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Metabolomics in Premature Labor: A Novel Approach to Identify Patients at Risk for Preterm Delivery

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Abstract

Objective—Biomarkers for preterm labor and delivery can be discovered through the analysis of the transcriptome (transcriptomics) and protein composition (proteomics). Characterization of the global changes in low molecular weight compounds which constitute the “metabolic network” of cells (metabolome) is now possible by using a “metabolomics” approach. Metabolomic profiling has special advantages over transcriptomics and proteomics since the metabolic network is downstream from gene expression and protein synthesis, and thus more closely reflects cell activity at a functional level. This study was conducted to determine if metabolomic profiling of the amniotic fluid can identify women with spontaneous preterm labor (PTL) at risk for preterm delivery, regardless of the presence or absence of intra-amniotic infection/inflammation (IAI).

Study Design—Two retrospective cross-sectional studies were conducted, including 3 groups of pregnant women with spontaneous PTL and intact membranes: 1) PTL who delivered at term; 2) PTL without IAI who delivered preterm; and 3) PTL with IAI who delivered preterm. The first was an exploratory study that included 16, 19 and 20 patients in groups 1, 2 and 3, respectively. The second study included 40, 33 and 40 patients in groups 1, 2 and 3, respectively. Amniotic fluid metabolic profiling was performed by combining chemical separation (with gas and liquid chromatography) and mass spectrometry. Compounds were identified by using authentic standards. The data were analyzed using Discriminant Analysis for the first study and Random Forest for the second.

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Results—1) In the first study, metabolomic profiling of the amniotic fluid was able to identify patients as belonging to the correct clinical group with an overall 96.3% (53/55) accuracy; 15 of 16 patients with PTL who delivered at term were correctly classified; all patients with PTL without IAI who delivered preterm neonates were correctly identified as such (19/19), while 19/20 patients with PTL and IAI were correctly classified. 2) In the second study, metabolomic profiling was able to identify patients as belonging to the correct clinical group with an accuracy of 88.5% (100/113); 39 of 40 patients with PTL who delivered at term were correctly classified; 29 of 33 patients with PTL without IAI who delivered preterm neonates were correctly classified. Among patients with PTL and IAI, 32/40 were correctly classified. The metabolites responsible for the classification of patients in different clinical groups were identified. A preliminary draft of the human amniotic fluid metabolome was generated and found to contain products of the intermediate metabolism of mammalian cells as well as xenobiotic compounds (e.g. bacterial products and Salicylamide).

Conclusion—Among patients with spontaneous PTL with intact membranes, metabolic profiling of the amniotic fluid can be used to assess the risk of preterm delivery in the presence or absence of infection/inflammation.

Keywords

preterm labor; preterm delivery; intra-amniotic inflammation; pregnancy; amniocentesis; microbial invasion of the amniotic cavity; MIAC; cytokines; chorioamnionitis; high-dimensional biology; “omics” sciences; intraamniotic infection

INTRODUCTION

Preterm parturition is one of the “Great Obstetrical Syndromes”. This term was coined to indicate that obstetrical disorders have: 1) multiple etiologies; 2) a long preclinical phase; 3) frequent fetal involvement; 4) be adaptive in nature; and 5) be the result of gene-environment interactions. [1–4] Progress in the understanding of the causes of preterm labor has relied on hypothesis-driven research. [5–73] This approach has yielded important information. However, the development of high-dimensional biology promises to provide a comprehensive description of the biological processes in complex diseases such as the preterm parturition syndrome[4,74,75] and identify biomarkers with prognostic and diagnostic implications, which may be more difficult to identify using a reductionist approach.

High-dimensional biology refers to the use of high throughput techniques which allow simultaneous examination of changes in the genome (DNA), transcriptome (mRNA), proteome (proteins), lipidome (lipids) and other biochemical components in a biological sample with the goal of understanding the physiology or mechanisms of disease. [76–78] The term “ome” refers to an abstract entity, group of mass, while “omics” sciences refer to the study of entities in aggregate. [79,80] Such techniques have included genomics, [81–85] transcriptomics, [86–88] proteomics, [89,90] and lipidomics, [91–93] and their fundamental principle is that a complex system can be understood more completely by considering its entirety, allowing for a global description of changes in biological samples.

Metabolites, which have small molecular weights, are part of primary and intermediate metabolism, and are estimated to be represented by more than 2,000 compounds in the human metabolome, [94–97] which refers to the catalog of those molecules in a specific organism or compartment, such as human metabolome, plasma metabolome, or amniotic fluid metabolome. Metabolomics is the study of the repertoire of non-proteinaceous, small molecules present in an organ, tissue or fluid. [98] Like other “omics” sciences, this discovery-based approach does not require a specific hypothesis and it is presumably

unbiased in nature. So far, examination of the cervico-vaginal and amniotic fluid proteome has been employed in order to identify biomarkers for spontaneous preterm labor. [99–110] The objective of this study was to determine, for the first time, if amniotic fluid metabolic profiling could be used to identify women with preterm labor and intact membranes at risk for preterm delivery, regardless of the microbial and inflammatory state of the amniotic cavity.

MATERIALS AND METHODS

Study design and population—We conducted a cross-sectional study by searching our clinical database and bank of biological samples to identify patients admitted to the hospital with the diagnosis of spontaneous preterm labor (PTL) with intact membranes, and that underwent amniocentesis for the assessment of the microbial state of the amniotic cavity and/or fetal lung maturity. Inclusion criteria included: 1) singleton gestation; 2) gestational age between 22–35 weeks; 3) live fetus; 4) intact membranes; and 5) signed informed consent approved by the Institutional Review Board of the Sotero del Rio Hospital (an affiliate of the Pontificia Universidad Catolica de Chile, Santiago, Chile,) or Wayne State University (Detroit, Michigan, USA) and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH, DHHS. Women with multiple pregnancies as well as those with fetal chromosomal and/or congenital anomalies were excluded.

