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Original Research Article

Associating prenatal phthalate exposure with childhood autistic traits: Investigating potential adverse outcome pathways and the modifying effects of maternal vitamin D

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ABSTRACT

The association between prenatal phthalate mixture exposure and the risk of autism spectrum disorder (ASD) in children, as well as the potential mechanism and impact of maternal vitamin D, remains unclear. We analyzed data from 3209 mother–child pairs. The associations between prenatal phthalate exposure and autistic traits in children aged 1.5, 3, 5, and 6 years were explored. Furthermore, the modifying effects of maternal vitamin D and the adverse outcome pathway, which elucidates the contribution of phthalates to ASD, were estimated. Exposure to a phthalate mixture was associated with an increased risk of ASD in children aged 1.5–6 years. For mothers with 25(OH)D deficiency, an exposure–response relationship was observed between phthalate mixtures in early to mid-pregnancy and autistic traits in children aged 3 years. However, this association was not observed for mothers with sufficient prenatal 25(OH)D levels. The potential mechanism of action of di(2-ethylhexyl) phthalate (DEHP) exposure may involve affecting GRIN2B, inhibiting NMDAR in the postsynaptic membrane, disrupting synaptic function, and impairing learning and memory, ultimately leading to ASD development. Importantly, maternal vitamin D supplementation was demonstrated to mitigate the risk of ASD associated with phthalate exposure. Reducing phthalate exposure during pregnancy may be associated with a decreased risk of autistic traits in children. Furthermore, adequate vitamin D supplementation could potentially mitigate the impact of phthalates on these traits. Additionally, the proposed biological mechanism provides insight into how phthalate exposure may contribute to the development of ASD.

1. Introduction

Phthalates, known as plasticizers, are commonly used chemicals. The pervasive utilization of phthalates in plastic products has raised growing concerns about the presence of phthalates in the environment. People are consistently exposed to phthalates through ingestion, inhalation, and dermal absorption [1], indicating widespread environmental pollution by phthalates. The regulation and use of phthalates vary greatly from country to country [2,3]. In European Union countries, phthalates are more heavily regulated, while regulations in the United States and China are less stringent. However, human exposure to phthalates remains a serious concern globally, especially among vulnerable populations such as pregnant women [4,5]. In a previous study, we found that nearly 100% of pregnant Chinese women had detectable levels of multiple phthalate

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metabolites in their urine [6]. Prenatal phthalate exposure has been associated with potential adverse effects, including neurotoxicity, such as poorer psychomotor development, a lower intelligence quotient (IQ), attention and hyperactivity problems, and poorer social communication [7–9]. These risks are significant and warrant attention.

Autism spectrum disorder (ASD) has garnered significant attention due to its unknown causes and diverse symptoms. Typically, ASD, which is detected in childhood, is characterized by traits such as repetitive movements, limited interest, and difficulties in social interaction [10,11]. According to the Global Burden of Disease Study 2019, ASD ranks 23rd in disability-adjusted life years and 56th in loss of health years among children aged 0–14 years [12]. A systematic review of 71 global studies reported an ASD incidence of 1.19% (ranging from 0.11‰ to 4.36%) [13]. There is a consistent trend of increasing prevalence across different countries and regions. The exact cause of this rapid growth is not fully understood [14,15], and nearly 75% of autism cases are of unknown cause [16]. It is crucial to identify modifiable environmental factors (e.g., phthalate exposure) associated with ASD and implement preventive measures to promote the healthy development and well-being of children.

Animal studies have shown that prenatal exposure to di(2-ethylhexyl) phthalate (DEHP) leads to autistic traits and ASD-like behaviors in offspring [17,18]. The mechanism underlying this association involves the impact of DEHP on brain signaling pathways and neuronal development [17,18]. Limited research in humans suggests a positive correlation between early life phthalate exposure and ASD risk in children [19]. However, there is still a need to explore the cumulative effects of phthalate mixtures, exposure–response relationships, vulnerable periods, sexual dimorphism, and underlying mechanisms [20].

Interestingly, among pregnant women who have consumed prenatal vitamins, exposure to phthalates is linked to a decreased risk of childhood ASD [21]. 1,25(OH)2D, the active metabolite of vitamin D, shares a molecular structure similar to that of classical steroid hormones. The impact of phthalates, a well-known environmental endocrine disruptor, on vitamin D levels has received more attention than that of other vitamins [22,23]. There is no consensus on the definition of vitamin D sufficiency for pregnant women. Typically, a circulating 25(OH)D concentration greater than 30 ng/mL is recommended [24]. The prevalence of maternal vitamin D insufficiency and deficiency is notably high in various regions, as follows: the Americas (64%, 9%), Europe (57%, 23%), the Eastern Mediterranean (46%, 79%), Southeast Asia (87%, data not available), and the Western Pacific (83%, 13%) [25]. A meta-analysis of prospective studies has revealed that children with maternal or neonatal vitamin D deficiency/insufficiency have a 54% greater risk of developing ASD [26]. Given these findings, it is important to understand whether vitamin D supplementation mitigates the increased risk of ASD associated with phthalate exposure.

Traditional toxicity testing methods rely heavily on expensive and time-consuming animal and cell experiments. In 2007, the National Research Council released the "Toxicity Testing in the 21st Century (TT21C): Vision and Strategy" report, which introduced a novel toxicology research model centered around toxicity pathways [27,28]. Building upon this concept, Ankley et al. at the United States Environmental Protection Agency were the first to propose a conceptual framework for adverse outcome pathways (AOPs) [29]. The AOP framework helps to extrapolate information from upstream to downstream events in chemical-induced diseases [29]. Searching for possible mechanisms in the literature can be extremely challenging. The integration of information from public databases, such as the Comparative Toxicogenomics Database (CTD), Computational Toxicology (CompTox) Chemicals Dashboard, DisGeNET, and MalaCards, has been demonstrated to be an efficient method for AOP development [30]. Researchers have successfully used the AOP framework to investigate the mechanisms underlying the associations between phthalate exposure and reproductive toxicity [31,32], and gestational diabetes mellitus [33]. To date, however, no studies have used the AOP framework to elucidate the causal pathways involved in the association between phthalates and ASD.

On the basis of the Ma'anshan birth cohort (MABC), the present study aimed to achieve the following three objectives: (1) to examine the association between prenatal phthalate coexposure and the risk of autistic traits in children; (2) to investigate how maternal vitamin D modifies these associations; and (3) to propose the mechanisms underlying the associations using an AOP framework.

2. Materials and methods

2.1. Overview

The first aim of the MABC study was to explore the link between early life environmental exposures and maternal-child health. Detailed information regarding the study design, recruitment, follow-up procedures, and sample collection has been provided in our previous publications [34,35]. In summary, the MABC study enrolled 3474 eligible pregnant women during the first trimester (around ten weeks of gestation) as research participants from May 2013 to September 2014. Among these, 201 pregnant women were not followed up for various reasons, including induced abortion for medical reasons (n = 30), spontaneous abortion (n = 120), stillbirth (n = 10), ectopic pregnancy (n = 2), and multiple pregnancies (n = 39). A total of 3273 women gave birth to singleton live-born infants. During the initial trimester (at enrollment), mid-pregnancy (mean 26 weeks of gestation), and late pregnancy (mean 34 weeks of gestation) follow-ups, pregnant women completed questionnaires and provided spot urine samples. All spot urine samples were collected between 8:00 and 10:00 a.m. 7 days after completing the questionnaires. To detect phthalate exposure, a total of 3118, 3148, and 3030 urine samples were collected during early, mid-, and late pregnancy, respectively.

