

ORIGINAL ARTICLE

Food Allergy and Gastrointestinal Disease

Early-life fecal metabolomics of food allergy

Kathleen A. Lee-Sarwar^{1,2} | Yih-Chieh Chen^{1,2} | Jessica Lasky-Su¹ | Rachel S. Kelly¹  | Robert S. Zeiger³ | George T. O'Connor⁴ | Leonard B. Bacharier⁵ | Xiaojiong Jia² | Avraham Beigelman^{6,7} | Diane R. Gold^{1,8} | Nancy Laranjo¹ | Supinda Bunyavanich^{9,10} | Scott T. Weiss² | Augusto A. Litonjua¹¹  | Patrick J. Brennan²

¹Channing Division of Network Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA

²Division of Allergy & Clinical Immunology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA

³Department of Clinical Science Kaiser Permanente Bernard J. Tyson School of Medicine, Pasadena, California, USA

⁴Pulmonary Center and Department of Medicine, Boston University School of Medicine, Boston, Massachusetts, USA

⁵Department of Pediatric Allergy, Immunology, and Pulmonary, Vanderbilt Children's Hospital, Vanderbilt University Medical Center, Nashville, Tennessee, USA

⁶Schneider Children's Medical Center of Israel, Tel Aviv University, Tel Aviv, Israel

⁷Division of Pediatric Allergy, Immunology & Pulmonary Medicine, Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri, USA

⁸Department of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA

⁹Division of Allergy and Immunology, Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York, New York, USA

¹⁰Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, New York, USA

¹¹Division of Pediatric Pulmonary Medicine, Golisano Children's Hospital at Strong, University of Rochester Medical Center, Rochester, New York, USA

Correspondence

Kathleen A. Lee-Sarwar, Channing Division of Network Medicine, Brigham and Women's Hospital, 181 Longwood Ave, Boston, MA 02115, USA.
Email: klee-sarwar@partners.org

Present address

Yih-Chieh Chen, Vertex Pharmaceuticals, Boston, Massachusetts, USA

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Abstract

Background: Intestinal microenvironmental perturbations may increase food allergy risk. We hypothesize that children with clinical food allergy, those with food sensitization, and healthy children can be differentiated by intestinal metabolites in the first years of life.

Methods: In this ancillary analysis of the Vitamin D Antenatal Asthma Reduction Trial (VDAART), we performed untargeted metabolomic profiling in 824 stool samples collected at ages 3–6 months, 1 year and 3 years. Subjects included 23 with clinical food allergy at age 3 and/or 6 years, 151 with food sensitization but no clinical food allergy, and 220 controls. We identified modules of correlated, functionally related metabolites and sought associations of metabolite modules and individual metabolites with food allergy/sensitization using regression models.

Results: Several modules of functionally related intestinal metabolites were reduced among subjects with food allergy, including bile acids at ages 3–6 months and 1 year, amino acids at age 3–6 months, steroid hormones at 1 year, and sphingolipids at age 3 years. One module primarily containing diacylglycerols was increased in those with food allergy at age 3–6 months. Fecal caffeine metabolites at age 3–6 months, likely

Augusto A. Litonjua and Patrick J. Brennan contributed equally to this work.

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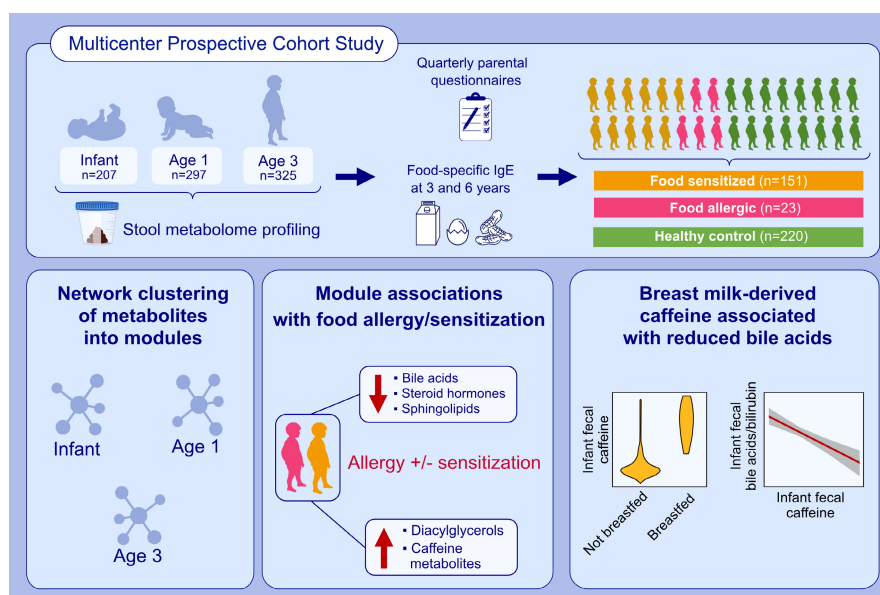
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derived from breast milk, were increased in those with food allergy and/or sensitization (beta = 5.9, 95% CI 1.0–10.8, $p = .02$) and were inversely correlated with fecal bile acids and bilirubin metabolites, though maternal plasma caffeine levels were not associated with food allergy and/or sensitization.

Conclusions: Several classes of bioactive fecal metabolites are associated with food allergy and/or sensitization including bile acids, steroid hormones, sphingolipids, and caffeine metabolites.

