Neonatal metabolome of caesarean section and risk of childhood asthma

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Birth by caesarean section influences the risk of asthma, partially by gut microbial colonisation and perturbed immune responses reflected by dysregulations in bile acid and tryptophan metabolism during early life https://bit.ly/3mFuqt0

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Abstract

Background Birth by caesarean section is linked to an increased risk of developing asthma, but the underlying mechanisms are unclear.

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

Methods We investigated the influence of caesarean section on liquid chromatography mass spectrometry 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 caesarean section metabolic profile, gut microbiome data and frequency of cord blood regulatory T-cells (Tregs) at 1 week of age.

Results In COPSAC2010, a partial least square discriminant analysis model showed that children born by caesarean section *versus* natural delivery had different metabolic profiles (area under the curve (AUC)=0.77, $p=2.2\times10^{-16}$), which was replicated in COPSAC2000 (AUC=0.66, $p=1.2\times10^{-5}$). The metabolic profile of caesarean section was significantly associated with an increased risk of asthma at school age in both COPSAC2010 (p=0.03) and COPSAC2000 (p=0.005). Caesarean section was associated with lower abundance of tryptophan, bile acid and phenylalanine metabolites, indicative of a perturbed gut microbiota. Furthermore, gut bacteria dominating after natural delivery, *i.e. Bifidobacterium* and *Bacteroides* were correlated with caesarean section-discriminative microbial metabolites, suggesting maternal microbial transmission during birth regulating the newborn's metabolism. Finally, the caesarean section metabolic profile was associated with frequency of cord blood Tregs.

Conclusions These findings propose that caesarean section programmes the risk of childhood asthma through perturbed immune responses and gut microbial colonisation patterns reflected in the blood metabolome at birth.

Introduction

Caesarean section is a risk factor for several chronic immune-mediated diseases such as asthma [1, 2]. Caesarean section 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 caesarean section [3], which has been linked to the risk of developing asthma [4]. Furthermore, infants born by caesarean section 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 caesarean section 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 caesarean section increases the risk of childhood asthma. We compared newborn DBS metabolic signatures after natural birth *versus* caesarean section 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 caesarean section 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

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 on the participants was obtained during scheduled visits at the COPSAC research clinic. Data on delivery mode were 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 paediatricians based on a quantitative symptom-driven, validated diagnostic algorithm [11, 12], requiring verified diary recordings of at least five episodes of troublesome lung symptoms within 6 months, each lasting at least three 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 β_2-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 pre-processing and metabolite annotation

Metabolic profiles of the DBS samples from COPSAC2010 and COPSAC2000 were acquired by liquid chromatography mass spectrometry (supplementary text S1). The data pre-processing was performed using XCMS and MZmine and data quality was evaluated based on pooled sample distribution (supplementary text S2 and figure S1). Pre-processed tandem mass spectrometry profiles were submitted to Global Natural Products Social Molecular Networking Platform [13] and MolNetEnhancer workflow [14] for compound annotation (supplementary text S3 and tables S1–S2).

Gut microbiome data

Fecal samples (n=532) were collected from the infants 1 week after birth for 16S rRNA profiling as described elsewhere [8]. Previously, we identified 15 bacterial genera reflecting caesarean section 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 analysed 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 (supplementary text S4) [15]. 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 variable (LV) scores from the PLS-DA model reflect each infant's metabolic profile of delivery mode, where higher LV scores are assigned to caesarean section. Therefore, LV1 scores are referred to in the manuscript as caesarean section scores, which are subsequently used for association with the risk of asthma by Cox regression survival analysis (survival R package) and illustrated using 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 caesarean section scores [4]. Statistics of average causal mediation effect (ACME) were investigated for assessment of the mediation.

Results

Baseline characteristics

The demographics of children born by caesarean section or natural birth are summarised in table 1, showing that children born naturally had higher gestational age than those born by caesarean section in both cohorts. Almost all mothers giving birth by caesarean section 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).

