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Early-life exposure to per- and polyfluoroalkyl substances and infant gut microbial composition

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Background: Human milk is rich in essential nutrients and immune-activating compounds but is also a source of toxicants including per- and polyfluoroalkyl substances (PFAS). Evidence suggests that immune-related effects of PFAS may, in part, be due to alterations of the microbiome. We aimed to identify the association between milk PFAS exposure and the infant gut microbiome.

Methods: PFAS [perfluorooctane sulfonic acid (PFOS) and perfluorooctanoate (PFOA)] were quantified in milk from ~6 weeks postpartum using high-performance liquid chromatography with tandem mass spectrometry. A molar sum (ΣPFAS) was calculated. Caregivers collected infant stool samples at 6 weeks (n = 116) and/or 1 year postpartum (n = 119). Stool DNA underwent metagenomic sequencing. We estimated the association of PFAS with diversity and relative abundances of species with linear regression. Single- and multi-PFAS models adjusted for potential confounders in complete case analyses and with imputed missing covariate data for 6-week and 1-year microbiomes separately. We assessed sensitive populations with stratification.

Results: PFOS and PFOA were detected in 94% and 83% of milk samples, respectively. PFOS was associated with increased diversity at 6 weeks among infants fed exclusively human milk [β = 0.24 per PFOS doubling, (95% CI = 0.03, 0.45), P = 0.03] and born to primiparous mothers [β = 0.37 (0.06, 0.67), P = 0.02]. Estimates were strongest in multi-PFAS models and among complete cases. ΣPFAS was associated with *Bacteroides vulgatus* relative abundance at 1 year [(β = –2.34% per doubling (–3.63, –1.05), FDR q = 0.099].

Conclusions: PFAS may increase infant gut microbiome diversity and alter the relative abundance of biologically relevant bacteria. Additional analyses may identify related health outcomes.

Keywords: Per- and polyfluoroalkyl substances, Microbiome, Metagenomics, Human milk

Introduction

Human milk is an essential source of immunoactive ingredients that shape an infant's developing immune system,[1] with growing evidence of a modulating role of the infant gut microbiome.[2,3] Milk can also carry toxic exposures such as per- and polyfluoroalkyl substances (PFAS).[4] PFAS are environmentally persistent chemicals that accumulate in adults and are passed to the fetus *in utero*, and transferred postnatally through milk.[5] Exposure to PFAS through water and food is nearly ubiquitous, and, based on evidence of health effects at lower doses than the 2016 health advisory, the US Environmental Protection Agency updated lifetime drinking water health advisories for four PFAS in June, 2022, including dramatically reduced interim exposure recommendations for two of the most frequently detected PFAS, perfluorooctane sulfonic acid (PFOS; 0.02 ppt from 70 ppt) and perfluorooctanoate (PFOA; 0.004 ppt from 70 ppt).[6] Infants may be particularly susceptible due to the relatively high body burden of exposure. Immunomodulation is one of the primary health effects seen with low doses of PFAS exposure that spurred this regulatory change for PFAS.[7,8] While PFOS and PFOA are known to suppress antibody response, the National Toxicology Program cited a need for research to elucidate additional mechanisms by which these chemicals may suppress immune function,[9] which may include changes in the gut microbiome.

The gut microbiome, or the community of bacteria, fungi, and viruses that inhabit the gastrointestinal system, is first colonized at birth and is relatively unstable until approximately 3 years of age.[10,11] During this window, when community dynamics are being established, bacteria may be particularly sensitive

What this study adds

The mechanisms by which per- and polyfluoroalkyl substances (PFAS) exert immunomodulatory effects are not fully elucidated. We aimed to understand whether early-life dietary PFAS exposure in human milk is associated with the infant gut microbiome. Perfluorooctane sulfonic acid (PFOS) was associated with increased early-life gut microbial diversity, which has been associated with increased risk of infection. This finding, among others, suggests that the infant microbiome is associated with PFAS exposure and may be a mechanism through which PFAS act on the host system.

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Sequencing data used in this study are available through the National Center for Biotechnology Information (NCBI) Sequence Read Archive (https://ncbi.nlm.nih.gov/sra) under accession number PRJNA296814. Publicly available human milk PFAS data used in this study was generated through grants supported by the National Institute of Environmental Health Sciences as part of the Children's Environmental Health Analysis Resource (CHEAR). Additional epidemiologic data are not publicly available due to their sensitive nature and identifiable nature but may be available upon request. Code is available upon request from Hannah E. Laue (Hannah.E.Laue@Dartmouth.edu).

to external insults (e.g., environmental exposures).[12,13] Two prior studies have examined the relationship between early-life exposure to PFAS and the infant gut microbiome, including one study that quantified PFAS exposure in human milk.[14,15] These studies utilized 16S rRNA sequencing, which limits the ability to examine bacterial species and gene pathways.[16] Additionally, underlying characteristics of the study populations (i.e., high proportion of operative births in Brazil and high proportion of preterm births in Norway) necessitate further inquiry in other populations.

In light of the increasing concern about PFAS exposure in the United States and globally we examined both a single time point and longitudinal associations between milk PFAS concentrations and the infant gut microbiome in a US pregnancy cohort. In doing so, we addressed gaps in the prior literature, by (1) using metagenomic sequencing to allow for more precise annotation of bacterial species and gene pathways; (2) examining short-term and longer-term associations; (3) identifying susceptible populations with analyses stratified by possible modifying factors.