This study was conducted in two phases and included patients divided in 3 groups: 1) PTL who subsequently had a delivery at term; 2) PTL without IAI who delivered preterm; and 3) PTL with IAI who delivered preterm. The first study included 16 pregnant women with PTL who delivered at term, 19 women with PTL without IAI who delivered preterm, and 20 patients with PTL and IAI. The second study included 40 pregnant women with PTL who delivered at term, 33 women with PTL without IAI who delivered preterm, and 40 patients with PTL and IAI.

Definitions—Spontaneous preterm labor was defined by the presence of regular uterine contractions occurring at a frequency of at least two every 10 minutes associated with cervical change before 37 completed weeks of gestation that required hospitalization. Preterm delivery was defined as birth before 37 weeks of gestation. Intra-amniotic infection was defined as a positive amniotic fluid culture for microorganisms. For the purpose of the first study, intra-amniotic inflammation was defined as an amniotic fluid white blood cell (WBC) count >100 cells/mm^3, [111,112] while for the second study, intra-amniotic inflammation was defined as an amniotic fluid interleukin (IL)-6 concentration >2.6 ng/mL. [113]

Sample collection—Amniotic fluid samples were obtained from transabdominal amniocentesis performed for evaluation of microbial status of the amniotic cavity and/or assessment of fetal lung maturity in patients approaching term. Samples of amniotic fluid were transported to the laboratory in a sterile capped syringe and cultured for aerobic/anaerobic bacteria and genital mycoplasmas. White blood cell count, glucose concentration and Gram-stain were also performed in the amniotic fluid shortly after collection as previously described. [112,114,115] The results of these tests were used for clinical management. Amniotic fluid IL-6 concentrations were used only for research purposes. Amniotic fluid not required for clinical assessment was centrifuged at 1,300 g for 10 minutes at 4°C and the supernatant was aliquoted and stored at −80°C until analysis. Many of these samples have been previously used to study the biology of inflammation, hemostasis, and growth factor concentrations in normal pregnant women and those with pregnancy complications.

Metabolomics methodology

Randomization of samples—amniotic fluid samples (n=168) were coded with a unique identifier for subsequent handling, and these identifiers were associated with a random number so that laboratory staff was blinded to the clinical diagnosis (including studies 1 and 2). The samples were processed in ascending order of their random numbers in two sets of samples. All samples were bar-coded throughout and received new barcodes at each step.

Extraction and preparation—The samples were removed from a −80° freezer and placed on ice to thaw. A 550 μl aliquot of pooled amniotic fluid (control sample) was placed on ice as well. A 100 μl aliquot of each sample was placed in individual wells of a 96-well deep-well plate, as were three 100 μl aliquots of the control sample and three 100 μl volumes of water (for blank controls). The sample placement was randomized and controlled by a platform-wide LIMS. Using a method described by Andreoli *et al.*, [116] a 400 μl volume of EtOAc/EtOH (1:1) was added to each sample and mixed on a Hamilton Star liquid-handling robot (Hamilton, Inc. Reno, NV, USA). The samples were transferred to a filter plate and a vacuum applied. Subsequent rinse solvents were similarly applied at 200 μl each (MeOH, MeOH/H2O (3:1), and DCM/MeOH (1:1), respectively). The resulting pooled extract was divided into two equal parts for LC/MS and GC/MS, respectively. Samples were concentrated on a Labconco Centrivap Concentrator 7810000, prior to lyophilization in a Labconco Freezone 6 lyophilizer (both Labconco Corp., Kansas City, MO, USA). The remaining samples were returned to a −80° freezer. Samples to be analyzed by liquid chromatography were re-dissolved in a 40% aquous ethanolic solution, while samples to be analyzed using gas chromatography were derivatized according to the procedure described by Gehrke and Leimer. [117] All samples had a final volume of 100 μl.

Mass spectroscopy and data processing—Samples subjected to LC/MS were analyzed on a Thermo-Finnigan LTQ-FT, a hybrid ion trap-Fourier transform mass spectrometer (Thermo Fischer Scientific, Inc. Waltham, MA, USA). The ion trap mass spectrometer (ITMS) and Fouier transform mass spectometry (FTMS) units were run at resolutions of 1,500 and 50,000, respectively, and tuned daily for these resolutions. Each sample carried a set of internal standards which were used to validate the performance of the procedures. An ITMS was scanned from 80 to 800 m/z, and FTMS analyzed from 200 to 2,000 m/z. A 10 μl aliquot of the re-dissolved extract was injected into the solvent path of a Perflourophenyl (PFP) column. The non-linear elution gradient began at water [0.1% formic acid (FA)] to 100% methanol (0.1% FA) at 7.5 minutes and ultimately to 100% acetone (0.1% FA) at 15 minutes. The column was re-equilibrated for 10 minutes before the next injection.

The samples were analyzed by GC/MS on a Thermo-Finnigan Mat95 (Thermo Fischer Scientific Inc, Waltham, MA, USA) coupled to an Ultra-Trace high resolution Gas Chromatograph (the Mat95 was tuned to a resolution of 10,000, and mass accuracy checked against the 131 ion of Perfluorokerosene) daily. The chromatographic conditions were non-linear on a RTX-5-sil column. The temperatures ranged from 60° to 340° C. A 1 μl aliquot of the derivatized extract was injected into a split/splitless injector (kept at 240° C) run in splitless mode. Each sample carried a set of internal standards which were used to validate procedural parameters.

