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Environmental Exposures to pesticides, phthalates, phenols and trace elements are associated with neurodevelopment in the CHARGE study

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Abstract

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Author Statement

We have no additional statement.

Competing interests

Authors declare they have no actual or potential competing financial interests.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval and consent to participate

The CHARGE study protocol and this study were approved by the institutional review boards for the State of California and the University of California-Davis (UC-Davis). Participants provided written informed consent before collection of any data.

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Objective: To determine if higher exposures measured in early childhood to environmental phenols, phthalates, pesticides, and/or trace elements, are associated with increased odds of having a diagnosis of Autism Spectrum Disorder (ASD), Developmental Delay (DD), or Other Early Concerns (OEC) compared to typically developing children (TD).

Methods: This study included 627 children between the ages of 2–5 who participated in the Childhood Autism Risks from Genetics and Environment (CHARGE) study. Urine samples were collected at the same study visit where diagnostic assessments to confirm diagnosis indicated during the recruitment process were performed. Adjusted multinomial regression models of each chemical with diagnosis as the outcome were conducted. Additionally, two methods were used to analyze mixtures: repeated holdout multinomial weighted quantile sum (WQS) regression for each chemical class; and a total urinary mixture effect was assessed with repeated holdout random subset WQS.

Results: Many urinary chemicals were associated with increased odds of ASD, DD or OEC compared to TD; however, most did not remain significant after false discovery rate adjustment. Repeated holdout WQS indices provided evidence for associations of both a phenol/paraben mixture effect and a trace element mixture effect on DD independently. In analyses adjusted for confounders and other exposures, results suggested an association of a pesticide mixture effect with increased risk for ASD. Results also suggested associations of a total urinary mixture with greater odds of both ASD and DD separately..

Conclusion: Higher concentrations of urinary biomarkers were associated with ASD, DD, and OEC compared to TD, with consistency of the results comparing single chemical analyses and mixture analyses. Given that the biospecimens used for chemical analysis were generally collected many months after diagnoses were made, the direction of any causal association is unknown. Hence findings may reflect higher exposures among children with non-typical development than TD children due to differences in behaviors, metabolism, or toxicokinetics.

Keywords

autism; mixtures; environmental phenols; weighted quantile sum; paraben; pesticide

Introduction

As rates of autism spectrum disorder (ASD) diagnosis have risen in the last quarter century, the burden on our communities has become a major public health concern. ASD affects about 1 in 54 children (Maenner, Shaw et al. 2020). Associations of ASD with environmental exposures such as air pollution (Lam, Sutton et al. 2016, Gong, Dalman et al. 2017, Raz, Levine et al. 2018, Ritz, Liew et al. 2018, Pagalan, Bickford et al. 2019) and nutrition (Schmidt, Hansen et al. 2011, Schmidt, Tancredi et al. 2012, Surén, Roth et al. 2013, Schmidt, Kogan et al. 2017, Goodrich, Volk et al. 2018) have been emerging rapidly, leading to increased recognition that environmental exposures likely contribute, either alone or in combination with genetic factors, to ASD risk (National Research Council 2000).

There is a growing body of epidemiologic evidence considering exposure to various classes of pesticides and ASD. Studies have found higher prenatal organophosphate, pyrethroid, and/or organochlorine exposures (Roberts, English et al. 2007, Shelton, Geraghty et al.

2014, von Ehrenstein, Ling et al. 2019) for children with ASD, when prenatal residences were linked to a curated database of nearby agricultural applications that has been validated in relation to measurements from concurrent air samples (Wofford, Segawa et al. 2014). Additionally, a study of pesticide metabolite levels in multiple urine samples collected during pregnancy in a high-risk ASD birth cohort, found that pyrethroid metabolite 3-phenoxy-benzoic acid (3-PBA) had a suggestive association with ASD (adjusted OR 1.5, 95% CI: 0.9, 2.5) (Barkoski, Philippat et al. 2020). In the same study population OP metabolite levels were associated with ASD only among girls (Philippat, Barkoski et al. 2018).

For phthalates, robust research demonstrates associations with neurodevelopmental outcomes generally, including cognitive impairments, internalizing and externalizing behaviors, and attention deficit-hyperactivity disorders, but only limited evaluations of ASD (Engel, Patisaul et al. 2021) (Zhang, Chen et al. 2019). In studies with laboratory animals, phthalates have been found to have neuro-developmental toxicities that mirror those found in humans (Engel, Patisaul et al. 2021). Sex differences in neurodevelopmental outcomes are commonly observed in relation to phthalate exposures (Engel, Patisaul et al. 2021). A Canadian study found that boys with higher *in utero* phthalate exposures, as measured by prenatal urinary phthalate concentrations, had more autistic behaviors, especially if mothers had inadequate folic acid supplementation (Oulhote, Lanphear et al. 2020). However, results in a study conducted in high risk populations did not show a relationship (Shin, Schmidt et al. 2018).

Less studied are the environmental phenols, such as BPA and parabens, which are also potential risk factors for child behavioral outcomes (Harley, Gunier et al. 2013, Braun, Muckle et al. 2017, Philippat, Nakiwala et al. 2017) and altered sex thyroid levels (Aker, Johns et al. 2018, Berger, Gunier et al. 2018). Recent work in a high-risk ASD birth cohort using weighted quantile sum regression (WQSR) showed that a mixture of prenatal urine metabolites of environmental phenols was suggestively associated with ASD, and significantly associated with children who were non-TD (but not ASD) compared to TD (Barkoski, Busgang et al. 2019). Another recent study suggested that associations between BPA and ASD have sex differences in dysregulated genes related to the hippocampus (Thongkorn, Kanlayaprasit et al. 2019).

Metals such as lead (Bellinger 2008), and mercury (Ruth and Zota 2019), have been well studied and an abundant literature of many decades has established adverse neurological effects of clinical relevance. The metalloid arsenic is associated with both ASD (Wang, Houssain et al. 2019) and adverse neurological effects more generally (Tyler and Allan 2014). In contrast, other trace elements have been less studied. Few studies have addressed elements such as cadmium or molybdenum and adverse neurological or neurodevelopmental outcomes (Ciesielski, Weuve et al. 2012, Vázquez-Salas, López-Carrillo et al. 2014, Sanders, Henn et al. 2015).

The goal of this project was to evaluate if exposures to pesticides, phthalates, phenols and trace elements at 2–5 years of age are associated with neurodevelopmental outcomes. For diagnostic outcomes, three main comparisons are made, based on distinct

neurodevelopmental case groups: ASD vs TD (typical development), DD (developmental delays other than ASD) vs TD, and Other Early Concerns (OEC) vs TD.

Methods

This investigation was conducted within the CHARGE study (Hertz-Picciotto, Croen et al. 2006), a case control study that recruits participants from three groups: children with an autism spectrum disorder (ASD), children with developmental delay but not ASD, and general population controls. The first two groups are primarily identified from the California Department of Developmental Services, which coordinates services for persons with developmental disabilities, and is inclusive of all residents of California regardless of place of birth, religion, or financial resources. General population controls are sampled from California birth files, with frequency-matching to ASD cases on age, sex and broad geographic regions encompassing up to 10 counties. Children from all three groups must be: a) aged 24–60 months at recruitment; b) living with a biologic parent who speaks English or Spanish; c) born in California; and d) residing in the study catchment area. Participants that were recruited to the study between 2006 and 2017 and had at least 16 ml of urine collected at their assessment and available for chemical analyses, constituted our study sample, comprising a total of 627 participants.

