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Authors

Barkoski, Jacqueline M

Philippat, Claire

Tancredi, Daniel

et al.

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In utero pyrethroid pesticide exposure in relation to autism spectrum disorder (ASD) and other neurodevelopmental outcomes at 3 years in the MARBLES longitudinal cohort

Jacqueline M. Barkoski[1], **Claire Philippat**[2], **Daniel Tancredi**[3], **Rebecca J. Schmidt**[1,4], **Sally Ozonoff**[4], **Dana Boyd Barr**[5], **William Elms**[1], **Deborah Bennett**[1,*], **Irva Hertz-Picciotto**[1,3]

[1]Department of Public Health Sciences, School of Medicine, University of California, Davis, CA, USA

[2]Institute for Advanced Biosciences, INSERM U1209, CNRS UMR 5309, University Grenoble Alpes, 38000 Grenoble, France

[3]Department of Pediatrics, School of Medicine, University of California, Davis, CA, USA

[4]MIND (Medical Investigations of Neurodevelopmental Disorders) Institute, University of California, Davis, CA, USA

[5]Rollins School of Public Health, Emory University, Atlanta, GA, USA

Abstract

Background: We assessed the relationships between prenatal pyrethroid pesticide exposure and autism spectrum disorders (ASD) or non-typical development (non-TD) at 3 years.

Methods: Participants were mother-child pairs (n=201) in the MARBLES (Markers of Autism Risk in Babies-Learning Early Signs) cohort. Because familial recurrence risk is high, MARBLES enrolls pregnant women with a family history of ASD. Children were clinically assessed at 3 years of age and classified into 3 outcome categories: ASD, typically developing (TD), or non-TD (and not meeting criteria for ASD). Repeated maternal second and third trimester urine samples were analyzed for pyrethroid metabolite 3-phenoxybenzoic acid (3-PBA). Multinomial logistic regression was used to obtain relative risk ratios (RRR) linking 3-PBA concentrations averaged

[*]**Corresponding Author:** Jacqueline M. Barkoski, MS1C, One Shields Ave, University of California, Davis, Davis, CA 95616, Telephone: 530-754-8282, Fax: (530) 752-3239, jmbarkoski@ucdavis.edu.

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Declaration of interests

None

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across each trimester and over pregnancy with child's outcome, either ASD or non-TD vs. TD. Models were adjusted for specific gravity, maternal pre-pregnancy BMI, prenatal vitamin use, birth year, home-ownership, and TCPy (3,5,6-trichloro-2-pyridinol) pregnancy concentrations.

Results: The median specific gravity corrected 3-PBA concentration of all samples was 1.46 ng/ml. Greater second trimester 3-PBA concentrations were associated with modestly elevated relative risk ratios of ASD (RRR: 1.50 (95% CI 0.89 to 2.51), p = 0.12). There were no differences between non-TD and TD.

Conclusions: This study found no evidence for differences in 3-PBA comparing non-TD with TD. A moderately elevated RRR was found comparing urinary 3-PBA concentrations for ASD versus TD; however, the confidence interval was wide and hence, these findings cannot be considered definitive.

Keywords

pyrethroid; autism; MARBLES; pesticide; neurodevelopment; pregnancy

1. Introduction

Autism spectrum disorder (ASD) is a severe neurodevelopmental disorder affecting one in 54 children in the United States (Maenner et al., 2016), and the prevalence has increased over the past 20 years. The etiology of ASD is not understood, but there are likely multiple contributing factors. Findings from studies on twins show genetic factors contribute considerably (Bailey et al., 1995; Folstein and Rutter, 1977), but environmental exposures also play a substantial role (Bai et al., 2019; Constantino et al., 2013; Gaugler et al., 2014; Hallmayer et al., 2011; Sandin et al., 2014). There is growing evidence to support the notion that environmental exposures such as air pollution (Yang et al., 2017), maternal metabolic conditions (Krakowiak et al., 2012; Li et al., 2016), and pesticides (Eskenazi et al., 2007; Roberts et al., 2007; Shelton et al., 2014a; von Ehrenstein et al., 2019) increase risk for ASD, and prenatal vitamin use may reduce risk (Guo et al., 2019; Levine et al., 2018; Schmidt et al., 2011; Schmidt et al., 2019; Schmidt et al., 2012; Surén et al., 2013).

Pyrethroids are a class of synthetic insecticides which act on voltage gated sodium channels in the nerve cell membrane (ASTDR, 2003). Pyrethroids cause persistent opening of the sodium ion channels allowing more sodium ions to pass and depolarizing the neuronal membrane (Shafer et al., 2005). In rodents pyrethroid exposures have been shown to affect neurotransmission, possibly altering brain development (Aziz et al., 2001; Malaviya et al., 1993). Pyrethroids have also been found to transcriptionally mimic autism in high-throughput screening using RALS-Seq methods (Simon et al., 2019). Animal studies demonstrated that although pyrethroids exhibit relatively low acute toxicity, there is potential for developmental neurotoxicity (Shafer and Hughes, 2010; Shafer et al., 2005; Soderlund, 2012).

