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Metabolomics and preterm birth: What biomarkers in cervicovaginal secretions are predictive of high-risk pregnant women?

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ABSTRACT

Prematurity is the most important cause of perinatal mortality and morbidity and has serious consequences for the health of women and child later in life. Until now, the best predictive test for spontaneous preterm birth is ultrasound measurement of uterine cervical length. However, the physiological mechanism related to preterm births is still unclear. Can we improve the prediction of spontaneous preterm birth? Are there measurable physiological chemical biomarkers in biological fluids that could be more reliable predictors of preterm birth? Our hypothesis is that release mechanisms involving multiple biochemical reactions play a specific role in precipitating the onset of labor. The cascade of these biochemical events leads to perturbations in the metabolome which can be detected with high resolution and sensitive instruments using robust technologies, such as mass spectrometry. The main objective of this project was to perform a pilot mass spectrometry metabolomic study of women who had a spontaneous preterm birth compared to controls using the uterine cervical length as a selective factor. Non-invasive collection of cervicovaginal secretions from 15 women was performed: 5 with a short cervix who had a spontaneous preterm birth compared to a control cohort with a short cervix ($n = 5$), and another control cohort with a long cervix ($n = 5$). A total of 1908 metabolites were detected in these women. Markers of interest were identified by multivariate analysis. Further studies are planned on larger numbers of women followed by characterization of biomarkers of interest by mass fragmentation, isolation, purification and NMR studies. This metabolomic approach may allow the development of new strategies for the management of women at high-risk for spontaneous preterm birth.

1. Introduction

Preterm birth, delivery before 37 weeks of gestation, is a major public health problem. A recent study showed that the frequency of preterm births is about 12–13% in the USA and 5–9% in other developed countries [1]. Moreover, the rate of preterm births has been increasing over the last 30 years [1]. Prematurity is the most important cause of perinatal mortality and morbidity and has serious consequences for the health of women and child later in life. The cost associated with preterm birth is overwhelming: an estimate of more than 7 billion dollars in the US alone [2,3].

There are two different types of preterm births: iatrogenic and spontaneous. Medically indicated preterm births are the cause of 35% of preterm births; spontaneous preterm births (sPTB) arise from spontaneous labor with intact membranes in 40–45% of cases, or with premature rupture of membranes (PROM) in another 30–35% [1]. The causes of preterm labor and PROM are not fully understood. Clinical and research evidence supports the concept that most spontaneous preterm deliveries result from one or more of these four pathophysiologic processes: (1) amniochorionic-decidual or systemic inflammation; (2) activation of the maternal or fetal hypothalamic–pituitary–adrenal; (3) decidual hemorrhage; (4) pathologic distension of the uterus. Even if different pathways have been described, modification of cervix and local inflammation appear as major factors of sPTB.

Until now, the best predictive test for sPTB is ultrasound measurement of uterine cervical length. A short cervical length (<30 mm) has been associated with sPTB [4]. The sensitivity and specificity of the cervical length is still low and the positive predictive value varies from 20% to 55% [5]. Inflammatory markers in cervicovaginal secretions, amniotic fluid, and maternal blood have also been associated with sPTB [6]. But, many questions have yet to be answered: Can we improve the prediction of sPTB? Are

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Table 1
Clinical information for 15 women under study. Five women with preterm births with short cervical length and 10 control women with short ($n = 5$) or long cervical length ($n = 5$). Women giving birth to twins (*). Women with streptococcus infection (**).

