Reproductive Sciences
2014, Vol. 21(6) 791-803

DOI: 10.1177/1933719113518987
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Amniotic Fluid Metabolomic Analysis in Spontaneous Preterm Birth

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Abstract

Objective: To identify metabolic changes associated with early spontaneous preterm birth (PTB; <34 weeks) and term births, using high-throughput metabolomics of amniotic fluid (AF) in African American population. **Method:** In this study, AF samples retrieved from spontaneous PTB (<34 weeks [n = 25]) and normal term birth (n = 25) by transvaginal amniocentesis at the time of labor prior to delivery were subjected to metabolomics analysis. Equal volumes of samples were subjected to a standard solvent extraction method and analyzed using gas chromatography/mass spectrometry (MS) and liquid chromatography/MS/MS. Biochemicals were identified through matching of ion features to a library of biochemical standards. After log transformation and imputation of minimum observed values for each compound, *t* test, correlation tests, and false discovery rate corrections were used to identify differentially regulated metabolites. Data were controlled for clinical/demographic variables and medication during pregnancy. **Results:** Of 348 metabolites measured in AF samples, 121 metabolites had a gestational age effect and 116 differed significantly between PTB and term births. A majority of significantly altered metabolites could be classified into 3 categories, namely, (1) liver function, (2) fatty acid and coenzyme A (CoA) metabolism, and (3) histidine metabolism. The signature of altered liver function was apparent in many cytochrome P450-related pathways including bile acids, steroids, xanthines, heme, and phase II detoxification of xenobiotics with the largest fold change seen with pantothenol, a CoA synthesis inhibitor that was 8-fold more abundant in PTB. **Conclusion:** Global metabolic profiling of AF revealed alteration in hepatic metabolites involving xenobiotic detoxification and CoA metabolism in PTB. Maternal and/or fetal hepatic function differences may be developmentally related and its contribution PTB as a cause or effect of PTB is still unclear.

Keywords
prematurity, liver functions, OMICs, African Americans, pregnancy

Introduction

The differences between mechanistic pathways of spontaneous preterm birth (PTB) and normal term births are still unclear. This lack of understanding contributes to limited number of markers for early diagnosis and prevention of preterm labor.[1] Several biomarker studies using maternal and fetal biological specimens have reported single or combination of biomarkers either as a predictor or as a mechanistic contributor to the preterm labor pathways.[2-4] Recent systematic reviews of patient metadata and PTB biomarkers have demonstrated the futility of predicting preterm labor risk using single biomarker test.[1,4] This is partly attributed to the involvement of multiple biomarkers, their redundancy in contributing to multiple PTB pathways, and unidentified risk factors that can influence the outcome, confirming the complexity of this syndrome.[5-7] Signals to initiate labor pathways at preterm and term involve multiple maternal–fetal organ systems. Our current understanding of contributing factors for PTB is mostly limited to inflammation and therefore the focus has mainly been on inflammatory markers.[8-10]

Biomarker discovery has progressed rather dramatically and improvements have been achieved in sensitivity and

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reproducibility of data. High-throughput technologies, a major progress in advancing targeted biomarker quantitation and validation, from standard enzyme-linked immunosorbent assays (ELISAs) to bead-based assays have improved our PTB biomarker knowledge base.[11-13] Additionally, proteomics technologies have also yielded valuable information on novel markers associated with PTB pathways and suggest mechanistic roles for these markers in PTB (or say, but provided limited insight into mechanisms they may play in PTB).[14-20] Most of these studies remain to be reproduced and confirmed in prospective studies either as a predictive marker or as a mechanistic factor associated with labor.

Recent advances in metabolomics provide another avenue to investigate biomarkers of interest in PTB mechanistic pathways.[21,22] Metabolomics is the study of all metabolites that are unique signature of all chemical reactions in an organ system that can be measured in biological samples (amniotic fluid [AF], plasma, urine, etc). The disease state or the physiologic state of intrauterine tissues is often better reflected in the metabolomic profile of biochemicals than transcriptome (messenger RNA) or proteome signature as metabolome represents a bridge between genome and phenotype.[20-23] Metabolomics utilizes high-throughput identification, quantitation, and characterization of biochemicals that are <1800 Da and are involved in various biological pathways. This approach can potentially identify over 2000 endogenous biochemicals using mass spectrometric analysis. Identified biochemicals can be further mapped into pathways and disease functions using bioinformatics approach.

Romero et al has reported the usefulness of metabolomic signature to diagnose women at risk of preterm labor by AF profiling of metabolites.[22] Here, we applied metabolic analysis to better understand the organ systems and biofunctions that contribute to PTB in an African American population where the risk of PTB is approximately double than other ethnic groups. Toward that end, we analyzed AF samples collected at the time of preterm or term delivery and found that global metabolite profiles differed significantly between those groups. Many metabolites affected by PTB reflect liver metabolism.

Methods

Patient recruitment and sample collection for this study were approved by the institutional review board (IRB) Tristar Nashville and Western IRB for the Perinatal Research Center, Nashville, Tennessee, for a parent study that examined racial disparity associated with genetic and biomarkers of PTB. Informed written consents were obtained from patients prior to enrollment, and reuse of those samples for this study was approved by IRB at The University of Texas Medical Branch (Galveston, Texas).

Study Population

Patients were recruited at the Centennial Medical Center (Nashville, Tennessee) between 2003 and 2008 as a part of Nashville Birth Cohort to study racial disparity in genetic and biomarkers in spontaneous PTB. Briefly, this is a tertiary care facility where many high-risk pregnancy referrals from middle Tennessee are treated. Therefore, our percentage of PTB will be slightly higher than basic population rates. Using a very stringent inclusion and exclusion criteria (for definition of the phenotype as spontaneous PTB with intact membranes), we have recruited $\sim$570 PTB (75% caucasians and 25% African Americans) and $\sim$1900 normal term births (61% caucasians and 39% African Americans) until 2008. Amniotic fluid samples were collected from approximately 30% of the PTB and 50% term births. All included pregnancies were singleton live births. Ethnicity was identified by self-report and a questionnaire that traces ancestry back 2 generations from the parents. Individuals who had more than 1 ancestral group were excluded from the study. Only African Americans were included in this study. We recruited mothers between the ages of 18 and 40. Gestational age was determined by last menstrual period and corroborated by ultrasound dating. The PTB were defined as delivery at $<34^{0/7}$ weeks gestation, and term births were defined as deliveries $>37^{0/7}$ with no medical or obstetrical complications during pregnancy. We excluded patients with multiple gestations, preeclampsia, preterm premature rupture of the membranes, placental previa, fetal anomalies, gestational diabetes, poly- and oligohydramnios, and other complications such as surgeries during pregnancy. Patients were consented and recruited upon admission to the hospital for labor. No selection criteria was used other than the inclusions and exclusions mentioned previously. Demographic data were collected from patient interviews, and clinical data were collected from patients' medical records. Data collected included age, socioeconomic status (education, yearly income, insurance status, and marital status), behavioral factors (smoking status), and a complete medical and obstetric history (tocolytic use, prior PTB, and infection status).

Amniotic fluid sample collection

In this study, AF samples were collected during labor (either preterm or term) by transvaginal amniocentesis where dilation was >6 cm. Samples were collected before cesarean section or before preterm or term vaginal delivery by puncture of intact membranes using a 22-gauge needle prior to artificial rupture of the membranes through the dilated cervical cavity. Amniotic fluid was centrifuged immediately for 10 minutes at 2000*g* to remove cellular and particulate matter. Aliquots of AF were stored at -70°C until analysis. Amniotic fluid samples for deliveries between $24^{0/7}$ and $34^{0/7}$ were included in PTB group and those between $40^{0/7}$ and $42^{0/7}$ were included as controls. The PTB had equal number of samples with and without intraamniotic infections (documented by microbiologic culture of AF or PCR for 16s ribosomal RNA) and clinical signs of chorioamnionitis (high fever, elevated C-reactive protein, abdominal tenderness, fetal tachycardia, mucopurulent vaginal discharge, histologic chorioamniotic, and funisitis). There was no difference in maternal age, SES factors, and parity between PTB and term births.

