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Maternal Serum Serpin B7 Is Associated With Early Spontaneous Preterm Birth

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Abstract

Objective—To identify serum biomarkers of early spontaneous preterm birth (SPTB) using semi-quantitative proteomic analyses.

Study Design—Nested case-control study of pregnant women with previous SPTB. Maternal serum was collected at 19 to 24 and 28 to 32 weeks gestation, and analyzed by liquid chromatography-multiple-reaction monitoring-mass spectrometry. Targeted and shotgun proteomics identified 31 candidate proteins that were differentially expressed in pooled serum samples from spontaneous preterm (<34 weeks - cases) and term deliveries (controls). Candidate

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protein expression was compared in individual serum samples between cases and controls matched by age and race groups, and clinical site. Protein expression was verified by Western blot in the placenta and fetal membranes from cases and controls.

Results—Serum samples were available for 35 cases and 35 controls at 19 to 24 weeks, and 16 cases and 16 controls at 28 to 32 weeks. One protein, serpin B7, yielded serum concentrations that differed between cases and controls. The mean concentration of serpin B7 at 28 to 32 weeks was 1.5-fold higher in women with subsequent preterm deliveries compared to controls; there was no difference at 19 to 24 weeks. Higher levels of serpin B7 at both gestational age windows were associated with a shorter interval to delivery, and higher levels of serpin B7 in samples from 28 to 32 weeks were associated with a lower gestational age at delivery. Western blotting identified serpin B7 protein in placenta, amnion, and chorion from cases and controls.

Conclusion—Targeted and shotgun serum proteomics analyses associated one protein, serpin B7, with early SPTB. Our results require validation in other cohorts and analysis of the possible mechanistic role of serpin B7 in parturition.

Keywords

preterm birth; proteomics; serine proteinase inhibitors

Introduction

Preterm birth, defined as birth before 37 weeks of gestation, is a leading cause of infant morbidity and mortality. In the US, approximately 12 percent of all births are preterm.[1] Despite decades of research, there has been little progress in developing effective interventions to prevent preterm birth. In fact, the rate of preterm birth has increased slightly over the last several decades.[2] The ultimate goal of the Genomic and Proteomic Network for Preterm Birth Research (GPNPBR) is to identify possible biomarkers that could predict the susceptibility to spontaneous preterm birth (SPTB) as well as to shed light on the molecular mechanisms involved in its etiologies. Understanding those mechanisms will help us predict SPTB and may facilitate the introduction of more effective prevention and treatment strategies.

We hypothesized that maternal proteomic profiles could prospectively identify women at risk for SPTB. In order to test this hypothesis, we developed a longitudinal cohort study of 500 women with a history of SPTB <37 weeks enrolled before 18 completed weeks of pregnancy at three GPN-PBR clinical sites. The previous preterm delivery criterion was chosen in order to enrich the cohort for preterm delivery cases in the current pregnancy. Maternal blood was collected at three study visits (enrollment visit between $10^{0/7}$ weeks and $18^{6/7}$ weeks gestation, second study visit between $19^{0/7}$ weeks and $23^{6/7}$ weeks, and third study visit between $28^{0/7}$ weeks and $31^{6/7}$ weeks) and at admission for delivery.

Recent advances in the application of various proteomics-based platforms have facilitated the discovery of novel protein SPTB biomarkers.[3-6] Mass spectrometry-based shotgun proteomics methodology has become a standard method for characterizing proteomes of biological fluids as well as tissues.[7-9] Further advancements in multidimensional protein

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separation techniques have made it possible to identify low-abundance proteins and thereby increase the dynamic range of detection in complex biological samples such as plasma and serum.[10, 11] In addition, the application of stable isotope labeling of amino acids in cell culture (SILAC) technology permits the generation of entire labeled proteomes that can be used as internal standards to facilitate accurate quantification of peptides and proteins thereof in biological samples.[12, 13] Finally, liquid chromatography-multiple reaction monitoring mass spectrometry (LCMRM/MS) methodology provides speed, sensitivity, selectivity and the ability to quantitate multiple peptides simultaneously.[14, 15] The present study was designed to probe human serum samples by combining 1) targeted proteomics utilizing the quantification capabilities of SILAC to generate a stable isotope labeled proteome (SILAP) standard and LC-MRM/MS methodology, and 2) shotgun proteomics utilizing the enhanced search capabilities of multidimensional LC tandem mass spectrometry (LC/MS/MS) to identify candidate protein SPTB biomarkers.

Materials and Methods

Longitudinal cohort

The cohort of 500 pregnant women with previous SPTB was projected to have 30 SPTB <34 weeks (six percent of the cohort) and 60 SPTB between 34 and 37 weeks (12 percent of the cohort; overall SPTB rate = 18 percent) that could be matched with term deliveries from the same cohort between 39 and 41 weeks for nested case-control analyses. The number of patients in each group was estimated based on other studies of similar nature and valid estimates of sample size.[16]

Women were eligible for enrollment at the three GPN-PBR clinical sites (University of Alabama at Birmingham, University of Texas Medical Branch at Galveston, and University of Utah) if they had a history of at least one SPTB of a singleton pregnancy at a gestational age between $20^{0/7}$ weeks and $36^{6/7}$ weeks in a previous pregnancy and a current singleton pregnancy less than $18^{6/7}$ weeks gestation. Study participants were enrolled during routine prenatal visits before 19 weeks of pregnancy. Gestational dating was based upon the first day of the patient's last menstrual period (LMP) and ultrasound examination performed before enrollment. If the LMP date was uncertain, the ultrasound measurements obtained at the participant's first ultrasound examination were used to determine the project gestational age. If the date of the LMP was certain and the ultrasound confirmed this gestational age within seven days, then the LMP-derived gestational age was used. If the ultrasound-determined gestational age did not confirm the LMP-generated gestational age within seven days, then the ultrasound was used to determine the project gestational age.