Pyrethrum is a substance produced by the C*hrysanthemum* flower, and is toxic to insects. Pyrethroids are synthetic forms that were developed to be more stable in sunlight allowing greater persistence in the environment. With decreased photodegradation pyrethroid residue

can persist in indoor environments for weeks after application (Starr et al., 2014). Pyrethroid pesticides are included in over 3,500 EPA registered products used in and around homes, on pets, in mosquito control and in agriculture (U.S. EPA, 2013). In 2001, the U.S. EPA banned the use of organophosphate pesticides (OP) in household products, which led to an increase of pyrethroid pesticides in this market (EPA, 2010). Pyrethroids are used, to a lesser extent, in agriculture particularly on crops such as cotton, vegetables, and nuts. Exposures occur through residential sources and residues on food (Lu et al., 2006a; Lu et al., 2006b).

Associations between organochlorine, organophosphate, and pyrethroid pesticides and ASD risk have been observed in large case-control studies (Roberts et al., 2007; Shelton et al., 2014b; von Ehrenstein et al., 2019). These studies have linked agricultural pesticide applications with maternal addresses during pregnancy assess pesticide exposures and studies utilizing biomarkers of pregnancy pesticide exposure and ASD have largely focused on OP's. In one study pregnancy OP exposures were associated with pervasive developmental disorder (a type of autism spectrum disorder) at 24 months (Eskenazi et al., 2007). More recently, pregnancy OP biomarker concentrations were linked with ASD among girls in a high-risk population but not in the full population (Philippat et al., 2018). The research on pesticides and ASD is growing but studies on pyrethroid pesticides and ASD are limited.

Findings for pyrethroid pesticide and child cognitive and behavioral outcomes are sparse. Pyrethroid biomarker concentrations from pregnancy urine samples have been associated with lower cognitive scores (Watkins et al., 2016), and increased behavioral difficulties (Viel et al., 2017) in early childhood. Detectable levels of pyrethroid biomarkers measured in prenatal urine were associated with a range of behavioral and executive functioning deficits (Furlong et al., 2017). Pyrethroids used on agricultural fields, in combination with other pesticides, near maternal residence was associated with lower IQs (Coker et al., 2017). In one study pregnancy air monitor samples of PBO (a synergist often in pyrethroid formulations) were associated with lower cognitive scores at 36 months (Horton et al., 2011). To our knowledge, prenatal pyrethroid metabolites have not yet been examined in relation to risk for ASD in the child.

Given the current state of knowledge on widespread exposures and growing use of pyrethroid pesticides and their neurotoxic properties, we investigated the effects of *in utero* pyrethroid pesticide exposure in relation to both children's diagnosis of ASD or non-typical development (non-TD) at 3 years. The study population consisted of mother-child pairs from the MARBLES (Markers of Autism Risk in Babies – Learning Early Signs) Study, a longitudinal cohort of younger siblings of children with ASD in northern California.

2. Materials and methods

2.1. Study Population

The present study included 201 mother-child pairs enrolled in the MARBLES Study from 2007 – 2014 with pesticide metabolite concentrations from urine samples collected during pregnancy and child neurodevelopmental assessments. The MARBLES Study enrolls pregnant women from northern California whose expected child or children have an elevated

risk for ASD. The vast majority of women had a previous child with ASD and a handful had a strong family history; these factors put the child they are expecting at higher risk to develop ASD Hertz-Picciotto et al., 2018). In this cohort, 23% of the children were diagnosed with ASD. Families were recruited from lists of children receiving services for ASD through the California Department of Developmental Services, from other studies at the University of California, Davis Medical Investigation of Neurodevelopmental Disorders (MIND) Institute, and from other referrals and self-referrals. Mothers were enrolled prior to or during pregnancy and were followed through pregnancy; infants were followed from birth to 3 years. Inclusion criteria for the full study were: (i) mother or father had one or more child(ren) with ASD and/or the gestating younger child had an older half-sibling or an equivalent or closer blood relative with ASD; (ii) mother was at least 18 years of age or older; (iii) mother was already pregnant or planning a pregnancy and biologically able to become pregnant; and (iv) mother lived within 2 hours of the Davis/Sacramento region at time of enrollment. The University of California, Davis (UCD) institutional review board approved the MARBLES Study and informed consent was obtained from each participant.

