Chapter 1

Spontaneous Unexplained Preterm Labor with Intact Membrane: Finding Protein Biomarkers through Placenta Proteome

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Abstract

Spontaneous unexplained preterm labor with intact membrane (sPTL-IM) remains as an unresolved challenge in obstetrics due to the complex syndromes involved during preterm birth. Two dimensional-gel electrophoresis (2D-GE) coupled with matrix-assisted laser desorption/ionization-time of flight/time of flight (MALDI TOF/TOF) mass spectrometry has become an alternative in screening for potential novel protein-based biomarkers and revealing the pathophysiology of sPTL-IM. To achieve this objective, protein extracted from fetal and maternal sides of the placenta obtained from sPTL-IM (n = 5) and the respective control (n = 10) groups were separated and compared using 2D-gel electrophoresis. MALDI-TOF/TOF mass spectrometry was utilized to identify the differentially expressed proteins between both groups, and the molecular functions of these proteins were studied. A total of 12 proteins were significantly differentiated in sPTL-IM over the control. Differentially expressed proteins were identified to have involved in structural/cytoskeletal components, immune responses, fetal and placenta development, and anticoagulation cascade. More proteins were found to be differentially expressed in the fetal side compared to the maternal side of the placenta. This postulates that the influence of sPTL-IM from fetus is greater than that of the mother. Ultimately, these results might lead to further investigations in elucidating the potential of these proteins as biomarkers and/or drug targets.

Keywords: comparative placenta proteomics, 2D-gel electrophoresis, MALDI-TOF/TOF, placenta tissues, proteomics, spontaneous unexplained preterm labor

1. Introduction

Spontaneous preterm labor (PTL) is one of the major causes of preterm birth (PTB), which accounts for 56% of the total PTB [1] and it possesses deteriorating effects, both long and short term, on the health of the mother and the offspring [2]. Moreover, statistics showed that 30% of pregnant women who experienced spontaneous PTL are often spontaneous with no known signs of PTL (herein termed as spontaneous unexplained PTL with intact membrane (sPTL-IM)) [3].

Current diagnosis for spontaneous PTL-IM was based on several predictors which include risk factors assessment, cervical measurement, and/or biochemical markers specifically fetal fibronectin and phosphorylated insulin-like growth factor-binding protein 1 [4]. These protein biomarkers possess a major limitation where they can only be detected 14 days prior to onset, providing a short time frame to plan for preventive care and treatment [5]. On the other hand, positive-predictive values of other newly found biomarkers including thioredoxin or interleukin 1 receptor antagonist had shown to be relatively poor [4, 5].

It has become evident that existing and newly found protein biomarkers often lack specificity and sensitivity to provide an effective prediction measure against sPTL-IM among pregnant women [6, 7]. Specific and sensitive predictive tool is important to allow the effective use of treatment or care for the risked mothers and avoid the use of unnecessary medical interventions, which further reduces medical costs. Furthermore, throughout every stages of pregnancy from conception to parturition, it is believed that levels of certain protein biomarkers fluctuate. Hence, the identification of these proteins that are associated with sPTL-IM is a sensational method to establish reliable novel biomarkers in assisting sPTL-IM prediction among pregnant women, as well as unwinding its underlying complex pathophysiologies [6, 7].

Since results from easily accessible biological fluids do not always reflect accurate localized information on the state of a pregnancy and impending labor, the use of gestational tissues can be advantageous. The placenta is an organ connecting developing fetus with the mother that plays roles during labor [8]. Interestingly, the study reported that protein expression in the fetally origin tissues (chorion) is different from the maternally origin tissues (decidua basalis) [9]. Thus, both the fetal and the maternal side of the placenta are a potential source of preliminary discovery of biomarker associated to the processes of sPTL-IM. Unfortunately, studies that applied proteomic approaches to discover protein alterations in the placenta mainly focused on pregnancies complicated with gestational diabetes mellitus and preeclampsia [10, 11] while little is known about the proteome from placenta in relation to sPTL-IM. Therefore, investigating protein levels in delivered placentas offers researchers not only to identify potential biomarker panels but also to study the delivery pathophysiology of sPTL-IM.

Given the clinical significance of sPTL-IM and the presence of proteomics technology, placenta has become a good source for proteome study associated with sPTL-IM. The purpose of this research was to discover protein biomarkers that could potentially be used to identify the risk of sPTL-IM among pregnant women. In addition, this will also allow us to understand any functional classes of proteins that are associated with sPTL-IM and disclose its

pathophysiological complications. For this, we have applied an existing proteomic approach (two dimensional-gel electrophoresis (2D-GE)-coupled matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS)) to identify differentially expressed protein during sPTL-IM by comparing the fetal and maternal placenta proteome between the sPTL-IM and spontaneous term labor with intact membrane (sTL-IM) placentas, respectively, prior to the study of their functions.

2. Materials and methods

2.1. Sample population

The study was approved by the Medical Research and Ethics Committee (NMRR-13-1660-18742) and written informed consent was obtained from all subjects prior to placenta collection.

Placenta was taken from five asymptomatic pregnant women who experienced sPTL-IM (22 and 36 + 6 weeks of gestation). The control group comprised pregnant women who underwent spontaneous labor with ≥37 completed weeks of gestation (n = 10) and had at least one previous history of spontaneous PTB. Gestational age was determined from the last menstrual period and by ultrasound measurements up to the second trimester. Another ultrasound measurement was performed during the second trimester to rule out congenital malformations.