Developmental Assessment:

We assessed for autism diagnosis to confirm diagnosis indicated through the recruitment process using two gold standard psychometric instruments, both of which are widely accepted for research; the Autism Diagnostic Interview-Revised (ADI-R) (Lord, Rutter et al. 1994, Lord, Pickles et al. 1997, Le Couteur, Lord et al. 2003) and the Autism Diagnostic Observation Schedules (ADOS) (Lord, Risi et al. 2000). The ADI-R is a semi-structured interview for the primary caregiver that reviews the child's development. The ADOS is a semi-structured assessment in which the researcher observes the social interaction, communication, play and imaginative use of materials by children suspected of having ASD (Lord, Risi et al. 2000). To assign final diagnoses of ASD, we used DSM-5, and followed established algorithms utilizing both the ADOS and ADI-R (Risi, Lord et al. 2006). Children from all three groups were administered the Mullen Scales of Early Learning (MSEL) and the Vineland Adaptive Behaviors Scores (VABS). Children who did not meet criteria for an ASD diagnosis and have scores on either the MSEL or VABS that fell below 1.5 SD lower than the mean, and had scores on the other instrument less than 2.0 SD lower than the mean were classified as DD. Children in both the developmental delay and general population groups were screened for ASD using the Social Communications Questionnaire to confirm that they do not have ASD (Rutter, Bailey et al. 2003). For those who screened positive, the ADI-R and ADOS was then administered to determine whether or not they have ASD. All other children who were enrolled because of a community diagnosis of ASD or DD, but were not confirmed for either of these two diagnoses, were grouped together as Other Early Concerns (OEC). Children enrolled as general population controls who did not meet criteria for either ASD or DD were classified as TD. All classification groups are mutually exclusive. All clinicians who psychometrically evaluated children achieved

research reliability on the instruments they administered. English and Spanish speaking clinicians were used in this study.

Exposure Assessment:

Urine samples were collected from the participating child at the time of the visit and immediately frozen at -20°C . Samples remained frozen until analysis (mean 7.4 years, 5–95% range 2.2 – 11.7 years). They were then shipped on dry ice to the New York State Department of Health's (NYSDOH) Wadsworth Center's Human Health Exposure Analysis Resource (HHEAR) Targeted Analysis Laboratory for analysis. The target phenolic compounds were analyzed in urine samples after enzymatic deconjugation, followed by liquid-liquid extraction, as previously described (Asimakopoulos, Thomaidis et al. 2014, Li, Xue et al. 2018). Briefly, 2.5 ng of isotopically labeled internal standards were spiked into 250 μL of urine. The samples were buffered with 400 μL of 1 M ammonium acetate containing 200 unit/mL of β -glucuronidase (MP Biomedicals, LLC) and incubated at 37°C for 12 h. Ethyl acetate (10 mL) was added, shaken for 60 min and centrifuged at 4500 rpm for 5 min. The supernatants were washed with water before being concentrated under a nitrogen stream. The extract was reconstituted with 250 μL of methanol, and analyzed by HPLC-MS/MS. HPLC-MS/MS parameters are described in detail elsewhere (Rocha, Asimakopoulos et al. 2018).

The method for the analysis of urinary phthalate metabolites (PhMs) entailed enzymatic deconjugation, followed by solid-phase extraction (SPE) and an isotope diluted method of quantification (Li, Martinez-Moral et al. 2019). Briefly, 250 μL of urine sample with 2.5 ng of labeled internal standard mixture was buffered with 300 μL of 1 M ammonium acetate containing 100 unit/mL of β -glucuronidase and was incubated at 37°C for 12 h. The samples were passed through ABS Elut-NEXUS SPE cartridges (60 mg 3 mL, Agilent, Santa Clara, CA) that were conditioned with acetonitrile (1.5 mL) and phosphate buffer (1.2 mL). Eluates of acetonitrile (1.2 mL) and ethyl acetate (1.2 mL) were evaporated to near-dryness under nitrogen and re-dissolved in 250 μL of acetonitrile/water (10:90 *v/v*) for further analysis by HPLC-MS/MS. Details of the instrumental methods are described earlier (Rocha, Asimakopoulos et al. 2017).

The six dialkylphosphate metabolites (DAPs) of organophosphates were extracted from urine samples by a SPE method. Briefly, urine samples (250 μL) were spiked with 2.5 ng of isotopically labelled internal standard mixture and mixed with 2% formic acid (750 μL). The samples were then passed through WAX cartridges (60 mg/3 mL; Biotage, Salem, NH, USA) that were conditioned with methanol (2 mL) and water (2 mL). The eluates (2 mL of 5% ammonia in methanol) were dried under a gentle stream of nitrogen, reconstituted with 250 μL of acetonitrile/20 mM ammonium acetate (90:10, *v/v*), and filtered (0.22 μm nylon, Spin-X, Costar; Corning, NY, USA) before analysis by HPLC-MS/MS, as described in Li et al. (2020) (Li, Banjabi et al. 2020).

Urine specimens were analyzed for trace elements in the Laboratory of Inorganic and Nuclear Chemistry at the Wadsworth Center using a well-established biomonitoring method based on ICP-MS (Minnich, Miller et al. 2008).

In brief, urine samples, and 4 different levels of urine quality control materials were diluted 1+19 with a reagent containing nitric acid, Triton X-100, and appropriate internal standards. Diluted samples were analyzed for As, Be, Cd, Mo, Tl, and U on a PerkinElmer® NexION® 300D ICP-MS instrument calibrated with NIST-traceable standards. Method accuracy was assured *via* analysis of NIST SRM 2668 Toxic Elements in Frozen Human Urine. Ongoing laboratory performance was monitored via satisfactory participation in numerous proficiency testing (PT) programs for trace elements in urine, including those operated by Le Centre de Toxicologie du Québec, the UK Trace Elements External Quality Assessment Scheme, and the NYSDOH Biomonitoring PT program for Trace Elements.

Urinary biomarkers were specific gravity (SG) corrected using the following formula: $P_c = P \times [(SG_p - 1)/(SG - 1)]$ (Hauser, Meeker et al. 2004), where P_c is the SG-corrected metabolite concentration (ng/mL), P is the measured metabolite concentration in ng/mL, SG is the specific gravity of the urine sample, and SG_p (1.0223) is the median specific gravity across CHARGE participants providing urine for this study with the full set of covariates. Specific gravity correction factors $[(SG_p - 1)/(SG - 1)]$ greater than 2 were assigned a value of 2 and for values below 0.5 were assigned 0.5.

Data Analysis –

Since all instrument software-generated values were provided, negative values can arise legitimately at or below the limit of detection (LOD) (as a result of random error). The calculation of the LOD represents a theoretical relative standard deviation (RSD) of $\pm 33\%$ of the signal, and this is equivalent to a relative uncertainty of $\pm 94\%$ in the concentration number reported, resulting in some negative values. For each urinary chemical with a minimum SG-corrected concentration of 0 or less (i.e. negative), the minimum concentration and a value of 0.01 were added to all values such that concentrations were shifted to a positive nonzero value. All chemicals were measured in ng/ml. To account for right skewedness of biomarker data, natural log transformed values were used.