Exclusion criteria were maternal uterine anomalies, planned cervical cerclage, multi-fetal gestation, fetal aneuploidy or lethal fetal anomalies, polyhydramnios (amniotic fluid index ≥25 cm or deepest vertical pocket ≥12 cm), planned or probable delivery at a non-network site, no availability for prospective specimen/data collection, and serious maternal medical conditions (such as renal disease, chronic liver disease, organ transplant recipients, spinal cord injuries, severe pulmonary disorders, severe heart disease, malignancy not in remission, antiphospholipid syndrome, genetic thrombophilia, diabetes mellitus class C or greater, hemoglobinopathy, isoimmunization, chronic conditions requiring medication for control

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[such as chronic hypertension, lupus, inflammatory bowel disease, and asthma], and HIV infection).

Following the enrollment visit, participants were seen again at $19^{0/7}$ weeks to $23^{6/7}$ weeks and at $28^{0/7}$ weeks to $31^{6/7}$ weeks. Maternal peripheral blood samples were collected in serum separator tubes at all study visits and at admission for delivery. The blood samples were allowed to coagulate at 4C for 30 minutes, then the samples were centrifuged at 1,200 *g* for ten minutes at room temperature. Aliquots of serum were placed in liquid nitrogen and stored at −80C.

Demographic and outcome data were recorded by trained research coordinators in a web-based database developed by the network's data coordinating center (DCC) at Yale University. Serum samples were shipped on dry ice to the network's analytical core at the University of Pennsylvania, where all proteomics assays were performed by laboratory personnel who were blinded to demographic and outcome data. Proteomics results and clinical outcomes were analyzed at the DCC at Yale University. The GPN-PBR scientific protocol was approved by the Investigational Review Boards at all five institutions.

Proteomics techniques

We utilized targeted and shotgun proteomics techniques to develop a panel of peptides to measure in serum samples from subjects in our longitudinal study.

Targeted proteomics were performed using SILAP released by four biologically relevant transformed cell lines: endocervical (End1) cells, vaginal mucosal (Vk2) cells, endometrial carcinoma (ECC1) cells, and placental choriocarcinoma (BeWo) cells. Transformed cells were obtained from American type Culture Collection (ATCC, Manassas, VA). Methods for SILAC-based targeted proteomics were described previously.[6] Briefly, cells were grown in stable isotope-labeled serum-free DMEM/F12 media containing [$^{13}C_6{}^{15}N_2$]-lysine and [$^{13}C_6{}^{15}N_1$]-leucine (Cambridge Isotopes, Cambridge, MA). Cells were passaged seven times and then supernatant was collected every other day, filtered through a 0.22 m filter, concentrated through a 5 kDa MW cutoff spin-filter (Millipore, Billerica, MA), pooled, and stored at −80C until analyzed. Supernatants were depleted of six high-abundance plasma proteins (albumin, transferrin, haptoglobin, anti-trypsin, immunoglobulin G [IgG], and IgA) using a multiple affinity removal system (MARS Hu6) affinity LC column (Agilent Technologies, Palo Alto, CA). Protein concentration was estimated by Coomassie Protein Assay (Thermo Scientific, Rockford, IL), and supernatants were stored at -80C. Equal amounts of protein (100 g/cell line) from the immunodepleted End1, Vk2, ECC1, and BeWo supernatants were mixed together to create the End1-Vk2-ECC1-BeWo SILAC secretome.

In order to identify proteins in the End1-Vk2-ECC1-BeWo SILAC secretome, proteins were precipitated using a standard methanol/chloroform protocol and digested with trypsin (Promega, Madison, WI).[6] Strong cation exchange (SCX) chromatography was performed on a PolySulfoethyl A column (The Nest Group, Southborough, MA) attached to an HP 1100 HPLC system (Agilent). For each sample, 32 two-minute fractions were collected and pooled into 9 fractions as previously described.[6] These 9 fractions were lyophilized and stored at -80C until further analysis. Individual SCX fractions were analyzed by microflow

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reversed phase LC-electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) using a high resolution LTQ Orbitrap-XL instrument (Thermo Scientific, San Jose, CA) operating at a resolution of 100,000 at *m/z* 400.

The MS/MS spectra were searched against an indexed human RefSeq database (version updated November 2007, 33,439 entries) with TurboSEQUEST (Thermo Scientific, Waltham, MA, version 27.12) and Mascot (Matrix Science, Boston, MA, version 2.2.03). Strict trypsin cleavage rules with maximum of two missed cleavages, mass accuracy of 1 Da for the precursor and fragment ion, and variable modifications of methionine oxidation, carboxyamidomethlyation on cysteine, [$^{13}C_6{}^{15}N_2$]-lysine and [$^{13}C_6{}^{15}N_1$]-leucine and were applied in the search criteria. The SEQUEST and Mascot output files were integrated into Scaffold version 2.01 (Proteome Software, Portland, OR) for validating MS/MS based peptide and protein identifications. Assignment of peptide sequences was performed using the PeptideProphet algorithm.[17] PeptideProphet accounts for the distribution of scores over an entire data set to calculate the probability of a correct assignment for every peptide. PeptideProphet calculates false-positive error rates at specific probability score cutoff values for each data set.[18] A minimum PeptideProphet probability score of ≥0.5 was used to remove low probability peptides. At this cutoff, the estimated false-positive error rate was 10.8 percent. Protein identifications were accepted with a minimum ProteinProphet probability score of ≥0.8 and at least two identified unique peptides.[19] For this data set, a ProteinProphet probability score of ≥0.8 corresponded to a false-positive error rate of four percent. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

