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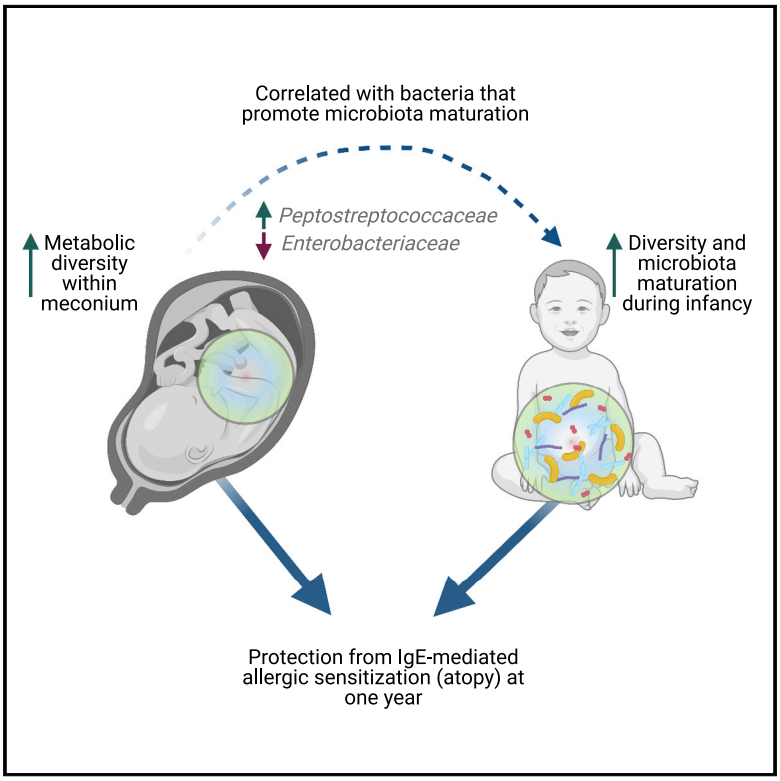
A rich meconium metabolome in human infants is associated with early-life gut microbiota composition and reduced allergic sensitization

Graphical abstract



Highlights

- Metabolic diversity is reduced in newborns that develop allergic sensitization
- Gut microbiota maturation is reduced within infants that develop allergic sensitization
- Meconium metabolites are associated with important taxa for microbiota maturation

Authors

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In brief

Petersen et al., reveal that newborns who develop allergic sensitization have a less-diverse gut metabolome at birth and delayed microbiota maturation during infancy. This suggests that both microbiota colonization and immune development are affected by metabolites present in the gut at birth.

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A rich meconium metabolome in human infants is associated with early-life gut microbiota composition and reduced allergic sensitization

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SUMMARY

Microbiota maturation and immune development occur in parallel with, and are implicated in, allergic diseases, and research has begun to demonstrate the importance of prenatal influencers on both. Here, we investigate the meconium metabolome, a critical link between prenatal exposures and both early microbiota and immune development, to identify components of the neonatal gut niche that contribute to allergic sensitization. Our analysis reveals that newborns who develop immunoglobulin E (IgE)-mediated allergic sensitization (atopy) by 1 year of age have a less-diverse gut metabolome at birth, and specific metabolic clusters are associated with both protection against atopy and the abundance of key taxa driving microbiota maturation. These metabolic signatures, when coupled with early-life microbiota and clinical factors, increase our ability to accurately predict whether or not infants will develop atopy. Thus, the trajectory of both microbiota colonization and immune development are significantly affected by metabolites present in the neonatal gut at birth.

INTRODUCTION

Microbiota maturation begins in earnest immediately after birth, when pioneering bacteria are introduced into the neonatal gut and form a niche capable of supporting successive colonizers that will comprise the infant microbiota.[1,2] Maturation of this microbial community continues through the first few years of life and occurs concomitantly with dramatic shifts in host immune function.[3–5] Despite what has evolutionarily been beneficial for both humans and microbes, increased prevalence of immunoglobulin E (IgE)-mediated (atopic) diseases in young children has occurred in the past half century, in association with changes in the infant gut microbiota.[6] Alarmingly, atopic disorders, including eczema (atopic dermatitis), food allergy, asthma, and allergic rhinitis, are currently estimated to affect up to 30% of the population, with many children experiencing a lifelong burden.[7–10]

Reduced colonization of beneficial early-life microbes is linked to a number of IgE-mediated disorders; however, how this early-life microbial community is formed is only just now being understood.[11–13] Furthermore, whether this phenomenon is limited to the postnatal early-life window has been challenged as studies reveal prenatal factors capable of influencing both microbiota composition and immune development. Meconium is the first stool sample passed after birth. The meconium metabolome reflects a wide range of fetal exposure as it begins forming in the fetal gut by gestational week 16 and is not passed until the first few days of life. Meconium is not only a rich source of metabolites reflecting perinatal influences but also contains the starting material for the initial microbiota.[12,14–18] In this study, we interrogated metabolic signatures within the meconium that might influence both microbiota maturation and immune development. We found that reduced meconium metabolic richness was associated with IgE-mediated allergic sensitization (atopy, as quantified by standardized skin prick testing [SPT]) to common allergens at 1 year of age. Moreover, select metabolites present within the meconium were associated with changes detected months later in key bacterial taxa important for driving microbiota maturation in