Autistic traits were evaluated using Part A of the Checklist for Autism in Toddlers (CHAT-23) at the ages of 1.5 and 3 years, as well as the modified Chinese version of the Clancy Autism Behavior Scale (C-CABS) at the ages of 5 and 6 years [35]. Part A of the CHAT-23 consists of 23 items with 7 core items. Children with autistic traits were defined as those with a total score > 6 and/or a score > 2 on the core items in the present study. The CARS consists of 14 items, and each item is divided into three frequency levels (scored from 0 to 3 points). Children were classified as having autistic traits if they had a total score greater than 14, fewer than 3 items scored 0, or more than 6 items scored 2. It is widely recognized that diagnosing ASD is more challenging in younger children, and clinicians typically rely more on CHAT-23 scores than on C-CABS scores. Early diagnosis and intervention for ASD children lead to better outcomes. In the present study, autistic traits at the age of 3 were considered the primary outcome, with assessments at ages 1.5, 5, and 6 as secondary outcomes. Of the initial participants, 64 individuals did not provide assessment results, resulting in 3209 mother–child pairs for the present study (Fig. S1).

All participants signed written informed consent before the survey. The study protocol was approved by the Ethics and Human Subject Committee of Anhui Medical University (No. 20131195).

2.2. Measurement of urinary phthalate metabolites and serum 25(OH)D

Urine and serum samples were collected at the Maternal and Child Health Care Center in Ma'anshan. The samples were subsequently transported under cold chain conditions to the Key Laboratory of Population Health Across Life Cycle (Anhui Medical University). The samples were then stored in a frozen state in the biological sample repository.

The concentrations of six phthalate metabolites in urine were measured using high-liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) at a university laboratory, as previously reported [36]. The process involved thawing the urine samples at room temperature, performing enzymatic hydrolysis, solid-phase extraction, rinsing, and elution before analysis using an HPLC–MS/MS instrument. Each analysis batch included two blank samples and two quality control samples

processed simultaneously. The recovery rates of the spiked concentrations (5, 50, and 200 ng/mL) ranged from 88.8% to 108.9%. The intraday precision ranged from 0.19% to 8.75%, and the interday precision ranged from 1.16% to 9.85%. Concentrations below the limit of detection (LOD) were treated as LOD/sqrt(2). Urinary creatinine (Cr) was used to adjust the metabolite concentrations. To achieve a normal distribution, a $\log_{10}$ transformation was applied to normalize the data. Due to the strong correlations among three DEHP metabolites, namely, mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydrohexyl) phthalate (MEHHP), and mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), as shown in Fig. S2, the molar sum of the DEHP metabolites was calculated ($\sum$DEHP = MEHP + MEHHP + MEOHP; μmol/L). Similarly, the molar sum of low-molecular-weight phthalate metabolites, including monomethyl phthalate (MMP), monoethyl phthalate (MEP), and monobutyl phthalate (MBP), was also calculated ($\sum$LMWP = MMP + MEP + MBP; μmol/L).

Serum total 25(OH)D levels were assessed using a DiaSorin kit (DiaSorin, Inc., Stillwater, MN, USA) and radioimmunoassay (RIA) following the manufacturer's guidelines. In each processing batch, two blank samples, two quality control samples, and five concentration standards (ranging from 5 to 100 pg/mL) were simultaneously processed. The linear regression equation for the concentration standards required a correlation coefficient (r) of ≥0.99.

2.3. Statistical analysis

The geometric mean of creatinine-corrected concentrations of phthalate metabolites was calculated as an average measure of pregnancy exposure. The concentrations of phthalate biomarkers were assessed for each trimester to investigate the trimester-specific effects of phthalate exposure on childhood autistic traits.

A Poisson regression model with a log link function was used. The analysis evaluated the associations between phthalate metabolite exposure and autistic traits in children aged 1.5, 3, 5, and 6 years. The results were expressed as a binary outcome, including risk ratios (RRs) along with their corresponding 95% confidence intervals (CIs). Children without autistic traits served as the reference group in the analysis. Individual phthalate metabolites were assessed for associations with ASD outcomes using single pollutant models (Poisson regression). Multiple hypothesis testing-corrected *P*-values were obtained by calculating the false discovery rate (FDR).

Next, an environment risk score (ERS) for all six phthalate metabolites was constructed by applying adaptive elastic net regularization (ENR) [37, 38]. An adaptive elastic net more effectively addresses collinearity than does a traditional elastic net. It addresses the collinearity problem over the elastic net and satisfies the asymptotic normality assumption, enabling statistical inference and hypothesis testing through the provision of large sample standard errors (SEs) and *P* values. Adaptive weights in elastic nets ensure that smaller coefficients shrink faster toward zero, while larger coefficients penalize less. The weight assigned to each phthalate pollutant can be determined by its corresponding beta coefficient obtained from the elastic net analysis [38]. The optimal tuning parameters (λ1 and λ2) were chosen based on 10-fold cross-validations to minimize prediction errors. The ERS was computed as a weighted sum of the selected nonzero predictors. All models were adjusted for confounders.

Poisson regression models were also used to estimate the associations of prenatal phthalate coexposure (indicator: ERS) with childhood autistic traits. Pregnant women were classified into three exposure groups based on ERS tertiles. The low ERS group included individuals with ERS levels below the 33.33rd percentile. The medium ERS group comprised individuals with ERS levels ranging from the 33.33rd to the 66.67th percentile. The high ERS group consisted of individuals with ERS levels above the 66.67th percentile [38]. RR and 95% CIs were calculated to assess the association between autistic traits and ERS as a categorical variable. Additionally, linear trend tests were conducted by treating the ERS category as a continuous variable in the models.

Following a literature review and the construction of a directed acyclic graph, a minimum set of covariates necessary for adjustment was identified (Fig. S3a). The covariates adjusted in all the models (Fig. S3b) included maternal age, prepregnancy body mass index (BMI), education, ethnicity, household income, smoking during pregnancy, drinking during pregnancy, primipara, blood lead exposure, and IQ.

To explore the effect modification of the association between phthalate metabolites and ASD by vitamin D, the mothers were categorized into three groups based on their 25(OH)D concentrations as follows: sufficient (>30 ng/mL), insufficient (21–29 ng/mL), and deficient (<20 ng/mL) [24]. For each subgroup of mothers with different 25(OH)D concentrations, the associations between prenatal ERS exposure and autistic traits were calculated. The analysis compared the results to those for the mother–child pairs in the low ERS group, using those below the 33.33rd percentile as the reference.

2.4. AOP proposal based on public databases

To investigate the potential underlying mechanisms, an AOP framework was proposed based on public databases. On July 23, 2023, we accessed the page for DEHP on the CTD because DEHP and its metabolites were found to be associated mainly with autistic traits in the present study. Using the “Gene” and “Phenotype” tags of the DEHP page in the CTD, candidate genes and phenotypes related to DEHP were collected and recorded as the DEHP-gene and the DEHP-phenotype, respectively. Moreover, under the “Disease” tag of the DEHP page in the CTD, a search was performed using the keyword “Autism spectrum disorder” to obtain annotated genes associated with DEHP and ASD (recorded as DEHP-gene-ASD). DEHP gene information was also collected from the CompTox database. Similarly, ASD-gene data were collected from DisGeNET and MalaCards. Additionally, ASD-phenotype information was obtained from MalaCards. A summary of the collected genes and phenotypes is provided in Table S1.

