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Neonatal metabolome of cesarean section and risk of childhood asthma

**Authors:** Gözde Gürdeniz¹, Madeleine Ernst², Daniela Rago¹, Min Kim¹, Julie Courraud², Jakob Stokholm¹, Klaus Bønnelykke¹, Anders Björkbom², Urvish Trivedi³, Søren J Sørensen³, Susanne Brix⁴, David Hougaard², Morten Rasmussen¹, Arieh S Cohen², Hans Bisgaard¹*, Bo Chawes¹

¹COPSAC, Copenhagen Prospective Studies on Asthma in Childhood, Herlev and Gentofte Hospital, University of Copenhagen, 2820 Copenhagen, Denmark

²Section for Clinical Mass Spectrometry, Department of Congenital Disorders, Danish Center for Neonatal Screening, Statens Serum Institut, 2300 Copenhagen, Denmark

³Department of Biology, University of Copenhagen, 2200 Copenhagen, Denmark

⁴Department of Biotechnology and Biomedicine, Technical University of Denmark, 2800 Lyngby, Denmark

⁵Section of Chemometrics and Analytical Technologies, Department of Food Science, University of Copenhagen, 1958 Frederiksberg C, Denmark

* Correspondence:

Professor Hans Bisgaard, MD, MSc

E-mail: bisgaard@copsac.com

Website: [www.copsac.com](http://www.copsac.com)

**Keywords:** Cesarean section; LC-MS/MS metabolomics; newborn dried blood spot; asthma; gut microbiome; cord blood Treg
ABSTRACT

Background: Birth by cesarean section (CS) is linked to an increased risk of developing asthma, but the underlying mechanisms are unclear.

Objective: To elucidate the link between birth by CS and asthma using newborn metabolomic profiles and integrating early life gut microbiome data and cord blood immunology.

Methods: We investigated the influence of CS on liquid chromatography mass spectrometry (LC-MS) metabolomic profiles of dried blood spots from newborns of the two independent Copenhagen Prospective Studies on Asthma in Childhood cohorts, i.e., COPSAC2010 (n=677) and COPSAC2000 (n=387). We assessed the associations between the CS metabolic profile, age one-week gut microbiome data and frequency of cord blood Tregs.

Results: In COPSAC2010, a partial least square-discriminant analysis (PLS-DA) model showed that children born by CS vs natural delivery had different metabolic profiles (AUC=0.77, p=2.2e-16), which was replicated in COPSAC2000 (AUC=0.66, p=1.2e-5). The metabolic profile of CS was significantly associated with an increased risk of asthma at school-age in both COPSAC2010 (p=0.03) and COPSAC2000 (p=0.005). CS was associated with lower abundance of tryptophan, bile acid and phenylalanine metabolites, indicative of a perturbed gut microbiota. Further, gut bacteria dominating after natural delivery, i.e., *Bifidobacterium* and *Bacteroides* were correlated with CS-discriminative microbial metabolites, suggesting maternal microbial transmission during birth regulating the newborn’s metabolism. Finally, the CS metabolic profile was associated with frequency of cord blood Tregs.
Conclusions: These findings propose that CS is programming the risk of childhood asthma through perturbed immune responses and gut microbial colonization patterns reflected in the blood metabolome at birth.

INTRODUCTION

Cesarean section (CS) is a risk factor for several chronic immune-mediated diseases such as asthma (1, 2). CS bypasses the normal transmission of microbes to the fetus from the birth canal and maternal gut microbiome, leading to a perturbed bacterial microflora in the gut of infants born by CS (3), which has been linked to the risk of developing asthma (4). Furthermore, CS born infants lack alterations in stress hormones, which may play a role in adaptation to the extra-uterine environment (5). Previous studies linking early life gut microbiota dysbiosis with asthma have investigated these associations (6, 7), while underlying causal biochemical mechanisms are still to be elucidated.

Metabolites are tightly regulated end-products and intermediates of cellular metabolism that reflect the genetic background, environmental factors, and host-microbiome interactions. Therefore, neonatal metabolic profiling may be a powerful tool to investigate alterations in metabolic pathways related to perinatal events such as CS to elucidate the mechanisms leading to increased risk of disease later in life. In Denmark, blood is routinely collected as dried blood spot (DBS) samples from every infant a few days after birth to screen for inborn errors of metabolism. These DBS samples are stored at the Danish National Biobank, which offers a unique resource for metabolic profiling.

The aim of this study was to investigate mechanisms whereby CS increases the risk of childhood asthma. We compared newborn DBS metabolic signatures after natural birth vs. CS and associated these with asthma development later in childhood in the Copenhagen Prospective Studies on Asthma in Childhood 2010 (COPSAC2010) birth cohort and sought
replication in the independent COPSAC2000 cohort. Furthermore, we investigated the association between CS metabolites and gut microbial profiles reflecting mode of delivery (4) and evaluated the possible impact on the developing immune system by integrating cord blood T cell distribution data (8).

MATERIALS AND METHODS

Study participants

The COPSAC2010 is a population-based mother-child cohort including 738 pregnant women and their 700 children (9), whereas COPSAC2000 is a mother-child cohort of 411 children born to mothers with a history of asthma (10). COPSAC2010 included preterm and late-term infants (30-42 weeks), whereas COPSAC2000 only included term infants (36-42 weeks). All information of the participants was obtained during scheduled visits at the COPSAC research clinic. Data on delivery mode data was further validated against the National Registries.

