Environment International 131 (2019) 105002

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Organophosphate pesticide metabolite concentrations in urine during pregnancy and offspring attention-deficit hyperactivity disorder and autistic traits

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ARTICLE INFO

Handling Editor: Heather Stapleton

Keywords:
Prenatal exposure
Organophosphate pesticides
Biomarkers
Attention-deficit hyperactivity disorder
Autism spectrum disorder
Population-based birth cohort

ABSTRACT

Background: Prenatal exposure to organophosphate (OP) pesticides has been associated with altered neuronal cell development and behavioral changes in animal offspring. However, the few studies investigating the association between prenatal OP pesticide exposure and neurodevelopmental outcomes such as Attention-Deficit Hyperactivity Disorder (ADHD) and autistic traits in children produced mixed findings.

Objective: The objective of the present study was to examine whether maternal urinary concentrations of OP pesticide metabolites are associated with ADHD and autistic traits in children participating in the Generation R Study, a population-based birth cohort from Rotterdam, the Netherlands.

Method: Maternal concentrations of 6 dialkylphosphates (DAPs) were measured using gas chromatography coupled with tandem mass spectrometry in urine samples collected at < 18 weeks, 18-25 weeks, and > 25 weeks of gestation in 784 mother-child pairs. DAP metabolite concentrations were expressed as molar concentrations divided by creatinine levels and $\log_{10}$ transformed. ADHD traits were measured at ages 3, 6, and 10 years using the Child Behavior Checklist (CBCL) (n = 781) and autistic traits were measured at age 6 years using the Social Responsiveness Scale (SRS) (n = 622). First, regression models were fit for the averaged prenatal exposure across pregnancy. Second, we investigated associations for each collection phase separately, and applied a mutually adjusted model in which the effect of prenatal DAP concentrations from each time period on ADHD and autistic traits were jointly estimated. All associations were adjusted for relevant confounders.

Results: Median DAP metabolite concentration was 309 nmol/g creatinine at < 18 weeks, 316 nmol/g creatinine at 18–25 weeks, and 308 nmol/g creatinine at > 25 weeks of gestation. Overall, DAP metabolite concentrations were not associated with ADHD traits. For instance, a log10 increase in averaged total DAP concentrations across gestation was not associated with a lower ADHD score (-0.03 per SD 95 CI: −0.28 to 0.23). Similarly, no associations between maternal DAP concentrations and autistic traits were detected.

Conclusions: In this study of maternal urinary DAP metabolite concentrations during pregnancy, we did not observe associations with ADHD and autistic traits in children. These are important null observations because of the relatively high background DAP concentrations across pregnancy, the relatively large sample size, and the 10-year follow-up of the offspring. Given the measurement error inherent in our OP pesticide exposure

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https://doi.org/10.1016/j.envint.2019.105002
Received 30 November 2018; Received in revised form 14 June 2019; Accepted 8 July 2019
Available online 29 July 2019

biomarkers, future studies using more urine samples are needed to accurately measure OP pesticide exposure over pregnancy in relation to ADHD and autistic traits.

1. Introduction

Organophosphate (OP) pesticides are a class of insecticides commonly used in agriculture. Some of the active OP pesticides may remain on or in food after they are applied to food crops (Eaton et al., 2008) and the exposure of non-occupationally exposed individuals occurs most likely through their diet (Llop et al., 2017; Lu et al., 2008; Sokoloff et al., 2016; van den Dries et al., 2018). After ingestion, most OP pesticides undergo bioactivation, during which the toxic oxon form is established, followed by detoxification, which produces up to 6 non-specific dialkyl phosphate (DAP) metabolites (Duggan et al., 2003). Preformed DAP metabolites also exist in the food supply (Clune et al., 2012; Lu et al., 2005; Quirós-Alcalá et al., 2012). It is therefore uncertain to what degree total DAP metabolite concentrations reflect actual OP pesticide exposure or the ingestion of possibly less toxic DAP metabolites (Krieger et al., 2012). However, the estimation of urinary DAP metabolite concentrations is considered a non-invasive and useful biomarker for OP pesticide exposure (Bravo et al., 2004), and thus, the most-used method of estimating exposure to this class of compounds in general populations (Engel et al., 2016).

High OP pesticide exposure is neurotoxic for both animals and humans (Costa, 2006; Pope et al., 1992; Rosenstock et al., 1991). However, both animal and human studies have suggested that even low-dose OP pesticide exposure may have negative health consequences (Jaga and Dharmani, 2003). Animal studies investigating exposure to the OP pesticides chlorpyrifos, diazinon and malathion (of which the residues were frequently being detected on fruit and vegetables between 2004 and 2006 in the Netherlands (ChemKap, 2017)) have shown that exposure levels below the threshold for acetylcholinesterase inhibition can induce changes in neurochemistry and behavior (Savy et al., 2015), result in cognitive impairments (dos Santos et al., 2016; Terry et al., 2012) and change the expression of genes related to mental disorders (Savy et al., 2018). Low-dose OP pesticide exposure in animal studies also changed neuronal cell development (Slotkin et al., 2008), induced oxidative stress (Slotkin and Seidler, 2010; Zafiropoulos et al., 2014), and affected the thyroid and the reproductive systems (Androutsopoulos et al., 2013; De Angelis et al., 2009; Haviland et al., 2010). Moreover, animals prenatally exposed to these OP pesticides had higher activity rates, greater motor agitation and hyperactivity signs, lower level of social behavior, and animals were limited in their exploration of novel objects (Grabovska and Salyha, 2015; Lan et al., 2017).

Because the human brain is particularly susceptible to neurotoxicity during fetal life (Rice and Barone, 2000), and because OP pesticides can cross the placental barrier and the blood-brain barrier (Bradman et al., 2003), most epidemiological studies of low-dose OP pesticide exposure focus on prenatal exposure in relation to neurodevelopment (González-Alzaga et al., 2014). Several of these studies have found prenatal OP pesticide exposure to be associated with or suggestive of poorer reflexes in neonates (Engel et al., 2007; Young et al., 2005), mental and psychomotor developmental delays in the offspring aged 1 to 3 years (Engel et al., 2011; Eskenazi et al., 2007; Rauh et al., 2006), and decreased intellectual functioning in children aged 6 to 9 years (Bouchard et al., 2011; Engel et al., 2011; Jusko et al., 2019; Rauh et al., 2011). Yet, other studies have not observed associations with neurodevelopmental outcomes (González-Alzaga et al., 2014). For example, Cartier et al. (2016) did not find evidence for an association between prenatal OP pesticide exposure and intellectual functioning in children aged 6 years.

