Integrative analysis of the intestinal metabolome of childhood asthma

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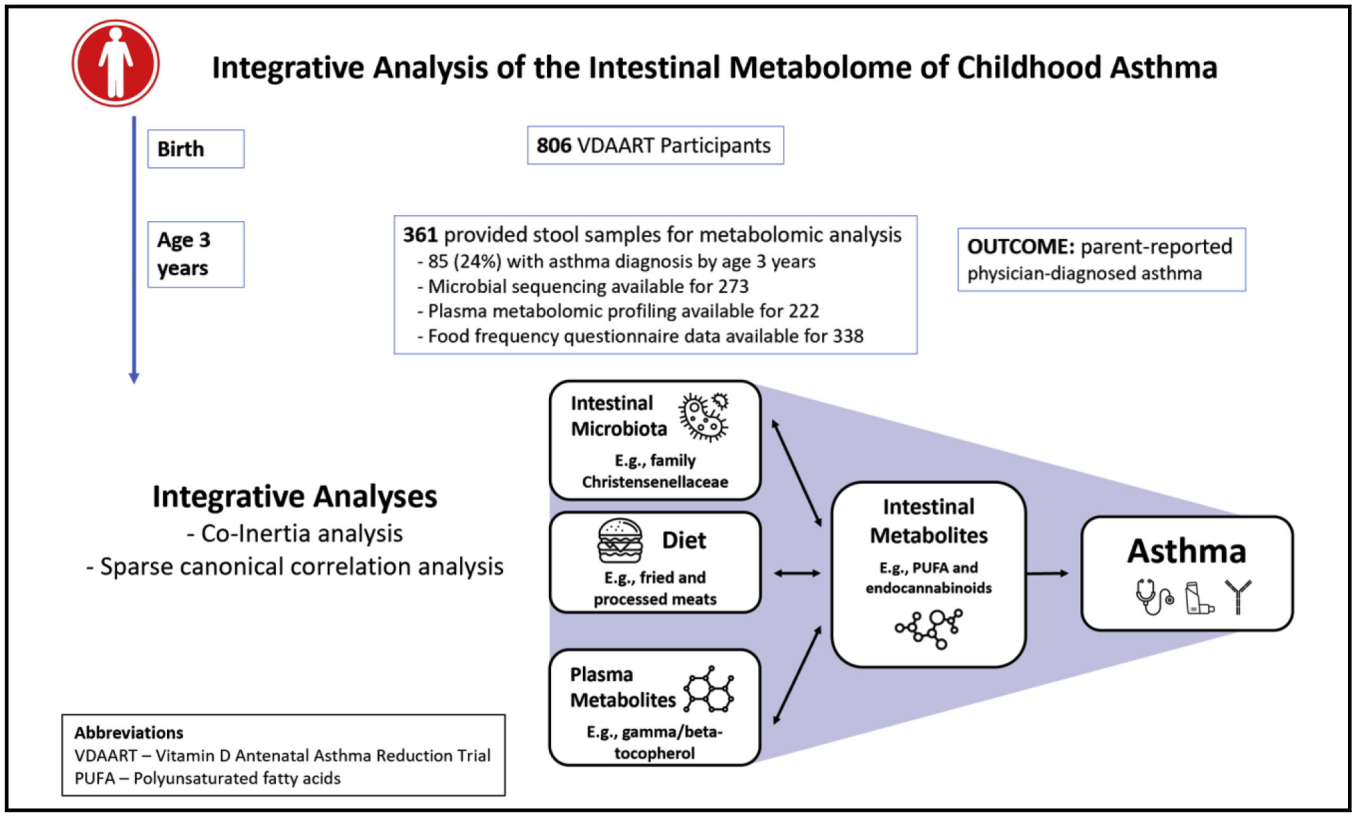
GRAPHICAL ABSTRACT



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Background: The intestinal metabolome reflects the biological consequences of diverse exposures and might provide insight into asthma pathophysiology.
Objective: We sought to perform an untargeted integrative analysis of the intestinal metabolome of childhood asthma in this ancillary study of the Vitamin D Antenatal Asthma Reduction Trial.
Methods: Metabolomic profiling was performed by using mass spectrometry on fecal samples collected from 361 three-year-old subjects. Adjusted logistic regression analyses identified metabolites and modules of highly correlated metabolites associated with asthma diagnosis by age 3 years. Sparse canonical correlation analysis identified associations relevant to asthma between the intestinal metabolome and other "omics": the intestinal microbiome as measured by using 16S rRNA sequencing, the plasma metabolome as measured by using mass spectrometry, and diet as measured by using food frequency questionnaires.
Results: Several intestinal metabolites were associated with asthma at age 3 years, including inverse associations between asthma and polyunsaturated fatty acids (adjusted logistic regression $\beta = -6.3$; 95% CI, -11.3 to -1.4; $P = .01$) and other lipids. Asthma-associated intestinal metabolites were significant mediators of the inverse relationship between exclusive breast-feeding for the first 4 months of life and asthma (P for indirect association = .04) and the positive association between a diet rich in meats and asthma ($P = .03$). Specific intestinal bacterial taxa, including the family Christensenellaceae, and plasma metabolites, including γ-tocopherol/β-tocopherol, were positively associated with asthma and asthma-associated intestinal metabolites.
Conclusion: Integrative analyses revealed significant interrelationships between the intestinal metabolome and the intestinal microbiome, plasma metabolome, and diet in association with childhood asthma. These findings require replication in future studies. (J Allergy Clin Immunol 2019;144:442-54.)

Key words: *Asthma, microbiome, Christensenellaceae, metabolome, vitamin E, nutrition, diet, breast-feeding*

Asthma is the most common chronic disease in children,[1] and each year, approximately 400,000 persons die from asthma worldwide.[2] Asthma is a complex disease, with dietary, genetic, microbial, and environmental factors all contributing to risk through mechanisms that remain incompletely understood.[3] These diverse exposures are reflected by the intestinal metabolome,[4] which provides a functional readout of their consequences.[5]

Intestinal metabolites are small molecules that can be derived from host, microbiota, or exogenous sources, including the diet. Metabolomics analysis has yielded novel insights into the molecular mechanisms of several human diseases.[6] Integrative analyses of the human intestinal metabolome with the diet, the intestinal microbiome, and the circulating metabolome reveals intricate relationships, providing insight into the basic physiology of clinically relevant metabolites and their contributions to pathogenic changes.[7] For example, in the context of cardiovascular disease, the metabolite trimethylamine-N-oxide is a product of intestinal microbial metabolism of dietary phosphatidylcholine that enters the systemic circulation and has been linked to development of atherosclerosis.[8] There might be similar undiscovered mechanisms that contribute to the pathophysiology of childhood asthma.

Abbreviations used
PUFA: Polyunsaturated fatty acid
VDAART: Vitamin D Antenatal Asthma Reduction Trial

We sought to gain insight into asthma pathophysiology by performing an untargeted analysis of the intestinal metabolome of childhood asthma in this ancillary study of the Vitamin D Antenatal Asthma Reduction Trial (VDAART), hypothesizing that asthma is associated with perturbations to the intestinal metabolome. In addition to characterizing the asthma-associated intestinal metabolome at age 3 years, we conducted integrative analyses to investigate relationships between the intestinal metabolome and other asthma-related "omics," including the intestinal microbiome, plasma metabolome, and diet.

METHODS

All methods are described in detail in the Methods section in this article's Online Repository at www.jacionline.org.