The COPSAC studies are conducted according to the principles of the Declaration of Helsinki and are approved by the local Ethics Committee (KF 01-289/96, H-B-2008-093) and the Danish Data Protection Agency (2015-41-3696). Both parents gave oral and written informed consent before enrolment.

Asthma diagnosis

The diagnosis of asthma was exclusively performed by the COPSAC pediatricians based on a quantitative symptom-driven, validated diagnostic algorithm (11, 12), requiring verified diary recordings of at least 5 episodes of troublesome lung symptoms within 6 months, each lasting at least 3 consecutive days; symptoms typical of asthma, including exercise-induced symptoms, prolonged nocturnal cough, and/or persistent cough outside of common colds; rescue use of inhaled β2-agonist; and response to a 3-month course of inhaled corticosteroids and relapse
upon ending treatment. Children fulfilling all the above criteria at age 0-3 years were defined as having persistent wheeze, whereas asthma was diagnosed in children fulfilling the criteria and still requiring inhaled corticosteroids by age 6 years.

*Newborn DBS samples*

DBS samples from COPSAC2010 and COPSAC2000 were collected at age 2-3 days and 1-12 days after birth, respectively, and were stored at -20°C at the Danish National Biobank until analysis.

*Metabolic profiling, data preprocessing and metabolite annotation*

Metabolic profiles of the DBS samples from COPSAC2010 and COPSAC2000 were acquired by LC-MS (Text S1). The data preprocessing was performed using XCMS and MZmine and data quality was evaluated based on pooled sample distribution (Text S2, Figure S1). Preprocessed tandem mass spectrometry (MS2) profiles were submitted to Global Natural Products Social Molecular Networking Platform (GNPS) (13) and MolNetEnhancerWorkflow (14) for compound annotation (Text S3, Table S1-S2).

*Gut microbiome data*

Fecal samples (N=532) were collected from the infants one week after birth for 16S rRNA profiling as detailed elsewhere (8). Previously, we identified 15 bacterial genera reflecting CS delivery (4), which were included in the analysis.

*Cord blood regulatory T cells*

Cord blood was collected by needle puncture of the umbilical vein and regulatory T cells (Tregs) were analyzed using a lyse-no-wash procedure and detected using flow cytometry (8).
Statistics

Multivariate data analysis was performed in the PLS_Toolbox (version 6.5, Eigenvector Research) built in MATLAB R2018b and statistical analyses were conducted using R version 4.0.2. A partial least square-discriminant analysis (PLS-DA) was applied to differentiate the metabolic profiles of the COPSAC2010 DBS samples by delivery mode using double cross-validation (15) (see details in Text S4). Replication in COPSAC2000 was done by investigating the performance of the PLS-DA model with the selected metabolites from COPSAC2010 for predicting delivery mode in COPSAC2000 and was evaluated based on area under the curve (AUC).

The latent variables (LV) scores from the PLS-DA model reflect each infant’s metabolic profile of delivery mode, where higher LV scores are assigned to CS. Therefore, LV1 scores are referred to in the manuscript as CS scores, which are subsequently used for association with the risk of asthma by Cox regression survival analysis (survival R package) and illustrated with Kaplan-Meier curves.

The complex heatmap R package was used for illustration of the correlations (Spearman Rank) between metabolites and gut bacterial abundances and hierarchical cluster analysis was applied using the complete agglomeration method.

Causal mediation analysis (R package (16)) was performed to assess whether the effect of delivery mode on the metabolome was mediated through Tregs or microbiome CS scores (4).
RESULTS

Baseline characteristics

The demographics of children born by CS or natural birth are summarized in Table 1, showing that children born naturally had higher gestational age than CS-born children in both cohorts. Almost all mothers giving birth by CS received intrapartum antibiotics, which was the case for 13% of mothers giving natural birth. Intrapartum antibiotics did not influence the DBS metabolic profiles within the natural birth strata (PLS-DA, AUC=0.57, P=0.06).

Metabolome of delivery mode

The total number of metabolites passing quality control was 677 for COPSAC2010 and 387 for COPSAC2000, while the data preprocessing led to 2,041 and 2,355 features, respectively.

CS and naturally born infants from COPSAC2010 had differential metabolic profiles based on PLS-DA (AUC=0.77±0.06, p=2.2e-16) and 32 metabolites were associated with delivery mode. A LV1 vs LV2 scores plot showed a clear separation between CS and natural birth (Figure 1A) and the influence of each of the 32 selected metabolites for discriminating between delivery modes is illustrated in Figure 1B. Most of the annotated metabolites (Figure S2) suggested modulations in the tryptophan, bile acid and phenylalanine metabolism, which are mostly of microbial origin and were lower among infants born by CS. Others were annotated at family level using MolNetEnhancer workflow (Figure S3).

In COPSAC2000, 24 of the 32 selected metabolic features from COPSAC2010 were detected, which included tryptophan and phenylalanine metabolites while among bile acids only cholic acid (CA) was present (Figure S4). A PLS-DA model using the delivery mode differential metabolites from COPSAC2010 also discriminated between CS vs natural birth in COPSAC2000 (AUC=0.66, p=1.2e-5).
Further, the DBS metabolic profiles also differed between emergency vs elective CS vs natural birth based on PLS-DA models (AUC>0.65) with the best classification performance found in the elective CS vs natural birth model (AUC=0.83) (Figure S5).

