trials.⁴⁴

This study provides the most comprehensive analysis to date of differential protein profiles in the CVF of asymptomatic control subjects compared to those in PTL who deliver at term and those with SPTB. MudPIT and 2D-DIGE both revealed several proteins that were significantly differentially abundant in PTL and SPTB samples. The findings of this study, however, are based on a limited number of samples and must be validated in a larger cohort. Furthermore, despite the biological plausibility of several of our observations, the possibility exists that some of our findings are due to random biological variation. To minimize this possibility, we considered only proteins in which the spectral count and fold change yielded a p -value < 0.001 and performed pairwise comparisons among control, PTL, and SPTB groups as well as a trend analysis to identify differential expression from control < PTL < SPTB. The findings of this study have potential implications for the clinical practice of obstetrics if one or more of these proteins can be modeled for clinical use. Currently, the most widely utilized cervical-vaginal marker for SPTB is fFN, a biomarker with good specificity but poor sensitivity. We identified several proteins with greater differential expression than fFN when comparing both the asymptomatic group and PTL group to the SPTB group. Among these are several of the proteins discussed above, including calgranulin C, α -1-acid glycoprotein, α -1-antitrypsin precursor, and annexin V.

The identification of novel protein biomarkers of SPTB represents an important step forward in advancing our understanding of the physiologic perturbances that lead to preterm birth. We acknowledge that our findings should be considered preliminary until validated in a larger cohort. However, to reverse the trend of the last 25 years, which has seen the rate of SPTB steadily rise in the U.S., innovative treatment strategies based upon the reliable identification of women at high-risk for preterm birth must be developed.

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Supporting Information Available: Table of CVF proteins identified from the analysis of 10 824 Ms/MS spectra using multiple search engines. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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