The End1-Vk2-ECC1-BeWo secretome was developed so that it could be used as a SILAP standard to quantitate candidate protein levels in human serum samples by LC-MRM/MS using a high sensitivity Vantage TSQ Mass Spectrometer (Thermo Scientific). The protocol for targeted proteomics in serum samples is illustrated in Figure 1A. Serum samples collected from women during the second and third study visits and at admission for delivery were pooled in two comparison groups – five women who experienced SPTB <34 weeks and five women who experienced uncomplicated term deliveries at 39 to 41 weeks. Protein (15 [.proportional]g) from pooled serum samples from each study visit was added to equal amounts of protein (15 [.proportional]g) from the End1-Vk2-ECC1-BeWo SILAP secretome, and each sample was digested with trypsin and analyzed by LCMRM/MS. A total of 264 proteins were identified consistently in the pooled serum samples, and the quantity of each protein was determined by computing its L/H ratio (L=light [endogenous] peptide amount; H=heavy [SILAP] peptide amount). The L/H ratio of three proteins was significantly higher at second/third study visits and delivery visits among pooled serum samples from women who delivered preterm, while the L/H ratio of five proteins was significantly lower at second/third study visits and delivery visits among pooled serum samples from women who delivered preterm ($P<0.10$, Mann-Whitney rank sum test, Table 1). We selected these eight proteins for inclusion in our overall protein panel for comparison among individual serum samples from SPTB and term deliveries in the longitudinal cohort. We also added fibronectin and thrombospondin proteins, which have potentially important biological relevance[6] and were detected in the End1-Vk2-ECC1-BeWo SILAP secretome

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and pooled serum samples (but not at different levels between SPTB and term deliveries), to the list of targeted proteins (Table 1).

Shotgun proteomics was performed by comparing pooled serum samples from SPTB with pooled serum samples from term deliveries. This made it possible to expand the list of candidate biomarker proteins for comparisons between individual SPTB cases and term delivery controls (Figure 1B). Serum samples from each study visit were pooled for five SPTB cases and for five term delivery controls (5 women/group x 3 visits/woman = 15 samples pooled/group). Pooled serum samples underwent IgY-14 immuno-depletion LC (IgY-14 column, Seppro Protein Depletion, Sigma Life Science, St. Louis, MO) to remove the 14 most abundant plasma proteins, trypsin digestion, SCX fractionation into ten fractions, and LC-MS/MS analysis. A total of 336 proteins were identified in pooled serum samples from SPTB, while 448 proteins were identified in pooled serum samples from term deliveries. A total of 21 proteins were expressed differentially between pooled samples from SPTB and pooled samples from term deliveries ($P<0.10$, Mann-Whitney rank sum test, Table 2).

The 21 proteins identified by shotgun proteomics and the ten proteins identified by targeted proteomics formed the panel of 31 candidate proteins the levels of which were compared by LC-MRM/MS analysis (Figure 2) in individual serum samples from nested cases and controls. Briefly, 10μL of serum from individual SPTB cases and term delivery controls was diluted in 490 μl phosphate-buffered saline (approximate protein concentration = 1 μg/μL), and 40 μL of diluted serum was combined with 8 μg End1-Vk2-ECC1-BeWo SILAP secretome. Proteins in each sample were precipitated, reduced, and digested with trypsin. The levels of digested peptides in each sample were measured by LC-MRM/MS (5 μL injections, two peptides/protein, three MRM/peptide). The shotgun proteomics data has been deposited and is freely available at the Proteomics, Identifications (PRIDE) database of the European Bioinformatics Institute, Hinxton, UK (http://www.ebi.ac.uk/pride/).

Western blotting

Western blots were performed to measure levels of proteins identified in maternal serum proteomics assays in amnion, chorion, and placenta samples collected from SPTB cases and term delivery controls in a separate GPN-PBR observational study. Biospecimens were collected at cesarean delivery in approximately 120 women in six clinical groups (n=20 in each group): 1) preterm delivery without labor (maternal or fetal indications for delivery at 24 and 0/7 weeks to 34 and 6/7 weeks); 2) preterm delivery following idiopathic preterm labor; 3) preterm delivery following preterm premature rupture of membranes (PPROM) without labor at 24 and 0/7 weeks to 34 and 6/7 weeks; 4) preterm delivery following PPROM and labor; 5) term delivery without labor (fetal malpresentation or elective repeat cesarean delivery at 39 and 0/7 weeks to 41 and 6/7 weeks); and 6) term delivery following labor. Protein levels were measured in amnion, chorion, and placenta biopsy samples from five cases in clinical groups 1, 2, 5, and 6 (preterm delivery +/− labor and term delivery +/−labor).

Briefly, protein was extracted from amnion, chorion, and placenta samples using RIPA buffer (G-Biosciences, St. Louis, MO) with Complete Mini tablet (Roche, Indianapolis, IN).

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Protein quantification was performed using Pierce BCA Protein Assay Kits (Thermo Scientific, Rockford, IL). Western blots were performed using 20 μg of protein for each sample loaded into Mini-Protean TGX Precast Gels 12% (BioRad Laboratories, Hercules, CA). All blots were transferred to Immobilon PVDF membranes (Millipore Corporation, Billerica, MA), which were washed in diH_2O and blocked with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE). Blots were then probed for protein expression using primary rabbit polyclonal antibodies (1:5000) and anti-beta-tubulin antibody (loading control, 1:5000, ab6046, Abcam, Cambridge, MA). After incubating blots with anti-rabbit IgG (goat) IRDye secondary antibody in Odyssey buffer for IR blots, the blots were read using the Odyssey CLx Infrared Imaging System (LICOR).

Protein levels in Western blots were calculated by densitometric analysis of infrared fluorescent signals, which were directly proportional to the amount of antigen on Odyssey Western blots. Normalization was performed against the internal control protein (beta-tubulin).

