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Gestational exposures to mixtures of multiple chemical classes and autism spectrum disorder in the MARBLES study

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Abstract

Background: Previous epidemiologic studies on gestational chemical exposures and autism spectrum disorder (ASD) often lack analysis of chemical mixtures or are limited to investigating certain chemical classes.

Objective: We examined the impact of multi-class chemical mixtures on ASD risk, using data from the MARBLES (Markers of Autism Risks in Babies-Learning Early Signs) cohort.

Methods: Children were clinically assessed at age 3 and classified as ASD, typical development (TD), or non-TD with other neurodevelopmental concerns. In blood or urine from 105 pregnant mothers, we quantified 42 biomarkers across 5 chemical classes: per- and polyfluoroalkyl substances (PFAS), parabens, phenols, phthalates, and organophosphate esters (OPEs). We only analyzed 30 biomarkers detected in >50 % of the sample. After identifying clusters with similar chemical profiles via hierarchical clustering, we applied linear discriminant analysis (LDA) to compute LDA exposure summary scores. In covariate-adjusted models, we used LDA scores to assess co-adjusted, multipollutant associations (relative risk [RR]) with ASD or non-TD, via

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Jeong Weon Choi: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Hyuna Jang:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Jordan R. Kuiper:** Writing – review & editing, Methodology. **Deborah H. Bennett:** Writing – review & editing, Methodology, Funding acquisition. **Rebecca J. Schmidt:** Writing – review & editing, Methodology, Funding acquisition. **Hyeong-Moo Shin:** Writing – review & editing, Methodology, Funding acquisition.

Ethical approval

The MARBLES study protocol and this study were approved by the Institutional Review Boards (IRB) for the State of California and the University of California-Davis (UC-Davis). Participants provided written informed consent before collection of any data. The analysis of coded specimens at the Centers for Disease Control and Prevention (CDC) laboratory was determined by CDC not to constitute engagement in human subject research.

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quasi-Poisson regression. We further examined overall mixture effect and chemical interactions with Bayesian kernel machine regression.

Results: We identified four distinct clusters: PFAS (Cluster 1), OPEs (Cluster 2), parabens and triclosan (Cluster 3), and phthalates and bisphenol A (Cluster 4). Relative to TD, LDA scores for each cluster were associated with increased risk of ASD (RR [95 % CI]: 1.14 [1.03, 1.25], 1.12 [1.01, 1.24], 1.17 [1.07, 1.29], 1.17 [1.07, 1.28] for Cluster 1–4, respectively), whereas clusters 2 and 4 were associated with non-TD (1.07 [1.01, 1.14] and 1.12 [1.05, 1.19], respectively). Cumulative exposure across the four clusters was linked to increased risk of both ASD and non-TD. Potential interactions within and between clusters were observed.

Conclusion: This study shows that considering multiple chemical classes resulted in stronger associations with ASD and non-TD risk, compared to when investigated separately in our previous studies.

Keywords

Autism; Chemical exposure; Gestational exposure; Interaction; Mixture

1. Introduction

Autism spectrum disorder (ASD) is an increasing public health concern, with 1 in 36 children in the U.S. diagnosed with ASD. Its prevalence rate has increased by more than three times over the past two decades (Maenner et al., 2023). Although the causes of the increase are not fully understood, gestational or early-life chemical exposures are increasingly known as contributing factors (Braun, 2017; Lyall et al., 2017). Endocrine-disrupting chemicals, such as parabens, per- and polyfluoroalkyl substances (PFAS), phenols, phthalates, and organophosphate esters (OPEs), have been detected in the biospecimens of pregnant women, implying gestational chemical exposures to the developing fetus (Adibi et al., 2008; Lee et al., 2018b; Li et al., 2023; Monroy et al., 2008; Song et al., 2020; Zheng et al., 2022). Many epidemiological studies have reported associations between gestational exposures to these chemicals and ASD or ASD-related behaviors (Ames et al., 2023; Barkoski et al., 2019; Haggerty et al., 2021; Kim et al., 2021; Oh et al., 2021; Shin et al., 2018, 2020a). However, prior studies have investigated only one or a few chemical classes or lack an analysis of chemical mixture effects such as additive, synergistic, or antagonistic interactions across chemical classes.

While many studies have examined associations between combined exposure to multiple chemicals during pregnancy and child neurodevelopment (Brennan Kearns et al., 2024; Guo et al., 2020; Hamra et al., 2019; Jedynak et al., 2021; Kalloo et al., 2021; Oskar et al., 2024; Tanner et al., 2020; Tsai et al., 2023; van den Dries et al., 2021; Vuong et al., 2020; Yonkman et al., 2023), studies on the chemical mixture effects on ASD are limited. These studies have targeted two to seven chemical classes and focused on various neurodevelopmental outcomes, including ASD, intellectual disability, cognitive ability, and behavior problems. Only three studies investigated associations between gestational exposures to chemical mixtures and child ASD or autistic behaviors (Hamra et al., 2019; Tsai et al., 2023; van den Dries et al., 2021). A U.S. cohort study investigated associations

between ASD and a mixture of 25 chemicals in five classes (polybrominated diphenyl ethers [PBDEs], polybrominated biphenyls [PBBs], polychlorinated biphenyls [PCBs], organochlorine pesticides [OCPs], PFAS) but found no significant associations (Hamra et al., 2019). A Dutch study examined associations between autistic behaviors and a mixture of 17 chemicals in three classes (phthalates, bisphenol, organophosphate pesticides [OPPs]) and also found no significant associations (van den Dries et al., 2021). A Taiwanese study investigated associations between autism spectrum problems and mixtures of 15 chemicals in metals and phthalates (Tsai et al., 2023). Notably, this Taiwanese study found that the mixtures of metal and phthalates were associated with autism spectrum problems, although the outcomes were not associated with individual chemical biomarkers, suggesting the need to consider combined exposure to multiple chemical classes. These studies applied multiple statistical methods such as the Bayesian approach and quantile-based g-computation to examine exposure to mixtures. However, little is known about interactions between chemicals within various chemical mixtures, or across chemical classes, which may elucidate additive, synergistic, or antagonistic effects of chemical mixtures on ASD.

Previous epidemiologic studies of ASD in the MARBLES (Markers of Autism Risk in Babies – Learning Early Signs) cohort examined gestational exposures to individual chemical classes in association with childhood ASD. However, these studies have been limited in performing comprehensive mixture analyses (Barkoski et al., 2019, 2021; Dou et al., 2024; Oh et al., 2021; Philippat et al., 2018; Shin et al., 2018). While these studies showed statistically significant or marginal associations between ASD and a few compounds within each chemical class, they provide limited understanding of the overall impact of gestational chemical exposures on ASD. Therefore, this current study aims to investigate the impact of mixtures of multiple chemical classes on ASD by leveraging the MARBLES data (Hertz-Picciotto et al., 2018). Given our small sample size of mother-child pairs (n = 110), we first utilized a two-step approach – clustering followed by dimension reduction – to identify combinations of chemical exposures (i.e., clusters) that summarize exposure levels within each cluster. Then, we evaluated the associations between summarized scores of these clusters and the outcome. Additionally, we examined the interactions within and across clusters.

2. Materials and methods

2.1. Study population

For this current study, we used the data from the MARBLES cohort (Hertz-Picciotto et al., 2018). Since 2006, the MARBLES study has recruited pregnant women who already had a child diagnosed with ASD and were thus at elevated likelihood (~20 %) of having another child who may develop ASD (Hertz-Picciotto et al., 2018; Ozonoff et al., 2011, 2024). Most participants were recruited from a list of families receiving state-funded services for children with ASD. Participants were eligible if they *i)* had one or more children with ASD; *ii)* were at least 18 years old; *iii)* spoke, read, and understood English; and *iv)* lived within 2.5 h of the Davis/Sacramento region. Study protocols were approved by the Institution Review Boards for the University of California Davis (UC Davis) and the State of California. All data were collected with informed consent of participants. Details of the study design,

recruitment methods, and data collection are available elsewhere (Hertz-Picciotto et al., 2018).