First, a Venn diagram analysis was conducted using the “VennDiagram” package in R [39] to examine the overlaps among the four datasets as follows: the DEHP-gene from CTD and CompTox and the ASD-gene from DisGeNET and MalaCards. The genes obtained from the intersections of these four groups were subsequently combined with the DEHP-gene-ASD dataset, resulting in the creation of the target gene set. Subsequently, the target gene set was subjected to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) enrichment analyses using the “clusterProfiler” package in R [40]. These analyses were specifically focused on *Homo sapiens* as the source organism. Finally, the target phenotypes were identified by comparing the significant results ($P < 0.05$) obtained from the GO and KEGG enrichment analyses with the DEHP-phenotypic data from the CTD and ASD phenotypic data from MalaCards. Phenotypes that appeared in at least two independent datasets were defined as target phenotypes.

Cytoscape 3.8.0 (https://cytoscape.org/) was used to visualize the DEHP-target gene–target phenotype-ASD network and interactions among the genes, incorporating information from various sources, such as the CTD, CompTox, DisGeNET, MalaCards, and enrichment analysis results. Additionally, AOP-Wiki (https://aopwiki.org) was searched to identify relevant adverse outcome pathways (AOPs) associated with autism. By integrating these gathered pieces of information, a putative AOP was proposed, serving as a potential clue to understanding the mechanism through which DEHP may contribute to the development of ASD.

3. Results

3.1. Population characteristics

No significant differences were observed between the mother–child pairs included in the present study (n = 3209) and those excluded (n = 64) in terms of population characteristics (*P*-value > 0.05), indicating no evidence of selection bias due to attrition related to follow-up

(Table 1 and Table S2). All the characteristics of the participants in the present study are shown in Table 1. In the present study, the maternal mean age and prepregnancy BMI were 26.63 years and 20.87 kg/m^2, respectively. In addition, 57.49% of the mothers had a junior high education, and 98.50% of the pregnant women were Han ethnicity. Moreover, 88.63% of the participants were primiparous women, and the vast majority of the women did not smoke (99.81%), did not drink alcohol (91.99%), did not have GDM (86.91%), and did not have HDP (93.83%) during pregnancy. Among the offspring, there were 1639 boys and 1570 girls, with an average birth weight and gestational age of 3365.85 g and 39.45 months, respectively.

3.2. Distribution of phthalate metabolites, maternal vitamin D levels, and childhood autistic traits

The urinary concentrations of phthalate metabolites in pregnant women from the MABC study have been previously reported [36]. The distributions of concentrations among the participants are shown in Table 2 and Fig. S2.

Regardless of the trimester, the exposure levels of MBP were the highest, with a median concentration of 58.32 μg/g Cr (Table 2). The next highest exposure level was observed for the MMP, for which the median concentration was 18.76 μg/g Cr. The two secondary metabolites of DEHP, namely, MEOHP and MEHHP, exhibited relatively higher exposure levels, with median concentrations of 8.75 and 7.16 μg/g Cr, respectively. MEP and MEHP had relatively lower exposure concentrations, with median concentrations of 6.72 and 4.31 μg/g Cr, respectively, during pregnancy. As expected, DEHP metabolites demonstrated high within-trimester correlations. Within trimester correlations of the other metabolites were low to moderate (Fig. S2).

Among pregnant women, the frequency of vitamin D deficiency ranged from 44.09% to 62.29%, while the frequency of vitamin D sufficiency ranged from 8.05% to 18.0% (Table 2). Screening for autistic traits using the CHAT-23 revealed that 13.28% of 1.5-year-old children and 8.57% of

Table 1
Characteristics of the Ma'anshan Birth Cohort (MABC) study population with singleton births and the current study.

Characteristics	Participants (n = 3209)		
	Overall	Boys (n = 1639)	Girls (n = 1570)
Maternal variables			
Age category (years) (mean ± SD)	26.63 ± 3.64	26.67 ± 3.75	26.60 ± 3.53
Prepregnancy BMI (kg/m^2) (mean ± SD)	20.87 ± 2.82	20.83 ± 2.84	20.91 ± 2.79
Education [n (%)]			
Less than middle school	640 (19.94)	329 (20.07)	311 (19.81)
High school	724 (22.56)	367 (22.39)	357 (22.74)
Junior school	991 (30.88)	513 (31.30)	478 (30.45)
University of above	854 (26.61)	430 (26.24)	424 (27.01)
Ethnic [n (%)]			
Han	3161 (98.50)	1616 (98.60)	1545 (98.41)
Other	48 (1.50)	23 (1.40)	25 (1.59)
Per capita monthly income of households (RMB) [n (%)]			
Less than 2,499	847 (26.39)	445 (27.15)	402 (25.64)
2,500–4,000	1373 (42.79)	693 (42.28)	680 (43.34)
More than 4,001	989 (30.82)	501 (30.57)	488 (31.08)
Smoking during pregnancy [n (%)]			
Yes	6 (0.19)	5 (0.31)	1 (0.06)
No	3203 (99.81)	1634 (99.69)	1569 (99.94)
Drinking during pregnancy [n (%)]			
Yes	257 (8.01)	142 (8.66)	115 (7.32)
No	2952 (91.99)	1497 (91.34)	1455 (92.68)
Primipara [n (%)]			
Yes	2844 (88.63)	1446 (88.22)	1398 (89.04)
No	365 (11.37)	193 (11.78)	172 (10.96)
Gestational weight gain (kg) (mean ± SD)	17.82 ± 5.10	17.73 ± 5.08	17.91 ± 5.11
Diabetes mellitus [n (%)]			
None	2789 (86.91)	1428 (87.13)	1361 (86.69)
GDM	408 (12.71)	204 (12.45)	204 (12.99)
Diabetes before pregnancy	12 (0.38)	7 (0.42)	5 (0.32)
Hypertension [n (%)]			
None	3011 (93.83)	1543 (94.14)	1468 (93.50)
Gestational hypertension	134 (4.18)	68 (4.15)	66 (4.20)
Preeclampsia	57 (1.78)	24 (1.46)	33 (2.10)
Chronic hypertension	6 (0.19)	4 (0.24)	2 (0.13)
Missing	1 (0.03)	0 (0.00)	1 (0.06)
Intelligence quotient (mean ± SD)	95.88 ± 10.67	95.90 ± 10.65	95.86 ± 10.69
Lead exposure (ppb) (mean ± SD)	1.06 (0.66, 1.88)	1.05 (0.65, 1.91)	1.07 (0.67, 1.83)
Delivery mode [n (%)]			
Vaginal	1586 (49.42)	840 (51.25)	740 (47.13)
Cesarean	1622 (50.55)	799 (48.75)	823 (52.42)
Missing	1 (0.03)	0 (0.00)	1 (0.06)
Child variables			
Gestational age (weeks) (mean ± SD)	39.45 ± 1.35	39.38 ± 1.35	39.52 ± 1.31
Birth weight (g) (mean ± SD)	3365.85 ± 447.84	3394.88 ± 456.44	3335.54 ± 436.77
Breastfeeding [n (%)]			
Never	419 (13.06)	384 (23.43)	351 (22.36)
Less than one year	2054 (64.01)	1036 (63.21)	1018 (64.84)
More than one year	735 (22.90)	219 (13.36)	200 (12.74)
Missing	1 (0.03)	0 (0.00)	1 (0.06)

BMI, body mass index; GDM, gestational diabetes mellitus; SD, standard deviation. There were no significant differences observed between the mother–child pairs included in the present study and those excluded.

Table 2
Distribution of phthalate metabolite exposure, maternal vitamin D intake, and childhood autistic traits.

