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## **Prenatal exposure to organophosphate pesticides and risk of autism spectrum disorders and other non-typical development at 3 years in a high-risk cohort**

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1 **Prenatal exposure to organophosphate pesticides and risk of autism spectrum disorders**  
2 **and other non-typical development at 3 years in a high-risk cohort**

3  
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41 Abbreviations

42 ADI-R: Autism Diagnostic Interview-Revised

43 ADOS: Autism Diagnostic Observation Schedule,

- 44 BMI: Body Mass Index
- 45 DEP: diethylphosphate
- 46 DETP: diethylthiophosphate
- 47 DEDTP: diethyldithiophosphate
- 48 DMP: dimethylphosphate
- 49 DMTP: dimethylthiophosphate
- 50 DMDTP: dimethyldithiophosphate
- 51 OP: Organophosphate pesticides
- 52 SCQ: Social Communication Questionnaire
- 53  $\Sigma$ DAP: molar sum of DEP, DETP, DMP, DMTP and DMDTP
- 54  $\Sigma$ DEP: molar sum of DEP and DETP
- 55  $\Sigma$ DMP: molar sum of DMP, DMTP, and DMDTP
- 56
- 57

58 **Abstract**

59 Introduction: Organophosphates are widely used pesticides that have been show to affect child  
60 neurodevelopment. Previous studies that explored their potential effects on Autism Spectrum  
61 Disorder (ASD) either relied on proxies of external exposure or on questionnaires completed by  
62 the parents to identify autism-like behaviors but do not provide a clinical diagnosis of ASD.

63 Aims: We studied the associations between prenatal biologic markers for exposure to  
64 organophosphate pesticides and the risk of having a child with ASD or non-typical development  
65 (NTD).

66 Method: We analyzed 203 mother-child pairs of the ongoing MARBLES (*Markers of Autism*  
67 *Risk in Babies – Learning Early Signs*) mother-child cohort, which enrolls mothers who are  
68 either pregnant or planning a pregnancy and having an elevated risk for the expected child to  
69 develop ASD. Seven metabolites of organophosphate pesticides were assessed in repeated urine  
70 samples collected during pregnancy. At 36 months, children were assessed with measures of  
71 cognitive function and adaptive behaviors, and with two gold-standard diagnostic instruments for  
72 ASD: the Autism Diagnostic Observation Schedule and the Autism Diagnostic Interview-  
73 Revised. Children were classified in one of the following groups: ASD (n = 46), non-typical  
74 development (NTD, n = 55) and typically developing (TD, n = 102).

75 Results: After adjustment for potential confounders, organophosphate metabolite concentrations  
76 were not associated with an increased risk of ASD or NTD when boys and girls were studied  
77 together. After stratification by sex, dimethylthiophosphate (DMTP) pregnancy concentration  
78 tended to be associated with an increased ASD risk among girls (OR for a doubling in the DMTP  
79 concentration: 1.64 (95%CI, 0.95; 2.82)) but not among boys (OR: 0.84, 95%CI: 0.63; 1.11).

80 Discussion: This is the first study of clinically confirmed diagnoses of ASD that utilized repeated  
81 measurements of organophosphate metabolites during pregnancy to explore the associations  
82 between these pesticides and ASD risk in children. The association we observed among girls, as  
83 well as the lack of association in boys, need to be replicated in further studies with similar design  
84 and larger sample size. In light of the higher baseline risk for ASD in this cohort, generalizability  
85 to children lacking a first degree relative affected by ASD is unknown.

86 Keywords: Autism spectrum disorder, Biomarkers, Developmental concerns, Prenatal exposure,  
87 Prospective cohort, Organophosphate pesticides