Metabolomic analysis of samples

The analysis was conducted by Metabolon, Inc (Durham, North Carolina).

Sample preparation

The sample preparation process was carried out using the automated MicroLab STAR system from Hamilton Company (Reno, NV). Recovery standards were added prior to the first step in the extraction process for QC purposes. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into 2 fractions; 1 for analysis by liquid chromatography (LC) and 1 for analysis by gas chromatography (GC). Samples were placed briefly on a TurboVap (Zymark, Sparta, NJ) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/mass spectrometry (MS) or GC/MS.

Quality assurance/quality control

For QA/QC purposes, a number of additional samples were included with each day's analysis. Furthermore, a selection of QC compounds was added to every sample, including those under test. These compounds were carefully chosen so as not to interfere with the measurement of the endogenous compounds. These QC samples were primarily used to evaluate the process control for each study as well as aiding in the data curation.

Liquid chromatography/mass spectrometry (LC/MS, LC/MS2)

The LC/MS portion of the platform was based on a Waters ACQUITY UPLC (Milford, MA) and a Thermo-Finnigan LTQ (Thermo Fisher Scientific, USA) mass spectrometer, which consisted of an electrospray ionization source and linear ion-trap (LIT) mass analyzer. The sample extract was split into 2 aliquots, dried, and then reconstituted in acidic or basic LC-compatible solvents, each of which contained 11 or more injection standards at fixed concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in 2 independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol both containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5 mmol/L ammonium bicarbonate. The MS analysis alternated between MS and data-dependent MS2 scans using dynamic exclusion.

Gas chromatography/MS

The samples destined for GC/MS analysis were redried under vacuum desiccation for a minimum of 24 hours prior to being derivatized under dried nitrogen using bistrimethyl-silyl-triflouroacetamide. The GC column was 5% phenyl, and the temperature ramp is from 40°C to 300°C in a 16-minute period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. The instrument was tuned and calibrated for mass resolution and mass accuracy on a daily basis. The information output from the raw data files was automatically extracted as discussed subsequently.

Accurate Mass Determination and MS/MS Fragmentation (LC/MS, LC/MS/MS)

The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ-FT mass spectrometer, which had a LIT front end and a Fourier transform ion cyclotron resonance mass spectrometer backend. For ions with counts greater than 2 million, an accurate mass measurement could be performed. Accurate mass measurements could be made on the parent ion as well as fragments. The typical mass error was less than 5 ppm. Ions with less than 2 million counts require a greater amount of effort to characterize. Fragmentation spectra (MS/MS) were typically generated in data-dependent manner, but if necessary, targeted MS/MS could be used, such as in the case of lower level signals.

Bioinformatics

The informatics system consisted of 4 major components, the Laboratory Information Management System (LIMS), the data extraction and peak-identification software, data processing tools for QC and compound identification, and a collection of information interpretation and visualization tools for use by data analysts. The hardware and software foundations for these informatics components were the LAN backbone, and a database server running Oracle 10.2.0.1 Enterprise Edition.

Data Extraction and Quality Assurance

The data extraction of the raw mass spectrometric data files yielded information that could be loaded into a relational database and manipulated without resorting to BLOB manipulation. Once in the database, the information was examined and appropriate QC limits were imposed. Peaks were identified using Metabolon proprietary peak integration software, and component parts were stored in a separate and specifically designed complex data structure.

Compound Identification

Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards. As of this writing, more than 1000 commercially available purified standard compounds had been acquired registered into LIMS for distribution to both the LC and GC platforms for determination of their analytical characteristics. The combination of chromatographic

properties and mass spectra gave an indication of a match to the specific compound or an isobaric entity. Additional entities could be identified by virtue of their recurrent nature (both chromatographic and mass spectral). These compounds have the potential to be identified by future acquisition of a matching purified standard or by classical structural analysis.

Curation

A variety of curation procedures were carried out to ensure that a high-quality data set was made available for statistical analysis and data interpretation. The QC and curation processes were designed to ensure accurate and consistent identification of true chemical entities, and to remove those representing system artifacts, misassignments, and background noise. We used proprietary visualization and interpretation software to confirm the consistency of peak identification among the various samples. Library matches for each compound were checked for each sample and corrected if necessary.

Normalization

For studies spanning multiple days, a data normalization step was performed to correct variation resulting from instrument interday tuning differences. Essentially, each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each data point proportionately. For studies that did not require more than 1 day of analysis, no normalization is necessary, other than for purposes of data visualization.

Statistical Analysis

For pairwise comparisons, Welch *t* tests and/or Wilcoxon rank-sum tests were performed. For classification, random forest (RF) analyses were used. Random forests give an estimate of how well we can classify individuals in a new data set into each group, in contrast to a *t* test, which tests whether the unknown means for 2 populations are different or not, RFs create a set of classification trees based on continual sampling of the experimental units and compounds. Then each observation is classified based on the majority votes from all the classification trees. Statistical analyses are performed with the program "R" http://cran.r-project.org. Multiple comparisons were performed using false discovery rate (FDR). The FDR for a given set of compounds can be estimated using the *q* values. In order to interpret the *q* value, first sort the data by the *P* value then choose the cutoff for significance (typically $P < .05$). The *q* value gives the FDR for the selected list (ie, an estimate of the proportion of false discoveries for the list of compounds whose *P* value is below the cutoff for significance). We used a cutoff of 0.05 in this study.

Data Quality

Instrument and process variability. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (ie, noninstrument standards) present in 100% of the samples, which are technical replicates of pooled samples. Median RSD for instrument variability using QC samples was 7%, and total process variability measured using endogenous biochemicals was 11% in this study that are well within the acceptable ranges of visibilities.

Table 1. Demographic Characteristics of Patients.

Subject Characteristics	Preterm Birth (PTB), N = 25	Normal Term Birth, N = 25
Gestational age, days	228.8 ± 22.48	278.8 ± 3.60[a]
Birth weight, g	1369 ± 461.4	3237 ± 318.5[a]
Maternal age, years	26.6 ± 6.4	26.36 ± 6.08
Cigarette smoking	4% (1/25)	16% (4/25)
Marital status	11 married	5 married
	14 single or divorced	15 single or divorced
Fetal sex	13 female	10 female[b]
	12 male	13 male

[a]Significant difference as expected between PTB and normal term births.
[b]Two data points are missing.

Results

Demographic details of the patients used for this study are included in Table 1. As expected, gestational age and birth weight were significantly different between PTB and term births. Other maternal demographic details were not statistically different.

The goal of this study was to identify small metabolite biomarkers associated with mechanisms of preterm and term labor by comparing the global metabolic profile of AF samples. We analyzed 50 AF samples (25 PTB; 25—normal term labor and delivery) from African Americans for this study. Correlation analysis was used to identify individual metabolites with levels that correlated significantly (*P* value <.05) with gestational age. Comparison of global biochemical profiles revealed several key metabolic differences between samples collected from full term versus PTB. Of particular interest, several metabolites that differed between PTB and term births are connected by their relationship to liver function.