Study design and clinical outcome ascertainment

Subjects were offspring of participants in VDAART, a multisite, randomized, double-blind, placebo-controlled trial of vitamin D supplementation during pregnancy for the prevention of asthma and other allergic diseases in offspring conducted in the United States (NCT00920621).[9] The study protocol was approved by the institutional review boards at each participating institution and at Brigham and Women's Hospital. All participants provided written informed consent. The asthma outcome was based on parental report of a physician's diagnosis of asthma in the child's first 3 years of life. A flow diagram is displayed in Fig 1.[10]

Metabolomic profiling

VDAART participants followed after birth (n = 806) were asked to provide blood and stool samples at age 3 years. Stool samples were provided by 361 (45%) and blood samples were provided by 411 (51%) for metabolomics analysis. Stool was not collected if the child had used antibiotics in the past 7 days. For both stool and plasma samples, metabolomic profiling was performed at Metabolon (Research Triangle Park, NC), as previously described.[11] Additionally, 245 subjects, 156 of whom had available stool metabolomics, were selected for a prior analysis of plasma metabolomics using an additional quantitative lipids platform at Metabolon.[12] These were included in limited correlation analyses of intestinal and plasma lipids.

Bacterial microbiome profiling

DNA extraction and sequencing of the bacterial 16S V4 hypervariable region using the Illumina MiSeq platform (San Diego, Calif) were performed at Partners Healthcare Personalized Medicine (Boston, Mass). Additional processing of microbiome data was performed by using Qiime[13] and the Phyloseq package for R software.[14] Because it was recently shown that quantitative microbiome profiling is preferable to relative abundance profiling in co-occurrence analyses and in seeking disease associations,[15] quantitative PCR with universal 16S rRNA primers was performed at Partners Healthcare Personalized Medicine to estimate bacterial biomass concentration per stool sample for use in estimating quantitative species-level taxa abundances.

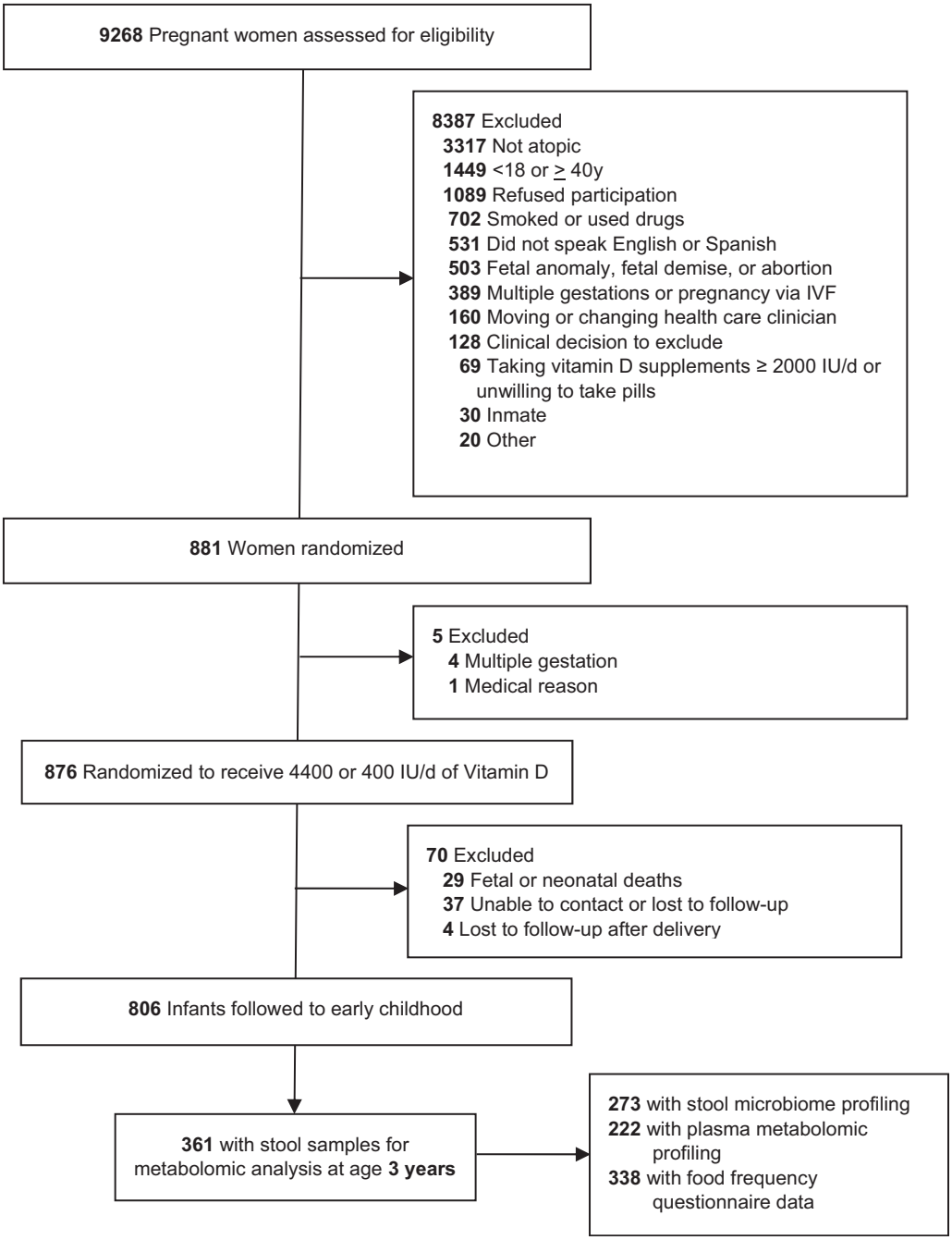

FIG 1. Flow diagram of subjects included in the study. A portion of this figure has been published previously.[10]

Dietary ascertainment

The child's diet was evaluated at age 3 years, when parents completed a modified version of a semiquantitative 87-item food frequency questionnaire that was previously validated in preschool-aged children.[16] Food frequency questionnaire responses were used to estimate total daily calorie intake, as detailed in the Methods section in this article's Online Repository.

Statistics

Details of statistical analyses are provided in the Methods section in this article's Online Repository. Statistical analyses were conducted with R (version 3.5.0; R Foundation for Statistical Computing, Vienna, Austria). Modules of highly correlated and likely functionally related intestinal metabolites were identified by using the weighted gene correlation network analysis R package (version 1.61).[17] Eigenvalues summarizing relative abundances of metabolites of each module for each subject were used in subsequent analyses. Multivariable logistic regression was used to determine the associations of metabolites and metabolite module eigenvalues with asthma. Covariates in adjusted analyses were selected on the basis of significant ($P < .05$) bivariate associations between these variables and asthma (Table I) and included sex, race/ethnicity, study center, maternal education, and gestational age, which has reported associations both with early microbiome development[18,19] and

TABLE I. Baseline characteristics tabulated for subjects who provided stool samples for metabolomics analysis and for the entire VDAART offspring cohort