	Maternal age (years)	BMI (kg/m^2)	Gestational age at sampling (weeks)	Gestational age at delivery (weeks)	Cervical length (mm)	Tobacco usage	Medication during pregnancy			
							Antibiotic therapy	Tocolysis	Corticotherapy	Prometrium
Preterm 1	29.9	15.4	31.0	32.6	12.0	Yes	Yes	Yes	Yes	Yes
Preterm 2	28.2	26.7	32.3	33.1	24.4	No	Yes	No	Yes	No
Preterm 3*	25.9	22.3	33.0	34.1	26.9	No	Yes	No	Yes	No
Preterm 4	30.4	28.4	32.6	34.9	28.8	No	No	No	No	No
Preterm 5	33.7		32.1	35.9	15.1	No	No	No	Yes	Yes
Control 1**	25.6	33.8	33.1	39.1	9.1	No	Yes	No	Yes	No
Control 2	27.2		33.3	41.0	24.1	No	Yes	No	No	No
Control 3	32.7	23.8	31.1	39.9	27.2	No	No	No	No	No
Control 4	22.8	22.1	32.6	38.7	29.0	Yes	No	No	No	No
Control 5*	35.7	39.6	32.4	37.0	21.2	No	No	No	No	Yes
Control 6	29.2	21.9	31.0	39.1	42.2	No	No	Yes	Yes	Yes
Control 7**	27.7	19.5	31.1	39.3	38.8	No	No	No	No	No
Control 8	24.0	21.9	32.3	38.0	48.2	No	No	No	No	No
Control 9	29.6	21.0	31.9	38.0	35.8	No	Yes	No	No	No
Control 10**	26.2	23.8	30.9	40.3	40.4	No	No	No	No	No

there measurable biomarkers in biological fluids that could be reliable predictors of preterm birth? Are there non-invasive biological fluid collections for pregnant women which might prevent clinical complications? We believe the answers rest in the domain of metabolomics.

Metabolomics is the study of the thousands of low-molecular-weight molecules found in biological fluids and tissues of different individuals, whether normal or afflicted with disease, which reflect changes in biological functions [7–10]. After identification of disease-specific biomarkers, determination of the concentrations is performed. These biomarker levels are closely related to biochemical, physiological, environmental, and genetic status of an organism and are regarded as the ultimate outcome of cellular regulation, resulting in the visible phenotypes. Metabolic alterations are thus the most proximate indicators of changes in the body in response to a disease process [11], drug therapy [12] and stressful physiological events. The evaluation and comparison of biological matrices by performing metabolic profiling and fingerprinting using sophisticated technologies, such as mass spectrometry (MS) or nuclear magnetic resonance (NMR), in women giving birth to full-term babies (>37 weeks of gestation) compared to those giving birth prematurely might be the solution.

We postulate that release mechanisms involving multiple biochemical reactions play a specific role in precipitating the sPTB, and that the cascade of these biochemical events leading to perturbations in the metabolome can be detected with high resolution and sensitive instruments using robust technologies. Serial sampling of the mother may be performed just before, during and after preterm birth. Data analysis of the metabolites occurring in women giving preterm birth can be compared to control reference values obtained for women delivering at full-term. One metabolomic study recently reported the use of amniotic fluid for the identification of patients at risk for preterm delivery [13]. In our view, the collection of cervicovaginal secretions is a less invasive method than the use of amniotic fluid to determine women at-risk of giving birth prematurely. To our knowledge, there are no published metabolomic studies using cervicovaginal secretion samples for prediction of sPTB.

The main objective of this study was to evaluate women who had a spontaneous preterm birth compared to controls in order to detect biomarkers that would predict prematurity. We preferred to use cervicovaginal secretions because they can be collected easily in a non-invasive and repetitive way, at different times of gestation and parturition in order to perform the mass spectrometry analyses. Two groups of women were studied: those with preterm and those with at-term deliveries. The women were stratified according to the length of the cervix: 5 women with a short cervix who had a spontaneous preterm birth compared to control groups having a short cervix ($n = 5$) or a long cervix ($n = 5$) delivering at-term.

2. Materials and methods

The Ultra performance liquid chromatography-Quadrupole time-of-flight mass spectrometer system (UPLC-QTof Synapt MS) (Waters Corp., Milford, MA, USA) was used to carry out our metabolomic studies.