TABLE 1 Demographic characteristics of children in the Copenhagen Prospective Studies on Asthma in Childhood cohorts (COPSAC2010 and COPSAC2000) with dried blood spot metabolic profiles grouped by delivery method

	COPSAC2010 (n=677)				COPSAC2000 (n=387)			
	Natural birth	EM-CS	EL-CS	p-value	Natural birth	EM-CS	EL-CS	p-value
Total	533 (79)	79 (12)	64 (9)		309 (80)	49 (13)	29 (7)	
Child								
Male[#]	268 (50)	31 (39)	33 (51)	0.34	149 (48)	31 (63)	10 (34)	0.58
Birth								
Gestational age, weeks[¶]	40.1±1.5	39.4±2.6	39.1±0.8	<0.01	40±1.4	39.9±2.1	38.6±1.1	0.01
Weight, kg[¶]	3.6±0.5	3.5±0.8	3.5±0.5	0.46	3.5±0.5	3.5±0.8	3.3±0.4	0.30
Intrapartum antibiotics[#,+]	70 (13)	76 (96)	64 (100)	<0.01				
Season at birth[#]				0.04				0.31
Autumn	109 (20)	24 (30)	14 (22)		88 (28)	18 (37)	7 (24)	
Spring	143 (27)	17 (22)	13 (20)		61 (20)	9 (18)	10 (34)	
Summer	106 (20)	20 (25)	19 (30)		82 (27)	14 (29)	8 (28)	
Winter	175 (33)	18 (23)	18 (28)		78 (25)	8 (16)	4 (14)	
Prenatal exposures								
Smoking[#]	41 (8)	8 (10)	4 (6)	0.92	81 (26)	6 (12)	8 (28)	0.17
Fish oil supplementation[#,§]	263 (49)	45 (57)	30 (47)	0.53				
Maternal asthma[#,f]	149 (28)	30 (38)	26 (41)	0.01	309 (100)	49 (100)	20 (100)	

Data are presented as n (%) or mean±SD, unless otherwise stated. EM-CS: emergency caesarean section; EL-CS: elective caesarean section. [#]: Pearson Chi-squared test, caesarean section *versus* natural birth; [¶]: unpaired t-test, caesarean section *versus* natural birth; [+]: antibiotics given either to the mother or proband; [§]: fish oil intervention was only conducted in COPSAC2010; [f]: maternal asthma was an inclusion criterion in COPSAC2000.

Metabolome of delivery mode

The total number of metabolites passing quality control was 677 for COPSAC2010 and 387 for COPSAC2000, while the data pre-processing led to 2041 and 2355 features, respectively.

Caesarean section and naturally born infants from COPSAC2010 had differential metabolic profiles based on PLS-DA (AUC=0.77±0.06, $p=2.2\times10^{-16}$) and 32 metabolites were associated with delivery mode. A LV1 *versus* LV2 scores plot showed a clear separation between caesarean section 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 (supplementary 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 caesarean section. Others were annotated at family level using the MolNetEnhancer workflow (supplementary 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 was present (supplementary figure S4). A PLS-DA model using the delivery mode differential metabolites from COPSAC2010 also discriminated between caesarean section *versus* natural birth in COPSAC2000 (AUC=0.66, $p=1.2\times10^{-5}$).

Furthermore, the DBS metabolic profiles also differed between emergency *versus* elective caesarean section *versus* natural birth based on PLS-DA models (AUC>0.65) with the best classification performance found in the elective caesarean section *versus* natural birth model (AUC=0.83) (supplementary figure S5).

Caesarean section metabolome and risk of asthma

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

Adjusting the analyses for sex and season of birth did not change the findings in either of the cohorts (p<0.05). However, the association between the caesarean section metabolic score and asthma only remained significant in COPSAC2000 after adjusting for gestational age (HR 1.21, 95% CI 1.06–1.38;