Few studies have explored the association between OP pesticide exposure and Attention-Deficit Hyperactivity Disorder (ADHD) and autistic traits in children, and have reported inconsistent findings. A prospective birth cohort study among ethnic minorities from inner-city New York observed associations between prenatal OP pesticide exposure and ADHD traits in 228 children aged 3 years (Rauh et al., 2006). Also, another birth cohort study among low-income participants from farmworking communities in California observed associations with ADHD traits in 322 children aged 5 years (Marks et al., 2010). Yet, Eskenazi et al. (2007) did not observe these associations among children aged 2 years using data from the same cohort as Marks et al. (2010). These cohort studies were also used to assess the association between prenatal OP pesticide exposure and pervasive developmental disorder (PDD), which includes Autism Spectrum Disorders (ASDs) and found prenatal exposure to OP pesticides to be predictive of PDD at 2 to 3 years (Eskenazi et al., 2007; Rauh et al., 2006), but the number of PDD cases was small in one of the studies (Rauh et al., 2006). Next, a study using data from the same cohort as Eskenazi et al. (2007) and Marks et al. (2010) found that children prenatally exposed to higher levels of OP pesticides had more autistic traits as measured with the Social Responsiveness Scale (SRS) in 246 children aged 14 years (Sagiv et al., 2018). Yet, another study using data from 224 mother-child pairs from a metropolitan area in Ohio found that prenatal OP pesticide exposure did not increase autistic symptoms at age 8 years (Millenson et al., 2017). Only in subgroups, Furlong et al. (2014) observed an association between OP pesticide exposure and autistic symptoms among Black ($n = 42$) and male children ($n = 66$) using 136 mother-child pairs from New York. Similarly, Philippat et al. (2018), using data from a cohort study of women at high risk for having a child with ASD, did not observe an overall association among 203 children aged 3 years. However, after stratifying by sex prenatal OP pesticide exposure was associated with an increased risk of ASD among girls ($n = 78$) (Philippat et al., 2018).

This heterogeneity may be explained by differences in study areas and study populations. For example, 3 studies took place in California in a farmworker community where the use of insecticides is abundant (Eskenazi et al., 2007; Marks et al., 2010; Sagiv et al., 2018), whereas other studies took place in urban areas (Furlong et al., 2014; Millenson et al., 2017; Rauh et al., 2006) where the source and route of OP pesticide exposure may be different. Next, several of these studies were small in sample size. This may have reduced the power to detect associations and perform interaction analyses. Also, most of these studies included 1 or 2 urine specimens per subject to measure OP pesticide exposure. Analyzing multiple urine specimens per subject is of importance, because the urinary concentration of DAP metabolites reflects only recent exposure, and individual exposure differs substantially from day-to-day, depending on diet (Needham, 2005; Sokoloff et al., 2016).

Therefore, much uncertainty still exists about the relationship between fetal exposure to OP pesticides and the development of ADHD and ASD. The Generation R Study provides suitable data to address these research gaps because of the large sample size, 3 repeated measurements of maternal urinary concentrations of OP pesticide metabolites, repeated neurobehavioral measurements, and the availability of detailed demographic information. Furthermore, as documented elsewhere, the median total DAP concentrations among the Generation R Study mothers was more than 2-fold higher compared with background-exposed pregnant women in the U.S living in non-agricultural communities, which suggests a greater range of exposure and thus statistical power with which to evaluate exposure—disease associations (Ye et al., 2008). The objective of the present study was to examine whether maternal urinary concentrations of OP pesticide metabolites is

related to ADHD and autistic traits in young children.

2. Methods

2.1. Study population and follow-up

Generation R is a prospective population-based birth cohort designed to identify early environmental and genetic determinants of development (Kooijman et al., 2016). Briefly, all mothers who resided in the study area in Rotterdam, the Netherlands, and had a delivery date between April 2002 and January 2006 were eligible. Mothers were enrolled during pregnancy or in the first months after the birth of their child when newborns attended child health centers for routine visits. The study protocol underwent human subjects review at Erasmus Medical Center, Rotterdam, the Netherlands (IRB Registration no.: IRB00001482, MEC 198.782.2001.31, MEC 217.595/2002/202, MEC-2007-413, MEC-2012-165). Mothers provided written informed consent for themselves and their children.

Among the 9778 mothers who participated in the study, 8879 (91%) were enrolled during, as opposed to after, pregnancy. Between February 2004 and January 2006, spot urine specimens during early, middle, and late pregnancy (< 18, 18–25, > 25 weeks of gestational age, respectively) were collected at the time of routine ultrasound examinations when in total, 4918 women were enrolled. Of these, 2083 women provided a complete set of 3 urine specimens. From birth until the age of 4 years, data collection was performed by mailed questionnaires and by routine child health center visits. At child age 6 and 10 years, families were invited to participate in an in-person follow-up to collect neurobehavioral data, additional biospecimens, and sociodemographic and health data. We selected samples based on follow-up data with relevant outcomes, which was obtained for 1449 children of the 2083 women with a complete set of urine specimens. The availability of follow-up data permitted studies of prenatal OP pesticide exposure and child health, including neurodevelopment. From these 1449, 800 mother-child pairs were selected at random for lab analyses of DAP metabolites in the maternal and child urine samples. Of those, 784 had a sufficient volume of urine for analyses. The final analytic sample included 781 mother-child pairs with exposure and data on ADHD traits (from age 3 to 10 years), and 622 mother-child pairs with exposure and data on autistic traits (at age 6 years).

2.2. Urine collection and analysis of DAP metabolites

Maternal spot urine specimens were collected during early, mid-, and late pregnancy (< 18, 18–25, > 25 weeks of gestational age, respectively). Child spot urine specimens were collected when mother-child pairs attended the 6-year examination. Details of urine specimen collection have been described elsewhere (Kruithof et al., 2014). All urine samples were collected between 8 am and 8 pm in 100 ml polypropylene urine collection containers that were kept for a maximum of 20 h in a cold room (4 °C) before being frozen at −20 °C in 20 ml portions in 25 ml polypropylene vials. Measurements of 6 non-specific DAP metabolites of OP pesticides were conducted at Institut National de Santé Publique (INSPQ) in Quebec, Canada, using gas chromatography coupled with tandem mass spectrometry (GC–MS/MS) (Health Canada, 2010). Three dimethyl (DM) metabolites (dimethylphosphate (DMP), dimethylthiophosphate (DMTP), and dimethyldithiophosphate (DMDTP)) were determined, as well as 3 diethyl (DE) metabolites (diethylphosphate (DEP), diethylthiophosphate (DETP), and diethyldithiophosphate (DEDTP)). The limit of detection (LOD) was 0.26 μg/l for DMP (0% < LOD), 0.40 for DMTP (2–4% < LOD), 0.09 for DMDTP (18–20% < LOD), 0.50 for DEP (3–5% < LOD), 0.12 for DETP (12% < LOD) and 0.06 for DEDTP (81–85% < LOD). Measured concentrations below the LOD were included in the data analysis. The inter-day precision of the method during this project, expressed as the coefficient of variation (CV%), varied between 4.2 and 8.8 for DEDTP, 4.1–7.2 for DEP, 5.0–9.1 for DETP, 5.5–7.1 for DMDTP, 5.3–8.0 for DMP and 5.5–7.7 for DMTP based on reference materials (clinical check-urine level II 637 E-495 and MRM E-459) (van den Dries et al., 2018). Molar concentrations were used to compare our results with those from other studies, based on the following molecular weights: DMP 126.0, DMTP 142.1, DMDTP 158.2, DEP 154.1, DETP 170.2, and DEDTP 186.2 g/mol. To account for urinary dilution, creatinine concentrations were determined based on the Jaffe reaction (Butler, 1975; O'Brien et al., 2015). The LOD for creatinine was 0.28 mmol/l, and the day-to-day CV% varied between 3.0 and 3.3 (van den Dries et al., 2018).