2.1. Ethics approval

This project was approved by the Research Ethics Board (REB) of the Faculty of Medicine and Health Sciences and the Centre hospitalier universitaire de Sherbrooke (CHUS).

2.2. Patients and controls specimen collection

After informed consent was obtained, we used specimens and data collection from a cohort study of referred women for transvaginal ultrasonography (Colibri Study presented at the 30th Annual Meeting of the Society for Maternal and Fetal Medicine) [14] and created a nested-case control study to identify predictive factors of preterm birth.

We selected 15 women among 97 referred for transvaginal ultrasonography. Cervicovaginal secretion samples were collected from these 15 women during the ultrasound exam: 5 had a spontaneous preterm birth with a short cervical length (<30 mm), 5 controls with a short cervix, and 5 controls with a long cervical length (>30 mm). The samples were collected with two Dacron swabs (MedPro: Cat. 018-430, AMG Médical Inc., Montreal, QC) attached to the transvaginal ultrasound probe. The swabs were lying on the posterior fornix during the ultrasound exam (duration: 5 min) to achieve saturation with the vaginal fluid and then carefully removed. Gestational age at sampling varied from 31 to 33 weeks of gestation for preterm women and from 30.9 to 33.3 for controls, whereas gestational age at delivery varied from 32.6 to 35.9 weeks for preterm women and from 37 to 41 weeks for controls (Table 1).

Table 1 also presents clinical information for these 3 different cohorts. Two women (1 patient and 1 control woman) smoked cigarettes during their pregnancy. No illicit drugs were used by

Table 2
Mass spectrometry acquisition parameters.

Mode	Electrospray
Polarity	Positive
Capillary	3.2 kV
Sampling cone	35 V
Extraction cone	5 V
Source temperature	120 °C
Desolvation temperature	450 °C
Cone gas flow	30 L/h
Desolvation gas flow	700 L/h
Analyzer mode	V mode
Dynamic range	Extended
Mass range	100–1000 Da
Scan time	0.1 s
Data format	Centroid
Collision energy	6 V
Lock mass interval	10 s

any of the participants. Two women had twins: one in the preterm group and one in the control group. Six women received antibiotics: 3 in the preterm group and 3 women in the control group. Three women from the control group had streptococcus group B infection (one in the short cervix length group and 2 in the long cervix length group). All women were caucasian and non-vegetarian.

2.3. Chemicals and reagents

All chemicals used were analytical reagent grade. Laboratory water was purified to ultra pure grade with the use of a Nanopure Infinity water purification system (Ultrapure, 18.3 MΩ, Barnstead, Dubuque, IA, USA). Phosphate buffered saline (PBS), bovine serum albumin (BSA), and acetaminophen, caffeine, sulfadimethoxine, erythromycin, nortriptyline and terfenadine were purchased from Sigma–Aldrich (St Louis, MO, USA). Chromatographic grade methanol and acetonitrile were obtained from Fisher Scientific (Nepean, Ontario, Canada).

2.4. Processing of specimens

After collection of the cervicovaginal secretions, the Dacron swabs were deposited in 1 mL of phosphate buffered saline at pH 7.4 containing 1% bovine serum albumin. Sample solutions were stored at −80 °C until analysis. Processing of samples was done as follows: 400 μL of each sample were diluted twice with water and loaded on Oasis HLB cartridges (30 mg, 30 μm, Waters Corp.). A water (500 μL) wash step was followed by elution with 500 μL methanol. The organic phase was evaporated under nitrogen. Reconstitution was performed with 200 μL of 20% methanol in water.