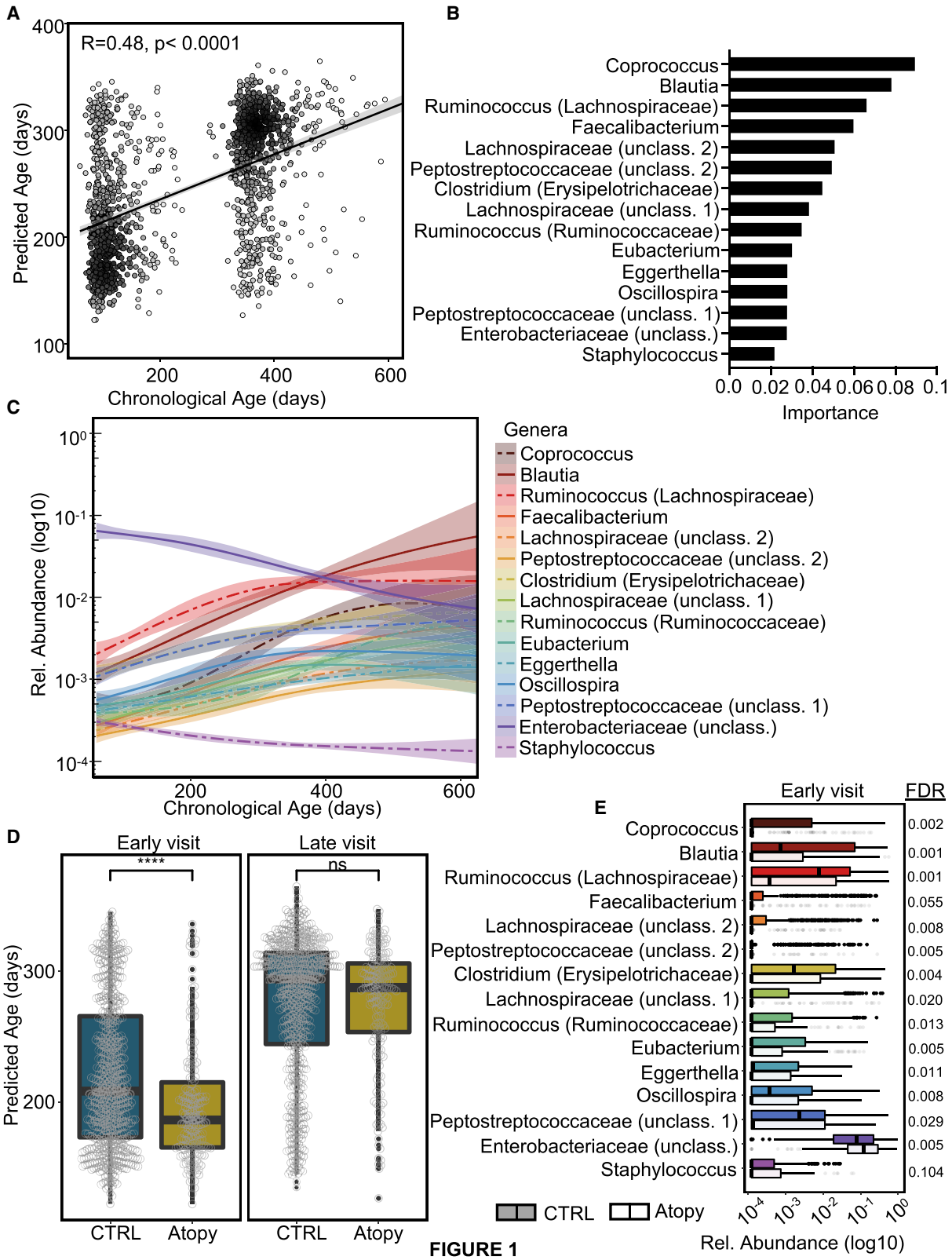

FIGURE 1

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the first year of life as well as the atopy phenotype. Thus, deficiency in microbiota maturation and immune development likely begins *in utero*, and these findings may be valuable to identifying at-risk infants and revealing directly modifiable metabolic targets to prevent allergic sensitization.

RESULTS

Microbiota maturation is reduced before atopy

We obtained 16S rRNA amplicon-sequencing data on stool collected from 950 healthy infants enrolled in the Canadian Healthy Infant Longitudinal Development (CHILD) cohort, following a protocol in which sequential stool samples were collected within the first 2 years of life; an early sample (median age, 3 months) and a later sample (median age, 1 year). Using a random forest regressor with a nested 10-fold cross-validation (NCV), we observed that the microbiota composition could accurately predict the chronological age of the infants at the time of sample collection (Figure 1A; $p < 0.0001$). Based on that model, we identified the top-15 bacterial taxa, ranked by importance for predicting microbiota maturation. When we observed their colonization over time, we saw a remarkable dynamic fluctuation in the relative abundance of these key taxa during early life (Figures 1B and 1C). Although members of *Enterobacteriaceae* and *Staphylococcus* decreased in relative abundance over time, increasing abundance was detected in most of the top-15 taxa. These included large increases in the relative abundance of *Blautia*, as well as smaller increases in *Coprococcus*, *Faecalibacterium*, and *Ruminococcus* relative abundance, as infants aged.

Aberrant immune development has previously been associated with changes to gut microbial composition early in life.[19–23] We, therefore, examined microbiota maturation in children who developed atopy, which was defined as a positive allergy SPT at 1 year of life (212 of the original 979 sequenced infants). Infants with an atopic response at 1 year had a significantly less-mature microbiota than healthy non-atopic infants had at early time points but not at the time of the SPT administration (Figures 1D and S1A). This was not due to differences in chronological age between the atopic and control groups at stool collection (Figure S1B) but can, instead, be attributed to differences in the composition of the top-15 most-important taxa for determining microbiota maturation. Indeed, when we compared colonization of all genera between control and atopic infants, we observed a striking overlap between those taxa that were significantly different between the groups at the early visit and those that ranked highly in predicting microbiota maturation (Figures 1E and S1C; Table S1). Notably 13 of 15 taxa were decreased in relative abundance in the atopic infants compared with the controls.

Atopic infants have a less-diverse neonatal niche

The largest difference in microbiota maturation between atopic and control infants was detected in early samples (Figure S1A). To identify potential drivers of those differences, we investigated the neonatal niche present within meconium at birth. Meconium begins to accumulate by gestational week 16 and is passed shortly after birth. Bacterial DNA can be detected within the meconium, and many studies have debated the initial timing of bacterial colonization, with some researchers finding evidence of colonization in a subset of samples by gestational week 22.[12,24–27] Most colonizing bacteria, however, are likely not introduced until after birth because bacterial DNA is very low in relation to human DNA for the first few hours after birth.[2] We, therefore, chose to perform a metabolic profile of the neonatal niche that would not be susceptible to contamination issues surrounding low-microbial biomass and could, instead, encompass both maternal sources of metabolites during fetal development and those that may present from any pioneering bacteria at birth.