KEYWORDS

bile acids, caffeine, food allergy, food sensitization, metabolomics



GRAPHICAL ABSTRACT

This analysis identifies associations between the fecal metabolome at ages 3–6 months, 1 and 3 years with food allergy and/or sensitization. Fecal metabolites including bile acids, sphingolipids, and caffeine metabolites are associated with food allergy/sensitization. Results suggest that breast-milk derived intestinal caffeine may impact other fecal metabolites and thereby be associated with risk of food allergy/sensitization.

1 | INTRODUCTION

Food allergy is prevalent, costly and has a major impact on quality of life.¹ It is a complex disease with diverse risk factors, and a growing body of literature supports causal and likely interconnected roles of dietary factors² and the intestinal microbiome³ in food allergy pathogenesis. The intestinal metabolome refers to the collection of small molecules present in the gut, and reflects diverse exposures relevant to food allergy risk, including the diet⁴ and microbiome.⁵ Interrogation of the intestinal metabolome and its associations with food allergy could provide insight into mechanisms of food allergy pathogenesis, including progression from food sensitization to development of clinical food allergy. Prior studies of the metabolomics of food allergy have identified associations with fecal diacylglycerols⁶ and lipids,^{7,8} and with circulating microbial-derived metabolites

including bile acids and sphingolipids⁹; however, in part due to study heterogeneity, a metabolomic signature of food allergy has not been established.

We previously reported that in participants in the Vitamin D Antenatal Asthma Reduction Trial (VDAART), fecal metabolites at age 3–6 months, including a depletion in *Bacteroides*-derived sphingolipids, were associated with food allergy by age 3 years.¹⁰ Here, we extend our analysis to include a larger number of stool samples with metabolomic profiling from ages 3–6 months, 1 year and 3 years, and leverage clinical data from up to age 6 years to determine food allergy and sensitization phenotypes in a larger subsample of VDAART participants. We identified reductions in fecal metabolite classes with biologically plausible roles in allergy inception, including bile acids, steroid hormones, and sphingolipids in association with food allergy. In addition, we found that caffeine metabolites that are likely

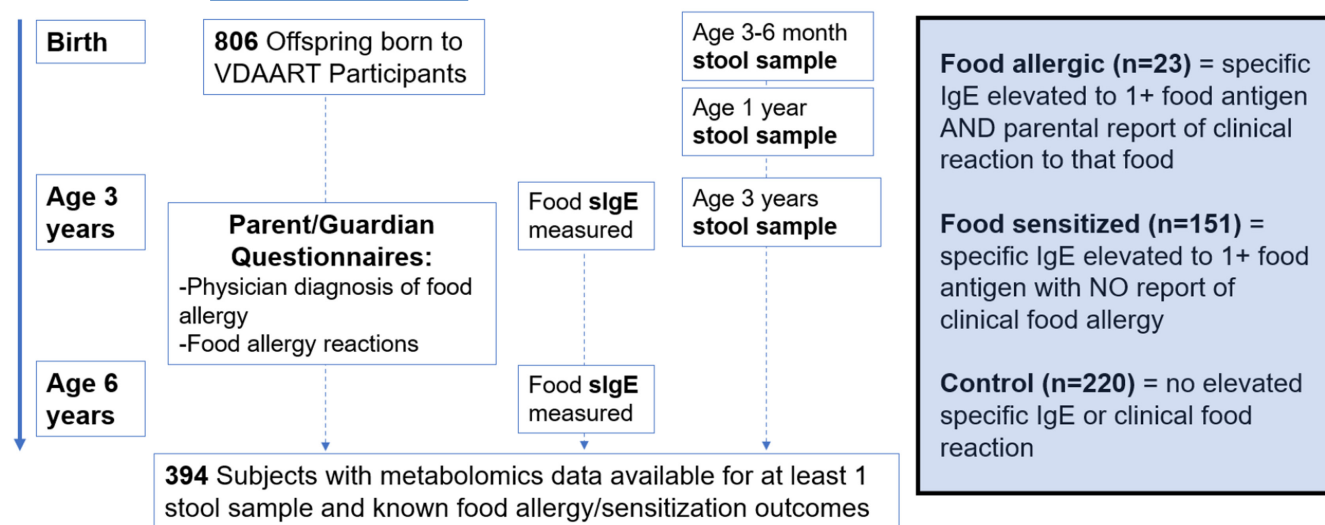


FIGURE 1 Study design overview. sIgE, serum specific IgE to foods.

derived from maternal breast milk are enriched in stool samples of infants who developed food allergy and/or sensitization compared to control subjects and may impact disease risk via effects on other metabolites including bile acids.

2 | METHODS

2.1 | Study design

Detailed methods are available in the Appendix S1. Subjects were offspring of participants in VDAART, a randomized controlled trial of Vitamin D supplementation during pregnancy to prevent asthma in offspring conducted in the United States (St. Louis, Boston, and San Diego; NCT00920621).¹¹ 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.

All subjects were asked to provide a blood sample at ages 3 and 6 years for measurement of serum specific IgE to egg white, walnut, milk, peanut, soybean, and wheat. Parents completed questionnaires quarterly between birth and age 3 years and then annually in which they reported whether subjects had been diagnosed with food allergy by a healthcare provider, which food the subject was allergic to, and symptoms of interim food allergy reactions. Food sensitization was defined as specific IgE ≥ 0.35 kU/L to at least one food allergen and no parent-reported food allergy reactions. Food allergy was defined as parental report of diagnosed allergy to milk, egg, wheat, soy, peanut, or other nuts with IgE ≥ 0.35 kU/L to the same food. Control subjects had neither food sensitization nor food allergy at any time point.