Two regression approaches were used for assessing the chemicals of interest in relation to child diagnoses: single chemical and mixture models. Additionally, the mixture models were applied in two ways: to combine individual compounds within a chemical class, and to combine across all classes of chemicals. Chemicals used in regression analyses had at least 60% of measured concentrations above the study wide LOD prior to SG correction.

All models were adjusted for covariates selected *a priori* or that were related to the exposure and outcome ($p < 0.20$). These included child's sex, year of birth, age in months at time of diagnosis, and race, as well as parental homeowner status, and maternal metabolic conditions during pregnancy. We strove to use the most parsimonious model that still adjusted for important confounders, and thus selected a single variable, parental homeowner status, to represent SES. Year of birth was centered by subtracting the mean birth year.

Preliminarily, single chemical multinomial logistic regression models were used for each individual chemical, to simultaneously estimate three regression coefficients, representing the strength of associations with ASD, DD, or OEC versus TD. This approach has been used to analyze chemical exposures in a high-risk autism cohort, for example comparing ASD to

TD (Barkoski, Busgang et al. 2019). A false discovery rate (FDR) correction was applied to p-values per outcome and chemical class to account for multiple comparisons.

Weighted quantile sum (WQS) regression was used to test for a mixture effect of chemicals on the outcome while accommodating for a complex correlation pattern among the chemical components of that mixture. Analyses were conducted by focusing the inference in a positive direction (i.e., increased risk) using one sided confidence limits. The 5th percentile (PCT) was used to define the lower limit. An advantage of WQS regression is that estimation of the weights can be conducted while focusing the inference in a specified direction with a powerful 1 degree of freedom test, thereby improving the stability in estimation due to the complex exposure correlation pattern. Forty percent of the participants were used to estimate the weights associated with each chemical; next the WQS index, using these estimated weights, was calculated for the remaining 60% of participants and the association between the index and diagnosis group was tested in this validation group. When the WQS index was significant, the weight contribution of each chemical was displayed and important chemicals in the mixture were identified if they contribute a weight greater than $1/c$ where c is the number of chemicals in the mixture (Carrico, Gennings et al. 2015). We refer to $1/c$ as the chemical of concern threshold moving forward. We implemented this approach using multinomial WQS for individual chemical classes. To increase robustness of our estimates, we applied repeated holdout validation, randomly splitting the participants 100 times, to examine the distribution of effect estimates and associated weights (Tanner, Bornehag et al. 2019). When at least 95% of the holdouts resulted in increased odds, per diagnosis group, chemical weight distributions were displayed. Chemicals with 90% of repetitions above the chemical of concern threshold were defined as being probable contributors to the mixture effect and those with fifty to less than 90% of repetitions above threshold were defined as possible contributors. Chemicals with more than 90% of repetitions below the threshold were defined as probably not contributors and those with fifty to less than 90% below the threshold were defined as possibly not contributors.

Additionally, a total chemical mixture was tested, combining across all urinary chemical classes, using random subset WQS (rsWQS). This approach randomly selects a subset of a selected number of predictors and estimates weight parameters across multiple ensemble steps with varying predictor subsets and averages the weight contributions across 1000 subsets (Curtin, Kellogg et al. 2019). For the current study, six chemicals were included in each subset such that each chemical's weight was estimated approximately 140 times across all subsets. Similar to the bootstrap WQS, we used repeated holdout validation with 100 random splits of the data where 60% of subjects were selected into the training dataset and the remaining 40% were used for validation.

An alpha of 0.05 was the criterion for statistical significance. For mixture analyses, WQS models were constrained in the positive direction to assess the hypothesized harmful effects of chemical mixtures and significance was assessed using a one-sided test. The interpretation of the odds ratio (X) for a WQS index is that for every 1 decile increase in the weighted index there is an X increase in odds of ASD compared to TD. All statistical analyses were conducted with SAS statistical analysis software version 9.4 (SAS, Cary, NC, USA) and an independent programmer validated the results in this report.

Results

The population included 237 TD, 224 ASD, 81 DD, and 85 OEC participants. The population had a greater fraction of male participants, as ASD is more prevalent in males. The population was 49% non-Hispanic White, followed by 30% Hispanic, and 21% non-White, non-Hispanic. Sample characteristics are stratified by diagnosis in Table 1 as well as the association of each covariate with log transformed methyl paraben (MEPB), selected as a representative compound as it was widely detected and had an association with all three outcomes to help select model covariates. Supplementary Table S1 provides the chemical names and abbreviations of all the chemicals analyzed, stratified by chemical class. Supplementary Table S2 provides the % above LOD for each chemical to indicate which chemicals met our threshold of above 60% to be included in the remaining analyses as described above. Based on this threshold, urinary concentrations of 30 phenols, 20 phthalate metabolites, 6 pesticide metabolites, and 6 trace elements, per outcome, are displayed in Supplementary Table S3. Matrixes of correlation coefficients between compounds are presented in Supplementary Figures S1 and S2.

For each chemical class, we present results from the repeated holdout WQS as well as the single chemical analysis. We highlight chemicals that were probably or possibly strong contributors in the WQS. Due to the large number of results of the single chemical analysis, we focused our discussion on results that were statistically significant at $p < 0.05$, or had sizable (non-null) effect size ($OR > 1.1$ or < 0.9), noting that significance should not be the only measured considered.

Phenols and parabens:

The repeated holdout WQS indicated a significant association between the phenol/paraben index and DD diagnosis [average $OR = 2.40$, (5th PCT=1.05); Table 1, Figure S3]. A weaker but still significant mixture effect was also found for diagnosis of ASD with the phenol/paraben index [average $OR = 1.50$, (5th PCT=1.04)]. There was a positive trend with the phenol index and OEC diagnosis, however the signal of that association was relatively weak [average $OR = 1.65$, (5th PCT=0.94)]. The compounds contributing to these associations can be seen in Figures 1A and 1B, which presents how often a chemical exceeded the concern threshold, and Table 3, which indicates compounds with either a probable or possible contribution in the mixtures analysis.

MEPB was a probable contributor to the mixture effect for the relationship between the phenol/paraben index and ASD diagnosis. Additionally, the single chemical multinomial regression indicated higher concentrations of MEPB in urine were associated with increased odds of ASD to TD [$OR = 1.19$, 95% $CI = (1.08 - 1.32)$] (See Table S4), and remained significant after correcting for false discovery rate (FDR) (FDR-corrected p -value 0.014). Natural log-transformed single chemical results are summarized in Figure 2 using a volcano plot to indicate directionality and magnitude of the association and chemicals are labeled if it passes the 0.05 significant threshold, with similar plots for all other compound classes available in the Supplementary Information, Figure S4. The quartile analysis used quartiles ordinally such that the results can be interpreted as an increase odds per quartile increase. Single chemical analysis with quartiles supported associations with MEPB [$OR = 1.40$ (1.18–

1.68), FDR-corrected p-value 0.004] (see Table S5). The alignment of the measures for MEPB can be seen in Table 3, which includes all compounds for which there are any single compound odds ratios that are significant, or greater or equal to 1.10 or less than or equal to 0.90, or for which there are probable or possible contribution in any mixture analysis.