2.5. Instrumentation and experimental parameters

Analysis of cervicovaginal secretions was performed using the UPLC-QTof Synapt MS and data recorded with MassLynx software (Waters Corp.). Mass spectrometry acquisition parameters are presented in Table 2. We targeted the 100–1000 Da mass range. Fig. 1 shows the time profile of the eluent gradient for the ultra performance liquid chromatography analysis. Samples and standard mixtures were injected on a HSS-T3 C18 column (2.1 mm × 50 mm, 1.8 μm particle size, Waters Corp.). The flow rate was 0.5 mL/min, without splitting the eluent. Formic acid was the additive used in the eluent and terfenadine (1 μM) was the reference compound (lock mass) providing mass accuracy (see Fig. 2). Marker selection in study samples using MarkerLynx software (Waters) was defined with the following parameters: an intensity threshold of 25 counts, a retention time window of 0.2 min and a mass window of 0.05 Da. A marker corresponds to a particular mass, at a precise retention

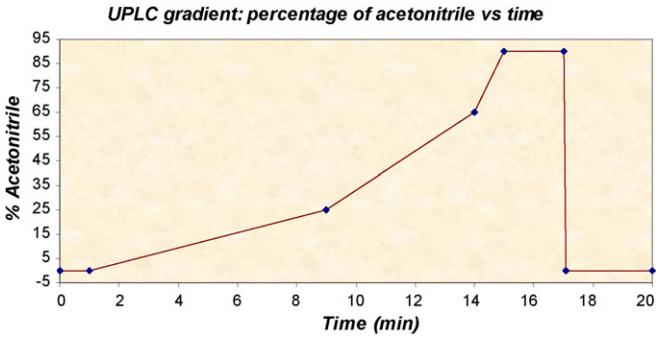

Fig. 1. Time profile of the eluent gradient.

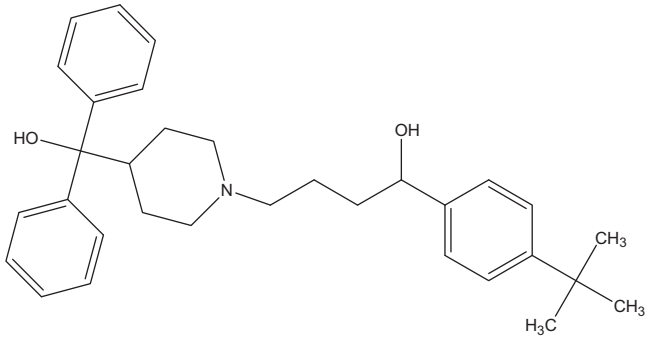

Fig. 2. Terfenadine chemical structure used as a lock mass standard in positive ion mode on the UPLC-QTof Synapt system.

time with a specific intensity. It is well established that multivariate analysis (MVA) allows the analysis of more than one statistical variable at a time. In particular, partial least square-discriminant analysis (PLS-DA) can provide a quantitative model of how different the groups of samples of this study are from each other and which markers make them different. MVA was performed on EZinfo software (Umetrics).

3. Results and discussion

Raw data from the injection of study samples and a standard mixture (5 compounds, see Table 3) were processed with MarkerLynx software. No peak shape spoiling or drift of retention times (CV% below 1%) were observed for spiked standards which bracketed patient samples. Moreover, differences between theoretical mono-isotopic mass and experimental mass-to-charge ratio (m/z) for standards were below 1.3 mDa (see Table 3). Experimental observations obtained from spiked standards support the reliability of the study samples.

A total of 1908 metabolites (markers) were detected by MarkerLynx in samples obtained from 15 women. After MVA using a PLS-DA model, a total of 1878 metabolites were detected from 10 cervicovaginal secretions from 10 pregnant women: 5 women with

Table 3
Mass accuracy for 5 spiked standards for the UPLC-QTof MS.

Mass spectrum results	Experimental mass[a] (m/z)	Theoretical mass (M+H)	Delta (mDa)
Acetaminophen	152.0699	152.0712	1.3
Caffeine	195.0882	195.0882	0.0
Sulfadimethoxine	311.0816	311.0814	0.2
Erythromycin	734.4696	734.4691	0.5
Nortriptyline	264.174	264.1752	1.2

[a] Average of 6 samples processed by MarkerLynx.