Classification of PTB and Term Births

Random Forest analysis was used to (1) assess the capacity to accurately categorize preterm versus term patient groups based on the global metabolic profile of AF and (2) identify biochemicals important to the group classification accuracy. The RF analysis gave a mean prediction accuracy of 90%, a large improvement over the 50% accuracy that would be expected from random assignment to 1 of the 2 groups (Figure 1). It indicates that metabolite profiles differ greatly between groups and can be used to classify individuals into their correct groups with

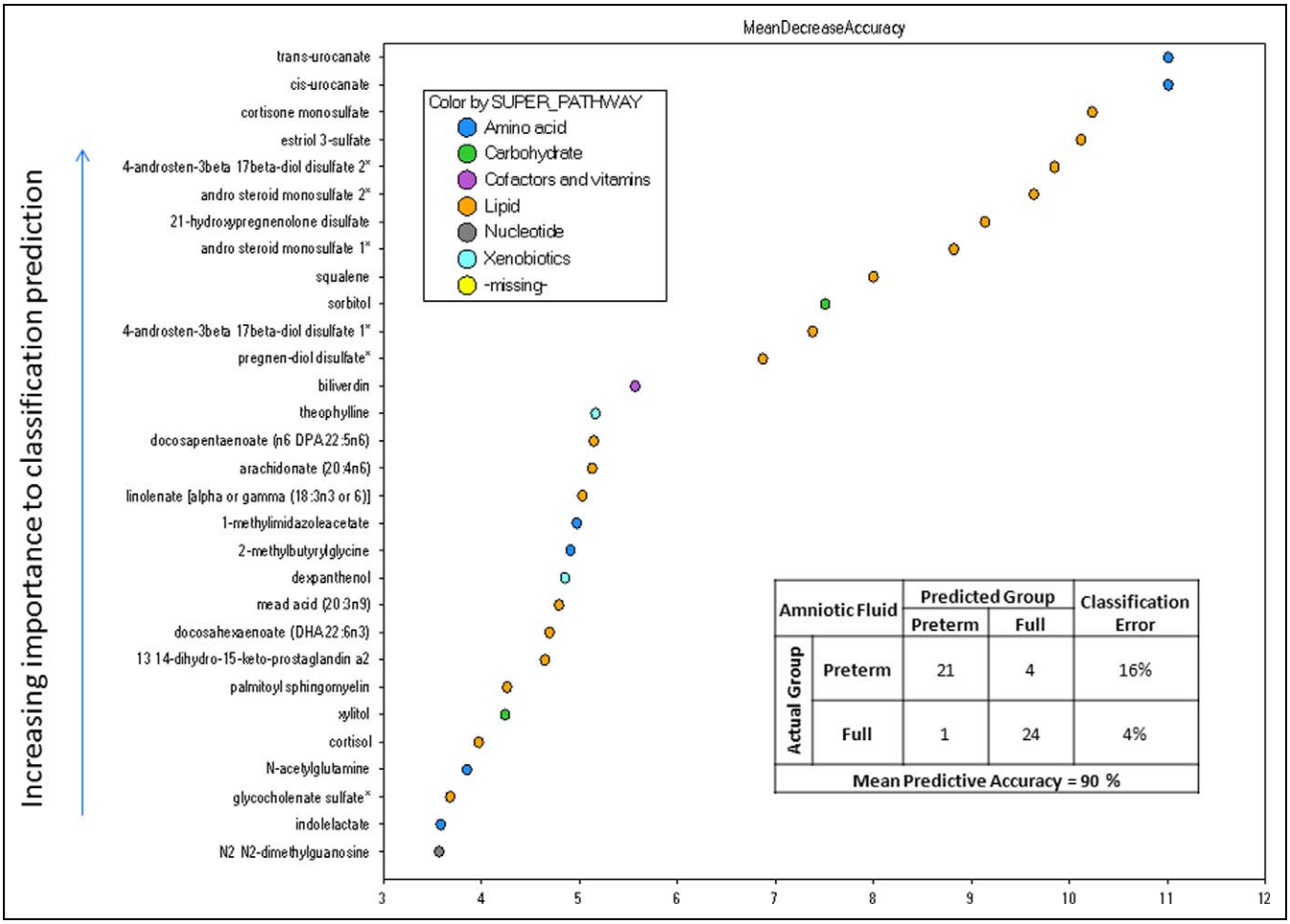

Figure 1. Random forest plot to document PTB and term birth classification. Various biochemicals are shown in color dots, and mean predictive accuracy of PTB versus term birth status based on metabolomic profiling is shown in table (insert). PTB, preterm birth.

good accuracy. Heat map demonstrating biomarker changes arranged by gestational age, where blue indicate metabolites that are less abundant and yellow is the most abundant (Figure 2). Dysregulated biochemicals are displayed based on PTB versus term birth status, intake acetaminophen by patients and changes correlated with gestational age. The following are categories of differentially regulated metabolites between PTB and term births:

Histidine Metabolites

Histidine metabolites are the top contributing biochemicals and some of the strongest *P* values were observed in histidine metabolites including cis-urocanate, trans-urocanate, and 1-methylimidazoleacetate.

Many of the differentially regulated metabolites important to classification accuracy are related to liver metabolism and are described further.

Phase II Detoxification of Xenobiotics

Metabolism and elimination of xenobiotics generally proceeds in 3 phases: (I) modification, often by cytochrome P450 oxidases that introduce reactive groups, (II) conjugation with glutathione, sulfate, glucuronate or glycine, and (III) excretion. In this study, several metabolites associated with liver metabolism differed significantly between preterm and full-term samples, including several metabolites of acetaminophen (Figure 3). Acetaminophen is a very common pain reliever used by pregnant women during pregnancy and labor. Acetaminophen is known to be metabolized by glurcuronidation, sulfation, and hydroxylation to yield nontoxic products that are excreted by kidneys. A toxic form (NAPQI) can also be formed. Although acetaminophen metabolites were not detected in all samples, the data suggest that 4-acetamidophenol, 2-methoxyacetaminophen sulfate, 3-(cysteine-S-yl) acetaminophen, 3-(N-acetyl-L-cystein-S-yl) acetaminophen, and p-acetamidophenylglucuronide were elevated in samples from PTB (Figure 1). Given that the liver is the main site of phase II detoxification, these metabolite differences could cause or result from altered liver activity of the maternal or fetal liver. The human fetal liver possesses well-developed metabolism of xenobiotics, from as early as 8 to 10 weeks.[24,25] Acetaminophen metabolism in fetal liver is mainly by sulfation as opposed to conjugation with UDPGA in adults.[26] However, due to small size of the fetus, the relative contribution of the fetus to