2.3. Assessment of child ADHD traits

Child emotional and behavioral problems were assessed by maternal report with the Child Behavior Checklist (CBCL) 1.5–5 (Achenbach and Rescorla, 2000) during the assessments at child age 3, and 6 years, and with the CBCL 6–18 at child age 10 years (Achenbach and Rescorla, 2001). The CBCL is an internationally validated and reliable measure of emotional and behavioral problems (Achenbach and Rescorla, 2001). The CBCL measures emotional and behavioral problems on a continuous severity scale and research has shown that symptom scores predict psychiatric disorders as defined by the DSM in adulthood (Hofstra et al., 2002; Roza et al., 2003). Each item (CBCL 1.5–5: 99 items, CBCL 6–18: 112 items) within different scales (CBCL 1.5–5: 7 scales, CBCL 6–18: 8 scales) is scored on a 3 point rating scale 0 = 'not true', 1 = 'somewhat or sometimes true', and 2 = 'very true or often true', based on the preceding 2 months for the CBCL 1.5–5 and the preceding 6 months for the CBCL 6–18. The scales were found to be generalizable across 23 countries, including the Netherlands (Ivanova et al., 2010). From the CBCL checklist, we used the standardized sum score of the Diagnostic and Statistical Manual of Mental Disorders (DSM)-oriented ADHD traits across childhood for our analyses. The sum scores of ADHD traits were standardized to make the CBCL 1.5–5 (6 items) and the CBCL 6–18 (7 items) comparable, with a higher score indicating a higher level of ADHD traits.

2.4. Assessment of child autistic traits

At age 6 years, the SRS was administered to obtain a measure of autistic traits (Constantino and Gruber, 2005). The SRS provides a valid quantitative measure of subclinical and clinical autistic traits and assesses various dimensions of interpersonal behavior, communication and repetitive/stereotypic behavior characteristic of ASD (Constantino et al., 2003). The SRS represents the mothers' observation of the child's social behavior during the previous 6 months. The SRS is a useful screening tool to identify children who need further ASD-specific diagnostic assessment. The SRS has excellent correspondence to ASD classification according to the Developmental, Dimensional, and Diagnostic interview (3Di) and the Autism Diagnostic Observation Schedule (ADOS) (Duvekot et al., 2015). We used an abbreviated version of the SRS with a total of 18 items (Roman et al., 2013) to reduce participant burden. These items cover 3 domains: social cognition, social communication, and autistic mannerism. Previous studies have shown high correlations ($r > 0.90$) between the total scores of the abbreviated version of the SRS and the complete version of the SRS (Constantino and Todd, 2003). We used the SRS total score as a continuous measure in our analyses.

2.5. Additional data collection

Maternal reproductive, sociodemographic, and cognitive data were assessed by multiple questionnaires and instruments throughout the study. During pregnancy, data on maternal height and weight were collected, as was information on maternal age, maternal psychopathology (0 = no problems, 1 = borderline: GSI score > 80%

(Ettema and Arrindell, 2003)), parity (0, 1, or 2+), smoking (no smoking during pregnancy, smoked until pregnancy recognized, and continued smoking during pregnancy), alcohol intake during pregnancy (no alcohol consumption during pregnancy, alcohol consumption until pregnancy recognized, continued occasionally (< 1 glass/week), and continued frequently (1+ glass/week)), marital status (married/partner or single), household total net income (< 1200 euro per month (i.e., below the Dutch social security level), 1200–2000 euro per month, > 2000 euro per month), highest completed education level (low: < 3 years of high school; intermediate: 3+ years of secondary education; and, high: university degree or higher vocational training), and ethnicity (Dutch national origin, other-Western and non-Western). Further, body mass index (BMI) was calculated and categorized into 4 groups (< 18.5, 18.5– < 25, 25– < 30, and ≥30).

After birth, mothers reported on the duration of breastfeeding by postal questionnaire when the child was 2 months, 6 months and 12 months old. Mothers were asked whether they ever breastfed their child, and if yes, duration of any breastfeeding was assessed by asking at what age of the infant they stopped breastfeeding (in months). Next, an adapted Infant/Toddler Home Observation for Measurement of the Environment (IT-HOME) inventory (Bradley and Caldwell, 1984) was administered during a home visit at approximately 3 months of age (SD = 1.17 months). The validated 29-item version of the IT-HOME was used to measure the events, objects, and social interactions experienced by the child in the family context (Rijlaarsdam et al., 2012). Higher scores on the IT-HOME indicate a more enriched environment. Maternal IQ was measured when mother-child pairs attended the 6-year examination, and was assessed using a computerized Ravens Advanced Progressive Matrices Test, set I (Prieler, 2003). The test is a 12-item reliable and validated short version of the Raven's Progressive Matrices to assess non-verbal cognitive ability (Chiesi et al., 2012).

2.6. Statistical methods

The 3 DM metabolites (DMP, DMTP, and DMDTP) were summed as total DM (nmol/l) and the 3 DE metabolites (DEP, DETP, and DEDTP) were summed as total DE (nmol/l). Total DAP concentrations (nmol/l) were calculated by summing the 6 metabolites. Urinary concentrations were expressed on a volume and creatinine basis (nmol/g creatinine) and log10 transformed. For DAP metabolites, a small number of concentrations were missing due to insufficient samples or machine errors (≤5 measurements for any visit for DMs; ≤23 for DEs; ≤5 per visit for creatinine). Missing DAP metabolite values and missing covariate data were 10 times imputed with the Multivariate Imputation by Chained Equations (MICE) method in R (R core Team, 2015; van Buuren and Groothuis-Oudshoorn, 2011). DAP metabolite concentrations were log10 transformed prior to the multiple imputation (MI) procedure to approach normality. Both ADHD traits scores and autistic traits scores were included as predictors for the imputation of covariates, but were not imputed.

To address our primary research objective, we created linear mixed effects models (LMM) using averaged DM, DE, and DAP concentrations over pregnancy in relation to repeated measures of ADHD and used linear regression models to assess the association between averaged DM, DE, and DAP concentrations and autistic traits. Because urinary DAP levels are highly variable over time, this average is likely a better estimate of each participant's exposure than any single exposure measurement (Spaan et al., 2015). As a secondary approach, we investigated the DAP – ADHD traits and the DAP - autistic traits association for each collection phase (gestational age < 18 weeks, 18–25 weeks, and > 25 weeks) separately, and applied a mutually adjusted model in which the effect of prenatal DAP concentration from each time period on ADHD and autistic traits was jointly estimated. This additional approach was chosen to identify possible windows of vulnerability and to be able to compare our results with other studies that used a single spot urine sample in pregnancy to determine OP pesticide exposure.