Comprising ingested materials, including skin and gut cells, amniotic fluid, vernix, and lanugo hair as well as excreted fetal metabolites, the metabolites found within the meconium encapsulate a wide range of fetal exposures during gestation and have the potential to directly influence both gut microbiota and immune development.[1] To understand whether the neonatal niche in the guts of atopic infants was associated with the detected changes in their early-life microbiota and susceptibility to atopy at 1 year, we performed a global metabolomics analysis of meconium samples in a subset of 100 infants. Importantly, this subset of 100 infants was representative of the 950 infants with microbiota data with regard to key demographic and clinical variables (Table S2).

Overall, 714 metabolites were detected across a wide range of metabolic pathways within the samples, including predominately lipids, followed by amino acids, xenobiotics, and vitamins and cofactors (Table S3; Figure S2A). We have previously demonstrated that bacterial diversity within early-life microbiota is associated with protection against the development of an atopic response at 1 year.[28] Replicating that fundamental observation within the 100 infants selected for the meconium analysis, we detected a significant reduction in bacterial alpha-diversity (amplicon sequence variant [ASV] richness) at early time points in atopic infants compared with that of non-atopic controls ($p = 0.01$; Figure 2A).

To define the factors that may affect microbiota development, we quantified the number of detected metabolites in the meconium and used that measure of metabolite richness to estimate diversity within that niche. Strikingly, atopic infants had a significantly less metabolically rich meconium at birth compared with that of non-atopic infants, suggesting that differences in the niche that supports microbiota development and, ultimately,

Figure 1. The microbiota matures more slowly in atopic (Atopy) relative to control (CTRL) infants

(A) Predicted age of infant based on 16S microbiota sequencing compared with true chronological age at the time of sampling (early visit, n = 765; late visit, n = 761). R and p values were derived from comparing linear regression of the predictive age to the chronological age. Grey scale reflects sample density.

(B) Top-15 most-important taxa for predicting age of infants based on 16S sequencing.

(C) Relative abundance of top-15 important taxa from (B) compared with chronological age of infant at the time of sampling.

(D) Predicted age of healthy controls (CTRLs) and atopic infants at early visit (CTRL, n = 574; atopy, n = 191) and late visit (CTRL, n = 573; atopy, n = 188).

(E) Relative abundance of top-15 important taxa in the stool of healthy control and atopic infants at early visit (CTRL, n = 574; atopy, n = 191).

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ using a two-tailed, unpaired Student's t test (D) and Wilcoxon test (E), followed by the false-discovery rate (FDR) correction against all genera present within sequenced samples. Boxplot error bars represent 25th (bottom) and 75th (upper) quartiles.

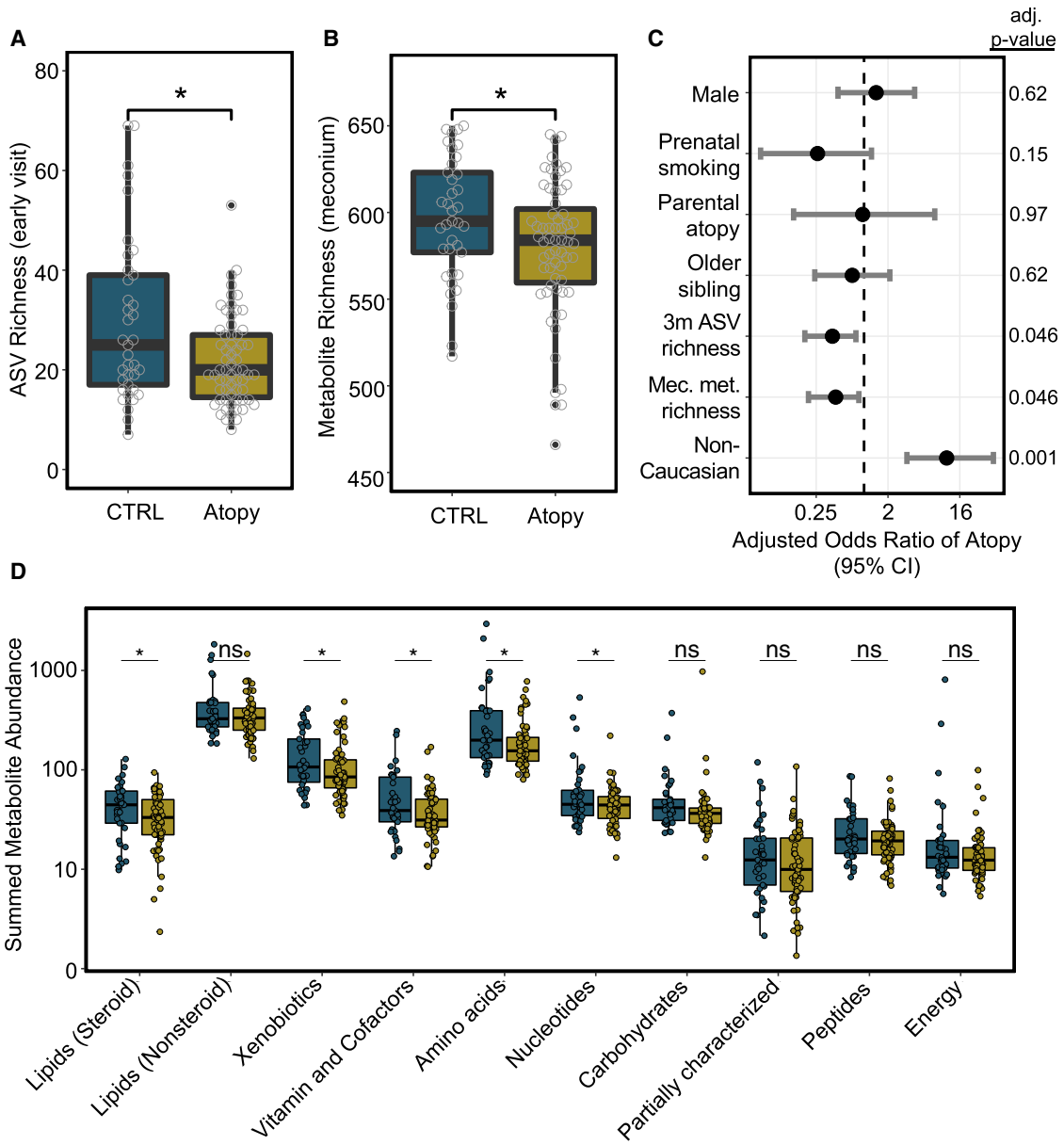


Figure 2. Metabolic richness in the meconium is associated with protection from atopy at 1 year

(A) ASV richness (number of observed ASVs per sample) between healthy controls and atopic infants at early visit (CTRL, n = 37; atopy, n = 63).