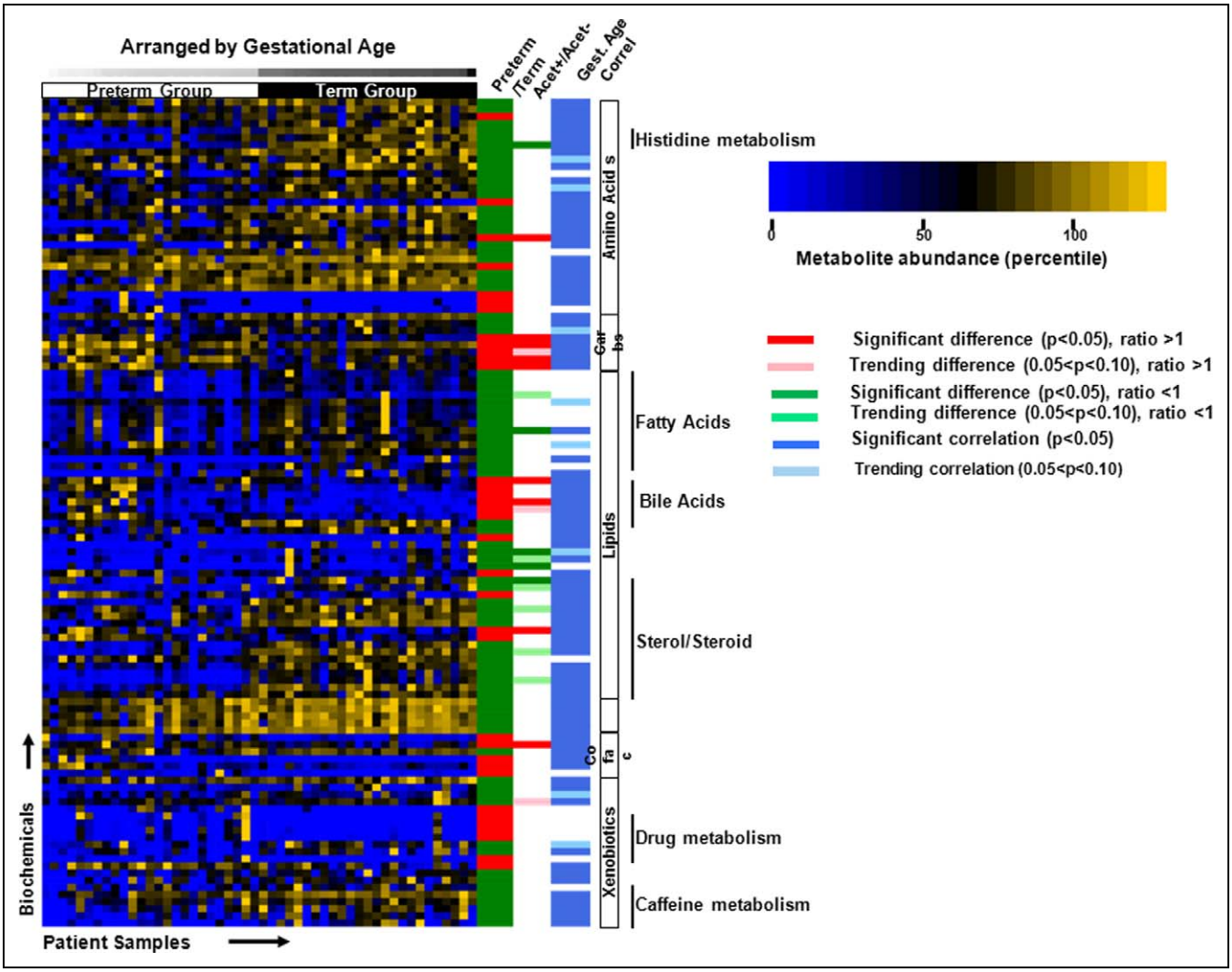

Figure 2. Heat map representation of metabolites that differed significantly between preterm and full term samples. Each block represents the abundance of 1 metabolite from 1 sample. Samples are sorted by gestational age at birth, with increasing age moving from left to right. Cells are conditionally formatted so that the minimum value measured (or imputed) for each metabolite is blue; the maximum value is yellow; the 15th percentile is black. Blocks in columns to the right are colored according to the legend shown in the figure for comparisons between preterm and full term groups, patient samples testing positive or negative for acetaminophen metabolites and metabolite correlation with gestational age. Metabolite pathways discussed in the main text are marked at the right.

overall gestational pharmacokinetics of drugs may be minor.[27] In order to identify metabolic correlates of acetaminophen use, we regrouped PTB and full-term patients based on detection (or not) of acetaminophen metabolites in AF samples on the metabolomics platform. Next, we applied *t* tests to identify metabolites that differed significantly between the 2 groups. Results of this analysis show that few of the metabolites that differed between PTB and full-term groups differed with acetaminophen use. These results suggest that differences in acetaminophen metabolites, like the other signatures of altered liver function described subsequently are a symptom of altered liver function, not a driving force. Other sulfated metabolites (p-cresol sulfate, phenol sulfate, glycocholenate sulfate, and 3-indoxyl suflate) were less abundant in preterm samples, possibly suggesting altered metabolic activity in the liver for other substrates as well.

Steroid Metabolites

Steroids are synthesized from cholesterol to control numerous physiological processes including development, osmotic balance, metabolism, and stress response (Figures 4 and 5). The maturation and activation of fetal hypothalamic–pituitary–adrenal axis has been well studied as a mechanism to initiate the process of parturition. The increase in production of C19 steroid precursor DHEAS and cortisol from fetal adrenal gland plays a major role in initiation of labor process, while placental progesterone production from cholesterol maintains quiescence during pregnancy.[28-30] The pathway of steroid biosynthesis is depicted in Figure 4. Two cholesterol precursors (squalene and lathosterol) were less abundant in preterm AF (Figure 5). Stress steroids (cortisol

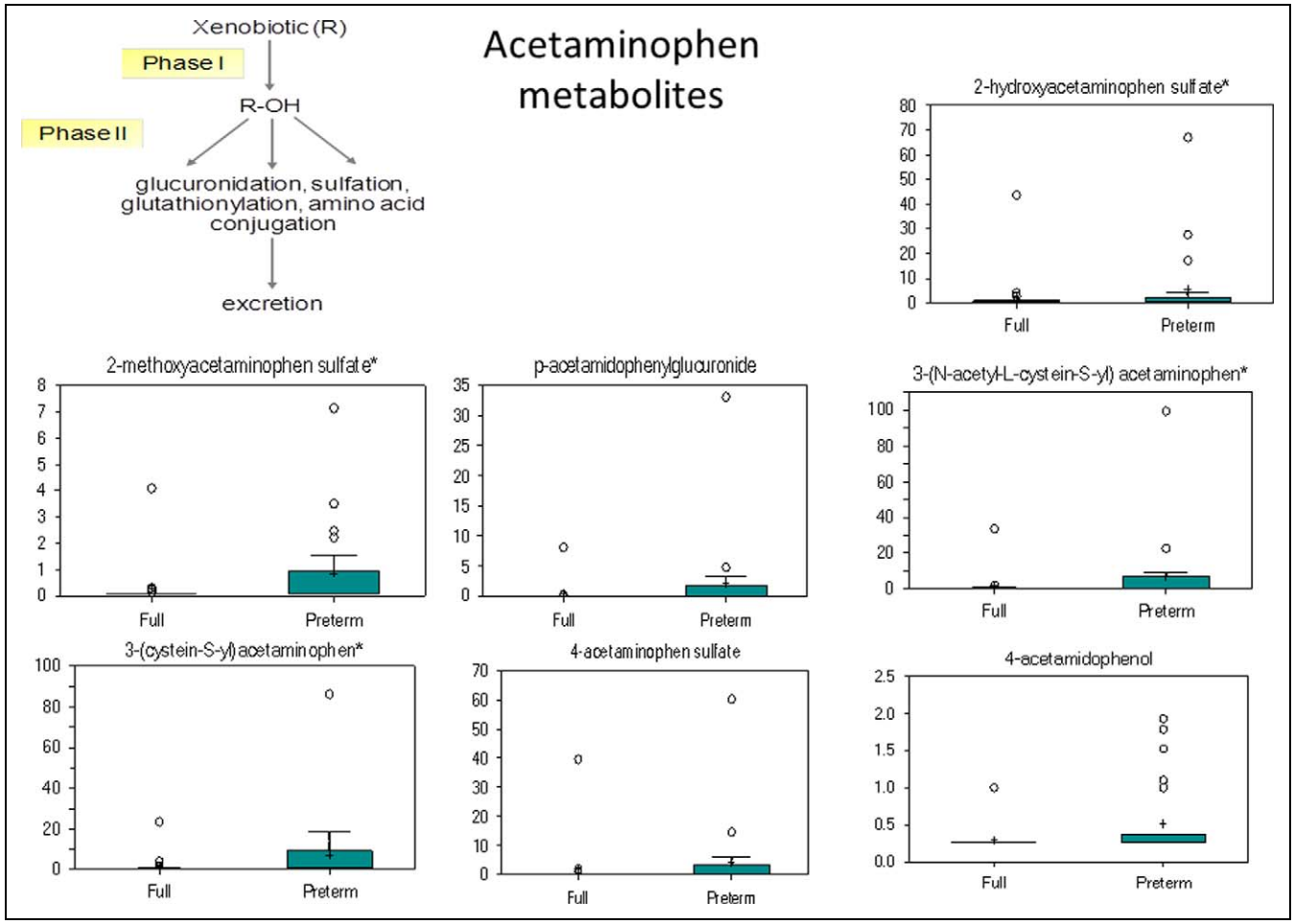

Figure 3. Two phases of xenobiotic metabolism by liver are shown in the pathway. Acetaminophen is a common nonprescription pain medication consumed by pregnant women, and its increase in preterm patients is expected. However, a principle component analysis and data adjusted for its usage suggest that changes related to acetaminophen metabolite dysregulation is not related to consumption of acetaminophen. Figure shows key acetaminophen metabolites differentially present in amniotic fluid between full-term and preterm amniotic fluids.

and cortisone) were less abundant in PTB, while the mineral corticoid (progesterone) was more abundant in PTB (Figure 4). Steroid metabolite conjugates also differed between sample cohorts and included changes in sulfated metabolites of placental progesterone (of androsterone, andro steroid, estriol, and cortisone), disulfated metabolites (of 21-hydroxypregnenolone, pregnen-diol, 5α-pregnan-3β,20α-diol, and 4-androsten-3β,17β-diol), and pregnanediol-3-glucuronide (Figure 5). Collectively, these differences could result from altered synthesis/metabolism or transport of steroids. Many of these metabolites had strong gestational age effects, suggesting that the differences in PTB versus full-term samples may result from difference in their gestational age and not necessarily a different mechanism/pathway. This would be in corroboration with the placental clock theory of timing of parturition.[31,32] This would imply that other extrinsic factors could lead to acceleration of the clock thereby leading to preterm parturition, the final mechanism of which may not be necessarily different from term parturition.