Methods

Study population

eFigure 1 (http://links.lww.com/EE/A211) depicts the flow of participants included from the New Hampshire Birth Cohort Study (NHBCS) and sample sizes. The NHBCS recruited individuals at pregnancy visits to 10 prenatal clinics in New Hampshire.[17] Participants were eligible for enrollment if they were (1) 18–45 years of age, (2) receiving routine prenatal care at one of the study clinics, (3) consuming drinking water from a private source at their place of residence, with no plans to move prior to delivery, (4) pregnant with a singleton, and (5) able to speak English. Over 2500 mother-child dyads have been enrolled starting in 2009, with recruitment ongoing. Beginning in 2014, at the regularly scheduled 6-week postpartum visit, mothers who fed their infant human milk were asked to provide a milk sample and a stool sample from their child. Caregivers also provided a stool sample from their child at their 1-year wellness check. Throughout the first-year postpartum, caregivers completed regular questionnaires that included questions regarding lifestyle and sociodemographic characteristics. Caregivers provided written and informed consent. All study protocols have been approved by the Center for the Protection of Human Subjects at Dartmouth, and all methods were carried out in accordance with relevant guidelines and regulations.

Human milk PFAS exposure quantification

Mothers of infants who received any amount of human milk as part of their diet were asked to collect a milk sample (~60 mL) from each breast following a standardized protocol that included refraining from the use of soaps, lotions, or other substances. Samples of whole milk from each breast were stored in the participants, home refrigerator. Within 24 hours, whole milk was transferred into 5 mL aliquots by study staff and stored at –80°C. Aliquots were retrieved from –80°C, thawed at room temperature, transferred into 1 mL sub-aliquots, and refrozen at 80°C. Subsequently, 1 mL milk aliquots were arbitrarily chosen from left or right breast, randomized and shipped frozen, overnight on dry ice for PFAS analysis at the Minnesota Children's Health Exposure Analysis Research (CHEAR) Exposure Assessment Hub. For quality control (QC) purposes, 5% of the samples were blinded and duplicated to assess relative percent difference (RPD) and intraclass correlation (ICC) of the analytical method. PFAS concentrations [PFOS, PFOA, perfluorononanoate (PFNA), perfluorodecanoate (PFDA), perfluoroundecanoate (PFUnDA), perfluorobutane sulfonic acid (PFBS), perfluoroheptanoate (PFHpA), perfluoroheptane sulfonic acid (PFHpS), and perfluorohexanoate (PFHxA), perfluorohexane sulfonic acid (PFHxS)] were quantified as previously described.[18] Briefly, a validated liquid chromatography tandem mass spectrometry (LC/MS/MS) method with stable-isotope labeled internal standards was employed using 400 µL aliquots of milk.[19] Reagent blanks and low- and high-concentration QC materials were run with each batch of 20 unknowns and were used to assess batch performance. Accuracy for the method, indicated by % recovery, was determined through matrix spikes (n = 8), and ranged from 72% to 103%. Precision for the method, indicated by relative standard deviation (%RSD), was determined by repeated measurements of in-house prepared QC materials (n = 20) 3% to 18%. Only PFHxS, PFOA, and PFOS yielded results in which both duplicate aliquot concentrations were above the limit of quantification (LOQ). For these analytes the RPD was 6%, 6%, and 12%, and the ICC was 0.919, 0.918, and 0.978, respectively. Across all samples, PFOS and PFOA consistently had concentrations above the LOQ (both 0.01 ng/mL) and were used in analyses.[18] Concentrations below the LOQ were imputed as the LOQ/√2. A molar sum of PFAS exposure for each individual (ΣPFAS) was calculated as: $\sum PFAS = \left(\frac{C_{PFOS}}{500.13} + \frac{C_{PFOA}}{414.07}\right) * 10^{-9}$

where C_{PFOS} is the concentration of PFOS, C_{PFOA} is the concentration of PFOA, and 500.13 and 414.07 are the molar weights of PFOS and PFOA, respectively. Exposures were $\log_2$-transformed to reduce the impact of extreme values on effect estimates.

Fecal microbiome metagenomic sequencing

As previously described, stool samples were collected from infants at approximately 6-week and 1-year postpartum in a diaper provided by study staff (aliquoted and frozen at –80°C within 24 hours of receipt), and microbial DNA was isolated by staff at the Biorepository Core of the Center for Molecular Epidemiology at Dartmouth College.[20] Samples underwent metagenomic sequencing at the University of Chicago's Marine Biological Laboratory at Woods Hole, Massachusetts, using the NextSeq Illumina platform with libraries prepared using Nugen's Ovation Ultralow V2 protocol. Raw sequences were prepared using the BioBakery suite of tools,[21] as described previously.[22] KneadData (v0.7.7) was applied to trim sequences and remove reads matching the human genome. Using the CHOCOPhlAn database of marker genes, the MetaPhlAn (v3.0.7) algorithm generated taxonomic profiles. HUMAnN (v3.0.0a4) functionally profiled metagenomes, which were mapped to Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologies (KOs).[23–25] Bacterial alpha diversity was calculated at the species level with the Shannon[26] (primary) and Inverse Simpson[27] (secondary) Indices, and beta diversity was quantified with Bray-Curtis Dissimilarities.[28]

Covariates

Covariates were selected *a priori* based on their potential to confound the association between milk PFAS concentrations