For this study, we included 110 mother-child pairs with complete chemical measurement data from both urine and blood samples collected during pregnancy, as well as neurodevelopmental diagnostic data for the children. A flow chart for the study sample selection from the full cohort is shown in Fig. 1. Note that not all mothers had measurements of blood and urine samples during pregnancy and some mothers only had measurements of either urine or blood throughout the entire pregnancy. Because chemical analyses were conducted across different research projects in different years (from 2016 to 2021), the number and type of the samples used for the analysis of each chemical class vary, resulting in the reduced number of mother-child pairs, compared to the full cohort. Among the 110 mother-child pairs, five mothers participated in the study for two separate pregnancies, resulting in a total of 105 unique mothers in the study population.

2.2. Child neurodevelopmental assessment

At approximately 3 years of age, children were administered the Autism Diagnostic Observation Schedule (ADOS), which is a standardized diagnostic tool for ASD (Lord et al., 2000; Ozonoff et al., 2005). ADOS calibrated severity scores (CSS) are normalized scores for language ability and age, obtained from raw scores of the two ADOS sub-scales. The CSS has been utilized as a measure of ASD symptom severity and ranges from 1 to 10, with higher scores reflecting greater ASD symptom severity (Gotham et al., 2009). Children were also assessed for cognitive development using the Mullen Scales of Early Learning (MSEL), a standardized instrument for ages from birth to 36 months (Mullen, 1995). We classified child neurodevelopmental outcomes into ASD, typical development (TD), or non-typical development (non-TD) with other neurodevelopmental concerns using an algorithm based on *Diagnostic and Statistical Manual of Mental Disorders, 5*th *Edition* (DSM-5) criteria as well as scores of ADOS (Lord et al., 2000; Ozonoff et al., 2005) and MSEL (Mullen, 1995). Children classified with ASD met *DSM-5* criteria for ASD and had ADOS CSS ≥4 (n = 25). Children classified as non-TD (n = 20) did not meet *DSM-5* criteria for ASD but had an ADOS CSS ≥3 and/or had two or more MSEL subscale scores ≥1.5 standard deviation (SD) below the mean, and/or had one more MSEL subtest score ≥2 SD below the mean. Children were classified as TD (n = 65) if they did not meet any of the above criteria (Ozonoff et al., 2014) (Fig. S1).

2.3. Sample collection and chemical quantification

In MARBLES, both maternal blood and urine samples were collected during each trimester of pregnancy. For blood, study staff members collected a blood sample from all mothers in each trimester. For urine, each mother was instructed to collect three first morning void (FMV) samples for three consecutive weeks and a 24-h urine sample in each trimester. Samples were kept in a home freezer until the staff members came to retrieve them. Then, they were brought to the laboratory at UC Davis, aliquoted, and then stored at −80 °C until analysis. For whole blood, the samples were centrifuged for serum separation before storage.

For phthalates, phenols, and parabens, when mothers provided three or more urine samples within a trimester, we selected the first FMV as an individual sample and pooled all remaining samples for that trimester and quantified them to reduce analytical costs. Among mothers who had measurements for all chemical classes included in this current study, only 2nd and 3rd trimester samples remained. For OPEs, due to the limited analytical budget, we selected only the earliest 3rd trimester sample for each mother. However, if the samples were collected in the second half of the 3rd trimester, we selected the last sample from the 2nd trimester which is closer to the first half of the 3rd trimester. Details of sample collection, transport, and storage are described elsewhere (Hertz-Picciotto et al., 2018; Shin et al., 2019). Sample type and collection times for each chemical class are shown in Supporting Information: Table S1.

A total of 42 biomarkers, including 9 PFAS in blood, 14 phthalate metabolites, 5 phenols, 4 parabens, and 10 OPE metabolites in urine were analyzed (Table S2). Both PFAS and phthalates were analyzed at the Center for Disease Control and Prevention (CDC). Phenols and parabens were analyzed at the Laboratory of Exposure Assessment and Development for Environmental Research (LEADER), Rollins School of Public Health, Emory University. OPEs were analyzed at the Wadsworth Center-Human Health Exposure Analysis Resources (HHEAR) laboratory.

Using online solid-phase extraction coupled to high-performance liquid chromatography-isotope dilution tandem mass spectrometry (LC-MS/MS), we quantified nine PFAS in serum, including perfluorohexane sulfonate (PFHxS), PFOS, PFOA, perfluorononanoate (PFNA), perfluorodecanoate (PFDA), perfluoroundecanoate (PFUnDA), perfluorododecanoate (PFDoDA), 2-(N-methyl-perfluorooctane sulfonamido) acetate (MeFOSAA), and 2-(N-ethyl-perfluorooctane sulfonamido) acetate (EtFOSAA). We quantified 14 metabolites of eight phthalates in urine including monoethyl phthalate (MEP), mono-n-butyl phthalate (MBP), mono-hydroxy-n-butyl phthalate (MHBP), mono-isobutyl phthalate (MiBP), mono-hydroxy-isobutyl phthalate (MHiBP), monobenzyl phthalate (MBzP), mono(3-carboxypropyl) phthalate (MCPP), mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP), monoisononyl phthalate (MNP), mono-carboxyisooctyl phthalate (MCOP), and mono-carboxyisononyl phthalate (MCNP).

We also quantified five phenols, four parabens, and 10 OPE metabolites in urine. Analyzed phenols include bisphenol A (BPA), bisphenol F (BPF), bisphenol S (BPS), and triclosan (TCS) as well as triclocarban (TCC). Analyzed parabens include methyl paraben (MEPB), ethyl paraben (ETPB), propyl paraben (PRPB), and butyl paraben (BUPB).

Analyzed OPEs include diethyl phosphate (DEP), dipropyl phosphate (DPRP), sum of di-n-butyl phosphate and di-iso-butyl phosphate (DBUP/DIBP), bis(butoxyethyl) phosphate (BBOEP), bis(2-ethylhexyl) phosphate (BEHP), bis(2-chloroethyl) phosphate (BCEP), bis(1-chloro-2-propyl) phosphate (BCPP), bis(1,3-dichloro-2-propyl) phosphate (BDCPP), diphenyl phosphate (DPHP), and bis(2-methylphenyl) phosphate (BMPP). Details of

analytical methods and quality assurance and quality control (QA/QC) are shown in Supporting Information: 1. Additional Information on Chemical Quantification.

2.4. Exposure metric

For biomarker concentrations below the limit of detection (LOD), we used a proxy value (LOD/√2) (Hornung and Reed, 1990). For compounds or metabolites measured in urine, to account for urinary dilution, we measured specific gravity (SG) using a digital handheld refractometer (ATAGO Co., Ltd., Tokyo, Japan) and then corrected urinary biomarker concentrations by using the following formula: $C_{SG} = C \times [(1.012–1)/(SG – 1)]$, where C_{SG} is the SG-corrected concentration (ng/mL), C is the measured biomarker concentration in urine (ng/mL), 1.012 is the median SG of all analyzed samples, and SG is the specific gravity of each sample. Because mothers did not provide the same number of urine samples, we computed a weighted average of biomarker concentrations (C_{avg}) for phthalates, phenols, and parabens using the following formula (Barkoski et al., 2019; Shin et al., 2018): $C_{avg} = (C_{ind} + C_{pooled} \times N_{pooled})/(1 + N_{pooled})$, where C_{ind} is the biomarker concentration in an individual sample, Cpooled is the biomarker concentration in a pooled sample, and N_{pooled} is the number of composites in a pooled sample. For di(2-ethylhexyl) phthalate (DEHP), we used the molar sum of four DEHP metabolites (∑DEHP = MEHP + MEHHP + MEOHP + MECPP, nmol/mL) as a DEHP biomarker instead of individual DEHP metabolites in statistical analyses due to the high correlations among four DEHP metabolites (Spearman's rho = 0.85 to 0.99; data not shown). The SG-corrected concentrations were used to calculate the C_{avg}. For blood PFAS with long elimination half-lives in a body, we used the arithmetic average of available PFAS concentrations for mothers who provided multiple samples.

2.5. Statistical analysis

We conducted univariate statistics and compared participant characteristics among children with TD, non-TD, and ASD using Pearson's chi-squared test. The descriptive statistics of each biomarker concentrations are available in Table S3, and we compared biomarker levels among children with TD, non-TD, and ASD using the Mann-Whitney test (Table S4). For further statistical analyses, we only included 30 biomarkers detected in >50 % of the samples, including 3 parabens, 2 phenols, 11 phthalate biomarkers (with 14 measured metabolites, including the sum of 4 DEHP metabolites as the DEHP biomarker [∑DEHP]), 7 OPE metabolites, and 7 PFAS. We used continuous ln-transformed concentrations due to their right-skewed distributions and created a correlation heatmap for the ln-transformed concentrations of these 30 biomarkers using Pearson's correlations (Fig. S2, Table S5).