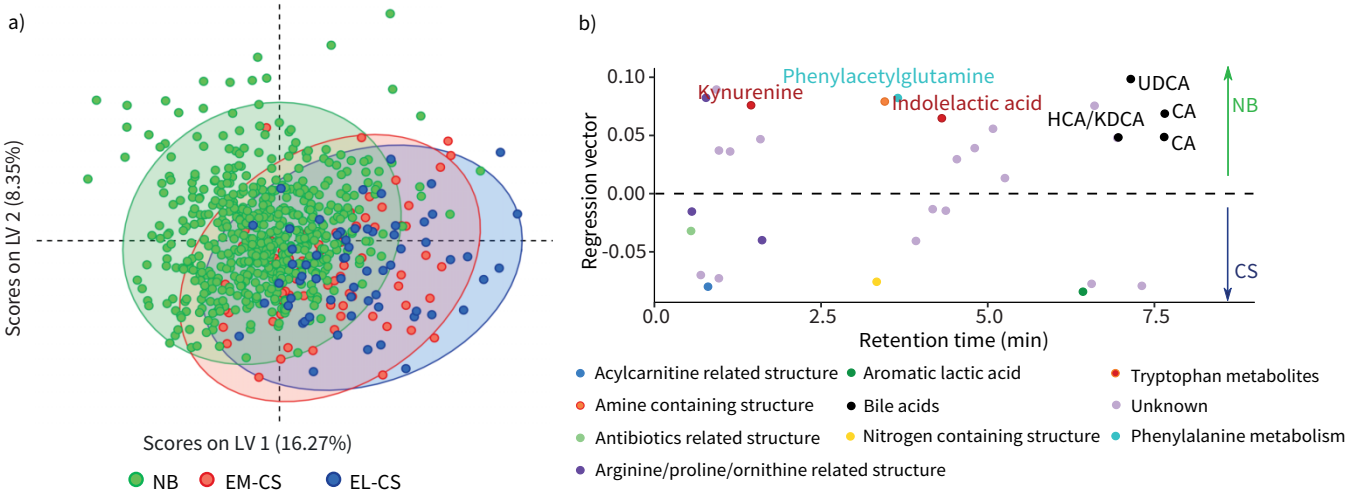

FIGURE 1 Two component partial least square discriminant analysis (PLS-DA) models discriminating between caesarean section (CS) and natural birth (NB) using the measurements of 32 selected blood metabolites from the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC2010). a) Latent variables (LV)1 and LV2 scores plot. The CS group is shaded according to 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 *versus* 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.

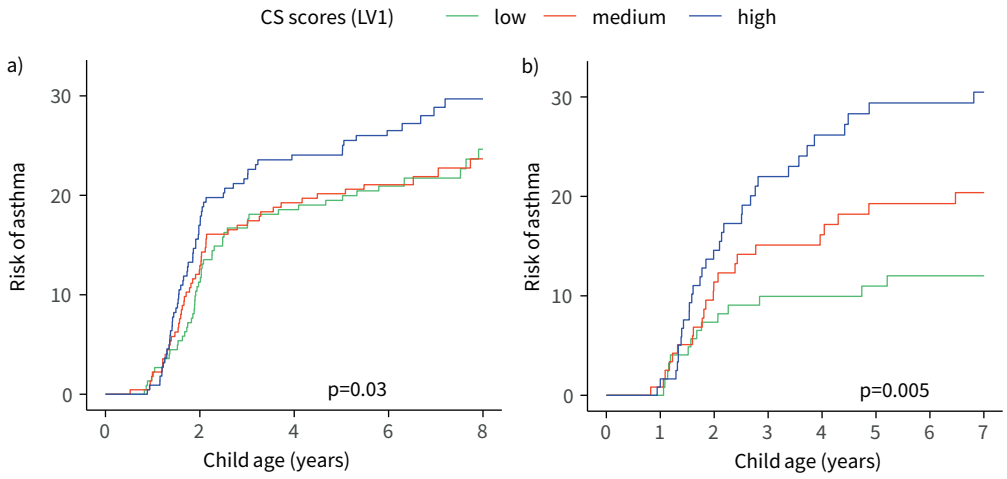

FIGURE 2 Kaplan–Meier curve of the caesarean section (CS) scores divided into tertiles and risk of developing asthma by age of 6 and 7 years, for a) children of Copenhagen Prospective Studies on Asthma in Childhood (COPSAC)2010, where the CS score of each child is calculated based on the partial least square discriminant analysis (PLS-DA) model with 32 metabolites reflecting delivery mode; and b) children of COPSAC2000, where the CS score of each child is predicted based on PLS-DA model of COPSAC2010. Cox regression survival analysis was performed on continuous CS scores, to calculate the statistics.

p=0.005). Gestational age-adjusted analysis of individual metabolites reflecting delivery mode revealed decreased effect size for certain metabolites (supplementary figure S6), which suggests that the metabolic consequence of a low gestational age is partly similar to being born by caesarean section.

Among the infants delivered by caesarean section, the risk of asthma was significantly higher for elective caesarean section compared to emergency caesarean section (HR 1.10, 1.07–1.42; p=0.003).

DBS metabolome and gut microbiome

Naturally born infants were characterised by several highly abundant microbial metabolites. Furthermore, delivery mode has previously been associated with specific gut microbial colonisation 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.* caesarean section and natural birth characterised by 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 caesarean section scores mediated 10% of the influence of delivery mode on the DBS metabolome (ACME β=0.29, 95% CI 0.08–0.50; p=0.006). This suggests that particularly for infants born naturally the dominating gut microbial population regulates the metabolic composition present in the blood.