(B) Metabolite richness (number of observed metabolites per sample) between healthy controls and atopic infants (CTRL, n = 37; atopy, n = 63).

(C) Adjusted odds ratio of developing atopy at 1 year based on multivariate generalized linear regression analysis. p values were further adjusted using an FDR correction for multiple comparisons.

(D) Summed metabolite abundance of metabolites within a metabolic pathway after scaling to median abundance of each metabolite.

*$p < 0.05$, **$p < 0.01$ using a two-tailed, unpaired Student's t test (A, B, and D) and multivariate linear regression (C). Boxplot error bars represent 25th (bottom) and 75th (upper) quartiles.

influences immune development may already exist at birth (p = 0.02; Figure 2B). Furthermore, we demonstrated that increases in both meconium metabolic and microbiota richness early in life were significantly associated with protection against developing atopy (ASV-richness interquartile adjusted odds ratio [aOR] = 0.40; 95% confidence interval [CI]: 0.18–0.79; adj. p = 0.046; metabolite-richness interquartile aOR = 0.44; 95% CI: 0.20–0.86; adj. p = 0.046; Figure 2C). Notably, this loss of metabolite abundance within the meconium was not global but was, instead, restricted to certain pathways, including steroids, vitamins and cofactors, amino acids, and nucleotides (Figure 2D). Thus, atopy at 1 year is associated with a less metabolically rich meconium at birth, as well as being associated with reductions in microbiota diversity and maturation early in life.

Specific meconium metabolites are reduced in atopic infants

To identify specific metabolic shifts in meconium that were associated with atopy at 1 year, we clustered all 714 detected metabolites into 18 highly correlated modules using weighted-correlation network analysis (WGCNA) (Table S3).[29] Two-thirds of those modules contained metabolites from a predominately metabolic pathway (for example, module 4 was predominately saturated fatty acids, whereas module 10 comprised ceramides). However, one third contained a mixture of metabolites that would not have been functionally binned together using pathway analysis alone, highlighting the benefits of unsupervised clustering when analyzing patterns within metabolomic datasets. Modules 5 and 8 contained 41 and 38 metabolites, respectively, from multiple pathways and were significantly reduced in atopic infants (Figure 3A). Despite the diversity of metabolites within those modules, metabolic set enrichment analysis (MSEA) revealed which metabolic pathways were least-represented within atopic infant meconium. Those pathways involved amino acid metabolism, including glycine, serine, and tyrosine; glutathione metabolism; nicotinamide metabolism; estrone metabolism; and lipid metabolism (Figures 3B and 3C). Within module 5, nicotinamide, tyrosine metabolites, and estriol, in particular, were all significant and contained high module membership, indicating their inter-relatedness within the module, as well as their influence on overall module significance (Figure 3D), whereas the significance of module 8 was not driven by any specific metabolites (Figure 3E). Thus, these data reveal that, in addition to overall reductions in diversity, specific reductions in metabolites associated with amino acid metabolism, vitamins, and hormones are reduced in infants that develop an atopic response by 1 year.

Metabolic metabolites influence taxa important for microbiota maturation

Meconium metabolites can be further metabolized and fermented by the microbes that will form the early-life microbiota. A number of early-life exposures heavily influence microbiota composition; however, researchers can detect sustained colonization of certain bacterial strains found within meconium up to 1 month later, suggesting that the neonatal niche itself may have a lasting effect on early-life colonization.[2,20,30,31] We performed a Procrustes analysis on the Euclidean distances of both the meconium metabolomic composition and infant stool microbiota composition to quantify any correspondence that may exist between the metabolic niche at birth and the infant microbiota composition (Figure 4A).[32,33] Indeed, there was a modest correspondence between the meconium metabolome and the microbiota composition at the early visit when we performed a Procrustes analysis ($p = 0.03$ based on 1,000 Monte Carlo iterations). This correspondence was not significant later in infancy (Figure S3A; $p = 0.09$ based on 1,000 Monte Carlo iterations). To identify metabolites that may be influencing the microbiota maturation, we correlated meconium module eigenvalues with the relative abundance of the top-15 important microbes for predicting microbiota maturation (Figure 4B). *Peptostreptococcaceae* abundance at the early visit was significantly and positively correlated with a number of meconium modules containing non-steroidal lipids, including saturated fatty acids, acyl carnitines, and phospholipids as well as one module containing multiple amino acid metabolites (Figure 4B; Table S3). Interestingly, module 5, whose members were reduced in meconium from atopic infants, was also negatively correlated with the relative abundance of *Enterobacteriaceae* early in life. Many metabolites within module 5 were negatively associated with *Enterobacteriaceae* from early collection time points, some, but not all, of which overlapped with those that were significantly reduced in newborns that later developed atopy (Figure 4C). As facultative anaerobes, members of the *Enterobacteriaceae* family are one of the first colonizers following birth. Overall richness of the meconium metabolome was also significantly and negatively correlated with *Enterobacteriaceae* abundance early in life but not with predicted age (Figures S3B and S3C). Together, these data suggest the colonization of bacterial taxa important for microbiota maturation may be influenced by the gut metabolic niche at birth.

Metabolic signatures in meconium help to identify infants at risk of atopy

A number of children who develop allergic sensitization experience a lifelong burden, with almost no curative therapies currently existing. Therefore, identification of at-risk infants early in life may be particularly beneficial in preventing the development of allergic diseases. We next sought to determine whether these metabolic modules within the meconium could increase our ability to predict allergic sensitization objectively assessed as a positive SPT response at 1 year. We selected a subset of clinical variables based on key demographics and perinatal factors that were statistically associated with the atopy phenotype in our cohort (Table S4). These included parental atopy, prenatal smoking, gender, having an older sibling, and ethnicity. Using the machine-learning least-absolute shrinkage and selection operator (LASSO)-logistic-regression approach, the clinical variables alone achieved a receiver operating characteristic cross-validation area under the curve statistics (CV-AUC) of 0.69 (Figures 5A and S4). Using the relative abundance of the top-15 most-important taxa contributing to predicting microbiota maturation at 3 months, LASSO-logistic regression selected 13 taxa (both *Ruminococcus* genera were removed), which had an CV-AUC of 0.59 (Figures 5A and S4). Combining the eigenvalue information for modules 5 and 8 into a LASSO-logistic score had an CV-AUC of 0.60 (Figures 5A and S4). The addition of either the microbiota or the meconium panels to the clinical variables each statistically increased the accuracy to CV-AUC of 0.74. Combining all three panels improved predictability to CV-AUC of 0.76. Collectively, these data demonstrate that, along with clinical factors and the early-life microbiota composition, the meconium metabolic niche is a contributing factor to our ability to identify infants at risk of developing allergic sensitization.