	Stool metabolomics available				Entire VDAART cohort			
	All children (n = 361)	Asthma (n = 85)	No asthma (n = 276)	*P* value	All children (n = 806)	Asthma (n = 125)	No asthma (n = 623)	*P* value
Sex, no. (%)				.24				**.02**
Male	203 (56)	53 (62)	150 (54)		421 (52)	79 (63)	318 (51)	
Female	158 (44)	32 (38)	126 (46)		385 (48)	46 (37)	305 (49)	
Race/ethnicity, no. (%)				**.001**				**<.001**
Black, non-Hispanic	161 (45)	53 (62)	108 (39)		317 (39)	73 (58)	229 (37)	
White, non-Hispanic	71 (20)	13 (15)	58 (21)		161 (20)	17 (14)	130 (21)	
Hispanic or other	129 (36)	19 (22)	110 (40)		328 (41)	35 (28)	264 (42)	
Gestational age (wk), mean (SD)	38.7 (2.5)	37.8 (2.8)	39.0 (2.0)	**<.001**	39.0 (2.0)	38.3 (2.6)	39.1 (1.8)	**<.001**
VDAART treatment group, no. (%)				.87				.64
4400 IU/d vitamin D	182 (50)	44 (52)	138 (50)		405 (50)	60 (48)	316 (51)	
400 IU/d vitamin D	179 (50)	41 (48)	138 (50)		401 (50)	65 (52)	307 (49)	
Study center, no. (%)				**.006**				**.001**
Boston	82 (23)	19 (22)	63 (23)		240 (30)	39 (31)	175 (28)	
St Louis	178 (49)	53 (62)	125 (45)		292 (36)	62 (50)	227 (36)	
San Diego	101 (28)	13 (15)	88 (32)		274 (34)	24 (19)	221 (35)	
Maternal education, no. (%)				**.04**				**.005**
Less than high school	50 (14)	16 (19)	34 (12)		100 (12)	23 (18)	73 (12)	
High school or technical school	106 (29)	27 (32)	79 (29)		241 (30)	40 (32)	181 (29)	
Some college	84 (23)	24 (28)	60 (22)		192 (24)	35 (28)	137 (22)	
College graduate or higher	121 (34)	18 (21)	103 (37)		273 (34)	27 (22)	232 (37)	
Household income, no. (%)				**.02**				**.004**
<$30,000	118 (33)	35 (41)	83 (30)		236 (29)	47 (38)	176 (28)	
$30,000-$49,999	42 (12)	10 (12)	32 (12)		105 (13)	17 (14)	78 (13)	
$50,000-$74,999	38 (11)	9 (11)	29 (11)		97 (12)	12 (10)	77 (12)	
$75,000-$99,999	29 (8)	3 (4)	26 (9)		79 (10)	5 (4)	68 (11)	
$100,000-$149,999	38 (11)	4 (5)	34 (12)		62 (8)	6 (5)	53 (9)	
>$150,000	16 (4)	0 (0)	16 (6)		31 (4)	0 (0)	29 (5)	
Refused to say or unknown	80 (22)	24 (28)	56 (20)		196 (24)	38 (30)	142 (23)	
Total IgE, kU/L, mean (SD)	135 (494)	286 (941)	87 (183)	**<.001**	123 (434)	273 (882)	96 (271)	**.003**
Allergic sensitization, no. (%)	145 (48)	49 (67)	96 (42)	**<.001**	253 (47)	55 (63)	198 (44)	**.001**
Eczema, no. (%)	100 (28)	38 (45)	62 (22)	**<.001**	172 (23)	43 (36)	117 (19)	**<.001**
Recent steroid use, no. (%)	31 (9)	25 (29)	6 (2)	**<.001**	48 (6)	39 (31)	9 (1)	**<.001**

P values are for *t* tests for gestational age and total IgE levels and for χ^2 tests for all other comparisons. Total IgE levels were log-transformed before comparison by using the *t* test. Recent steroid use pertains to use of inhaled or systemic steroids in the 3 months before the study visit at age 3 years, during which stool samples were collected. Data on asthma status were missing for 58 subjects, none of whom provided stool samples for metabolomics. Gestational age was unavailable for 2 children. Total and serum specific IgE levels were missing for 272 and 265 subjects, respectively, including 62 and 59 subjects with stool metabolomics. Eczema at age 3 years was unavailable for 55 subjects, none of whom provided stool samples for metabolomics. Boldface indicates statistical significance.

asthma.[20,21] Total IgE levels, allergic sensitization, and eczema were associated with asthma but were considered characteristic of the outcome rather than confounders and as such were not included as covariates.

There are currently no consensus standards for multiple testing correction in metabolomics; methods such as Bonferroni correction and even more liberal corrections are considered too stringent for metabolomics data because of the high correlation of functionally related metabolites. We used a false discovery correction using a "number of effective tests" method that accounts for the highly collinear nature of metabolites[22,23]: we used principal components analysis of the metabolites to determine how many components are required to explain 50% of the variance in the data, with the rationale that correlated metabolites will load similarly to the same components. We consider the number of components, in this case 17, to be the number of effective tests and use this number to compute a multiple testing corrected *P* value (.05 divided by 17 = .0029).

Multivariable linear regression analyses were performed to determine associations between potential predictors of the intestinal microenvironment (eg, breast-feeding and antibiotics exposure) and asthma-associated metabolites and metabolite modules. Pearson correlation was used to analyze associations between plasma metabolites and corresponding intestinal metabolite module eigenvalues. Mediation analyses were performed for 2 purposes: (1) to estimate the direct association between breast-feeding and asthma and the indirect association mediated through intestinal metabolites and (2) to estimate the direct association between dietary variables and asthma and the indirect association mediated through intestinal metabolites. All tests were 2-sided, and the significance level was prespecified at a *P* value of less than .05, except where otherwise specified.

Integrative analyses

Coinertia analysis was used to compare global associations of the plasma metabolome, intestinal metabolome, and diet with the intestinal metabolome. Sparse canonical correlation analysis was used to identify intestinal metabolites that are associated with features of other data types (plasma metabolites, intestinal microbes, and dietary items) and with asthma. These methods are described in detail in the Methods section in this article's Online Repository.

RESULTS

Subjects' characteristics

To assess the intestinal metabolome of childhood asthma, we analyzed stool samples collected from offspring of VDAART participants. Stool samples were provided by 361 three-year-old

subjects, including 85 (24%) given a diagnosis of asthma by age 3 years (Table I). Asthmatic patients differed from those without asthma on several baseline characteristics among the entire VDAART cohort, and similar differences were seen among the subset who provided stool samples. Of subjects who provided stool samples, those with asthma versus no asthma were significantly more likely to be male (62% vs 54%), black (62% vs 39%), born at earlier gestational ages (mean [SD], 37.8 [2.8] vs 39.0 [2.0] weeks), and born to mothers with lower education (21% vs 37% with a college degree or higher) and lower-income households (41% vs 30% with income <$30,000). Asthma status was associated with study site (Boston, St Louis, or San Diego), and this was likely due to differences in race/ethnicity and socioeconomic status between sites. Asthma was also associated with atopy, including increased serum specific IgE levels (≥0.35 kU/L) to at least 1 common food or environmental allergen (67% vs 42%), diagnosis of eczema (45% vs 22%), and recent steroid use (29% vs 2%).

Early-life intestinal metabolites are associated with childhood asthma

A total of 737 intestinal metabolites were measured by using mass spectrometry and analyzed from stool samples collected at age 3 years. Logistic regression analyses adjusted for sex, race/ethnicity, study center, maternal education, and gestational age identified 45 metabolites that were significantly ($P < .05$) associated with asthma by age 3 years (see Table E1 in this article's Online Repository at www.jacionline.org). Of note, all but 3 of these 45 metabolites were inversely associated with asthma. Five of the 45 metabolites remained significant after correcting for multiple testing through a number of effective test methods, including a polyunsaturated fatty acid (PUFA), omega-6 docosapentaenoic acid, and 2 diacylglycerols (see Table E1).

Because steroid medication use could affect the intestinal metabolome and is expected to be greater in asthmatic patients, we added a covariate to the model to account for use of either inhaled or systemic steroids in the 3 months before turning 3 years old (see Table E1). Per parental report, 44 (12% of subjects) had used inhaled steroids, 15 (4%) had used systemic steroids, and a total of 48 (13% of subjects) had used either inhaled or systemic steroids. Associations between metabolites and asthma were not significantly changed after adjusting for steroid use (see Table E1).

To query the functional significance of these metabolites, we constructed a network of highly correlated and likely functionally related intestinal metabolites. Network construction was performed on all metabolites with no filtering on association with asthma. Analyses identified 54 modules of highly correlated intestinal metabolites (see Table E2 in this article's Online Repository at www.jacionline.org). Three modules of predominantly lipid metabolites ranging in size from 9 to 24 metabolites were inversely associated with asthma based on their eigenvectors, including a module of primarily diacylglycerols ($\beta = -6.5$; 95% CI, −11.8 to −1.4; $P = .01$), a module of primarily endocannabinoids ($\beta = -6.5$; 95% CI, −11.6 to −1.6; $P = .01$), and a module of primarily PUFAs ($\beta = -6.3$; 95% CI, −11.3 to −1.4; $P = .01$; Fig 2 and see Table E3 in this article's Online Repository at www.jacionline.org).