Bisphenol F (BPF) was both a probable contributor to the mixture effect on ASD and was borderline significant in the single chemical linear model [OR=1.14 (0.99–1.31)]. Probable contributor ethyl paraben (ETPB) was not significant in the linear model [OR=1.07 (0.95–1.20)] but was significant in the quartile analysis [OR=1.19 (1.01–1.42)]. This relationship was not significant after FDR correction. Henceforth, we only include FDR correction values in the main text if they are significant or boarderline significant. Bisphenol S (BPS) was a possible contributor to the mixture effect but was not significant in linear or quartile single chemical analysis [linear OR=1.07 (0.90–1.28)]. There were compounds that were possible contributors to the mixture effect and singifcnat in quartile but not significant in linear single chemical analyses, specifically ethyl paraben (ETPB) [quartile OR=1.19 (1.01, 1.42)] and 4-hydroxybenzoic acid (HB4) [quartile OR=1.19 (1.00–1.41)] There were additional compounds found to be significant in the single chemical analysis, specifically, 3,4-dihydroxy benzoic acid (DHB34) [linear OR=1.28 (1.02–1.61)] and propyl paraben (PRPB) [linear OR=1.13 (1.02–1.24), quartile OR=1.22 (1.03–1.45)], that were not significant in the mixtures model. The compounds 2,2'-dihydroxy-methoxybenzophenone (BP8) and protocatechuic acid ethyl ester (OHETP) were associated with decreased odds of ASD in both the linear and quartile single compound analysis, with OHETP remaining significant after FDR-correction, but there was no evidence of a mixture of compounds associated with decreased odds.

For the DD diagnosis, MEPB, BPS, 3,4-dihydroxy-benzoic acid methyl ester (DHB34), and 2,4,6-trichlorophenol (TCP246) had probable, or possible contributions in the mixture analysis (Figure 1B) and had significant results in the single chemical analysis [MEPB linear OR=1.31 (1.14–1.50), FDR corrected p-value 0.002, quartile OR=1.63 (1.27–2.10), FDR corrected p-value 0.003], [linear BPS OR=1.27 (1.01–1.60)], [linear DHB34 OR=1.37 (1.01, 1.87), quantile OR=1.27 (1.00, 1.60)] and [quantile TCP246 OR=1.32 (1.03–1.67)] respectively. There were additional compounds that were “possible contributors” in the mixtures analysis, but not significant in either of the single compound analysis, specifically 4,4'-(1-phenylethylidene) bisphenol (BPAP) [linear OR=1.17 (0.82–1.67)] BPF [linear OR=1.06 (0.88–1.27)]. Compounds with significant findings in the single compound analysis but were not likely contributors in the mixtures analysis include DCP24 [linear OR=1.49 (1.02–2.19)], OHMEP [linear OR=1.22 (1.00–1.47)], and PRPB [linear OR=1.20 (1.06–1.37), quantile OR=1.37 (1.08–1.74)].

For OEC, BUPB [linear OR=1.26 (1.07–1.49)], MEPB [linear OR=1.20 (1.04–1.37)], and PRPB [linear OR=1.20 (1.05–1.38)] all having a borderline significant effect after FDR correction in the linear model, with MEPB and PRPB also having significant results in the quartile model.

Phthalates: The WQS analysis resulted in no significant mixture effect for phthalate metabolites on any of the outcomes (Table 2). All effect estimates were positive, on

average, with at least 75% of the repetitions greater than 1; however, the signals were noisy, decreasing our confidence in a significant association (Figure S3). Quartiled results show that higher levels of mono-carboxy isononyl phthalate (MCINP) were associated with increased odds of ASD [OR=1.19 (1.00–1.41)]. There were multiple single chemical analyses associated with increased odds of DD compared to TD, including mono (3-carboxypropoyl phthalate (MCP) [linear OR=1.46 (1.07–1.97), quartile OR=1.29 (1.01–1.65)], mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) [quartile: OR=1.35 (1.05–1.73)], and mono (2-ethyl-5-oxohexyl) phthalate (MEOHP) [quartile OR=1.31 (1.01–1.69)]. For the OEC group, the only significant finding in the single chemical analysis was for the quantile analysis for MCIOP [OR=1.31 (1.03–1.67)].

Pesticides: Mixture analyses resulted in a statistically significant association between the DAP pesticide metabolites of organophosphate pesticides mixture and ASD [(average OR=1.10, 5th PCT=1.02); Table 3, Figure 1]. The pesticide mixture was positive, on average, for DD but the confidence interval overlaps with 1, likewise for OEC. In the mixture associated with ASD, diethylphosphate (DEP) was a contributor in all but one hold-out set (Figure 1C), and was also significant in the single chemical analysis [linear: OR=1.20 (1.02–1.42), quartile: OR=1.37 (1.15–1.64), 0.002 FDR corrected P-value]. Diethylthiophosphate (DETP) is a possible contributor to the mixture effect and was also significant in the single chemical analysis [linear: OR=1.16 (1.00–1.35), quartile: OR=1.19 (1.00–1.42)]. There were no significant single chemical results for the DD group, and only one for the OEC group, specifically DEP [quartile OR=1.33 (1.04–1.64)].

Trace Elements: The trace element mixture was significantly associated with increased odds of DD compared to TD [(average OR=1.75, 5th PCT=1.19); Table 2, Figure 1]. No one chemical showed strong evidence of contribution, such that Cd, Mo, Tl and U are possibly important (Figure 1D). Thus, it appears that four different individual elements each contribute only modestly, but together the impact is noticeable. Of these, U was significant in single element models [linear: OR=2.25 (1.17–4.35) 0.077 FDR-corrected p-value, quartile models: OR=1.30 (1.02–1.65) 0.084 FDR-corrected p-value], while Tl was only significant in quartile models [OR=1.32 (1.04–1.67) 0.084 FDR-corrected p-value].

Although the mixture effect was not significant for ASD, U was associated with increased odds of ASD compared to TD and was borderline significant after FDR correction in the linear model [linear: OR=1.96 (1.16–3.32) 0.060 FDR-corrected p-value, quartile models: OR=1.22 (1.03–1.45)]. There were no significant findings for the OEC group.

Mixtures: A total mixture effect, combining all urinary chemicals (44 chemicals in total) was tested with each outcome discretely using random subset WQS with repeated holdout samples. The mixture effect was positively associated with ASD (average OR=1.84, 5th PCT=1.08), DD (average OR=3.44, 5th PCT=1.43), and OEC (average OR=2.20, 5th PCT=1.07) diagnoses (Table 2, Figure 3). Figure 4 displays chemical contributions per outcome and are classified by color based on the chemical class. DEP, MEPB, PRPB, ETPB, MCINP, and U are probably important contributors to the total mixture effect on ASD diagnosis (Table 2). MEPB, TCP246, DHB34, MCP, MEHHP, and Tl are probably important contributors to the total mixture effect on DD diagnosis. DEP, MEPB, and MCIOP

are probably important contributors to the total mixture effect on OEC diagnosis. These compounds all had indications of being important contributors, either because they were identified as contributors in the single class mixtures, or because they were significant in single chemical analysis. There were many other chemicals that were possible contributors.