Statistics

Clinical data and protein expression levels were compared between SPTB and term deliveries using chi square tests for categorical data, Student's *t* tests for normally distributed continuous data, and rank sum tests for non-normally distributed continuous data. The primary analysis was the comparison of maternal serum protein levels at all three study visits between SPTB cases and term delivery controls. Initial analyses indicated that first visit serum samples did not yield informative protein levels for further analyses, so more extensive analyses were performed using specimens obtained from second and third study visits. Secondary analyses included: 1) correlation between protein levels and days from study visit to delivery; 2) correlation between protein levels and gestational age (days) at delivery; 3) correlation between changes in protein level between second and third study visits and SPTB; and 4) combinations of proteins (i.e., biomarker panel) that were associated with SPTB.

An overall difference in protein levels in Western blots was compared among the four clinical groups by one-way analysis of variance (ANOVA), and differences in protein levels between individual clinical groups were compared using the Newman-Keuls Multiple Comparison Test.[20]

Results

Among the 500 women enrolled in the high-risk cohort, 39 women had a subsequent SPTB before 34 weeks gestation (39/500 = 7.8 percent incidence, range $23^{6/7}$ to $33^{5/7}$ weeks). Serum samples from second study visits (19-24 weeks) were available from 35 of these women, who were matched to the next subject (according to subject number) by age (+/−4 years), race, and clinical site and who delivered at 39-41 weeks gestation without complications (35 controls). Serum samples from the third study visit (28-32 weeks) were available for only 16 SPTB cases, largely because many of these women delivered before the third study visit (Figure 3). Demographic characteristics for the 35 SPTB cases before 34 weeks gestation and 35 term delivery controls are listed in Table 3.

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The L/H ratios (endogenous peptide area/SILAP-derived peptide area) were calculated in each maternal serum sample for the ten proteins (20 peptides, Table 1) identified by targeted proteomics techniques. Maternal serum levels of the 21 proteins (41 peptides, Table 2) identified by shotgun proteomics techniques were quantified by calculating the endogenous peptide area/SILAP- derived peptide area. No peptides from the second study visit maternal serum samples were secreted at significantly different levels between preterm birth cases and controls, and only one peptide (serpin B7, peptide 2 [YVEVFFPQFK]) from third study visit maternal serum samples was secreted at a significantly greater level in preterm birth cases compared to controls (Table 4).

Secondary analyses were conducted to study the relationship between maternal serum protein expression and: 1) interval (days) from study visit to delivery; 2) gestational age (days) at delivery; and 3) changes in protein levels between the second and third study visit and SPTB. During the second study visit, an elevated maternal serum level of only one peptide (serpin B7, peptide 1 -ADLSGIASGGR) was associated significantly with a shorter interval to delivery (P=0.02), and during the third study visit, an elevated maternal serum level of serpin B7, peptide 2 (YVEVFFPQFK) was associated significantly with a shorter interval to delivery (P=0.001). No peptides identified during the second study visit were associated significantly with the gestational age at delivery, but an elevated maternal serum level of one peptide (serpin B7, peptide 2 - YVEVFFPQFK) during the third study visit was associated significantly with an earlier gestational age at delivery (P=0.004). More specifically, greater maternal serum levels of the serpin B7 peptide at the third study visit were associated with an earlier gestational age at delivery. Finally, changes in peptide levels between the second and third study visits were not associated with SPTB.

We assessed if expression changes in a set of proteins in maternal serum samples earlier in pregnancy might predict SPTB. In addition to the association between higher concentrations of serpin B7, peptide 2 (YVEVFFPQFK) from the third study visit with SPTB (P=0.02), we observed a trend toward significance between greater levels of calreticulin precursor, peptide 2 (EQFLDGDGWTSR) in maternal serum samples from the third study visit and SPTB (P=0.06, Table 4). Thus, we studied serpin B7and calreticulin precursor as a potential biomarker panel associated with SPTB. We observed that both peptide levels were elevated above the mean level in 5/16 women who delivered preterm (sensitivity 0.31) and in 0/16 women who delivered at term (specificity 1.00, Table 5). Meanwhile, both peptide levels were below the mean level in 4/16 women who delivered preterm and in 10/16 women who delivered at term. We observed that the relationship between SPTB and three categories of peptide levels (no elevated levels, one elevated level, two elevated levels; P=0.02) and the relationship between SPTB and two categories of peptide levels (no elevated levels versus one or two elevated levels; P=0.04) were statistically significant. No other predictive biomarker panels were identified.

Western blots were performed to measure serpin B7 expression in amnion, chorion, and placenta samples from 10 preterm deliveries (24 and 0/7 weeks to 34 and 6/7 weeks) and 10 term deliveries (39 and 0/7 weeks to 41 and 6/7 weeks). Serpin B7 was detected in all amnion, chorion, and placenta samples (Figure 4). Among the four clinical groups, serpin B7 levels were greatest in samples from women who delivered preterm (24 weeks 0 days to

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34 weeks 6 days) without labor and were significantly greater than in women delivering preterm with labor and term with and without labor (Figure 5).

Comment

Utilizing targeted and shotgun based proteomics techniques, we developed a panel of 31 proteins with potential biological significance and expressed at low abundance in maternal serum. We observed that only one protein, serpin B7, was detected at higher levels in serum samples from women who delivered preterm compared to women who delivered at term. In serum samples obtained at the second study visit (19 and 0/7 weeks to 23 and 6/7 weeks), elevated serpin B7 L/H ratios were associated with a shorter interval to delivery. In serum samples obtained at the third study visit (28 and 0/7 weeks to 31 and 6/7 weeks gestation), elevated serpin B7 peptide levels were associated with SPTB at <34 weeks, a shorter interval to delivery, and an earlier gestational age at delivery. A biomarker panel combining maternal serum serpin B7 and calreticulin precursor levels was not more closely associated with SPTB than serpin B7 levels alone, although elevated levels of both peptides was more discriminatory by excluding all pregnancies that delivered at term (sensitivity 0.3, specificity 1.0). No other useful biomarker panels could be developed. Western blot experiments, confirmed that serpin B7 was expressed in pregnancy-related tissues (placenta and fetal membranes).