From these analyses, we concluded that several intestinal metabolites were associated with childhood asthma, with the majority of associated metabolites decreased in asthmatic patients. Modules of highly correlated asthma-associated lipid metabolites included PUFAs, endocannabinoids, and diacylglycerols.

Breast-feeding is a potential determinant of asthma-associated intestinal metabolites

To identify determinants of the asthma-associated intestinal metabolome, we examined potential predictors of the early-life intestinal microenvironment (Table II). In bivariate analyses those with asthma versus no asthma were more likely to have been exposed to perinatal antibiotics (52% vs 40%), less likely to have been exclusively breast-fed for the first 4 months of life (14% vs 37%), and more likely to have attended daycare by age 3 years (62% vs 50%). We identified no association between asthma and pet dog ownership, mode of delivery, or having at least 1 older sibling. In a logistic regression analysis adjusted for all potential predictors of the early-life intestinal microenvironment and for sex, race/ethnicity, maternal education, study site and gestational age, the only independent predictor of asthma was exclusive breast-feeding for the first 4 months of life, which was inversely associated with asthma (odds ratio, 0.36; 95% CI, 0.18-0.67; $P = .002$).

To investigate the possibility that intestinal metabolites mediate the association between breast-feeding and asthma, we looked for associations between asthma-associated metabolites and breast-feeding in linear regression analyses adjusted for potential confounders. Twelve intestinal metabolites were associated with asthma and breast-feeding in a directionally consistent manner (see Table E4 in this article's Online Repository at www.jacionline.org). Mediation analyses estimated that individual metabolites accounted for 9.0% to 23.2% of the association of breast-feeding with asthma, and 8 of the 12 metabolites were significant (P for indirect effect < .05) mediators of the association of breast-feeding with asthma (see Table E4). Seven of the 12 were diacylglycerols, and accordingly, the diacylglycerol metabolite module from network analysis was inversely associated with asthma and positively associated with breast-feeding ($\beta = 0.02$; 95% CI, 0.01-0.03; $P = .004$) in adjusted analyses. Mediation analysis estimated that 13% of the relationship between breast-feeding and asthma was mediated by the diacylglycerol intestinal metabolite module (P for indirect association = .04). In sum, this analysis identified lack of exclusive breast-feeding for the first 4 months of life as a risk factor for asthma that can act in part through intestinal metabolites, including diacylglycerols.

The intestinal metabolome is globally associated with the intestinal microbiome, plasma metabolome, and diet

We next sought to determine the extent to which the intestinal metabolome associates with other relevant "omics," including the intestinal microbiome, plasma metabolome, and diet. The intestinal bacterial microbiome was profiled by using quantitative 16S rRNA DNA sequencing on the same stool samples that underwent metabolomic profiling, plasma metabolomic profiling was performed by using mass spectrometry, and diet was assessed based on food frequency questionnaire responses. The number of subjects with each data type is shown in Fig 1.

Coinertia analysis revealed significant global similarities between the intestinal metabolome and other "omics" ($P < .02$ for

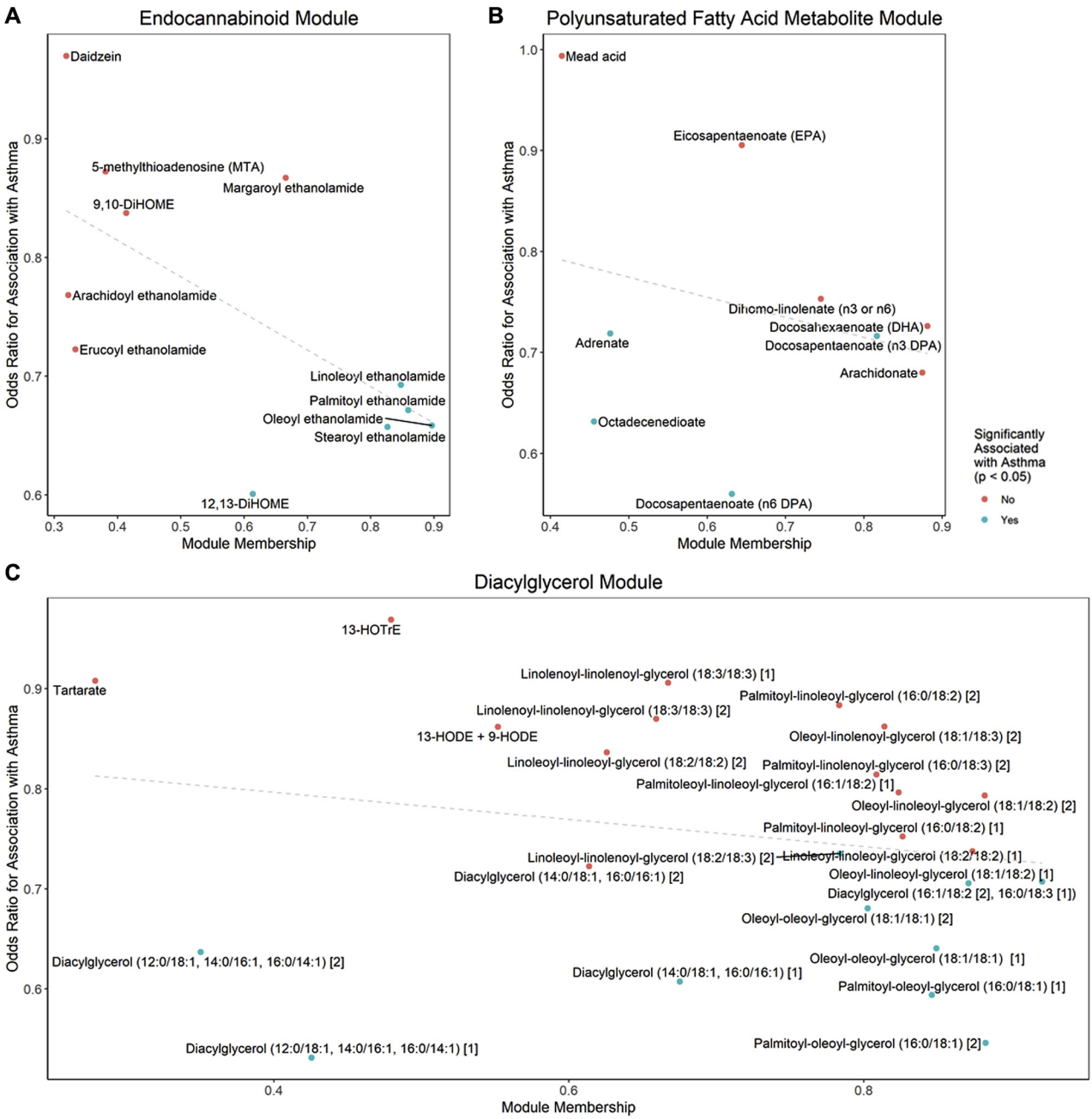

FIG 2. Members of the endocannabinoid **(A)**, PUFA **(B)**, and diacylglycerol **(C)** metabolite modules, which were inversely associated with asthma at age 3 years and are displayed by their association with asthma (*y-axis*, adjusted odds ratio) and by module membership (correlation of individual metabolite with module eigenvalue, *x-axis*).

all pairwise "omics" comparisons). This method generates an RV score that ranges from 0 to 1, with higher scores indicating greater global similarity between a pair of data sets. Diet and the plasma metabolome had similar associations with the intestinal metabolome (RV score, 0.22 and 0.21, respectively), and the intestinal microbiome had the greatest similarity with the intestinal metabolome (RV score, 0.35). These results suggest that the intestinal metabolome engages in the most significant interrelationships with the intestinal microbiome compared with the diet or plasma metabolome.