Discussion

This mixture analysis demonstrates associations of phenols/parabens, pesticide metabolites, and trace metals as chemical classes associated with increased odds of ASD and/or DD. When all 44 individual chemicals were analyzed many of the same individual chemicals that were prominent in their contribution to their respective chemical class mixture's association with outcome also appeared to be the probable important contributors to the total mixture associations, and were often significant in either the linear or quartile single chemical analysis. The same chemicals were often associated with all three outcomes, although the strength of the association often varied between the three outcomes. Still, this is indicative of the compounds impacting overall neurodevelopment, and not necessarily limited to one particular outcome. Findings were weaker for the OEC group, which is somewhat expected as this group was closer to the typically developing group.

The compound with the most consistent results was MEPB, with associations with both ASD and DD that were significant after FDR-correction for both linear and quartile models, and probable significance in both the phenol and overall mixture. ETPB, a structurally similar compound, was also a probable contributor to ASD. Interestingly, these compounds were also noted as contributors to increased risk of either ASD or non-typical development as compared to those developing typically in a high risk ASD cohort (Barkoski, Busgang et al. 2019).

For other compounds, the evidence was not as clear. For example, neither the linear or quartile models reached statistical significance for BPF in association with ASD, yet this compound was a probable contributor in the class and a possible overall mixture. In this case, as well as most other cases, the OR was positive. The opposite example can also be found, for example, PRPB was significant in both the linear and quartile single chemical models in association with ASD, and was not even a probable contributor in the class mixture.

In interpreting these results, first and foremost is the fact that measurements were made after the child initially received the diagnoses, or in a rare number of instances, concurrently with the timing of the diagnosis. Additionally, urinary measurements of the organic compounds examined here represent recent exposures due to a half-lives on the order of days in the human body, and should ideally be accessed with multiple repeated urine samples (Hoppin et al. 2002, Barr et al. 2005, Perrier et al 2016,). For trace elements, the half-lives range from days to years (Barbosa et al. 2005). Therefore, the study does not provide evidence as to whether these chemicals contributed to the diagnoses of concern. Additionally, for some of these chemicals there can be considerable day-to-day variation in metabolite levels (Barkoski et al. 2018, Shin et al. 2019). The higher urinary levels may, however, indicate differences in recent external exposures. For example, if children with ASD, DD or OEC

spend more time indoors, they may have higher exposures to certain chemicals used in household furnishings or products (phenols, parabens, phthalates). Similarly, food-borne chemicals such as pesticides in fruits or fruit juices or phthalates in food packaging could be higher in children with non-typical development if they consume more than TD children of certain foods. Also, some children with ASD or DD have other conditions as well and may be prescribed one or more medications, including medications to address behavioral concerns, and phthalates are used in enteric coatings of medications.

Confounders were selected for the analysis based on them being confounders for only one chemical (specifically MEPB). This combination was selected as it had one of the strongest associations in the uncorrected model. However, there could be other confounders that were important in other associations, but it was beyond the scope of this study to select a different set of confounders for each model. Therefore, there is the possibility of residual confounding due to unmeasured confounders.

Additionally, genetic differences by neurodevelopmental condition may result in differences in metabolism, distribution or excretion of certain chemicals. A number of studies have observed metabolomic differences between ASD and TD, suggesting that such pathways may explain some of the findings reported here (Orozco et al. 2019, Liu et al. 2019, Liang et al. 2020, Ming et al. 2012, and Glinton et al. 2019). Further work characterizing toxicokinetic differences by neurodevelopmental outcome would shed light on the mechanisms and directionality underlying the associations that emerged from our mixture analyses. As the children continue to mature, an additional future direction is to examine the chemicals measured in early childhood for potential influence on their long-term trajectories, which may diverge over time. Such analyses would provide appropriate temporality of measurements to support a known direction of potential causal interpretations.

Conclusions

Higher concentrations of urinary biomarkers increased the odds of ASD, DD, and OEC compared to TD for several compounds, with consistent results comparing single chemical analyses and mixture analyses. Findings were particularly consistent for MEPB for both ASD and DD, ETPB for ASD, and DEP for ASD. Biospecimens used for chemical analysis were collected many months after diagnoses were made the direction of any causal association is unknown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

3-PBA	3-phenoxy-benzoic acid
ADI-R	Autism Diagnostic Interview-Revised
ADOS	Autism Diagnostic Observation Schedules
As	Arsenic
ASD	Autism Spectrum Disorder
BP1	Benzophenone-1
BP3	Benzophenone-3
BP8	2,2'-dihydroxy-methoxybenzophenone
BPA	Bisphenol A
BPAP	4,4'-(1-phenylethylidene)bisphenol
BPF	Bisphenol F
BPP	4,4'-(1,4-Phenylenediisopropylidene) bisphenol
BPS	Bisphenol S
BUPB	Butyl Paraben
Cd	Cadmium
CHARGE	Childhood Autism Risks from Genetics and Environment
DCP24	2,4-Dichlorophenol
DD	Developmental Delay
DEP	diethylphosphate
DETP	diethylthiophosphate
DHB34	3,4-Dihydroxy benzoic acid
DMP	Dimethylphosphate

DMTP	Dimethylthiophosphate
ETPB	Ethyl paraben
FDR	false discovery rate
HB4	4-Hydroxybenzoic acid
HHEAR	Human Health Exposure Analysis Resource
LOD	Limit of Detection
MCINP	mono-carboxy isononyl phthalate
MCIOP	mono-carboxy isooctyl phthalate
MCPP	mono (3-carboxypropyl) phthalate (multiple)
MECPP	mono-(2-ethyl-5-carboxypentyl) phthalate
MEHHP	mono (2-ethyl-5-hydroxyhexyl) phthalate
MEOHP	mono (2-ethyl-5-oxohexyl) phthalate
MEPB	Methyl Paraben
MHPP	mono-2-heptyl phthalate
MIBP	mono-isobutyl phthalate (DiBP)
Mo	Molybdenum
MSEL	Mullen Scales of Early Learning
OEC	Other Early Concerns
OH4BP	4-hydroxybenzophenone
OHETP	Protocatechuic acid ethyl ester
OHMEP	3,4-Dihydroxy-benzoic acid methyl ester
PCP	Pentachlorophenol
PhMs	phthalate metabolites
PRPB	Propyl paraben
PT	proficiency testing
rsWQS	random subset WQS
SG	specific gravity
SPE	solid-phase extraction
TCP246	2,4,6-Trichlorophenol

TCS	Triclosan
TD	typically developing
TI	Thallium
U	Uranium
VABS	Vineland Adaptive Behaviors Scores
WQS	weighted quantile sum
WQSR	weighted quantile sum regression

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Highlights

- Included 627 children between the ages of 2–5 from CHARGE, a case-control study.
- Measured 44 environmental compounds and metabolites in urine.
Estimated risks of autism spectrum disorder (ASD) and developmental delay (DD).
- Conducted a weighted quantile sum regression analysis and single chemical analyses.
- Higher concentrations increased odds of ASD and DD compared to typical.