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and gut microbiome diversity and composition, or as predictors of microbiome diversity and composition. Birth mode (vaginal/operative), child's sex (male/female) and gestational age at delivery were abstracted from infant and maternal medical records. Maternal parity (primiparous/multiparous), maternal education (less than college degree, college degree, any postgraduate), marital status (married/unmarried), maternal age at enrollment, and maternal prepregnancy BMI (ppBMI) were derived from prenatal and postnatal questionnaires. Feeding habits at 6 weeks, approximately the age of milk sample collection, were determined from questionnaires and infants were classified as either being fed exclusively human milk or fed a mixture of human milk and formula. Missing covariate data were multiply imputed using multivariate imputation by chained equations (*mice* v3.14).[29–31] Forty datasets were imputed with 20 iterations using logistic regression with bootstrapping for binary variables (feeding mode, marital status, parity) and polytomous logistic regression for maternal education; no continuous variables had missingness. For more complex statistical analyses (beta diversity, species, KOs), we created a single imputed dataset from the 40 using the mode of imputed variables as in our prior work.[32] Our primary analysis utilized imputed data and a complete case analysis was conducted for comparison.

Statistical approach

We restricted to term births (≥37 weeks gestation) and infants with milk samples collected contemporaneous to 6-week stool samples (i.e., no more than 1 week after stool sample collection). We first conducted our analyses with limited confounder adjustment (birth mode, gestational age, and age at stool sample collection)—the basic model. In our full model, we additionally adjusted for parity, maternal education, maternal marital status, child's sex, maternal age, and maternal ppBMI. Feeding mode and proportion of meals that were human milk are the source of exposure under investigation and therefore only considered in sensitivity analyses for comparability to prior studies.[14,15]

We hypothesized that the associations between milk PFAS concentrations and features of the gut microbiome may differ based on underlying characteristics; thus, we conducted stratified analyses to examine possible effect modification in both complete cases and multiply imputed datasets. Stratifying variables included child's sex—a known modifier of health effects due to environmental exposures[33]; delivery mode—which is a strong driver of the early-life gut microbiome,[20,34] including microbial susceptibility to environmental exposures[22,35]; and feeding status and parity—both of which are related to PFAS concentrations in milk (i.e., multiparous mothers, who excreted PFAS to a prior fetus, are expected to have lower concentrations of PFAS for the index participant; mothers who feed their infant exclusively human milk excrete more PFAS than those who supplement with formula, and thus are expected to have lower milk PFAS concentrations by 6 weeks postpartum).[36] Previously, we have observed differences in associations between environmental toxicants and the infant gut microbiome, suggesting nutrient components of human milk may counteract the harmful effects of contaminants.[13] Infants born to multiparous mothers have different microbial development, which may result in microbiomes that are more or less susceptible to environmental toxicants.[37] In addition to testing for effect modification, stratifying by feeding method reduced exposure misclassification. Due to limited sample size, we were unable to analyze associations separately among infants delivered operatively or infants who received any formula.

To test whether PFAS exposure was associated with gut microbial diversity, we linearly regressed $\log_2$-transformed PFAS concentrations against metrics of alpha diversity using the *pool* function in the "mice" R package to pool estimates and variances from 40 parallel regressions using Rubin's rules.[30,38] Our primary models for all analyses included both PFOS and PFOA or the molar sum (multipollutant), but we also examined single-pollutant models. We then examined the marginal contribution of PFAS concentrations to the dissimilarity of sample structure using "adonis2" from the *vegan* R package (v2.6-2).[39] For visual purposes only, we conducted a principal coordinates analysis ["pco" from the *ecodist* R package (v2.0.9)] on the dissimilarity matrix and plotted each sample and median centroids for the first and second components with exposures dichotomized at their medians.[40] For alpha and beta diversity analyses $P < 0.05$ was considered statistically significant. To test whether the relative abundance of bacterial features (species or KOs) was associated with PFAS exposure, we used the *MaAsLin2* algorithm (v1.7.3),[41] which linearly regresses exposures and covariates against log-transformed feature relative abundance. We reduced multiple testing by first filtering to features that were present in at least 10% of samples included in the analysis. In feature analyses, we considered a Benjamini-Hochberg adjusted false discovery rate of $q < 0.1$ to be statistically significant and a $P < 0.05$ to be nominally significant.[42] All analyses were conducted in R v4.2.0 using the RStudio v2022.02.3+492 environment.[43,44]

Results

A total of 134 infants were included in either or both 6-week (n = 116) or 1-year analyses (n = 119). As expected, gut bacterial diversity increased between the two time points (**Table 1**). Milk PFOS and PFOA concentrations were 3 ± 1.7 ng/dL and 2.2 ± 1.5 ng/dL, respectively, which was similar to the whole cohort.[18] PFOS and PFOA were detected in 94% and 83% of milk samples, respectively, and their concentrations were moderately and statistically significantly positively correlated ($\rho = 0.63$, $P < 0.001$; eFigure 2, http://links.lww.com/EE/A211). Less than 3% of observations were missing data on parity or maternal education and approximately 30% were missing data on feeding status. As expected, PFAS concentrations were higher among primiparous individuals and those who did not feed their infant exclusively human milk (eFigure 3, http://links.lww.com/EE/A211). In contrast, concentrations did not differ depending on the infant's sex or birth mode. We report results from our primary models (with missing covariate data imputed, fully adjusted, multipollutant) unless indicated otherwise. Results did not vary in other models (complete cases, minimal adjustment, single pollutant) unless otherwise noted.