Intestinal bacterial taxa correlate with intestinal metabolites and with childhood asthma

We next sought to identify bacterial taxa that correlate with intestinal metabolites and asthma using sparse canonical

TABLE II. Bivariate analyses of the associations of asthma with potential determinants of the early-life intestinal metabolome

	Stool metabolomics available				Entire VDAART cohort			
	All children (n = 361)	Asthma (n = 85)	No asthma (n = 276)	*P* value	All children (n = 806)	Asthma (n = 125)	No asthma (n = 623)	*P* value
Maternal antibiotics during or child antibiotics after delivery, no. (%)	157 (43)	45 (53)	112 (41)	.06	337 (42)	65 (52)	248 (40)	**.01**
Birth by cesarean section, no. (%)	113 (31)	24 (28)	89 (32)	.57	239 (30)	40 (32)	185 (30)	.65
Exclusive breast-feeding until age 4 mo, no. (%)	109 (31)	12 (15)	97 (36)	**.001**	247 (33)	16 (14)	218 (37)	**<.001**
Daycare by age 3 y, no. (%)	178 (50)	47 (57)	131 (49)	.24	378 (53)	73 (62)	290 (50)	**.02**
Pet dog during infancy, no. (%)	88 (25)	17 (20)	71 (26)	.38	168 (22)	21 (18)	142 (24)	.20
At least 1 living child previously born to mother, no. (%)	205 (57)	50 (59)	155 (56)	.76	435 (54)	67 (54)	344 (55)	.82

Analyses were performed for subjects who provided stool samples for metabolomics analysis and for the entire VDAART offspring cohort. *P* values are for χ^2 tests. Perinatal antibiotics and mode of delivery data were unavailable for 3 subjects. Exclusive breast-feeding until age 4 months was unavailable for 68 subjects, including 13 with available stool metabolomics. Daycare status was unavailable for 99 subjects, including 8 with available stool metabolomics. Household dog status was unavailable for 49 subjects, including 4 with available stool metabolomics. Boldface indicates statistical significance.

correlation analysis, a method that identifies features of 2 data types (ie, intestinal metabolome and microbiome) available for the same set of subjects that are correlated with each other and with a clinical outcome (ie, asthma). This method generates a canonical variate by assigning nonzero canonical loadings to a limited number of intestinal metabolites and taxa, with the direction and magnitude of each feature's loading indicating the direction and strength of association with asthma and with other features with nonzero loadings. In this analysis, rather than seeking bacteria that are most strongly associated with asthma, we aim to identify highly or uniquely metabolically active bacteria that, by way of metabolic activity, associate with asthma.

Among stool samples collected at age 3 years, a canonical variate was based on 37 intestinal metabolites and 29 bacterial taxa that were associated with each other and with asthma. Canonical loadings for these metabolites and taxa were used to calculate intestinal metabolite and bacterial taxa canonical scores for each subject. Intestinal metabolite and bacterial taxa scores were positively associated with asthma after adjustment for sex, race/ethnicity, study center, maternal education, and gestational age (adjusted logistic regression $\beta = 0.20$ and .0002; $P = .005$ and .047, respectively) and were correlated with each other (Pearson $\rho = 0.65$, $P < .001$). Increased abundance of species of the family Christensenellaceae was most strongly associated with asthma and asthma-associated intestinal metabolites (Fig 3).

When modules of highly correlated metabolites were analyzed instead of individual metabolites, the canonical variate included the same 29 bacterial taxa with similar loadings (see Table E5 in this article's Online Repository at www.jacionline.org) in association with 8 modules of highly correlated metabolites. These 8 modules included the PUFA and endocannabinoid modules that were previously identified as associated with asthma and that loaded negatively on the canonical variate, which is consistent with their inverse association with asthma.

These analyses show that there is a strong association between the intestinal metabolome and microbiome that influences asthma risk at age 3 years. We found evidence that PUFAs, endocannabinoids, and Christensenellaceae family species participate in relevant microbiome-metabolome interactions.

Intestinal and plasma metabolomes associate with childhood asthma

We also applied sparse canonical correlation analysis to identify 41 intestinal and 37 plasma metabolites from age 3 years that correlated with each other (Pearson $\rho = 0.61$, $P < .001$) and with asthma (adjusted logistic regression $\beta = 0.25$ and 1.65; $P = .02$ and .003, respectively; Fig 4). Interestingly, γ-tocopherol/β-tocopherol was the highest-loading feature among both intestinal (loading = 0.31) and plasma (loading = 0.38) metabolites. However, this form of vitamin E, analyzed as an individual metabolite, was not significantly associated with asthma in adjusted logistic regression analyses either in stool (OR, 1.43; 95% CI, 0.83-2.56; $P = .21$) or plasma (OR, 1.05; 95% CI, 0.11-9.39; $P = .97$).

Recently, we found that plasma PUFAs were inversely associated with asthma at age 3 years in VDAART participants.[24] Given the present findings of inverse associations between intestinal PUFAs and other lipid metabolite modules and asthma, we hypothesized that plasma and intestinal lipids can be correlated. However, we found no correlation between plasma PUFAs (total, omega-3 or omega-6), endocannabinoids, or diacylglycerols and their corresponding intestinal metabolite module eigenvalues (see Table E6 in this article's Online Repository at www.jacionline.org). These findings suggest that intestinal and plasma lipids might relate to asthma risk through different mechanisms.

From these analyses, we concluded that interrelationships between the intestinal and plasma metabolomes associate with asthma at age 3 years. Vitamin E in the form of γ-tocopherol/β-tocopherol might, in concert with coexposures, have a significant influence on the asthma-associated metabolome.

Meat intake correlates with intestinal metabolites and childhood asthma

Finally, sparse canonical correlation analysis identified 7 foods in the diet and 41 intestinal metabolites that correlated with each other (Pearson $\rho = 0.40$, $P < .001$) and with asthma (adjusted logistic regression $\beta = 0.19$ and 0.50; $P = .01$ and .02, respectively; Fig 5). Asthma associations were preserved after adding body mass index and estimated total daily calorie intake to regression