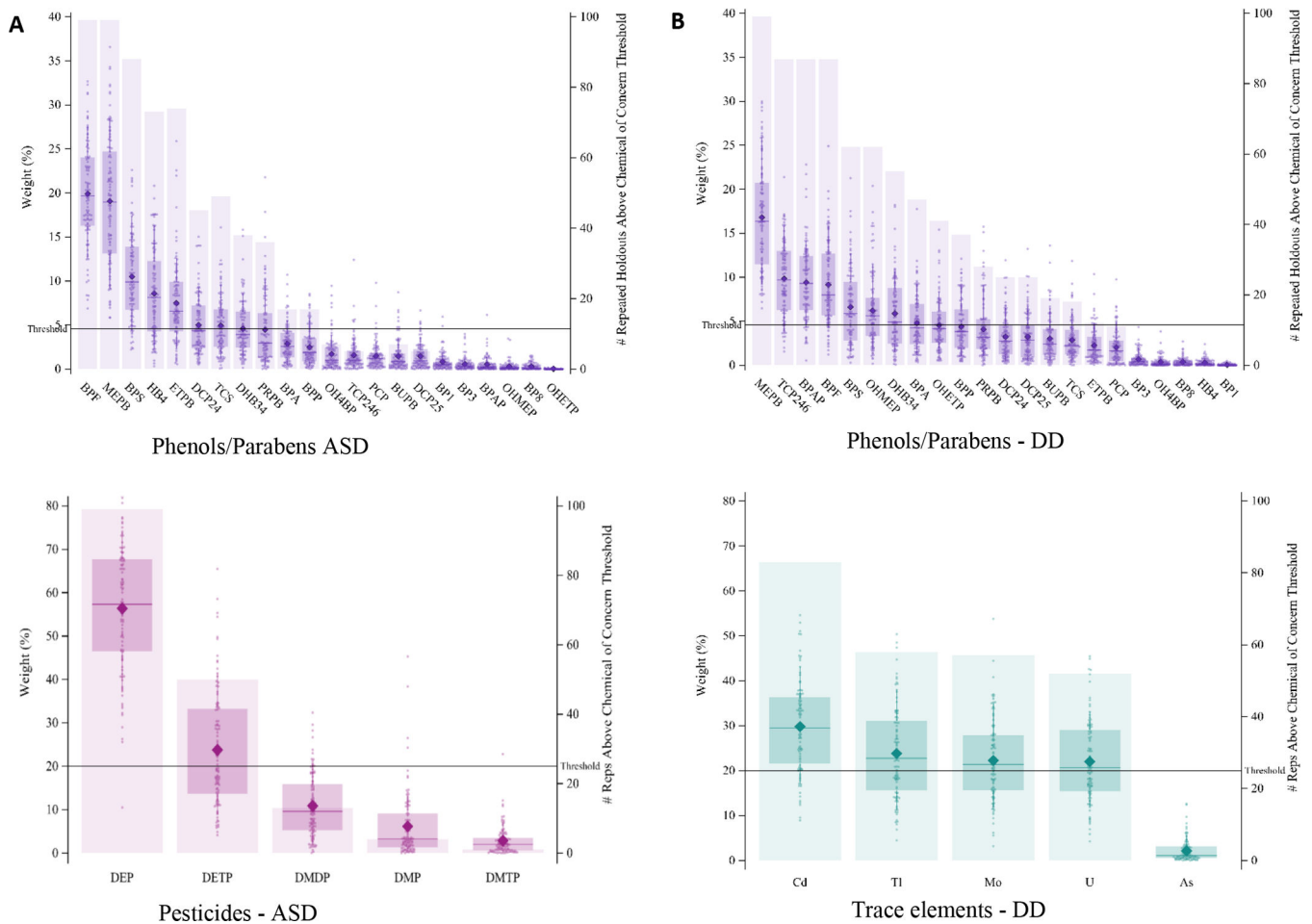


Figure 1: Weight distributions of each chemical from each 100 repetitions (left axis) and the number of repetitions in which the chemical exceeded the 1/c threshold contribution of concern for class mixture multinomial WQS models with more than 90% repetitions above the null, where weights associated with ASD for the phenol/paraben mixture are shown in A, DD for the phenol/paraben mixture are shown in B, ASD for the pesticides mixture are shown in C, DD for the trace elements mixture shown in D.

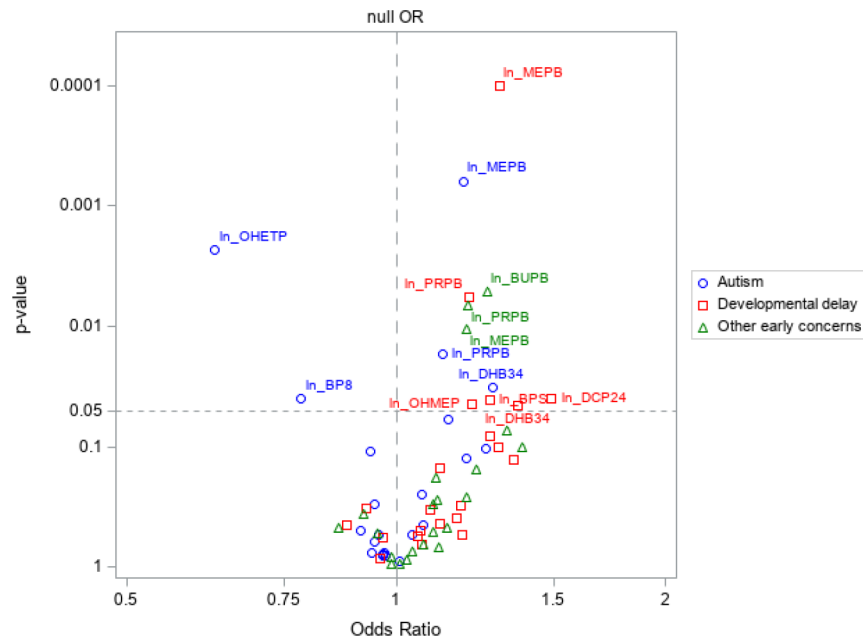


Figure 2: Volcano plot of raw p-values of log transformed phenols and parabens in association with ASD (blue), DD (red), and OEC (green) compared to TD. The x-axis shows the direction and magnitude of the OR, while the y-axis shows raw p-values.