The data analysis was chemocentric; therefore, the resulting data streams were processed to identify as many compounds as could be seen by the Metabolyzer software. [118] All identified peaks had a signal-to-noise ratio of >6, and an area of >50,000 ions. All named compounds were compared to authentic compounds. Compounds seen with regularity, but for which no standard was available, were assigned unique identifiers. Quantitation was

relative to internal standards specific to each data stream. Data analyzed included both relative and normalized (Z-score) concentration. The identification of Mass spectral peaks for both the LC and GC platforms was accomplished by comparison to authentic standards.

Data mining—Metabolomic profiles in the amniotic fluid were defined as the sets of biochemical compounds and their concentrations that best distinguish PTL patients in one group from the other two groups. Compounds that are significantly high or low in one group could be discovered by algorithms that have been used to build “class predictors”. The class predictors use the concentrations of these informative compounds to distinguish the profiles of PTL patients in one group from profiles of individuals in the other groups. Broadly speaking, this is “supervised learning”, a type of data mining in which a learning algorithm is presented with known “classes” and then attempts to derive a set of rules (a class predictor) that will predict the class membership of new samples. In investigations of this kind, class predictors are typically trained on a random subset of the data, the “training set”. Our study was conducted in two phases. The first set included 16 women with PTL who delivered at term, 19 women with PTL without IAI who delivered preterm, and 20 patients with PTL and IAI. The second set included 40 women with PTL who delivered at term, 33 women with PTL without IAI who delivered preterm, and 40 patients with PTL and IAI. This allowed us to rigorously assess the degree to which the class predictors have captured signatures that truly distinguish the different PTL groups. We used three approaches to class prediction: partial least square discriminate analysis (PLS-DA), relative class association/ weighted voting, and scatter analysis.

Statistical analysis—Linear discriminant function analysis, which constructs linear separation boundaries, was used to explore the relationship between the metabolomic profile of amniotic fluid and clinical classification of patients for study #1. Random Forest, a classification tree-based algorithm, was used for the analysis of study #2. Single classification tree method is useful for selecting from a small collection of predictor variables those that best explain a phenotype. Some of the features of this technique are: 1) ease of interpretation; 2) flexibility for the inclusion of interactions among predictors; and 3) ability to handle heterogeneity among samples. Classification trees use all variables and relevant cases when creating nodes in a single tree that represents the outcome of the learning process. However, single classification tree algorithms may easily over-fit data in the presence of a large number of predictor variables.

Alternatively, Random Forest builds a large number of classification trees, each grown using a subset of a bootstrap sample, while the remaining are left out for prediction. Each bootstrap sample was drawn randomly and of the same size from the original data with replacement. The variables used for splitting nodes in a tree are randomly chosen, much smaller sub-samples of the complete set of variables. The introduced double randomness (bootstrap sample and variable selection) and internal validation ultimately leads to an unbiased estimation of misclassification error. Over-fitting in presence of a large number of variables can be therefore avoided. Importantly, random Forest calculates importance scores which estimate the importance of a variable by looking at how much node impurities or prediction accuracy decreases when data for that variable are permuted while all other data are left unchanged. The Importance Score is a useful tool that organizes the metabolites according to the degree of contribution in classifying the cases into well-defined clinical phenotypes. Importance Scores are used to prioritize the variables in this model, and this information is used to determine the patterns that are informative with respect to the phenotype, creating a classification for each case. The results of classification are compared with actual clinical statuses. As the result of this process, matrices (true-negative, false-positive, true-positive, and false-negative) are created showing the number of patients

classified properly. Classification accuracy is calculated as the proportion of total of correct classifications (number of true-negative and true-positive classifications).

In this study, the three classes were preterm labor and delivery with and without IAI, and preterm labor delivered at term.. Rpackage "randomForest"[119] was applied to classify the three classes based on metabolomic profiling.

Gini Index calculation: The variable importance calculation depends slightly upon the specific algorithm used to split nodes in growing a trees in Random Forest. A splitting rule takes all the cases at a particular node in a decision tree and divides those cases so that some go to the left branch and others go to the right branch. There are a very large number of possible splitting rules – one for each explanatory variable and every possible split-point for that variable. At each node in the current tree, Classification and Regression Trees (CART) does a computer-intensive search to find the particular splitting rule that most helps in the classification of the cases. Our analysis used the Gini impurity criterion to determine the best splitting rule at each (non-terminal) node in the tree, because of its fast but generally robust performance in classification problems. Alternative rules include the entropy criterion and the misclassification rate (see Brienan for a comparative study). [120] The rules are usually consistent with each other. Gini criterion seeks the split such that the cases that go to the left are mostly of one category (or a small set of categories), while the cases that go to the right are mostly of another category (or small set of other categories). One says that the best split maximally reduces the "impurity" at that node. In Random Forest, this splitting process continues until all of the cases in a node have the same classification, and thus the node is "pure".

There are many possible measures of impurity. The most widely used Gini index at a node t is:

$$\Phi(t)=\sum_{J=1}^{K} p_j\,(1-p_j)$$

where there are k different categories among the cases at node t, and P_j is the proportion of cases in the node belonging to category j. CART searches among all the possible splits that divide cases in a parent node t into a left and right child nodes to find the one that maximizes

$$\Phi(t)-P_L\,\Phi(t_L)-P_R\,\Phi(t_R)$$

where P_L is the proportion of cases assigned to the left child node, P_R is the proportion of cases assigned to the right child node and $\Phi(t_L)$, $\Phi(t_R)$ are the Gini indices for the left and right child nodes.