DISCUSSION

Formed early in the second trimester, meconium is a collection of ingested and excreted metabolites that reflects a wide range of fetal exposures during gestation and serves as the initial metabolic niche for microbes entering the gut. We investigated this critical link between prenatal development and the early-life microbiota composition to identify factors associated with allergic

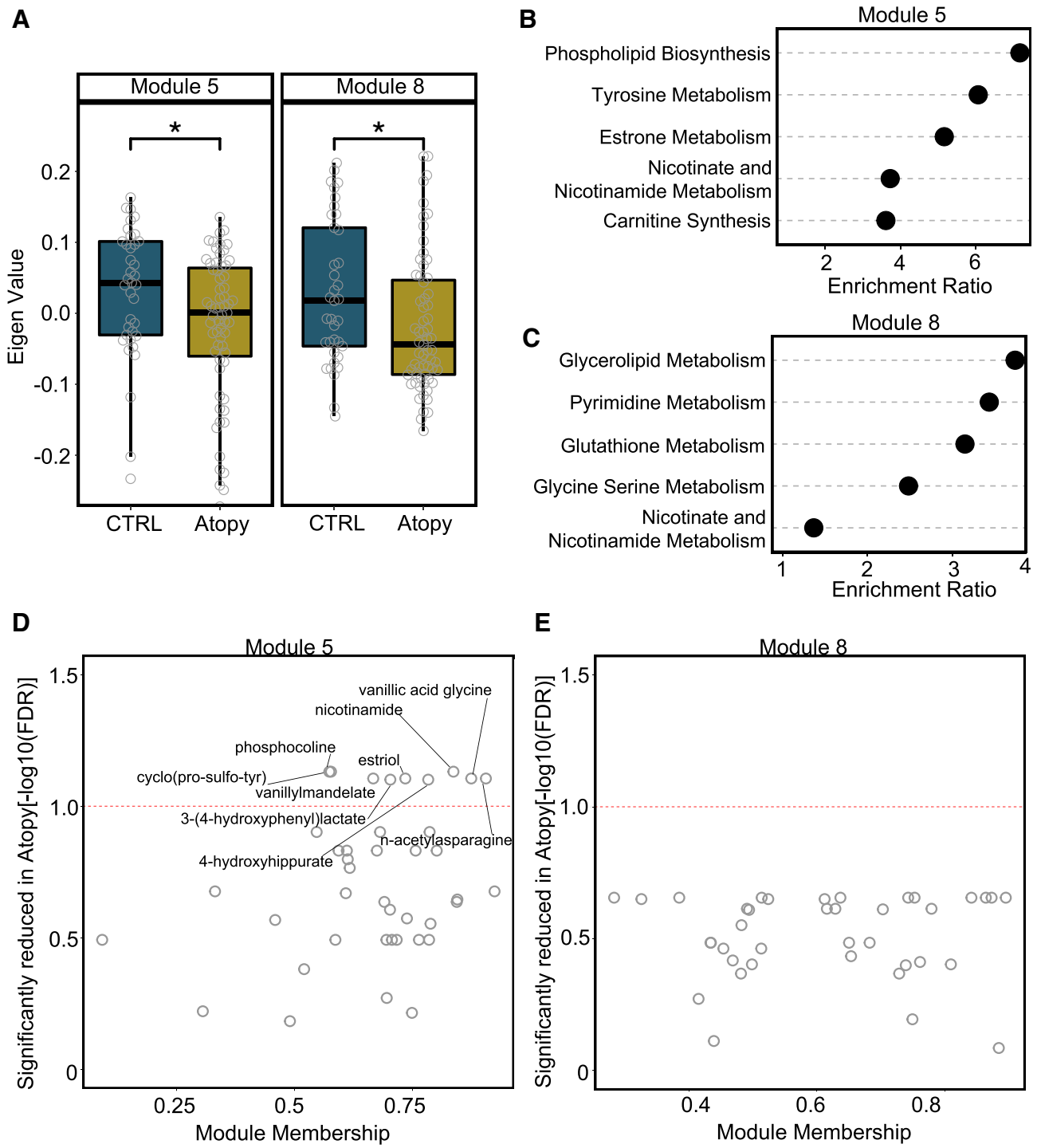


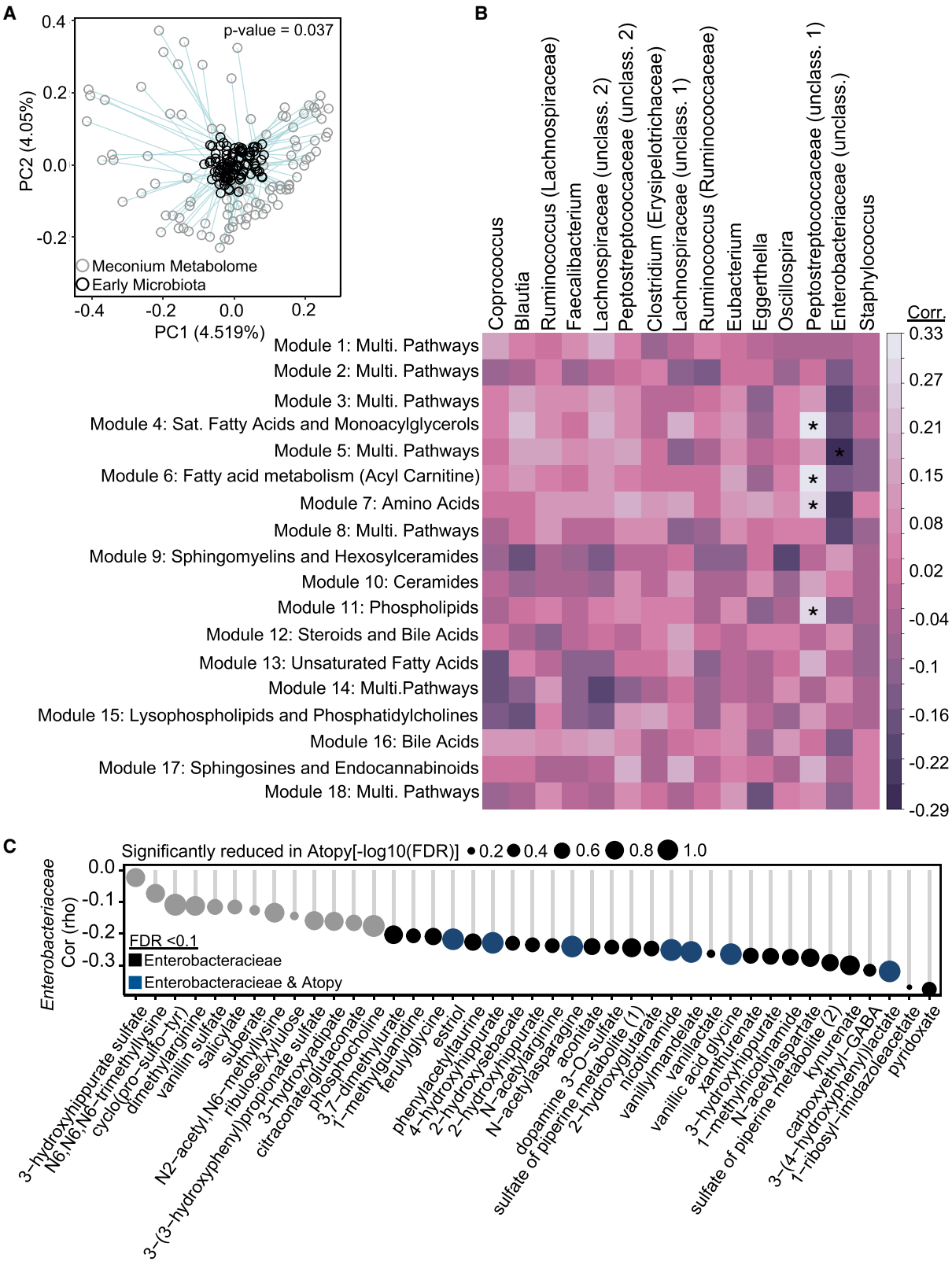
Figure 3. Two metabolic modules within the meconium are associated with protection from atopy at 1 year

(A) Eigenvalues of modules 5 and 8 for each sample between healthy controls and atopic infants (CTRL, n = 37; atopy, n = 63).

(B and C) Top-five metabolic Small Molecule Pathway Database (SMPDB) pathways from module 5 (B) and module 8 (C) that were enriched in control meconium compared with atopic meconium, according to MSEA. Enrichment ratio represents the ratio of the Q-statistic for each pathway to the expected statistic.

(D and E) Scatterplot of metabolite significance and module membership (defined as the Pearson correlation of each metabolite's abundance and respective to its module eigenvalue) of each metabolite within module 5 (D) and module 8 (E); red-dashed line indicates an FDR cutoff of 10%, which was the cutoff for statistical significance. All labeled metabolites are statistically significant.

*$p < 0.05$, **$p < 0.01$ using a two-tailed, unpaired Student's t test (A). Boxplot error bars represent 25th (bottom) and 75th (upper) quartiles.



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sensitization. In addition to having a less-mature microbiota early in life (Figure 2A), infants who developed atopy by 1 year of age had a less-diverse metabolic niche within their meconium at birth (Figure 2B). Reductions in diversity were limited to specific metabolic pathways, including steroids, amino acids, vitamins and cofactors, and nucleotides (Figure 2C). Using unsupervised clustering, we further identified highly correlated metabolite modules that were associated with an atopic response and/or colonization of the early-life microbiota.