Metabolomic profiling was performed by Metabolon, Inc. on stool samples collected at age 3–6 months, 1 year and 3 years as previously described.^{12,13} Plasma metabolomic profiling was performed

on maternal blood samples collected during the third trimester of pregnancy and when offspring were 1 year old.

2.2 | Statistical methods

Because race/ethnicity was associated with food allergy/sensitization, it was included as a covariate in all analyses unless otherwise specified. We additionally adjusted for exact age at stool sample collection in analyses of samples collected at age 3–6 months. False discovery rate (FDR) correction was applied to address multiple testing in analyses of individual metabolites and FDR < 0.05 was considered statistically significant. In all other analyses, a threshold of $p < .05$ determined statistical significance.

As metabolites belonging to the same class or with similar functions are expected to be correlated, we constructed a network of highly correlated metabolites using the weighted gene correlation network analysis (WGCNA) R package.¹⁴ We identified associations of individual metabolites and metabolite modules with clinical outcomes in pairwise adjusted logistic regression analysis. Subsequent analyses focused on an infant fecal metabolite module consisting of predominantly caffeine metabolites that was associated with food allergy/sensitization. We identified associations of the infant fecal caffeine metabolite module with breastfeeding status using t tests. We used Pearson correlation to seek associations between maternal plasma caffeine relative abundance in the third trimester of pregnancy and when offspring were 1 year old with one another and with the infant fecal caffeine metabolite module. We sought associations between maternal plasma caffeine and food allergy/sensitization in adjusted logistic regression analyses. Finally, to identify potential confounders of the association between early-life caffeine exposure and clinical outcomes, we sought associations of caffeine exposures (maternal plasma and infant fecal caffeine) and of food allergy/sensitization with maternal characteristics using

Wilcoxon rank sum tests and Spearman correlation for categorical and continuous variables, respectively.

3 | RESULTS

3.1 | Subject characteristics

A total of 394 subjects had known food allergy/sensitization status and fecal metabolomics data available from at least one stool sampling time point, and these subjects were similar in baseline characteristic to the overall VDAART cohort (Figure 1, Table 1). There were 220 (56%) control subjects who were neither food sensitized nor food allergic, 23 (6%) subjects with food allergy and 151 (38%) with food sensitization. Table S1 displays the clinical history and allergen-specific IgE for subjects with food allergy. No subjects with food allergy were diagnosed before 9 months of age, so the age 3–6 month stool sample collection time point preceded disease onset.

The majority of subjects with food allergy or sensitization were of black race (54% of sensitized, 52% of allergic vs 38% of control, Fisher exact test $p = .03$, Table 1). Accordingly, subsequent analyses were adjusted for race/ethnicity. Otherwise, subjects with food allergy, sensitization or healthy controls exhibited comparable baseline characteristic distributions (Table 1). There were no significant differences between groups in microbiome-modifying environmental exposures including mode of delivery, perinatal antibiotic exposure, pet dog or older siblings, breastfeeding, or daycare history (Table 1). As expected, subjects with food allergy were most likely to have eczema (86% vs 55% of sensitized and 56% of control, Fisher exact test $p = .01$), and asthma (70% vs 31% of sensitized and 28% of control, Fisher exact test $p < .01$).

Stool samples were collected for fecal metabolomics profiling at ages 3–6 months ($n = 207$ samples profiled in 2 batches), 1 year ($n = 292$ samples), and 3 years ($n = 325$ samples, Figure S1). The number of named metabolites detected at each stool sampling time point ranged from 647 to 751. The majority of metabolites were amino acids or lipids; metabolite class distributions are displayed in Figure S2A.

3.2 | Fecal metabolite associations with food allergy/sensitization

In pairwise comparisons of phenotypes (food allergic vs sensitized; food sensitized vs control; and food allergic vs control), we identified numerous metabolites nominally ($p < .05$) associated with outcomes, though none remained statistically significant after correction for multiple testing ($FDR > 0.05$, Figure S3). Short-chain fatty acid metabolites including acetate, propionate and butyrate were not associated with food allergy or sensitization and were not included in subsequent analyses ($p > .05$ for pairwise phenotype comparisons at each time point, Figure S2B).

Absence of highly significant associations with individual metabolites does not preclude the possibility that groups of functionally

related fecal metabolites could be associated with food allergy or sensitization. To identify groups of functionally related metabolites, we performed weighted gene correlation network analysis (WGCNA),¹⁴ which yielded 26 metabolite modules from samples collected at age 3–6 months, 30 from age 1 year and 27 from age 3 years (Table S2). Modules that differed by food allergy/sensitization phenotype (adjusted logistic regression $p < .05$) are displayed in (Figures S4 and S5). At age 3–6 months, 3 fecal metabolite modules were associated with clinical food allergy: one module included bilirubin metabolites and bile acids that were reduced in association with food allergy compared to both controls (module OR = 0.26, 95% CI 0.08–0.66, $p = .01$) and those with food sensitization (module OR = 0.41, 95% CI 0.17–0.87, $p = .03$); another module included amino acids (including 16 of the 20 proteinogenic amino acids) and the sphingolipid 3-ketosphinganine, which were lower in those with food allergy compared to control subjects (module OR = 0.48, 95% CI 0.21–0.96, $p = .048$); and a final module included several diacylglycerols that were increased in those with food allergy compared to control subjects (module OR = 2.53, 95% CI 1.17–6.20, $p = .02$). Only one fecal metabolite module from age 3–6 months differed between subjects with food sensitization and control subjects; most of the metabolites in this module were caffeine metabolites that were increased in subjects with food sensitization compared to control subjects (OR = 1.66, 95% CI 1.03–2.68, $p = .04$) and also borderline increased in subjects with food allergy compared with control subjects (OR = 2.92, 95% CI 0.94, 11.23, $p = .08$).