Each LMM included a random intercept, a random slope of time (the age of the child at the outcome ascertainment in years), and an Autoregressive (order 1) covariance structure which improved the model fit based on a lower Akaike information criteria (AIC). The inclusion of an interaction term between time and exposure did not improve the model AIC significantly. For the DAP - autistic traits analyses the autistic traits score was square root transformed to approach normality of the residuals.

All analyses consisted of an unadjusted model and an adjusted model. The adjustment variables were maternal age, psychopathology score, ethnicity, education, income, marital status, alcohol consumption during pregnancy, non-verbal IQ, BMI, height, parity, smoking during pregnancy, and child sex. Potential adjustment variables were selected a priori defined with a Directed Acyclic Graph (DAG) using the Dagitty software (Textor et al., 2017). The DAG was based on previous studies of OP pesticide exposure and child neurodevelopment and on biologically plausible covariate–exposure and covariate–outcome associations observed in our data (See Supplementary Fig. S1). Additionally, adjusting for the possible confounders breastfeeding in months and IT-HOME score did not change the effect estimates meaningfully and were not included in the models.

2.7. Sensitivity analyses

Several sensitivity analyses were performed. First, potential effect modification by sex was explored via interaction terms, stratification, and augmented product terms (Buckley et al., 2017), because other studies have reported sex specific effects of the association between prenatal DAP metabolite concentrations with ADHD and autistic traits (Furlong et al., 2014; Marks et al., 2010; Philippat et al., 2018). Second, we refit models with using dichotomous ADHD and autistic traits scores because several studies investigated the association between DAP metabolite concentrations with the use of clinical cases (Eskenazi et al., 2007; Shelton et al., 2014). The dichotomization was based on the borderline clinical cut-off score for ADHD (> 93rd percentile) (Achenbach and Rescorla, 2000, 2001) and SRS cut-off value for screening in the population (consistent with weighted scores of ≥1.078 for boys and ≥1.000 for girls) (Constantino et al., 2003), and based on the > 80th percentile of the ADHD and autistic traits scores (to increase power). Third, we used inverse probability weighting to correct for loss to follow-up and to account for potential selection bias because participants in our study sample were more likely be Dutch, older, have a higher level of education, and a higher income compared with the full cohort (Table S1). Fourth, we substituted values below the LOD with LOD/√2 rather than the use of the measured concentrations below the LOD, which were included in the primary analysis. The replacement of values below the LOD with LOD/√2 is a common substitution method in environmental exposure studies (Baccarelli et al., 2005). Fifth, we refit models with metabolite concentrations expressed as nmol/l with creatinine concentration added as a separate covariate which is another common method to adjust for creatinine concentrations. Sixth, because DAP metabolite concentrations demonstrated only weak to moderate reliability over pregnancy (e.g., intraclass correlation coefficient (ICC) for DAP metabolites = 0.30) (Spaan et al., 2015), we examined the effect of adjusting for measurement error by applying regression calibration (Hardin et al., 2003). Seventh, because preformed DAP metabolites may exist on fruits, and fruit intake is associated with DAP metabolite concentrations (van den Dries et al., 2018) in our study population, we additionally stratified the main analyses for maternal fruit intake (assessed using a modified version of a validated semi-quantitative food frequency questionnaire) (Steenweg-de Graaff et al., 2012). The stratification was based on dichotomizing intake at the first quantile (75 g per day). Eight, we also adjusted for season of urine collection because there may be seasonal variation in food consumption and OP pesticides use which could affect DAP concentrations (Attfield

Kathleen et al., 2014). Ninth, since previous studies have suggested that families with low social economic status (SES) are more vulnerable to OP pesticide exposure (Cartier et al., 2016; Donauer et al., 2016; Stein et al., 2016), we explored potential effect modification by education as a proxy of SES. Finally, we explored potential associations of child DAP metabolite concentrations with ADHD and autistic traits and investigated potential effect modification by sex, since few studies have reported these associations (Bouchard et al., 2010; Lizardi et al., 2008). The child - DAP analyses were adjusted for maternal education, maternal age, maternal smoking, averaged prenatal DAP metabolite concentrations across pregnancy, child sex, child BMI at age 6 years, child ethnicity, household income at child age 6 years, and marital status of the mother at child age 6 years.

3. Results

3.1. Sample characteristics

Table 1 presents the maternal and infant characteristics at time of enrollment. The age at enrollment of the mothers participating in this study averaged 31 years (sd = 5 years). Women included in the present analysis were older, had lower BMIs, nulliparous, Dutch, highly educated, married, occasional consumers of alcoholic beverages during pregnancy, less likely to smoke, and had higher incomes and lower maternal psychopathology scores compared with those not included.

3.2. DAP concentrations

Total DAP metabolites comprised mostly DM metabolites, and the median concentrations were fairly similar across the 3 sampling periods (Table 2). The total DAP metabolite concentrations measured between 18-25 weeks of gestation (median = 316 nmol/g creatinine) was slightly higher compared with the DAP metabolite concentrations measured at < 18 weeks of gestation (median = 309 nmol/g creatinine) and > 25 weeks of gestation (median = 308 nmol/g creatinine). The ICC (estimated by using a 2-way mixed-effects model with absolute-agreement) for DAP metabolite concentrations varied between 0.22 and 0.26 for a single-measurement and 0.51 and 0.54 for the mean of the 3 measurements (Table S2). Moreover, the DAP metabolite concentrations across pregnancy showed weak correlations (r = 0.18–0.35) (Table S3).

3.3. ADHD and autistic traits descriptive statistics

Table 3 presents the descriptive statistics of the ADHD and autistic traits score. The mean ADHD score measured at age 3 years (m = 3.1, sd = 2.3) and at 6 years (m = 3.2, sd = 2.6) was slightly higher than the mean ADHD score measured at 10 years (m = 2.6, sd = 2.7). The percentage of participants within the ADHD borderline clinical range was 6.8% at 3 years, 5.3% at 6 years, and 6.6% at 10 years. The mean autistic traits score was 4.1 (sd = 4.2) and 1.9% of the participants were within the clinical range of autism.

3.4. Primary analyses of DAP - ADHD and autistic traits associations

There was no association between the averaged DAP, DE, and DM metabolite concentrations across pregnancy and ADHD traits (Table 4). Further, the autistic traits score did not differ by averaged maternal DAP, DM, and DE metabolite concentrations across pregnancy (Table 5).

3.5. Secondary analyses of DAP - ADHD and autistic traits associations

No association between DAP, DE, and DM metabolite concentrations and ADHD traits was observed in any of the 3 urine collection periods during pregnancy (Table 4). Next, similar to the separate regressions, the mutually adjusted DAP and DM metabolite concentrations were not statistically significantly associated with ADHD traits. However, we observed an inverse association between DE metabolite concentrations measured at < 25 weeks of gestation in the mutually adjusted model. A 10-fold higher level of DE metabolite concentrations at < 25 weeks of gestation was associated with a 0.15 standard deviation lower level of in ADHD traits (95% confidence interval (CI) = −0.28, −0.01).

No association between DAP, DE, and DM metabolite concentrations and autistic traits was observed in any of the 3 urine collection periods during pregnancy (Table 5). Moreover, similar to the separate regressions, the mutually adjusted DAP, DE, and DM metabolite concentrations were not statistically significantly associated with autistic traits.