Cord blood regulatory T-cells

The relationship between cord blood T-cell subsets and caesarean section metabolic scores (n=66) and merged caesarean section metabolic–gut microbial profiles (n=56) were investigated in COPSAC2010. The frequency of Tregs showed association with the caesarean section metabolic score (R=0.24, p=0.03), which was stronger for the merged caesarean section 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 β=0.23, 95% CI 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 caesarean section metabolic profile was associated with an increased risk of asthma development in both cohorts, suggesting that caesarean section leads to early-life

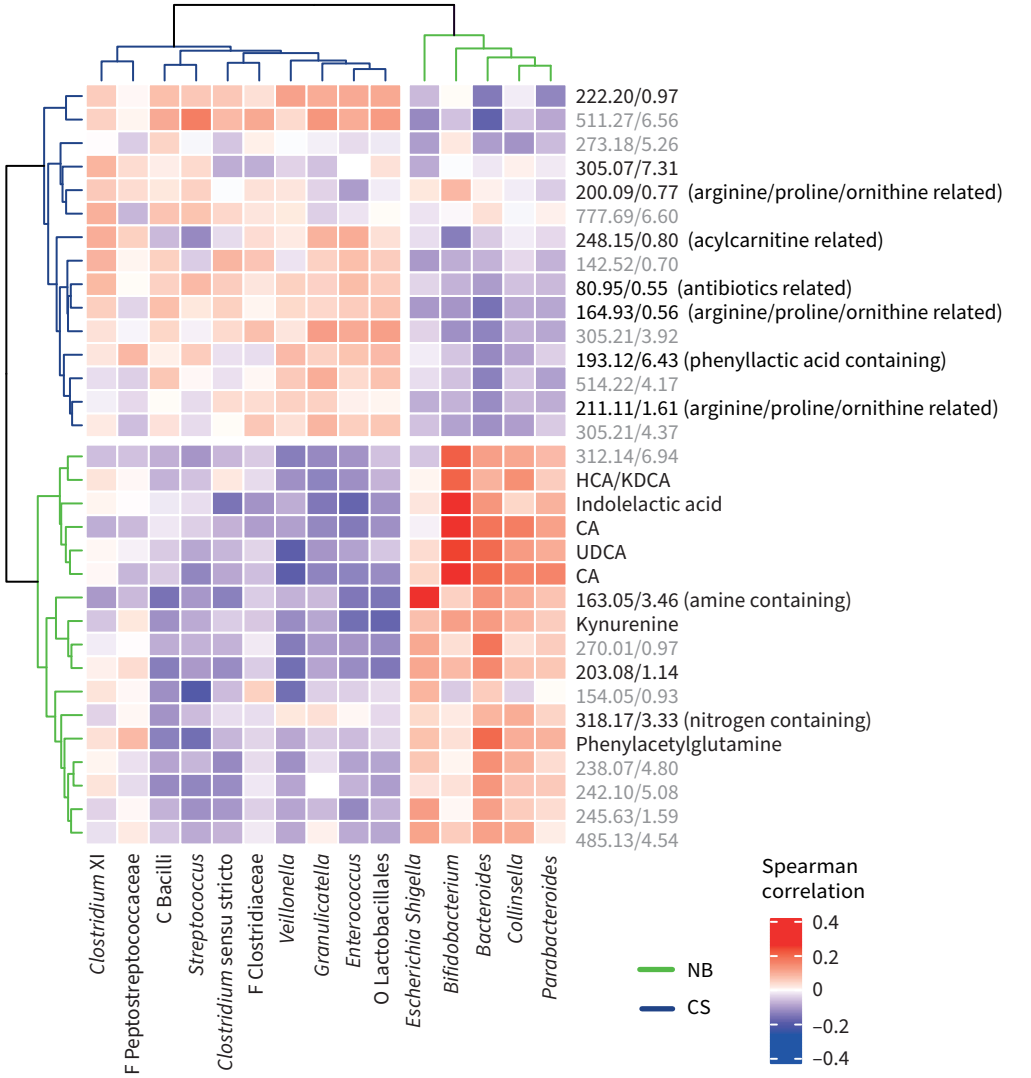

FIGURE 3 Heatmap describing the relationship between the selected 32 blood metabolites and 15 gut bacteria at 1 week of age based on Spearman rank correlation. Metabolites are presented with either name or mass to charge ratio and retention time (min), and structural information obtained from the MolNetEnhnacer workflow. For metabolites with grey font, only mass spectrometry 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 characterised by metabolites and bacteria associated either with natural birth (NB) or caesarean section (CS). The metabolites and bacteria associated with each other within each cluster. HCA: hyocholic acid; KDCA: ketodeoxycholic acid; CA: cholic acid; UDCA: ursodeoxycholic acid.

metabolic perturbations mediating the link between delivery mode and asthma. The caesarean section metabolic profile was also associated with gut microbial dysbiosis and number of cord blood Tregs, which adds important mechanistic insight into the effects of caesarean section on asthma development (figure 5).