Bile Acids

Bile acids are synthesized from cholesterol in the liver, further metabolized by the gut microbiome, and released to facilitate absorption of dietary fats. In this study, several bile acids were elevated in PTB samples and included glycocholate, taurocholate, taurochenodeoxycholate, taurodeoxycholate, and glycodeoxycholate (Figure 6). Bile acids are removed from circulation by the maternal liver, and high circulating levels are known to cause fetal stress. Again a strong gestational age effect was seen in these metabolites, as well with higher concentrations of bile acid metabolites in preterm samples. This would be another consequence of alteration in maternal liver function, as the mother is mostly responsible for clearing the bile acids. Bile acid metabolism, secretion from liver, and liver reuptake can be disrupted by hormonal increases associated with pregnancy (cholestasis).[33] Also consistent with cholestasis and altered liver function, heme metabolites (bilirubin [Z, Z], bilirubin [E, E], and biliverdin) were elevated in PTB. Likewise, steroid sulfates are known to be elevated with cholestasis.

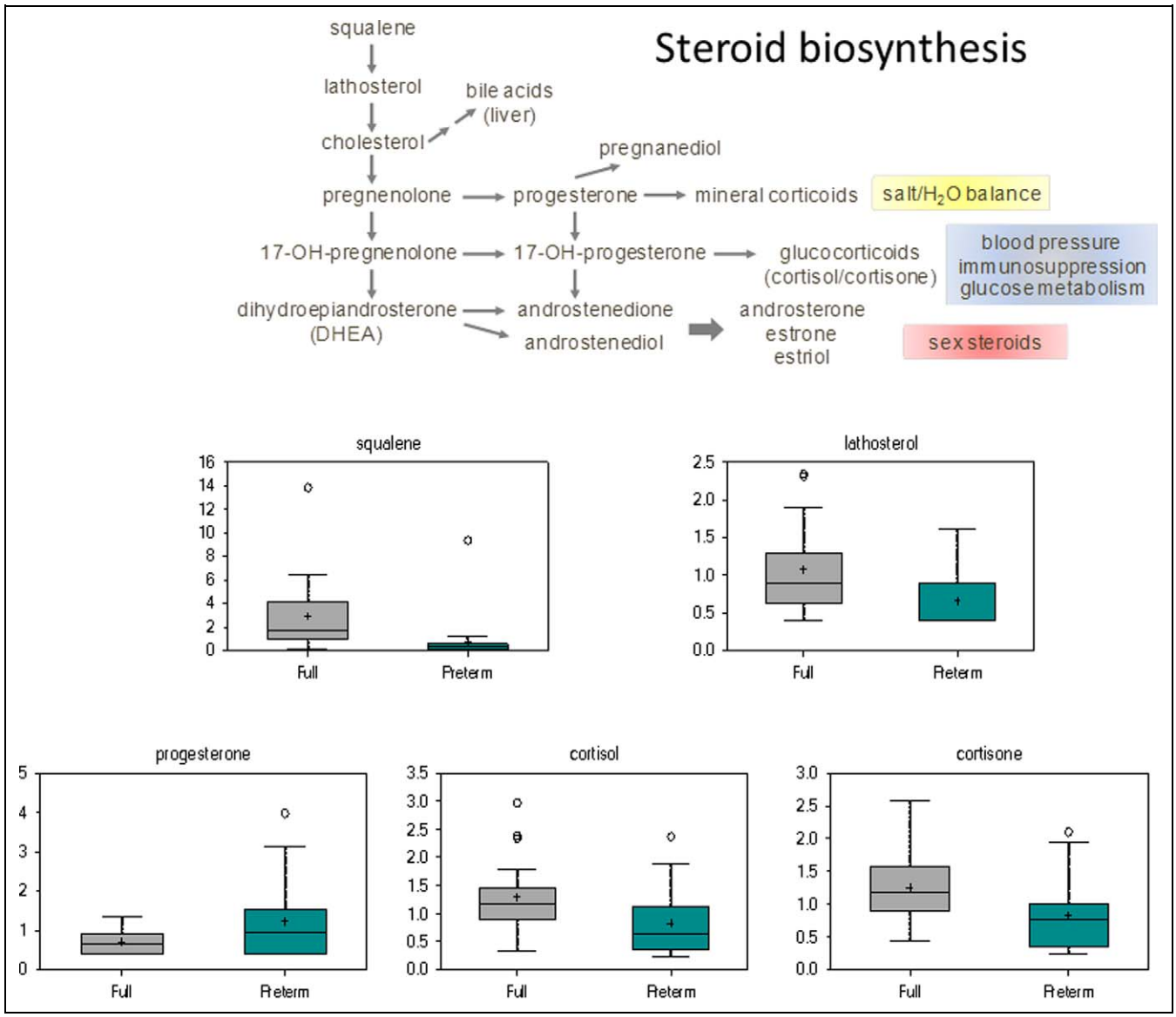

Figure 4. A pathway steroid biosynthesis and dysregulated steroid conjugates in amniotic fluid of women with preterm and term (full-term) birth.

Although cholestasis is a pathologic condition of pregnancy due to maternal liver dysfunction precipitated by pregnancy in susceptible women, its association with PTB is not reported and is associated with stillbirth.[34-36] Figure 6 also details the bile acid metabolism in the liver.

Xanthine Metabolites

In this study, several metabolites related to caffeine (theobromine, theophylline, 1-methylurate, 1,7-dimethylurate, 1,3,7-trimethylurate, and 7-methylxanthine) were less abundant in PTB. Like many metabolites described previously (bile acids, steroids, and acetaminophen), these signaling molecules are metabolized by cytochrome P450s in the liver. Changes in these metabolites could reflect altered consumption of caffeine or altered liver metabolism. We did not have caffeine consumption reporting from mothers to further examine contributing factors to these metabolite differences.

Fatty Acid Metabolism

The results from this study also suggested altered fatty acid metabolism between PTB and full term. Several long-chain fatty acids (LCFAs) and essential fatty acids (EFA) were less abundant in PTB. Since EFAs are obtained through diet, lack of EFA metabolites likely indicate deficiency of these factors (omega-3 fat and its derivative docosahexaenoic acid), associated with PTB.[37-39] 3-Hydroxybutyrate (BHBA), a ketone body formed from fatty acid catabolism made mainly in the liver, was significantly less abundant in PTB, suggesting decreased catabolism of fatty acids as an energy source. Together the results of this analysis suggest that the observed decrease in

