Metabolomic Profile of Amniotic Fluid and Wheezing in the First Year of Life—A Healthy Birth Cohort Study

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Objectives To apply metabolomic analysis of amniotic fluid in a discovery cohort to see whether a specific biochemical-metabolic profile at birth is associated with the subsequent onset of wheezing over the first year of life.

Study design This prospective exploratory study was conducted in a healthy term-born Dutch cohort recruited at 2 hospitals in Utrecht (UMCU, Utrecht, and Diakonessenhuis, Utrecht), The Netherlands. A metabolomic approach based on mass spectrometry was applied to analyze 142 amniotic fluid samples collected at birth. The infants were followed up during their first year of life with recording any respiratory symptoms daily, and they were classified according to the onset of wheezing.

Results Orthogonally constrained projection to latent structures discriminant analysis was used to investigate differences in the metabolic profiles of the infants with (n = 86) and without (n = 56) wheezing. A search of the available databases for amniotic fluid metabolites identified by stability selection, combined with pathway analysis, highlighted the possible metabolic perturbations involved in this condition. The model built using 16 relevant variables with plausible biological significance, showed an area under the curve of 0.82 ($P < .001$) and an area under the curve calculated by 7-fold full cross-validation of 0.72 ($P = .003$), with the steroid hormone biosynthesis and the 2-phenylalanine metabolism emerging as probably perturbed pathways.

Conclusions Infants who will or will not experience wheezing in their first year of life have distinct amniotic fluid metabolomic profiles at birth. Changes occurring in biochemical-metabolic pathways in late intrauterine life may have a pathogenic role in early-onset wheezing. *(J Pediatr 2018;■■:■■-■■).*

Previous longitudinal studies have demonstrated that an individual's lung function trajectory is established very early in life.[1] A number of factors, primarily early viral respiratory infections, can have profound effects on subsequent lung function and the development of chronic or recurrent respiratory symptoms.[2] There is also evidence of prior lung function impairment predisposing to recurrent respiratory symptoms in postnatal life.[3-5] A growing body of data supports the effect of multiple factors existing before and after conception, throughout childhood and into adulthood, on lifelong lung health.[6] Studying the influence of the intrauterine environment and perinatal exposure is fundamental to our understanding of the lung's development and health throughout life, as recently established at a workshop sponsored by the National Heart, Lung, and Blood Institute.[6]

The fetal lung is first exposed to the amniotic fluid and its constant contact with the amniotic fluid influences its growth and development, with potential effects on postnatal respiratory health.[6,7] Being rich in low-molecular-weight metabolites, the amniotic fluid is an appropriate biological matrix for the application of metabolomics, and several metabolites in the amniotic fluid have already been identified using this approach.[8,9]

Metabolomics involves the use of spectroscopic techniques to analyze the low-molecular-weight metabolites in biological samples. Being guided by no a priori hypothesis, untargeted metabolomics can lead to the identification of metabolic patterns characteristic of a given pathologic condition.[10] Our aim in this proof-of-concept study was to apply metabolomics to see whether specific biochemical-metabolic characteristics of the amniotic fluid at birth are associated with the subsequent onset of wheezing symptoms in the first year of life in a healthy birth cohort study.

AUC_{pred}	Area under the ROC curve of the outcome predicted for the samples
ESI	Electrospray ionization
LC-MS	Liquid chromatography mass spectrometry
oCPLS2-DA	Orthogonally constrained PLA-DA
PCA	Principal component analysis
PLS-DA	Projection to latent structures discriminant analysis
ROC	Receiver operating characteristic
RT	Retention time
UPLC	Ultraperformance liquid chromatography

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Methods

This prospective study was conducted at 2 hospitals (1 secondary and 1 tertiary) in Utrecht (UMCU, Utrecht, and Diakonessenhuis, Utrecht), The Netherlands, between January 2006 and November 2008, as described elsewhere.[7] Women delivering at term vaginally or by elective cesarean section were recruited if their pregnancy had been uncomplicated (any pregnancy-related medical conditions were mild and self-limiting). Given the purposes of the metabolomic analysis, only women delivering between 38 and 42 weeks of gestation were considered to limit any variability related to gestational age in the metabolomic arrangement of amniotic fluid. The following conditions potentially associated with amniotic fluid modifications were also excluded: prolonged rupture of membranes (>24 hours), intrapartum fever, and, in the newborns, early-onset sepsis or major congenital organ abnormalities (such as spina bifida or congenital heart disease).