3.6. Sensitivity analyses

No effect modification by sex was observed (*P*-value for interaction > 0.1) with regard to the associations between log10 transformed DAP metabolite concentrations and ADHD traits (Table S4) or autistic traits (Table S5). Further, the results with the use of the clinical cut-off score (Tables S6 and S7), the inverse probability weighted results (Table S8), the results with concentrations below the LOD substituted by LOD/√2 (Table S9), the results with metabolite concentrations expressed as nmol/l with creatinine concentration added as a separate covariate (Table S10), were all similar to the main analyses. When we adjusted for the measurement error in our exposure biomarkers by assessing averaged urinary DAP concentrations on ADHD and autistic traits with the application of regression calibration, we observed that the effect estimates were stronger (i.e., further away from the null) compared to the results from the primary model, but that the standard errors were increased (Table S11). We observed no difference in the associations when stratified by fruit intake (Tables S12 and S13), and the results with additional adjustment for season of urine collection were similar to the main results (Table S14). Next, no effect modification by SES was observed (Tables S15 and S16). Finally, no associations of child DAP metabolite concentrations with ADHD and autistic traits were detected (Table S17), and these associations did not differ by sex (Table S18).

4. Discussion

In this large population-based study, higher maternal urinary concentrations of DAP metabolites during pregnancy were not associated with more ADHD traits in 3 to 10 year old children or with autistic traits in 6 year old children. Moreover, no effect modification by sex was observed. Finally, those exposed to higher urinary concentrations of DAP metabolites in childhood also did not have more ADHD traits and autistic traits.

Our results were consistent with a previous study from the Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS) cohort (Eskenazi et al., 2007) investigating the association between prenatal OP pesticide exposure and ADHD in children aged 2 years, but not consistent with to 2 other studies, which suggested that prenatal OP pesticide exposure was associated with ADHD (Marks et al., 2010; Rauh et al., 2006). Marks et al. (2010), using data from the same birth cohort study as Eskenazi et al. (2007), found prenatal OP pesticide exposure to be associated with more ADHD traits at child age 5 years. Rauh et al. (2006), using data from a study population from inner-city New York, found that prenatal exposure to chlorpyrifos measured in blood was associated with offspring's ADHD traits at the age of 3 years.

Our null results for autistic traits are not in line with the results in children aged 14 years from a previous study of the CHAMACOS cohort (Sagiv et al., 2018), but consistent with findings of the Mount Sinai Children's Environmental Health Study (Furlong et al., 2014) and Health Outcomes and Measures of the Environment (HOME) Study (Millenson et al., 2017) in children aged 8 years that also measured autistic traits with the use of the SRS in children.

Table 1
Characteristics of all participants of the Generation R Study and of the participants included in the analysis.

Characteristics	Generation R cohort (n = 9778) [c]	Included in the ADHD analyses[a] (n = 781) [c]	Included in the autistic traits analyses[b] (N = 622)[c]
Infant characteristics			
Sex of infant at birth			
Male	50.6%	50.8%	51.4%
Female	49.4%	49.2%	48.6%
Missing, n	153	–	
Mother characteristics			
Age in years			
< 20	4.2%	1.8%	1.1%
20– < 25	15.9%	10.0%	7.4%
25– < 30	26.4%	26.6%	25.6%
30– < 35	36.9%	46.0%	49.0%
≥35	16.6%	15.7%	16.9%
Missing, n	–	–	–
BMI			
< 18.5	2.1%	2.3%	2.4%
18.5– < 25	57.9%	65.9%	67.3%
25– < 30	26.3%	23.5%	22.3%
≥30	13.8%	8.3%	8.1%
Missing, n	899	4	2
Height in cm (quartiles)			
< 161	23.6%	15.7%	13.0%
161– < 168	27.4%	30.6%	29.1%
168– < 173	24.6%	26.2%	27.9%
≥173	24.4%	27.5%	30.0%
Missing, n	934	1	1
Parity (previous births)			
0	55.1%	62.4%	63.5%
1	30.2%	26.6%	26.0%
≥2	14.7%	11.0%	10.5%
Missing, n	378	4	2
Ethnicity			
Dutch	50.0%	57.6%	63.3%
Other Western	11.6%	8.9%	9.0%
Non-Western	38.4%	33.5%	27.7%
Missing, n	694	–	–
Education[d]			
Low	26.5%	14.8%	11.9%
Intermediate	30.7%	30.2%	27.8%
High	42.8%	55.0%	60.2%
Missing, n	1221	25	11
Household income in euro's			
< 1200 per month	20.7%	12.6%	9.1%
1200–2000 per month	18.5%	16.6%	15.5%
> 2000 per month	60.8%	70.8%	75.4%
Missing, n	3066	101	62
Marital status			
Married/living with partner	85.5%	89.8%	91.4%
No partner	14.5%	10.2%	8.6%
Missing, n	1213	29	18
Non-verbal IQ score			
≤85	29.7%	17.9%	15.7%
> 85–≤100	25.7%	22.8%	23.3%
> 100– < 115	34.7%	31.2%	31.3%
≥115	19.8%	28.0%	29.7%
Missing, n	5430	20	171
Psychopathology (quartiles)			
< 0.08	24.1%	24.9%	26.4%
0.08– < 0.17	25.2%	29.7%	29.6%
0.17– > 0.38	25.7%	24.4%	24.9%
≥0.38	25.0%	21.1%	19.1%
Missing, n	3128	95	72
Smoking			
No smoking during pregnancy	73.4%	77.1%	79.7%
Until pregnancy recognized	8.6%	8.9%	9.1%
Continued during pregnancy	18.0%	14.0%	11.2%
Missing, n	1534	63	51

(*continued on next page*)

Table 1 (*continued*)

Characteristics	Generation R cohort (n = 9778) [c]	Included in the ADHD analyses[a] (n = 781) [c]	Included in the autistic traits analyses[b] (N = 622)[c]
Alcohol beverage consumption			
No alcohol consumption during pregnancy	48.0%	36.7%	33.7%
Until pregnancy recognized	13.2%	17.5%	17.2%
Continued occasionally (< 1 glass/week)	31.6%	39.3%	42.0%
Continued frequently (1 or more glass/week for at least two trimesters)	7.2%	6.5%	7.1%
Missing, n	1870	40	29

[a] ADHD traits were measured at child mean ages 3, 6 and 10 years.
[b] Autistic traits were measured at child mean age 6 years.
[c] Values shown are percentages.
[d] Low: no education finished, primary education, lower vocational training, intermediate general school or < 3 years at general. Intermediate: ≥3 years of secondary education, intermediate vocational training or first year of higher vocational training. High: university degree or higher vocational training.

Several other researchers found some evidence for an association between prenatal OP pesticide exposure and autism in studies of clinical cases (Roberts et al., 2007; Shelton et al., 2014) or PDD cases aged 2 to 3 years (Eskenazi et al., 2007; Rauh et al., 2006). However, often the associations were limited to subgroups (Furlong et al., 2014; Marks et al., 2010; Philippat et al., 2018). Marks et al. (2010) reported an association between OP and ADHD traits, only in boys, Furlong et al. (2014) between prenatal DE metabolite concentrations and autistic traits (SRS-score), only in boys and in black children (aged 5 years), and Philippat et al. (2018), between DMTP metabolites and ASD in girls (aged 3 years) using data from the Markers of Autism Risk in Babies - Learning Early Signs (MARBLES) mother-child cohort. We did not replicate these findings.