The study had two major strengths. First, rigorous, semi-quantitative proteomics techniques were utilized that enabled us to study potentially clinically relevant proteins detected at low levels in maternal serum samples. Specifically, SILAC-based assays allowed us to study proteins that were secreted by cells derived from reproductive tissues, while our shotgun-based techniques allowed us to focus on 21 proteins that were expressed differentially in pooled serum samples from SPTB compared to term deliveries. Second, the careful enrollment, sample collection, and data entry procedures that were employed by experienced research coordinators at the GPN-PBR clinical sites ensured accurate clinical phenotyping of study subjects and preserved the integrity of biospecimens used in the network's studies. In addition, enrollment of subjects at multiple sites and the diversity of subjects enrolled in the network studies enhanced the generalizability of our results.

The major limitation of the study is that our targeted proteomics study focused exclusively on proteins that were secreted by biologically relevant transformed cell lines (endocervical [End1] cells, vaginal mucosal [Vk2] cells, endometrial carcinoma [ECC1] cells, and placental choriocarcinoma [BeWo] cells). However, all proteomics platforms are limited in the analysis of complex mixtures containing different protein classes with specific physicochemical properties, and our complementary targeted and shotgun proteomics techniques utilized labeling-based methods that yielded accurate protein identification and semi-quantitative results.[6, 21, 22] Quantitative proteome profiling is critical for comparative analysis of proteins from normal and diseased patients, as similar proteins may be present in both states but at significantly different concentrations.[21, 22] Because we were more concerned that these methods were more likely to exclude potentially interesting proteins (false negative results) than include false positive results, we did not correct for multiple comparisons. Hence, these discovery-based results require validation in other cohorts.

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Over the past decade, other investigators have employed proteomics techniques to study biomarkers of SPTB in amniotic fluid, maternal serum, and maternal cervicovaginal fluid samples.[4-6, 23-26] Earlier studies also investigated protein expression in amniotic fluid samples in relation to intra-amniotic infection.[27, 28] Limitations to previous studies using mass spectrometry techniques to identify proteins in maternal serum samples include the age of samples used (more than ten years old) and restricted analyses of low molecular weight proteins.[4, 26] Two groups of investigators employed surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry to identify protein profiles in amniotic fluid samples that were associated with risk of preterm delivery, but the proteins were not identified in either study.[23, 25] Two previous studies identifying proteins in maternal cervicovaginal fluid produced promising results but were limited by small sample sizes.[5, 6]

In our serum proteomics assays, the only protein we discovered at elevated levels in women who later delivered preterm was serpin B7, which originally was identified in 1998 in human glomeruli.[29] Serpin B7 (or megsin) is a serine proteinase inhibitor, clade B (ovalbumin), member 7. It is postulated that increased levels of serpin B7 may inhibit protease (plasmin) activity that normally leads to extracellular matrix degradation.[30] Elevated levels of serpin B7 have been detected in the glomeruli of patients with IgA nephropathy.[31] Consequently, maternal serum levels of serpin B7 may be elevated in response to extracellular matrix degradation, but serpin B7 expression has not been studied previously in relation to SPTB, and upstream mediators of matrix degradation might be better biomarkers of SPTB. In our study, serpin B7 protein expression was detected at highest levels in amnion, chorion, and placenta samples from women who delivered preterm without labor. Although all samples were obtained following cesarean delivery to exclude mode of delivery as a potential confounder, the expression of serpin B7 in these samples should be considered in relation to the pregnancy complications that led to these iatrogenic preterm deliveries (i.e., severe preeclampsia, fetal growth restriction and abnormal fetal heart rate patterns).

In summary, serpin B7 was identified as a potential biomarker associated with SPTB. These semi-quantitative targeted and shotgun proteomics results require validation in other cohorts, including low-risk women with no history of SPTB, and analysis of the possible mechanistic role of serpin B7 in parturition. Meanwhile, a similar proteomics approach can be utilized with other maternal specimens (e.g., urine, cervicovaginal fluid, saliva) to identify biomarkers associated with spontaneous preterm birth.

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Condensation

Semi-quantitative proteomics analyses using second trimester maternal serum samples associated serpin B7 with early spontaneous preterm delivery.