All subjects were patients who sought for prenatal care and delivered in Hospital Serdang, Malaysia, during 2014–2015 and represented the Malay population in Peninsula Malaysia who carried a singleton pregnancy. Risk factor was at least one prior spontaneous PTB of a singleton infant due to spontaneous PTL-IM (>20 to <37 weeks of gestation). Pregnant women with known obstetrical complications such as multiple gestations, chronic infective diseases, hypertension, preeclampsia, pregestational diabetes, severe anemia, placenta previa, known malignancy, known or suspected congenital malformation of the fetus, symptoms/signs of PTL, fetal loss in second trimester, intrauterine growth restriction (IUGR), had an immunodeficiency virus, cervical incompetence, planned cervical cerclage, uterine malformation, antepartum hemorrhage, and previous cervical surgery were excluded from the investigation. Subjects with uncertain gestation were also excluded from this study. Moreover, obstetric complications needing iatrogenic and preterm prerupture of membranes (PPROM) deliveries were also not recruited in the study. It is believed that PPROM did not share the same condition as spontaneous PTL [12]. Each patient was given an anonymized ID number. Demographic and clinical characteristics of these pregnant women from whom preterm and term samples utilized in this study were collected and are presented in **Table 1**.

2.2. Reagents

The chemicals used in this study include phosphate buffer saline (PBS, Sigma-Aldrich, USA, P4417), protease inhibitor mix (GE Healthcare, USA, 80-6501-23), β-mercaptoethanol (Invitrogen, US, 11528926), urea (GE Healthcare, USA, 17-1319-01), thiourea (GE Healthcare, USA, RPN6301), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, GE

Characteristics	Case (n = 5)	Control (n = 10)	P value
Estimated gestational age (weeks)	35.4 ± 0.89	38.5 ± 0.53	–
Maternal age (years)	33.2 ± 5.89	32.7 ± 5.93	NS
Parity	3 (1–6)	4 (1–6)	NS
Fetus weight (kg)	2.32 ± 0.40	3.22 ± 0.25	0.02*
Placenta weight (g)	384 ± 20.74	488.5 ± 11.56	<0.002*

NS, not significant; data are presented as means ± SEM, except parity data are presented as median (range).*$P < 0.05$ when compared to the other group.

Table 1. Demographic and clinical details of women participated in this study.

Healthcare, USA, 17-1314-01), Tris (Bio-Rad, USA, 1610716), biolyte 3/10 ampholyte (Bio-Rad, USA, 1632094), dithiothreitol (DTT, GE Healthcare, USA, 17-1318-02), hydrochloric acid solution (HCl, Fisher Chemical, UK, SA48-500), ethylenediaminetetraacetic acid (EDTA, Sigma, USA, E9884), potassium chloride (KCl, Sigma-Aldrich, USA, P9541), sucrose (Fisher Reagent, USA, S25590), methanol (Fisher Reagent, USA, A4521), dichloromethane (DCM, Fisher Reagent, USA, AC326600025), lithium chloride (LiCl, Sigma-Aldrich, USA, 13013), 2D quant kit (GE Healthcare, USA, 80-6483-56), glycerol (Sigma-Aldrich, USA, G5516), sodium dodecyl sulfate (SDS, Bio-Rad, USA, 1610302), bromophenol blue (Bio-Rad, USA, 1610404), pre-stained BLUeye (Gene Direx, USA, PM007-0500), acrylamide/bis mixed solution (37.5:1) (Nacalai tecque, Japan, 06121), wide-range gel preparation buffer (Nacalai tecque, Japan, 07831-94), N,N,N′,N′-tetramethylethylenediamine (TEMED, GE Healthcare, USA, 17-1312-01), ammonium persulfate (APS, Sigma-Aldrich, USA, 09913), agarose NA (GE Healthcare, USA, 17-0554-01), glycine (Bio-rad, USA, 1610718), DNase I (New England Biolabs, UK, M0303 L), immobiline dry strip pH 3-10 non-linear, 13 cm (GE Healthcare, USA, 17-6001-15), dry strip cover fluid (GE Healthcare, USA, 17-1335-01), iodoacetamide (IAA, Bio-Rad, USA, 1632109), formaldehyde solution (Sigma-Aldrich, USA, F8775), silver nitrate (Sigma-Aldrich, USA, 209139), sodium carbonate (Sigma-Aldrich, USA, S7795), sodium thiosulfate pentahydrate (Sigma-Aldrich, USA, 217247), glacial acetic acid (Sigma-Aldrich, USA, 320099), ethanol (EtOH, Fisher Chemical, USA, A955-4), phosphoric acid (Fisher Chemical, USA, A260-500), ammonium sulfate (Fisher Chemical, USA, A702-500), Coomassie brilliant blue G-250 (Sigma, UK, 27815), acetonitrile (Fisher Chemical, UK, A998-212), acetic acid (Fisher Chemical, USA, A35-500), ammonium hydrogen citrate (Merck, UK, 101154), trifluoroacetic acid (TFA, Sigma-Aldrich, UK, 302031), and α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, UK, C8982).

Milli-q water (Milli-Q Integral 3 water purification system, Merck Millipore, USA) was used in preparing all the chemical solutions required.

2.3. Tissue collection, processing, and storage

To improve the homogeneity and comparability, placentas selected in this study were collected from pregnant women who were experiencing sPTL-IM or sTL-IM with prior history of spontaneous PTL-IM among Malay ethnicity only.

Placentas with attached fetal membranes and umbilical cords were collected within 10 min of delivery and thoroughly washed in ice-cold PBS to remove the blood. Placenta tissues were collected from six different locations from the side facing the basal plate (maternal side) as well as the side facing the chorionic plate (fetal side). Relative consistency was maintained during collection by selecting six closest locations between all collected placentas; a grid indicated with six locations was overlaid as a guide. Moreover, the site with calcium deposits, visible connective tissue, and blood clots were avoided. The chorion with adherent decidua was separated from the amnion using sterile forceps. All collected tissues were snapped frozen in liquid nitrogen and stored at −80°C until protein extraction.

Based on the histology evidence by a trained histologist, no evidence of histological infection in placenta parenchyma was reported in all samples.