Although overlap existed among some of these metabolites, most showed distinct patterns between the development of an atopic response and the early-life microbiota, suggesting independent influences on microbiota maturation and immune development. One example of this was the nicotinamide pathway, which was enriched in healthy controls in two separate metabolic modules (Figures 3B and 3C). Although nicotinamide has antioxidant properties that would support anaerobic colonization in the neonatal gut, it is also a downstream metabolite of the tryptophan-kynurenate pathway.[34,35] Kynurenate, which was also reduced in atopic meconium (false-detection rate [FDR]-corrected p value = 0.12), influences immune responses through Aryl hydrocarbon receptor (AhR) ligation and acts as a reactive oxygen species scavenger.[36] Similarly, mechanistic links can be proposed for steroids, which have been demonstrated to affect both microbes and immune function. Multiple bacteria are capable of using steroids as an energy source, including *Micrococcaceae* members, which may colonize fetal meconium in a limited capacity and influence immune development *in utero*.[12,37] Estriol, however, which is reduced within atopic meconium, has also been shown to skew immune cells toward tolerogenic responses.[38] Lastly, multiple amino acid metabolism pathways were reduced within the atopic cohort, including tyrosine, glycine, and serine. Amino acid metabolism and fermentation by pioneering bacteria at birth has been suggested to be a vital step in creating short-chain fatty acids, which support successive colonization of strict anaerobes within the neonatal gut.[2] Although a reduction in steroids and amino acid metabolism might directly affect neonatal immune development, altered levels of those metabolites may also affect keystone microbes that will influence the early microbiota community structure. Understanding prenatal factors determining infant meconium composition and uncoupling the direct versus indirect effects of different meconium metabolites on immune development and bacterial colonization within neonates could lead to timely interventions to prevent the development of allergic sensitization.