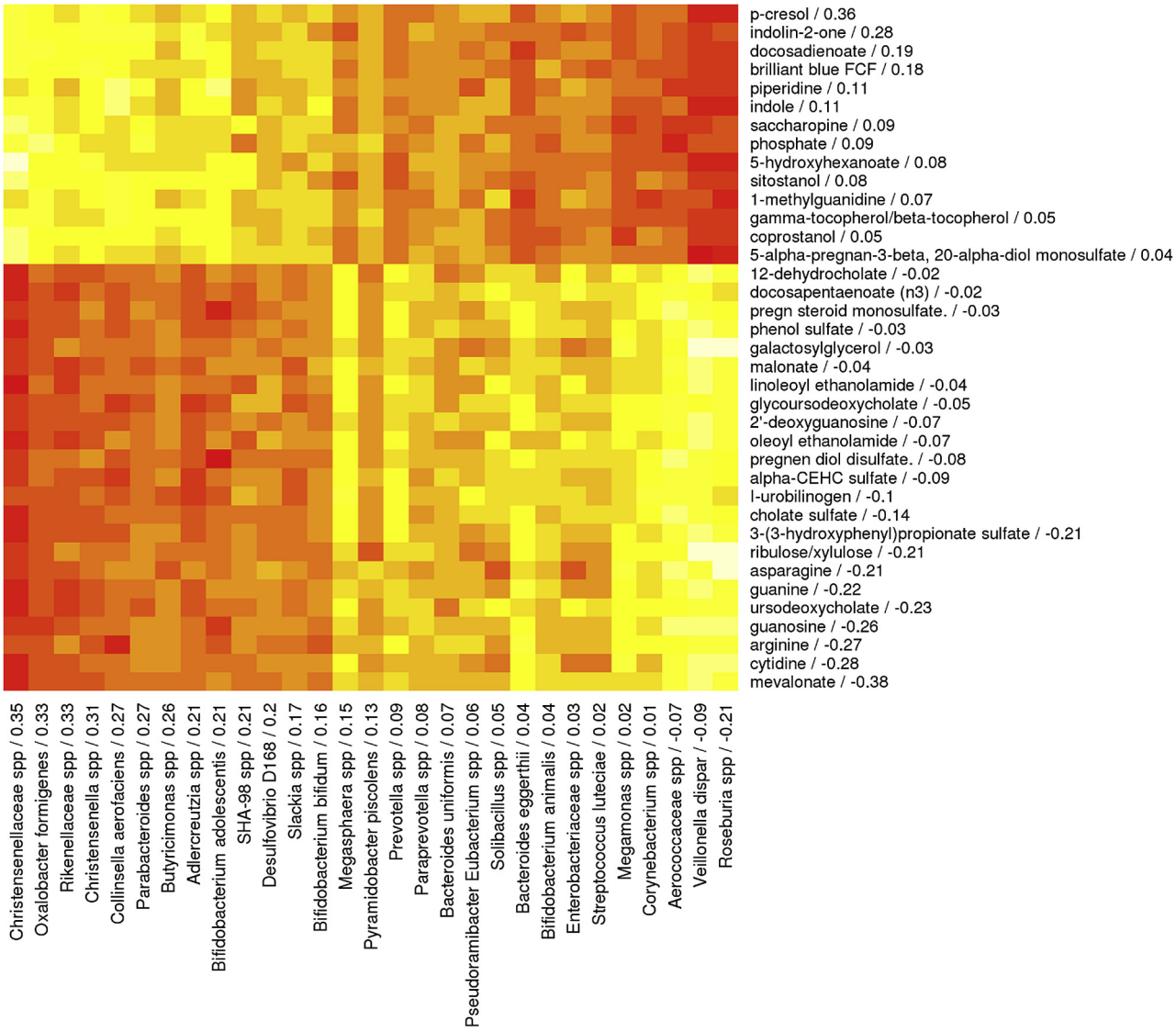

FIG 3. Spearman correlation heat map of intestinal metabolites *(rows)* and intestinal bacterial taxa *(columns)* that are correlated with each other and with asthma at age 3 years in sparse canonical correlation analysis. Canonical loadings are given for each feature after the feature name, with greater loadings indicating greater contribution to the canonical variate, and sign indicating direction of association. Red indicates negative correlations, and yellow indicates positive correlations. This analysis included 273 subjects with both plasma and stool metabolomics data available.

models ($\beta = 1.06$, $P = .003$ for foods and $\beta = 0.22$, $P = .002$ for metabolites). Foods with the highest positive loadings, indicating the most robust positive associations with asthma and intestinal metabolites, included fried and processed meats, such as fried chicken, chicken nuggets, hot dogs, and hamburgers (Fig 5). When modules of highly correlated metabolites were analyzed instead of individual metabolites, the canonical variate included the same 7 foods with similar loadings (see Table E7 in this article's Online Repository at www.jacionline.org) in association with 8 modules of highly correlated metabolites. These 8 modules included the diacylglycerol module that was previously identified as associated with asthma and that loaded negatively on the canonical variate, which is consistent with its inverse association with asthma. Mediation analysis estimated that 17% of the relationship between dietary score on these 7 foods and asthma was mediated by intestinal metabolite score (P for indirect association = .04). These analyses highlighted an association between a diet rich in meats, especially fried or processed meats, and asthma, with evidence that this association is mediated in part by intestinal metabolites.

Few intestinal metabolites correlate with the diet, intestinal microbiome, plasma metabolome, and asthma

Two metabolites were inversely associated with asthma and had negative loadings in all 3 sparse canonical correlation analyses (of associations between the intestinal metabolome

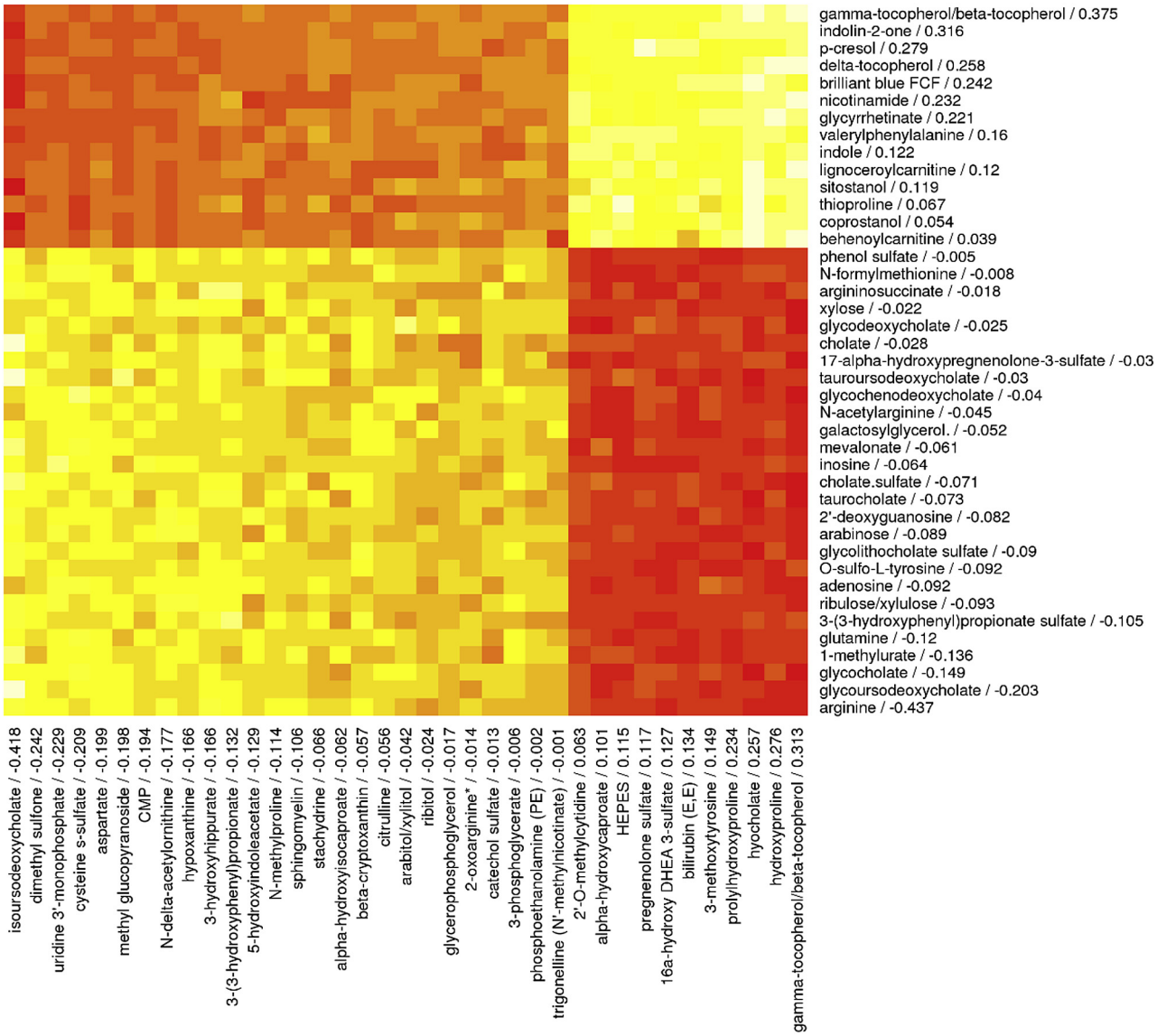

FIG 4. Spearman correlation heat map of intestinal metabolites *(rows)* and plasma metabolites *(columns)* that are correlated with each other and with asthma at age 3 years in sparse canonical correlation analysis. Canonical loadings are given for each feature after the feature name, with greater loadings indicating greater contribution to the canonical variate, and sign indicating direction of association. Red indicates negative correlations, and yellow indicates positive correlations. This analysis included 222 subjects with both plasma and stool metabolomics data available.

and the diet, plasma metabolome, and intestinal microbiome): phenol sulfate and galactosylglycerol. These might be central metabolites in asthma pathophysiology; however, given that only 2 such metabolites were found, in general, the intestinal metabolome is likely perturbed in different ways by asthma-associated plasma, dietary, and microbiome changes.