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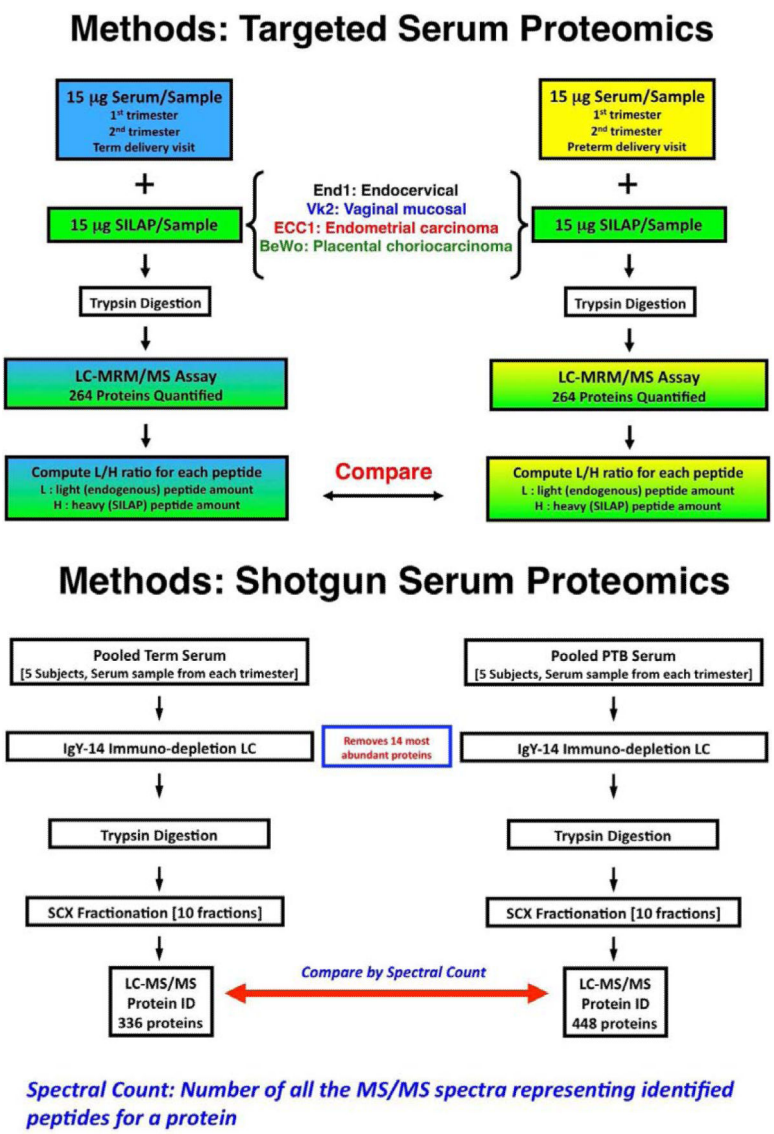

Figure 1.

Protocols for targeted (1A) and shotgun (1B) proteomics comparing pooled maternal serum samples from five spontaneous preterm births and five term deliveries.

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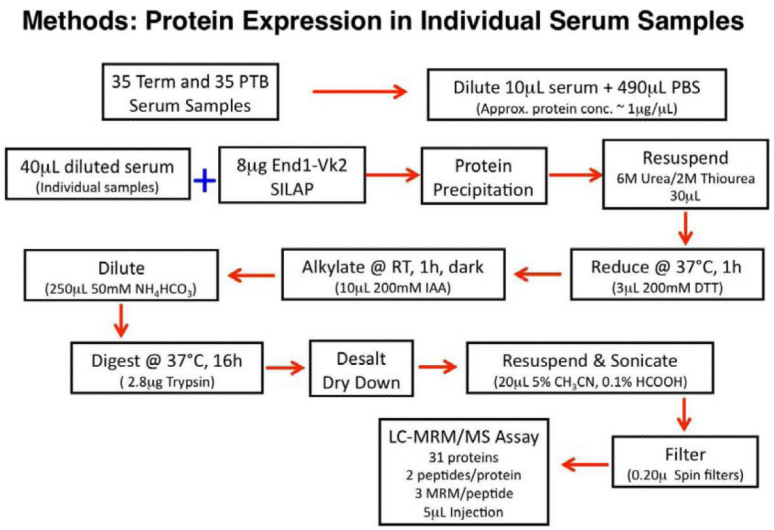

Figure 2.

Protocol for comparing the levels of 31 candidate proteins by liquid chromatography-multiple reaction monitoring mass spectrometry in individual maternal serum samples from nested preterm birth cases and controls.

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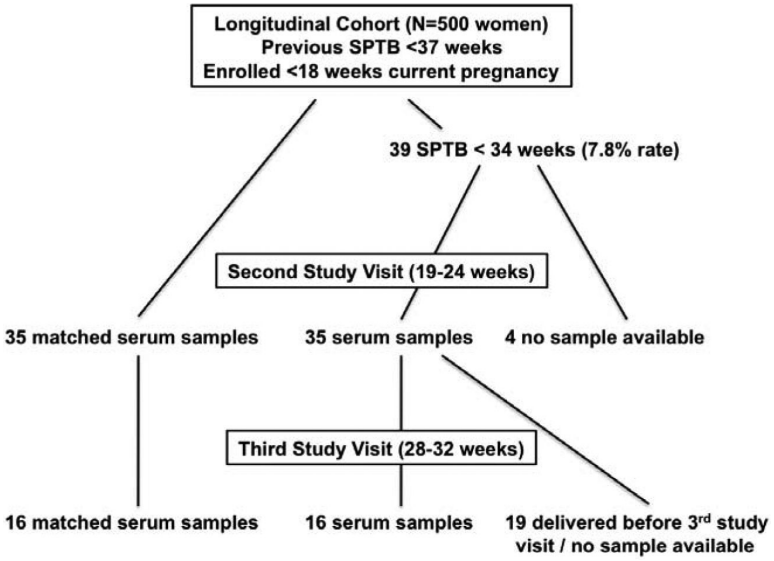

Figure 3.

Flow chart of patients in the longitudinal cohort whose serum samples from second and third study visits were used in proteomic analyses. SPTB = spontaneous preterm birth.