2.4. Protein extraction by DNase/lithium chloride-dense sucrose homogenization-coupled dichloromethane-methanol precipitation

For each individual sample, a total of 500 mg placenta tissues were randomly chosen and pulverized in a mortar with pestle containing liquid nitrogen. Powdered placenta tissues were subjected to protein extraction using DNase/lithium chloride-dense sucrose, and then cleaned up with dichloromethane-methanol precipitation as described previously by Tan et al. [13].

These purified protein samples were stored at −80°C until 2D-GE was performed.

2.5. Protein quantification

The total placentas protein concentration of the purified placentas tissue lysates from both case and control (maternal and fetal sides) were quantified by 2D Quant kit (GE Healthcare) according to the manufacturer's instruction. Bovine serum albumin was used as a standard throughout all the independent experiments.

2.6. 2D gel electrophoresis

The 2D-placenta proteome of the fetal and maternal sides of sPTL-IM was compared to their term controls, respectively. The purified tissue lysates from both case and control placentas (maternal and fetal sides) were diluted in a rehydration buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 0.2% biolyte 3–10, 20 mM DTT, 40 mM Tris, 0.002% (w/v) bromophenol blue to a final concentration of 266 μg, and a total volume of 250 μL. The protein samples were passively rehydrated at room temperature onto the 13 cm, pH 3–10 nonlinear IPG strips (GE Healthcare) for 16 h. Strips were overlaid with a dry strip cover fluid and focused using IPGphor II IEF system (Ettan IPGphor 3 IEF, GE Healthcare, USA) at 20°C and 50 μA/strip of maximum current limit using the following program: step and hold at 500 V for 500 Vh, gradient at 1000 V for 750 Vh, gradient at 8000 V for 11,250 Vh, and step and hold at 8000 V for 7333 Vh.

After completion of the first dimension IEF, IPG strips were equilibrated for 15 min at room temperature in a buffer containing 6 M urea, 50 mM Tris/HCl pH 8.8, 40% (v/v) glycerol, 2% (w/v) SDS, 0.02% bromophenol blue, and 65 mM DTT, followed by a 15-min incubation at

room temperature in the same buffer containing 135 mM IAA in place of DTT. After equilibration, IPG strips were placed and sealed on top of a 1.0-mm thick 10% wide-range PAGE with 0.5% agarose containing 0.02% bromophenol blue dye for second dimension wide-range PAGE analysis. The gels were run using the SE 600 Ruby electrophoresis tank (GE Healthcare). Each gel was run at a constant current of 10 mA for 15 min at 15°C followed by 25 mA for the remainder of the run until the tracking dye migrated to within 1 cm of the bottom of the gel.

To minimize gel-to-gel variation, placenta tissue lysates from both sPTL-IM and control placentas were ran simultaneously in a quart gel electrophoresis system (Ruby, GE Healthcare) for the second dimension and stained.

2.7. Protein visualization, statistical analysis, and spot detection

Case and control 2D gels (maternal and fetal sides) were visualized by silver nitrate [14] or colloidal Coomassie blue staining [15]. After staining, silver-stained 2D gels were scanned using the Image Scanner III imager (Image scanner III, GE Healthcare, USA). Gels stained with colloidal Coomassie blue were kept in 1% (v/v) acetic acid at 4°C until spot picking for protein identification.

All gel images were imported, and the protein spots were quantified by using ImageMaster 2D platinum software, version 7 (GE Healthcare Amersham Biosciences) program for comparative analysis. Protein spots were quantified in terms of their relative volume where the individual spot volume is divided by the total volume of the whole set of gel spots.

2D-gel images of sPTL-IM (maternal and fetal sides) were compared with term controls (maternal and fetal sides), respectively. Automatic gel image alignment was applied first and adjusted manually. Every single spot detected by the software was reviewed, and artifacts were excluded. To avoid variation in analysis, the same parameters were used for each gel. Besides, a single master gel image with the best quality and containing all the spots was prepared in each group as the reference gel. Normalization of the spot intensities was conducted according to the total optical density of the gel. After determining the percentage of intensity for each spot, the mean intensity of each spot between the cases and controls was analyzed by Student's *t*-test. Only protein spots with complete reproducibility across the sample set, expression intensity more than twofold changes (increase or decrease) as well as the *P*-value less than 0.05 were considered statistically significant for protein identification.

2.8. In-gel tryptic digestion

The protein spots from colloidal Coomassie blue-stained wide-range PAGE selected for further protein identification were excised manually using a sterile surgical blade. These gel plugs were cut into smaller pieces and transferred to a microcentrifuge tube filled with gel plaque storage buffer (7% (v/v) acetic acid and 10% (v/v) methanol) and stored at 4°C until in-gel digestion.

Gel plugs were washed with 150 μL of 100 mM ammonium bicarbonate for 10 min, de-stained and dehydrated in 150 μL of 50% acetonitrile in 50 mM ammonium bicarbonate for five times (15 min each time), and rehydrated in 150 μL of 10 mM DTT in 100 mM ammonium bicarbonate at room temperature for 30 min. DTT solution was removed and the gel plugs were

alkylated in 150 μL of 55 mM iodoacetamide in 100 mM ammonium bicarbonate at room temperature for 20 min in the dark. The gel plugs were dehydrated twice in 150 μL of 50% acetonitrile (20 min each time), then 50 μL of 50% acetonitrile (15 min); following acetonitrile removal, the gel plugs were completely dried by vacuum centrifugation.

Each protein spot was "in-gel" digested in 25 μL of 7 ng/μL trypsin (Promega, cat# V5111) in 200 mM ammonium hydrogen citrate for overnight at 30°C; 25 μL of 50% acetonitrile was added to each protein spot, incubated for 15 min (room temperature), quick spin, trypsin digestion solutions were collected, and dried by vacuum centrifugation. The pellet was reconstituted with 10 μL of 0.1% TFA for their mass spectrometric analysis.