In analyses of fecal metabolite modules from stool samples collected at ages 1 and 3 years, we found several modules of metabolites that were reduced in subjects with food allergy compared with those with food sensitization and control subjects (Figure S5). Of modules from age 1 year, 3 modules consisted of metabolites that were reduced in association with food allergy: two modules of bile acids (bile acid module 1: OR = 0.15, 95% CI 0.05–0.40, $p < .001$ and OR = 0.12, 95% CI 0.04, 0.36, $p < .001$; bile acid module 2: OR = 0.27, 95% CI 0.06–0.91, $p = .06$ and OR = 0.27, 95% CI 0.06–0.90, $p = .05$ for comparison to controls and sensitized, respectively), and a module consisting of a mix of bile acids and steroid hormones (OR = 0.39, 95% CI 0.15–0.98, $p = .05$ and OR = 0.14, 95% CI 0.03–0.51, $p = .004$ for comparison to controls and sensitized, respectively). Finally, a module from stool samples collected at age 3 years included sphingolipids that were reduced in subjects with food allergy (OR = 0.24, 95% CI 0.08–0.66, $p = .01$ and OR = 0.26, 95% CI 0.09–0.64, $p = .01$ for comparison to controls and sensitized, respectively). In sensitivity analyses excluding 3 subjects with unknown food allergy reaction signs/symptoms (subjects 10, 11, and 14 in Table S1), metabolite module associations with food allergy/sensitization were preserved, albeit with attenuated statistical significance as expected with reduced sample size (Table S3).

We previously reported on a group of fecal sphingolipids from age 3–6 months that was higher in subjects with food sensitization than those with food allergy or controls, in a pilot analysis utilizing a subset of the current data (fecal metabolomics data from 83 stool samples collected at age 3–6 months with clinical follow-up only

TABLE 1 Baseline characteristics of subjects with food allergy phenotype information and fecal metabolome data from at least one time point and the overall VDAART cohort

	Analyzed subjects n = 394	Food allergic n = 23	Food sensitized n = 151	Control n = 220	p value	VDAART cohort n = 806
Male sex - number (%)	220 (56)	14 (61)	83 (55)	123 (56)	.88	421 (52)
Race/ethnicity - number (%)						
Black, non-Hispanic	176 (45)	12 (52)	81 (54)	83 (38)	.03	317 (39)
White, non-Hispanic	72 (18)	5 (22)	23 (15)	44 (20)		161 (20)
Hispanic or other	146 (37)	6 (26)	47 (31)	93 (42)		328 (41)
Maternal education - number (%)						
High school or lower	177 (45)	12 (52)	69 (46)	96 (44)	.71	341 (42)
Some college or higher	217 (55)	11 (48)	82 (54)	124 (56)		465 (58)
Study center - number (%)						
Boston	90 (23)	6 (26)	31 (21)	53 (24)	.07	240 (30)
St. Louis	183 (46)	13 (57)	81 (54)	89 (40)		292 (36)
San Diego	121 (31)	4 (17)	39 (26)	78 (35)		274 (34)
Treatment assignment - number (%)						
High dose vitamin D	200 (51)	11 (48)	75 (50)	114 (52)	.90	405 (50)
Low dose vitamin D	194 (49)	12 (52)	76 (50)	106 (48)		401 (50)
Gestational age - mean (SD)	38.9 (2.2)	38.8 (2.5)	38.9 (1.9)	38.9 (2.3)	.77	39.0 (2.0)
Preterm (<37 weeks gestation) - number (%)	44 (11)	3 (13)	15 (10)	26 (12)	.75	71 (9)
Birth by cesarean section - number (%)	118 (30)	9 (39)	43 (28)	66 (30)	.57	239 (30)
Perinatal antibiotics - number (%)	173 (44)	15 (65)	62 (41)	96 (44)	.10	337 (42)
Pet dog during infancy - number (%)	97 (25)	5 (22)	31 (21)	61 (28)	.28	168 (22)
Older sibling(s) - number (%)	218 (55)	13 (57)	88 (58)	117 (53)	.62	435 (54)
Exclusive breastfeeding for first 4 months - number (%)	116 (30)	7 (32)	39 (27)	70 (33)	.44	247 (33)
Daycare in first 3 years - number (%)	197 (51)	14 (64)	82 (56)	101 (47)	.13	378 (53)
Asthma by age 6 years - number (%)	125 (32)	16 (70)	47 (31)	62 (28)	<.01	198 (25)
Eczema by age 6 years - number (%)	222 (57)	19 (86)	83 (55)	120 (56)	.01	370 (52)

Note: Missingness: Dog in home for 3 subjects; breastfeeding missing for 11 subjects; daycare missing for 10 subjects; eczema missing for 5 subjects. *p* value for gestational age is for Kruskal-Wallis test; all other *p* values are for Fisher exact test.

Bold values indicate *p* values of < .05.

through age 3 years). We observed similar associations between relevant sphingolipids and food allergy/sensitization in our expanded dataset (Table S4).