Cesarean section metabolome and risk of asthma

The CS metabolic scores of COPSAC 2010 and the predicted CS metabolic scores for COPSAC2000 were both associated with an increased risk of asthma till 6 and 7 years of age: HR=1.08, [95% CI: 1.00-1.15], p=0.03 and HR=1.21, [1.06-1.38], p=0.005, respectively (Figure 2). The risk of developing asthma during childhood was higher for children having a more CS-like metabolic profile, i.e., higher CS scores in both cohorts.

Adjusting the analyses for sex and season of birth did not change the findings in either of the cohorts (p-values<0.05). However, the association between the CS metabolic score and asthma only remained significant in COPSAC2000 after adjusting for gestational age (HR=1.21, [1.06-1.38], p=0.005). Gestational age adjusted analysis of individual metabolites reflecting delivery mode revealed decreased effect size for certain metabolites (Figure S6), which suggests that the metabolic consequence of a low gestational age is partly similar to being born by CS.

Among the CS delivered infants, the risk of asthma was significantly higher for elective CS compared to emergency CS (HR=1.10, [1.07-1.42], p=0.003).

DBS metabolome and gut microbiome

Naturally born infants were characterized by several highly abundant microbial metabolites. Further, delivery mode has previously been associated with specific gut microbial colonization patterns in the COPSAC2010 infants at 1 week of age (4). Therefore, we explored the relationship between the selected DBS metabolites and gut bacterial abundances (Figure 3). Hierarchical cluster analysis revealed two major clusters, i.e., CS and natural birth
characterizing the metabolites and microbial taxa. The strongest associations were between microbial metabolites, i.e., bile acids and indolelactic acid and abundance of *Bifidobacterium* and *Bacteroides* within the natural birth cluster. Gut microbial CS scores mediated 29% of the influence of delivery mode on the DBS metabolome (B=0.96 [0.73-1.22], p<2e-16). This suggest that particularly for infants born naturally the dominating gut microbial population regulates the metabolic composition present in the blood.

*Cord blood Tregs*

The relationship between cord blood T cell subsets and CS metabolic scores (n=66) and merged CS metabolic-gut microbial profiles (n=56) were investigated in COPSAC2010. The frequency of Tregs showed association with the CS metabolic score (R=0.24, p=0.03), which was stronger for the merged CS metabolic-gut microbial profiles (R=0.37, p=0.003) (*Figure 4*). Furthermore, frequency of Tregs modestly mediated the association between delivery mode and the metabolome (ACME = 0.23, [0.002-0.29], p=0.05).

**DISCUSSION**

**Primary findings**

This study demonstrates a prominent influence of delivery mode on newborn blood metabolites derived from the tryptophan, bile acid and phenylalanine metabolism in COPSAC2010, which was replicated in the independent COPSAC2000 cohort. The CS metabolic profile was associated with an increased risk of asthma development in both cohorts, suggesting that CS leads to early life metabolic perturbations mediating the link between delivery mode and asthma. The CS metabolic profile was also associated with gut microbial dysbiosis and number of cord blood Tregs, which adds important mechanistic insight into the effects of CS on asthma development (*Figure 5*).
Interpretation of the findings

We showed that the metabolic profile in newborns reflecting delivery by CS was associated with an increased risk of childhood asthma in two independent cohorts. The majority of the annotated metabolites, i.e., tryptophan metabolites and bile acids have previously been associated with asthma endpoints (17). Nonetheless, our study is the first to suggest that perturbations of tryptophan and bile acid metabolism are linked to delivery mode, which may explain the underlying biochemical mechanism whereby CS increases the risk of developing childhood asthma.

We found that indolelactic acid, which is an intermediate of the tryptophan metabolism was lower in CS delivered newborns. The correlation between indolelactic acid and *Bifidobacterium* aligns with earlier findings that indolelactic acid is mainly produced from *Bifidobacterium* during early life (18) and that natural birth is associated with higher *Bifidobacterium* abundance (3, 19). Indolelactic acid act as ligands for the aryl hydrocarbon receptor (AhR) found in intestinal immune cells, which regulates innate and adaptive immune responses crucial for intestinal homeostasis (20). In a mouse study, microbially derived AhR ligands were maternally transmitted and shown to shape the early immune system of the offspring (21). Particularly during infancy, indolelactic acid produced by gut *Bifidobacterium* spp. has been demonstrated as a key determinant of AhR dependent signaling (22). Therefore, we speculate that *Bifidobacterium* is transferred from mother to infant during natural birth, subsequently colonizes the infant’s gut and leads to higher abundance of blood indolelactic acid, which contributes to development of intestinal barrier functions and the immune system in early life.

Perturbated kynurenine metabolism was also found to associate with CS in our study. The kynurenine pathway modulates pro-inflammatory and anti-inflammatory responses in the gastrointestinal tract via catabolism of indoleamine 2,3-dioxygenase (IDO1) on T cells (23, 24).
Notably, the gut microbiota plays a central role in regulating IDO1 activity, which has been demonstrated in germ-free and antibiotic-treated mice (24). In the absence of gut microbiota (i.e., germ-free mice), an increase in plasma tryptophan reduced the kynurenine-to-tryptophan ratio, which could explain our finding of lower abundance of kynurenine in CS-born infants due to a lack of microbial exposure during natural birth. Both *Bifidobacterium infantis* and *Lactobacillus johnsonii* have been shown to modulate the kynurenine pathway (23, 25), and kynurenine can also interact with AhR (26). Therefore, microbiota seeded from mother to infant during natural delivery may directly or indirectly modulate tryptophan metabolism and tryptophan metabolites possibly via AhR activation educating the immune system in early life, which may impact the risk of developing asthma.