Before examining the mixture effects, we conducted a two-steps analysis due to the small sample size (110 mother-child pairs) and many predictors (30 biomarkers). First, we performed hierarchical clustering (h-clustering), an agglomerative clustering algorithm, to reduce the dimensionality of the biomarker and identify clusters with similar chemical profiles. We selected the optimal number of clusters (k) based on ≥ 80 % stability (i.e., the consistency of the cluster structure when the algorithm is applied to slightly perturbed versions of the data) and a high average silhouette coefficient (i.e., a measure of how well each data point fits within its assigned cluster compared to other clusters) (Batool and Hennig, 2021; Chavent et al., 2011). The clustering results were displayed

using a dendrogram. Second, we conducted a linear discriminant analysis (LDA) to obtain LDA components for each cluster identified from h-clustering. The LDA is a method for feature extraction or dimension reduction for classification (Abdulhafedh, 2022) by finding a linear combination of biomarkers that provides maximal separation between groups (Balakrishnama and Ganapathiraju, 1998). From the LDA analysis, we defined LDA components as LDA scores (LDAS) which summarize the exposure level (i.e., biomarker concentrations) of each cluster:

$$LDAS_{cluster_i} = \sum_{j=1}^{K-1} D_j X_{c_i}, i = 1, 2, \ldots, k$$

where LDAS is the exposure summary score vector obtained via the LDA, K is the number of outcome groups in each combination ($K = 2$ in our case as shown below), k is the optimal number of clusters obtained from h-clustering, D is the linear determinant coefficient vector of the j^{th} component, X is a $n \times c_i$ exposure matrix, c_i is the number of chemicals in the cluster i, and n is sample size. We conducted two separate LDA analyses with the following combinations of child neurodevelopmental outcome groups: *i)* children with TD or ASD and *ii)* children with TD or non-TD. We compared the distributions of LDA scores between the two outcome groups using a t-test to assess whether the estimated scores distinguished between each combination. For each combination, we also calculated coefficients of linear discriminants to determine the contribution (i.e., weight) of each chemical within each cluster, indicating how much each chemical influences the separation of the outcome group in its respective cluster.

Then, we performed quasi-Poisson regression using LDAS as an exposure variable to examine multipollutant associations with ASD or non-TD compared to TD, estimating relative risk (RR) while adjusting for selected covariates and LDAS of all clusters:

$$y = \beta_0 + \beta_1 LDAS_{cluster_1} + \ldots + \beta_k LDAS_{cluster_k} + Z$$

where LDAS is the LDA score, k is the number of clusters ($k = 4$ which is determined from h-clustering), and Z is the covariance matrix. We constructed a directed acyclic graph (DAG) to identify potential confounders (Fig. S3), which included *a priori* selected variables based on previous MARBLES studies (Barkoski et al., 2019, 2021; Dou et al., 2024; Oh et al., 2021; Philippat et al., 2018; Shin et al., 2018). The selected covariates include child's sex (male, female), year of birth (2009–2010, 2011–2014), maternal age at delivery (<35, ≥35 years), maternal race/ethnicity (non-Hispanic White; other, which included Hispanic, Asian, Black, and multiracial persons), parity (1, ≥1, missing), maternal pre-pregnancy body mass index (BMI) (<25.0, ≥25.0 kg/m^2), maternal education (less than bachelor's degree, bachelor's degree or more), and homeownership (no, yes, missing).

To investigate possible interactions between and within clusters, we used bivariate exposure-response distributions of Bayesian Kernel Machine Regression (BKMR) with a multiple parallel chains model. The distributions show the effect of one exposure conditional on different percentiles (25th, 50th, 75th, and 90th percentiles) of another exposure, while the

rest of the exposures were fixed at the 50th percentile (Bobb et al., 2015). The Bayesian approach in BKMR is more flexible with small sample sizes compared to frequentist methods, and we applied multiple chains to obtain stable estimation. The BKMR model with probit link function was fitted using Markov Chain Monte Carlo (MCMC) with five chains of 10,000 iterations after a burn-in sample of 1000 iterations, and default parameter settings were used for modeling.

Using BKMR, we examined univariate exposure-response curves between biomarker concentrations and LDAS as well as between biomarker concentrations and risk of ASD or non-TD. Furthermore, the overall effects of the clusters were examined by analyzing the changes in RR when LDAS of all clusters were set at different percentiles (10th to 80th percentiles, with 10th percentile increase) compared to when those of all clusters were set at the 50th percentile (Bobb et al., 2015).

For the mixture analyses, we converted the ln-transformed biomarker concentrations to z-scores to account for differences in the scales of biomarker concentrations; these z-scores were also used in the h-clustering and LDA. Statistical analyses were performed in R version 4.3.1 (R Core Team, https://www.R-project.org/). For the BKMR analysis, we used a "*bkmrhat*" package. The significance level was set at $\alpha = 0.05$ for most analyses, except for Pearson's chi-squared test ($\alpha = 0.10$) to account for our limited sample size and minimize the risk of missing meaningful associations.

3. Results

3.1. Participant characteristics

Among the 110 mother-child pairs included in this study, 65 (59 %), 25 (23 %), and 20 (18 %) children were classified as TD, ASD, and non-TD, respectively (Table 1). More male children than female children were included in the current study (63 %). More mothers of non-TD children tended to deliver a baby at 35 years old or younger (70 %), compared to those of ASD and TD children (55 % and 52 %, respectively). The participants retained in this study had similar characteristics to those not included from the full cohort (Table S6).

3.2. Maternal prenatal biomarker concentrations

Three out of 4 parabens, 7 out of 9 PFAS, 2 out of 5 phenols, all 7 OPE metabolites and 14 phthalate metabolites were detected in more than 50 % of the samples (Table S3). Compared to mothers of TD children, mothers of ASD children had lower BCPP concentration, while mothers of non-TD children had higher phthalate biomarker concentrations (∑DEHP, MCPP, MNP, MCOP) (p-value <0.05) (Table S4). Pearson's correlations coefficients of ln-transformed concentrations ranged from 0.13 to 0.76 among parabens and phenols, from −0.17 to 0.65 among PFAS, from 0.36 to 0.96 among phthalates, from 0.17 to 0.61 among OPEs (Fig. S2, Table S5). Overall, biomarker concentrations within the same chemical class showed higher correlations each other compared to those across chemical classes. We also observed notable cross-chemical class correlations among phthalates, parabens, BPA, and OPEs. For example, most phthalate biomarkers had at least one moderate positive correlation with parabens (r = 0.30 to 0.52), phenols (r = 0.32 to 0.71), and OPE biomarkers

(r = 0.31 to 0.52). Also, MEPB showed moderate correlations with TCS (r = 0.33), BPA (r = 0.39) and OPE biomarkers (e. g., DIBP/DBUP, BDCPP, DPHP) (r = 0.30 to 0.41), while PRPB had moderate correlations with TCS (r = 0.36), BPA (r = 0.31) and DPHP (r = 0.34). BPA had moderate correlations with some OPEs (i.e., DBUP/DIBP, BBOEP, BDCPP, DPHP) (r = 0.31 to 0.39).

3.3. Chemical clustering and exposure summarization

From h-clustering of the 30 biomarkers quantified in prenatal maternal samples of 110 mother-child pairs, we identified four distinct clusters that are reasonably well-separated from each other (Fig. 2). This number met our selection criteria of ≥80 % stability and high average silhouette width. Clusters 1 through 4 include PFAS, OPEs, parabens/TCS, and phthalates/BPA, respectively. This indicates that h-clustering discriminates clusters with similar chemical functions or uses.

Using LDA, we estimated LDA scores for each cluster in two outcome group combinations: ASD vs. TD (Fig. 3A) and non-TD vs. TD (Fig. 3B). When comparing LDA score distributions between outcome groups, we found significant differences (p-value <0.05) in most cases, except for Cluster 3 of non-TD vs. TD. Overall, outcomes tended to be separated well across clusters, implying that each outcome has different exposure characteristics. For most clusters, mothers whose children had ASD or non-TD diagnosis tended to have higher LDA scores compared to mothers who had TD children, implying certain chemical combinations might be associated with ASD or non-TD.