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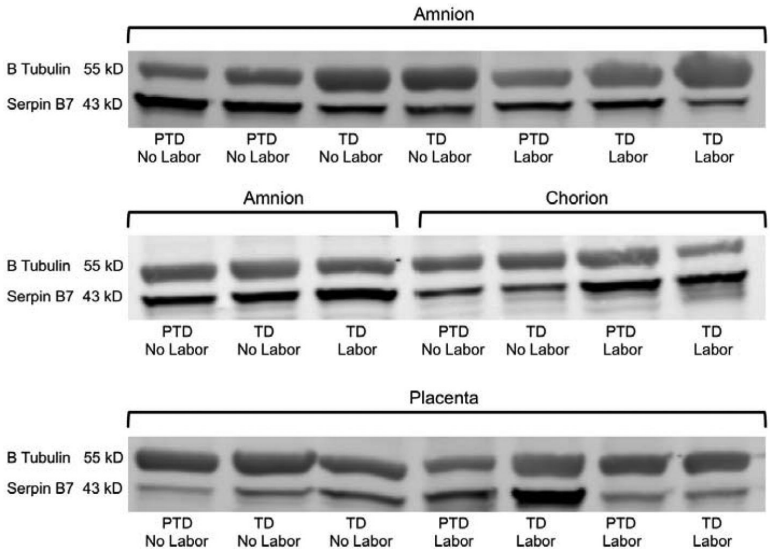

Figure 4.

Western blots demonstrating serpin B7 and beta-tubulin (loading control) expression in representative amnion, chorion, and placenta samples. The blots were probed for serpin B7 expression using a rabbit polyclonal antibody to a 330-amino acid peptide of human serpin B7 (ab47740, Abcam). Samples were mixed in each gel to enhance objectivity of assessment. TD = term delivery. PTD = preterm delivery.

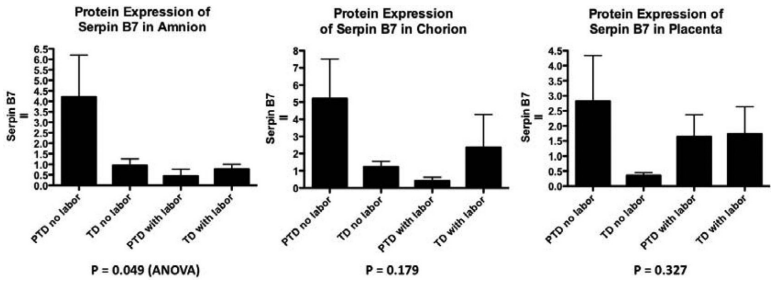

Figure 5.

Bar graphs demonstrating serpin B7 expression (normalized to beta-tubulin expression) in amnion, chorion, and placenta samples from preterm deliveries (PTD) and term deliveries (TD), +/− labor preceding delivery.

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Table 1

Ten proteins in End1-Vk2-ECC1-BeWo SILAC secretome were detected at different levels in pooled serum samples from five spontaneous preterm delivery cases and five term delivery controls. Levels of these proteins were measured by LC-MRM/MS in individual serum samples from spontaneous preterm delivery cases and term delivery controls.

PROTEIN	PEPTIDE
Cadherin-1; type 1 preprotein	DTANWLEINPDTGAISTR
	NTGVISVVTTGLDR
Calreticulin precursor	FYALSASFEPFSNK
	EQFLDGDGWTSR
Serpin B7	ADLSGIASGGR
	YVEVFFPQFK
Proteasome subunit β type-5 isoform 3	ATAGAYIASQTVK
Glia-derived nexin isoform c precursor	VLGITDMFDSSK
	TIDSWMSIMVPK
Galectin-3 binding protein	LADGGATNQGR
	ELSEALGQIFDSQR
Peptidylprolyl isomerase 3	VSFELFADK
	FEDENFILK
Heat shock protein β1	VSLDVNHFAPDELTVK
	LATQSNEITIPVTFESR
Thrombospondin-1 precursor[1]	TIVTTLQDSIR
	SITLFVQEDR
Fibronectin-1 isoform 1 preprotein[1]	FLATTPNSLLVSWQPPR
	NTFAEVTGLSPGVTYYFK
	DLQFVEVTDVK

[1]Thrombospondin and fibronectin were detected in the End1-Vk2-ECC1-BeWo SILAC secretome and in pooled maternal serum samples, but their levels were not significantly different between pooled samples from spontaneous preterm delivery cases and term delivery controls. These proteins were added to the panel of proteins identified by targeted proteomics techniques because of their potential biological importance.

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Table 2

Twenty-one proteins were detected by shotgun proteomics techniques at different levels in pooled serum samples from five spontaneous preterm delivery cases and five term delivery controls. Levels of these proteins were measured by LC-MRM/MS in individual serum samples from spontaneous preterm delivery cases and term delivery controls.

PROTEIN	PEPTIDE
Zinc-α2-glycoprotein	YSLTYIYTGLSK
	AYLEEECPATLR
Histidine-rich glycoprotein	ADLFYDVEALDLESPK
	SGFPQVSMFFTHTFPK
Pappalysin-1	SPAVITGLYDK
	SFDNFDPVTLSSCQR
Carboxypeptidase N	LSNNALSGLPQGVFGK
	DHLGFQVTWPDESK
Serum amyloid P-component	VGEYSLYIGR
	GYVIIKPLVWV
Apolipoprotein A-IV	SELTQQLNALFQDK
	LGPHAGDVEGHLSFLEK
Apolipoprotein E	SWFEPLVEDMQR
	GEVQAMLGQSTEELR
α2-antiplasmin isoform b	DSFHLDEQFTVPVEMMQAR
	WFLLEQPEIQVAHFPFK
Ficolin-3 isoform 1	ALPVFCDMDTEGGGWLVFQR
	LLGEVDHYQLALGK
Corticosteroid-binding globulin	WSAGLTSSQVDLYIPK
	GTWTQPFDLASTR
Kininogen-1 isoform 1	ENFLFLTPDCK
	DIPTNSPELEETLTHTITK
Hyaluronan-binding protein-2 isoform 1	FCEIGSDDCYVGDGYSYR
	NPDADEKPWCFIK
Plasminogen isoform 1	VIPACLPSPNYVVADR
	FVTWIEGVMR
N-acetylmuramoyl-L-alanine amidase	TDCPGDALFDLLR
	EFTEAFLGCPAIHPR
Insulin-like growth factor c-binding protein	APGWDPLCWDECR
Pregnancy-zone protein	ALLAYAFSLLGK
	NALFCLESAWNVAK
Prothrombin	HQDFNSAVQLVENFCR
	IVEGSDAEIGMSPWQVMLFR
Retinol-binding protein-4	LLNLDGTCADSYSFVFSR