88

89

90 **Introduction**

91 Organophosphate (OP) pesticides are a class of insecticides widely used throughout the world.  
92 While they were phased out for most residential uses by the U.S. Environmental Protection  
93 Agency in the early 2000s (Clune et al. 2012), they are still applied in agriculture for insect  
94 control on food crops (Shelton et al. 2012), on golf courses and some other uses. In the general  
95 population, exposure to OP mainly occurs through inhalation from agricultural spray drift and  
96 ingestion of residues on food products. An interventional study in 23 children showed that  
97 replacing their conventional diet with organic food items for 5 consecutive days led to significant  
98 reduction in urinary OP pesticide metabolite concentrations (Lu et al. 2006). OP pesticides were  
99 initially developed as nerve gases for chemical warfare and then adapted for insect control at  
100 lower doses. They affect mammalian and insect nervous systems by irreversibly inhibiting the  
101 enzyme acetyl cholinesterase (AChE) that breaks down the neurotransmitter acetylcholine.  
102 Inhibition of AChE leads to accumulation of acetylcholine in the synapses, that can result in  
103 acute neurologic symptoms. In addition to high-dose effects on AChE inhibition, toxicological  
104 studies have suggested that OP pesticides can down-regulate serotonin receptors, induce  
105 oxidative stress and alter calcium and potassium homeostasis (reviewed by Shelton et al, 2012  
106 (Shelton et al. 2012)). In addition, a magnetic resonance imaging study in 40 children aged 5.9–  
107 11.2 years found brain morphological changes in the group with high cord blood concentrations  
108 of chlorpyrifos (Rauh et al. 2012).

109

110 Regarding the effects of OP pesticides on child neurodevelopment, epidemiological studies that  
111 measured the parent compounds in blood or their metabolites in urine reported associations with  
112 abnormal reflexes in neonates (Engel et al. 2007; Young et al. 2005; Zhang et al. 2014), poorer

113 mental development in toddlers assessed with the Bayley scales of early learning (Eskenazi et al.  
114 2007)), and poorer working memory and intellectual quotient in children (Bouchard et al. 2011;  
115 Horton et al. 2012; Rauh et al. 2011). Only a few studies have looked at Autism Spectrum  
116 Disorder (ASD), a neurodevelopmental disorder characterized by impairments in social  
117 interactions and communication and a pattern of stereotyped behaviors or sensory sensitivities,  
118 for which increased prevalence has been observed in the past decades in the United States  
119 (Centers for Disease Control and Prevention 2012). One study did not report an association  
120 (Millenson et al. 2017) while the five others reported increased risk of an ASD diagnosis or ASD  
121 like symptoms in association with OP pesticide exposure during pregnancy (Eskenazi et al. 2007;  
122 Furlong et al. 2014; Rauh et al. 2006; Roberts et al. 2007; Shelton et al. 2014). These studies on  
123 ASD were limited by the fact that they relied on 1) mandated reports of commercial pesticide use  
124 near the houses of the participants as a surrogate of exposure (Roberts et al. 2007; Shelton et al.  
125 2014) which does not take into account exposures occurring through other sources like food; or  
126 2) by the use of questionnaires completed by the parents to identify autism-like behaviors but do  
127 not provide a clinical diagnosis of ASD (Eskenazi et al. 2007; Furlong et al. 2014; Millenson et  
128 al. 2017; Rauh et al. 2006).

129 Our aim was to study prenatal exposure to OP pesticides in relation to children's diagnosis of  
130 ASD and non-typical development (NTD). We relied on the MARBLES prospective cohort,  
131 which obtained repeated urine samples during pregnancy to assess OP exposure and confirmed  
132 all diagnoses at 3 years using diagnostic gold-standard assessments for ASD.

## 133 **Methods**

### 134 *Population*

135 We analyzed the ongoing MARBLES cohort that started in 2006 and enrolls women at high risk  
136 for having a child with ASD. Selection criteria are 1) having a biological child diagnosed with  
137 ASD and so being at elevated risk to have another child with this disorder (close to 20%  
138 (Ozonoff et al. 2011) compared to about 1.5% in the general population); 2) being pregnant or  
139 planning a pregnancy and being biologically able to become pregnant; 3) living within an  
140 approximate 2 hour drive to the UC Davis MIND (Medical Investigation of Neurodevelopmental  
141 Disorders) Institute clinic; 4) being at least 18 years old. A few women (n = 2) did not match the  
142 first criteria of inclusion but were enrolled since they were at high risk of having a child with  
143 ASD (e.g., mother had an identical twin with an ASD child). Eligible families are identified  
144 using the list of families receiving state-funded services for a child with ASD, provided to us by  
145 the California Department of Developmental Services. Some families are also referred by other  
146 research studies at the UC Davis MIND Institute or by health and service providers, or learn  
147 about the study at outreach events. To confirm the ASD diagnosis of the older sibling, the study  
148 psychologist requests the record of an ASD diagnosis, i.e., an evaluation made by a psychologist  
149 using the ADOS (Autism Diagnostic Observation Schedule, (Lord et al. 2000)). If neither the  
150 parent nor the clinician provides such a record, then the study psychologist administers the  
151 ADOS to the child and the Social Communication Questionnaire (SCQ) to the mother (Rutter et  
152 al. 2003). Based on these assessments, the older sibling is either deemed to meet criteria for  
153 ASD, in which case the mother is enrolled, or not, in which case the mother is ineligible.