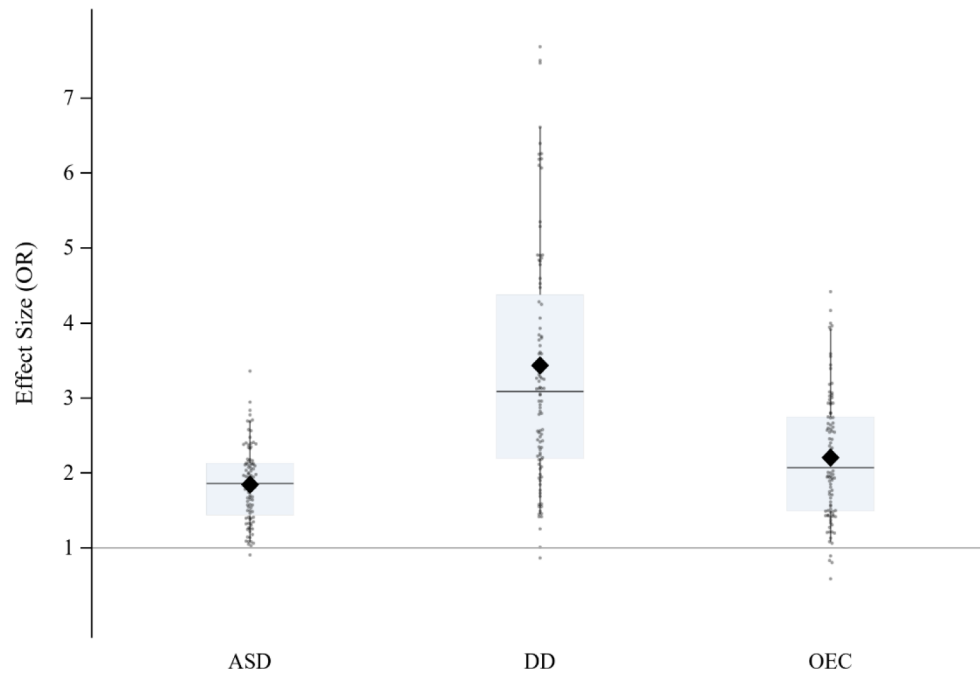


Figure 3: Visual representation of OR distributions from discrete full mixture rsWQS comparing odds of ASD, DD, and OEC to TD with positively constrained betas in estimating the weights, adjusted for covariates (sex, year of birth, homeowner status, child's race, child's age in months, and maternal metabolic conditions). The box represents 25th and 75th percentiles, the line represents the median, the closed diamond represents the mean, and the whiskers show the 5th and 95th percentiles.

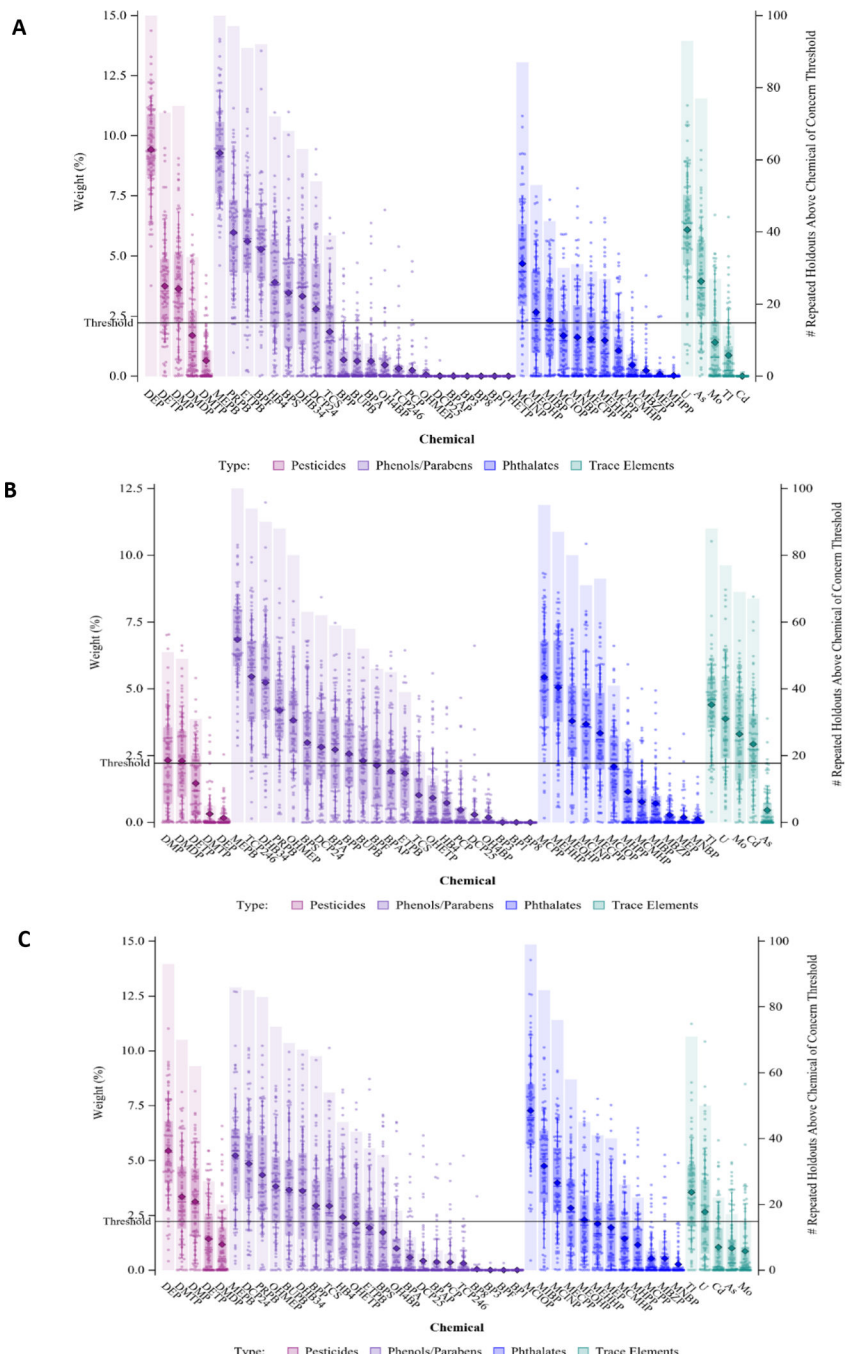


Figure 4: Weight distributions of each chemical from each 100 repetitions (left axis) and the number of repetitions in which the chemical exceeded the 1/c threshold contribution of concern (4.54%; right axis) for total mixture multinomial WQS models with more than 90% repetitions above the null, where weights associated with ASD are shown in A, DD are shown in B, and OEC are shown in C.

Table 1: Characteristics of mothers and children included in the analysis, by 3-year clinical outcome, from the CHARGE cohort (N=627)