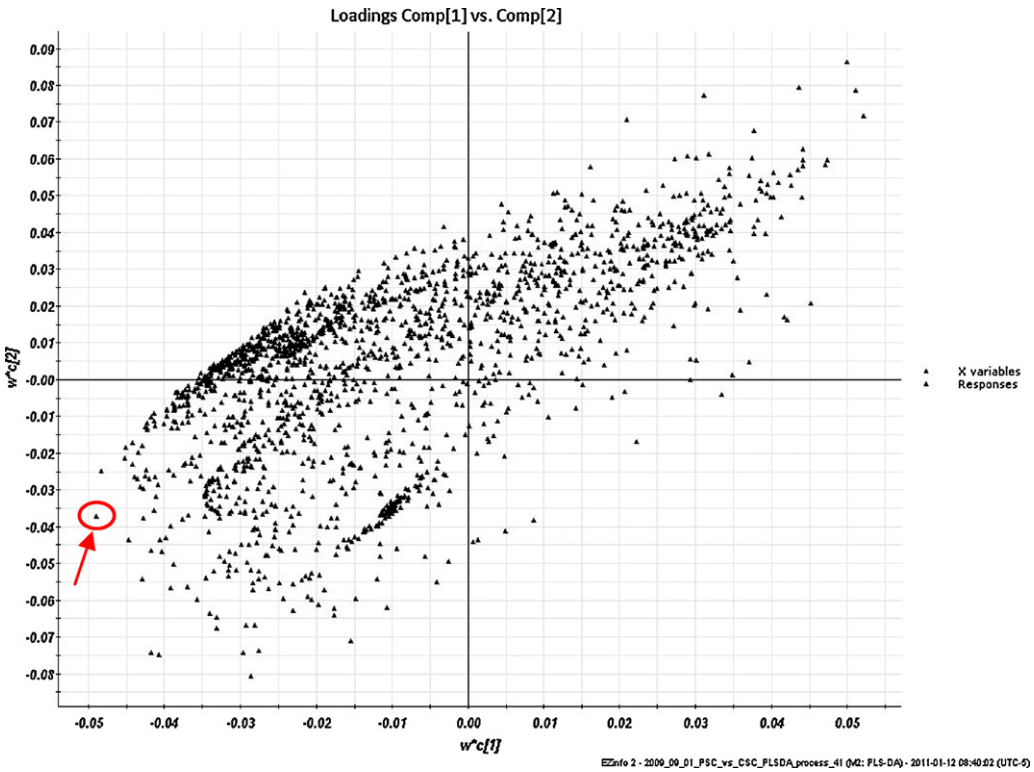

Fig. 3. Loading plot of 1878 biomarkers from cervicovaginal secretion samples obtained from 10 women with a short cervix: 5 had a spontaneous preterm birth and 5 controls had a full-term birth. A red arrow indicates the biomarker of interest. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

a short cervix experienced a spontaneous preterm birth and 5 control women with a short cervix delivered at full-term. Fig. 3 presents a loading plot of the scores for the markers obtained by PLS-DA using the two groups described above. Each point in this graph corresponds to a specific mass and retention time. Graphical axes in Fig. 3 correspond to component 1 versus component 2 where each component is the product of weights by coefficients. The scoring plot in Fig. 4 corresponds to a map of 10 samples in which each