2.2. Child Neurodevelopmental Assessment

Participants are assessed at the MIND Institute by expert examiners at four ages, including 36 months, when a comprehensive diagnostic assessment is conducted. The Mullen Scales of Early Learning (MSEL), a norm-referenced developmental measure, is administered at each of these time points. Four subscales (visual reception, fine motor skills, receptive language, and expressive language) are combined into an overall composite score (Mullen, 1995). During the child's 3-year visit, expert clinicians evaluate children on the Autism Diagnostic Observation Scale (ADOS), the gold-standard diagnostic tool (Lord et al., 2008). Three categories of children's outcomes at 3 years are defined using an algorithmic approach previously developed by the Baby Siblings Research Consortium (Chawarska et al., 2014; Ozonoff et al., 2014), which takes into account scores on clinical assessments including the ADOS and MSEL. The first is ASD, defined by a child scoring at or above the ASD cutoff on the ADOS and meeting Diagnostic and Statistical Manual of Mental Disorders −5 (DSM-5) criteria for ASD. The second diagnostic category, non-typical development (non-TD), consists of those who have two or more MSEL subscale scores that are more than ≥ 1.5 standard deviation (SD) below the normative mean, or at least one MSEL subscale score that is more than 2 SD below the normative mean, and/or an elevated ADOS within 3 points of the ASD cutoff. The third category is typical development (TD), defined as a child who scores above 2.0 SD below the normative mean on all MSEL subscales, no more than one MSEL subscale that is between 1.5 and 2.0 SD below the normative mean; and an ADOS higher than 3 points below the ASD cutoff.

2.3. Exposure Data Collection and Analysis

During each trimester of pregnancy, participants were instructed to collect three first morning void (FMV) urine samples (one week apart) and one 24-hour urine sample. Samples were stored in home freezers and then collected during home visits and transported to UCD where they were then stored in −80° C freezers.

For analytical efficiency and cost-effectiveness, we pooled samples for participants with three or more samples in a trimester (Barkoski et al., 2018; Philippat et al., 2018; Shin et al., 2018). Gestational age was calculated using the date of the mothers' last menstrual period (LMP) and, when available, pregnancy ultrasound information from medical records. The 1st trimester included gestational ages from LMP through 13 weeks, the 2nd trimester included gestational ages 14 – 27 weeks, and the 3rd trimester included gestational ages 28 weeks to birth. For each trimester the first sample was analyzed individually and the remaining samples were pooled. For participants with 1 – 2 samples in a trimester each sample was analyzed individually. Nine mothers had all of their spot and 24hr samples analyzed individually as part of a longitudinal evaluation of pesticide concentrations and their variability. Details of the collection methods and pooling strategy are also outlined in Barkoski et al. 2018 and Shin et al. 2018.

We had fewer samples from the 1st trimester because on average participants completed their first study visit early in the 2nd trimester (gestational week 18). Therefore, we only analyzed samples from the 2nd and 3rd trimesters. A total of 1,030 primary samples were collected from the 2nd and 3rd trimesters. After pooling samples we had 638 urine samples, representing 201 mother-child pairs, analyzed for pesticide concentrations.

Specific gravity (SG) was measured from urine samples with a handheld refractometer (Atago Urine Specific Gravity Refractometer, PAL 10-S) at UCD. Distilled water was used to calibrate between each measurement. Then, urine samples were shipped overnight on dry ice to Emory University's Rollins School of Public Health where they were analyzed for pesticide metabolite 3-phenoxybenzoic acid (3-PBA). Chemical analyses of 3-PBA were conducted according to previously established methods (Olsson et al., 2004). Briefly, 2 mL urine samples were spiked with an internal standard mixture consisting of isotopically labeled 3-PBA, and incubated with β-glucuronidase/sulfatase to liberate conjugated metabolites. The hydrolysates were extracted using mixed-mode solid-phase extraction cartridges and elutes were concentrated and analyzed using high-performance liquid chromatography/tandem mass spectrometry with both quantification and confirmation ions monitored (Barr et al., 2010). Samples were also analyzed for trans-2,2-(dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (tDCCA), but over 35% of samples were <LOQ, and the correlation between tDCCA and 3-PBA was 0.88, and thus they were not included in the analysis.

2.4. Statistical Analyses

The goal of this project was to examine the relationship of quantified prenatal pyrethroid exposure with the child's risk for either ASD or non-TD, as compared with TD. All statistical analyses were performed with SAS statistical software version 9.4 (SAS, 2002–2012). Pesticide concentrations below the limit of detection (LOD) were assigned a value of LOD/√2 where LOD for 3-PBA was 0.1 ng/mL. To account for urine dilution, metabolite concentrations were SG corrected using the formula described in Hauser et al. $Pc = P \times [(SGp - 1)/(SG - 1)]$ (Hauser et al., 2004), where Pc is the SG-corrected pesticide metabolite concentration (ng/mL), P is the measured metabolite concentration in ng/mL, SG is the specific gravity of the urine sample, and SGp (1.012) is the median specific gravity

across all of the MARBLES Study samples. The SG-corrected 3-PBA concentrations were included in all analyses as the main exposure variable.