Next, we explored whether children with higher DAP metabolite concentrations at age 6 years had higher levels of ADHD or autistic traits since few studies using data from NHANES and Children Pesticide Survey (CPS) reported these associations (Bouchard et al., 2010; Lizardi et al., 2008) in children aged 7 to 15 years. We found no evidence for

Table 2
Descriptive statistics of maternal urinary dialkyl phosphate metabolite concentrations of 781 mothers participating in this study.

	nmol/g creatinine					nmol/l				
	min	p25	p50	p75	max	min	p25	p50	p75	max
Dialkyl phosphates[a]										
< 18 weeks' gestation	15.4	188.1	309.4	499.3	6444.5	6.3	124.8	219.0	421.2	7798.7
18–25 weeks' gestation	41.0	206.8	316.1	485.9	3069.5	10.0	120.0	227.2	407.2	4607.7
> 25 weeks' gestation	21.0	194.1	308.0	489.3	3013.3	10.5	121.6	223.8	408.2	3332.6
Diethyl alkyl phosphates[b]										
< 18 weeks' gestation	0.0	25.0	43.1	79.4	3030.5	0.0	15.5	31.3	65.1	6818.6
18–25 weeks' gestation	0.0	23.3	41.6	74.5	660.5	0.0	12.6	27.9	57.0	1093.4
> 25 weeks' gestation	0.0	21.6	41.7	77.3	745.1	0.0	14.4	30.7	62.2	593.2
Dimethyl alkyl phosphates[c]										
< 18 weeks' gestation	6.7	148.6	242.4	416.1	6106.5	0.9	100.1	182.4	344.7	4221.0
18–25 weeks' gestation	24.8	169.4	268.6	415.4	2612.0	7.6	98.9	185.6	335.9	3902.2
> 25 weeks' gestation	12.2	157.1	248.3	398.9	2908.1	8.5	99.5	184.2	333.0	3300.5

[a] Total dialkyl phosphates is the sum of DEDTP, DETP, DEP, DMDTP, DMTP and DMP.
[b] Diethyl alkyl phosphates is the sum of DEDTP, DETP and DEP.
[c] Dimethyl alkyl phosphates is the sum of DMDTP, DMTP and DMP.

Table 3
Descriptive statistics of ADHD and autistic traits scores.

	Mean ± SD	Min	Max	Clinical cases (N (%))[a,b]	N
ADHD score at age 3 years	3.1 ± 2.3	0.0	11.0	42 (6.8%)	618
ADHD score at age 6 years	3.2 ± 2.6	0.0	12.0	41 (5.3%)	777
ADHD score at age 10 years	2.6 ± 2.7	0.0	12.0	39 (6.6%)	588
Autistic traits at age 6 years	4.1 ± 4.2	0.0	32	12 (1.9%)	622

[a] Any ADHD score that falls below the 93rd percentile is considered normal, scores above the 93 percentile are borderline clinical, or clinical cases (Achenbach and Rescorla, 2000, 2001).
[b] We utilized the cut-off values recommended by the authors of the SRS for screening in population-based studies (consistent with weighted scores of 1.078 for boys and 1.000 for girls) (Constantino et al., 2003).

Table 4
Difference in standardized ADHD score[a] (and 95% confidence interval) across childhood per 10-fold increase in maternal urine dialkyl phosphate metabolite concentrations in nmol/g creatinine, by timing of pregnancy urine sampling and degree of adjustment.

Dialkyl phosphate type	Childhood ADHD scores (N = 781)[b]											
	Unadjusted				Adjusted[c]				Mutually adjusted[d]			
	B	95% CI			B	95% CI			B	95% CI		
Dialkyl phosphates (total)[e]												
< 18 weeks' gestation	−0.15	−0.33	to	0.04	−0.06	−0.23	to	0.11	−0.07	−0.25	to	0.11
18–25 weeks' gestation	−0.03	−0.22	to	0.17	0.05	−0.14	to	0.23	0.08	−0.13	to	0.28
> 25 weeks' gestation	−0.12	−0.31	to	0.07	−0.02	−0.20	to	0.16	−0.03	−0.22	to	0.16
Mean of three urines	−0.20	−0.46	to	0.07	−0.03	−0.28	to	0.23				
Diethyl alkyl phosphates[f]												
< 18 weeks' gestation	−0.09	−0.22	to	0.05	−0.03	−0.15	to	0.10	−0.03	−0.15	to	0.10
18–25 weeks' gestation	−0.03	−0.16	to	0.11	0.05	−0.08	to	0.18	0.08	−0.05	to	0.22
> 25 weeks' gestation	−0.23	−0.37	to	−0.10	−0.13	−0.26	to	0.00	−0.15	−0.28	to	−0.01
Mean of three urines	−0.25	−0.45	to	−0.05	−0.08	−0.27	to	0.11				
Dimethyl alkyl phosphates[g]												
< 18 weeks' gestation	−0.11	−0.29	to	0.06	−0.04	−0.20	to	0.12	−0.05	−0.22	to	0.12
18–25 weeks' gestation	−0.03	−0.22	to	0.15	0.03	−0.15	to	0.20	0.03	−0.16	to	0.22
> 25 weeks' gestation	−0.05	−0.23	to	0.13	0.03	−0.14	to	0.20	0.02	−0.16	to	0.20
Mean of three urines	−0.14	−0.39	to	0.12	0.00	−0.24	to	0.25				

[a] Positive scores indicating more symptomatic behavior.
[b] 781 mother-child pairs and 1983 observations.
[c] Adjusted for maternal age, maternal psychopathology (0 = no problems, 1 = borderline: GSI score > 80%), sex of the child, ethnicity (Dutch, other-Western and non-Western), education (low, intermediate and high), income (low, middle and high), marital status, maternal alcohol consumption (no alcohol consumption during pregnancy, alcohol consumption until pregnancy was known, occasionally alcohol consumption during pregnancy and frequently alcohol consumption during pregnancy), non-verbal IQ of the mother, BMI categories (< 18.5, 18.5–25, 25–30, 30+), height of the mother, parity categories (0, 1, 2+), and smoking (no smoking during pregnancy, smoked until pregnancy was known, smoked during pregnancy).
[d] Adjusted model with the inclusion of the three exposures in one model.
[e] Total dialkyl phosphates is the sum of DEDTP, DETP, DEP, DMDTP, DMTP and DMP.
[f] Diethyl alkyl phosphates is the sum of DEDTP, DETP and DEP.
[g] Dimethyl alkyl phosphates is the sum of DMDTP, DMTP and DMP.

Table 5
Difference in autistic traits score[a,b] (and 95% confidence interval) per 10-fold increase in maternal urine dialkyl phosphate metabolite concentration in nmol/g creatinine, by timing of pregnancy urine sampling and degree of adjustment.