3.4. Multipollutant associations of chemical mixtures with ASD/non-TD

For all clusters, higher LDA scores were associated with increased risk of ASD relative to TD (RR ranged from 1.12 to 1.17, p-value <0.05) (Fig. 4A, Table S7). Based on the absolute coefficients of linear discriminants, separation of TD from ASD was most influenced by PFNA, BCPP, MEPB, and MHiBP in each cluster (Fig. 4B, Table S8). This indicates that gestational exposures to these compounds contributed the most to the differences between TD and ASD. When examining the relationship between chemical concentrations and LDA scores, PFNA concentrations showed a positive relationship with LDA scores (Fig. S4, bottom panel), while BCPP, MEPB, and MHiBP concentrations showed opposite trends (Figure S5–S7, bottom panels). These trends were similar to the relationship between chemical concentrations and the RR of ASD (Figure S4–S7, top panels).

For non-TD, higher LDA scores were associated with increased risk of non-TD relative to TD for Cluster 2 (OPEs) (RR = 1.07, 95 % CI: 1.01, 1.14) and Cluster 4 (phthalates and BPA) (RR = 1.12, 95 % CI: 1.05, 1.19) (Fig. 4C, Table S7). In Cluster 2 and Cluster 4, BCETP and MBP were the most influential components, respectively (Fig. 4D, Table S8), indicating that gestational exposures to BCETP and MBP contributed the most to the differences between TD and non-TD. BCETP had a negative relationship with LDA scores but showed a non-linear relationship with the RR of non-TD (Fig. S8). MBP had a non-linear relationship with LDA scores but showed a slightly decreasing trend with the RR of non-TD (Fig. S9).

When using BKMR to investigate the overall effects of the cluster mixtures on ASD or non-TD, increased cumulative exposures to mixtures of the four clusters were associated with increase in the risk for ASD and non-TD (Fig. 5).

3.5. Interactions between and within clusters for ASD/non-TD

We investigated interactions between clusters using BKMR bivariate plots (Figure S10). We assumed there is a potential interaction between biomarkers if the shape of the curves between one biomarker and the outcome is different for the different quantiles of another biomarker (e. g., if the curves intersect with one another at any point). Overall, we observed potential interactions between clusters, with one showing a decreasing slope of the curves at higher conditional exposure percentiles. Cluster 1 (PFAS) showed interactions with Cluster 4 (phthalates/BPA) for ASD (Fig. S10).

We also examined interactions between chemical biomarkers within each cluster that showed statistical significance with increased risk of ASD or non-TD, relative to TD (Figures S11–S16). In Cluster 1, we observed potential interactions between PFAS compounds, with noticeable interactions between PFHxS and Me-FOSAA for ASD. The relationship between PFHxS and ASD risk changed from inverse to positive when Me-FOSAA was at the 90th percentile concentration (Fig. 6A, Figure S11). In Cluster 2, we observed potential interactions between OPE compounds, with BCETP showing noticeable interactions with other OPEs and a non-monotonic dose-response trend (Figure S12). In Cluster 3, we observed interactions between MEPB and ETPB, where the inverse relationship between MEPB and ASD risk become less steep as ETPB concentrations increased. Additionally, PRPB and TCS showed interactions, where the slope of the curve flattened as TCS concentrations increased (Figure S13). Cluster 4 showed potential interactions between phthalates, with particularly noticeable interactions between MCNP and MCOP (Fig. 6B). Additionally, MCNP showed potential interactions with MBP, MHBP, and MNP (Figure S14). For non-TD, BCETP and BCPP showed noticeable interactions with other OPEs in Cluster 2. BCETP also displayed non-monotonic dose-response trend, similar to that observed for ASD (Figure S15). In Cluster 4, we observed noticeable interactions between MCNP and MNP, where the slope of the curve flattened as MNP concentrations increased (Fig. 6C, Figure S16).

4. Discussion

In the present study, we examined the associations between gestational exposures to five chemical classes and risk of ASD/non-TD in children. We used concentrations of 29 chemical biomarkers quantified in maternal samples collected during pregnancy. Based on the results of clustering, we chose four clusters corresponding to distinct chemical classes (PFAS [Cluster 1], OPEs [Cluster 2], parabens and TCS [Cluster 3], phthalates and BPA [Cluster 4]), indicating that chemicals with similar exposure characteristics (e.g., source, product use) are clustered together. When using the LDA scores of each cluster as exposure variables, we found that increased risks of both ASD and non-TD were associated with biomarkers represented in the chemical clusters of common plasticizers, including OPEs, phthalates, and BPA. Clusters of PFAS and parabens were additionally associated with ASD

risk. Furthermore, the cumulative exposure to chemicals in all clusters were associated with increased risks of ASD and non-TD. We observed the interactions of biomarkers between and within clusters, suggesting potential interactions among chemicals that have similar uses or exposure sources, such as food, indoor dust, or consumer products (Dodson et al., 2020; Li et al., 2019; Pacyga et al., 2019; Shin et al., 2020b; Tittlemier et al., 2007).

Some of our results were comparable to those of previous MARBLES studies that investigated the association of ASD with individual chemical compounds as well as with mixtures of certain chemical classes, including PFAS, parabens, and phenols (Barkoski et al., 2019; Oh et al., 2021; Shin et al., 2018). From this current study, we observed that ASD risk was associated with PFAS (Cluster 1) and PFNA had the largest discriminant ability to distinguish TD and ASD, consistent with our previous study finding that individual PFOA and PFNA were associated with increased risk of ASD, along with previous findings (Oh et al., 2021). From this current study, we observed increased risk of ASD with parabens and TCS (Cluster 3), with MEPB having the largest discriminant ability. Our previous MARBLES study used trinomial weighted quantile sum (WQS) regression to analyze a mixture of parabens and BPA (Barkoski et al., 2019) and reported that exposure to the mixture was associated with increased ASD risk, with borderline statistical significance. This previous study also reported statistically significant associations between the mixture and increased risk of non-TD, and MEPB was the most weighted compound. In the previous study, individual BPA was inversely associated with ASD risk, and the current study also showed that BPA had a similar inverse trend with ASD risk (Fig. S7). However, BPA contributed less than phthalates in the same cluster and did not show noticeable interaction with other phthalates. For phthalates, we observed increased risk both for ASD and non-TD in association with Cluster 4 from this current study, with MHiBP and MBP having the largest discriminant ability, respectively. Our previous MARBLES study with phthalates reported that individual phthalate biomarkers were not associated with ASD, but MEP was associated with increased risk for non-TD (Shin et al., 2018). MCPP and MCNP were also associated with increased risk for non-TD, with borderline significance. These results were comparable with our findings that MEP, MCPP, and MCNP have positive relationships with non-TD. However, we observed potential antagonistic effects between MCNP and MNP in the interaction analysis (Figure S16), suggesting a need to investigate interactions among compounds. A more comprehensive approach is necessary when examining exposure to chemical mixtures. We observed increased risk for ASD and non-TD with OPEs (Cluster 2) from the current study. In our previous study, we also observed increased risk for non-TD in relation to OPE mixture although this association did not reach statistical significance (Choi et al., 2024).

Three epidemiological studies have investigated the associations between mixtures of multiple chemical classes and ASD or ASD-related behaviors in children using different statistical approaches from the current study (Hamra et al., 2019; Tsai et al., 2023; van den Dries et al., 2021). Only one Taiwanese study reported similar findings with the current study that higher exposure to a mixture of phthalates and/or metals was associated with increased odds of ASD-related problems using quantile-based g-computation (Tsai et al., 2023). In the phthalate and metal mixture, cobalt, MEOHP, and lead contributed the most to positive weights. The other two studies found no significant associations with ASD or

ASD-related behaviors (Hamra et al., 2019; van den Dries et al., 2021). A U.S. cohort study investigated the association with a mixture of PBDEs, PBBs, PCBs, OCPs, and PFAS in children diagnosed with ASD using a Bayesian method (Hamra et al., 2019). A study from the Netherlands examined the association between a mixture of phthalates, bisphenols, and OPPs and autistic behaviors using quantile-based g-computation (van den Dries et al., 2021). Note that there was not much overlap for the compounds considered between these studies and this current study. Additionally, differences in population characteristics, neurodevelopmental assessment tools, and statistical methods might have contributed to varying results.