154

155 For the current study, we included all active MARBLES participants who were born between  
156 2006 and March 2014, for whom pregnancy urine samples were available and who did not drop  
157 out before the examination of the child at 3 years of age. A comparison of mother-child pairs



158 included in this analysis versus those not included is provided in the Supplemental Material,  
159 Table S3. Those not included lacked urine samples, dropped out before the 3-year visit or had an  
160 incomplete exam at 3 years. The MARBLES study has been approved by the institutional review  
161 boards for the State of California and the University of California Davis. The parents of all  
162 participants signed an informed consent before being enrolled in the study.

### 163 *Collection of urine samples*

164 Beginning at enrollment and throughout pregnancy, mothers of the MARBLES study were asked  
165 to collect for each trimester up to three first morning void urine samples (referred as spot  
166 samples in the manuscript) and one 24-hour urine sample collected on a different day than the  
167 spot samples. Mothers collected spot samples, each one week apart, and then stored these spot  
168 urine samples in their home freezer until collection by study staff during a home visit. The 24-  
169 hour urine samples included all urine voids for a 24-hour period starting at 8 am the day before  
170 the study staff made a home visit. Participants used collection hats that were emptied into the 24-  
171 hour container after each void. Twenty-four hour samples were stored in the mother's home  
172 refrigerator. The MARBLES Study personnel pick up urine samples during home visits and  
173 transported them to UC Davis for storage at -80°C. Urine samples were then thawed and  
174 aliquoted as follows: for a given trimester if the woman collected only one or two urine samples  
175 (spot or 24 hour sample) both samples were analyzed individually (not pooled). If three or more  
176 samples (spots and 24 hour samples) were available, generally the first sample of that trimester  
177 was analyzed as an individual sample and the remaining samples were pooled together (pooled  
178 within-trimester, within-subject). Pooling enabled us to take advantage of the repeated  
179 measurements per subject to reduce exposure measurement error while keeping the total cost of  
180 OP pesticide metabolite measurements reasonable..

181

182 *Assessments of OP metabolites*

183 In the present study, urine samples (pools, spots and 24-hour) from the 2<sup>nd</sup> and 3<sup>rd</sup> trimesters of  
184 pregnancy were shipped overnight on dry ice to Emory University's Rollins School of Public  
185 Health (RSPH) for OP metabolite quantification. Specific gravity was measured on all samples  
186 (spot, pools, 24-hour) prior to shipment. Dimethylphosphate (DMP), diethylphosphate (DEP),  
187 dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMDTP), diethylthiophosphate  
188 (DETP) and diethyldithiophosphate (DEDTP) along with 3,5,6-trichloro-2-pyridinol (TCPy), a  
189 specific metabolite of chlorpyrifos and chlorpyrifos-methyl, were measured using either gas-  
190 chromatography or high-performance liquid chromatography coupled with tandem mass  
191 spectrometry with both quantification and confirmation ions monitored. Full details on the  
192 methods have been previously published (Olsson et al. 2004; Prapamontol et al. 2014). DEDTP  
193 was not detected in any of the urine samples and was not included in our analysis.

194 *Assessment of child neurodevelopment and diagnostic outcome*

195 Children were assessed at 3 years of age using the ADOS, a semi-structured interview during  
196 which the clinician observes the child's social interaction, communication, play and imaginative  
197 use of materials (Lord et al. 2000), and the Mullen Scales of Early Learning (MSEL), using the  
198 four subscales that measure fine motor, visual reception, expressive and receptive language  
199 (Mullen 1995). In addition, the parents completed the SCQ to screen for symptoms of autism  
200 spectrum disorder (Berument et al. 1999). Parents of children demonstrating signs of ASD on  
201 either the ADOS or SCQ were additionally interviewed with the Autism Diagnostic Interview-  
202 Revised (ADI-R), a standardized instrument that probes for ASD symptoms throughout the  
203 child's life. Using their ADOS and MSEL scores, we classified the children in one of the

204 following groups: ASD (n = 46), non-typical development (NTD, n = 55) and typically  
205 developing (TD, n = 102). Criteria of inclusion in each group are detailed in Supplemental  
206 Material, Table S2.