Dialkyl phosphate type	Autistic traits score at 6 years (N = 622)											
	Unadjusted				Adjusted[c]				Mutually adjusted[d]			
	B	95% CI			B	95% CI			B	95% CI		
Dialkyl phosphates (total)[e]												
< 18 weeks' gestation	0.05	−0.20	to	0.30	0.15	−0.10	to	0.39	0.15	−0.11	to	0.40
18–25 weeks' gestation	−0.07	−0.35	to	0.20	0.02	−0.25	to	0.29	−0.03	−0.32	to	0.26
> 25 weeks' gestation	−0.09	−0.35	to	0.16	0.06	−0.20	to	0.32	0.05	−0.22	to	0.32
Mean of three urines	−0.07	−0.43	to	0.30	0.17	−0.20	to	0.54				
Diethyl alkyl phosphates[f]												
< 18 weeks' gestation	−0.05	−0.22	to	0.12	0.02	−0.15	to	0.18	0.04	−0.13	to	0.21
18–25 weeks' gestation	−0.12	−0.30	to	0.06	−0.05	−0.22	to	0.13	−0.02	−0.21	to	0.16
> 25 weeks' gestation	−0.28	−0.48	to	−0.07	−0.16	−0.37	to	0.04	−0.16	−0.37	to	0.05
Mean of three urines	−0.29	−0.56	to	−0.02	−0.12	−0.39	to	0.15				
Dimethyl alkyl phosphates[g]												
< 18 weeks' gestation	0.10	−0.14	to	0.34	0.17	−0.07	to	0.40	0.16	−0.08	to	0.40
18–25 weeks' gestation	−0.04	−0.30	to	0.22	0.05	−0.21	to	0.31	−0.03	−0.30	to	0.25
> 25 weeks' gestation	−0.02	−0.26	to	0.23	0.13	−0.12	to	0.38	0.12	−0.15	to	0.38
Mean of three urines	0.03	−0.32	to	0.39	0.25	−0.11	to	0.61				

[a] Square root transformed.
[b] Positive scores indicating more symptomatic behavior.
[c] Adjusted for maternal age, maternal psychopathology (0 = no problems, 1 = borderline: GSI score > 80%), sex of the child, ethnicity (Dutch, other-Western and non-Western), education (low, intermediate and high), income (low, middle and high), marital status, maternal alcohol consumption (no alcohol consumption during pregnancy, alcohol consumption until pregnancy was known, occasionally alcohol consumption during pregnancy and frequently alcohol consumption during pregnancy), non-verbal IQ of the mother, BMI categories (< 18.5, 18.5–25, 25–30, 30+), height of the mother, parity categories (0, 1, 2+), and smoking (no smoking during pregnancy, smoked until pregnancy was known, smoked during pregnancy).
[d] Adjusted model with the inclusion of the three exposures in one model.
[e] Total dialkyl phosphates is the sum of DEDTP, DETP, DEP, DMDTP, DMTP and DMP.
[f] Diethyl alkyl phosphates is the sum of DEDTP, DETP and DEP.
[g] Dimethyl alkyl phosphates is the sum of DMDTP, DMTP and DMP.

any association.

The inconsistency of results may be related to differences in OP pesticide exposure sources across study areas, the exposure mixture, or the exposure assessment methodology. Most previous studies were carried out in the US and the exposure mixture of OP pesticides in Europe is different than in the US due to differences in regulations regarding the use of OP pesticides. Within the US, the majority of the studies were conducted in California (Eskenazi et al., 2007; Marks et al., 2010; Philippat et al., 2018; Roberts et al., 2007; Sagiv et al., 2018; Shelton et al., 2014) where agricultural insecticides are extensively used. In contrast, the Generation R population lives in urban settings, where the main route of exposure is through the ingestion of food, and most likely fruits (van den Dries et al., 2018). Furthermore, DAP metabolites are non-specific biomarkers of OP pesticide exposure, thus it is possible that the mixture of parent compounds differed across cohorts and thus toxicity varies even if DAP levels were similar. Also, because preformed DAP metabolites are present on food crops and in the natural environment (Clune et al., 2012; Lu et al., 2005; Quirós-Alcalá et al., 2012), it is uncertain which amount of the total DAP metabolite concentrations is due to the ingestion of the less toxic DAP metabolites from the natural environment (Krieger et al., 2012). Finally, the majority of these studies, including our study, measured DAP metabolites in urine as a biomarker of OP pesticide exposure. However, some studies took a different approach. For example, 3 studies used maternal residence near agricultural pesticide applications to estimate exposure (Roberts et al., 2007; Sagiv et al., 2018; Shelton et al., 2014), and another study used parent compounds measured in umbilical cord blood collected at delivery to measure the level of OP pesticide exposure (Rauh et al., 2006). Differences in exposure sources, mixtures, routes, and assessments across studies complicate the comparison.

Discrepancies in the results of studies may be related to differences in study populations. For example, the majority of our study sample consisted of Dutch participants with a relatively high SES. Apart from the study of Millenson et al. (2017), that reported similar results, most previous studies mainly included participants from ethnic minorities or with low SES (Eskenazi et al., 2007; Furlong et al., 2014; Marks et al., 2010; Rauh et al., 2006; Sagiv et al., 2018). It is conceivable that these populations with low SES were exposed to unobserved background risk factors that are related to the likelihood of OP pesticide exposure and the risk for ADHD or autistic traits. Although these studies adjusted for SES related confounders, there may still be residual confounding. In our study, the unadjusted models predicting ADHD and autistic traits were almost all in a negative direction, suggesting a protective effect. After adjustment (including SES related confounders), the observed inverse associations were closer to the null or in the positive direction (albeit clearly non-statistically significant), suggesting the presence of some observed negative confounding (Mehio-Sibai et al., 2005). However, the inverse association for DE metabolite concentrations measured at > 25 weeks of gestation and ADHD traits in the mutually adjusted model remained. We must be cautious in interpreting this association which may be a result of multiple testing. Also, this finding is contrary to the findings of Eskenazi et al. (2007) and Marks et al. (2010) who observed that higher DE metabolite concentrations were associated with more PDD and ADHD problems. Although we included many potential confounders in the model, we cannot rule out that other underlying factors such as a healthier lifestyle among those exposed to OP pesticides, might have resulted in unobserved negative confounding. Millenson et al. (2017) and Cartier et al. (2016) observed a similar pattern of negative confounding in the association between maternal DE metabolite concentrations and child neurodevelopment among persons with relatively high SES. These studies showed a significant unadjusted association of maternal DE metabolite concentrations with autistic traits and verbal comprehensive score in an unexpected direction (i.e. higher DE metabolite concentrations - less problems). However, after adjustment the observed inverse associations were closer to the null.

Another notable difference between our study and other studies investigating the association between OP pesticide exposure and ADHD and autistic traits is that in this study the DAP metabolite concentrations (308–316 nmol/g creatinine) were 2–3 times higher than in previous studies (Cartier et al., 2015; Eskenazi et al., 2007; Llop et al., 2017; Philippat et al., 2018; Rauch et al., 2012; Sokoloff et al., 2016; Ye et al., 2009). This could be due to higher consumption of fruits and vegetables, higher SES (which is associated with more healthy food consumption), or due to the extensive farming practices in the Netherlands (OECD, 2015; van den Dries et al., 2018). Nevertheless, we did not find any evidence for an association between OP pesticide exposure and ADHD and autistic traits.