Diversity measures

Milk PFOS concentrations were associated with increased gut bacterial diversity at 6 weeks of age, although this estimate did not reach statistical significance [$\beta = 0.11$ change in Shannon Index per doubling PFOS exposure (–0.05, 0.27), $P = 0.17$ in fully adjusted multipollutant models]. Among infants fed exclusively human milk (no formula) [$\beta = 0.24$ (0.03, 0.45), $P = 0.03$] and first-born infants [$\beta = 0.37$ (0.06, 0.67), $P = 0.02$] this association was stronger and statistically significant (**Figure 1**, eTable 1, http://links.lww.com/EE/A212). These associations were slightly attenuated in single-pollutant models. Sensitivity analyses including six-week feeding status produced similar effect estimates (eTable 1, http://links.lww.com/EE/A212). In contrast, we did not observe associations with milk PFAS concentrations and microbiome diversity at 1 year of age (eTable 1, http://links.lww.com/EE/A212).

Milk PFOS concentrations were not clearly associated with beta diversity at 6 weeks overall. An association was observed among males in basic models ($R^2 = 0.028$, $P = 0.033$; eTable 2, http://links.lww.com/EE/A212), but this estimate was attenuated by inclusion of additional covariates and in single-pollutant models (**Figure 2**). Similarly, PFOS concentrations were associated

Table 1.

Characteristics of 134 New Hampshire Birth Cohort Study mother-child pairs included in analysis of PFAS and the infant gut microbiome [mean ± SD (% missing) or n (%)]

	6 week		1 year	
	Multiple imputation (n = 116)	Complete cases (n = 79)	Multiple imputation (n = 119)	Complete cases (n = 76)
Maternal characteristics				
Maternal age (y)	32.1 ± 4.5	32.2 ± 4	32.2 ± 4.4	32 ± 3.8
Maternal BMI (kg/m^2)	25.7 ± 5.9	25.7 ± 5.6	25.6 ± 5.4	25.6 ± 5
Marital status				
Not married	16 (13.8)	9 (11.4)	16 (13.4)	7 (9.2)
Married	98 (84.5)	70 (88.6)	101 (84.9)	69 (90.8)
Missing	2 (1.7)	–	2 (1.7)	–
Maternal education				
Less than college graduate	24 (20.3)	17 (21.5)	27 (22.7)	17 (22.4)
College graduate	44 (37.9)	28 (35.4)	43 (36.1)	27 (35.5)
Any postgraduate education	46 (39.7)	34 (43)	47 (39.5)	32 (42.1)
Missing	2 (1.7)	–	2 (1.7)	–
Parity				
Primiparous	55 (46.6)	38 (48.1)	57 (47.9)	38 (50)
Multiparous	58 (49.2)	41 (51.9)	59 (49.6)	38 (50)
Missing	3 (2.5)	–	3 (2.5)	–
Birth/infant characteristics				
Gestational age at delivery (w)	39.5 ± 1.1	39.5 ± 1.1	39.5 ± 1.2	39.5 ± 1.1
Birth mode				
Vaginal	88 (75.9)	59 (74.7)	88 (73.9)	57 (75)
C-section	28 (24.1)	20 (25.3)	31 (26.1)	19 (25)
Child's sex				
Female	53 (45.7)	35 (44.3)	47 (39.5)	30 (39.5)
Male	63 (54.3)	44 (55.7)	72 (60.5)	46 (60.5)
6-week feeding mode				
Any formula	13 (11.2)	13 (16.5)	13 (10.9)	13 (17.1)
Exclusive human milk	68 (58.6)	66 (83.5)	65 (54.6)	63 (82.9)
Missing	35 (30.2)	–	41 (34.5)	–
Microbiome				
Shannon Index	1.3 ± 0.5	1.3 ± 0.5	2.5 ± 0.4	2.5 ± 0.4
Simpson Index	3 ± 1.6	2.9 ± 1.5	8.4 ± 3.7	8 ± 3.3
Exposures				
Human milk PFOS (ng/dL)	3 ± 1.7	2.9 ± 1.8	3 ± 1.7	2.9 ± 1.8
Human milk PFOA (ng/dL)	2.2 ± 1.5	2.3 ± 1.7	2.2 ± 1.5	2.3 ± 1.6

with beta diversity at 1 year among males in basic, single-pollutant models (R^2 = 0.022, P = 0.046) but not after including PFOA or other covariates. In the full population, the molar sum of PFAS concentrations was associated with beta diversity at one year in basic models (R^2 = 0.014, P = 0.047), although this estimate was attenuated in fully adjusted models (R^2 = 0.12, P = 0.081).

Bacterial species

There were no clear associations between milk PFAS concentrations and the relative abundance of bacterial species at six weeks of age (eTable 3, http://links.lww.com/EE/A212). Although the molar sum of PFAS was negatively correlated with *Bacteroides vulgatus* relative abundance at 6 weeks [β = –0.75% per doubling PFAS (–1.65, 0.15), q = 0.974], this estimate did not even reach nominal significance (P = 0.11).

Milk PFAS exposure (molar sum) was associated with lower relative abundance of *Bacteroides vulgatus* at 1 year in fully adjusted models [β = –2.34% per doubling PFAS (–3.63, –1.05), q = 0.099; **Figure 3**, eTable 3, http://links.lww.com/EE/A212]. This association was similar after adjusting for feeding status at 6 weeks, in minimally adjusted models, and among complete cases (eTable 3, http://links.lww.com/EE/A212). In certain minimally adjusted models among complete cases, the molar sum of PFAS or PFOS concentrations were associated with increased relative abundance of *Lachnospira pectinoschiza* (β in all models ~ 1.15), although none of these estimates overcame the significance threshold after correction for multiple testing in imputed data models.