Because there are a varying number of urine samples per mother available for analysis, we calculated the average pesticide metabolite concentrations (C_{ave}) for each trimester. We analyzed the first sample individually, and all remaining samples from the trimester as a pooled sample. In order for the average trimester concentration to have equal contribution from each sample, we multiplied the concentration of the pooled sample by the number of samples in the pool prior to adding the value to the sample analyzed individually. The average pesticide metabolite concentration for each participant was calculated using the following formula: $C_{ave} = (C_{ind} + C_{pooled} \times N_{pooled})/(N_{ind} + N_{pooled})$, where C_{ind} is the pesticide metabolite concentration in the individual sample, C_{pooled} is the pesticide metabolite concentration in the pooled sample, N_{pooled} is the number of composites in the pooled sample, and N_{ind} is the number of the individual sample (=1). Due to a skewed distribution, average SG-corrected 3-PBA concentrations were natural log transformed.

Participants had variable compliance with the urine sample protocol and on average, participants collected 5 (Standard Deviation = 2.6) urine samples during the second and third trimesters. We used analytical weights to account for the unbalanced number of urine samples per pregnancy and the correlations among samples that were averaged together from each pregnancy to form that woman's average exposure, giving higher weights to more representative samples. These analytical weights reflected the "effective number" (Chih, 2011) of samples used to quantify a woman's average exposure for a given measurement: $weight_{ij} = n_i/(1 + (n_i - 1)ICC_j)$, where n_i is the number of individual urine samples that were pooled together for woman I (from a given trimester, if applicable) and ICC_j is the within-woman intraclass correlation coefficient for compound j. Intraclass correlation coefficients for each compound were estimated using compound-specific between-mother and within-mother variance component estimated from maximum-likelihood analysis of pooled-sample and spot-sample observations, using a user-written program in SAS PROC NLMIXED, accounting for the variable number of samples collected for each pregnancy. The ICC for 3-PBA from repeated spot and pooled urine samples was 0.16 and this value was used for analytical weights in regression models.

Potential confounders were selected based on the literature regarding associations of covariates with pyrethroid exposure and with child neurodevelopment. We used a Directed Acyclic Graph (DAG) to represent assumed causal relationships between the exposure, outcome and covariates (Supplemental Material, Fig. S1). Bivariate associations between natural log-transformed 3-PBA SG-corrected concentrations and demographic factors were assessed using Kruskal–Wallis tests of association for categorical and with linear regression for continuous demographic variables. Covariates were retained in the final multivariable regression model based on whether addition of the covariate to the model changed the exposure beta coefficient by 10% or more. The final models were adjusted for maternal pre-pregnancy BMI (continuous), homeownership (owner, non-owner), and self-reported prenatal vitamin use during the first month of pregnancy (yes, no). Child's birth year (continuous) also met criteria to be included. However, we also determined that there was a nonlinear association between year and natural log transformed 3-PBA concentrations based

on Akaike information criterion (AIC) and standard errors. Hence, in all adjusted regression models year was centered at 2010 and linear and squared centered-year terms were included as covariates. Finally, we also adjusted for organophosphate (OP) exposure using natural log transformed TCPy-SG corrected (chlorpyrifos metabolite) average pregnancy concentrations because pyrethroid and OP pesticides have been used interchangeably, a large body of evidence on prenatal OP pesticides, much of it based on chlorpyrifos, has demonstrated their associations with impairments in child neurodevelopment (Eskenazi et al., 2007; Hertz-Picciotto et al., 2018; Rauh et al., 2006), and chlorpyrifos was the most commonly and widely used OP pesticide in the U.S. during the period of these MARBLES pregnancies. Other covariates that were considered but did not meet the confounding criteria were maximum parental education (less than college, college degree, graduate or professional degree), mother's age at delivery (continuous years), child's race/ethnicity (white, non-white, Hispanic), mother's place of birth (US born, non-US born), parity (continuous), season of conception (warm, cold), and child's sex (male, female).

Multinomial logistic regression models were used to test the association between natural log-transformed urinary prenatal 3-PBA concentrations, overall and within the 2nd and 3rd trimesters, and child diagnosis at 3 years. We obtained crude and adjusted relative risk ratio (RRR) estimates from multinomial logistic regression models for ASD and non-TD where TD children served as the reference in all models. Analytical weights, described above, were applied to multinomial logistic regression models, using weight statements in SAS PROC SURVEYLOGISTIC (for multinomial logistic regression models).