Variable	During pregnancy	Trimester 1	Trimester 2	Trimester 3
Urinary creatine-adjusted concentration of phthalate metabolites [Median (25th percentile, 75th percentile)]				
MMP (μg/g)	18.76 (10.51, 32.64)	11.98 (7.05, 21.87)	33.04 (12.02, 91.47)	12.51 (6.41, 32.63)
MEP (μg/g)	6.72 (3.66, 13.15)	7.93 (3.99, 18.06)	6.33 (2.52, 17.42)	5.41 (2.39, 14.04)
MBP (μg/g)	58.32 (36.34, 92.58)	47.22 (23.52, 101.56)	63.22 (26.71, 154.00)	57.67 (31.87, 120.89)
LMWP (μmol/g)	0.51 (0.33, 0.77)	0.39 (0.22, 0.72)	0.62 (0.29, 1.47)	0.46 (0.27, 0.87)
MEHP (μg/g)	4.31 (2.72, 6.93)	2.50 (1.34, 4.51)	6.14 (3.02, 12.72)	5.07 (2.75, 9.31)
MEOHP (μg/g)	8.75 (6.38, 12.56)	6.61 (4.32, 10.08)	10.68 (5.76, 21.33)	8.91 (6.21, 12.97)
MEHHP (μg/g)	7.16 (4.84, 10.73)	4.80 (3.02, 8.13)	9.77 (4.91, 19.51)	7.32 (4.61, 12.32)
DEHP (μmol/g)	0.07 (0.05, 0.11)	0.05 (0.03, 0.08)	0.10 (0.05, 0.19)	0.08 (0.05, 0.12)
Maternal serum 25(OH)D [n (%)]				
Deficiency	1999 (62.29)	1415 (44.09)	1706 (53.16)	1732 (53.97)
Insufficiency	718 (22.37)	1048 (32.66)	757 (23.59)	1190 (37.08)
Sufficiency	300 (9.35)	578 (18.01)	426 (13.28)	284 (8.85)
Missing	192 (5.98)	168 (5.24)	320 (9.97)	3 (0.09)
Autistic traits [n (%)]	**1.5-year-old (CHAT-23)**	**3-year-old (CHAT-23)**	**5-year-old (C-CABS)**	**6-year-old (C-CABS)**
Scale-score	2.00 (1.00, 3.00)	8.00 (5.00, 11.00)	6.00 (3.00, 9.00)	4.00 (1.00, 7.00)
Autistic traits (+)	426 (13.28)	275 (8.57)	174 (5.42)	116 (3.61)
Autistic traits (−)	2655 (82.74)	2656 (82.77)	2342 (72.98)	2400 (74.79)
Missing	128 (3.86)	278 (8.66)	693 (21.59)	693 (21.60)

CHAT-23, the Checklist for Autism in Toddlers; C-CABS, the modified Chinese version of the Clancy Autism Behavior Scale; MMP, monomethyl phthalate; MEP, monoethyl phthalate; MBP, monobutyl phthalate; LMWP, low-molecular-weight phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; DEHP, di(2-ethylhexyl) phthalate; LMWP, the weighted molar sum of the phthalate metabolites MMP (molecular weight = 179) and MEP (molecular weight = 1) concentrations expressed in μmol/g creatinine; DEHP, the weighted molar sum of the DEHP metabolites MEHP (molecular weight = 272), MEOHP (molecular weight = 292) and MEHHP (molecular weight = 294) concentrations expressed in μmol/g creatinine.

3-year-old children exhibited autistic traits. Additionally, screening with the C-CABS indicated that 5.42% of 5-year-old children and 3.61% of 6-year-old children had autistic traits (Table 2). Compared to those at 3 years of age, the loss to follow-up rates for children aged 5 and 6 years increased by 33.37% and 24.24%, respectively.

3.3. Associations between phthalate metabolites and the risk of ASD

Prenatal exposure to DEHP (RR_{adj} = 1.99, 95% CI = 1.18–3.29) and its metabolites—MEHP (RR_{adj} = 1.57, 95% CI = 1.07–2.31), MEOHP (RR_{adj} = 1.92, 95% CI = 1.12–3.26), and MEHHP (RR_{adj} = 1.71, 95% CI = 1.07–2.70)—significantly elevated the risk of autistic traits in 3-year-old children (Table S3 and Fig. 1). This association was primarily driven by positive associations between DEHP (trimester 2: R_{adj} = 1.26, 95% CI = 0.94–1.67; trimester 3: R_{adj} = 1.54, 95% CI = 1.02–2.28) and its metabolite exposure MEOHP (trimester 2: RR_{adj} = 1.30, 95% CI = 0.98–1.17; trimester 3: RR_{adj} = 1.67, 95% CI = 1.06–2.60) during mid- to late-pregnancy and childhood autistic traits (Table S3 and Fig. 1). Most of the results remained marginally significant after adjusting for the FDR (Table S3).

In 5- and 6-year-old children, DEHP and MEHP exposure had a marginal association with an increased risk of autistic traits (Table S4 and Fig. S4; Table S5 and Fig. S5), with a slightly reduced effect intensity. Additionally, exposure to the MMP during mid-pregnancy increased the risk of autistic traits in children (Table S4 and Fig. S4). For autistic traits at 1.5 years of age, exposure to DEHP and its metabolites during early pregnancy elevated the risk of ASD (Table S6). Interestingly, negative correlations were observed between all six metabolites during late-pregnancy and the risk of ASD (Table S6 and Fig. S6). Overall, these findings suggested that prenatal exposure to phthalates, particularly DEHP, may contribute to an increased risk of ASD in childhood. Considering the higher loss to follow-up rates among children aged 5–6 years, caution should be exercised when comparing results across age-stratified models.

3.4. Associations between cumulative phthalate exposure and the risk of ASD

Compared to children in the low ERS group, those in the high ERS group exhibited a marginal increase in the risk of developing autistic traits at the age of 3 (RR_{adj} = 1.15, 95% CI = 0.99–1.35). This association was primarily driven by a positive correlation between ERS during mid-pregnancy and the risk of autism (medium ERS: RR_{adj} = 1.35, 95% CI = 0.96–1.92; high ERS: RR_{adj} = 1.68, 95% CI = 1.21–2.36) (Table S7

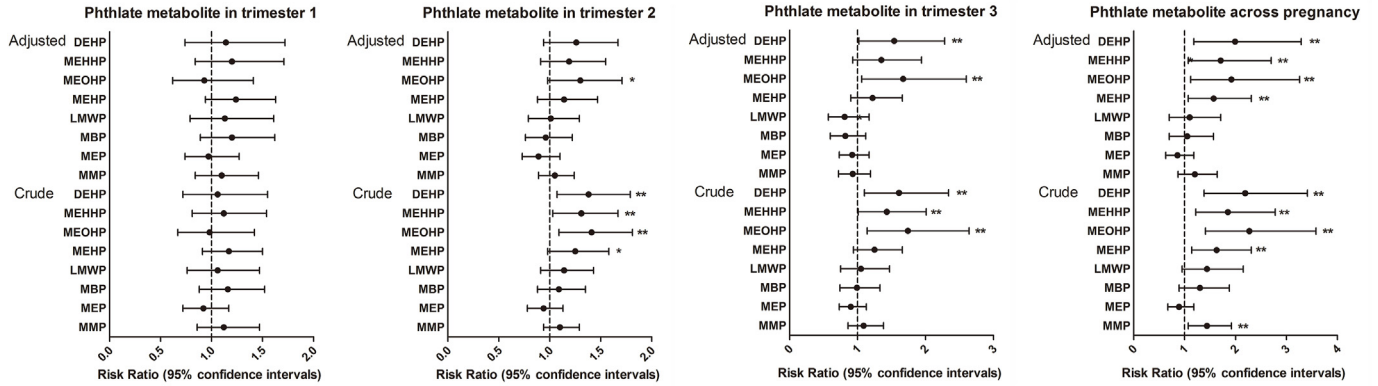

Fig. 1. Associations between prenatal individual exposure to phthalate metabolites and autistic traits in children aged 3 years. The adjusted model included maternal age, prepregnancy BMI, education, ethnicity, household income, smoking during pregnancy, drinking during pregnancy, primipara, lead exposure, and intelligence quotient as covariates.