Our study design has a number of important strengths, including the prospective data and sample collection approach of the CHILD cohort, the objective clinical assessment of allergic sensitization though the use of standardized SPT, and the application of multi-omic approaches to define both the meconium metabolome and the early-life gut microbiota. Although studies have shown promise in their ability to alter allergic outcomes by modifying the microbiota, inherent technical difficulties involved with modulating the microbiota composition have prevented that from being a universally beneficial treatment. Furthermore, questions remain regarding the timing and mechanistic interactions that precede allergic sensitization. Here, we have identified metabolic shifts that are already present at birth, which are associated with both early microbiota colonization and immune development. These findings highlight the potential for restorative interventions that directly modulate key metabolites *in utero* to promote beneficial microbiota community structure and healthy immune development before they even begin to manifest signs and symptoms of allergic disease.

Limitations of study

Recognizing that the CHILD cohort is an observational human study, it is important to acknowledge the limitations of our study design. In addition, although the microbiota stool analysis encompassed data from more than 979 infants, the meconium metabolic profiling was limited to only 100 of those infants. Here, we report compelling associations suggesting that the trajectory of both microbiota maturation and allergic immune skewing may be significantly affected by metabolites in the neonatal gut at birth; however, those associations require validation through rigorous experimental interrogation and replication in independent human populations.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- METHOD DETAILS
  - Stool 16S sequencing and preprocessing
  - Meconium metabolomics
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Figure 4. Meconium metabolome influences early-life microbiota composition

(A) Procrustes analysis of the Euclidean distances of the meconium metabolome (open gray circles) and the late-microbiota composition (open black circles); p value based on 1,000 Monte Carlo iterations.

(B) Heatmap of Spearman correlation between metabolite abundance and relative abundance of top-15 important taxa for predicting age during the early visit. *FDR-corrected $p < 0.1$.

(C) Spearman correlation of the abundance of module 5 metabolites and *Enterobacteriaceae* abundance; circle size reflects the $-\log_{10}$(FDR) for atopic significance, metabolites with gray circles were not significant for either *Enterobacteriaceae* correlation or the atopy phenotype, metabolites with black circles were significant (FDR-corrected $p < 0.1$) for *Enterobacteriaceae* correlation, and metabolites with blue circles were significant (FDR-corrected $p < 0.1$) for *Enterobacteriaceae* correlation and atopy phenotype.

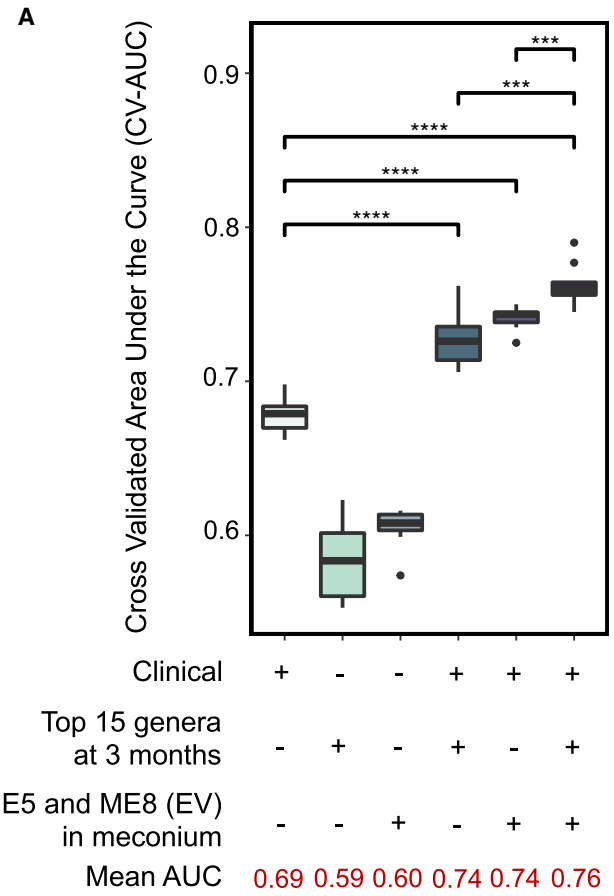

Figure 5. Meconium metabolome and microbiome composition can predict the development of atopy at 1 year

(A) Comparison of CV-AUC for clinical (parental atopy, ethnicity, gender, older sibling, and prenatal smoking), microbiome (top-15 important taxa relative abundance at 3 months), and meconium (eigenvalues for modules 5 [ME5] and 8 [ME8]) included in the LASSO-logistic model.

*p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001 using a Wilcoxon test (A). Boxplot error bars represent 25th (bottom) and 75th (upper) quartiles.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2021.100260.

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AUTHOR CONTRIBUTIONS

C.P., B.B.F., and S.E.T. conceived the synthesis of meconium metabolic profiling and early-life microbiota composition. C.P., D.L.Y.D., R.C.T.B., H.S., S.E.T., and B.B.F. planned the analyses and interpreted the data. M.R.S., T.J.M., A.B.B., M.B.A., P.J.M., P.S., and S.E.T. designed and performed the CHILD cohort study. All authors contributed to writing and reviewing the paper, have approved it for submission, and agree to be accountable for its content.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure ethnic or other types of diversity in the recruitment of human subjects. We worked to ensure that the study questionnaires were prepared in an inclusive way. One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. The author list of this paper includes contributors from the location where the research was conducted who participated in the data collection, design, analysis, and/or interpretation of the work.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Raw 16S rRNA sequence data	CHILD database	NCBI accession number: PRJNA657821
Oligonucleotides		
16S rRNA-Forward Primer 515F: GTGCCAGCMGCCGCGGTAA		N/A
16S rRNA-Reverse Primer 806R:GGACTACHVGGG TWTCTAAT		N/A
Software and algorithms		
GraphPad Prism 8	GraphPad	https://www.graphpad.com
QIIME2	Bolyen et al.[39]	https://qiime2.org/
RStudio	R	https://www.rstudio.com
WGCNA	Langfelder and Horvath[29]	https://cran.r-project.org/web/packages/WGCNA/WGCNA.pdf
ape	Pradis and Schliep[40]	https://cran.r-project.org/web/packages/ape/ape.pdf
vegan	Oksanen et al.[41]	https://cran.r-project.org/web/packages/vegan/vegan.pdf

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, B. Brett Finlay (bfinlay@msl.ubc.ca).

Data and code availability

The accession number for the demultiplexed 16s rRNA sequencing data reported in this paper is BioProject: PRJNA657821.