We observed cumulative exposure to the four chemical clusters identified through h-clustering, resulting in a greater overall effect of these clusters on the risk of ASD and non-TD. Underlying mechanisms of gestational exposures to chemicals and their impact on child neurodevelopment are still unclear and complicated. However, one common mechanism suggested for endocrine-disrupting chemicals (EDCs), such as the compounds included in our study, relates to thyroid hormone disruption during pregnancy (Braun, 2017; Ghassabian and Trasande, 2018). Maternal thyroid hormones play an important role in the normal brain development of offspring (Préau et al., 2015) and some birth cohort studies have reported that maternal thyroid hormone deficiency during pregnancy is associated with increased risk of ASD or autistic traits of children (Andersen et al., 2014; Getahun et al., 2018; Levie et al., 2018; Roman et al., 2013). Therefore, thyroid hormone disruption by EDCs may affect the neurodevelopment of children before or after birth. Several epidemiological studies have reported that prenatal exposures to these chemicals as either individual chemicals or a mixture were associated with maternal thyroid dysfunction during pregnancy (Berger et al., 2018; Choi et al., 2021; Derakhshan et al., 2021; Kato et al., 2016; Lebeaux et al., 2020; Preston et al., 2020; Romano et al., 2018). However, more studies are needed on prenatal exposures to mixtures of multiple chemical classes in relation to maternal thyroid dysfunction.

When investigating interactions within and between clusters, we observed complex chemical interactions among chemicals in relation to child neurodevelopmental outcomes, highlighting the need to account for exposures to multiple chemical classes in epidemiological studies. For example, BCPP, the most influential compound for ASD in Cluster 4, had an inverse relationship with ASD risk (Fig. S5). However, within the same cluster, BCPP showed both potential synergistic and antagonistic interactions, with dose-response patterns varying depending on co-exposures with other OPEs (Figure S12). Specifically, in the BCPP-BBOEP combinations, the slopes of BCPP became narrower as BBOEP percentiles increased, whereas in the BCPP-DPHP combination, the slopes of BCPP became steeper as DPHP percentiles increased, indicating a possible synergistic effect. These patterns suggest that BBOEP may attenuate and DPHP may amplify the effect of BCPP, which could be interpreted as potential antagonistic and synergistic interactions in a dose-dependent manner. Similarly, BCETP, the most influential chemical for non-TD, also showed both potential synergistic and antagonistic interactions with other OPEs mirroring the patterns observed for BCPP (Figure S15). However, given the complexity of mixture effects and the non-monotonic dose-response trends (i.e., curved shapes), these interpretations should be further validated in future studies. To our knowledge, limited

studies exist on the biological mechanisms underlying the interaction of chemicals within the same or across different classes. A few *in vitro* and *in vivo* studies on PFAS mixtures found antagonistic effects on neurotoxicity (Menger et al., 2020), synergistic effects on reproductive toxicity (Kjeldsen and Bonefeld-Jorgensen, 2013), and additive effects on developmental toxicity (Zhou et al., 2017). One study reported that the complex patterns of interactions within PFAS might depend on various factors such as dose levels, proportions of dose, or mixture components (Ojo et al., 2021). In human liver cells, most combinations of PFOS with other PFAS compounds such as PFOA, PFHxS, PFNA, PFDA, and PFHpA showed synergistic interactions; however, antagonistic effects were observed in some mixtures with PFOA (Ojo et al., 2020). Additionally, we observed non-monotonic dose-response relationship for some chemicals combined with interactions with other chemicals. This relationship has frequently been reported for EDCs in toxicological studies (Lagarde et al., 2015), highlighting the need to account for non-linear effects and interactions in epidemiological studies. Assuming monotonic trends may lead to misinterpretation of associations between chemical exposures and health outcomes. However, our findings may indicate that the interaction was spurious given the number of interactions tested and our small sample size.

The present study has several strengths. First, we improved the investigation of chemical exposures in relation to ASD by using clinical diagnosis data. Second, to address the challenge of a small sample size, we applied a two-step approach using h-clustering and LDA, which reduced the dimensionality and provided stable estimates. This approach offers advantages over commonly used mixture analysis methods, such as penalized regression (e.g., lasso, elastic net), which often suffer from variance overestimation and overfitting in small sample scenarios (Riley et al., 2021). In addition, LDA might be more statistically efficient for a small sample size compared to quantile-based g-computation used in other mixture studies (Tsai et al., 2023; van den Dries et al., 2021). LDA models the covariance structure within each group to maximize the difference between groups, while quantile-based g-computation estimates effects across multiple quantiles (Keil et al., 2020). Furthermore, using LDA scores as exposure measures rather than individual chemical biomarker concentrations may have minimized bias that may be caused by correlations between chemicals and reduce the risk of confounding effects from one chemical on the observed associations of another.

However, this study also has some limitations to be noted. First, our findings may not be generalizable to other populations because MARBLES is an enriched ASD likelihood cohort, with children who already have an older sibling diagnosed with ASD. Second, our sample size is small compared to other similar studies. Third, OPE biomarkers were measured in a single spot urine sample mostly collected in the 3rd trimester (92 %) unlike other urinary biomarkers. Considering short biological half-lives of OPEs, it may not represent average OPE exposure during pregnancy. A few prior studies observed intraclass correlation coefficients for some urinary OPE biomarkers in pregnant women, showing varied results depending on the study population and biomarkers, with weak to high reproducibility (0.16–0.95) (Hoffman et al., 2014; Romano et al., 2017). Fourth, this study did not consider postnatal exposure although chemicals measured in this study are also frequently observed in breastmilk, diet, and various environmental media, indicating

potential ongoing exposure during their first three years of life in addition to gestational exposures. Several studies have reported the associations between early postnatal exposure to chemicals and increased odds of ASD or other neurodevelopmental concerns (Harris et al., 2021; Lee et al., 2018a; Oh et al., 2022). The evaluation of longitudinal early life chemical exposure mixtures should be an area of future study. Lastly, this study did not consider other well-known neurotoxicants, such as heavy metals (e.g., methyl mercury, cadmium, and lead) and organophosphate pesticides.