2.9. Mass spectrometry

The resulting tryptic peptides obtained after in-gel digestion were analyzed by using a Bruker Ultraflexreme MALDI-TOF/TOF MS (Ultraflexreme; Bruker Daltonics GmbH, Breman, Germany) to give a peptide mass fingerprint and peptide sequence information. A solution of α-cyano-4-hydroxycinnamic acid (0.7 mg/mL) in 85% acetonitrile, 15% water, and 0.1% TFA was used as the matrix. Equal volumes of the matrix solution were mixed with tryptic digest peptides in 1:1 and spotted onto the MALDI plate (Achorchip plate). By routine, a standard peptide calibration mix in the mass range of 800–3200 Dalton (Bruker Daltonics, Leipzig, Germany) was analyzed for external calibration of the mass spectrometer.

MALDI-TOF/TOF mass spectra were acquired with smartbeam laser at 355 nm and operated in a positive and reflectron mode with 25-kV accelerating voltage; TOF/TOF tandem MS fragmentation spectra were acquired for each sample, averaging 5000 laser shots per fragmentation spectrum on each of the seven to 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions). The collision energy was set to 1 kV, and nitrogen was used as the collision gas.

The resulting peptide mass fingerprints were submitted to a computer equipped with the MASCOT search program (Matrix Science Ltd., UK) (www.matrixcsience.com) for identification of the protein present in the gel spot. Searches were performed without constraining to the protein molecular weight or isoelectric point. MASCOT search parameters were set as follows: carbamidomethylation of cysteine as a fixed modification, oxidation of methionine as a variable modification, trypsin as a proteolytic enzyme, residues up to one missed cleavage site with trypsin, and mass tolerance as 200 ppm. MS/MS spectra were searched as above using a peptide mass tolerance of 100 ppm and a fragment mass tolerance of ±0.2 Dalton. The protein identification database used was the National Center for Biotechnology Information (NCBInr), and the species selected for analysis was *Homo sapiens*. Results were scored using probability-based MOWSE scores (protein score is $-10 \times \mathrm{Log}_{10}(P)$, where P is the probability that the observed match is a random event). Protein scores greater than 67 were considered significant ($P < 0.05$).

2.10. Data analysis

Student's *t*-test (SPSS version 19.0) was applied on 2D quantification data to compare and evaluate the statistical significance of targeted protein among the study groups. *P*-value less than 0.05 was considered to be significant in both cases.

3. Results

3.1. Characteristics of the research population and body weight of fetus

Five pregnant women who were experiencing sPTL-IM were recruited in the study group while 10 pregnant women experiencing sTL-IM were recruited as the control group. Clinical data of these pregnant women were compared between both groups. As shown in **Table 1**, there were no statistical significant differences between sPTL-IM and sTL-IM groups in maternal age and parity. Pregnant women resulted in a PTB due to sPTL-IM have significantly lighter babies (at birth) and placentas than their control ($P < 0.05$, **Table 1**).

3.2. Comparative two-dimensional protein profile analysis of placenta tissues obtained from spontaneous unexplained preterm labor with intact membrane and its corresponding control

To further investigate the proteomic alterations in the sPTL-IM, the spot intensities in placenta samples (maternal and fetal sides) from sPTL-IM and sTL-IM were compared using ImageMaster 2D platinum software program.

A normalized amount of protein extracted from placenta tissues of sPTL-IM and sTL-IM was fixed on 13-cm pH 3–10 IPG strips. Second dimension separation was performed on 10% wide-range PAGE, as described under "Materials and Methods" section. Silver-stained 2D gels representing sPTL-IM and sTL-IM (fetal and maternal sides) are shown in **Figure 1A** and **B**, respectively. A comparison of the fetal side of the placenta tissues obtained from both the sPTL-IM and sTL-IM proteomes revealed highly similar protein profiles (**Figure 1AI** and **BI**) despite important differences being observed in the relative levels of several proteins as illustrated by the closer view of differentially expressed protein profiles (**Figure 1C**). These results were in parallel for the maternal side of the placenta tissues (**Figure 1AII**, **BII**, and **C**).

Student's *t*-test analysis revealed 12 differentially expressed proteins that are significant (two spots with greater staining and 10 spots with decreased staining; dotted circles in **Figure 1** indicate upregulated proteins, whereas solid circles indicate downregulated proteins, AI and BI, $P < 0.05$) in the fetal side of sPTL-IM with 100% reproducibility across the sample set. Whereas on the maternal side, only two gel spots with decreased staining showed at least 2.0-fold changes (solid circle in **Figure 1AII** and **BII**, $P < 0.05$) for the same comparison.

3.3. Identification of differentially expressed proteins associated with spontaneous unexplained preterm labor with intact membrane by MALDI-TOF/TOF MS

Identified spots with significant protein scores are shown in **Tables 2** and **3**. The spot numbers in **Tables 2** and **3** corresponded to the numbers in **Figure 1C**.

As indicated in **Tables 2** and **3**, S100-A9 and Ig kappa chain C (IGKC) region were overexpressed (**Table 2**), while vimentin, calpain small subunit 1 (CAPNS1), protein PP4-X, ferritin light subunit (FTL), septin 2, peroxiredoxin 3 (Prdx 3), cytokeratin 8, cytokeratin 1, and tropomodulin 3 (Tmod-3) were underexpressed in the fetal side of the placenta obtained from sPTL-IM compared to the control (two different spots were identified as the same protein;