All R code used in this paper is presented in Data S1 and S2.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

CHILD Cohort

The CHILD Cohort Study recruited 3621 pregnant women from four cities (Vancouver, Edmonton, Winnipeg, Toronto) across Canada. Eligible infants (n = 3455) had no congenital abnormalities and were born at a minimum of 34 weeks of gestation. CHILD Cohort Study children were followed prospectively and detailed information on environmental exposures and clinical measurements and assessments were collected using a combination of questionnaires and in-person clinical assessments.[42–44]

All infants enrolled in the CHILD cohort protocol were administered a skin prick test at the one year scheduled visit. Children were then diagnosed with IgE-mediated allergic sensitization (also referred to as atopy) based on skin prick testing to multiple common food and environmental inhalant allergens, using $\geq$ 2 mm average wheal size as indicating a positive test relative to the negative control. Allergens tested at the one year visit were German cockroach, *Alternaria alternata*, house dust mites (*Dermatophagoides psteronyssinus* and *Dermatophagoides farnae*), cat hair, dog epithelium, cow's milk, peanut, egg white and soybean. Glycerin and histamine served as the negative and positive controls, respectively.

In the CHILD cohort protocol, the first stool passed after birth was collected as the meconium sample, subsequently stool was also collected at an early visit (median age: three months) and/or late visit (median age: one year). In this study we analyzed the stool microbiota in 979 infants and obtained high quality 16 s sequencing data from 950 infants. In a subset of 100 infants the meconium metabolome was also analyzed. See Tables S2 and S4 for the demographic features of these infants.

METHOD DETAILS

Stool 16S sequencing and preprocessing

The gut microbiota of infants in the CHILD cohort was defined as previously described.[20,31] Briefly, the V4 hypervariable region of the 16S rRNA gene was sequenced using universal primers (V4-515f: V4-806r). Paired-end sequences were pre-processed using Dada2 in Qiime2 v.2018.6 (https://www.qiime2.org).[39] Taxonomic identity was assigned to the resulting Amplicon Sequence Variants (ASVs) by alignment to the Greengenes reference (v13.8) database at 99% sequence similarity. Sequences were further filtered to remove sequences that were present at less than 0.005% of the total sequences. Samples were rarefied in R to 8,000 reads for downstream analyses.

Meconium metabolomics

100 mg of meconium from 37 healthy control infants and 63 atopy infants were stored at −80°C and sent to Metabolon, Inc (Morrisville, NC, USA) for non-targeted metabolic profiling via their mView Global Metabolomics Profiling Platform using Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS). Metabolite compounds were identified by automated comparison of the ion features in the meconium samples to an in-house library of chemical standards that included retention time, molecular weight (m/z), and associated MS spectra, and were visually inspected and curated for quality control using software developed at Metabolon. Known chemical entities were identified by comparison to Metabolon's library of purified standards.

WGCNA and MSEA

Meconium metabolite abundances were log-transformed and then weighted gene coexpression network analysis (WGCNA) was performed using the WGCNA package in R.[29] Positively correlated metabolites were clustered together using 'signed hybrid' networks and biweight midcorrelation. Modules that correlated with each other at 0.85 or greater were merged. An eigen value was then applied to each module based on its position along the PC1 axis. Our Metabolite Set Enrichment Analysis (MSEA) was performed using Metaboanalyst v.4 (https://www.metaboanalyst.ca/) using quantitative enrichment analysis which considers the abundance of the metabolites for each sample. For each module, a filtered data table was used that only included metabolites within the respective module and their corresponding log-normalized abundance for each sample, which were labeled as either control or atopy. Metaboanalyst classified metabolites and pathways by using the Small Molecule Pathway Database (SMPDB) (https://smpdb.ca/) before calculating an enrichment score. Enrichment scores represent the ratio of the Q-statistic for each pathway to the expected statistic of that pathway.

Predictive models

Predicted age was created from aggregated genera abundance across all early and late visit samples using a nested 10-fold cross-validated Random Forest Regressor in Qiime2 (https://docs.qiime2.org/2020.2/plugins/available/sample-classifier/regress-samples-ncv/). Genera were then ranked by importance for predictive accuracy and given an 'Importance' score that represented their proportional impact on model accuracy (all 'Importance' scores totaled to 1).Genera were then ranked by importance for predictive accuracy.

Penalized LASSO-logistic regression model (glmnet R package) was applied to candidate clinical, microbiota, and meconium factors to build classifiers, which select a subset of variables that are relevant predictors of atopy at one year. Performance characteristics of the classifier was evaluated using area under the curve (AUC) statistics from receiver operating characteristics (ROC) curves using 10-fold cross validation repeated 10 times. Ensembling was used to combine classifiers by using average probabilities from the individual classifiers. Clinical characteristics, parental atopy, smoking, ethnicity, children and mother antibiotics use, breastfeeding status at 6 months, and mode of delivery, were compared between children with and without atopy at 1 year of age using the Wilcoxon rank sum test for continuous variables and Fisher's exact test for categorical variables (Tables S1 and S4). Significant variables were selected as candidate clinical factors including gender, parental atopy, prenatal smoking, having an older sibling, and ethnicity of the infants. Candidate Meconium and Microbiota factors initially included eigen values of Module 5 and Module 8 as well as the relative abundance of each of the top 15 most important taxa for predicting age.

Statistical analysis

With the exception of predictive age, all analyses were performed in R (version 3.5.1). Graphs were created using the R packages *ggplot2* and *ggpubr*. Parametric and non-parametric pairwise comparisons were made using the R package *rstatix*. Metabolic modules and bacterial correlations were evaluated using the R package *WGCNA*. Procrustes analysis was performed on the Euclidian distances of the meconium metabolome and the 16 s microbiota composition using packages *ape* and *vegan*. Statistical significance for Procrustes was determined using 'protest'. AUCs from LASSO-logistic regression model were created using the R package *ROCR*. Odds ratio of developing atopy at one year was calculated by first converting ASV richness and Metabolite richness values to units of interquartile change. Then the adjusted odds ratio (aOR) of each variable were calculated using a multivariate logistic regression using 'survival', 'MASS', 'lme4', and 'sjstats'. The x axis is the adjusted odds ratio for each variable illustrated on a log2 scale. Error bars represent the 95% confidence interval (CI). P values were based on the aORs.