The findings of a perturbed tryptophan metabolism in CS born infants and the influence of the early life gut microbiota may have importance for future primary prevention of asthma. A recent study demonstrated that microbial perturbations caused by CS can be restored by maternal fecal microbiota transplantation (27), which may have partly acted through the tryptophan metabolism. Thus, future studies are needed to investigate whether the lack of certain tryptophan metabolites can be provided to infants born by CS through e.g., a fortified formula or supplementation of breastfeeding mothers after birth.

Children born by CS also showed lower levels of primary (i.e., CA) and secondary (i.e., UDCA) bile acids. Further, we found a correlation between bile acids and *Bifidobacterium*, which may suggest triggered formation through the microbiota. Indeed, it has been shown that *Bifidobacterium* mediates deconjugation of bile salts (28), which could explain the correlation with deconjugated bile acids in our study. Bile acids circulate between the liver and ileum, facilitating lipid absorption, and thereby play an important role in host-microbiota and gut-liver crosstalk. Therefore, lower abundance of bile acids in infants delivered by CS may contribute to perturbations in metabolic and inflammatory pathways (29) during early life that could be of
importance in the inception of asthma. In support of this, we previously demonstrated that perturbations in the tryptophan and bile acid metabolism in urine samples from 4-week-old infants was associated with an increased risk of developing asthma (17).

Bile acids and tryptophan metabolites act as signaling molecules to regulate early life immune homeostasis (24, 30). A higher number of Tregs have been detected in infants born by CS and Tregs play an important role in preventing excessive immune response to the environmental changes faced by the newborn. We observed a correlation between the number of cord blood Tregs and the CS metabolic score in the newborn child, which was even stronger when we integrated gut microbial profiles. This suggests an interplay between the developing immune system, the gut microbiome and the blood metabolome, which adheres with previous studies on the role of the microbial community on immune development (31, 32). However, since the influence of delivery mode on the cord blood immune profile is prior to gut microbial colonization our study suggests that Tregs have a role in regulation of microbial colonization patterns and further studies are needed to evaluate the observed associations.

**Strengths and limitations of the study**

Here, we demonstrated that newborn blood routinely collected and stored as DBS during the Danish neonatal screening program is a valuable resource for clinical metabolomics applications. Despite the potential, only few studies have utilized newborn blood to investigate the association between the early life metabolic profiles and disease development (33).

CS born infants are known to have delayed initiation of breastfeeding (34), which potentially could impact the abundance of *Bifidobacterium* and tryptophan metabolites (35). However, adjusting our analyses for breastfeeding did not significantly affect the association between delivery mode and *Bifidobacterium* during early life (4, 19). Further, the DBS were collected only
1-3 days after the birth for COPSAC2010; therefore, we expect that the effect of feeding type did not influence our findings.

We also showed that infants with lower gestational age had higher CS metabolic scores and lower gestational age has been linked to an increased risk of asthma (36). However, the CS metabolic score of the COPSAC2000 infants all born with a gestational age >37 weeks was strongly associated with asthma. Therefore, our findings suggest a greater impact of preterm birth than lower gestational age within the normal range on risk of asthma. Subsequently, preterm infants born by CS may have the highest risk of developing asthma.

Similar to CS delivery, intrapartum antibiotics has been associated with risk of persistent wheeze (37). As prescription of intrapartum antibiotics and CS delivery are highly correlated it is difficult to disentangle one from the other. Previously, we have shown that natural born children, whose mothers were treated with intrapartum antibiotics had gut microbial profiles in between those of CS born children and natural born children, whose mothers were not treated with intrapartum antibiotics (4). Importantly, in the present study we did not observe an effect of intrapartum antibiotics on the newborn’s blood metabolome.

Finally, in this study only a limited number of compounds characterizing delivery mode were annotated. The unannotated compounds may highlight additional underlying adverse effects of CS leading to childhood asthma (38, 39).
Conclusion

Newborns with a CS metabolic profile had an increased risk of developing asthma in two independent cohorts, suggesting that the inferred risk of asthma from CS is mediated through early life metabolic perturbations. The underlying metabolites were particularly derivates from the tryptophan metabolism and bile acids, indicative of interactions between the early life gut microbiome and host immune responses, which adds important knowledge on the effect of CS on risk of asthma and may contribute to development of novel primary prevention strategies.

Author Contributions: GG, BC, HB and DR developed the concept and designed the overall study approach. ASC, AB and DH organized and conducted mass spectrometric analysis. ME and JC interpreted mass spectra. SJS and UT performed the gut microbiome analysis. SB provided cord blood immune data. DR, MK, ME, ASC, MR, JS, KB, HB and GG interpreted the data. GG, BC, HB and ME wrote the manuscript.

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Conflict of interest: All authors declare no potential, perceived, or real conflict of interest regarding the content of this manuscript. The funding agencies did not have any role in design and conduct of the study; collection, management, and interpretation of the data; or preparation, review, or approval of the manuscript. No pharmaceutical company was involved in the study.
**Governance:** We are aware of and comply with recognized codes of good research practice, including the Danish Code of Conduct for Research Integrity. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical Practice (GCP) as defined in the EU's Directive on Good Clinical Practice, the International Conference on Harmonisation's (ICH) good clinical practice guidelines and the Helsinki Declaration. Privacy is important to us which is why we follow national and international legislation on General Data Protection Regulation (GDPR), the Danish Act on Processing of Personal Data and the practice of the Danish Data Inspectorate.