3.3 | Infant fecal caffeine metabolites are associated with food allergy/sensitization

We focused subsequent analyses on the module of caffeine metabolites detected in stool samples collected at age 3–6 months. This metabolite module included 9 metabolites involved in steps across nearly the entire caffeine metabolism pathway (Figure 2A)

and was enriched among subjects with food allergy or sensitization compared with control subjects (Figure 2B). This finding raised the question of where these caffeine metabolites originated, and we hypothesized that they could be derived from maternal breastmilk in breastfed infants whose mothers consumed caffeine.

Consistent with this, infant fecal caffeine metabolites were highly significantly enriched in subjects who were breastfed and not ingesting formula at the time of stool sample collection, in whom there was a range of caffeine metabolite abundances likely reflecting the range of maternal caffeine intake (*t* test *p* < .001 for both batches, Figure 3). In contrast, among subjects who were not breastfed, most exhibited low fecal caffeine metabolite levels. Accordingly,

FIGURE 2 (A) Caffeine metabolic pathway. Metabolites in the fecal caffeine metabolite module are displayed with purple background. Figure modified from KEGG Caffeine metabolism reference pathway (B). Fecal caffeine metabolite module from age 3–6 months. Displayed by association of individual metabolites with food allergy/sensitization (y axis, adjusted odds ratio) and by module membership (correlation of individual metabolite with module eigenvalue, x axis).

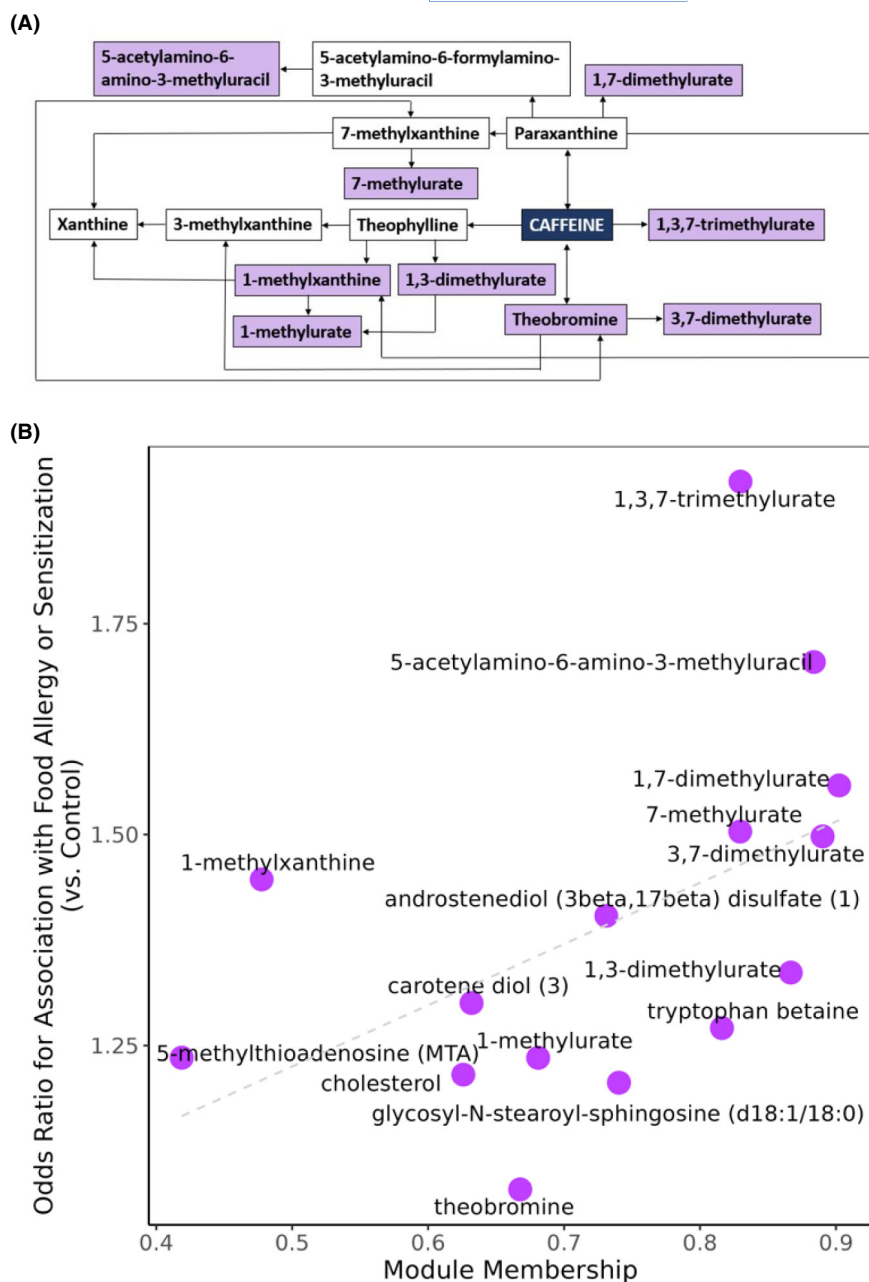
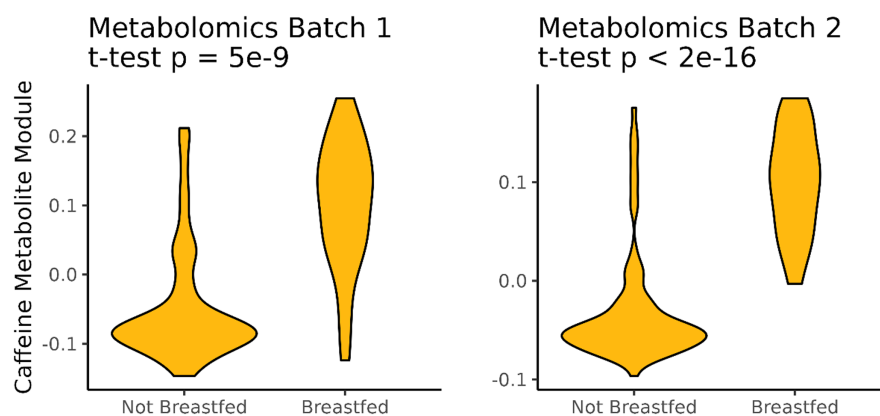