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PROTEIN	PEPTIDE
	DPNGLPPEAQK
Sex hormone-binding globulin isoform 3	IALGGLLFPASNLR
	DIPQPHAEPWAFSLDLGLK
Vitronectin	DVWGIEGPIDAAFTR
	SIAQYWLGCPAPGHL
Serpin peptidase inhibitor; clade G; member 1	HRLEDMEQALSPSVFK
	GVTSVSQIFHSPDLAIR

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Table 3

Demographic characteristics of 35 women with spontaneous preterm deliveries before 34 weeks gestation and 35 women with uncomplicated term deliveries at 39-41 weeks gestation in the nested case-control study.

Characteristic	Cases (n=35)	Controls (n=35)	P value[1]
Maternal age, mean±SD	26.34±5.67	26.71±5.08	0.7739
Maternal race (%)			
Black or African American	37.14	40.00	0.8060
Caucasian	54.29	54.29	1
Asian	2.86	2.86	1
Other	5.71	2.86	0.5551
Gravidity (%)			
1	31.43	31.43	1
2	17.14	34.29	0.1008
3	17.14	5.71	0.1329
4	25.71	17.14	0.3822
5 or 6	8.57	11.43	0.6903
Previous preterm delivery (%)			
1	68.57	71.43	0.7942
2	14.29	22.86	0.3565
>2	17.14	5.71	0.1329
Previous term delivery (%)			
0	60.00	65.71	0.6208
1	28.57	22.86	0.5844
>1	11.43	11.43	1
Maternal BMI (***lb/in^2?***), mean±SD	25.68±4.69	27.02±7.54	0.3838
Maternal education (%)			
K-5 (Elementary)	5.71	2.86	0.5551
6-8 (Middle School)	14.29	17.14	0.7426
9-12 (High School or GED)	51.43	45.71	0.6324
13-16 (College)	28.57	34.29	0.6066

[1] Chi-square tests were used for categorical variables, and *t* tests were used for continuous variables.

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Table 4

L/H ratios (endogenous peptide area/SILAP area) in maternal serum samples from the third study visit (28 weeks 0 days to 31 weeks 6 days gestation) for the ten proteins identified by targeted proteomics techniques.

PROTEIN	PEPTIDE	Term Births L/H[1] (N=16)	Preterm Births LH[1] (N=16)	P value[2]
Cadherin-1; type 1 preprotein	DTANWLEINPDTGAISTR	0.96±0.29	0.89±0.36	0.53
	NTGVISVVTTGLDR	0.81±0.23	0.83±0.25	0.83
Calreticulin precursor	FYALSASFEPFSNK	0.19±0.08	0.19±0.06	0.98
	EQFLDGDGWTSR	0.18±0.06	0.23±0.10	0.06
Serpin B7	ADLSGIASGGR	ND	ND	
	YVEVFFPQFK	0.04±0.02	0.06±0.02	0.02
Proteasome subunit β type-5 isoform 3	ATAGAYIASQTVK	ND[3]	ND	
Glia-derived nexin isoform c precursor	VLGITDMFDSSK	***0.53±0.07***	***0.48±0.05***	***0.28***
	TIDSWMSIMVPK	ND	ND	
Galectin-3 binding protein	LADGGATNQGR	ND	ND	
	ELSEALGQIFDSQR	***5.86±0.55***	***5.72±1.74***	***0.85***
Peptidylprolyl isomerase A	VSFELFADK	***0.18±0.06***	***0.16±0.06***	***0.71***
	FEDENFILK	***0.24±0.09***	***0.24±0.08***	***1.00***
Heat shock protein β1	VSLDVNHFAPDELTVK	ND	ND	
	LATQSNEITIPVTFESR	0.09±0.03	0.11±0.04	0.14
Thrombospondin-1 precursor	TIVTTLQDSIR	0.84±0.60	1.20±1.10	0.26
	SITLFVQEDR	0.79±0.38	0.87±0.61	0.66
Fibronectin-1 isoform 1 preprotein	FLATTPNSLLVSWQPPR	***5.28±1.83***	***3.18±0.89***	***0.33***
	NTFAEVTGLSPGVTYYFK	***3.65±1.27***	***2.57±0.65***	***0.37***
	DLQFVEVTDVK	ND	ND	

[1] L/H expressed as mean±SD for normally distributed L/H ratios and as median±SE for non-normally distributed L/H ratios (***bold italics***)

[2] P values determined by two-sample *t* tests with equal variances for normally distributed L/H ratios and by two-sample Wilcoxon rank-sum (Mann-Whitney) tests for non-normally distributed L/H ratios

[3] ND = not detected

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Table 5

Biomarker panel (serpin B7, peptide 2 [YVEVFFPQFK] and calreticulin precursor, peptide 2 [EQFLDGDGWTSR]) at third study visit and spontaneous preterm delivery at <34 weeks gestation. Peptide levels were considered elevated in individual maternal serum samples if the level exceeded the overall mean level for that peptide. The levels of both peptides were normally distributed in the nested case-control study.

	0 Elevated Peptide Levels	1 Elevated Peptide Levels	2 Elevated Peptide Levels
Term Deliveries (N=16)	10	6	0
Preterm Deliveries (N=16)	4	7	5
3 categories (no elevated levels, one elevated level, two elevated levels) P=0.02[1]			
2 categories (no elevated levels versus one or two elevated levels) P=0.04[1]			

[1] P values were calculated by Pearson chi square tests.