Interpretation of the findings

We showed that the metabolic profile in newborns reflecting delivery by caesarean section 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 end-points [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 caesarean section increases the risk of developing childhood asthma.

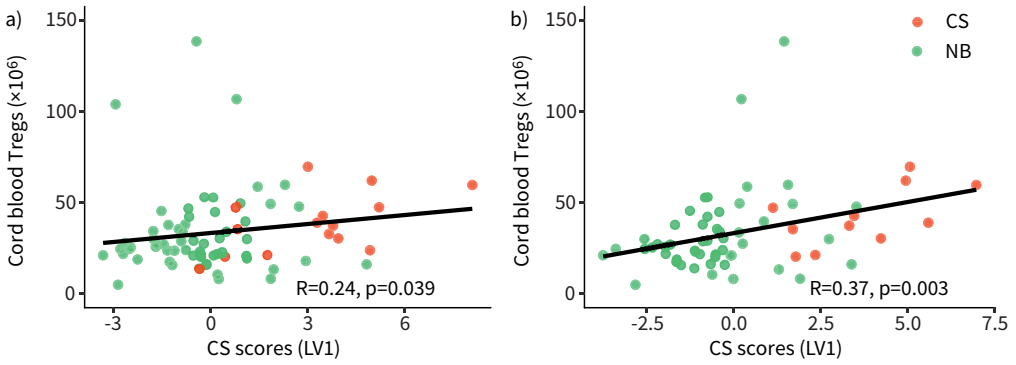

FIGURE 4 Spearman correlations between caesarean section scores and frequency of cord blood regulatory T-cells (Tregs). Caesarean section scores (latent variable (LV)1) were calculated based on a) the selected 32 metabolites from Copenhagen Prospective Studies on Asthma in Childhood (COPSAC)2010, and b) the merged 32 metabolites and 15 bacterial taxa from COPSAC2010.

We found that indolelactic acid, which is an intermediate of the tryptophan metabolism, was lower in caesarean section 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 acts as a ligand 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 signalling [22]. Therefore, we speculate that *Bifidobacterium* is transferred from mother to infant during natural birth, and subsequently colonises the infant's gut and leads to higher abundance of blood indolelactic acid, which contributes to the development of intestinal barrier functions and the immune system in early life.

Perturbated kynurenine metabolism was also found to associate with caesarean section in our study. The kynurenine pathway modulates pro-inflammatory and anti-inflammatory responses in the gastrointestinal tract *via* influence 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 caesarean section 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 caesarean section 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 caesarean section can be restored by maternal fecal microbiota transplantation [27], which may partly act 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 caesarean section through a fortified formula or supplementation of breastfeeding mothers after birth, for example.

Additionally, children born by caesarean section also showed lower levels of primary (*i.e.* cholic acid) and secondary (*i.e.* ursodeoxycholic acid) bile acids. Furthermore, 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 caesarean section may contribute to perturbations in