3. Results

MARBLES study population characteristics for 201 mother-child pairs with prenatal 3-PBA concentrations, from 638 urine samples, and a diagnosis at 36 months of age are reported in Table 1. The number of urine samples collected in a trimester ranged from 1 – 6 samples and the majority of mothers (71%) collected 2 samples in a trimester. The majority of children were boys (60.2%) and slightly more than half of the participants were white (55.2%). Births included in this study ranged from years 2007 – 2014 with the most children born in 2009. Year-to-year fluctuations are to be expected in this type of study, as the pool of potential participants in small, and the study requires a significant time commitment. In the present study population, mothers' mean age at delivery was 34 years and the majority of parents were college educated (68.8%) or owned a home (62.1%).

Participants 3-PBA urinary concentrations were detectable in 97% of samples with the median of average pregnancy concentrations 2.06 ng/mL (Table 2). We compared 3-PBA concentrations in MARBLES participants, whose pregnancies were in years 2007–2014, to concentrations from a sample of women of reproductive age from NHANES 2009 – 2010. When compared to the NHANES sample, the MARBLES participants had the highest concentrations and greatest proportion above the LOD. We found that pregnancies with male births had higher 3-PBA concentrations.

Of the 201 mother-child pairs with pregnancy 3-PBA concentrations and the 3-year clinical diagnosis, seven had missing information on covariates, leaving a total of 194 participants

with complete information on exposure, 3-year clinical outcome, and covariates. Results of the clinical assessment were 41 children with ASD, 52 in the non-TD group, and 101 classified as TD.

Adjusted RRRs for the pregnancy average 3-PBA concentrations and for trimester-specific averages are reported in Table 3, with non-adjusted model results in the Supplemental Material (Table S1). The reported relative risk ratios represent relative changes in the outcome for a 1-unit change in the corresponding natural log-transformed prenatal 3-PBA concentration, equivalent to an increase in concentration of approximately 2.7-fold. For natural-log average pregnancy 3-PBA concentrations we observed an elevated relative risk ratio for ASD compared to TD (RRR: 1.34 (95% CI 0.89 to 2.03)) and a lower relative risk ratio for non-TD compared to TD (RRR: 0.78 (95% CI 0.48 to 1.28)). The RRR's for 2nd trimester 3-PBA concentrations in relation to ASD (RRR: 1.50 (95% CI 0.89 to 2.51) p=0.12) and non-TD development (RRR: 1.17 (95% CI 0.69 to 1.98) p=0.56) were greater than estimates for 3rd trimester exposures or the combined pregnancy exposures.

4. Discussion

In the present study, greater *in utero* 3-PBA concentrations from 2nd trimester urine samples were associated with a modest increase (50%) in risk for ASD at 3-years, relative to their risk for TD. Previously published studies analyzing pyrethroids in association with ASD only considered exposures from agricultural pesticide use. In a record-linkage study of births in the California central valley from 1996–1998 and agricultural pesticide applications near the residence at birth, Roberts et al. found an association of ASD with prenatal exposures to bifenthrin, a pyrethroid (Roberts et al., 2007). Bifenthrin is the most stable pyrethroid, with an exceptionally long half-life outdoors, making it one of the most potent pyrethroid pesticides on the market (Cao et al., 2014). However, bifenthrin does not metabolize to 3-PBA and we did not assess a bifenthrin specific metabolite. In a similar study, von Ehrenstein et al. analyzed a wide array of pesticides in California births in the San Joaquin Valley from 1998–2010; nearby prenatal agricultural applications of permethrin, but not bifenthrin, were associated with a slight increase in ASD risk (OR=1.10, 95% CI=(1.01, 1.20)) (von Ehrenstein et al., 2019). Both of these studies relied on the database from California Pesticide Use Reports, (PUR) which undergoes extensive quality control (California Department of Pesticide Regulation, 2020), and have been validated by a high correlation with airborne concentrations at distances similar to the buffers used by these studies (Wofford et al 2014). Although these studies did not use biomarkers of pyrethroid exposure, the PUR data capture exposures over time, and in agricultural communities, which may represent the predominant pesticide exposures. Our study catchment area included some participants from agricultural communities, but also encompassed several metropolitan areas.

There are few published studies investigating prenatal pyrethroid exposure in relation to child cognitive development, and thus far the findings are inconsistent. In the ELEMENT study from Mexico City, Mexico, researchers reported a marginal association between prenatal 3-PBA concentrations and Bayley Scales of Infant Development (BSID) Mental Development Index (MDI) scores at 24 months but not at 36 months. As compared with the

lowest exposure category, participants in the medium and high 3-PBA categories had reduced MDI scores at 24 months (Watkins et al., 2016).