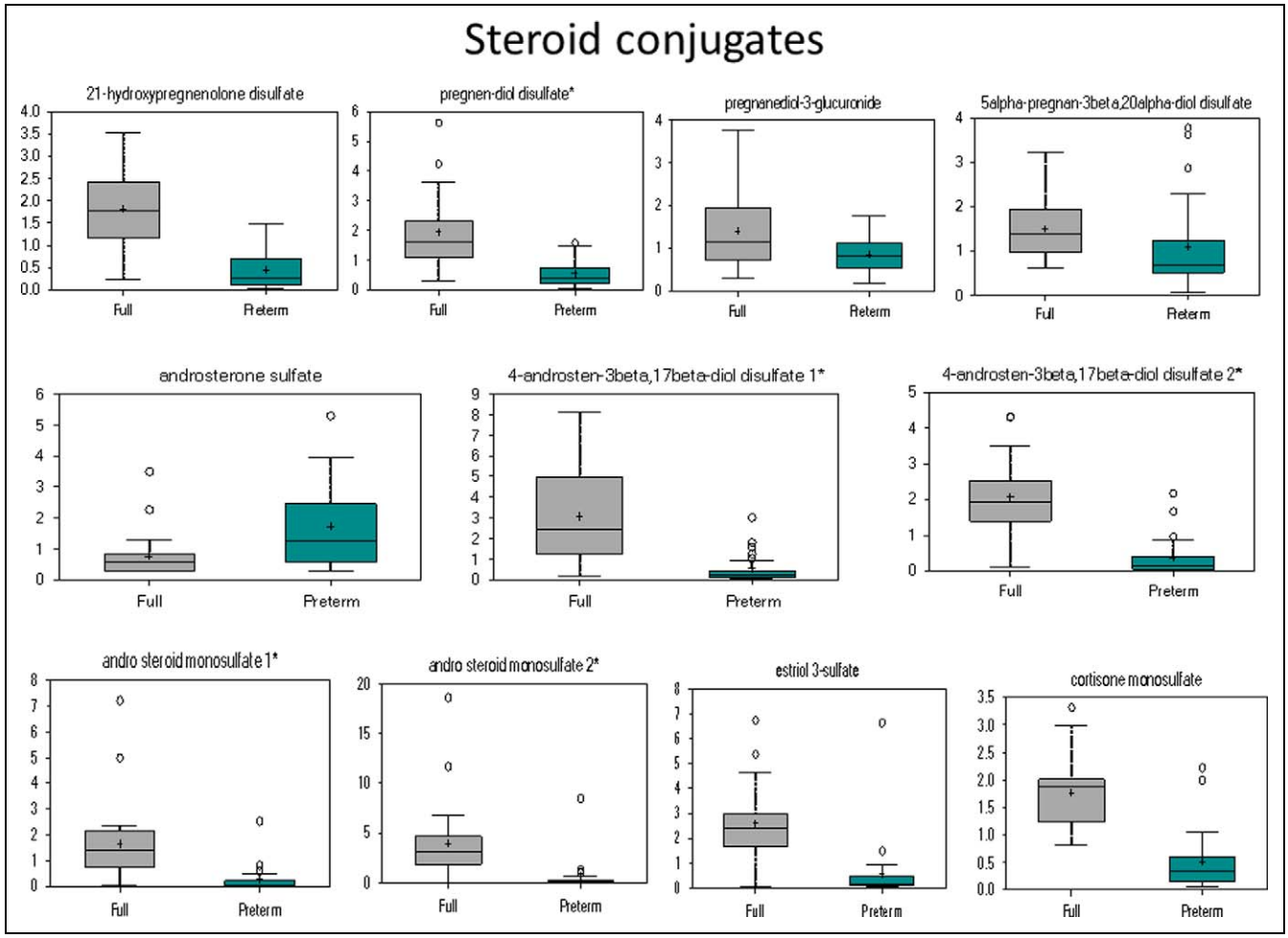

Figure 5. Dysregulated steroid conjugates in amniotic fluid of women with preterm and term (full-term) birth.

LCFA and EFA may result from decreased fat uptake, which could result from the aforementioned changes in bile acids.

Inflammation

Inflammation stimulates production of prostaglandins derived from phospholipase A2 activity toward membrane lipids to release C20 fatty acids. Several markers of inflammation were less abundant in PTB (Figure 7). Two C20 lipid sources for prostaglandins (arachidonate and mead acid) were less abundant PTB samples. C20 lipids are further metabolized by enzymes including COX-2 to generate prostaglandins. 13,14-Dihydro-15-keto-prostaglandin A2 and 12-HETE were also less abundant.

Cosmetic Formulation Chemicals

Besides the indicators of physiologic metabolic process, other biochemicals were also found to be altered in PTB compared to full-term samples. Pantothenol (dexapanthenol), a chemical commonly found in cosmetic products such as hair moisturizers, was much more abundant in preterm samples. This metabolite competes with pantothenate as a substrate for pantothenate kinase, an enzyme required to make coenzyme A (CoA).[40] The CoA is a cofactor for amino acid and fatty acid metabolism. Thus, reduced biosynthesis of CoA could relate to the observed differences in amino acid and fatty acid metabolism described previously. 1,2-Propanediol, also commonly added in cosmetic and pharmaceutical product formulations, was also elevated in PTB samples. Together these metabolite differences suggest that exposure or metabolism/excretion of environmental pollutants may impact PTB. However, it is important to note that 1,2-propanediol can also be made from BHBA or as a product of methylglyoxal detoxification, and further testing would be required to fully explore effects of these chemicals.

Discussion

This study was designed to determine the differences in metabolic abundances associated with preterm and term labor. The AF sampling from forebag when cervix is fully dilated prior to artificial rupture of membranes is suggestive of physiologic profiles representing metabolic derangements associated with

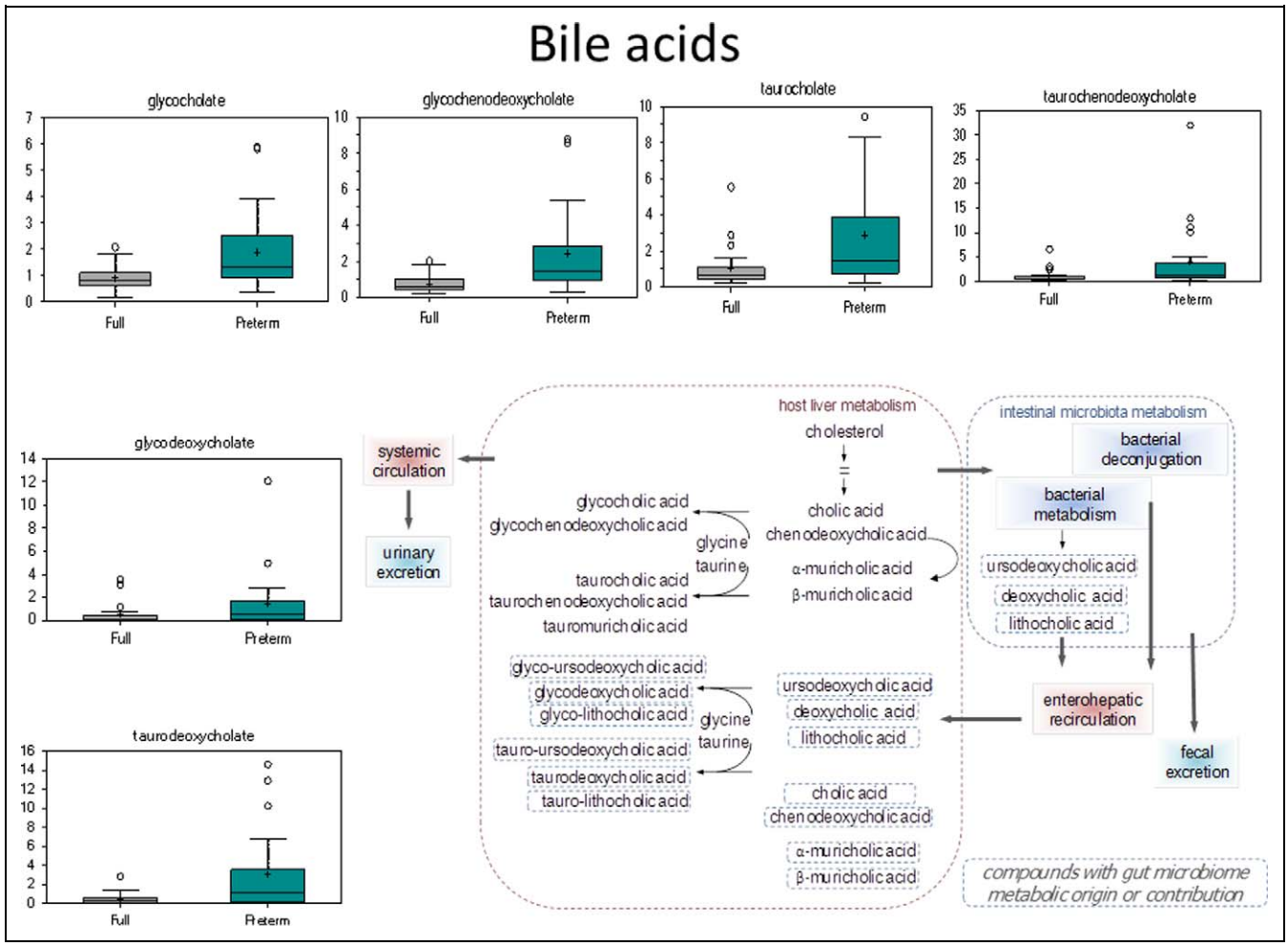

Figure 6. Dysregulated bile acid metabolites in amniotic fluid of women with preterm and term (full-term) birth. Flow diagram indicate bile acid metabolism by liver.

labor and delivery at preterm and term and provide insights into mechanistic aspects. Therefore, biomarkers, pathways, and organ system function identified in this study are not PTB risk predictors but provide insights into the dysfunctional status of certain organ systems that may be driving labor process at preterm compared to term. These studies are unbiased, and information gathered can be used to identify diagnostic markers and generate hypothesis for experiments to determine driving forces for preterm labor.