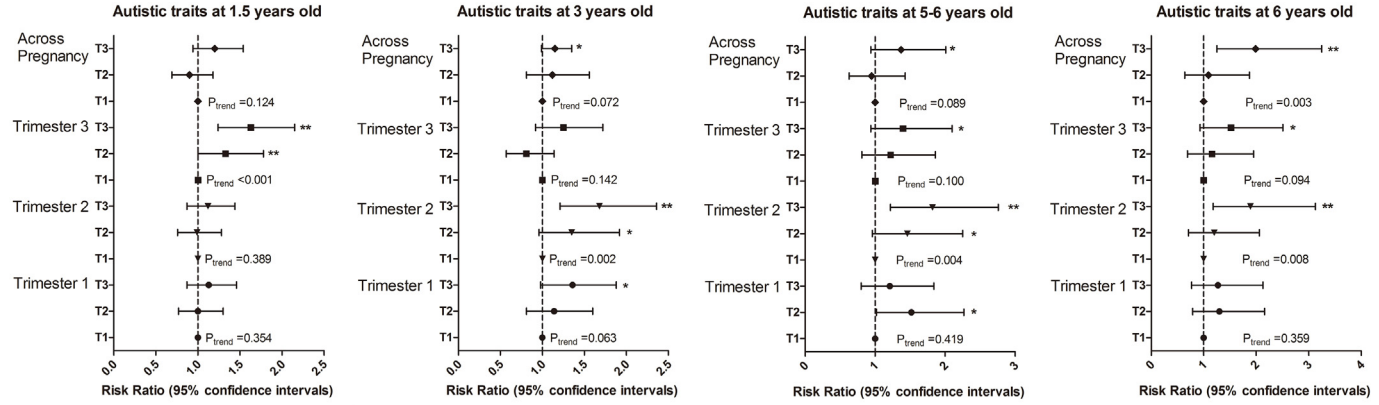

Fig. 2. Relationships of prenatal cumulative exposure to phthalate metabolites (measured by biomarker: ERS) with autistic traits in children aged 1.5–6 years; The adjusted model included maternal age, prepregnancy BMI, education, ethnicity, household income, smoking during pregnancy, drinking during pregnancy, primipara, lead exposure, and intelligence quotient as covariates. T1, first tertile; T2, second tertile; T3, third tertile.

and Fig. 2). As the ERS level increased, the risk of autistic traits in children also increased (*P*-value for trend = 0.002).

Using the ERS as an indicator of coexposure to phthalates, the weighted coefficients of metabolites for ERS calculation during pregnancy when modeling the association with autistic traits in 3-year-old children were as follows: MMP (0.154), MEP (−0.150), MBP (0.016), MEHP (0.127), MEOHP (0.343), and MEHHP (0.148). MEOHP was heavily weighted in the ERS, potentially being the most critical contributing substance. Additionally, two other metabolites of DEHP, namely, MEHHP and MEHP, as well as the MMP, had higher weights in the ERS group. These findings were similar to the results of the single-substance model mentioned earlier.

Similar patterns were observed at the ages of 5 years (Table S8 and Fig. 2) and 6 years (Table S9 and Fig. 2). For children aged 1.5 years, a positive exposure–response relationship was also detected between ERS during late pregnancy and the risk of autistic traits in children (Table S10 and Fig. 2). These findings suggested that cumulative exposure to phthalates during pregnancy may contribute to an increased risk of ASD in children.

3.5. Modifying effects of maternal vitamin D concentrations on associations between phthalate coexposure and ASD incidence

Among mother–child dyads in the sufficiency subgroup of maternal 25(OH)D levels, no significant associations were found between ERS exposure and the risk of autistic traits in children aged 1.5–6 years (Table S7-S10).

However, in mother–child pairs with 25(OH)D deficiency, exposure to ERS during early and mid-pregnancy consistently demonstrated a positive association with childhood autistic traits at ages 1.5–6 years, following an exposure–response pattern (Tables S7–S10). For instance, coexposure to phthalates in the first (medium ERS: RR_{adj} = 0.96, 95% CI = 0.59–1.55; high ERS: RR_{adj} = 1.62, 95% CI = 1.07–2.49; *P*-value for trend = 0.018) and second (medium ERS: RR_{adj} = 1.63, 95% CI = 1.04–2.62; high ERS: RR_{adj} = 1.75, 95% CI = 1.11–2.82; *P*-value for trend = 0.020) trimesters was associated with autistic traits at age 3 (Table S7).

In the insufficient subgroup of 25(OH)D levels, a positive exposure–response relationship was also observed between ERS exposure during mid-pregnancy and the risk of autistic traits at ages 1.5 (medium ERS: RR_{adj} = 1.04, 95% CI = 0.67–1.63; high ERS: RR_{adj} = 1.43, 95% CI = 0.94–2.20; *P*-value for trend = 0.087); 5 (medium ERS: RR_{adj} = 1.55, 95% CI = 0.80–3.11; high ERS: RR_{adj} = 2.32, 95% CI = 1.27–4.47; *P*-value for trend = 0.006); and 6 (medium ERS: RR_{adj} = 1.60, 95% CI = 0.73–3.68; high ERS: RR_{adj} = 2.16, 95% CI = 1.04–4.81; *P*-value for trend = 0.043) (Tables S8–S10). Furthermore, a marginal or significant positive exposure–response relationship was detected between ERS exposure during mid-to-late pregnancy and the risk of autistic traits at age 3 (Table S7 and Fig. 3).

3.6. Sexual dimorphism underlying the associations between phthalate coexposure and ASD

A sex-stratified analysis was also conducted to investigate potential sex differences in the associations between phthalate exposure and autistic traits in children (Table 3). In boys, there was a positive exposure–response relationship between prenatal phthalate exposure and the risk of autistic traits at ages 3 (medium ERS: RR_{adj} = 1.30, 95% CI = 0.84–2.04; high ERS: RR_{adj} = 1.73, 95% CI = 1.15–2.64; *P*-value for trend = 0.009) and 6 (medium ERS: RR_{adj} = 0.85, 95% CI = 0.40–1.78; high ERS: RR_{adj} = 1.89, 95% CI = 1.03–3.60; *P*-value for trend = 0.029). Among girls, a similar exposure–response relationship was observed between phthalate exposure during pregnancy, particularly in the middle to late stages, and an increased risk of autistic traits at ages 5 (medium ERS: RR_{adj} = 1.43, 95% CI = 0.77–2.73; high ERS: RR_{adj} = 1.78, 95% CI = 0.98–3.31; *P*-value for trend = 0.062) and 6 (medium ERS: RR_{adj} = 1.51, 95% CI = 0.68–3.47; high ERS: RR_{adj} = 2.29, 95% CI = 1.10–5.09; *P*-value for trend = 0.028). However, no statistically significant association was found between prenatal phthalate exposure and autistic traits at age 3 in girls. These findings suggested that prenatal phthalate exposure was associated with a sex-specific risk of ASD during childhood, with the differences primarily observed in the preschool period.