Of note, intestinal γ-tocopherol/β-tocopherol had positive loadings in all 3 sparse canonical correlation analyses, and plasma γ-tocopherol/β-tocopherol also had a positive loading; however, this form of vitamin E was not associated with asthma when analyzed as an individual metabolite. These findings suggest that this metabolite might act in concert with coexposures to influence systems-wide effects on asthma-associated "omics."

DISCUSSION

In this analysis of a relatively large and diverse sample of 3-year-old children, we characterized the intestinal metabolome of asthma. To our knowledge, this is the first report to integrate dietary, microbiome, and plasma metabolomics into an evaluation of the intestinal metabolome during childhood. Because the intestinal metabolome can be viewed as the functional readout of a variety of exposures, including the diet, the microbiome, and host physiology, we conducted integrative analyses to show that asthma-relevant interactions occur between the intestinal metabolome and the intestinal microbiome, plasma metabolome, and diet (Fig 6). Our findings, although biologically plausible, require replication in future studies.

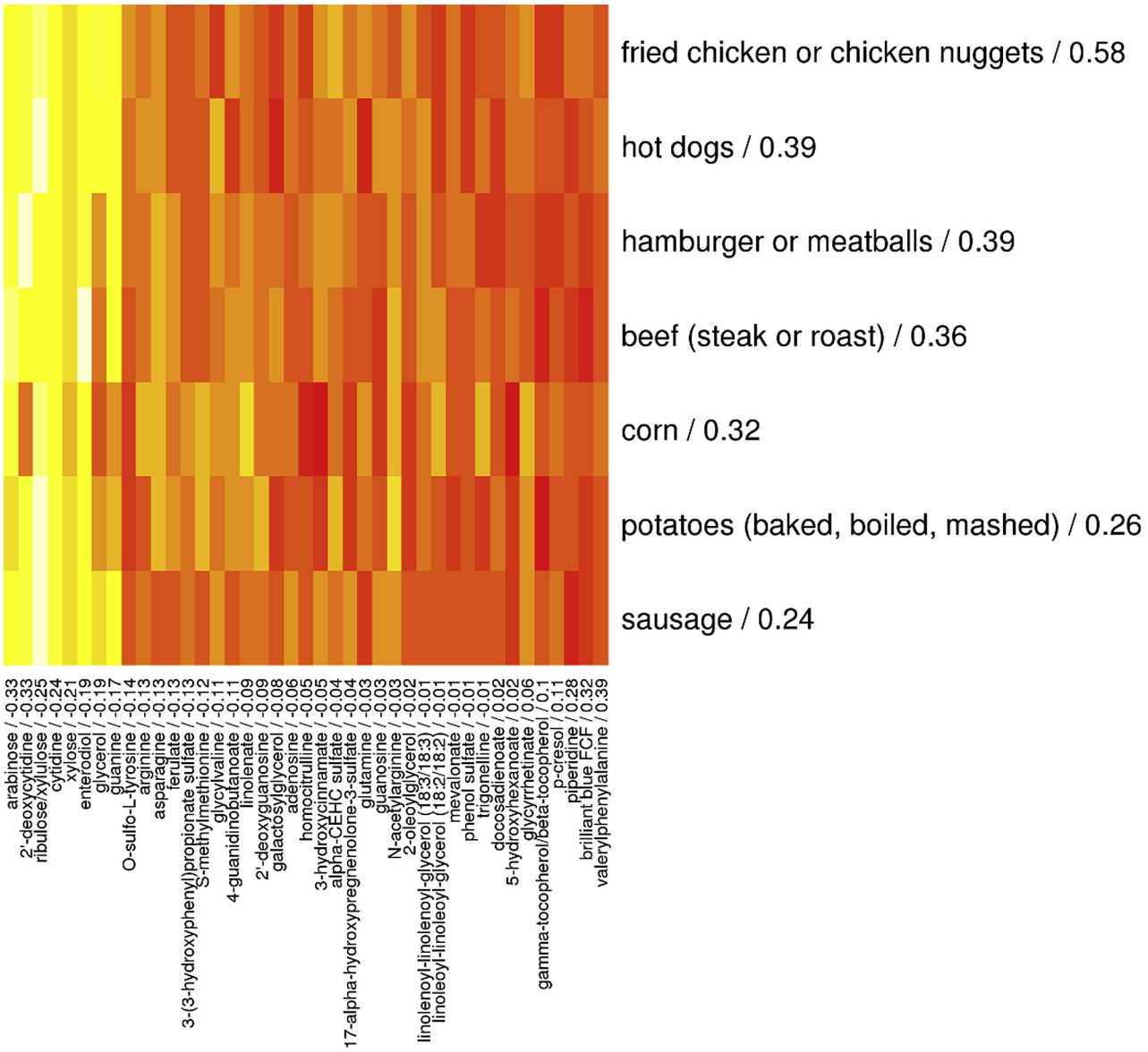

FIG 5. Spearman correlation heat map of foods *(rows)* and intestinal metabolites *(columns)* that are correlated with each other and with asthma at age 3 years in sparse canonical correlation analysis. Canonical loadings are given for each feature after the feature name, with greater loadings indicating greater contribution to the canonical variate, and sign indicating direction of association. Red indicates negative correlations, and yellow indicates positive correlations. This analysis included 338 subjects with food frequency questionnaire and stool metabolomics data available.

Most intestinal metabolites that were associated with asthma were reduced in asthmatic patients, which is consistent with prior literature showing early-life depletions of specific microbes, microbe functions and metabolites in children with asthma, atopy, or both.[25,26] Network analysis revealed inverse associations between asthma and 3 groups of intestinal lipids: PUFAs, diacylglycerols, and endocannabinoids. The role of PUFAs as precursors to immune-modulating eicosanoids and proresolving mediators lends biological plausibility to an association with asthma.[27] Indeed, dietary and circulating PUFAs have been associated with asthma, including in the VDAART cohort.[24] However, we found no association between plasma PUFAs and intestinal PUFAs in the present analysis. Although members of the human intestinal microbiota are not known to produce PUFAs, some participate in metabolism of PUFAs.[28,29] Therefore intestinal PUFAs likely represent dietary PUFAs, which are both in excess of the amount absorbed in the small intestine and not metabolized by the microbiota; that is, intestinal PUFA abundance is determined both by means of dietary intake and microbiota composition. Accordingly, we found that intestinal PUFAs correlated with the asthma-associated intestinal microbiome. These findings suggest that intestinal and plasma PUFAs relate to asthma risk through different mechanisms, with the former more reflective of bacterial dysbiosis.