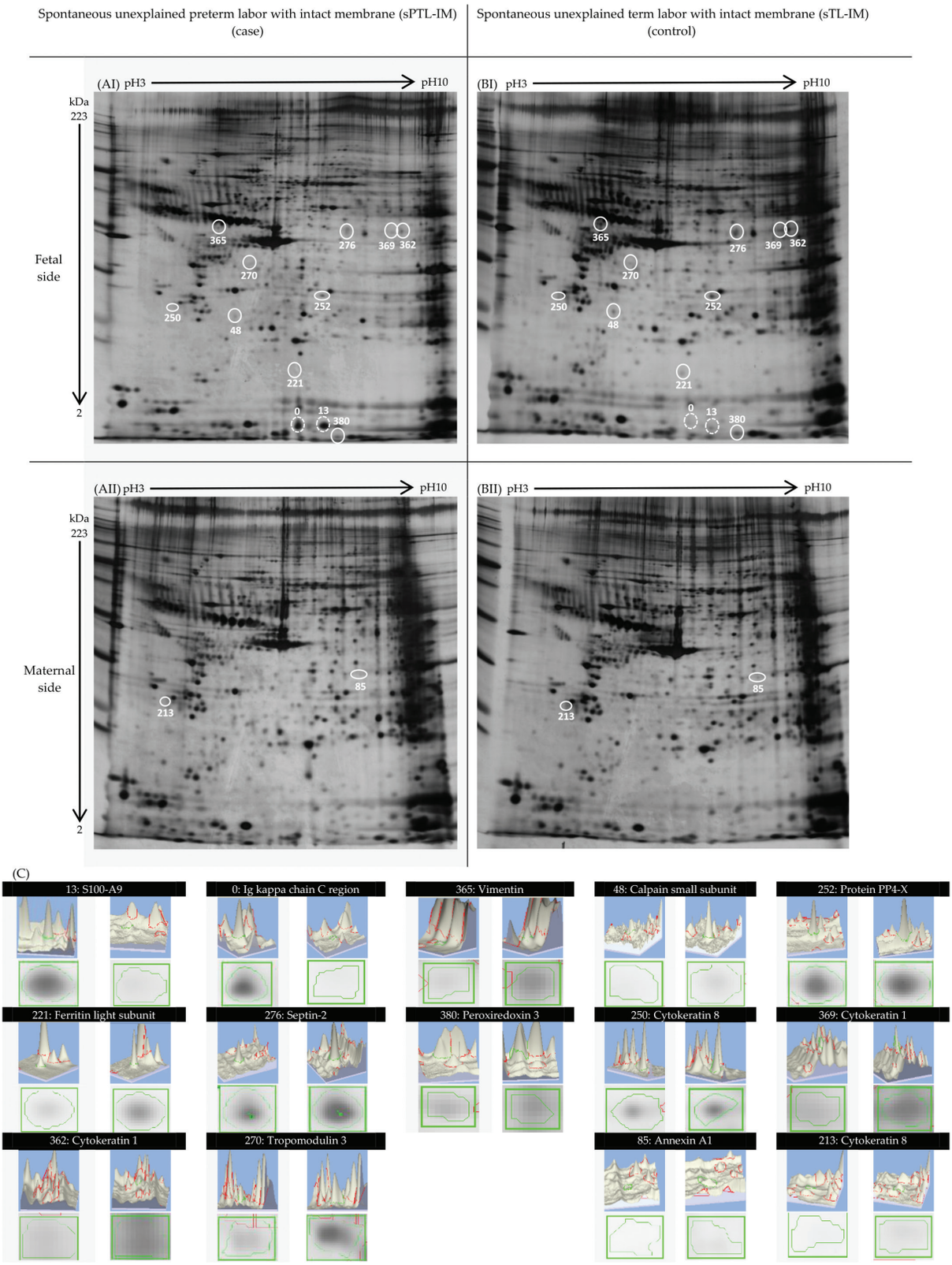

Figure 1. Representative 2D gels of placenta proteome. (AI and AII) Representative 2D gels of placenta tissue samples from sPTL-IM and (BI and BII) sTL-IM. The differentially expressed protein spots are circled on the gels and labeled with unique spot numbers. Dotted circles indicate overexpressed proteins in the gels analyzed using ImageMaster Software, while solid circles indicate underexpressed proteins. The spot number on the gels corresponds to the numbers in **Tables 2** and **3**. (C) A closer view of differentially expressed protein spots from 2D gels and their 3D images. The gray background boxes correspond to the sPTL-IM gels and the white background boxes correspond to the sTL-IM gels.

	Spot number[A]	NCBI accession #	Protein identified	Relative fold change[B]	Calculated pI/Mw (kDa)	P value	Mascot score[C]	Function	Sequence coverage (%)
Fetal side	13	NP_002956	Protein S100-A9	16.202	5.71/13.29	<0.001	70	Calcium binding protein	24
	0	P01834	Ig kappa chain C region	11.658	5.58/11.77	<0.001	120	Antigen binding agent	32

Accession #: accession numbers from SWISS-PROT database; A: arbitrary spot number supplied by Delta 2D. Numbers correspond to numbers in **Figure 1C**; B: fold change was calculated using ImageMaster 2D platinum software program; C: protein score generated by MS-MS identification platform, with a score higher than 67 being considered as statistically significant.

Table 2. Biochemical information of the overexpressed proteins in the placenta tissues of sPTL-IM.