5. Conclusion

In this current study leveraging the existing data of the MARBLES cohort, we observed that gestational exposures to mixtures of multiple chemical classes were associated with increased risk of ASD or non-TD. This is an important finding because the associations were more evident compared to those reported in previous studies that examined individual chemical classes separately. We also observed the potential interactions among chemicals and overall effects of these chemicals on increased risk of ASD or non-TD. Our findings highlight the importance of considering multiple chemical classes with similar mechanisms of action (e.g., thyroid disruption) in epidemiological studies. Future studies are needed to investigate a broader range of chemical classes in a low-familial ASD risk cohort with a large sample.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rebecca J. Schmidt reports a relationship with Beasley Allen Law Firm that includes: consulting or advisory and travel reimbursement. Rebecca J. Schmidt reports a relationship with Linus Biotechnology Inc that includes: consulting or advisory and travel reimbursement. Rebecca J. Schmidt reports a relationship with Simons Foundation that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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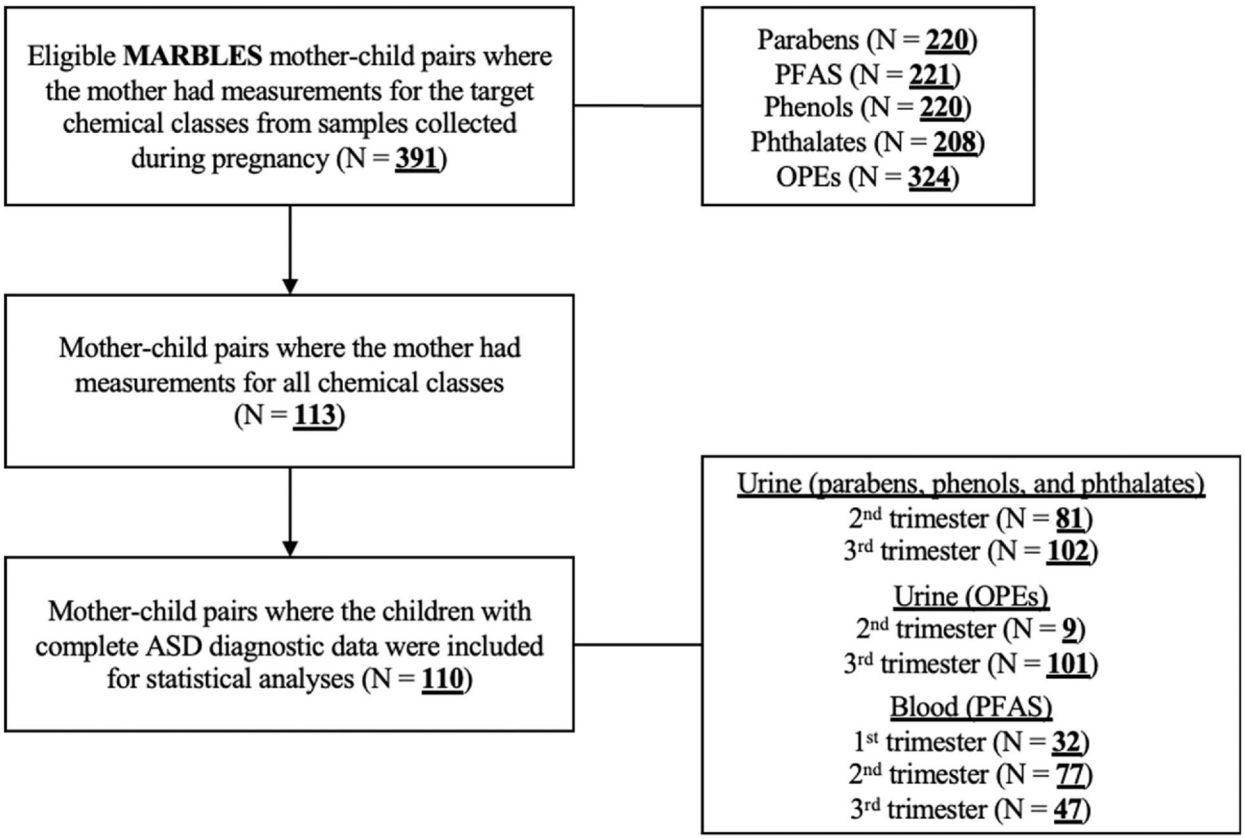

Fig. 1.

Flow chart for the study sample selection from the full cohort. Note: Not all mothers had measurements of blood and urine samples for every trimester and some mothers only had measurements of either urine or blood during the entire pregnancy. This study included mothers who provided at least one sample during the entire pregnancy.

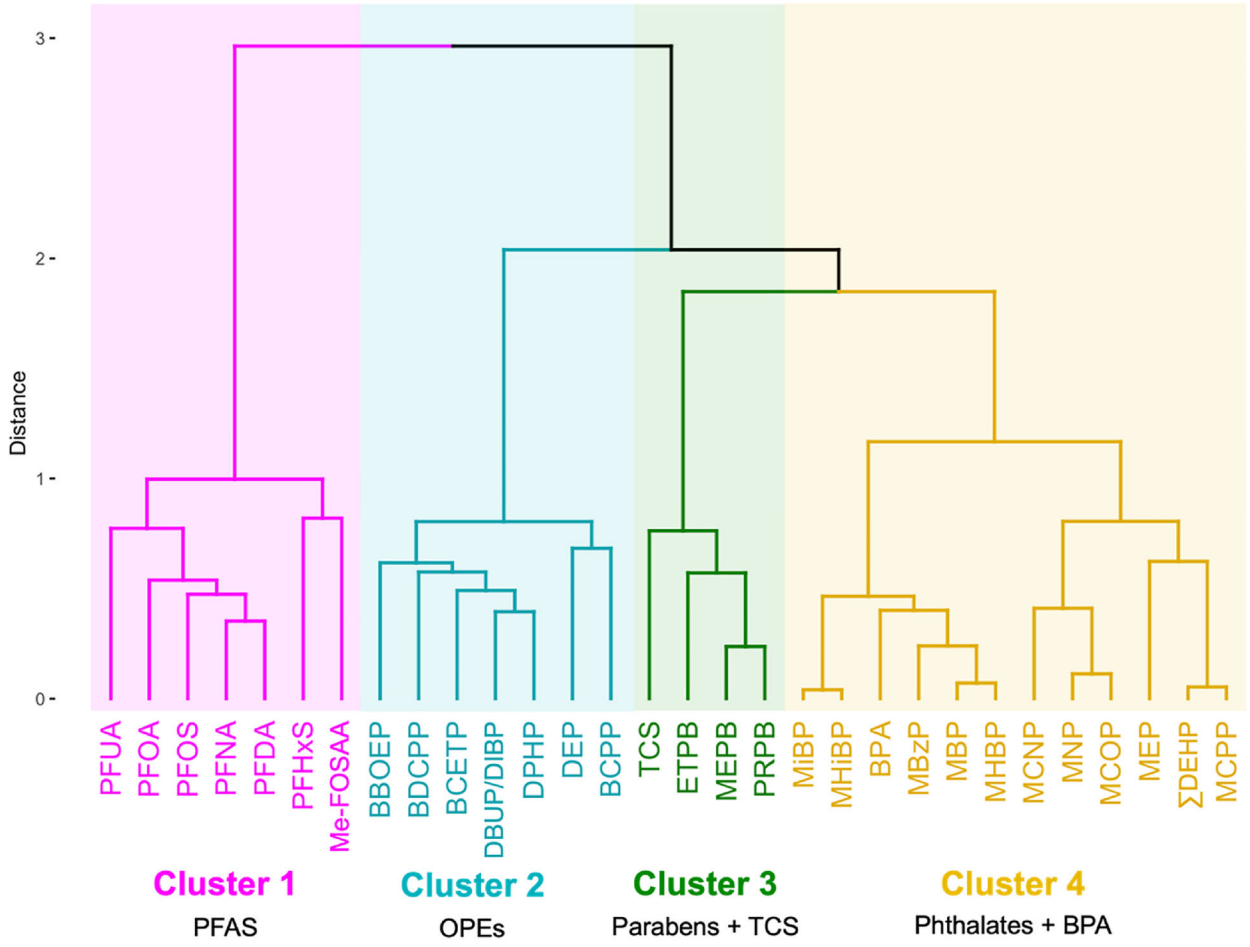

Fig. 2.

Four clusters of the 30 biomarkers identified from hierarchical clustering. This method works from bottom-up, starting with individual chemicals and iteratively merging the two most similar ones until a single large cluster remains. Similarity was measured using Euclidean distance (y-axis).