3.7. Potential mechanisms

A total of 23 genes and 16 phenotypes were identified (Figs. S7 and S8). Among these genes, the top four genes most strongly related to ASD were RELN (degree = 6), TNF (degree = 5), DLG4 (degree = 5), and GRIN2B (degree = 4) (Fig. S9). These genes were considered candidate molecular initiating events (MIEs) in the DEHP-induced AOP. Subsequently, twelve phenotypes (GO:0060964, GO:0019218, GO:0045444, GO:1903409, GO:0010893, GO:0048167, GO:0007611, GO:2000463, GO:0060291, GO:0007612, GO:0051968, and GO:0045211) that interact with these four genes were selected as probable phenotypes connecting DEHP-related genes with ASD.

To propose the AOP, it was necessary to identify putative MIEs, key events (KEs), and adverse outcomes (AOs) related to ASD. By integrating information from AOP-Wiki, a literature review [41], and our identified probable phenotypes, we selected “learning or memory” (GO:0007611) as the AO because learning and memory are usually impaired in individuals with ASD [42,43]. Due to the complex clinical manifestations

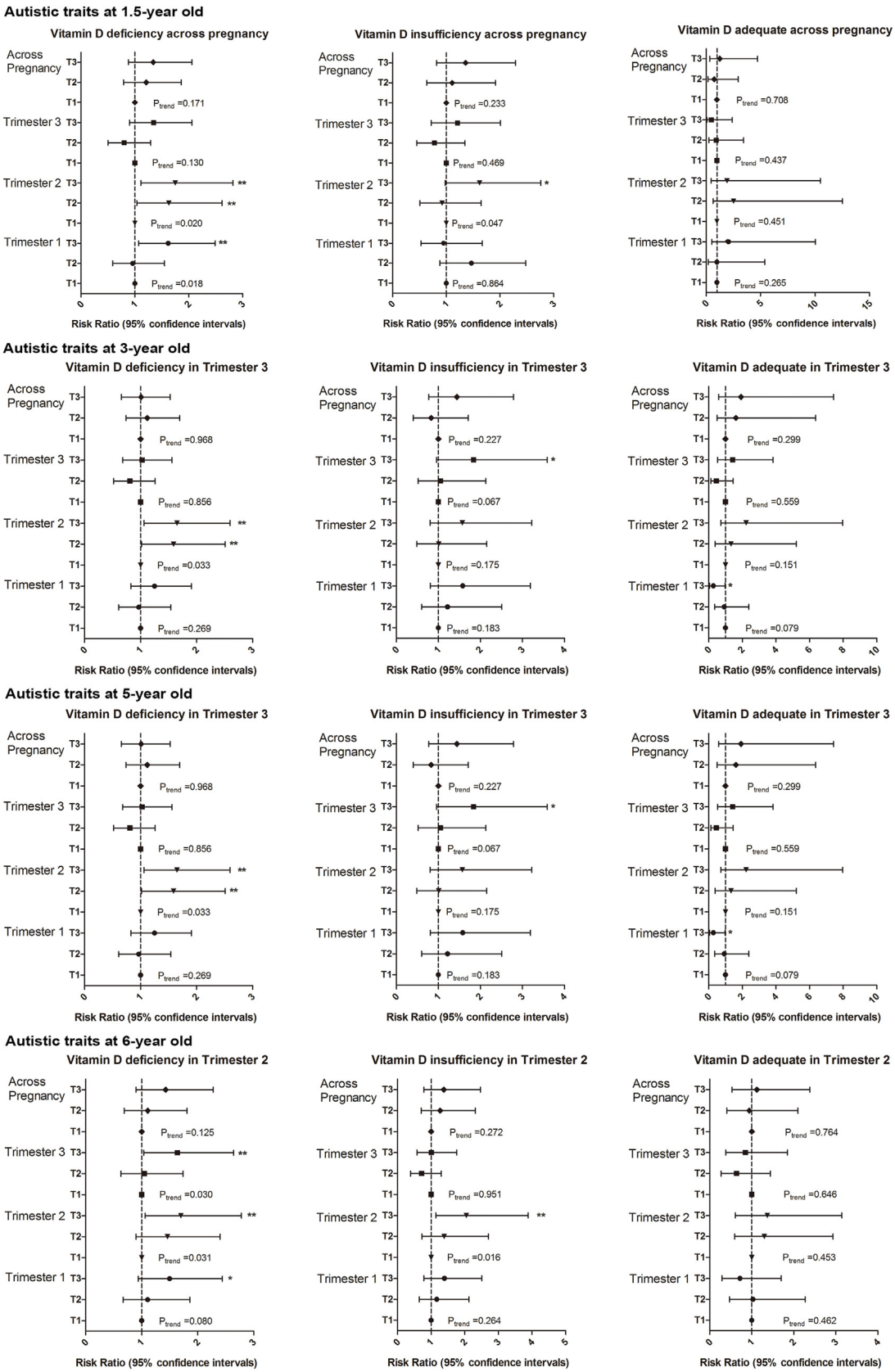

Fig. 3. Relationships of prenatal cumulative exposure to phthalate metabolites (measured by biomarker: ERS) with autistic traits in children aged 1.5–6.0 years stratified by maternal vitamin D status. The adjusted model included maternal age, prepregnancy BMI, education, ethnicity, household income, smoking during pregnancy, drinking during pregnancy, primipara, lead exposure, and intelligence quotient as covariates.

Table 3
Stratified analyses of the associations of phthalate mixture exposure (environmental risk score, ERS) with autistic traits according to child sex.