Based on the metabolomics signature of AFs, we report (1) liver functions are one of the key organ systems affected at preterm; (2) based on the metabolomics' signature of AFs, we report (a) liver functions are one of the key organ systems affected at preterm and (b) hepatic metabolites involving xenobiotic detoxification and CoA metabolism are differentially regulated and this may be very well developmentally related or it may be driving force in preterm labor. The study design will not allow us interpret the significance of this change as it needs more functional studies. The signature of altered liver function was apparent in many cytochrome P450-related pathways including bile acids, steroids, xanthines, heme, and phase II detoxification of xenobiotics with the most striking change seen with pantothenol, a CoA synthesis inhibitor that was 8-fold more abundant in PTB. Maternal acetaminophen intake was specifically investigated in our study to rule out that the usage of this drug during pregnancy as a potential confounder. We did not see any effect of the drug and confirmed that this increased acetaminophen is likely an indicator of altered hepatic metabolism.

Both maternal and fetal compartments can contribute AF metabolites and we cannot pinpoint the exact pathway causing these differences between preterm and term samples. Few of the metabolites can be traced back by the specificity of the pathways to maternal (eg, bile acid clearance) versus fetal (eg, placental steroids and fetal adrenal corticosteroids) compartments. However, the interrelation between the maternal and fetal response to these alterations and whether it is a "cause versus effect" cannot be delineated based on the results of this study.

One of the major pathophysiologic mechanisms associated with PTB is inflammation and candidate gene, and protein markers studies have adequately explained the role of inflammation. We did not identify any of the expected markers of

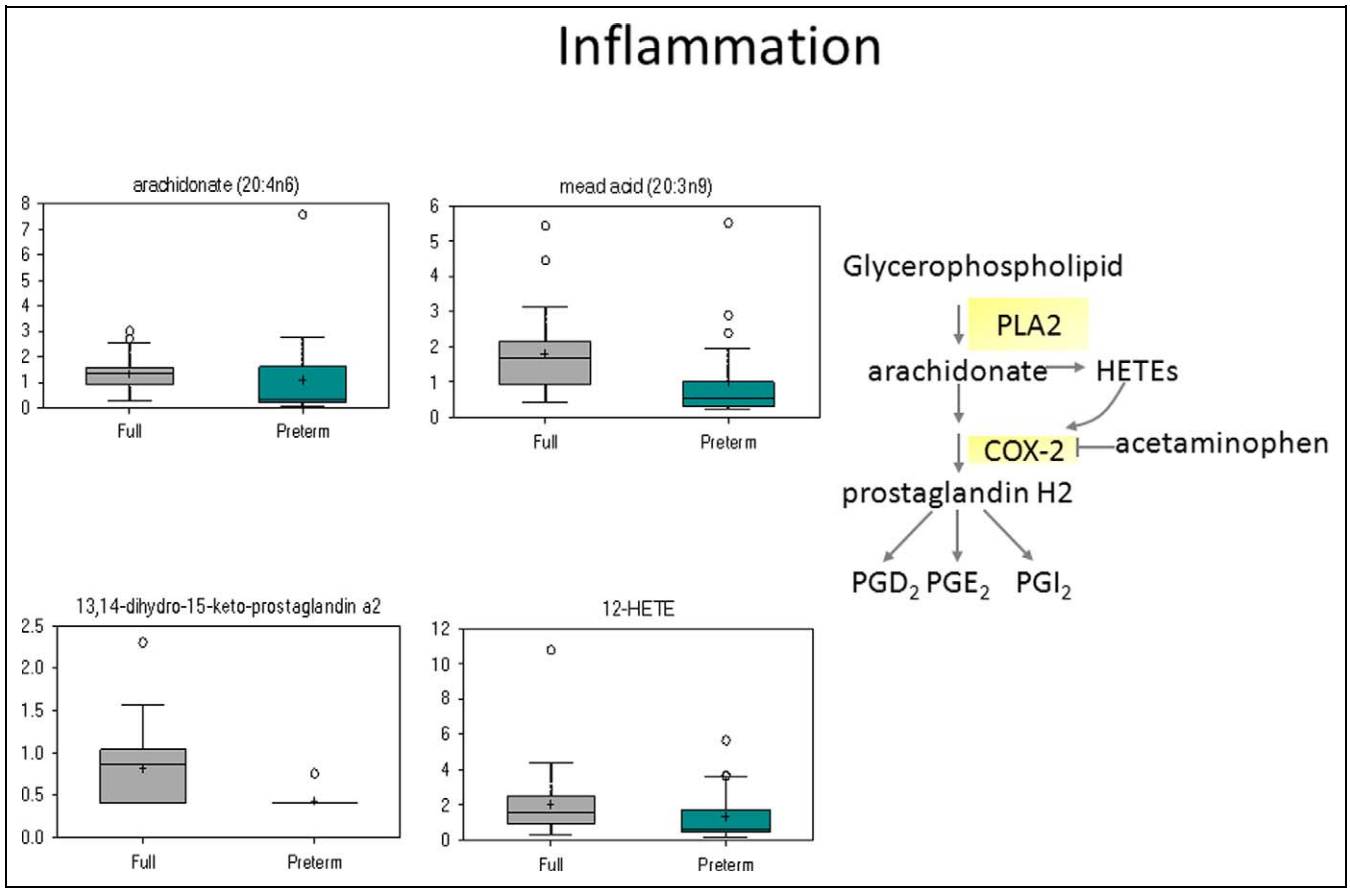

Figure 7. Changes in inflammatory metabolites. A few as shown in figure were different between the 2 groups. A pathway of uterotonin production from glycerophospholipid (cell membrane lipid) is shown in figure where acetaminophen may have a role in inhibiting some of the COX2-mediated prostaglandin production.

uterotonic activities or overwhelming inflammatory conditions associated with PTB as would normally expect. This will include prostaglandin and other uterotonic agents documented to be involved in labor. However, uterotonins are expected to increase during labor prior to delivery in both conditions and the differences in their metabolites may not be different to be detected in our analysis. This is in contradiction to our own reported findings of genetic and biomarkers (detected by ELISAs and spot arrays) associated with African American PTB. We speculate that the final effector metabolites may not differ in preterm and term births, and identification of differences in specific uterotonins (prostaglandins and eicosanoids) may require lipidomics approach or specific marker assays. Our approach is not sufficient to discriminate those markers. We also did not stratify PTB based on patient's intraamniotic infection status, as sample size was not sufficient. These data are consistent with data reported by Romero et al, where no documented uterotonins were seen in a study where data from PTB were analyzed after stratification based on intraamniotic infection.[22] Although those samples were collected from transabdominal amniocentesis at the time of diagnosis for preterm labor, we report similar findings from transvaginal amniocentesis samples prior to delivery during active labor.

Many of the significant differences between PTB and term births had a significant gestational age effect (eg, bile acids and steroids) while others did not (eg, acetaminophen metabolites). A future study could be designed to delineate the contribution of gestational age to the observed differences in preterm and full-term patient samples. Amniotic fluid could be collected at an earlier time point (20-26 weeks), and metabolite differences could be compared across groups once the outcome (preterm vs full term) is known. This study design could also identify early biomarkers that predict a forthcoming PTB, should there be differences present at the earlier time point.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship and/or publication of this article: This research was supported by the Swiss National Science Foundation (grant number 140202). This study is partially supported by funds from March of Dimes Perinatal Research Initiative Grant to R. Menon and also by R. Menon's Faculty development Funds from The University of Texas Medical Branch at Galveston, Texas.