Parents were informed about the study shortly before or after delivery, and amniotic fluid samples were only analyzed with their consent. The study was approved by the ethical review boards of both institutions and all parents gave their written informed consent to their involvement (study approval 05-112/K).

The clinical characteristics of the mother (maternal age at delivery, maternal conditions and pregnancy-related therapies, and smoking during the pregnancy) and her child (sex, gestational age at delivery, birth weight, Apgar scores at 1 and 5 minutes, and mode of delivery) were recorded. Gestational age was ascertained on the grounds of clinical history and the results of the earliest ultrasound examination. All these clinical data were included in the analysis as metadata.

Amniotic Fluid Sample Collection

For vaginal deliveries, the amniotic fluid sample was collected vaginally under nonsterile conditions at the time of the induced or spontaneous rupture of the membranes as explained elsewhere.[7,11] If spontaneous rupture of the membranes occurred outside the hospital, amniotic fluid was usually collected immediately after birth, because that is when a greater volume of amniotic fluid passes through the vagina. For elective cesarean section deliveries, the amniotic fluid was collected with a syringe directly after incision of the membranes. All amniotic fluid samples were stored at −80°C until analysis.

Follow-Up

Parents were asked to record their children's respiratory symptoms (including wheezing) in a daily log, previously developed and adopted in other published studies.[12,13] Based on these data, the infants were divided into 2 groups at the end of their first year of life, namely, children who had experienced at least 1 episode of wheezing in their first year (wheezing group), and children with no history of wheezing in their first year (control group). Data were also collected on any risk factors potentially related to this outcome, including the presence of siblings, daycare attendance, the presence of pets in the house, exposure to smoke during pregnancy and after birth, parental atopy, and maternal asthma.

Amniotic Fluid Metabolite Analysis by Mass Spectrometry Combined with Ultraperformance Liquid Chromatography

The amniotic fluid samples were analyzed at the mass spectrometry laboratory of the Women's and Children's Health Department of Padova University Hospital, at the Città della Speranza Institute for Pediatric Research in Italy.

As previously described,[14] the metabolic analysis on the amniotic fluid samples was performed with a Q-ToF Synapt G2 (Waters Corp, Milford, Massachusetts) high-resolution mass spectrometer interfaced with an ultraperformance liquid chromatography (UPLC) system (Acquity; Waters Corp). ULTRA grade liquid chromatography mass spectrometry (LC-MS) solvents and reagents (Sigma, St. Louis, Missouri) and MilliQ water of grade 1 purity were used for the analyses.

The MS analysis was conducted with an electrospray ionization (ESI) source in both positive (ESI+) and negative (ESI−) ionization modes.

The amniotic fluid samples were analyzed using both LC-MS and LC-MS^E for the purposes of our structural hypothesis. The latter is a nonselective fragmentation technique based on the passage of ionized molecules through the collision cell at high and low collision energy during chromatography. For further information please see the **Appendix** (available at www.jpeds.com).

Statistical Analyses and Identification of Relevant Variables

The UPLC-MS data were processed to obtain data tables using the MarkerLynx software (Waters Corp, Milford, Massachusetts). Data were normalized by median fold change normalization, log-transformed, and mean centered before performing the data analysis. An exploratory data analysis was run by means of principal component analysis (PCA), whereas discriminant analysis was performed by projection to latent structures discriminant analysis (PLS-DA) and orthogonally constrained PLA-DA (oCPLS2-DA).[15]

To pinpoint the subset of variables capable of explaining the differences between the wheezing and the control groups, a stability selection procedure was applied, based on Monte-Carlo sampling and VIP-based oCPLS2-DA (ie, oCPLS2-DA with variable selection based on the parameter called variable importance in projection)[16] (**Appendix**). A univariate data analysis based on the *t* test and receiver operating characteristic (ROC) curve analysis was also run and the results were combined with those obtained from the multivariate data analysis to highlight the subset of relevant variables to be identified.

PCA and PLS-DA were performed with SIMCA 14 (MKS Data Analytics Solutions, Malmö, Sweden), and oCPLS2-DA and its post-transformation, the stability-based selection and the univariate data analysis were implemented with the R 3.1.2 platform (R Foundation for Statistical Computing, Vienna, Austria).

To identify the relevant variables characteristic of each group, a search was run in the main metabolomic databases available

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