Phthalate coexposure	Trimester 1		Trimester 2		Trimester 3		During pregnancy	
	RR (95% CI)	*P* value	RR (95% CI)	*P* value	RR (95% CI)	*P* value	RR (95% CI)	*P* value
In subgroup of boys at 1.5 years old								
Low ERS	1.00	Ref.	1.00	Ref.	1.00	Ref.	1.00	Ref.
Medium ERS	0.97 (0.68,1.39)	0.885	0.90 (0.64,1.26)	0.544	1.08 (0.74,1.58)	0.693	0.84 (0.59,1.19)	0.326
High ERS	1.30 (0.93,1.82)	0.128	0.99 (0.70,1.38)	0.942	**1.53 (1.09,2.19)**	**0.016**	1.24 (0.90,1.73)	0.187
P value for trend	0.121		0.945		**0.013**		0.171	
In subgroup of boys at 3 years old								
Low ERS	1.00	Ref.	1.00	Ref.	1.00	Ref.	1.00	Ref.
Medium ERS	1.05 (0.67,1.66)	0.821	1.38 (0.86,2.22)	0.182	0.90 (0.57,1.43)	0.667	1.30 (0.84,2.04)	0.241
High ERS	**1.50 (0.99,2.30)**	**0.059**	**2.07 (1.35,3.25)**	**0.001**	**1.47 (0.98,2.23)**	**0.064**	**1.73 (1.15,2.64)**	**0.010**
P value for trend	**0.048**		**0.001**		**0.051**		**0.009**	
In subgroup of boys at 5 years old								
Low ERS	1.00	Ref.	1.00	Ref.	1.00	Ref.	1.00	Ref.
Medium ERS	1.44 (0.84,2.54)	0.194	1.41 (0.79,2.55)	0.249	0.88 (0.51,1.49)	0.629	0.71 (0.40,1.22)	0.216
High ERS	1.42 (0.82,2.49)	0.214	**1.93 (1.13,3.40)**	**0.019**	0.98 (0.59,1.63)	0.934	1.16 (0.71,1.90)	0.549
P value for trend	0.235		**0.017**		0.934		0.515	
In subgroup of boys at 6 years old								
Low ERS	1.00	Ref.	1.00	Ref.	1.00	Ref.	1.00	Ref.
Medium ERS	1.00 (0.49,2.04)	0.991	1.63 (0.81,3.38)	0.178	**2.06 (1.03,4.30)**	**0.045**	0.85 (0.40,1.78)	0.663
High ERS	1.24 (0.65,2.42)	0.510	**1.82 (0.93,3.72)**	**0.086**	1.74 (0.87,3.67)	0.126	**1.89 (1.03,3.60)**	**0.044**
P value for trend	0.492		**0.092**		0.152		**0.029**	
In subgroup of girls at 1.5 years old								
Low ERS	1.00	Ref.	1.00	Ref.	1.00	Ref.	1.00	Ref.
Medium ERS	1.03 (0.70,1.53)	0.870	1.10 (0.73,1.65)	0.645	**1.75 (1.13,2.77)**	**0.013**	1.01 (0.68,1.51)	0.948
High ERS	0.93 (0.63,1.39)	0.739	1.32 (0.89,1.96)	0.170	**1.75 (1.14,2.75)**	**0.012**	1.17 (0.80,1.72)	0.421
P value for trend	0.732		0.167		**0.016**		0.408	
In subgroup of girls at 3 years old								
Low ERS	1.00	Ref.	1.00	Ref.	1.00	Ref.	1.00	Ref.
Medium ERS	1.26 (0.76,2.13)	0.375	1.31 (0.79,2.21)	0.304	0.70 (0.41,1.18)	0.182	0.94 (0.57,1.55)	0.814
High ERS	11.16 (0.69,1.95)	0.578	1.21 (0.71,2.06)	0.483	0.96 (0.58,1.57)	0.864	0.93 (0.57,1.51)	0.759
P value for trend	0.590		0.505		0.827		0.759	
In subgroup of girls at 5 years old								
Low ERS	1.00	Ref.	1.00	Ref.	1.00	Ref.	1.00	Ref.
Medium ERS	1.60 (0.90,2.90)	0.115	1.58 (0.85,3.04)	0.156	**2.07 (1.06,4.29)**	**0.039**	1.43 (0.77,2.73)	0.265
High ERS	0.88 (0.45,1.72)	0.716	**1.70 (0.93,3.23)**	**0.091**	**2.53 (1.30,5.20)**	**0.008**	**1.78 (0.98,3.31)**	**0.062**
P value for trend	0.741		**0.098**		**0.008**		**0.062**	
In subgroup of girls at 6 years old								
Low ERS	1.00	Ref.	1.00	Ref.	1.00	Ref.	1.00	Ref.
Medium ERS	1.71 (0.83,3.72)	0.159	0.80 (0.34,1.86)	0.603	0.54 (0.23,1.22)	0.148	1.51 (0.68,3.47)	0.319
High ERS	1.20 (0.53,2.75)	0.662	**1.93 (0.97,4.01)**	**0.067**	1.47 (0.74,2.98)	0.275	**2.29 (1.10,5.09)**	**0.032**
P value for trend	0.687		**0.046**		0.281		**0.028**	

Bold type means *P*-value < 0.10.

of ASD and the wide range of diagnostic criteria and research characteristics, there was no direct scientific evidence supporting ASD as an AO. Therefore, "learning and memory" was chosen as a representative AO, and AOP 17, AOP 54, and AOP 13 were retrieved from AOP-Wiki based on this AO (Fig. S10). The MIEs identified in the linear AOPs included the binding of antagonists to NMDA receptors (AOP 13), thiol/selenoproteins involved in protection against oxidative stress (AOP 17), and the inhibition of the Na^+/I^- symporter (NIS) (AOP 54).

Based on its biological plausibility, GRIN2B was selected as the MIE because it encodes the glutamate ion receptor NMDA-type subunit 2B (GLU2B), which is considered one of the emerging risk genes for ASD [44]. Animal models with genetic defects in GRIN2B have been proposed and used in ASD-related studies [44]. We subsequently mapped DEHP and the four phenotypes (GO:0007611, GO:0048167, GO:0060291, and GO:0045211) that interact with GRIN2B as KEs to AOP 13 (OECD status: WPHA/WNT EndoSed). It should be noted that this AOP was applicable only during a specific period of brain development, which was the time of synaptogenesis. In humans, this period starts in the third trimester of pregnancy and continues for 2–3 years after birth.

Therefore, a putative AOP for DEHP-associated effects on neurodevelopment was proposed with a sequence of events described below. During pregnancy, exposure to DEHP leads to a downregulation of GRIN2B expression, which subsequently inhibits the function of postsynaptic membrane NMDARs, resulting in a decrease in calcium (Ca^{2+}) permeability. This reduction in intracellular Ca^{2+} levels further leads to a decrease in brain-derived neurotrophic factor (BDNF) secretion. Consequently, these molecular changes disrupt long-term potentiation (LTP) and synaptic plasticity, ultimately resulting in the formation of abnormal synapses and impaired hippocampal neural network function. These alterations in neural connectivity are believed to contribute to deficits in learning and memory processes (Fig. S11).

4. Discussion

Prenatal exposure to DEHP and its metabolites significantly increased the risk of autistic traits in 3-year-old children. Cumulative phthalate exposure was also linked to a higher risk of ASD in children. Maternal vitamin D levels modified the effects of phthalate coexposure, showing significant associations with autistic traits in children when vitamin D levels were deficient. Gender-specific differences were observed, with a positive exposure–response relationship primarily observed for boys and girls during different age periods. These findings highlighted the potential role of prenatal phthalate exposure in ASD development. Using an AOP framework, we propose a potential mechanism underlying this association.

The phthalate exposure pattern during pregnancy in the present study resembled that of previous studies in China. Among pregnant women from Wuhan, China (enrolled from 2014 to 2015), the median concentrations of M(n+i)BP (13.31 wk: 60.18 ng/mL; 24.13 wk: 44.84 ng/mL; 36.22 wk: 74.39 ng/mL) have been reported to be the highest, followed

by those of MEP and DEHP secondary metabolites [45]. However, the results from other countries differed from the present study. Compared to the data from the Health Outcomes and Measures of the Environment (HOME) cohort study (enrollment from 2003 to 2006), the MEP concentration in the present study was much lower than that in the USA (16/26 wk: 132 μg/g Cr). However, the MBP concentration in the present study was greater than that in the USA (16/26 wk: 31 μg/g Cr) [46]. The data from the Generation R cohort in the Netherlands (enrollment from 2002 to 2006) revealed the highest exposure levels of MEP (13.1 wk: 136.55 ng/mL; 20.4 wk: 72.64 ng/mL), followed by M(n+i)BP (13.1 wk: 37.01 ng/mL; 20.4 wk: 18.56 ng/mL), with lower exposure levels of the DEHP metabolite [47]. Data from the multicenter HELIX project in Spain, Norway, and France (enrollment from 2014 to 2015) showed that the exposure levels of MBP (20 wk: 40.4 μg/g Cr; 32 wk: 42.1 μg/g Cr) and DEHP metabolites—MEHP (20 wk: 3.2 μg/g Cr; 32 wk: 2.7 μg/g Cr), MEOHP (20 wk: 6.0 μg/g Cr; 32 wk: 5.7 μg/g Cr), and MEHHP (20 wk: 9.2 μg/g Cr; 32 wk: 8.3 μg/g Cr)—were similar to those in the present study, but the concentrations of MEP (20 wk: 47.1 μg/g Cr; 32 wk: 49.1 μg/g Cr) were slightly greater than those of MBP [48]. In summary, phthalate exposure levels varied across international cohorts, possibly due to differences in legislative actions in different countries, individual lifestyles, individual behaviors, the degree of environmental phthalate pollution, and the timing of data collection, etc.