Figure 1. Two component PLS-DA model discriminating between cesarean section (CS) and natural birth (NB) using the measurements of 32 selected blood metabolites from COPSAC2010. a) LV1 and LV2 scores plot. CS group is colored by type of CS i.e. emergency (EM-CS) and elective CS (EL-CS). b) The influence of the 32 selected metabolites in terms of discriminating between CS vs. NB presented by regression vectors for NB. The higher the regression coefficient the larger the influence of the feature to discriminate delivery mode. CA: cholic acid, UDCA: ursodeoxycholic acid, HCA: hyocholic acid, KDCA: ketodeoxycholic acid.
Figure 2. Kaplan-Meier curve of the cesarean section (CS) scores divided into tertiles and risk of developing asthma by age of 6 and 7 years, for a) children of COPSAC2010, where the CS score of each child is calculated based on the PLS-DA model with 32 metabolites reflecting delivery mode b) children of COPSAC2000, where the CS score of each child is predicted based on PLS-DA model of COPSAC2010. The Cox regression survival analysis was performed on continuous CS scores, to calculate the statistics.
Figure 3. Heatmap describing the relationship between the selected 32 blood metabolites and 15 gut bacteria at 1 week of age based on Spearman rank correlation. Metabolites are presented with either name or m/z and retention time (min), and structural information obtained from MolNetEnhancer workflow. For metabolites with grey font only MS1 was acquired. The order of microbial and metabolic profile is defined by hierarchical cluster analysis using the complete linkage agglomeration method and the Euclidean distance measure. The two major
clusters were characterized by metabolites and bacteria associated either with natural birth (NB) or cesarean section (CS). The metabolites and bacteria associated with each other within each cluster.
Figure 4. Spearman correlations between CS scores and frequency of cord blood regulatory T cells (Treg). CS scores (LV1) were calculated based on a) the selected 32 metabolites from COPSAC2010, and b) the merged 32 metabolites and 15 bacterial taxa from COPSAC2010.
Figure 5. Proposed mechanism of action of delivery mode on infants metabolic and microbial profiles associated with childhood asthma. Natural birth led to higher levels of tryptophan catabolites i.e. indolelactic acid and kynurenine, which are bioactive compounds acting on AhR and leading to improved immune regulation in the early life. Indolelactic acid production is likely controlled by Bifidobacterium in the gut which was highly abundant after natural birth. Under the influence of microbiota, kynurenine is produced by the IDO and TDO pathway. Natural birth promoted higher levels of bile acid some of which are possibly controlled by Bifidobacterium (e.g. deconjugation of primary bile acids produced in the liver). Bile acids are also regulated immune hemostasis. Cord blood Treg is associated with metabolic and microbial CS profiles. Overall, newborn metabolome reflects the mode of delivery and metabolic changes are partially mediated by the gut microbial community and cord blood Treg and metabolic fingerprint of cesarean section (CS) is associated with increased risk of childhood asthma.
Table 1. Demographic characteristics of children in the COPSAC2010 and COPSAC2000 cohorts with DBS metabolic profiles grouped by delivery method.

<table>
<thead>
<tr>
<th></th>
<th>COPSAC 2010 (n = 677)</th>
<th></th>
<th>COPSAC 2000 (n = 387)</th>
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</tr>
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<tbody>
<tr>
<td></td>
<td>Natural birth</td>
<td>EM-CS</td>
<td>EL-CS</td>
<td>P value</td>
</tr>
<tr>
<td>Total number n (%)</td>
<td>533(79)</td>
<td>79(12)</td>
<td>64(9)</td>
<td></td>
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<tr>
<td>Child Sex Male n (%)</td>
<td>268(50)</td>
<td>31(39)</td>
<td>33(51)</td>
<td>0.34</td>
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<tr>
<td>Birth Gestational age, weeks, mean±SD</td>
<td>40.1±1.5</td>
<td>39.4±2.6</td>
<td>39.1±0.8</td>
<td>&lt;0.01</td>
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<tr>
<td>Weight mean±SD</td>
<td>3.6±0.5</td>
<td>3.5±0.8</td>
<td>3.5±0.5</td>
<td>0.46</td>
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<tr>
<td>Intrapartum antibiotics* n (%)</td>
<td>70(13)</td>
<td>76(96)</td>
<td>64(100)</td>
<td>&lt;0.01</td>
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<tr>
<td>Season at birth n (%)</td>
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<td></td>
<td>0.31</td>
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<tr>
<td>Autumn</td>
<td>109(20)</td>
<td>24(30)</td>
<td>14(22)</td>
<td></td>
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<tr>
<td>Spring</td>
<td>143(27)</td>
<td>17(22)</td>
<td>13(20)</td>
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<td>Summer</td>
<td>106(20)</td>
<td>20(25)</td>
<td>19(30)</td>
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<td>Winter</td>
<td>175(33)</td>
<td>18(23)</td>
<td>18(28)</td>
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<tr>
<td>Prenatal exposures</td>
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<tr>
<td>Smoking n (%)</td>
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<td>4(6)</td>
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<td>Fish oil supplementation</td>
<td>263(49)</td>
<td>45(57)</td>
<td>30(47)</td>
<td>0.53</td>
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<tr>
<td>Maternal asthma n (%)</td>
<td>149(28)</td>
<td>30(38)</td>
<td>26(41)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*A Pearson χ² test, CS vs. natural birth.  
B Unpaired Student t test, CS vs. natural birth.  
C Antibiotics is given either to the mother or proband.  
D Fish oil intervention was only conducted in COPSAC2010.  
E Maternal asthma was an inclusion criterion in COPSAC2000.  
EM-CS: Emergency C-section, EL-CS: Elective C-section.
Online Data Supplement