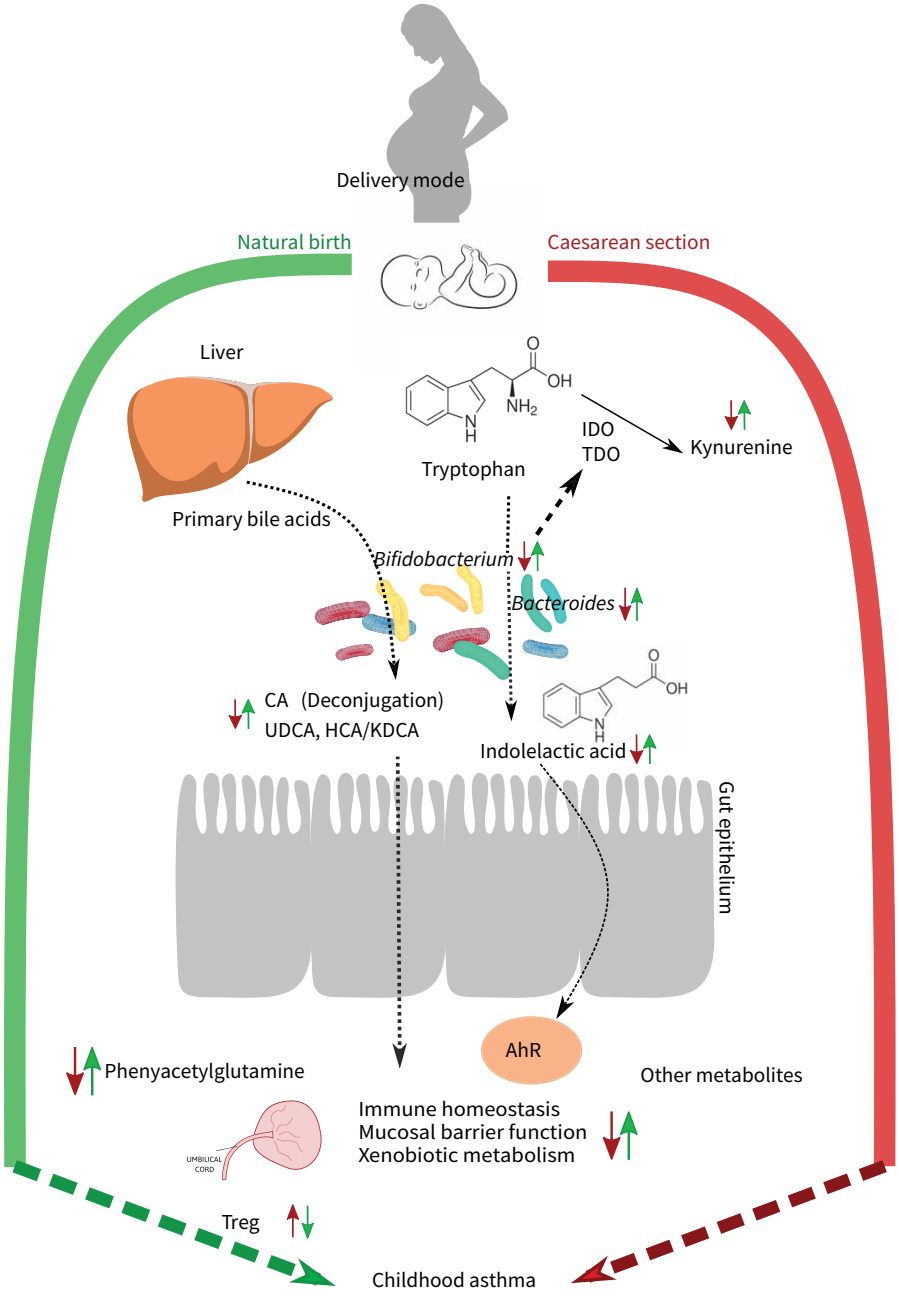

FIGURE 5 Proposed mechanism of action of delivery mode on infants metabolic and microbial profiles associated with childhood asthma. Natural birth led to higher levels of tryptophan catabolites, *i.e.* indolelactic acid and kynurenine, which are bioactive compounds acting on the aryl hydrocarbon receptor (AhR) and leading to improved immune regulation in early life. Indolelactic acid production is probably controlled by *Bifidobacterium* in the gut, which was highly abundant after natural birth. Under the influence of microbiota, kynurenine is produced by the indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) pathway. Natural birth promoted higher levels of bile acids, some of which are possibly controlled by *Bifidobacterium* (*e.g.* deconjugation of primary bile acids produced in the liver). Bile acids also regulate immune homeostasis. Cord blood regulatory T-cells (Tregs) are associated with metabolic and microbial caesarean section profiles. Overall, the newborn metabolome reflects the mode of delivery; metabolic changes are partially mediated by the gut microbial community; and cord blood Tregs and the metabolic fingerprint of caesarean section are associated with increased risk of childhood asthma. CA: cholic acid; UDCA: ursodeoxycholic acid; HCA: hyocholic acid; KDCA: ketodeoxycholic acid.

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 signalling molecules to regulate early-life immune homeostasis [24, 30]. A higher number of Tregs have been detected in infants born by caesarean section 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 caesarean section 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 colonisation, our study suggests that Tregs have a role in regulation of microbial colonisation 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 programme is a valuable resource for clinical metabolomics applications. Despite the potential, only few studies have utilised newborn blood to investigate the association between the early-life metabolic profiles and disease development [33].

Infants born by caesarean section 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]. Furthermore, 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 caesarean section metabolic scores and lower gestational age has been linked to an increased risk of asthma [36]. However, the caesarean section 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 caesarean section may have the highest risk of developing asthma.