A study from California's Salinas Valley investigated residential proximity to agricultural pesticide use and child intelligence quotient (IQ), as evaluated on the WISC, at 7 years. In this study Gunier et al. observed lower IQ scores for children whose mothers were living within 1 km of agricultural pyrethroid applications during pregnancy. Children had significantly lower full scale IQ (β: −2 (95% CI −3.7 to −0.3)), perceptual reasoning (β: −2.1 (95% CI −4.0 to −0.2)), and verbal comprehension (β: −1.8 (95% CI −3.4 to −0.3)) scores (Gunier et al., 2017). Agricultural applications would be a major source of pesticide exposure in the Salinas Valley because participants lived in an agricultural community.

Pyrethroid pesticide exposures have been studied in relation to child behaviors and cognition in the PELAGIE study, a prospective cohort study from France. Prenatal pyrethroid exposures, as measured in a single pregnancy urine sample, were not significantly associated with child cognitive development at 6 years (Viel et al., 2015). Prenatal *cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropant carboxylic acid (DCCA) concentrations were associated with internalizing behavioral difficulties at 6 years (Viel et al., 2017). In this French population *trans*-DCCA, which is a metabolite of permethrin and cypermethrin, was commonly detected. However, the majority of urine samples were below LOD for 3-PBA indicating this population of French mothers are exposed to different types of pyrethroids and possibly have different exposure routes compared to the US population. Detection limits for 3-PBA were lower in the PELAGIE cohort with LOD = 0.008 ng/mL compared to 0.1 ng/mL in the present study. Prenatal concentrations of 3-PBA were categorized due to the large number of samples below the LOD in the PELAGIE Study, and were not associated with childhood behaviors, despite higher proportions detected and significant associations between childhood 3-BPA and increased odds of behavioral disorders for the externalizing and reverse-scored prosocial behavior subscales. A variety of behavioral and executive functioning deficits were also found to be related to whether pyrethroid metabolites were detected prenatally in a study based in New York (Furlong et al., 2017).

Urinary pesticide concentrations from the MARBLES study population were higher than have previously been reported and the majority of samples in the MARBLES population were above LOD. This allowed us to investigate associations with a continuous term for 3-PBA exposures. Higher concentrations detected in MARBLES samples likely reflects growing use of pyrethroids since the 2001 EPA restriction of chlorpyrifos.

The use of pooled samples in the present study reduced exposure misclassification that can occur when participants are assigned to an exposure category based on urinary concentrations from a single spot sample (Barkoski et al., 2018). We reported an ICC = 0.16 for 3-PBA from multiple pregnancy urine samples indicating greater within- than between-person variability showing that a single urine sample from pregnancy may not capture long-term exposure for pyrethroids due to their quick metabolism. Participants in the present study could be exposed from residential and dietary sources with the latter contributing more variability in exposure. Since the restriction of chlorpyrifos, pyrethroids are commonly used

in residential pesticide products making home sources a primary exposure route (Atwood, 2017; Lu et al., 2006b; U.S. EPA, 2013).

Nevertheless, there are limitations with urinary pesticide metabolites. Even though we collected multiple urine samples from each trimester (1 – 6 samples), we still may have failed to capture exposures due to the rapid metabolism (about 7 hours) of these compounds (Ratelle et al., 2015). The literature on prenatal pyrethroid exposures and child neurodevelopment is inconsistent, which in part could be due to exposure misclassification in previous studies, which used a single spot urine sample to assess exposure. Published studies reporting associations for prenatal pesticide exposure and ASD have used agricultural pesticide applications as a proxy for pesticide exposure. The present study used a biomarker for pesticide exposures, which captures all exposure routes. However due to the rapid metabolism of pesticides, a urinary biomarker may fail to capture exposure over long periods. Pyrethroid metabolite 3-PBA is a valid biomarker for 6 common pyrethroid pesticides in humans but it may also reflect exposures to the metabolite in the environment. A potentially relevant finding was the detection of 3-PBA in house dust samples collected with floor wipes from homes in northern California (Trunnelle et al., 2014), indicating that metabolites from home applications may linger. Overall, we expect that the resulting errors in our use of urinary metabolites at various intervals throughout pregnancy would tend to be non-differential, particularly since the collection of urine samples occurred before the child was born and years before the diagnoses were made. In this circumstance, the bias in results would on average be towards the null and could increase the uncertainty, reflected in the confidence intervals, but bias away from the null is also possible.

Given the overall small sample size, the number of children affected by ASD or non-TD outcomes was only a few dozen of each. Precision is somewhat low, as reflected in the width of the confidence intervals. A larger sample would increase statistical power to detect smaller effects that would still have public health relevance and tighter confidence intervals.