Neonatal metabolome of Cesarean Section and Risk of Childhood Asthma

Authors: Gözde Gürdeniz¹, Madeleine Ernst², Daniela Rago¹, Julie Courraud², Jakob Stokholm¹, Klaus Bønnelykke¹, Anders Björkbom², Urvish Trivedi³, Søren J Sørensen³, Susanne Brix⁴, David Hougaard⁵, Morten Rasmussen¹⁺⁵, Arieh S Cohen², Hans Bisgaard¹, Bo Chawes¹
Text S1. Description of sample preparation and LC-MS/MS metabolic profiling

All extraction steps were performed on a Microlab STAR automated liquid handler (Hamilton Bonaduz AG, Bonaduz, Switzerland) using LC-MS grade solvents (Thermo Fisher Scientific, Waltham, MA, USA). Metabolites from DBS samples (3.2-mm-diameter punch) were extracted onto 96-well plates (analytical batches) in 100 µL 80% methanol. The supernatants (75 µL) were transferred onto new plates, dried under nitrogen, reconstituted in 75 µL 2.5 % methanol and transferred (65 µL) onto the final plates before injection. The samples from COPSAC2000 and COPSAC2010 were randomly distributed into six and ten batches, respectively and were analyzed subsequently. Each plate included eight water blanks, one internal standard mixture, four external controls, three paper blanks, four pooled samples (equal aliquots of all samples within a plate), two diluted pools and 74 cohort samples. A mixture of 24 isotope-labelled internal standards (amino acids and acylcarnitines) was added to the extraction solvents. The concentrations of the internal standards were the same in each sample and they were used to monitor the quality of each injected sample and to assess the performance of subsequent batch correction and normalization. Quality control pooled samples (PSs) were also analysed at regular intervals throughout the run to examine the variance observed in the sample preparation, data acquisition and preprocessing steps.

Metabolic profiles were acquired by a high resolution Thermo Scientific Q-Exactive Orbitrap mass spectrometer coupled to a Dionex Ultimate 3000 UPLC equipped with a quaternary pump, column oven and auto sampler (Thermo Fisher Scientific). Twenty µL of each sample were injected into a Acquity UPLC BEH reverse-phase C18 column (130 Å, 2.1 mm x 50 mm, 1.7 µm) preceded by a Acquity UPLC BEH C18 VanGuard pre-column (130 Å, 2.1 mm x 5 mm, 1.7 µm) (Waters Corporation, Waltham, MA, USA). The gradient operated 0-100 % solvent B over 9 min at 0.25 mL/min. The mobile phase was 1.25% MeOH, 1.25% ACN and 0.2% formic acid in water (solvent A) and 48.75% MeOH, 48.75% ACN and 0.2% formic acid in water (solvent B). Data dependent tandem mass spectrometric analysis was performed in positive ionization mode, with spray voltages +3.8 kV, capillary temperature 350°C, sheath gas flow rate 32 psi, auxiliary gas flow rate 8, and S-lens RF level 50%.

For full MS (MS1), the parameters were set as follows: microscans: 1, resolution: 35,000, AGC target: 1E6, maximum IT: 100 ms, number of scan ranges: 1, scan range from 70 to 1050 m/z and spectrum data type: profile. For dd-MS2 (MS/MS), the parameters were set as follows: microscans: 1, resolution: 17’500, AGC target: 1E5, maximum IT: 50 ms, loop count: 5, MSX
count: 1, TopN: 5, isolation window: 1.5 m/z, NCE: 35.0, stepped NCE: 50%, spectrum data type: profile. For dd, the parameters were set as follows: underfill ratio: 5%, intensity threshold: 1E5, apex trigger: 2 to 7 s, charge exclusion: 3-8 and above 8, peptide match: off, exclude isotopes: on and dynamic exclusion: 30s. Diisooctyl phthalate (m/z 391.2843) was used as lock mass. The stepped normalized collision energy was set as 17.5-35.0-52.5 units.

Text S2. LC-MS/MS data preprocessing

ThermoFisher .raw metabolic profiles were converted to the intermediate .mzML format with MSconvert v3.0.19171 (1) (http://proteowizard.sourceforge.net). The data preprocessing of fullscan spectra was performed using XCMS including automated peak detection and alignment algorithms preprocessing COPSAC2010 and COPSAC2000 data independently. Preprocessing of fullscan spectra resulted in a feature table in which the relative abundance of each ion in the samples is represented by the peak area. Features are removed if their average abundance among samples are lower than half of the average abundance within the blanks.