Bacterial functional potential

There were no clear associations between PFAS concentrations and KO relative abundance at 6 weeks among the full population in both basic and full models, but there were associations in certain subgroups (eTable 4, http://links.lww.com/EE/A212). Among female infants, PFOA concentrations were associated with differences in the relative abundance of several KOs (eTable 4, http://links.lww.com/EE/A212). This included, increased relative abundance of K06158, an ATP-binding cassette, at 6 weeks of age [β = 2.87% per doubling PFOA (1.65, 4.09), q = 0.058 in fully adjusted models]; decreased relative abundance of K06330, a spore coat protein [β = –3.69 (–5.43, –1.95), q = 0.09 in fully adjusted models]; and decreased relative abundance of K21011, a polysaccharide biosynthesis protein [β = –3.59 (–5.19, –1.99), q = 0.06 in fully adjusted models]. There were several associations between PFOA and KO relative abundance at 6 weeks among complete cases who were born vaginally, although most were no longer significant in fully adjusted models (eTable 4, http://links.lww.com/EE/A212). For example, each doubling of PFOA was associated with a 1.61 unit decrease in % relative abundance of K13542, uroporphyrinogen III methyltransferase/synthase [(0.95, 2.28), q = 0.03]. This KO was also either statistically significantly or nominally significantly associated with PFOA exposure in other complete case analyses, including the overall population, with similar effect size. The association was weaker in the overall population after multiple imputation of missing covariates (β = 1.13), and was nominally significant (P = 0.001), but failed to meet the threshold for multiple testing correction (q = 0.85). At

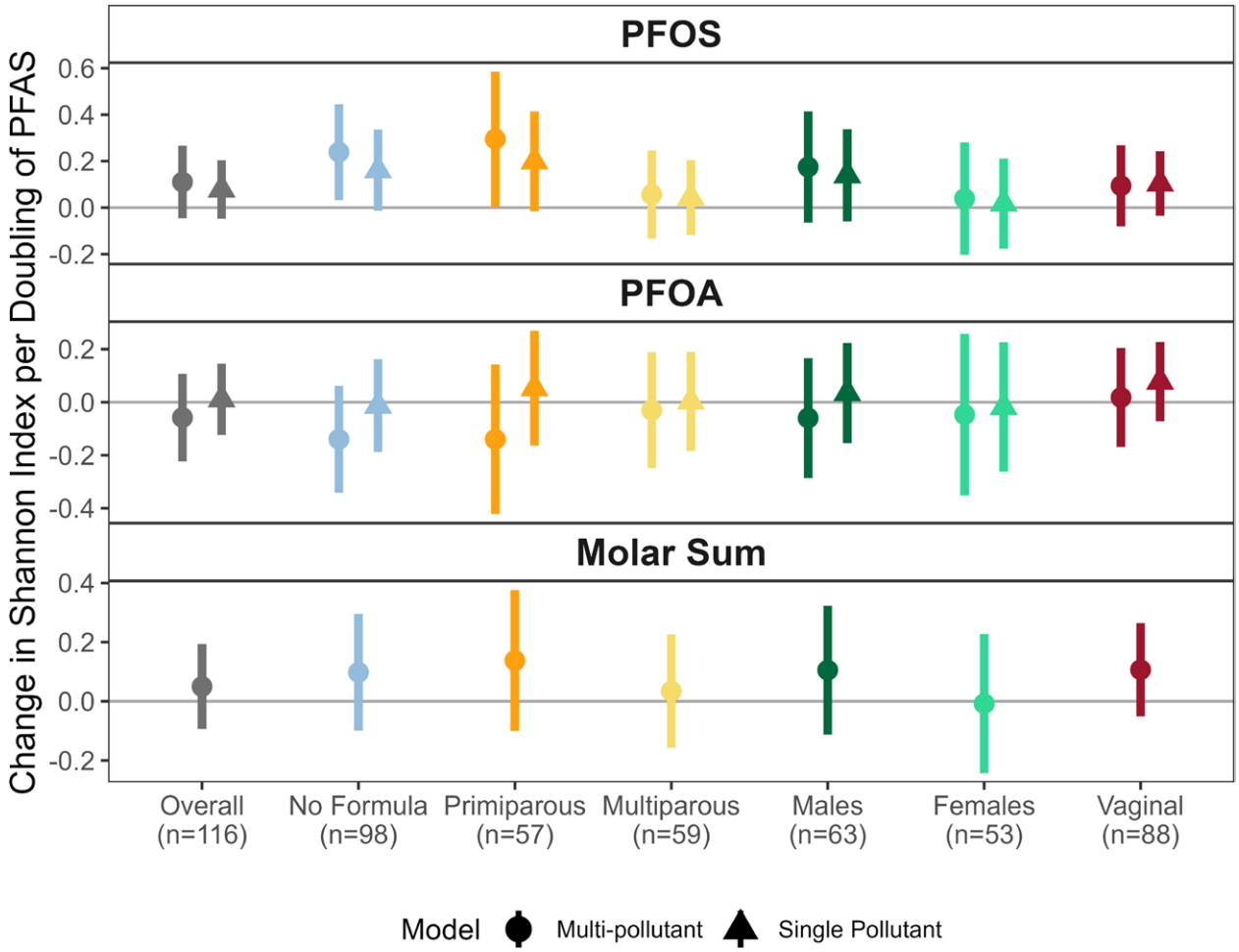

Figure 1. Associations of PFOS, PFOA, and their molar sum with the Shannon Index at 6 weeks. Models adjusted for birth mode (except for models among vaginally delivered), gestational age, age at stool sample collection, parity (except for models among primiparous or multiparous), maternal education, maternal marital status, child's sex (except for models among males and females), maternal age, and maternal prepregnancy BMI. Multipollutant models adjusted for PFOS and PFOA in the same model whereas only one pollutant was included in single-pollutant models. Missing covariate data were imputed.

one year, no KOs were associated with PFAS exposure among the full dataset, although there were some associations among complete cases (eTable 4, http://links.lww.com/EE/A212).