Reference

1. Menon R, Torloni MR, Voltolini C, et al. Biomarkers of spontaneous preterm birth: an overview of the literature in the last four decades. *Reprod Sci*. 2011;18(11):1046-1070.
2. Bastek JA, Elovitz MA. The role and challenges of biomarkers in spontaneous preterm birth and preeclampsia. *Fertil Steril*. 2013; 99(4):1117-1123.
3. Conde-Agudelo A, Romero R. Cervicovaginal fetal fibronectin for the prediction of spontaneous preterm birth in multiple pregnancies: a systematic review and meta-analysis. *J Matern Fetal Neonatal Med*. 2010;23(12):1365-1376.
4. Conde Agudelo A, Papageorghiou AT, Kennedy SH, Villar J. Novel biomarkers for the prediction of the spontaneous preterm birth phenotype: a systematic review and meta-analysis. *BJOG*. 2011;118(9):1042-1054.
5. Goldenberg RL, Andrews WW. Intrauterine infection and why preterm prevention programs have failed. *Am J Public Health*. 1996;86(6):781-783.
6. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet*. 2008;371(9606):75-84.
7. Romero R, Espinoza J, Kusanovic JP, et al. The preterm parturition syndrome. *BJOG*. 2006;113 suppl 3:17-42.
8. Bastek JA, Gomez LM, Elovitz MA. The role of inflammation and infection in preterm birth. *Clin Perinatol*. 2011;38(3):385-406.
9. Elovitz MA. Anti-inflammatory interventions in pregnancy: now and the future. *Semin Fetal Neonatal Med*. 2006;11(5):327-332.
10. Romero R, Espinoza J, Goncalves LF, Kusanovic JP, Friel L, Hassan S. The role of inflammation and infection in preterm birth. *Semin Reprod Med*. 2007;25(1):21-39.
11. Richens JL, Urbanowicz RA, Metcalf R, Corne J, O'Shea P, Fairclough L. Quantitative validation and comparison of multiplex cytokine kits. *J Biomol Screen*. 2010;15(5):562-568.
12. Kramer MS, Kahn SR, Platt RW, et al. Mid-trimester maternal plasma cytokines and CRP as predictors of spontaneous preterm birth. *Cytokine*. 2010;49(1):10-14.
13. Riedmaier I, Pfaffl MW. Transcriptional biomarkers—high throughput screening, quantitative verification, and bioinformatical validation methods. *Methods*. 2013;59(1):3-9.
14. Buhimschi IA, Buhimschi CS, Weiner CP, et al. Proteomic but not enzyme-linked immunosorbent assay technology detects amniotic fluid monomeric calgranulins from their complexed calprotectin form. *Clin Diagn Lab Immunol*. 2005;12(7):837-844.
15. Cobo T, Palacio M, Navarro Sastre A, et al. Predictive value of combined amniotic fluid proteomic biomarkers and interleukin-6 in preterm labor with intact membranes. *Am J Obstet Gynecol*. 2009;200(5):499-496.
16. Crawford JT, Pereira L, Buckmaster J, Gravett MG, Tolosa JE. Amniocentesis results and novel proteomic analysis in a case of occult candidal chorioamnionitis. *J Matern Fetal Neonatal Med*. 2006;19(10):667-670.
17. Gravett MG, Novy MJ, Rosenfeld RG, et al. Diagnosis of intraamniotic infection by proteomic profiling and identification of novel biomarkers. *JAMA*. 2004;292(4):462-469.
18. Gravett MG, Thomas A, Schneider KA, et al. Proteomic analysis of cervical-vaginal fluid: identification of novel biomarkers for detection of intra-amniotic infection. *J Proteome Res*. 2007; 6(1):89-96.
19. Hitti J, Lapidus JA, Lu X, et al. Noninvasive diagnosis of intraamniotic infection: proteomic biomarkers in vaginal fluid. *Am J Obstet Gynecol*. 2010;203(1):32-38.
20. Tambor V, Kacerovsky M, Lenco J, Bhat G, Menon R. Proteomics and bioinformatics analysis reveal underlying pathways of infection associated histologic chorioamnionitis in pPROM. *Placenta*. 2013;34(2):155-161.
21. Romero R, Espinoza J, Gotsch F, et al. The use of high-dimensional biology (genomics, transcriptomics, proteomics, and metabolomics) to understand the preterm parturition syndrome. *BJOG*. 2006;113 suppl 3:118-135.
22. Romero R, Mazaki Tovi S, Vaisbuch E, et al. Metabolomics in premature labor: a novel approach to identify patients at risk for preterm delivery. *J Matern Fetal Neonatal Med*. 2010;23(12):1344-1359.
23. Gracie S, Pennell C, Ekman Ordeberg G, et al. An integrated systems biology approach to the study of preterm birth using "-omic" technology—a guideline for research. *BMC Pregnancy Childbirth*. 2011;11:71.
24. Johnsrud EK, Koukouritaki SB, Divakaran K, Brunengraber LL, Hines RN, McCarver DG. Human hepatic CYP2E1 expression during development. *J Pharmacol Exp Ther*. 2003;307(1):402-407.
25. Pacifici GM, Rane A. Metabolism of styrene oxide in different human fetal tissues. *Drug Metab Dispos*. 1982;10(4):302-305.
26. Adjei AA, Gaedigk A, Simon SD, Weinshilboum RM, Leeder JS. Interindividual variability in acetaminophen sulfation by human fetal liver: implications for pharmacogenetic investigations of drug-induced birth defects. *Birth Defects Res A Clin Mol Teratol*. 2008;82(3):155-165.
27. Hukkanen J, Mantyla M, Kangas L, et al. Expression of cytochrome P450 genes encoding enzymes active in the metabolism of tamoxifen in human uterine endometrium. *Pharmacol Toxicol*. 1998;82(2):93-97.
28. Tuckey RC. Progesterone synthesis by the human placenta. *Placenta*. 2005;26(4):273-281.
29. Rabe T, Kalbfleisch H, Haun A, Runnebaum B. Influence of human lipoproteins on the progesterone synthesis of human term placenta in organ culture. *Biol Res Pregnancy Perinatol*. 1983; 4(2):75-83.
30. Hellig H, Gattereau D, Lefebvre Y, Bolte E. Steroid production from plasma cholesterol. I. Conversion of plasma cholesterol to placental progesterone in humans. *J Clin Endocrinol Metab*. 1970;30(5):624-631.
31. Smith R, Nicholson RC. Corticotrophin releasing hormone and the timing of birth. *Front Biosci*. 2007;12:912-918.
32. Challis JR. CRH, a placental clock and preterm labour. *Nat Med*. 1995;1(5):416.
33. Pathak B, Sheibani L, Lee RH. Cholestasis of pregnancy. *Obstet Gynecol Clin North Am*. 2010;37(2):269-282.
34. Coletta J, Simpson LL. Maternal medical disease and stillbirth. *Clin Obstet Gynecol*. 2010;53(3):607-616.
35. Simpson LL. Maternal medical disease: risk of antepartum fetal death. *Semin Perinatol*. 2002;26(1):42-50.
36. Fisk NM, Storey GN. Fetal outcome in obstetric cholestasis. *Br J Obstet Gynaecol*. 1988;95(11):1137-1143.

37. Dunlop AL, Taylor RN, Tangpricha V, Fortunato S, Menon R. Maternal micronutrient status and preterm versus term birth for black and white US women. *Reprod Sci*. 2012;19(9):939-948.
38. Mozurkewich EL, Klemens C. Omega-3 fatty acids and pregnancy: current implications for practice. *Curr Opin Obstet Gynecol*. 2012;24(2):72-77.
39. Jordan RG. Prenatal omega-3 fatty acids: review and recommendations. *J Midwifery Womens Health*. 2010;55(6):520-528.
40. Abiko Y.Investigations on pantothenic acid and its related compounds. IX. Biochemical studies. 4. Separation and substrate specificity of pantothenate kinase and phosphopantothenoylcysteine synthetase. *J Biochem*. 1967;61(3):290-299.