MZmine version 2.40.1 (2) was used to preprocess MS2 and full-scan spectra, MS2 fragmentation spectra (i.e. .mgf format) and MS2 filtered feature tables were exported. For MZmine, data was cropped, with chromatogram retention time from 0.4 to 9.0 min and m/z range from 10 to 20000 retained. Then mass lists were created with MS1 intensity above 1E4 and MS2 intensity above 0 retained. The chromatogram was built through the ADAP chromatogram builder (3) by using the following parameters, minimum group size of scans: 3, group intensity threshold: 5E4, minimum highest intensity: 3E5, and m/z tolerance: 0.01 m/z or 15 ppm. The chromatogram was further deconvoluted using the MEDIAN m/z center calculation, m/z range for MS2 scan pairing 0.02 Da and retention time range for MS2 scan pairing 0.01 min. The local minimum search algorithm was used for deconvolution with parameters set as follows: chromatographic threshold: 98%, search minimum in retention time range: 0.02 min, minimum relative height: 2.5%, minimum absolute height: 3E5, minimum ratio of peak top/edge: 2, peak duration range: 0.01-0.30 min. The peaks were deisotoped by using the isotopic peak grouper function, with parameters set as follows: m/z tolerance: 0.03 m/z or 25 ppm, retention time tolerance: 0.03 min, maximum charge: 2, representative isotope: most intense. Duplicate peaks were removed using the duplicate peak filter with the OLD AVERAGE filter mode, m/z tolerance: 0.2 or 50 ppm, and retention time tolerance: 0.1 min. Peaks from all samples were aligned, by using the join aligner function with parameters set as follows: m/z tolerance: 0.01 m/z or 15 ppm, retention time tolerance: 0.25 min, weight for m/z: 75, weight for retention time: 25. Features were only retained if found in at least 8.5% of all samples (61 out of 716). Finally, two feature tables were exported in the .csv format.
One feature table containing all extracted mass spectral features and another feature table filtered for mass spectral features with associated fragmentation spectra (MS2). A list of aggregated MS2 fragmentation spectra was exported in the .mgf format and submitted to mass spectral molecular networking through GNPS.

Features included in the MS2 filtered feature table were merged with the XCMS preprocessed peak table. The features were assigned to the same group if they are eluting within the range of 0.02 min and have correlating (R^2>0.7) relative intensities. From each feature group only the feature with the highest relative intensity was kept and the reduced dataset was used for the subsequent data analysis. To remove inter-batch variation, each feature was divided with the overall mean of its recordings included in the respective batch.

The quality of metabolomic data acquisition was controlled by assessing the samples’ internal standards signal and total ion count. Samples with obvious deviation or technical issues were excluded. Further quality control of analytical acquisition and data preprocessing was performed after preprocessing using principal component analysis (PCA) and observing clustering of pooled samples. Pooled samples clustered together in PC1 vs PC2 scores plot suggesting good data quality (Figure S1).

Text S3. Mass spectral molecular networking

A mass spectral molecular network was created through the Global Natural Products Social Molecular Networking Platform (GNPS) (http://gnps.ucsd.edu) using the feature based molecular networking workflow (4). The spectra in the network were then searched against all GNPS spectral libraries. All matches kept between network spectra and library spectra were required to have a mass spectral similarity score (cosine score) of above 0.7 and at least four matched peaks.

The data was filtered by removing all MS/MS fragment ions within +/- 17 Da of the precursor m/z. MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the +/- 50-Da window throughout the spectrum. The precursor ion mass tolerance was set to 0.02 Da and a MS/MS fragment ion tolerance of 0.02 Da. A network was then created where edges were filtered to have a cosine score above 0.7 and more than four matched peaks. Further, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other’s respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched
against GNPS’ spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least four matched peaks.

Substructure information was incorporated into the network using the GNPS MS2LDA workflow (https://ccms-ucsd.github.io/GNPSDocumentation/ms2lda/)(5–8). Information from in silico structure annotations from Network Annotation Propagation(8) and Dereplicator(8,9) were incorporated into the network using the GNPS MolNetEnhancer workflow (https://ccms-ucsd.github.io/GNPSDocumentation/molnetenhancer/) (10). Chemical class annotations were performed using the ClassyFire chemical ontology(11).

To further increase the number of annotated metabolites, MS2 profiles of the pooled samples were matched with the mzCloud database using Compound Discoverer version 2.1 (Thermo Fisher Scientific). Visual inspection of MS2 spectral similarity was performed using the mirror plot function of the Metabolomics Spectrum Identifier Resolver tool on GNPS (https://metabolomics-usi.ucsd.edu/).

In addition, chemical structural information of individual unknown features of interest was investigated through the in silico tool MS/MS on Sirius+CSI:FingerIDs (12).

Visual inspection of MS2 spectral similarity was performed using the mirror plot function of the Metabolomics Spectrum Identifier Resolver tool on GNPS (https://metabolomics-usi.ucsd.edu/).

The elution profile of the annotated bile acids was consistent with previous studies using similar chromatographic settings with earlier retention time for HCA (or KDCa) followed by UDCA and CA.
Text S4. PLS-DA double cross validation