In comparison with prior studies of pyrethroids, especially those examining ASD, we considered and adjusted for a wider array of potential risk and protective factors previously associated with ASD or neurodevelopmental outcomes in other studies. Nevertheless, bias from unmeasured, unknown, and/or omitted confounders cannot be ruled out. Another potential limitation was the lack of 1st trimester or postnatal urinary 3-PBA concentrations. Shelton et al. observed associations between pyrethroid exposures during both the 1st and 3rd trimester and increased risk for ASD (Shelton et al., 2014b); von Ehrenstein found associations with pyrethroid exposures in the first year of life (von Ehrenstein et al., 2019). We had fewer participants with urine samples from the 1st trimester therefore the present study focused on exposures from the latter two trimesters of pregnancy. Unique developmental processes in the brain occur in the early first trimester, beginning with the demethylation of both the maternal and paternal DNA, and when structures such as the neural tube form, migrational pathways are established, and even preconceptional exposures may influence the central nervous system. Nevertheless, later gestational and postnatal exposures can also have long-lasting effects on brain development.

Finally, the MARBLES Study population differs from the general population because of the design: the familial/sibling recurrence in this study was 23%, and despite a shared environment with their older affected sibling, many of the MARBLES children likely have an increased genetic susceptibility for ASD. Our results therefore may not be generalizable to populations with lower risk. In a population with a high genetic load, it is difficult to predict whether environmental contributions to ASD will be amplified (e.g., as a result of gene-by-environment interactions) or may be secondary to, or dwarfed by, strong genetic susceptibility that would confer higher background risk for ASD.

Major strengths of this study include exposure measurements from multiple biological specimens during pregnancy, blinding of laboratory personnel to the child outcomes, the high quality clinical data stemming from administration of child developmental assessments by highly trained clinicians with research reliability, and collection and adjustment for a number of potential risk/protective covariates, including sociodemographic information, that reduced confounding and potential selection bias. Additionally, the majority of urine samples were above the LOD. This allowed us to assess linear relationships between continuous measures for the exposure and outcomes. Maternal urine samples were pooled within a trimester to reduce analytical costs and to improve exposure assessment. We have previously reported that pooled samples can reduce exposure misclassification in this population (Barkoski et al., 2018).

This may be the first study to assess a biomarker of pyrethroid exposure, based on up to 6 urine samples from both the 2nd and 3rd trimesters during pregnancy, in relation to clinical ASD diagnosis at 3-years. Larger studies in general population cohorts with repeated urinary measures starting earlier in pregnancy and that consider gene-environment interactions should be conducted to clarify the potential for adverse neurodevelopmental effects of pyrethroid pesticides.

5. Conclusions

Exposures to pyrethroid pesticides *in utero* were evaluated as 3-PBA concentrations from multiple samples collected during the 2nd and 3rd trimesters in a cohort at high risk for developing ASD, the MARBLES Study, due to a sibling with ASD. During the 2nd trimester, a higher concentration of urinary 3-PBA was associated with a modest increased risk for ASD at 3-years, but these results are not definitive, in part due to the relatively small number of cases. Our findings do indicate exposures to pyrethroid pesticides are common among pregnant women in northern California, Given the growing use of these pesticides, determining whether pregnancy exposures pose a risk to the developing brain, and if so, at what levels, is increasingly critical. In the interests of healthy development for future children, studies of pesticides with accurate exposure ascertainment and larger sample sizes are needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- First study of pregnancy pyrethroid pesticide biomarker and risk of ASD at 3-years.
- Higher concentration of urinary 3-PBA associated with modest increased risk for ASD.
- Pesticide exposure characterization difficult due to temporal variability and multiple sources.

Table 1:

Characteristics of mother-child pairs from the Markers of Autism Risks in Babies – Learning - Early Signs (MARBLES) cohort with pesticide biomarker concentrations from urine samples collected during pregnancy and child neurodevelopmental assessments (n=201, 2007 – 2014.