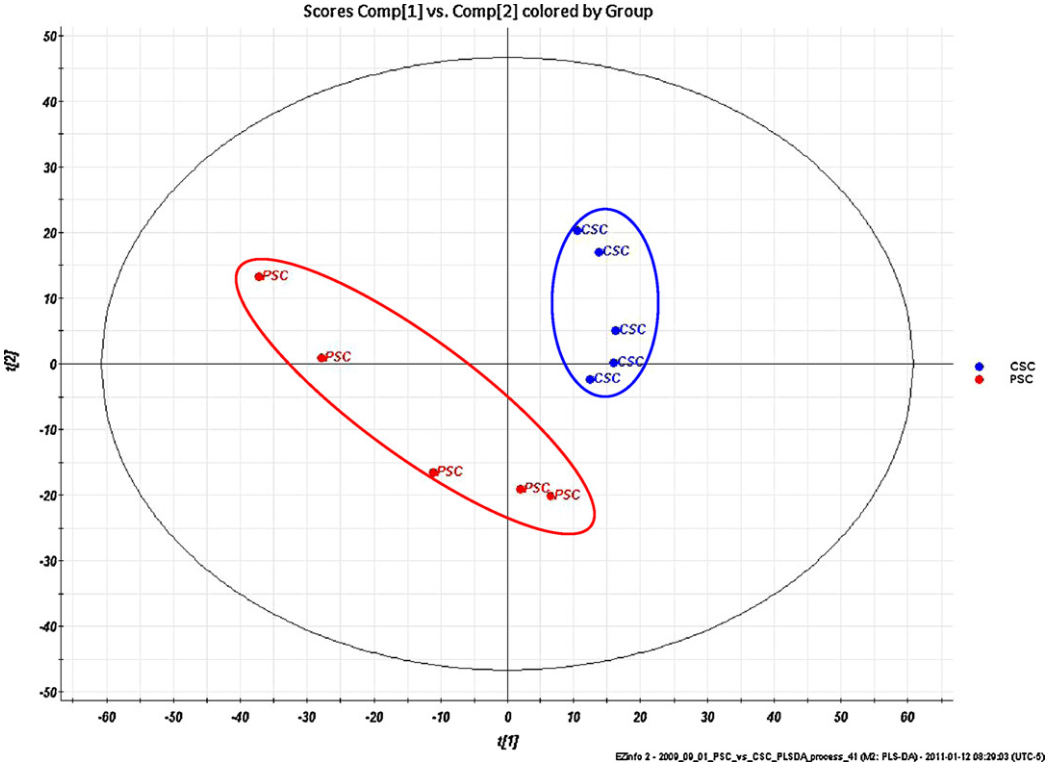

Fig. 4. Scoring plot for 10 cervicovaginal secretion samples obtained from 10 women: 5 women with a short cervix experienced a spontaneous preterm birth (=● psc) and 5 women with a short cervix had a full-term pregnancy (=● csc).

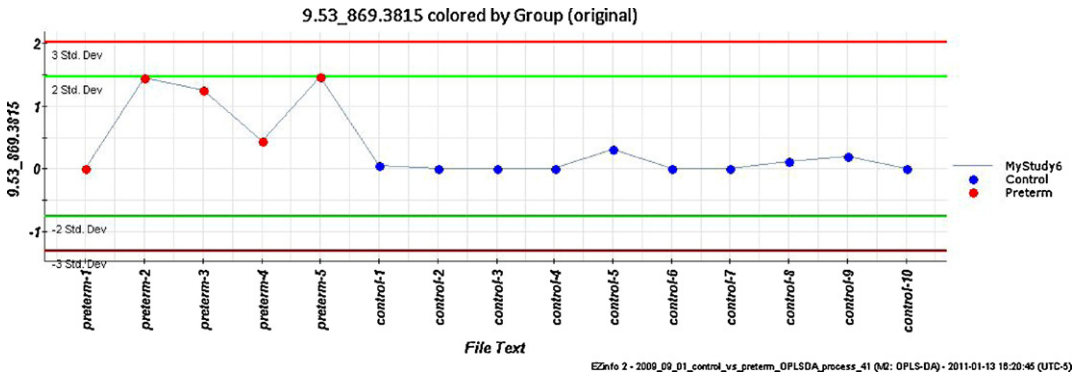

Fig. 5. Trend plot of m/z 869.3815 at retention time of 9.53 min recorded from 15 cervicovaginal secretion specimens: 10 control women who gave birth at-term =● (5 with short cervix and 5 with long cervix) and 5 women who gave birth prematurely = ● .