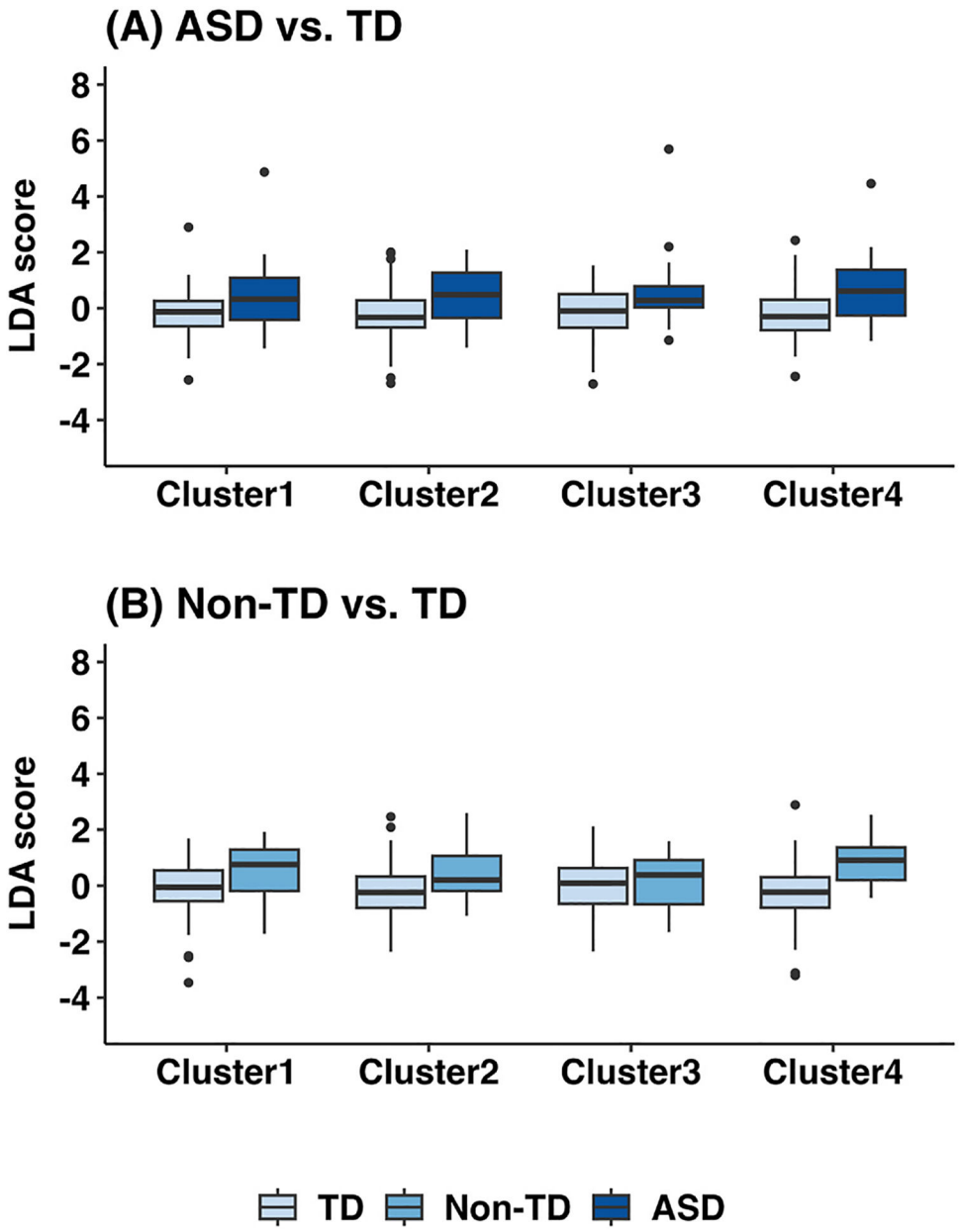

Fig. 3.

Comparison of the linear discriminant analysis (LDA) scores between (A) TD (n = 65) and ASD (n = 25) groups and (B) TD and non-TD (n = 20) groups, respectively. Three horizontal lines in box plots represent 25th, 50th, and 75th percentiles, respectively.

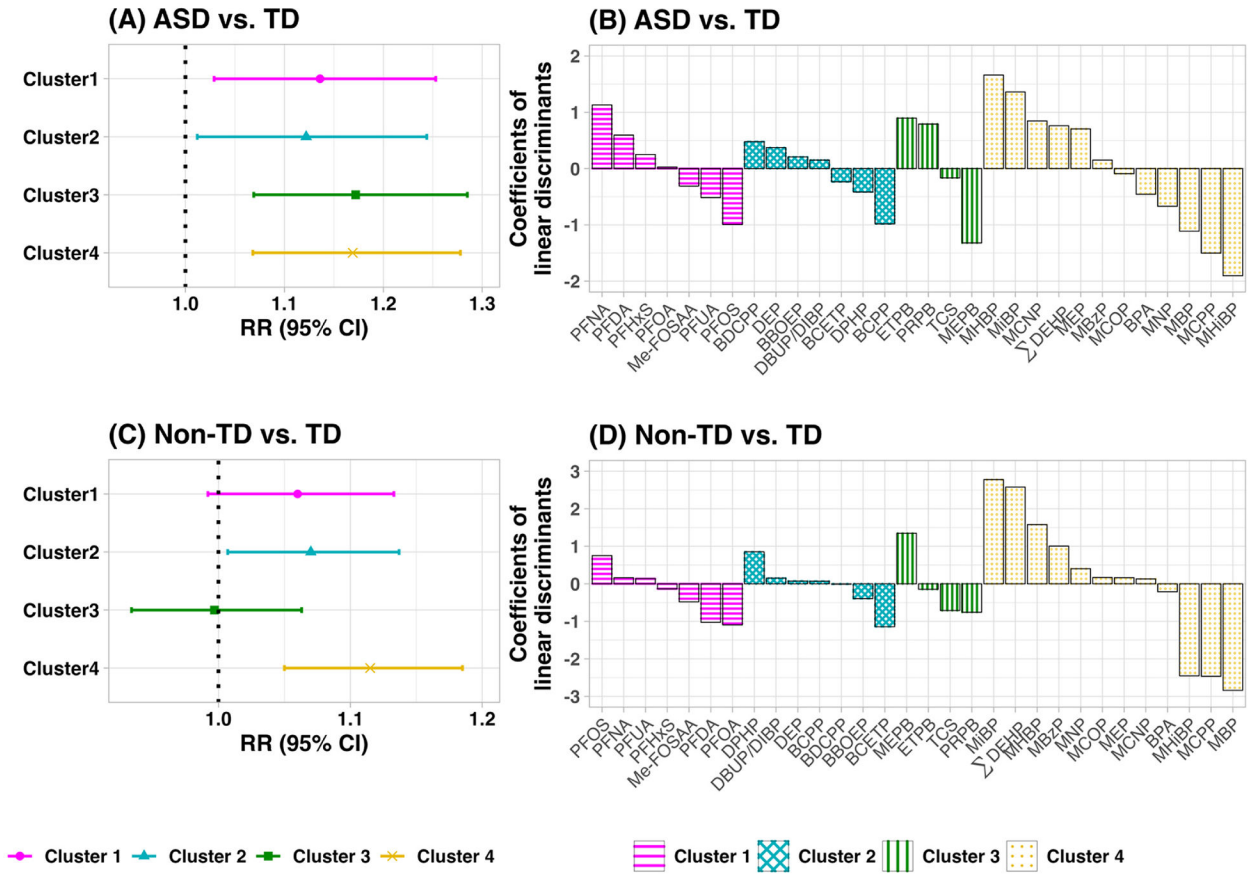

Fig. 4.

(A) Adjusted relative risk (RR; dots) and 95 % CI (error bars) of ASD (n = 25) vs. TD (n = 65) in relation to LDA scores and (B) the coefficients of linear discriminants of each chemical biomarker in each cluster, estimated in the linear discriminant analysis (LDA). (C) Adjusted RR (symbols) and 95 % CI (error bars) of non-TD (n = 20) vs. TD (n = 65) in relation to LDA scores and (D) the coefficients of linear discriminants of each chemical biomarker in each cluster, estimated in the LDA. Note: Each model was adjusted for child sex, year of birth, maternal age, maternal race/ethnicity, parity, maternal pre-pregnancy BMI, maternal education, and homeownership. Coefficients of linear discriminants are also provided in Supporting Information: Table S8.