Participant Characteristics	N	%	ASD	Non-TD	TD
Child's sex					
Boys	121	60.2	33 (73.3%)	34 (63.0%)	54 (52.9%)
Girls	80	39.8	12 (26.7%)	20 (37.0%)	48 (47.1%)
Birth year					
2007 –2008	48	23.9	4 (8.9%)	11(20.4%)	33 (32.4%)
2009	48	23.9	11 (24.4%)	13 (24.1%)	24 (23.5%)
2010 – 2011	50	24.9	13 (31.1%)	12 (22.2%)	24 (23.5)
2012 – 2014	55	27.4	16 (35.6)	18 (33.3%)	21 (20.5%)
Child's race/ethnicity					
White	111	55.2	21 (46.7%)	27 (50.0%)	63 (61.8%)
Non-white (Black or multiracial)	31	15.4	8 (17.8%)	11 (20.4%)	12 (11.8%)
Hispanic	59	29.4	16 (35.6%)	16 (29.6%)	27 (26.5%)
Gestational age at delivery (weeks)					
≤ 37 weeks	20	10.0	2 (4.4%)	4 (7.4%)	14 (13.7%)
> 37 weeks	181	90.0	43 (95.6%)	50 (93.6%)	88 (86.3%)
Parity					
1 *	83	43.0	14 (33.3%)	22 (42.3%)	47 (47.5%)
> 1	110	57.0	28 (66.7%)	30 (57.7%)	52 (52.5%)
Season of birth (months)					
Cold	100	49.8	20 (44.4%)	24 (44.4%)	56 (54.9%)
Warm	101	50.3	25 (55.6%)	30 (55.6%)	46 (45.1%)
Maternal pre-pregnancy BMI					
Normal/ underweight	106	53.0	20 (45.5%)	27 (50.0%)	59 (57.8%)
Overweight	49	24.5	13 (29.6%)	13 (24.1%)	23 (22.6%)
Obese	45	22.5	11 (25.0%)	14 (25.9%)	20 (19.6%)
Mom's age at delivery					
< 35 years	105	52.2	19 (42.2%)	29 (53.7%)	57 (55.9%)
≥ 35 years	96	47.8	26 (57.8%)	25 (46.3%)	45 (44.1%)
Prenatal vitamin use					
Yes	109	55.9	15 (35.7%)	30 (57.7%)	64 (63.4%)
No	86	44.1	27 (64.3%)	22 (42.3%)	37 (36.6%)
Parental education					
Less than college	62	31.2	13 (30.2%)	20 (37.0%)	29 (28.4%)
Bachelor degree	89	44.7	21 (48.8%)	21 (38.9%)	47 (46.1%)
Graduate or professional degree	48	24.1	9 (20.9%)	13 (24.1%)	26 (25.5%)
Homeowner					
Yes	121	62.1	23 (54.8%)	31 (59.6%)	67 (66.3%)

Participant Characteristics	N	%	ASD	Non-TD	TD
No	74	37.9	19 (45.2%)	21 (40.4%)	34 (33.7%)

* Two children had parity equal to zero, and were high risk due to the parents having siblings with ASD. ASD=autism spectrum disorder, non-TD= non-typical development, TD= typical development

Table 2.

Distribution of 3-PBA concentrations (ng/mL) measured from urine samples collected during pregnancy from 201 subjects from the MARBLES cohort, 2007 – 2014, and comparison to NHANES pesticide metabolite concentrations (ng/mL) from adult women 2009 – 2010.

				Selected Percentiles			
			N	25th	50th	75th	95th
MARBLES Study							
Pregnancy[1]							
	by sample	ng/mL - SG	638[2]	0.84	1.46	2.73	10.53
	by participant	ng/mL - SG	201[3]	1.25	2.06	3.50	9.96
2nd trimester							
	by sample	ng/mL - SG	270[2]	0.81	1.45	2.54	10.53
	by participant	ng/mL - SG	134[3]	1.00	1.81	3.04	9.05
3rd trimester							
	by sample	ng/mL - SG	368[2]	0.85	1.46	2.86	10.29
	by participant	ng/mL - SG	190[3]	1.13	1.93	3.55	13.22
NHANES							
Adult women (2009 – 2010)		ng/mL	1392	NR	0.4	1.06	6.5

CI= Confidence Interval, SG=specific gravity, 3-PBA=3 Phenoxybenzoic acid, NR=not reported,

[1]Samples from the 2nd and 3rd trimesters

[2]Total samples,

[3]Average 3-PBA concentration among women included in the present study

Table 3:

Adjusted multinomial logistic regression analysis assessing natural log-transformed prenatal 3-PBA concentrations overall and within the 2nd and 3rd trimester, in relation to children's 3-year diagnosis (n=194).

		ASD[1]			Non-Typical[1]		
Timing	**N**	**RRR**	**95% CI**	***p*-value**	**RRR**	**95% CI**	***p*-value**
Pregnancy average (2nd and 3rd trimesters)	194	1.34	(0.89 to 2.03)	0.16	0.78	(0.48 to 1.28)	0.33
2nd trimester	133	1.50	(0.89 to 2.51)	0.12	1.17	(0.69 to 1.98)	0.56
3rd trimester	183	1.31	(0.85 to 2.02)	0.23	0.77	(0.49 to 1.20)	0.25

[1]Typically developing children served as the reference, models adjusted for pre-pregnancy BMI, birth year, birth year squared, homeownership, prenatal vitamin use, average TCPy, RRR=Relative Risk Ratio, CI=Confidence Interval, ASD=autism spectrum disorder

Note: The reported relative risk ratios pertain to a 1-unit change in the corresponding natural log-transformed prenatal 3-PBA concentration, equivalent to an increased concentration of approximately 2.7-fold.