Similar to caesarean section delivery, intrapartum antibiotics has been associated with risk of persistent wheeze [37]. As prescription of intrapartum antibiotics and caesarean section 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 children born by caesarean section 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 characterising delivery mode were annotated. The unannotated compounds may highlight additional underlying adverse effects of caesarean section, leading to childhood asthma [38, 39].

Conclusion

Newborns with a caesarean section metabolic profile had an increased risk of developing asthma in two independent cohorts, suggesting that the inferred risk of asthma from caesarean section 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 caesarean section on risk of asthma and may contribute to development of novel primary prevention strategies.

Author contributions: G. Gürdeniz, B. Chawes, H. Bisgaard and D. Rago developed the concept and designed the overall study approach. A.S. Cohen, A. Björkbom and D. Hougaard organised and conducted mass spectrometric analysis. M. Ernst and J. Courraud interpreted mass spectra. S.J. Sørensen and U. Trivedi performed the gut microbiome analysis. S. Brix provided cord blood immune data. D. Rago, M. Kim, M. Ernst, A.S. Cohen,

M. Rasmussen, J. Stokholm, K. Bønnelykke, H. Bisgaard and G. Gürdeniz interpreted the data. G. Gürdeniz, B. Chawes, H. Bisgaard and M. Ernst wrote the manuscript.

Governance: We are aware of and comply with recognised 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 European Union'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.

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References

1 Kero J, Gissler M, Grönlund M-M, *et al.* Mode of delivery and asthma – is there a connection? *Pediatr Res* 2002; 52: 6–11.
2 Sevelsted A, Stokholm J, Bisgaard H. Risk of asthma from cesarean delivery depends on membrane rupture. *J Pediatr* 2016; 171: 38–42.
3 Stokholm J, Thorsen J, Chawes BL, *et al.* Cesarean section changes neonatal gut colonization. *J Allergy Clin Immunol* 2016; 138: 881–889.
4 Stokholm J, Thorsen J, Blaser MJ, *et al.* Delivery mode and gut microbial changes correlate with an increased risk of childhood asthma. *Sci Transl Med* 2020; 12: eaax9929.
5 Evers KS, Wellmann S. Arginine vasopressin and copeptin in perinatology. *Front Pediatr* 2016; 4: 75.
6 Arrieta M-C, Stiemsma LT, Dimitriu PA, *et al.* Early infancy microbial and metabolic alterations affect risk of childhood asthma. *Sci Transl Med* 2015; 7: 307ra152.
7 Stokholm J, Blaser MJ, Thorsen J, *et al.* Maturation of the gut microbiome and risk of asthma in childhood. *Nat Commun* 2018; 9: 141.
8 Thysen AH, Larsen JM, Rasmussen MA, *et al.* Prelabor cesarean section bypasses natural immune cell maturation. *J Allergy Clin Immunol* 2015; 136: 1123–1125.
9 Bisgaard H, Vissing NH, Carson CG, *et al.* Deep phenotyping of the unselected COPSAC2010 birth cohort study. *Clin Exp Allergy* 2013; 43: 1384–1394.
10 Bisgaard H. The Copenhagen Prospective Study on Asthma in Childhood (COPSAC): design, rationale, and baseline data from a longitudinal birth cohort study. *Ann Allergy Asthma Immunol* 2004; 93: 381–389.
11 Bisgaard H, Stokholm J, Chawes BL, *et al.* Fish oil-derived fatty acids in pregnancy and wheeze and asthma in offspring. *N Engl J Med* 2016; 375: 2530–2539.
12 Bisgaard H, Hermansen MN, Buchvald F, *et al.* Childhood asthma after bacterial colonization of the airway in neonates. *N Engl J Med* 2007; 357: 1487–1495.
13 Wang M, Carver JJ, Phelan VV, *et al.* Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nat Biotechnol* 2016; 34: 828–837.
14 Ernst M, Kang KB, Caraballo-Rodríguez AM, *et al.* MolNetEnhancer: enhanced molecular networks by integrating metabolome mining and annotation tools. *Metabolites* 2019; 9: 144.
15 Smit S, van Breemen MJ, Hoefsloot HCJ, *et al.* Assessing the statistical validity of proteomics based biomarkers. *Anal Chim Acta* 2007; 592: 210–217.
16 Tingley D, Yamamoto T, Hirose K, *et al.* mediation: r package for causal mediation analysis. *J Stat Soft* 2014; 59: 1–38.
17 Chawes BL, Giordano G, Pirillo P, *et al.* Neonatal urine metabolic profiling and development of childhood asthma. *Metabolites* 2019; 9: 185.
18 Aragozzini F, Ferrari A, Pacini N, *et al.* Indole-3-lactic acid as a tryptophan metabolite produced by *Bifidobacterium* spp. *Appl Environ Microbiol* 1979; 38: 544–546.