Diacylglycerols are crucial intracellular signaling second messengers involved in functions, including immune cell signaling and cell growth.[30] Intestinal extracellular diacylglycerols influence colonic epithelial cell growth in the setting of

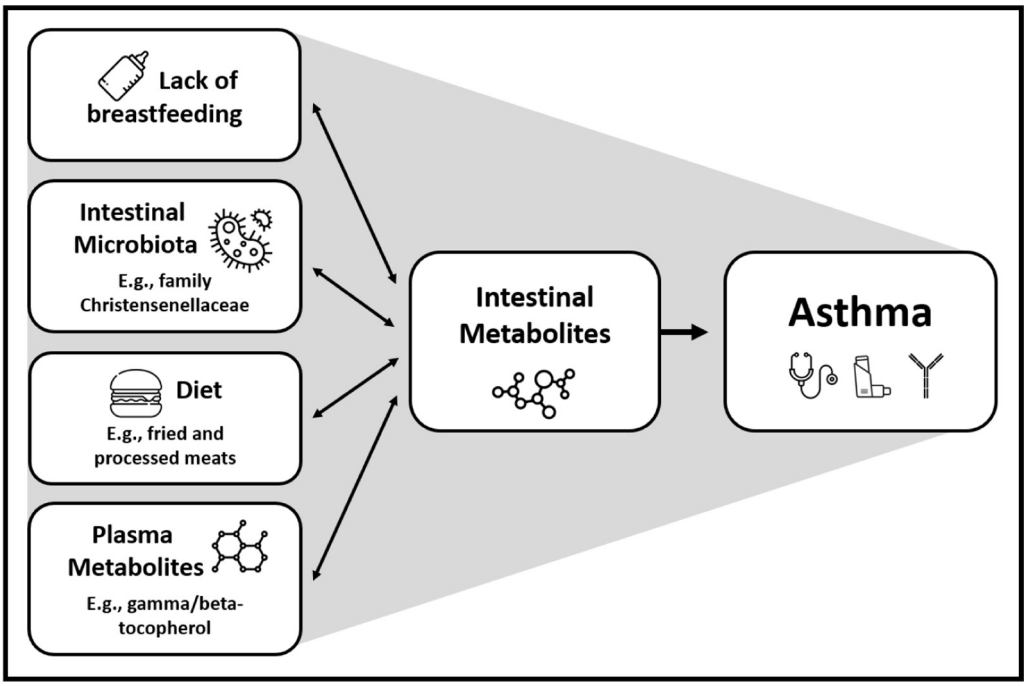

FIG 6. Schematic of results of integrative analyses of the asthma-associated intestinal metabolome. Note that a proposed causal pathway is depicted here and is one of several potential causal pathways that could explain the observed associations.

malignancy[31] and can be taken up from the intestinal lumen by host cells in other contexts. We report a novel association between intestinal diacylglycerols and childhood asthma and provide evidence that intestinal diacylglycerols mediate associations between dietary factors and asthma. Specifically, fecal diacylglycerols were mediators of the inverse association between breast-feeding and asthma. Breast-feeding has long-term influences on the intestinal microenvironment, lasting at least to school age.[32] Although there have been several reports of inverse associations between breast-feeding and asthma, this association has been inconsistent.[33-36] Some of this inconsistency could be explained by differences in other determinants of the intestinal microenvironment, such as the microbiota and subsequent diet, both of which influence fecal diacylglycerol abundances.[37] Consistent with this, we found that intestinal diacylglycerols were associated with an asthma-associated diet rich in fried and processed meats, providing evidence that both breast-feeding and subsequent diet can influence asthma risk by way of their effects on the intestinal metabolome. Diacylglycerols are used as emulsifiers in a wide range of foods, although we cannot determine whether the fecal diacylglycerols in our sample are of dietary, microbial, or host origin.

Endocannabinoids have both dietary and endogenous sources and are involved with broad systemic processes, including regulation of immune and metabolic functions.[38] In addition to an inverse association between intestinal endocannabinoids and asthma, we found that intestinal endocannabinoids correlated with the asthma-associated microbiome. The mechanism of these novel potential associations is unclear, and one could speculate involvement of effects on mast cell activation,[39] peroxisome proliferator–activated receptor α activity,[40] or eicosanoid mediators.[41]

We found that the intestinal microbiome had a strong global association with the intestinal metabolome, which is in keeping with other reports.[5] In particular, bacteria of the family Christensenellaceae were associated with asthma and asthma-associated intestinal metabolites. Christensenellaceae have been most strongly associated with lean (vs obese) weight.[42,43] To our knowledge, Christensenellaceae family species have not been previously associated with asthma, and this might be in part because prior analyses of the intestinal microbiome of asthma have focused on the microbiome in infancy[25,26,44,45] or adulthood,[46] which is in contrast to our study of early childhood. Interestingly, an analysis of TwinsUK data found that the family Christensenellaceae was the most heritable taxon analyzed,[42] raising the possibility that it could be a marker or consequence of genetic variants that associate with asthma.

We identified a potential multisystem role for intestinal γ-tocopherol/β-tocopherol, which had positive loadings in all sparse canonical correlation analyses; however, effects are likely indirect because this form of vitamin E was not associated with asthma when analyzed as an individual metabolite. Vitamin E has antioxidant properties and has been inconsistently linked to reduced risk of asthma.[47] In contrast and consistent with our findings, the γ-tocopherol form, which has increased in Western diets over recent decades,[48] has been associated with increased asthma risk or severity,[48-50] although some analyses find anti-inflammatory effects.[51] The systems effects of dietary vitamin E intake on the microbiome and metabolome are worthy of further investigation and might have relevance to asthma.

Finally, we found that a diet rich in fried and processed meats was associated with asthma and asthma-associated intestinal metabolites. Several prior studies have found positive associations between processed or fried meat consumption and asthma,[52-54] and one recent systematic review found that hamburger consumption had a particularly strong association

with asthma.[55] Our findings support a causal pathway whereby intestinal metabolites mediate at least part of the association between fried and processed meat consumption and asthma.

Some study limitations occurred. The outcome of asthma was based on parent-reported asthma diagnosis by age 3 years. Although preschool asthma is clinically important, this outcome might not accurately capture subjects whose asthma persists later in life.[56] We expect this potential misclassification to result in a bias toward the null and reduced power to detect relevant associations. Generalizability can be limited by the VDAART study inclusion criteria, which included parental history of asthma, eczema, or allergic rhinitis and maternal nonsmoking status. Intestinal metabolomics were not available for all VDAART subjects, although baseline characteristics were similar between subjects with available intestinal metabolomics and the overall cohort (Table I). Although subjects who had used antibiotics in the week before stool sample collection were excluded and we have performed sensitivity analyses to account for steroid use, we cannot rule out residual confounding by medication use. Although we adjusted for maternal education, we acknowledge the possibility of residual confounding by other socioeconomic factors. Current metabolomics analysis techniques do not reflect the entire intestinal chemical repertoire. Some metabolites, including most short-chain fatty acids, are too volatile or low concentration to be measured by using our metabolomic profiling method. Dietary analysis by means of food frequency questionnaire, although widely used, is subject to measurement bias. Causality cannot be inferred from these cross-sectional associations in 3-year-old subjects, and identified associations might not be relevant earlier or later in life. As VDAART participants continue to age, it will be important to follow-up our findings with longitudinal analyses.

In summary, we found that intestinal metabolites are associated with asthma at age 3 years. Lack of exclusive breast-feeding for the first 4 months of life was associated with increased risk of asthma and might act through perturbation of the intestinal metabolome. Integrative analyses revealed that the intestinal metabolome is strongly associated with the intestinal microbiome, and with regard to asthma, species of the family Christensenellaceae might play a role in this relationship. A diet rich in fried or processed meats correlates with asthma in an association that might be mediated in part by intestinal metabolites. Finally, vitamin E in the form of γ-tocopherol/β-tocopherol might have indirect but influential systems-wide effects on asthma risk. These findings are situated within a prior literature supporting their biological relevance and are worthy of further exploration to generate novel asthma biomarkers and treatment targets.

Key messages

- **Intestinal metabolites are associated with childhood asthma.**
- **The intestinal metabolome and microbiome correlate strongly.**
- **Associations of lack of breast-feeding and a meat-rich diet with asthma might be mediated by the intestinal microbiome through intestinal metabolites.**

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