Discussion

In our cohort study of human milk PFAS and the infant gut microbiome, we found associations of exposure with bacterial diversity and the relative abundance of biologically meaningful bacterial species (e.g., associated with PFAS-related health outcomes). Specifically, PFOS was associated with higher diversity at 6 weeks, and the molar sum of PFAS concentrations was associated with lower relative abundance of *Bacteroides vulgatus* at 1 year.

Our finding that PFOS was associated with more diversity, especially among first-born infants or those who were fed exclusively human milk is similar to that of Naspolini et al, who described a positive association between cord blood PFAS concentrations (nonmolar sum of PFOS and PFOA) and gut bacterial diversity in samples from the first-, third-, and sixth-month postpartum in a Brazilian cohort.[15] As in our study, their results were stronger among infants exclusively fed human milk. While different exposure assessment methods were used in our studies, cord blood and milk PFAS concentrations are likely correlated, as evidenced by strong correlations between maternal plasma PFAS and milk PFAS concentrations.[18] Iszatt et al reported a negative association between milk PFOS concentrations and measures of diversity in a cohort oversampled for preterm births in Norway.[14] Their models adjusted for maternal gut bacterial diversity, a potential mediator of the association between PFAS and the microbiome. We also observed a stronger association between PFOS and increased bacterial diversity among first-born infants compared to the overall population. Neither of the prior studies examined differences in associations by parity. It is possible our findings indicate a nonlinear dose response, with stronger associations at higher levels of exposure, although our preliminary analyses did not suggest nonlinearity. First-born infants are also exposed to higher prenatal PFAS doses,[45] which may alter their physiology so that they are more susceptible to postnatal exposure. Data from other populations may be informative in interpreting differences in associations from current studies.

Infants have lower gut bacterial diversity than adults due to limited exposure to colonizing bacteria, with most having a microbiome comparable to adults around age 3 years when dietary patterns are established.[11,46] As yet, the impact of increased diversity in early life on later health outcomes is not well-understood,[47] but higher diversity may not be as beneficial, as it is assumed to be in adults.[48] For example, in prior research in the NHBCS, an association between higher gut microbiome diversity in early life and increased odds of upper respiratory tract infection by age 1 year was observed.[49] This finding is notable, given that PFAS exposure has been linked to weakened vaccine response[7,50–52] and increased risk of infection.[8,51,53,54] Future mediation analyses may clarify the role of the gut microbiome in associations of PFAS and infection.