The challenge of identifying autistic traits in young children is the difficulty in determining the exact timing of ASD onset after birth, which poses a potential confounding relationship between early childhood phthalate exposure and ASD risk, despite some studies emphasizing the collection of phthalate samples before assessing ASD incidence. Therefore, studying the potential risk of ASD in children associated with prenatal (in utero) phthalate exposure based on a birth cohort aligns better with causal inference. Cunha et al. systematically searched phthalate-autism-related literature until November 17, 2022 [49]. Associations between prenatal phthalate exposure and autistic traits were investigated in nine cohort studies as follows [21,48,50–56]: four of these studies reported positive associations between exposure to specific phthalate metabolites and autistic traits [50,53–55]; one study identified a negative association with MEP exposure [48]; and four studies revealed nonsignificant relationships in all the children [21,51,52,56]. These nine studies included sample sizes ranging from 77 to 782 children, with ages varying from 3 to 9 years; most of the studies used the Social Responsiveness Scale (SRS) to assess autistic traits, and three studies collected two to three urine samples to estimate phthalate exposure [49]. Additional cohort studies have studied this association since the literature review in 2022. A study based on 408 children from Taiwan region indicated that prenatal coexposure to metals and phthalates may increase the risk of ASD by 2.11-fold at 4 years of age, as identified by the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) [57]. Phthalate metabolites were measured in a multicenter cohort of 501 women in early and late pregnancy, and behavior was assessed in children aged 4–5 years by the Behavioral Assessment System for Children (BASC-2) and SRS-2. Additionally, a phthalate mixture in early pregnancy has been associated with an increased SRS-2 total score without sex differences [58]. These findings support the conclusion that phthalate exposure during pregnancy increases the risk of ASD in offspring. However, Emma et al. recruited 437 participants from two prospective birth cohorts and reported that phthalate mixture exposure is not associated with SRS scores at ages 3–8 years [59]. The reasons for the inconsistency of the findings compared to those of the present study are unclear, but differences in population characteristics, exposure measurements, and outcome assessments cannot be excluded. For instance, prenatal vitamin use [21], including folic acid [59] and vitamin D supplementation, may mitigate the effects of phthalate exposure on ASD risk in children. In addition, the sample sizes of previous studies were generally small, which may have affected the efficiency of the statistical analysis, especially due to the possibility of exploring sexually dimorphic effects. Furthermore, as described in the previous paragraph, phthalate exposure varies from country to country, and several studies [50–52,54] have used only a single urine sample to assess phthalate exposure; therefore, bias resulting from exposure misclassification may be introduced. Finally, compared to the DSM-5, tools for assessing autism at different ages, such as the SRS, BASC-2, and CHAT-23, have different sensitivities and specificities.

The present study suggested a putative AOP in which, during pregnancy, exposure to DEHP downregulates GRIN2B, leading to the inhibition of NMDAR function in the postsynaptic membrane. This ultimately results in impaired learning and memory, possibly due to the important role of NMDARs in synaptic plasticity and memory formation. NMDAR activation is known to enhance the release of BDNF, promoting neuronal survival, differentiation, and synaptogenesis [60,61]. GRIN2B, which is considered an emerging risk gene for ASD, plays a role in cortical neuron migration and synaptic maturation [62]. Synaptic pruning, which is implicated in neurodevelopmental disorders, such as autism spectrum disorders, may be affected when GluN2B is absent postsynaptically [63].

There are concerns that the current legislation for restricting the use of phthalates may not be sufficient. A scoping review has shown that interventions, such as dietary changes and the replacement of personal care products, can effectively reduce exposure to phthalates. Even a few days of intervention can lead to a decrease in phthalate concentrations in urine [64]. However, reducing exposure for short periods of time seems to be insufficient. It is challenging for the general public to find consumer information about the presence of these chemicals, and changing behavior in one aspect may not necessarily reduce the body's overall burden. In addition, replacing personal care products and avoiding food packaging can be cost-prohibitive for many people. These findings suggest that widespread public health regulation to reduce phthalate exposure is critical, but implementing new regulatory measures or commitments can also be time-consuming. Interestingly, the present study revealed that adequate vitamin D intake during pregnancy can alter the risk of phthalate-related autistic traits in children. Promoting vitamin D supplementation during pregnancy not only improves pregnancy and birth outcomes but also has potential neuroprotective effects against environmental endocrine disruptors [65–67]. This is a cost-effective intervention worth considering, especially in light of the high prevalence of vitamin D deficiency in pregnant women and the associated health risks.

One important observation is that the majority of single pollutant models conducted at 1.5 years of age revealed negative correlations between late pregnancy exposure to phthalate metabolites and autistic traits. However, exposure to the ERS during late pregnancy had opposite associations with ASD symptoms. While we do not have a definitive explanation, it is worth considering the potential influence of the complex effects resulting from exposure to a mixture of metabolites. Furthermore, we propose that the manifestation of ASD symptoms in children at 1.5 years of age may not occur immediately following a single phthalate exposure during the late trimester but rather exhibit a gradual emergence over time.

A limitation of the present study was the use of the M-CHAT and C-CARS scales to assess autistic traits in children. Although these scales are suitable for large-scale epidemiological investigations, they have limited sensitivity and specificity. Future research should focus on clinical diagnoses using screening results and the DSM-V criteria for accurate identification of ASD patients. Furthermore, among the 3209 mother–child pairs included in the present study, 58.06% and 67.19% of the children were followed up at the ages of 5 and 6 years, respectively. More than 30% of the children had no scores available for the screening of ASD traits, which may have introduced bias into the interpretation of the model results stratified by age. Comparisons of results across age-stratified models should be performed with caution. Second, urinary metabolite detection of phthalates was performed only once during each trimester, which may have introduced some potential exposure bias. In addition, mono(2-ethyl-5-carboxypentyl) phthalate (MECPP) is one of the most prevalent secondary metabolites of DEHP in human urine, but it was not measured and included in the present study which may have resulted in an underestimation of DEHP exposure. Furthermore, although

maternal spot urine samples were obtained within 7 days in the morning between 8:00 and 10:00 a.m., variations in concentrations dependent on the time of collection are likely because of the fast metabolism and excretion of phthalates. The study population was mainly from Ma'anshan, Anhui Province, indicating that caution should be exercised when extrapolating the results to other populations. While this study suggested that adequate vitamin D may mitigate autistic traits in pregnant women due to phthalate exposure, further evidence from intervention trials is needed.

5. Conclusion

Prenatal exposure to multiple phthalate metabolites increases the risk of ASD in preschool children. This risk is particularly associated with late pregnancy exposure to DEHP and a mixture of all phthalate metabolites. The association showed a sex-specific pattern. DEHP may downregulate GRIN2B, inhibit postsynaptic membrane NMDARs, induce synaptic aberrations, impair learning and memory, and contribute to ASD. Prenatal vitamin D supplementation may mitigate the ASD risk associated with phthalate mixture exposure.

CRediT authorship contribution statement

H.G.: conceptualization, methodology, writing–original draft and funding acquisition; C.Z. and B.B. Z.: software, formal analysis and visualization, and writing–original draft; M.L.G., J.T., Y.Z.: investigation, data curation, and experiment for detection of phthalate metabolites; Z.X.Z., D.W.: bioinformatics analysis; K.H.: validation and project administration; F.B.T.: resources, supervision, funding acquisition and writing–review & editing.

Declaration of competing interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eehl.2024.01.007.

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