	Typical Development N=237	Autism Spectrum Disorder N= 224	Developmental Delay N=81	Other early concerns or high risk N=85	Mean MEPB (ng/ml)	Association with In MEPB p-value ^c	Outcome p- value ^b
	Freq (%)	Freq (%)	Freq (%)	Freq (%)			
Sex							
Male	193 (81.43)	181 (80.80)	60 (74.07)	50 (58.82)	568.0	0.680	<0.001
Female	44 (18.57)	43 (19.20)	21 (25.93)	35 (41.18)	389.9		
Race							
White (non-Hispanic)	128 (54.01)	106 (47.32)	32 (39.51)	41 (48.24)	416.6	0.010	0.140
Non-White (non-Hispanic)	46 (19.41)	55 (24.55)	16 (19.75)	15 (17.65)	904.7		
Hispanic (any race)	63 (26.58)	63 (28.13)	33 (40.74)	29 (34.12)	443.5		
Highest education in household							
High school diploma/GED or less	6 (2.53)	28 (12.50)	18 (22.22)	10 (11.76)	760.2	0.756	<0.001
Some college (inc. vocational, 2 yr degree)	82 (34.60)	62 (27.68)	28 (34.57)	31 (36.47)	606.5		
Bachelor's degree	90 (37.97)	81 (36.16)	26 (32.10)	28 (32.94)	440.3		
Graduate or professional degree	59 (24.89)	53 (23.66)	9 (6.57)	16 (18.82)	448.0		
Homeowner							
No	57 (24.05)	73 (32.59)	36 (44.44)	38 (44.71)	706.4	0.154	<0.001
Yes	180 (75.95)	151 (67.41)	45 (55.56)	47 (55.29)	441.1		
Prenatal Vitamin Use in 3 months before or during 1 st month of pregnancy (31 Missing)							
No	67 (29.13)	74 (34.58)	32 (41.56)	22 (29.33)	431.7	0.473	0.190
Yes	163 (70.87)	140 (65.42)	45 (58.44)	53 (70.67)	537.7		
Maternal metabolic conditions Healthy weight and no metabolic conditions							
	119 (50.21)	101 (45.09)	31 (38.27)	39 (45.88)	508.9	0.074	0.006

	Typical Development N=237	Autism Spectrum Disorder N= 224	Developmental Delay N=81	Other early risk N=85	Mean MEPB (ng/ml)	Association with In MEPB p-value ^a	Outcome p- value ^b
Overweight and no metabolic conditions	Freq (%) 58 (24.47)	Freq (%) 43 (19.20)	Freq (%) 18 (22.22)	Freq (%) 10 (11.76)	519.7		
Obese and no other metabolic conditions	34 (14.35)	30 (13.39)	15 (18.52)	10 (11.76)	566.8		
Any hypertensive disorder or diabetes at any BMI	26 (10.97)	50 (22.32)	17 (20.99)	26 (30.59)	551.4		
Mother's birthplace						0.557	0.024
USA	202 (85.23)	164 (73.21)	64 (79.01)	68 (80.00)	565.4		
Mexico	10 (4.22)	18 (8.04)	9 (11.11)	8 (9.41)	239.5		
Outside the US and Mexico	25 (10.55)	42 (18.75)	8 (9.88)	9 (10.59)	456.6		
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)			
Child age at assessment (months)	46.02 (9.04)	48.67 (8.70)	47.88 (7.99)	48.00 (9.25)		0.015	0.013
Child's year of birth	2006.5 (2.90)	2007.3(3.22)	2006.1 (2.73)	2006.4 (2.87)		0.172	0.006
Mother's age at time of child's birth (years)	30.23 (5.27)	30.67 (5.74)	30.11 (6.41)	31.00 (5.79)		0.160	0.617
Pre-pregnancy BMI	25.71 (5.46)	26.40 (6.17)	26.64 (6.23)	27.35 (7.83)		0.569	0.183

^aFor categorical variables with only two levels, p-values are shown from t-tests; for categorical characteristics with more than two levels, p-values are shown from ANOVA tests; for continuous characteristics, p-value are shown from univariate linear models.

^bFor categorical characteristics, p-values are shown from Chi square test; for continuous characteristics, p-values are shown from univariate multinomial logistic models.

Results from the multinomial WQS for each of the 4 classes of compounds, as well as for all compounds in a discrete full mixture rsWQS models, comparing relative odds of ASD, DD, and OEC with TD, with positively constrained betas in estimating the weights, adjusted for covariates (sex, year of birth, homeowner status, child's race, child's age in months, and maternal metabolic conditions). In the discrete full mixture rsWQS models, weights were estimated by randomly selecting 6 chemicals across 1000 subsets. The table provides the distribution of OR estimates across 100 splits for each chemical class, and for the mixture of all chemical classes combined, in relation to each of the developmental outcomes, and for the mixture of all chemical classes combined.

Table 2:

Chemical Class Mixture		ASD vs TD				DD vs TD				OEC vs TD			
		Mean	Median	5th PCT	95th PCT	Mean	Median	5th PCT	95th PCT	Mean	Median	5th PCT	95th PCT
				PCT	PCT			PCT	PCT			PCT	PCT
Phenols/Parabens	OR	1.50	1.46	1.04	2.10	2.40	2.25	1.05	4.59	1.65	1.58	0.94	2.50
Phthalates	OR	1.12	1.08	0.88	1.42	1.33	1.29	0.89	1.93	1.30	1.26	0.90	1.74
Pesticides	OR	1.10	1.11	1.02	1.19	1.05	1.05	0.93	1.19	1.11	1.11	0.99	1.25
Trace Elements	OR	1.17	1.16	0.88	1.46	1.75	1.68	1.19	2.51	1.22	1.18	0.80	1.77
Total Mixture	OR	1.84	1.86	1.08	2.70	3.44	3.09	1.43	7.04	2.20	2.07	1.07	3.93

Chemicals included in the analysis with either a significant association or sizable effect size with either ASD, DD or OEC, or that were a contributor to a mixture that was associated with either ASD, DD, or OEC.

Table 3:

Chemical Class	Chemical Abbreviation	ASD			DD			OEC [‡]					
		Linear	Quartile	Class Mixture	Overall Mixture	Linear	Quartile	Class Mixture	Overall Mixture	Linear	Quartile	Overall Mixture	
Phenols/Parabens	BP1										-		
	BP3		-								- (*)		
	BP8		-								-		
	BPA										+		
	BPAP										+		
	BPF		+	probable	possible							- (*)	
	BPP										+		
	BPS		+	possible	possible								
	BUPB										*		
	DCP24		+		possible						+		
	DHB34		*								+		
	ETPB			possible	probable						+		
	HB4		+	possible	possible						+		
	MEPB		**	**	probable	probable					*		
	Phthalates	OH4BP										+	
OHETP			- (**)										
OHMEP										*			
PCP										+			
PRPB			*		probable					*			
TCP246										+			
TCS										+			
MCINP			*		probable					+			
MCIOP										+			

Chemical Class	Chemical Abbreviation	ASD				DD				OEC ^f		
		Linear	Quartile	Class Mixture	Overall Mixture	Linear	Quartile	Class Mixture	Overall Mixture	Linear	Quartile	Overall Mixture
Pesticides	MCPP					*	*		probable	+		
	MECPP	+				+	+		possible	+	+	possible
	MEHHP	+				+	*		probable	+	+	
	MEOHP	+			possible	+	*		possible	+	+	
	MHPP					+						
	MIBP	+	+		possible					+	+	possible
Pesticides	DEP	*	**	probable	probable					+	*	probable
	DETP	*	*	possible	possible		+				+	
	DMP		+		possible		+			+	+	possible
	DMTP									+	+	possible
Trace Elements	As	+	+		possible					+		
	Cd		-				+	possible	possible	-		
	Mo						+	possible	possible	+		
	Tl						+	possible	probable	+	+	possible
	U	*	*		probable		*	possible	possible	+	+	possible

⁺ = OR > 1.10, but not significant

⁻ OR < 0.90, significance indicated

^{*} = OR increased and value significant

^{**} = OR increased, FDR correction value also significant

gray fill = indicates there was no relationship between the class mixture and the outcome

^f = For the OEC group, there was no relationship between any class mixture and the outcome