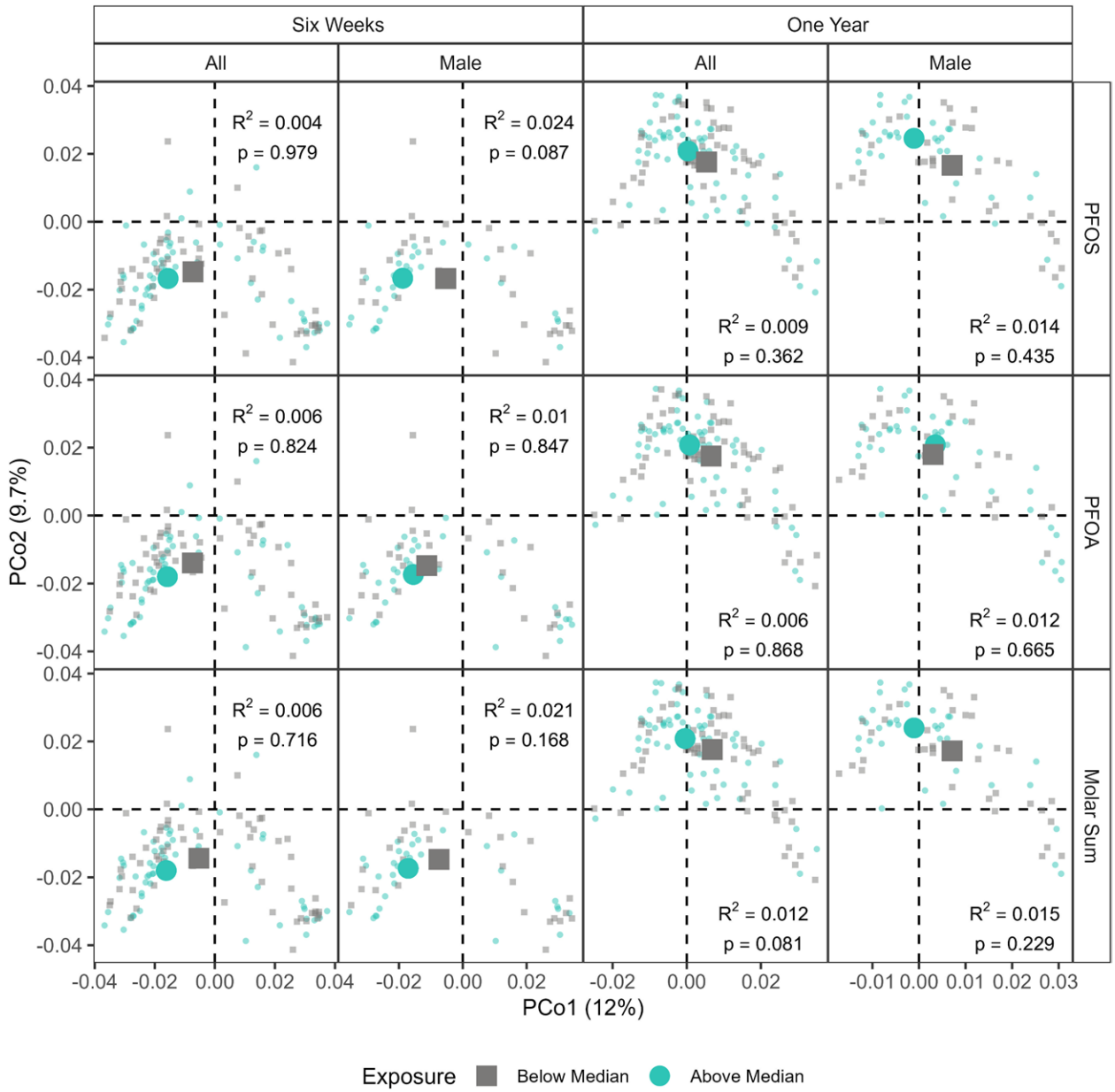

Figure 2. First two PCo of Bray-Curtis dissimilarity. Small points are individual samples; large points are median centroids, calculated based on population, age, and exposure dichotomized at the median. Percent variability explained by each PCo is displayed in parentheses on the axis label. R^2 and *P* values derive from *adonis2* PERMANOVAs including both PFOS and PFOA or Molar Sum concentrations treated continuously. Models adjusted for birth mode, gestational age, age at stool sample collection, parity, maternal education, maternal marital status, child's sex (except for models among males), maternal age, and maternal prepregnancy BMI. Missing covariate data were imputed.

Our finding that the molar sum of PFAS concentrations was associated with lower relative abundance of *Bacteroides vulgatus* at 1 year is significant in the context of *Bacteroides vulgatus*-related health effects. Exposure to PFAS, especially PFOA,[55] has been associated with lower risk of developing diabetes, with the strongest effects observed for type I diabetes (T1D).[56] The study authors posit that this apparent benefit of PFAS exposure is related to suppression of the immune system, although this hypothesis has not been tested. In contrast, *Bacteroides vulgatus* and the metabolically similar *Bacteroides dorei*, have been associated with T1D, with a spike in *Bacteroides vulgatus-dorei* relative abundance at 7.5 months of age, approximately 9 months before the average age of seroconversion.[57] A reduction in *Bacteroides vulgatus* relative abundance resulting from PFAS exposure may partially explain the negative association between PFAS and T1D risk. Similarly, food allergy is negatively associated with *Bacteroides vulgatus* relative abundance[58] and positively associated with PFAS exposure.[59] This suggests *Bacteroides vulgatus* may mediate the association between PFAS exposure and food allergy. Additional mechanistic research may elucidate our understanding of these associations.

To begin to elucidate the mechanistic underpinnings of the associations between PFAS and immune-related health effects, we examined whether PFAS concentrations were associated with the relative abundance of bacterial gene pathways (KOs). Although there were no cross-sectional or prospective associations among the overall population, there were some differences among subpopulations (females, vaginally delivered) at 6 weeks. The biological relevance of changes in the relative abundance of these gene pathways is not yet understood. It possible our mostly null findings result from limited annotation of bacterial genes (median 16% unmapped) and limited grouping to KOs