RESULTS

Tables 1 and 2 display the demographic and clinical characteristics of patients included in the first and second study, respectively. Microorganisms isolated from the amniotic fluid in the first and second study are presented in Table 3.

First Study (Exploratory study)

The purpose of the first study was to examine whether the metabolic profile of amniotic fluid could classify patients according to phenotype. A supervised analysis with the results

of the metabolic profiling of amniotic fluid using linear discriminant function analysis was conducted. The results displayed in Figure 1 show that the technique (metabolomics) had promise to classify patients into the three groups. Table 4 displays the classification of patients according to their metabolomic profile. The accuracy of classification was 96.36% (53/55). Recognizing that this excellent diagnostic performance could have been due to over-fitting, we conducted a second study in an independent and larger set of samples using more robust-to-overfitting method to identify metabolomes separating the three groups.

Second Study (Validation study)

Figure 2 shows the raw data of the second study in a heatmap, in which every single row represents a metabolic "fingerprint" of a particular patient. A cursory inspection of the graph displays differences among groups. Tables 5, 6 and 7 display the differentially expressed metabolites in the amniotic fluid of patients with PTL who delivered at term, PTL without IAI who delivered preterm, and that of those with PTL with IAI, respectively. Random Forest analysis of the metabolic profiling of the amniotic fluid accurately predicted 39 of 40 patients who delivered at term and 29 of 33 patients who delivered preterm without IAI. However, it performed less well in patients with preterm delivery with IAI (32 out of 40). The overall accuracy for prediction of clinical classes was 88.5% (100/113) (Table 8). To identify the metabolites that contributed to the separation of the groups, we calculated the Gini index (Table 9).

A list of the metabolites identified in study #1 and study #2 is provided as a supplementary table.

DISCUSSION

Principal findings of the study

1) The metabolome of amniotic fluid can be characterized by using parallel techniques; 2) a group of compounds previously identified as part of the human intermediate metabolism were identified in the amniotic fluid. Notably, we also identified compounds that could be considered xenobiotic, such as Salicylamide and bacterial products; 3) the amniotic fluid metabolome can be analyzed for the identification of metabolites differentially expressed in patients with PTL using a combination of liquid chromatography and gas chromatography coupled with mass spectrometry; 4) metabolomic profiling was able to identify patients as belonging to the correct clinical group with a 88.5% precision (100/113); 5) patients who deliver preterm without IAI have a relative decrease in both carbohydrates and amino acids; and 6) in contrast, those with IAI have a more substantial decrease in compounds in the carbohydrate cluster, and a relative increase in amino acids.

The results of the study reported herein indicate that the presence of IAI is associated with an altered amniotic fluid metabolite composition. Specifically, amniotic fluid of women with IAI contains less carbohydrates and more amino acids compared to patients with PTL who delivered at term. Interestingly, while a decrease in carbohydrates was associated with preterm delivery in the presence or absence of IAI, an increase in amino acid metabolites is a unique feature of PTL with IAI (Table 10). Indeed, carbohydrates such as mannose, galactose and fructose were relatively increased in patients with PTL who delivered at term, but decreased in patients with PTL and IAI. The opposite was true with amniotic fluid amino acids. A decrease in alanine, glutamine and glutamic acid was noted in patients with PTL who delivered at term, while all of these amino acids were increased in the presence of IAI.

The cross-sectional nature of our studies does not allow us to infer a causal or a temporal relationship between alterations in amniotic fluid metabolites and IAI. In addition, it is not

clear what the specific source of each metabolite is. Fetal urine is a major contributor of amniotic fluid and both amnion cells and amniotic fluid WBC secrete their metabolites into the amniotic cavity. Nevertheless, a possible explanation for the shift from carbohydrate-rich to amino-acid-predominant metabolites could be a fetal catabolic state associated with a fetal systemic response syndrome (FIRS). [121–126] Catabolic states are associated with an increased demand for carbohydrates and decreased protein synthesis. This, in turn, can affect plasma and urine concentrations of these metabolites. Indeed, early neonatal sepsis (within 48 hours from delivery) in preterm neonates born to mothers with chorioamnionitis is characterized by an increased demand for carbohydrates. [127] Likewise, term neonates with infection have a higher consumption rate of carbohydrates than non-infected term newborns that can not be reconciled by changes in caloric intake. [128–130] The increase in amniotic fluid amino acids further supports the hypothesis that fetal infection and/or inflammation induces a catabolic state. Sepsis is associated with changes in metabolism that result in net proteolysis and negative nitrogen balance. [131,132] Of interest, both non-essential (e.g. alanine, glutamate) and essential (e.g. isoleucine) amino acids were included among the differentially expressed metabolites among patients with and without IAI. The latter finding is of special importance since the source of the essential amino acids in amniotic fluid should be the maternal circulation. The increase in the amniotic fluid concentration of amino acids in patients with PTL and IAI may reflect either changes in maternal availability of these amino acids or altered placental-amino acid transport. [133]