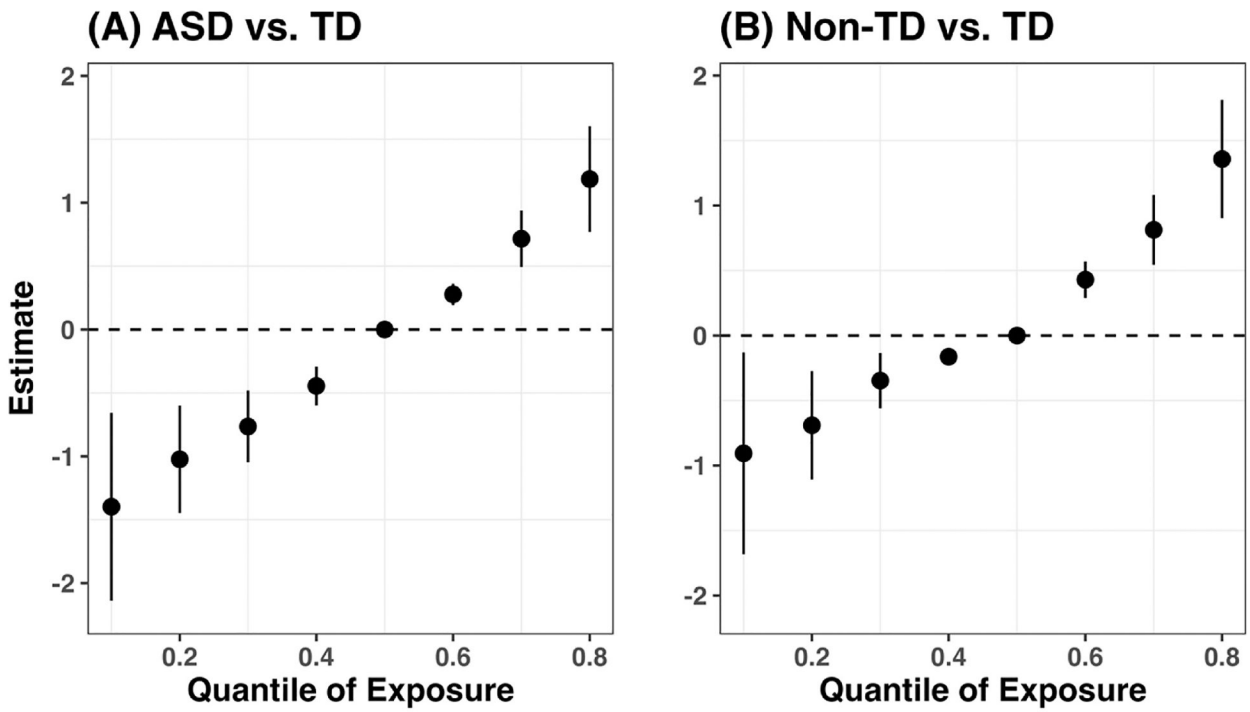

Fig. 5.

Overall effect of the cluster mixture (estimates and 95 % confidence intervals) on (A) ASD or (B) non-TD estimated by Bayesian kernel machine regression, illustrating estimated changes in relative risk when all exposures are at a certain percentile (ranging from 10th to 80th percentiles) compared to when all exposures are at the 50th percentile.

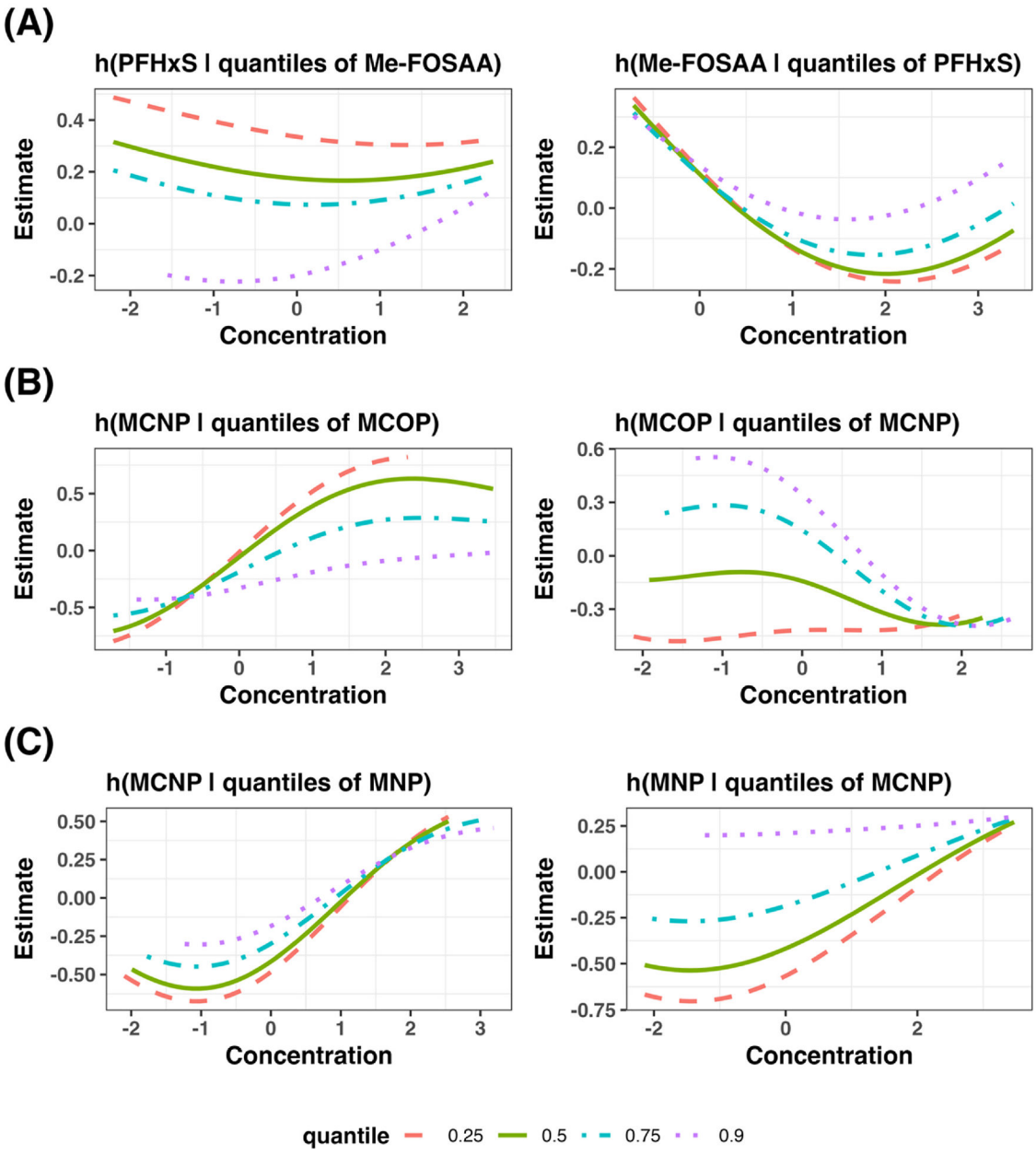

Fig. 6.

Interaction effects of (A) PFHxS and Me-FOSAA, (B) MCNP and MCOP for ASD vs. TD model, and (C) MCNP and MNP for non-TD vs. TD model. Bivariate exposure-response function using Bayesian kernel regression, illustrating the effects of one chemical conditional on varying quantiles (25th, 50th, 75th, or 90th percentiles) of the other chemical, while keeping the rest of the mixtures fixed at their 50th percentile. Concentrations are ln-transformed and converted to z-scores.

Table 1

Characteristics of the study participants (n [%]).

Characteristics	All (n = 110)	TD (n = 65)	ASD (n = 25)	Non-TD (n = 20)	P-value[a]
Child's sex					0.239
Male	69 (63)	37 (57)	19 (76)	13 (65)	
Female	41 (37)	28 (43)	6 (24)	7 (35)	
Year of birth					0.576
2009–2010	54 (49)	34 (52)	10 (40)	10 (50)	
2011–2014	56 (51)	31 (48)	15 (60)	10 (50)	
Maternal age at delivery (years)					0.136
< 35	55 (50)	29 (45)	12 (48)	14 (70)	
≥ 35	55 (50)	36 (55)	13 (52)	6 (30)	
Maternal pre-pregnancy BMI (kg/m^2)					0.446
< 25.0	52 (47)	34 (52)	10 (40)	8 (40)	
≥ 25.0	58 (53)	31 (48)	15 (60)	12 (60)	
Maternal education					0.449
Less than bachelor's degree	59 (54)	32 (49)	14 (56)	13 (65)	
Bachelor's degree or more	51 (46)	33 (51)	11 (44)	7 (35)	
Maternal race/ethnicity					0.377
Non-Hispanic White	62 (56)	40 (62)	13 (52)	9 (45)	
All other races and ethnicities[b]	48 (44)	25 (38)	12 (48)	11 (55)	
Parity					0.409
1	45 (41)	28 (43)	8 (32)	9 (45)	
> 1	63 (57)	37 (57)	16 (64)	10 (50)	
Missing	2 (2)	0 (0)	1 (4)	1 (5)	
Homeownership					0.263
No	42 (38)	22 (34)	10 (40)	10 (50)	
Yes	66 (60)	43 (66)	14 (56)	9 (45)	
Missing	2 (3)	0 (0)	1 (4)	1 (5)	

Abbreviation: autism spectrum disorder (ASD), body mass index (BMI), non-typical development (non-TD), typical development (TD).

[a]P-value from the Pearson's chi-squared test.

[b]Includes Hispanic persons, Black persons, Asian persons, and person with other races.