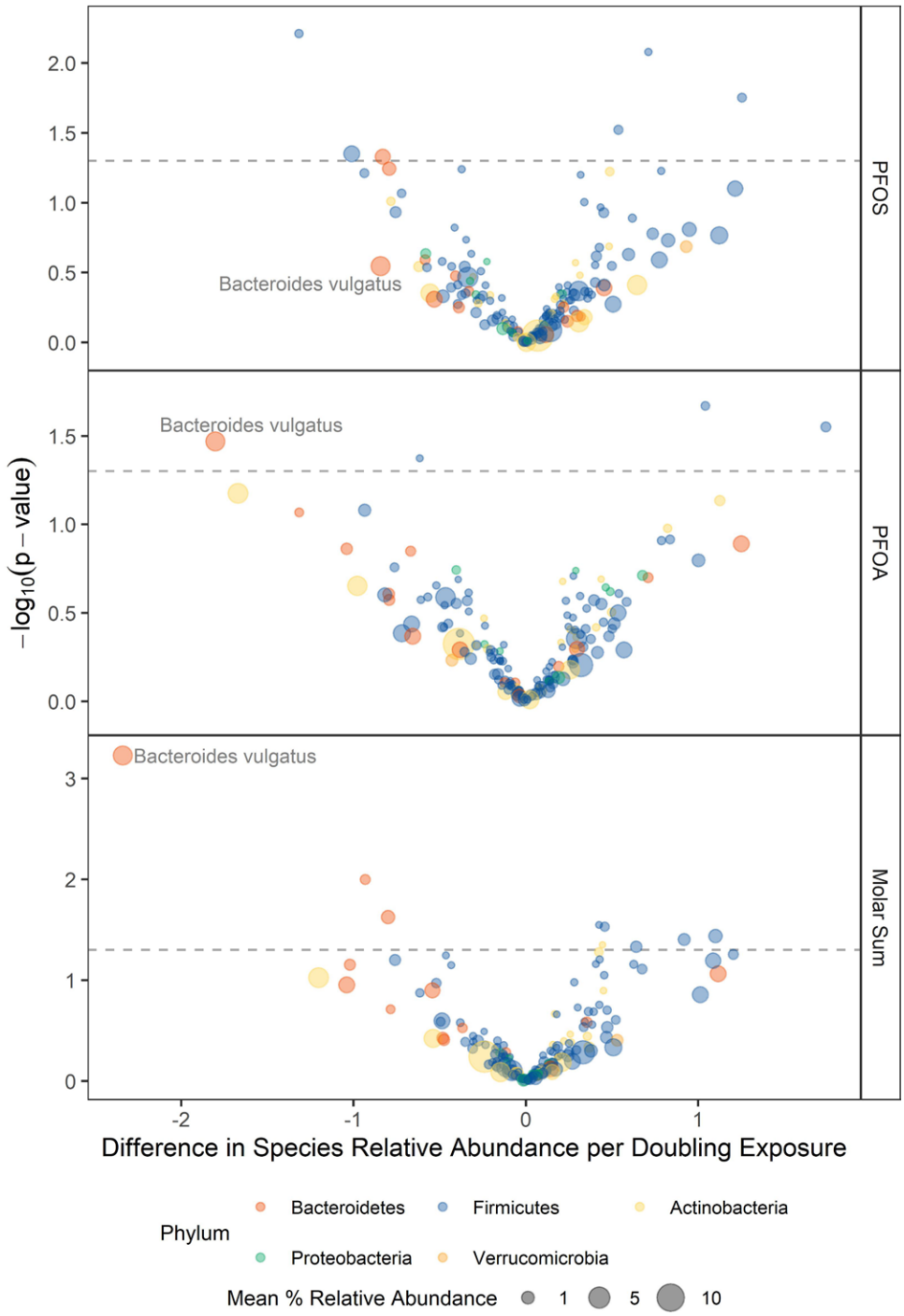

Figure 3. Volcano plots of associations between PFOS, PFOA, and their molar sum with bacterial species relative abundance at 1 year. Each point represents one PFAS-species association, with the point size reflective of mean species relative abundance in the 1-year-old population and point color reflective of the bacterial phylum to which the species belongs. The dashed gray line indicates nominal significance ($P = 0.05$). Points are labeled if the estimate is statistically significant ($q < 0.1$) for at least one exposure. Estimates and *P* values derive from *MaAsLin2* models including both PFOS and PFOA or Molar Sum concentrations with missing covariate data imputed. Models adjusted for birth mode, gestational age, age at stool sample collection, parity, maternal education, maternal marital status, child's sex, maternal age, and maternal prepregnancy BMI.

(median 77% ungrouped). Additionally, changes in gene relative abundance does not always reflect changes in gene transcription. Metatranscriptomic analyses would allow for better understanding of functional changes related to PFAS exposure. Alternatively, our null findings may be true and other pathways may underlie associations among PFAS, the microbiome, and health outcomes.

This study has inherent limitations as well as strengths. Our sample size was limited by the number of participants with fecal metagenomic sequencing, particularly in stratified analyses. Larger studies may provide more insight into sensitive populations, such as infants born operatively. Milk concentrations of PFAS capture ingested exposure, but extensive feeding habit data (*e.g.*, number of feedings per day, duration of each feed) or estimation of daily intake using weight could allow for quantification of internal dose. Milk is the main source of PFAS exposure among infants, but formula mixed with contaminated water could be a secondary source. Our analyses

among infants who were fed exclusively human milk reduced exposure misclassification, as suggested by the stronger association between PFOS and bacterial diversity among infants fed exclusively human milk compared with the overall study population. It is possible that our findings are reflective of PFAS in circulation at delivery rather than dietary intake of PFAS through human milk due to strong correlations between maternal serum PFAS concentrations and concentrations in their milk.[18] PFAS exposure in the NHBCS, while similar to the United States, is low, which may limit the generalizability of our findings to highly exposed populations and prevented quantification of other PFAS in milk samples. Our findings are likely subject to residual confounding by other PFAS or other endocrine disrupting chemicals.

Our study had the advantage of measuring human milk concentrations of PFAS, which provide an estimate of ongoing postpartum transmission of these contaminants to vulnerable infants. Our use of metagenomic sequencing allowed for inference of bacterial species at the species level and annotation of bacterial gene function. By utilizing fecal samples from two early-life timepoints, we uncovered both concurrent and prospective associations. The selection of covariates for our primary results was based on expert knowledge of the confounding structure, and we conducted sensitivity analyses to produce results comparable to prior studies. Yet, residual confounding remains a possibility. Our consideration of individual PFAS and their molar sum allowed us to elucidate compound-specific and mixture effects, which will inform policy to reduce PFAS exposures. Currently, there are no clear standards or guidelines related to PFAS concentrations in human milk. Our findings may inform much needed policy to reduce infant exposure.

Conclusion

Our findings support the hypothesis that changes in the gut microbiome could contribute to the health effects of PFAS exposure in early life, although additional mediation and mechanistic studies are required. Future analyses may elucidate the mediating role of the infant gut microbiome in the association between early-life PFAS exposure and host health outcomes and identify methods to reduce PFAS exposure in human milk.

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