An additional explanation for the changes in metabolite content of amniotic fluid in patients with PTL and IAI relates to the presence of bacteria in the amniotic cavity. Utilization of carbohydrates as nutrients by bacteria may result in decreased concentrations of these molecules. Several lines of evidence support this view: 1) a low amniotic fluid glucose concentration is a well-established biomarker of IAI. [134–137] Low glucose concentrations are also associated with microbial invasion of other biological fluids including cerebrospinal[138,139] and synovial fluid;[140] 2) Fusobacterium species contain a galactose-binding protein, and it is possible that this protein may contribute to the decrease concentrations of galactose in infected amniotic fluid;[141] 3) Ureaplasmal lipoglycans are constituted primarily of mannose, glucose, and galactose, [142] suggesting that these carbohydrates may be consumed with the replication of this microorganism in the amniotic fluid; and 4) glucose is also required as a fuel by activated neutrophils engaged in the process of microbial phagocytosis and killing. [143]

Methyladenine was the most important classifier as determined by Random Forest analysis (Gini Index). N^6-methyl-adenine (m6A) is found in the genomes of many fungi, bacteria and protists, [144] as well as in archaeal DNA. [145] N^6-methyl-adenine is involved in many fundamental bacterial cell processes including: 1) bacterial defense against bacteriophages and transposons; 2) regulation of chromosome replication, chromosome segregation, and reorganization of the nucleoid after DNA replication; 3) DNA-strand discrimination for mismatch repair; 4) regulation of conjugal transfer of plasmids; 5) packaging of phage DNA into capsids; and 6) transcriptional regulation of fimbrial operons and other virulence genes. [146] This is the first study to report the detection of methyladenine in amniotic fluid. Tavazzi et al. [147] used the highly sensitive, ion-pairing High Performance Liquid Chromatography (HPLC) method with UV detection to identify several purines, pyrimidines, amino acids and other molecules in amniotic fluid of 10 normal pregnant women. Despite the high sensitivity of HPLC, methyladenine was not detected. It is possible that the presence of methyladenine in amniotic fluid is due to bacterial destruction. This explanation can account for the importance of this metabolite in the classification of the study groups, as well as for the negative results reported by Tavazzi et al. [147] in normal pregnant women.

The second most important metabolite in the classification of the study groups was heptanedioic acid, also know as pimelic acid. [148] Diamino pimelic acid (DAP) is a component of the bacterial cell-wall. Specifically, DAP is a key cross-linking constituent of the peptidoglycan layer. Most bacteria require either lysine or its biosynthetic precursor, DAP, as a component of the peptidoglycan layer of the cell wall. [149] Importantly, mammals do not produce or utilize DAP and require L-lysine as a dietary component. These observations led to the hypothesis that inhibitors of the DAP biosynthetic pathway would not be expected to show mammalian toxicity and made the biosynthesis of L-lysine via DAP as a target for naturally occurring antibiotics. [150–152] Recently, a DAP residue [γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP)] was identified as the minimal structure in cell wall peptidoglycan capable of stimulating Nucleotide-binding oligomerization domain protein 1 (NOD1, an important component of the innate immune system). [153] iE-DAP is known to exist only in particular bacteria including common Gram-negative bacteria, such as *Escherichia coli*, and several Gram-positive bacteria, such as *Listeria monocytogenes*. [154] Of note, both *Escherichia coli* and *Listeria monocytogenes* were identified in the amniotic fluid of patients in our study. Consistent with this, IL-6 production by folliculostellate cells in the normal pituitary gland is stimulated by diamino pimelic acid. It is tempting to postulate that this metabolite is released to the amniotic cavity following the destruction of bacterial cell-wall.

Several metabolites that have been shown to have a prognostic value for the diagnosis of IAI and/or preterm labor such as prostaglandins[155–163] and leukotrienes[164–166], were not identified as differentially expressed in the present study. Low concentrations of these metabolites, their biochemical diversity, as well as extraction method that is not optimized for these compounds, can account for this limitation. This issue needs to be addressed with an additional analytical platform, such as specifically optimized lipidomics, to cover a broad range of metabolites.

Metabolomics in pregnancy—High-dimensional biology techniques have been used to study normal pregnancy and parturition, as well as pregnancy complications, largely through the application of genomics, [167–176] transcriptomics[177–198] and proteomics. [99,101,105,107,152,199–232] Yet, few studies have reported the use of metabolomics in reproduction and pregnancy complications. It has been proposed that metabolomics may play an important role in the management of male infertility[233] as well as in assisted reproductive technology; indeed, the metabolomic profiling of embryo culture media and follicular fluid has been used to assess oocyte and embryo quality and viability. [234–242]

Recently, Heazell et al. [243] used gas-chromatography-mass spectroscopy to examine the metabolic profile of conditioned culture media and tissue lysates of placental villous explants from normal pregnant women cultured at different oxygen tensions. This study demonstrated that detection of the placental metabolic footprint is feasible, and that this metabolic footprint was different among placental samples as a function of the oxygen tension used for culture. Hexadecanoic acid, threitol or erythritol, and 2-deoxyribose were among the most differentially expressed metabolites. The same authors[244] conducted a similar study using Ultra Performance Liquid Chromatography Mass Spectrometry (LC-MS) in serum-conditioned culture medium of villous trophoblast from placentas of normal pregnant women and patients with preeclampsia. The relative concentration of 154 metabolites was significantly different in culture medium from normal pregnancies between normoxic and hypoxic conditions. Interestingly, 47 metabolites in preeclampsia-derived conditioned media cultured under normoxic conditions had a comparable relative concentration to that of those from normal placentas cultured in hypoxic conditions, suggesting that hypoxia may have a role in preeclampsia. Three metabolic pathways (glutamate and glutamine, tryptophan metabolism and leukotriene or prostaglandin

metabolism) were considered of interest for future studies to determine their role in the pathophysiology of preeclampsia. [244]