Double cross validation is applied to validate the PLS-DA models (12). Initially, 100 different test sets each comprising data from randomly selected samples (10% of the total) were set aside, whereas the remaining samples, i.e. the paired training sets, were used for selection of discriminating features. The feature selection was performed by iteratively removing the variables with selectivity ratio and variable importance in projection values lower than 1.0 until no further increase in the cross-validation (eight-fold) classification errors could be observed. Final models with the selected variables were evaluated using the test sets error rate and area under the receiver operating characteristic curve (AUC). The features that were selected in at least 80% of the test-training sets pairs were investigated further as features reflecting mode of delivery.
Figure S1. A PC1 vs. PC2 scores plot of dried blood spots metabolic profiles of 677 newborns from the COPSAC2010 birth cohort (S) and pooled samples (PSs) based on 2041 features. PSs were clustered close to each other and to the center of the samples showing analytical reproducibility and representativeness. B PC1 vs. PC2 scores plot of DBS metabolic profiles of 387 newborns from the COPSAC2000 birth cohort and pooled samples based on 2355 features. PSs were clustered close to the center of the samples showing analytical reproducibility.
Among the 32 features reflecting delivery mode, MS2 was recorded for 17 of the features and seven of them *i.e.* kynurenine (tryptophan metabolism), indolelactic acid (tryptophan microbial catabolism), phenylacetylglutamine (phenylalanine pathway), and four bile acids were annotated based on GNPS spectral libraries. Mirror plots of MS2 associated with delivery mode (top) and matched MS2 retrieved from GNPS library (bottom) with a spectral similarity score (cosine score) $\geq 0.7$. The metabolomics USI tool ([http://metabolomics-usi.ucsd.edu/](http://metabolomics-usi.ucsd.edu/)) was used to extract MS2 and mirror plots were obtained using the spectrum_utils package (13) in Python 3.7. Matching peaks: black (experimental) and green (library); non-matching peaks: grey. (AS A SEPARATE FILE)
Figure S3. Molecular families and structural hypotheses retrieved from a molecular network created with the MolNetEnhancer workflow using MS2 profiles of DBS samples from COPSAC2010. Colored nodes represent mass spectral features significantly associated with delivery mode. Nodes with identical colors were grouped as one molecular feature for statistical analysis based on co-elution and high correlation of the respective MS1 intensities of the parent masses (Spearman’s Rho > 0.7). (A) Molecular family containing phenylacetylglutamine. (B)
Molecular families including arginine/ornithine/proline related substructures. (C) Molecular family of bile acids. (D) Molecular families including aromatic lactic acids. (E) Molecular family of Kynurenine [M+H] and in-source fragment [M-NH₃+H]. (F) Molecular family of acylcarnitine related compounds. (G) Polar, glycosylated or heterocyclic compounds previously found in serum of mice treated with antibiotics or prebiotics, and genetically modified *Nicotiana benthamiana*. We hypothesize that these compounds could be related to xenobiotics or antibiotics. (H) Molecular families for which no structural hypothesis could be retrieved. Structural hypotheses were retrieved by combining information from *in silico* annotation (Network Annotation Propagation), GNPS molecular networking and spectral library searches, substructure discovery through MS2LDA, and mass spectrometry searches using MASST. (AS A SEPARATE FILE)
Figure S4. Out of 32 metabolites reflecting delivery mode in COPSAC2010, 24 of them were detected also in COPSAC2000. Therefore, PLSDA model is performed with COPSAC2010 sample profiles of 24 metabolites. (a) LV1 vs LV2 scores describing the separation between CS and natural birth and (b) regression vector describing the influence of 24 metabolites for separation. The higher positive values indicate metabolites associated with natural birth while negative values describe the metabolites associated with CS. (c) The predicted LV1 and LV2 scores of COPSAC2000 samples based on PLSDA model with 24 metabolites of COPSAC2010.
Figure S5. AUC and ROC curves compare the performance of the selected feature combination to separate different subtypes of delivery mode for COPSAC2010 and COPSAC2000. (a) Distribution of AUC for 100 test sets describing the performance of PLS-DA models to separate different types of delivery mode in COPSAC2010. (b) Replication by evaluating the prediction of delivery mode for COPSAC2000 with the selected metabolites from COPSAC2010. EM-CS: emergency CS, EL-CS: elective CS
Figure S6. Association between gestational age and cesarean section (CS) scores (LV1) for each delivery mode stratum. Correlations were carried out between (a) gestation age (weeks) and CS scores from PLS-DA based on the selected 32 metabolites from COPSAC2010 DBS (from Figure 1A) and (b) predicted scores using the 24 detected metabolites from COPSAC2000 DBS. The correlation between gestational age and CS scores was significant (p<0.01, Spearman rank coefficients) for each delivery mode strata besides CS stratum of COPSAC2000. (C) The effect of type of delivery, i.e. natural birth vs CS, on 32 selected metabolites on COPSAC2010. The coefficient estimates are calculated at 95% CI using linear regression without adjustment and with adjusting for gestational age.
Table S1. Results of overall and delivery-mode-associated compound annotation diagnostics using MolNetEnhancer workflow and Compound Discoverer.

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<td>4147/2355</td>
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<td># merged features after</td>
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<tr>
<td>grouping (merged XCMS</td>
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<td>and MZmine)</td>
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<tr>
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<td>2347/1664</td>
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<td>(44.0)</td>
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<td>Total # annotated features with MS2 database matching (%)</td>
<td>125 (9.1)</td>
<td>168 (10.0)</td>
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<td>GNPS</td>
<td>79 (63.2)</td>
<td>110 (65.5)</td>
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<tr>
<td>Compound Discoverer</td>
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<td>21 (12.5)</td>
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<td>GNPS &amp; Compound Discoverer</td>
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<td>37 (22.0)</td>
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<td>MolNetEnhancer structural annotations</td>
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<td>892 (53.6)</td>
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<tr>
<td># features with MS2</td>
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### Table S2. GNPS MS2 match diagnostics for annotated metabolites.

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<td>M+H-NH3</td>
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<td>206.081</td>
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<td>M+H</td>
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References