FIGURE 3 Violin plots depict associations between the fecal caffeine metabolite module and whether subjects were ingesting breast milk and not ingesting formula at the time of stool sample collection. In metabolomics batch 1, 27 subjects were breastfed and 55 were not breastfed; in batch 2, 31 were breastfed and 92 not breastfed.



associations between caffeine metabolites and food allergy/sensitization were most prominent among subjects who were ingesting breastmilk at the time of stool sample collection (Figure S6).

While we did not directly ask participating mothers about caffeine intake, mothers did provide blood samples for plasma metabolomics both during the third trimester of pregnancy and again when offspring were 1 year old. There was a moderate and highly statistically significant correlation between circulating caffeine relative abundance in mothers between these two time points (Pearson $\rho = 0.47$, $p < 2e-16$), indicating that these measures of maternal caffeine intake are reasonable proxies for maternal caffeine when offspring infant stool samples were collected at age 3–6 months. Maternal circulating caffeine levels both in pregnancy and when offspring were 1 year old were positively correlated with infant fecal caffeine metabolites and as expected, this pattern was strongest in infants who were breastfed at the time of stool sample collection (mean Pearson $\rho = 0.37$ among breastfed and 0.17 among non-breastfed, Figure S7). Mothers of infants who were breastfed at the time of infant stool sample collection had higher circulating caffeine both in the 3rd trimester of pregnancy (t -test $p = .01$) and when offspring were 1 year old (t -test $p < .01$). However, given that there was no direct association between breastfeeding and food allergy/sensitization (Table 1), the association between fecal caffeine metabolites and food allergy/sensitization is unlikely to be due to breastfeeding itself. Of note, there was no direct association between maternal caffeine and offspring food allergy or sensitization (adjusted logistic regression $p > .05$ for pairwise comparisons of phenotype groups for both maternal caffeine measurement time points, Figure S8).

We considered that the observed association between infant fecal caffeine metabolites and food allergy/sensitization is due to confounding by a maternal characteristic associated with caffeine intake. Associations of caffeine exposures (infant fecal caffeine metabolites and maternal plasma caffeine) with maternal characteristics are displayed in Figure S9. Across all measures of caffeine exposure, caffeine levels were reduced in association with black race, lower education, younger maternal age, poor diet quality, and increased household tobacco smoke exposure (Wilcoxon rank sum or Spearman correlation $p < .05$). Infant fecal caffeine metabolites were lower among offspring of mothers with asthma only in one batch of

metabolomics (Wilcoxon rank sum $p = .02$) and otherwise caffeine levels did not differ between mothers with and without asthma or eczema. Infant fecal caffeine metabolites were negatively correlated with maternal BMI, though this reached statistical significance in only one metabolomics batch (batch 1 Spearman $\rho = -0.31$, $p = .005$; batch 2 $\rho = -0.11$, $p = .25$). Caffeine exposure was not associated with VDAART study treatment assignment (Wilcoxon test $p > .05$). However, with the exception of race/ethnicity, which was included as a covariate in models of metabolite associations with food allergy/sensitization, none of these maternal factors were associated with food allergy/sensitization ($p > .05$ in Wilcoxon rank sum or Fisher tests and race/ethnicity-adjusted logistic regression), arguing against confounding by these caffeine-associated maternal characteristics. We considered the possibility that race could modify the association of fecal caffeine with food allergy/sensitization; however, in analyses stratified by race, the association between the infant fecal caffeine metabolite module with food allergy/sensitization was preserved (among all subjects with adjustment for race/ethnicity: OR 1.65, 95% CI 1.05–2.61, among black subjects: OR 1.65, 95% CI 0.62–4.39, among non-black subjects: OR 1.67, 95% CI 1.04–2.77, Table S5).

Finally, because caffeine has a known impact on bile acid production and absorption¹⁵ and bilirubin levels,^{16–18} we hypothesized that infant fecal caffeine may be associated with reductions in intestinal bile acids and bilirubin. Accordingly, we found that the fecal caffeine metabolite module was negatively correlated with the bile acid/bilirubin metabolite module associated with reduced food allergy risk (metabolomics batch 1 Pearson $\rho = -0.49$, $p = 2e-6$, batch 2 $\rho = -0.33$, $p = .0002$, Figure S4, Figure 4). These results suggest that presence of caffeine in the gut may impact food allergy risk through its impact on other intestinal metabolic pathways, though validation of our findings in other cohorts, ideally including absolute quantification of relevant metabolites, is needed to make this conclusion. The caffeine metabolite module was associated with three other infant fecal metabolite modules (Spearman $\rho > |0.30|$ and $p < .001$ in both metabolomics batches; positive correlation with magenta and blue modules and negative correlation with turquoise module with module members provided in Table S2). However, none of those three additional metabolite modules were associated with food allergy/sensitization (all logistic regression $p > .05$).