19 Reyman M, van Houten MA, van Baarle D, *et al.* Impact of delivery mode-associated gut microbiota dynamics on health in the first year of life. *Nat Commun* 2019; 10: 4997.

20 Lamas B, Natividad JM, Sokol H. Aryl hydrocarbon receptor and intestinal immunity. *Mucosal Immunol* 2018; 11: 1024–1038.

21 Gomez de Agüero M, Ganal-Vonarburg SC, Fuhrer T, *et al.* The maternal microbiota drives early postnatal innate immune development. *Science* 2016; 351: 1296–1302.

22 Henrick BM, Rodriguez L, Lakshmikanth T, *et al. Bifidobacteria*-mediated immune system imprinting early in life. *Cell* 2021; 184: 3884–3898.

23 Dehhaghi M, Kazemi Shariat Panahi H, Guillemin GJ. Microorganisms, tryptophan metabolism, and kynurenine pathway: a complex interconnected loop influencing human health status. *Int J Tryptophan Res* 2019; 12: 1178646919852996.

24 Zelante T, Iannitti RG, Cunha C, *et al.* Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity *via* interleukin-22. *Immunity* 2013; 39: 372–385.

25 Desbonnet L, Garrett L, Clarke G, *et al.* The probiotic *Bifidobacteria infantis*: an assessment of potential antidepressant properties in the rat. *J Psychiatr Res* 2008; 43: 164–174.

26 Stone TW, Stoy N, Darlington LG. An expanding range of targets for kynurenine metabolites of tryptophan. *Trends Pharmacol Sci* 2013; 34: 136–143.

27 Korpela K, Helve O, Kolho K-L, *et al.* Maternal fecal microbiota transplantation in cesarean-born infants rapidly restores normal gut microbial development: a proof-of-concept study. *Cell* 2020; 183: 324–334.

28 Tanaka H, Doesburg K, Iwasaki T, *et al.* Screening of lactic acid bacteria for bile salt hydrolase activity. *J Dairy Sci* 1999; 82: 2530–2535.

29 Li B, Selmi C, Tang R, *et al.* The microbiome and autoimmunity: a paradigm from the gut-liver axis. *Cell Mol Immunol* 2018; 15: 595–609.

30 Hang S, Paik D, Yao L, *et al.* Bile acid metabolites control T_H17 and T_{reg} cell differentiation. *Nature* 2019; 576: 143–148.

31 Scharschmidt TC, Vasquez KS, Truong H-A, *et al.* A wave of regulatory T cells into neonatal skin mediates tolerance to commensal microbes. *Immunity* 2015; 43: 1011–1021.

32 Ruohtula T, de Goffau MC, Nieminen JK, *et al.* Maturation of gut microbiota and circulating regulatory T cells and development of IgE sensitization in early life. *Front Immunol* 2019; 10: 2494.

33 Donovan BM, Ryckman KK, Breheny PJ, *et al.* Association of newborn screening metabolites with risk of wheezing in childhood. *Pediatr Res* 2018; 84: 619–624.

34 Rowe-Murray HJ, Fisher JRW. Baby friendly hospital practices: cesarean section is a persistent barrier to early initiation of breastfeeding. *Birth* 2002; 29: 124–131.

35 Laursen MF, Sakanaka M, von Burg N, *et al. Bifidobacterium* species associated with breastfeeding produce aromatic lactic acids in the infant gut. *Nat Microbiol* 2021; 6: 1367–1382.

36 Liu X, Olsen J, Agerbo E, *et al.* Birth weight, gestational age, fetal growth and childhood asthma hospitalization. *Allergy Asthma Clin Immunol* 2014; 10: 13.

37 Rusconi F, Galassi C, Forastiere F, *et al.* Maternal complications and procedures in pregnancy and at birth and wheezing phenotypes in children. *Am J Respir Crit Care Med* 2007; 175: 16–21.

38 Hashimoto F, Nishiumi S, Miyake O, *et al.* Metabolomics analysis of umbilical cord blood clarifies changes in saccharides associated with delivery method. *Early Hum Dev* 2013; 89: 315–320.

39 Marchioro L, Shokry E, Geraghty AA, *et al.* Caesarean section, but not induction of labour, is associated with major changes in cord blood metabolome. *Sci Rep* 2019; 9: 17562.