Nuclear Magnetic Resonance spectroscopy has been used for more than 10 years to explore the metabolomic profile of the amniotic fluid to determine fetal lung[245,246] and kidney maturation, [247] as well as in other pathologic conditions such Down syndrome, [247] and open spina bifida. [247,248] Recently, 1H NMR coupled with LC-NMR/MS has been applied to further characterize the metabolic composition of the amniotic fluid[249,250] and to determine its possible role as a diagnostic method for fetal malformations. The advantage of NMR analysis is that it is non-destructive of the samples. On the other hand the metabolomics technique described in the present manuscript has a superior sensitivity. Graça et al. [251] studied amniotic fluid from midtrimester genetic amniocenteses of 51 normal pregnant women and 12 pregnancies with fetal anomalies. Maternal age, fetal gender, and maternal age at amniocentesis did not change the metabolic profile of amniotic fluid of normal pregnant women. Using Principal Component Analysis, no differences were observed between the amniotic fluid samples from normal pregnancies and those with fetal malformations. However, orthogonal variant Partial Least Squares-Discriminant Analysis differentiated the two groups by identifying changes in glucose, succinate, and eight amino acids, as well as changes in the protein fraction and in the ratio of free to protein-interacting lactate. The authors suggest that these changes may represent a shift in energy production towards glycolysis due to hypoxia. [251]

In conclusion, this is the first draft of the human amniotic fluid metabolome in patients with spontaneous preterm labor with intact membranes. A stereotypic pattern of metabolites was identified in clinical groups with different outcomes, and global changes in the metabolic profile of the amniotic fluid allowed classification of patients at risk for preterm delivery (88% accuracy). Characterization of the human amniotic fluid metabolome can serve as the basis for the development of rapid tests to identify the patient at risk for impending preterm birth from the patient who will deliver at term, in the presence or absence of intra-amniotic infection/inflammation. Further studies with more sensitive tools could increase the number of metabolites identified in amniotic fluid and improve the draft of the amniotic fluid metabolome reported herein. In addition to the study of amniotic fluid, metabolomics characterization of maternal plasma, urine and cervico-vaginal fluid can be of great importance. Insights gained from these studies could help improve the understanding of metabolism of the fetus and fetal membranes in normal and pathologic conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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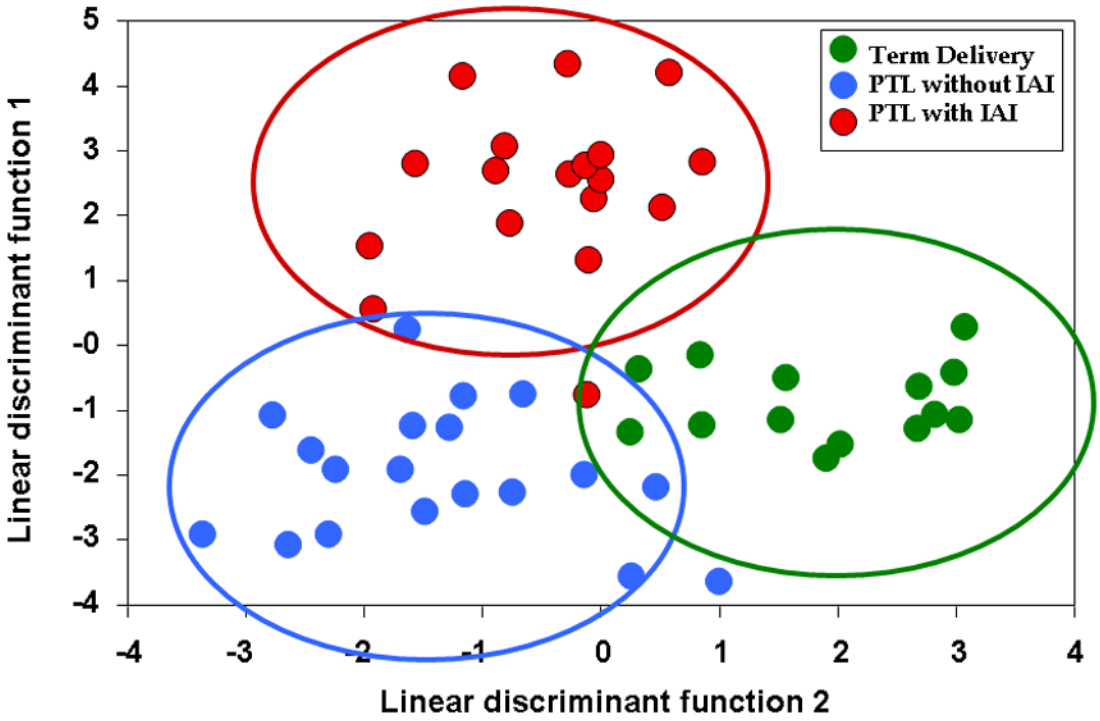

Figure 1.
Supervised analysis for classification of patients according to their metabolic profile of amniotic fluid. In green, patients who presented with preterm labor but delivered at term; in blue, those with preterm labor who delivered preterm without intra-amniotic inflammation and in red, those with intra-amniotic inflammation. (PTL – preterm labor; IAI – intra-amniotic infection/inflammation)