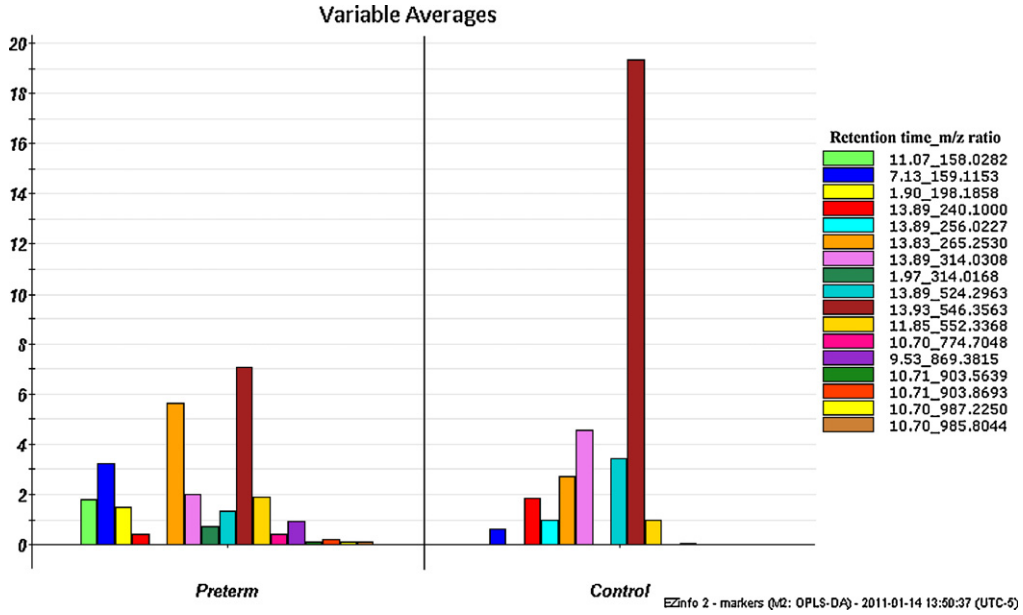

Fig. 6. Mean intensities of 17 variables (markers) at different retention time and m/z ratios for women giving birth prematurely ($n = 5$) compared to control women with at-term delivery ($n = 10$).

sample has a score obtained by PLS-DA treatment. Scores for components 1 and 2 are t1 and t2, respectively, as seen on the graphical axes of Fig. 4. The score plot defines which samples are similar (near each other) and which are dissimilar (far away of each other). We were clearly able to distinguish both groups described earlier. These MVA allowed us to target one marker of particular interest as presented in the trend plot in Fig. 5. This marker is also indicated by a red arrow in Fig. 3 with a specific m/z of 869.3815 recorded at a retention time of 9.53 min. This marker was observed in greater quantities in 4 out of 15 samples analyzed, and was found specifically in women with a short cervix experiencing spontaneous preterm birth. We plan to repeat assay with a larger cohort in order to assess the relevancy of m/z of 869.3815.

Fig. 6 shows the mean intensities of 17 variables (markers) at different retention times and mass/z ratios for women giving birth prematurely ($n = 5$) compared to control women delivering at-term ($n = 10$). We can clearly distinguish the differences between the two groups under study.

These preliminary results demonstrate the feasibility of the analysis of cervicovaginal secretion analysis and the detection, using this UPLC-QTof mass spectrometry technology, of metabolites that may be useful predictive of spontaneous preterm birth. The next step will be to collect data analysis on larger cohorts using different sample processing methods and establish a preclassification of analytes using different chromatographic systems and mass spectrometry conditions. The characterization of targeted biomarkers will be undertaken by performing mass fragmentation studies, isolation, purification and NMR studies. After the elucidation of the structures of the compounds of interest, specific mass spectrometric quantitative methods will be developed to measure the concentration of these biomarkers in cervicovaginal secretions.