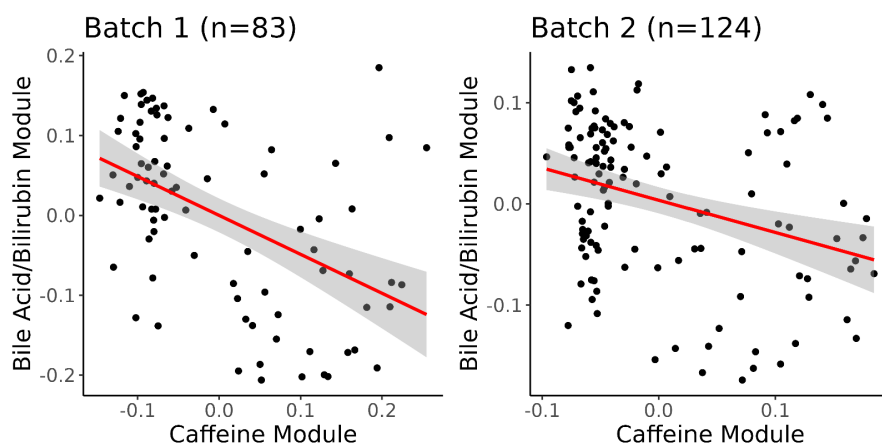


FIGURE 4 Scatterplots depict associations between food allergy/sensitization-associated fecal intestinal bile acid/bilirubin and caffeine modules from stool samples collected at age 3–6 months. Metabolomics Batch 1 Pearson $\rho = -0.49$, $p = 2e-6$, Batch 2 $\rho = -0.33$, $p = .0002$.

4 | DISCUSSION

We identified several classes of fecal metabolites associated with food allergy and/or sensitization over the first years of life. While some, including sphingolipids and bile acids, were associated with food allergy and/or sensitization when measured at multiple time points between infancy and age 3 years, the individual metabolites in these classes differed between time points and our findings largely suggest that different metabolites are relevant at different points in the inception and maintenance of food allergy.

Bile acids appear to be associated with food allergy throughout the disease course: we previously found in offspring of VDAART participants that fecal bile acids at age 3–6 months were reduced among subjects who later developed food allergy¹⁰ and here report that inverse associations of fecal bile acids with food allergy persist in stool from age 1 year. Additionally, a cross-sectional analysis by Crestani, et al found differences in serum bile acids between children with food allergy and those with asthma.⁹ Bacterially modified bile acids modulate T-cell differentiation and promote generation of T regulatory cells.^{19,20} Thus deficiencies in bile acids could impair immune system development. In our study, of bile acids in stool samples from age 3–6 months, those associated with reduced food allergy were all secondary microbially-modified bile acids, suggesting perturbation of microbial bile acid metabolism. In contrast, bile acids in stool samples from age 1 year that were associated with reduced food allergy included 2 primary bile acids (chenodeoxycholate and cholate), 4 conjugated bile acids (e.g., glycocholate, taurocholate), and 11 secondary microbially-modified bile acids, suggesting broader involvement of the bile acid pathway in the early maintenance of food allergy.

We found in both our prior¹⁰ and current analyses that bilirubin metabolites D-urobilin and I-urobilinogen were the members of a metabolite module detected in stool samples from age 3–6 months that were most strongly associated with reduced risk of food allergy. These findings are in accordance with a recent report of inverse associations between unconjugated bilirubin and childhood asthma and wheeze, though that study found no association with allergic sensitization including to food allergens.²¹ Bilirubin has been hypothesized to impact risk of asthma and atopy via its effects on lysophosphatidic acid metabolism and antioxidant activity.²¹

We previously detected an association between fecal *Bacteroides*-derived sphingolipids at age 3–6 months and protection from food allergy in VDAART participants.¹⁰ Associations of metabolites proximal in the de novo sphingolipid production pathway with food allergy were preserved in this analysis, and in particular, the proximal sphingolipid metabolite 3-ketosphinganine was part of a metabolite module that was reduced in association with food allergy in stool samples collected at age 3–6 months. In contrast, sphingolipid metabolites including lactosylceramides and hexosylceramides, which are products of distal steps in sphingolipid metabolism, were inversely associated with food allergy at age 3 years. These findings are consistent with findings of Crestani et al of reduced sphingolipid metabolite products of ceramide⁹ in serum of subjects with food allergy and suggest that different parts of the sphingolipid

pathway are relevant to food allergy development and maintenance. Mechanistically, sphingolipids might impact food allergy via differential regulation of the invariant natural killer T (iNK T) cell compartment,¹⁰ though sphingolipids may exert protective effects on intestinal homeostasis through other immune mechanisms²² including effects on mast cell responsiveness.²³ Microbial sphingolipids are produced by gut symbiont *Bacteroidetes*, and it is possible that the sphingolipid signal that we and other have observed marks a larger immune-protective role for this phylum.

We found evidence that in mothers who ingest caffeine, caffeine metabolites are present in breastmilk and appear in infant feces, where they are enriched in association with both food sensitization and allergy. Coffee and energy drinks are major sources of caffeine (63–235 mg per serving), with tea, soft drinks and chocolate containing lesser amounts (6–47 mg per serving).²⁴ Our results are consistent with a recent report on Japanese mother–child pairs that found that maternal caffeine intake in the middle or highest tertile during pregnancy was associated with increased food allergy in offspring compared to the lowest tertile.²⁵ However, that study did not stratify by breastfeeding status or analyze postpartum caffeine intake, and both that study and our study contrast with reports of inverse associations of caffeine intake with other atopic outcomes including asthma in adults.^{26–29} Caffeine may have a different effect on asthma than food allergy due to specific effects on airway smooth muscle.³⁰ Caffeine is used to treat respiratory conditions including apnea in premature infants,^{24,31} though this is unlikely to explain our results, as stool samples were collected starting at age 3 months and the frequency of prematurity was comparable between those with food allergy, sensitization and controls (Table 1).