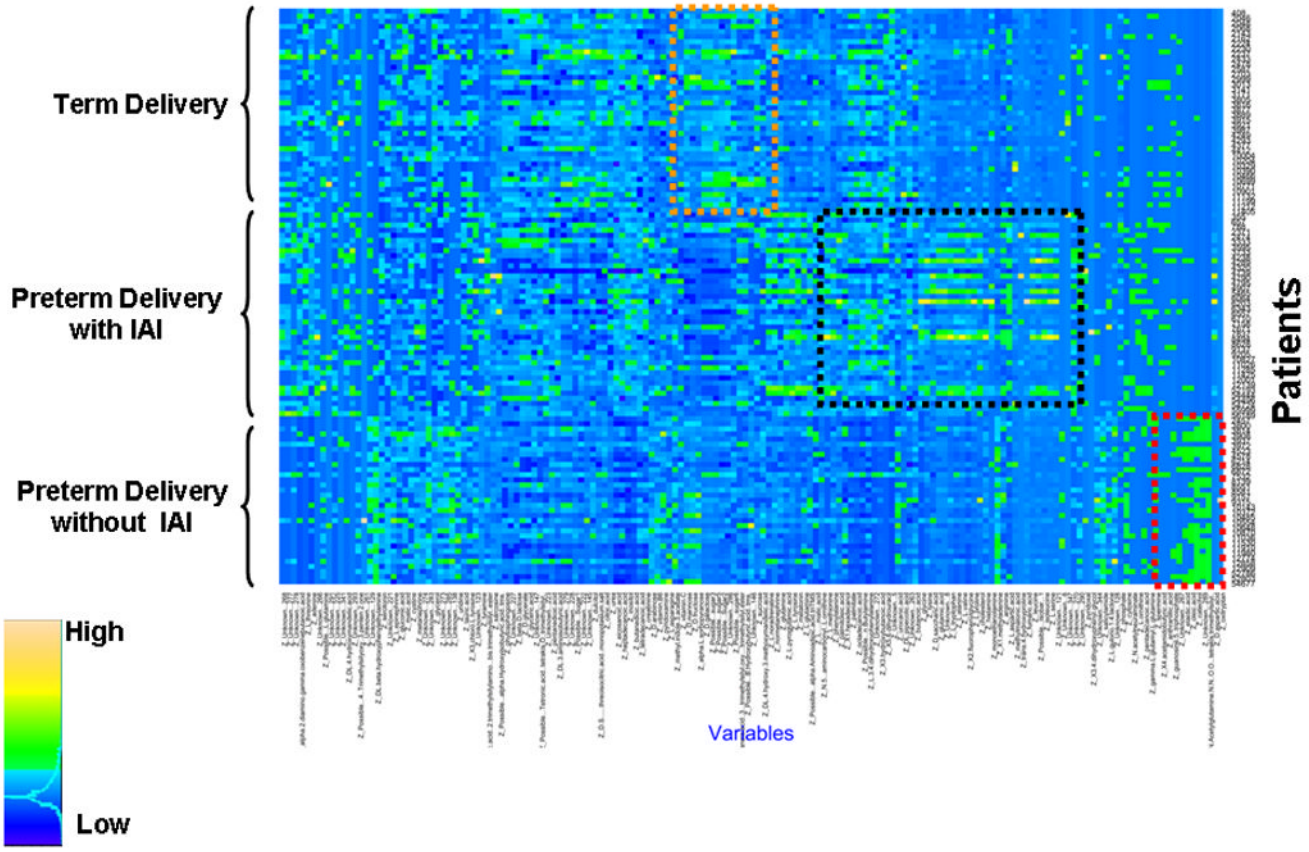

Figure 2.

Each horizontal line is a fingerprint of the metabolomic profile of amniotic fluid in an individual patient. Each vertical line represents the concentration of a metabolite in amniotic fluid. The color scale provides an index of abundance. Three patient groups are included in the study (see left column), and the metabolic fingerprinting characterizing each group is within a colored box. (IAI – intra-amniotic infection/inflammation)

Table 1

Demographic and clinical characteristics of the study population (First study).

	Term Delivery (n=16)	PTL without IAI (n=19)	PTL with IAI (n=20)
Maternal age (years)	25 (16 – 38)	21 (17 – 45)	31 (19 – 41)
Nulliparity	47 (9/19)	56 (9/16)	25 (5/20)
Gestational age at amniocentesis (weeks)	27 (22 – 32)	28 (23 – 33)	26 (22 – 33)
Cervical dilatation (cm)	0 (0 – 1)	1.5 (0 – 5)	0 (0 – 5)
Amniocentesis to delivery interval (days)	86 (49 – 130)	1 (0 – 7)	3 (0 – 7)
Gestational age at delivery (weeks)	39 (37 – 42)	29 (23 – 33)	27 (22 – 34)
Sample storage time (months)	49 (15 – 73)	63 (22 – 80)	33 (8 – 63)

Values are presented as median (range) of percentage (number)

PTL: preterm labor; IAI: intra-amniotic infection/inflammation

Table 2

Demographic and clinical characteristics of the study population (Second study).

	Term Delivery (n=40)	PTL without IAI (n=33)	PTL with IAI (n=40)
Maternal age (years)	21 (15 – 41)	22 (14 – 40)	22 (16 – 41)
Nulliparity	50 (20/40)	52 (17/33)	50 (20/40)
Gestational age at amniocentesis (weeks)	31 (24 – 33)	30 (23 – 33)	29 (23 – 33)
Cervical dilatation (cm)	0 (0 – 4)	0 (0 – 5)	1 (0 – 7)
Amniocentesis to delivery interval (days)	55 (33 – 109)	5 (0 – 7)	1 (0 – 7)
Gestational age at delivery (weeks)	39 (37 – 41)	31 (23 – 34)	29 (23 – 34)
Sample storage time (months)	71 (17 – 83)	32 (1 – 144)	57 (4 – 114)

Values are presented as median (range) of percentage (number)

PTL: preterm labor; IAI: intra-amniotic infection/inflammation

Table 3

Microorganisms isolated from the amniotic fluid in the first and second study.