A few limitations of the present study need to be considered. As mentioned above, DAP metabolites also exist in the food supply (Clune et al., 2012; Lu et al., 2005; Quirós-Alcalá et al., 2012). It is therefore uncertain to what extent the total DAP metabolite concentrations are due to the OP pesticide exposure or due to the ingestion of the less toxic DAP metabolites (Krieger et al., 2012). Further, DAP metabolite concentrations provide non-specific information about the cumulative exposure to a class of OP pesticides rather than a single OP pesticide (Wessels et al., 2003). It is therefore unknown to which specific OP parent pesticide(s) our study population was exposed. However, the estimation of urinary DAP metabolite concentrations is considered an appropriate and useful tool to identify and compare levels of OP pesticide exposure among various population (Bravo et al., 2004).

Urinary DAP metabolites have a short half-life. These metabolites are excreted in urine within approximately 24 h, which implies that the measured DAP concentrations may vary from day-today within each subject (Needham, 2005) giving rise to chance findings. It would be ideal to collect a broad range of urine specimens more often during pregnancy (Perrier et al., 2016), especially since the ICCs of DAP metabolite concentrations are modest (Spaan et al., 2015). To achieve excellent reliability of OP pesticide exposure over pregnancy, 4 weekly pools of 15 to 20 urine samples would be needed (Casas et al., 2018). The present study includes 3 spot-urine measures of maternal DAP metabolite concentrations per subject from a large sample. Although our sampling frequency is higher than that of most other studies of maternal urinary DAP metabolite concentrations and neurodevelopment (González-Alzaga et al., 2014), the urinary measurement variability may have still resulted in attenuated estimates due to measurement error in our biomarkers (Perrier et al., 2016). Indeed, adjusting for measurement error with regression calibration resulted in effect estimates that were further from the null albeit more imprecise (Carroll et al., 2006).

Another limitation of this study is the absence of information about the exact time of spot urine sampling. Because the urine spot samples were collected between 8 am and 8 pm, there may have been a combination of first morning and random spot samples. Concentrations of chemicals, urine volume and the rate of excretion vary with fluid intake, time of day, and other factors (Barr et al., 2005; Boeniger et al., 1993; Cornelis et al., 1996). Although, time of sample collection is unlikely to confound the association between DAP metabolite concentrations and child neurodevelopmental outcomes, the use of DAP metabolite concentrations without the adjustment of timing of urine sampling may have resulted in high intra-individual variability and less precise associations. To increase precision, we adjusted the main analyses for season of urine collection. The results were essentially the same.

Although, the percentage of participants above the SRS cut-off value for screening in the population (1.9%) (Constantino et al., 2003) was similar to the overall prevalence of ASD (0.6–2.2%) in children aged 8 years (Baio, 2014), the percentages of participants considered borderline clinical or clinical ADHD cases (Achenbach and Rescorla, 2000, 2001) in our study (5.3–6.8%) was lower than the prevalence of ADHD (7–14%) (Davidovitch et al., 2017). Because the children in our study have parents with a higher education, more income, and who were less

likely to smoke during pregnancy, it is possible that the children were generally healthier than the source population. This could have resulted in lower variability in ADHD and autistic traits which may explain our null findings. Also, the differences in characteristics between our study sample and the source population, i.e., the total Generation R cohort, may have introduced selection bias. Yet, we used inverse probability weighting to account for potential selection bias and the results were essentially the same.

A strength of this study is the evaluation of ADHD and ASD phenotype on a continuous spectrum rather than the use of a clinical cut-off (presence versus absence of disease). For example, several other studies used a clinical diagnosis of ASD (Philippat et al., 2018; Roberts et al., 2007; Shelton et al., 2014) or clinical cut-off (Eskenazi et al., 2007; Rauh et al., 2006). Other studies, including ours, relied on the CBCL and SRS measured on a continuous scale. Although the use of the SRS and CBCL does not provide a clinical diagnosis, it has many advantages. For instance, the use of a continuous scale increases power and allow us to account for children with fewer symptoms who may not have met the diagnostic criteria when a clinical cut-off is used, and reduces the impact of outcome misclassification (Sagiv et al., 2015). Further, the use of repeated measures of ADHD traits is another strength. Repeated measures of ADHD traits increase statistical power by allowing for missing observation at various time points, and gave us the ability to include a random slope for time to account for varying effects of time on ADHD across subjects (Baayen, 2008; Harrison et al., 2018; Matuschek et al., 2017; Nakagawa and Freckleton, 2011; Pinheiro and Bates, 2006). Finally, the availability of a broad range of contextual information for confounder adjustment is another strength of this study.

In conclusion, in this study of maternal urinary DAP metabolite concentrations during pregnancy, we did not observe associations with ADHD and autistic traits in children. These are important null observations because of the relatively high background DAP concentrations across pregnancy, the relatively large sample size, and the 10-year follow-up of the offspring. Given the measurement error inherent in our OP pesticide exposure biomarkers, future studies using more urine samples are needed to accurately measure OP pesticide exposure over pregnancy in relation to ADHD and autistic traits. Further, the Generation R Study is representative of an urban population with varying ethnicities, SES, and educational level, and therefore less generalizable to populations where the OP pesticide exposure sources may differ.

Declaration of Competing Interest

The authors did not declare any competing interests.

Acknowledgement

The Generation R Study is conducted by the Erasmus Medical Center, Rotterdam in close collaboration with the Faculty of Social Sciences of the Erasmus University Rotterdam, the Municipal Health Service, Rotterdam Homecare Foundation and Stichting Trombosedienst & Artsenlaboratorium Rijnmond. The authors would like to thank the contribution of participating parents and their children, general practitioners, hospitals, midwives, and pharmacies. Further, the authors would like to thank Dr. Frank Pierik for laying the groundwork for the NIEHS-TNO-Erasmus University collaboration and Dr. Russ Hauser for alerting MPL to the possibility of this project.

Funding

This research received financial support from the National Institutes of Health Grants K12 ES019852 and P30 ES001247, and from the intramural research program of the National Institute of Environmental Health Sciences, National Institutes of Health. Henning Tiemeier was supported by a grant of the Netherlands Organization for Scientific Research (NWO grant No. 024.001.003, Consortium on Individual Development, and NWO/ZonMW grant 016.VICI.170.200). Mònica Guxens is funded by a Miguel Servet fellowship (MS13/00054, CPII18/00018) awarded by the Spanish Institute of Health Carlos III. ISGlobal is a member of the CERCA Programme, Generalitat de Catalunya. Dr. Hanan El Marroun was supported by Stichting Volksbond Rotterdam, the Dutch Brain Foundation (De Hersenstichting, project number GH2016.2.01) and the European Union's Horizon 2020 research and innovation programme (grant agreement No. 733206 LifeCycle).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105002.

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