	Spot number[A]	NCBI Accession #	Protein identified	Relative fold change[B]	Calculated pI/Mw (kDa)	P value	Mascot score[C]	Function	Sequence coverage (%)
Fetal side	365	AAA61279	Vimentin	−9.454	5.03/53.74	0.035	188	Stabilization of cytoskeletal interactions	9
	48	NP_001740	Calpain small subunit 1	−2.397	5.05/28.47	0.004	142	Stabilized calpain heterodimers	16
	252	AAC41689	Protein PP4-X	−2.356	5.65/36.26	0.022	150	Membrane fusion	9
	221	AAA52440	Ferritin light subunit	−2.494	5.65/16.44	0.080	227	Iron homeostasis and inflammatory response	30
	276	NP_004395	Septin-2	−2.311	6.15/41.69	0.015	70	Filament-forming cytoskeletal GTPase	7
	380	EAW49398	Peroxiredoxin 3	−2.025	6.06/11.16	<0.001	103	Mitochondrial dysfunction	13
	250	CAA31376	Cytokeratin 8	−2.104	4.91/30.84	0.026	162	Filament reorganization	12
	369	AFA52002	Cytokeratin 1	−6.141	8.15/66.18	0.011	207	Filament reorganization	13
	362	AFA52002	Cytokeratin 1	−2.759	8.15/66.18	132	Filament reorganization	13	
	270	AAF31670	Tropomodulin 3	−3.083	5.08/39.73	0.027	161	Maintaining cytoskeletal architecture	17

	Spot number[A]	NCBI Accession #	Protein identified	Relative fold change[B]	Calculated pI/Mw (kDa)	P value	Mascot score[C]	Function	Sequence coverage (%)
Maternal side	85	1AIN_A	Annexin A1	−2.003	7.77/35.25	0.005	95	Anti-inflammatory properties	7
	213	AAA35748	Cytokeratin 8	−2.896	5.30/53.66	0.005	118	Filament reorganization	9

Accession #: accession numbers from SWISS-PROT database; A: arbitrary spot number supplied by Delta 2D. Numbers correspond to numbers in **Figure 1C**; B: fold change was calculated using ImageMaster 2D platinum software program; C: protein score generated by MS-MS identification platform, with a score higher than 67 being considered as statistically significant.

Table 3. Biochemical information of the underexpressed proteins in the placenta tissues of sPTL-IM.

cytokeratin 1) (**Table 3**), whereas two proteins namely annexin A1 (ANXA1) and cytokeratin 8 were underexpressed (**Table 3**) in the maternal side of the placenta obtained from sPTL-IM compared to the control.

Interestingly, in both the fetal and maternal sides of the placentas, cytokeratin 8 was reduced in expression in relation to sPTL-IM (**Table 3**). There were no overexpressed proteins found in the maternal side of the placenta.

Of the total 12 proteins identified, four functional groupings emerge (**Figure 2**). Cytokeratin 8, cytokeratin 1, vimentin, Tmod-3, septin 2, and CAPNS1 are grouped under structural and cytoskeletal components (53.8%), S100-A9, IGKC, and ANXA1 are grouped under immunomodulatory proteins (23.1%), FTL and Prdx 3 are grouped under placenta-modulatory proteins (15.4%), and lastly, protein PP4-X is grouped under anticoagulation cascade (7.7%).

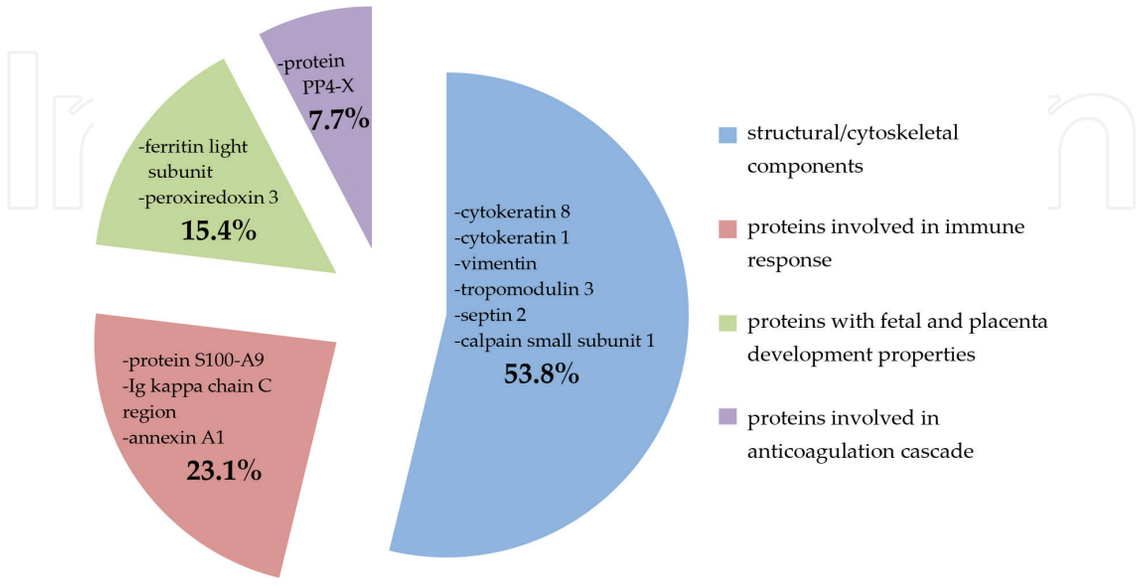

Figure 2. Functional grouping of differentially expressed proteins identified in the placentas obtained from pregnant women experiencing sPTL-IM.

4. Discussion

Current understanding of placenta association with sPTL-IM leading to PTB is strikingly limited, which further limits our ability to accurately predict the occurrence of this event. Despite great strides in neonatal care, infant survival rates remained low partly due to sPTL-IM nonetheless. This imparts a significant risk of various childhood diseases/disorders among premature infants. Thus, placenta proteome analyses of sPTL-IM allowed the identification of potential biomarkers and the exploration of the underlying molecular pathophysiology related to placenta insufficiency leading to this syndrome, where the study employing placenta proteome on this syndrome is still limited. As such, this report is designed to demonstrate the feasibility of using placenta proteome to study its association to sPTL-IM while making the initial findings widely available. In essence, we have identified 12 potentially important protein differences in the placenta of sPTL-IM.

It is possible that limitations exist in the 2D-GE technology. This is due to the presence of proteins that are extremely acidic and basic, hydrophobic, large molecular weights, and low copy numbers [16]. Furthermore, limitations were observed when identifying and quantifying proteins as the protein spots need to be excised out from the 2D gels and digested with proteases.