Importantly, we did not observe a direct association between maternal circulating caffeine and offspring food allergy/sensitization. This could be explained by measurement bias given that we did not ask mothers directly about caffeine intake at the time of infant stool sample collection. While the half-life of caffeine in adults is around 3.5 h, infants under age 5 or 6 months clear caffeine much more slowly, allowing for caffeine's half-life to extend up to 80 h.²⁴ So, infant fecal caffeine likely more accurately reflects routine caffeine exposure than maternal plasma caffeine measurement. The detection of caffeine, which is largely excreted by the kidney, in the infant gut may be due to different kinetics of caffeine metabolism in infants or possibly due to reduced renal excretion.³² We did observe detectable caffeine metabolites in some infants who were not breastfed (Figure 3), which could reflect intentional or inadvertent ingestion of caffeine-containing foods such as chocolate or chocolate milk. It is possible that oral ingestion of caffeine is responsible for detectable fecal caffeine metabolites in some infants who were breastfed as well. Alternatively, maternal caffeine intake, which likely has both good and bad effects on maternal and child health,²⁴ may truly not have an overall effect on risk of food allergy/sensitization but may exert effects specifically via infant gastrointestinal exposure that do increases risk. Consistent with this, we found that subjects with higher fecal caffeine exhibited lower levels of a fecal bile acid/bilirubin metabolite module that was associated with

reduced food allergy risk. Caffeine is known to impact bile acid flow and absorption¹⁵ and circulating bilirubin.^{16–18} Specifically, coffee consumption stimulates cholecystokinin production, which stimulates gallbladder contractility and bile production.³³ The mechanism of the association between caffeine and bilirubin is unknown and may relate to effects of caffeine on enzymes involved in bilirubin metabolism.^{17,18} Our findings suggest that intestinal caffeine exposure may lead to a reduction in these metabolites that predisposes to food allergy.

Short-chain fatty acids are microbial-derived metabolites of dietary fiber fermentation that have been associated with protection from allergic diseases including atopic asthma,^{8,34,35} allergic rhinitis⁸ and food allergy.⁸ We found no associations between fecal short-chain fatty acids and food allergy/sensitization, possibly because all VDAART participants are at elevated genetic risk of atopy and may not reflect the full range of short-chain fatty acid levels in the general population.

Our study had strengths, including diverse participants with sampling at multiple time points and both clinical and laboratory data to distinguish food sensitization from clinical food allergy. However, there were study limitations. All VDAART subjects are at elevated genetic risk of atopic disease, which may limit generalizability of our findings. The relatively small number of subjects with food allergy limited power to detect associations. Food allergy was ascertained based on parental report of diagnosis for practical reasons, which could result in measurement bias compared to the gold standard of oral food challenge. Additional misclassification of the food allergy phenotype could have occurred, including due to lack of information on whether subjects were ingesting allergenic foods, i.e., sensitized infants who had never eaten the food might have had an allergic reaction had they been exposed to the food. We were unable to evaluate maternal caffeine consumption at the exact time of infant stool sample collection, and use of measurements of maternal plasma caffeine before and after the time of infant stool sample collection represents a potential source of measurement bias. Caffeine metabolites were quantified as relative, not absolute abundances; however, these measurements do appear to reflect the range of systemic caffeine exposure: fecal caffeine metabolites in infants were correlated with maternal plasma caffeine metabolites in our study, and in a study of adult participants in the TwinsUK study, fecal caffeine metabolites measured using the same method were correlated with both self-reported coffee intake and serum caffeine levels.⁵ While we performed analyses to identify potential confounders of the association between caffeine and food allergy/sensitization, confounding by unmeasured confounders is still possible. Given the strong correlation between food allergy and eczema, it is possible that the observed associations with food allergy may actually be associations with eczema or may reflect pathobiology that is shared between these common comorbidities. Despite these limitations, our results could be used to inform future studies focused specifically on food allergy development.

In conclusion, our findings add to the literature pointing to a role for bile acids, sphingolipids, and bilirubin metabolites in food allergy pathogenesis and suggest a more novel pathway whereby exposure

to caffeine metabolites in breast milk may predispose to food sensitization and food allergy via modulation of the intestinal microbiome. Future epidemiologic and mechanistic studies, including in other human cohorts, are needed to validate these findings and, hopefully, will lead to new preventive and management strategies for food allergy.

AUTHOR CONTRIBUTIONS

Funding Acquisition: DRG, STW, AAL; Concept & Design: KAL, PJB, STW, RSZ, GTO, LBB, AAL; Data Collection: STW, RSZ, GTO, LBB, AAL, NL; Data Analysis: KAL, YCC; Statistical Support: KAL, RSK, STW, AAL; Manuscript – Original Draft: KAL; Manuscript – Review & Editing: YCC, JL-S, RSK, RSZ, GTO, LBB, XJ, AB, DRG, SB, STW, AAL, PJB.

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CONFLICT OF INTEREST

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ORCID

Rachel S. Kelly  <https://orcid.org/0000-0003-3023-1822>

Augusto A. Litonjua  <https://orcid.org/0000-0003-0422-5875>

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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