4. Conclusion

Preterm birth is a major public health problem with implications for both the mother and the neonate. Spontaneous preterm birth is even more complex. The study presented here reports a mass spectrometric approach to compare cervicovaginal secretions in women delivering at-term compared to those who gave birth prematurely. It is a novel non-invasive approach which may allow the development of new strategies for the management of women at high-risk for sPTB to reduce the incidence of preterm birth and perinatal morbidity. Further multivariate analysis having the potential to identify more putative biomarkers according to other parameters of interest (e.g. inflammation-type biomarkers) will also be undertaken.

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References

[1] R.L. Goldenberg, J.F. Culhane, J.D. Iams, R. Romero, Preterm birth 1. Epidemiology and causes of preterm birth, The Lancet 371 (9606) (2008) 75–84.
[2] World Health Organization (WHO), Global Program to Conquer Preeclampsia/Eclampsia, 2002, http://www.preeclampsia.org/statistics.asp.
[3] W.M. Gilbert, T.S. Nesbitt, B. Danielsen, The cost of prematurity: quantification by gestational age and birth weight, Obstet. Gynecol. 102 (3) (2003) 488–492.
[4] M.T. Mella, V. Berghella, Prediction of preterm birth: cervical sonography, Semin. Perinatol. 33 (5) (2009) 317–324.
[5] M. Van den Hof, J. Crane, Ultrasound cervical assessment in predicting preterm birth, J. SOGC 102 (2001) 1–4.
[6] S.Q. Wei, W. Fraser, Z.C. Luo, Inflammatory cytokines and spontaneous preterm birth in asymptomatic women: a systematic review, Obstet. Gynecol. 116 (2 Pt 1) (2010) 393–401.
[7] O. Fiehn, B. Kristal, B. van Ommen, L.W. Sumner, S.A. Sansone, C. Taylor, N. Hardy, R. Kaddurah-Daouk, Establishing reporting standards for metabolomic and metabonomic studies: a call for participation, OMICS 10 (2) (2006) 158–163.
[8] R.P. Horgan, O.H. Clancy, J.E. Myers, P.N. Baker, An overview of proteomic and metabolomic technologies and their application to pregnancy research, BJOG 116 (2009) 173–181.
[9] M. Mamas, W.B. Dunn, L. Neyses, R. Goodacre, The role of metabolites and metabolomics in clinically applicable biomarkers of disease, Arch. Toxicol. 85 (1) (2011) 5–17.
[10] J.D. Stewart, H.M. Bolt, Metabolomics: biomarkers of disease and drug toxicity, Arch. Toxicol. 85 (1) (2011) 3–4.
[11] C. Auray-Blais, C.R. Gagnon, J.T.R. Clarke, S.P. Young, D.S. Millington, Metabolomics, biomarker discovery and Fabry disease: an efficient platform is necessary!, Clin. Therapeut. 31 (2009) S24–S25.
[12] G.D. Lewis, A. Asnani, R.E. Gerszten, Application of metabolomics to cardiovascular biomarker and pathway discovery, J. Am. Coll. Cardiol. 52 (2) (2008) 117–123.
[13] R. Romero, S. Mazaki-Tovi, E. Vaisbuch, J.P. Kusanovic, T. Chaiworapongsa, R. Gomez, J.K. Nien, B.H. Yoon, M. Mazor, J. Luo, D. Banks, J. Ryals, C. Beecher, Metabolomics in premature labor: a novel approach to identify patients at risk for preterm delivery, J. Matern. Fetal Neonatal. Med. 23 (12) (2010) 1344–1359.
[14] E. Raiche, A. Ouellet, J. Poulin, M. Berthiaume, E. Rousseau, J.-C. Pasquier, Prediction of preterm delivery by supracervical and cervical ultrasound assessment associated with detection of vaginal inflammation (Colibri study), Am. J. Obs. Gyn. Suppl. (2009) S192.