The differentially expressed proteins identified from sPTL-IM can be generally classified into four functional classes, which were (1) structural/cytoskeletal components, (2) proteins involved in immune response, (3) proteins with fetal and placenta development properties, and (4) proteins involved in anticoagulation cascade.

4.1. Structural or cytoskeletal components

A majority of the identified proteins were proteinaceous components of structural or cytoskeletons (**Figure 2**) which is not surprising since the cytoskeletal proteins are known as major regulators of the endothelial function and signaling processes [17]. A total of six proteins were grouped under this function. Half of the proteins that fall into this class were intermediate filaments namely cytokeratin 8, cytokeratin 1, and vimentin (**Figure 2**). Cytokeratin 8 was found to be differentially expressed in both the fetal and maternal sides from the sPTL-IM placenta while the expression of cytokeratin 1 and vimentin was observed only at the fetal side. Commonly, keratin types I and II copolymerize to form intermediate filaments in epithelia [18], and vimentin form type III filaments in endothelial cells [19]. As the placenta is a highly vascularized system of epithelial membranes, the presence of these components is expected.

Dysregulation of keratin expression has been reported in many pregnancy disorders. Studies had showed that dysregulation of keratins 7, 8, 18, and 19 expressions was associated with pregnancy disorder, with the results contradicting each other about up- or downregulation of these keratins [20, 21]. The reason for this discrepancy is still not certain, and it may be explained by the severity of cases, unhealthy women might be from different backgrounds, or there is a more complex effect of this pregnancy complication on placenta intermediate filaments. Most importantly, changes in keratin expression level might influence cell architectural properties such as cell polarity and shape.

Vimentin was presumed to play a vital role in anchoring and positioning organelles in the cytosol, maintenance of cell integrity, and stabilization of cytoskeletal interactions [22, 23]. Downregulation of vimentin was observed in the obese placenta as well as in human-aborted placenta tissues [24, 25]. A deficiency of this protein could result in the dramatic reorganization of vimentin filaments in the cytoplasm, leading to morphological changes and cellular fragmentation and ultimately destabilizes the overall cytoplasm architecture which might lead to PTL [23].

Meanwhile, the remaining half of the identified proteins that fell into this functional group includes cytoskeletal and cytoskeleton-associated proteins namely Tmod-3, septin 2, and CAPNS1 (the fetal side of placenta tissues) (**Figure 2**). Studies also demonstrated that pregnancy maintenance is regulated by various cytoskeletal and cytoskeleton-associated proteins [26, 27].

Tmod-3 is one of the components of membrane-associated tropomyosin-filamentous actin (TM-F-actin) networks, which is responsible for the polarization of plasma membranes of epithelial cell and sarcoplasmic reticulum membranes of skeletal muscle [28, 29]. This protein is also involved in endothelial cell migration, erythroblast enucleation and erythroblast-macrophage adhesion in erythropoiesis, and insulin-stimulated GLUT4 trafficking in adipocytes [30–32]. Moreover, Tmod-mediated stabilization of TM-F-actin is critical for membrane morphology, mechanics, and physiology [29]. It is thus conceivable that alteration in Tmod-3 levels may contribute to the deregulation of actin filaments of the placenta.

Septin is known to play roles in vesicle trafficking, cytoskeletal remodeling, and apoptosis through the organization of actin-myosin contractile ring [33]. It regulates ring contraction or the attachment of actin-myosin contractile ring to the plasma membrane [34]. Septin was also proposed to have additional functions in mammals such as cell surface organization and vesicle fusion processes [33, 34].

CAPNS1 is a regulatory subunit that is involved in calpain heterodimers (calpain-1 and calpain-2) stabilization and operation. CAPNS1 proteolytic activities had been reported to involve in various cellular functions including apoptosis, proliferation, migration, adhesion, and autophagy. *Capns1* deficiency was reported to be the factor of calpain-1 and -2 zero expression that resulted in embryonic lethality around embryonic day 10.5 due to possible cardiac defects and hemorrhages [35].

Collectively, alterations in the expression of proteins involved in the intermediate filaments and cytoskeletal network can lead to significant reorganization of the placenta cytoskeletal, as well as defects in placenta endothelial cell migration and tubule formation. Such cytoskeleton rearrangement and defects in endothelial cell signals transduction and function may be contributing factors to the characteristic disorganization of myofiber in placentas, where tissue structural capacity for stretch was altered, leading to sPTL-IM. This cause-and-effect interpretation is consistent with the higher incidence of PTL during multiple versus single gestations [36].

One possible justification for more proteins from this functional group was dysregulated in fetal compared to maternal which could be that the fetal involved significantly in the process of cytoskeleton reorganization. This observation is consistent with the findings by Tonami et al. [37], which have shown that microtuble-stabilizing protein is highly expressed in the fetal side of the placenta.

4.2. Proteins involved in immune response

Immunomodulatory proteins were also identified in placenta tissues from pregnant women with sPTL-IM. These include S100-A9, IGKC (fetal side of placenta tissues), and ANXA1 (maternal side of placenta tissues) (**Figure 1C**). Inflammation has been attributed to the occurrence of PTL in human [38]. This is possible because the presence of inflammatory cytokines cause cervical ripening and the maturation of the chorioamnion, leading to labor [38]. In addition, Pereira et al. also observed the presence of calgranulins and annexins in cervical-vaginal fluid from patients with spontaneous PTL [39].

S100-A9 (also known as calgranulin B) is a calcium-binding protein that regulates inflammatory and immune responses which function extracellularly as an antimicrobial agent [40, 41]. Various studies have reported significantly elevated levels of calgranulins A and B in amniotic fluid from intra-amniotic-infected women [41], as well as in vaginal fluid from 86% of patients who were presented with PTL without obvious evidence of genital tract infection or inflammation [42]. Women in labor secondary to intra-amniotic inflammation have also been found to have increased *S100-A8* and *S100-A9* in amniotic fluid and maternal blood [41, 43]. Moreover, calgranulin B can interact directly with β2 integrins in forming "activated receptor" epitopes and plays a role in cytoskeletal reorganization [44].