First Study	Second Study
Ureaplasma urealyticum	*Ureaplasma urealyticum*
Candida albicans	*Candida albicans*
Fusobacterium sp.	*Fusobacterium sp.*
Listeria monocytogenes	*Escherichia coli*
Peptostreptococcus sp.	*Gardnerella vaginalis*
Prevotella sp.	*Mycoplasma hominis*
Streptococcus viridans	*Streptococus agalactiae*
Bacteroides sp.	*Vellonella sp.*
	Prevotella sp.
	Clostridium sp.
	Capnocytophaga sp.

Table 4

Prediction of the Clinical Class According to the Amniotic Fluid Metabolic Profile (First study; supervised).

		Predicted Class		
		PTL Term Delivery	**PTL without IAI**	**PTL with IAI**
	PTL Term delivery	15	1	0
	PTL without IAI	0	19	0
True Class	**PTL with IAI**	0	1	19

Data are presented as number.

PTL: preterm labor; IAI: intra-amniotic infection/inflammation

Table 5

Differentially regulated metabolites in patients with PTL who delivered at term (Second study).

Increase		Decrease	
Carbohydrates	**Others**	**Amino Acids**	**Others**
Galactose	Urea	Alanine	Possible heptanedioic acid
Hexose cluster 5	3-hydroxybutanoic acid	Glutamine	Possible alpha aminoadipic acid
Hexose cluster 3	Unknown 286	Pyroglutamic acid	Pentanedioic acid
Mannose	Palmitate	Isoleucine	Normetanephrine
Hexose cluster 2	Threo-isocitric acid	Glutamic acid	Unknown 8
Hexose cluster 6	Glycerol	Serine	Unknown 121
Fructose	Citric acid	Tyrosine	Unknown 221

Table 6

Differentially regulated metabolites in patients with PTL without IAI who delivered preterm (Second study).

Increase		Decrease		
Carbohydrates	**Others**	**Amino Acids**	**Carbohydrates**	**Others**
Hexose cluster 6	Urocanic acid	Alanine	Galactose	Urea
Dulcitol	Possible N-Acetyl glutamine	Pyroglutamic acid	Hexose cluster 5	3-hydroxybutanoic acid
	1-methyladenine	Proline	Hexose cluster 3	Palmitate
	Butanoic acid	Glycine	Mannose	Octadecanoic acid
	Beta hydroxyphenylethyamine	Glutamine	Inositol	Butanedioic acid
	Vitamin B6			
	Salicylamide			
	Oleic acid			
	Unknown 128			
	Unknown 276			
	Unknown 285			
	Unknown 283			
	Unknown 123			
	Unknown 344			

Table 7

Differentially regulated metabolites in patients with PTL with IAI (Second study).

Increase		Decrease	
Amino Acids	**Others**	**Carbohydrates**	**Others**
Alanine	Palmitate	Galactose	Glycerol
Pyroglutamic acid	Urea	Hexose cluster 3	Gluconic acid
Glutamine	Inositol	Hexose cluster 5	Threo-isocitric acid
Leucine	Octadecanoic acid	Mannose	Unknown 221
Proline	Possible heptanedioic acid	Hexose cluster 6	Unknown 286
Isoleucine	Possible alpha aminoadipic acid	Hexose cluster 2	
Valine	Possible butanoic acid 3oxy	Hexose cluster1	
Glutamic acid	Butanedioic acid	Fructose	
Glycine	Unknow 270		
Tyrosine	Unknown 8		
	Unknown 121		

Table 8

Prediction of the Clinical Class According to the Amniotic Fluid Metabolic Profile (Second study).

		Predicted Class		
		PTL Term Delivery	**PTL without IAI**	**PTL with IAI**
True Class	**PTL Term delivery**	39	0	1
	PTL without IAI	2	29	2
	PTL with IAI	7	1	32

Data are presented as number.

PTL – preterm labor; IAI – intra-amniotic infection/inflammation

Table 9

Metabolites Important in Classification Determined by Random Forest (Gini Index) ([*] - Amino acids; [#] - carbohydrates)

	Metabolite	Gini Index
1	Methyladenine	3.066
2	Heptanedioic acid	3.005
3	N Acetylglutamine [*]	2.939
4	Beta hydroxyphenylethylamine [*]	2.698
5	Unknown 128	2.389
6	Hexose cluster 5[#]	1.878
7	Hexose cluster 1[#]	1.874
8	Leucine [*]	1.836
9	glycerol	1.729
10	Isoleucine [*]	1.687
11	Inositol[#]	1.541
12	Methionine [*]	1.506
13	Glycine [*]	1.501
14	Galactose[#]	1.464
15	Hexose cluster 3[#]	1.463
16	Catechol	1.442
17	Salicylamide	1.431
18	Succinic acid	1.410
19	Unknown 5	1.263
20	Mannose[#]	1.220
21	Unknown 8	1.217
22	Glutamic acid [*]	1.130
23	Phenylalanine [*]	1.104
24	Alpha sorbopyranose[#]	1.077
25	Cholesterol	1.045
26	Unknown 289	1.039
27	Fructose[#]	1.031
28	Hexose cluster 2[#]	0.995
29	Tryptophan [*]	0.883
30	Eicosanoic acid	0.816

[*] Amino acids;

[†] Carbohydrates

Table 10

Relative abundance of carbohydrate and amino acids in the study groups.

	Carbohydrates	Amino Acids
PTL Term delivery	↑	↓
PTL without IAI	↓	↓
PTL with IAI	↓↓	↑