IGKC functions as an antigen-binding, immune regulation, and disposal of immune complexes [45]. Several lines of evidence suggested that during late pregnancy, recruitment of circulating maternal innate and adaptive leukocytes into the cervix and/or myometrium, and then to the decidua interface via chemotactic cascades induces a pro-inflammatory state which leads to labor [46].

ANXA1 is a calcium-dependent phospholipid-binding protein that is involved in cell proliferation, differentiation, mediating inflammation, apoptosis, and maintenance of pregnancy [42, 47, 48]. It has been reported that *ANXA1* deficiency leads to changes in the inflammatory response and the anti-inflammatory effects of glucocorticoids [48]. Moreover, anti-inflammatory effects by the potent inhibition of neutrophil trafficking were observed from mice that were administered with ANXA1 [49]. A decrease of ANXA1 expression can result in the liberation of phospholipase A2 activity, making more arachidonic acid (precursor of prostaglandins) available for prostaglandin generation [50], which is crucial in uterine contraction and cervical ripening.

Therefore, it remains possible that the premature activation of this inflammatory pathway can lead to a breakdown of feto-maternal tolerance and play a role in the induction of labor, which subsequently can result in spontaneous PTL.

4.3. Proteins with fetal and placenta development

Identified proteins that are associated to this function were Prdx 3 and FTL where both were identified from the fetal side of placenta tissues (**Figure 1C**).

Prdx 3 is a mitochondria antioxidant enzyme that was known to involve in apoptosis and response to oxidative stress [51]. Previous studies have shown that a lower expression of Prdx 3 in human placenta tissues triggered oxidative stress and caused dysfunction of mitochondria in trophoblast cells [52]. From our results, the downregulation of this protein in placentas of sPTL-IM patients suggested the stimulation of fatty acid cascade, such as arachidonic acid and cell cycle arrest by oxidative stress [53, 54]. Both processes are potentially synergistic mechanisms that contribute to preterm contractions, cervical dilatation, and PTL.

Ferritin is a multimeric protein that consisted of two subunits: ferritin heavy chain (FTH) and FTL, which share about 50% sequence homology and is mainly found in the villous stroma of the placenta [55]. FTL has been reported to play a critical role in iron storage as well as regulation of intracellular iron homeostasis and inflammatory response [56, 57]. Mutation in the *FTL* has also been demonstrated to cause neuroferritinopathy and neurodegeneration [58, 59]. On the other hand, anti-inflammatory activities were observed from FTL overexpression where Fan et al. [56] reported inhibition on lipopolysaccharide (LPS)-induced transcription of anti-inflammatory factor at the feto-maternal interface of the placenta leading to PTL [60]. Therefore, it is possible that the production of inflammatory mediators induced by LPS at feto-maternal interface of the placenta can contribute to FTL degradation and synergized to increase intracellular free iron. This leads to increased oxidative stress. Furthermore, elevated placenta oxidative stress was thought to play a key role in stimulating the release of factors, further leading to maternal endothelium activation [61].

4.4. Proteins involved in anticoagulation cascade

Only one identified protein was classified in this group. Protein PP4-X (also known as annexin IV (ANX4)) was downregulated in sPTL-IM placentas when compared to the control placentas at the fetal side (**Figure 1C**). This protein has been known to be involved in multiple cellular functions including endocytosis, exocytosis, ion fluxes, and apoptosis [62, 63]. This protein is also one of the important components of biological cascades regulating coagulation which makes it an important factor for the maintenance of pregnancy [63]. Placenta structural abnormality and dysfunction in IUGR fetuses were reported to be caused by reduction in two members of annexins (ANX4 and ANX8) [64]. Furthermore, coagulative disorder and antiphospholipid antibody syndrome have been associated with an increased incidence of pregnancy complications, including PTL [65]. Several cases of increased polymorphism rate in genes related to coagulation have also been associated with PTB [66]. Therefore, it is conceivable that alterations in ANX4 levels may contribute to a failure of placenta anticoagulation and structural abnormality, leading to impaired utero placenta development.

Interestingly, more proteins were differentially expressed in the fetal side than in the maternal side of the placenta tissues obtained from pregnant women who experienced sPTL-IM (**Tables 2** and **3**); this interpretation is consistent with the signals from the fetus where they are capable of initiating the onset of parturition process [67]. The work by Reinl and England [67] demonstrated a possible interplay between placenta corticotrophin-releasing hormone and fetal surfactant protein-A that drives parturition in human. Moreover, another study indicated that nuclear receptor coactivators 1 and 2 regulate the transcription of surfactant protein-A and consequently the induction of labor [68].

5. Conclusion

The underlying mechanisms leading to sPTL-IM are not fully understood. This leads to limited prediction and prevention of sPTL-IM due to its complex pathophysiological process. Placenta was an excellent biological tissue that might facilitate the discovery of potential biomarkers as well as to assess dysfunction in the framework of sPTL-IM. Moreover, sPTL-IM

may alter selected placenta functions that lead to PTL. For this, proteomic approaches have enabled the discovery of potential biomarkers in the placenta and unveiled the pathophysiological pathways associated to sPTL-IM. The identified proteins involved in four distinct functional classes include inflammation, oxidative stress, anticoagulation, and extracellular matrix remodeling leading to preterm parturition. These results can help in the future selection of more meaningful potential biomarkers for the early detection of sPTL-IM that might appear in easily accessible body fluid. Lastly, it is postulated that signals for the initiation of sPTL-IM are more likely to arise from the fetus and to a lesser extent from the mother due to more proteins differentially expressed in the fetal side of the placenta of sPTL-IM.

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Conflict of interest

The authors reported no conflict of interest.

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