### 207 *Statistical analysis*

208 We performed multiple imputation using the *mi impute chained* command in Stata 14 (White et  
209 al. 2011) to impute OP concentrations that were below the limit of detection. Imputation models  
210 included the OP concentrations along with the following variables: home owner vs. renter status,  
211 season and date of birth, maternal height and weight, maternal age and specific gravity. We  
212 restricted the imputed values to be in the following range:  $\geq 0$  and  $< \text{LOD}$ . OP pesticide  
213 metabolite concentrations were then standardized for specific gravity (sg) using the following  
214 formulae:  $C_{\text{SG}} = C \times [(\text{SG}_{\text{mean}} - 1)/(\text{SG} - 1)]$ , where  $C_{\text{SG}}$  is the specific gravity corrected  
215 biomarker concentration,  $\text{SG}_{\text{mean}}$  is the arithmetic mean of the specific gravity in our study  
216 population, and  $C$  is the measured OP concentration. We computed the following sum of the  
217 molar concentrations:  $\sum\text{DEP}$  (molar sum of the diethylphosphate metabolites: DEP and DETP),  
218  $\sum\text{DMP}$  (molar sum of the dimethylphosphate metabolites: DMP, DMTP, DMDTP) and  $\sum\text{DAP}$   
219 (molar sum of DEP, DETP, DMP, DMTP and DMDTP). Some organophosphorus insecticides  
220 such as Malathion can be metabolized into the three DAP included in  $\sum\text{DMP}$  or  $\sum\text{DEP}$ .  
221 However, degradation of others such as Naled and Diazinon only lead to one or two DAPs  
222 (Supplementary Material, Table S6). For these compounds, it might be relevant to study  
223 individual metabolite in addition to the molar sums that include some of their metabolites but  
224 also metabolites that are not related to them. For this reason, in this manuscript we also presented  
225 results for individual OP metabolites that were detected in more than 60% of the samples  
226 (namely DEP, DMTP).

227 We fit adjusted multinomial logistic regression models predicting the odds of ASD and NTD,  
228 each relative to TD. We used the mean of the log<sub>2</sub>-transformed sg-standardized biomarker  
229 concentrations as a proxy of the pregnancy exposure. In addition to studying the mean of the OP  
230 concentrations over the 2<sup>nd</sup> and 3<sup>rd</sup> trimester we also computed the average OP concentration in  
231 mid (corresponded to the 2<sup>nd</sup> trimester) and late pregnancy (3<sup>rd</sup> trimester).

232 Compliance to the urine sampling protocol was variable and on average, women collected 5  
233 (Standard Déviation = 2.6) urine samples (spot and / or 24-hour) over the second and third  
234 trimesters (Supplemental Material, Table S1). To take into account the unbalanced number of  
235 urine samples per participant and the correlations among samples that were averaged together  
236 from the same woman to form that woman's (possibly trimester-specific) average exposure, we  
237 used analytical weights. Weights reflected the "effective number" (Ching-Ping 2011) of samples  
238 used to quantify a given woman's average exposure for a given measurement:  $weight_{ij} = n_i / (1 +$   
239  $(n_i - 1) ICC_j)$ , where  $n_i$  is the number of individual urine samples that were pooled together for  
240 woman  $i$  (from a given trimester, if applicable) and  $ICC_j$  is the within-woman intraclass  
241 correlation coefficient for compound  $j$  measurements from individual urine samples. Intraclass  
242 correlation coefficients for each compound were estimated using compound-specific between-  
243 mother and within-mother variance component estimates (for hypothetical individual urine  
244 sample measurements) from maximum-likelihood analysis of pooled-sample and spot-sample  
245 observations, using a user-written program in SAS PROC NLMIXED that accounted for the  
246 variable number of samples in each of these observations. The estimated ICCs were 0.09 for  
247 TCPy; 0.12 for DMTP; 0.19 for DEP and  $\sum DMP$ ; and 0.18 for  $\sum DMP$  and  $\sum DAP$ .

248 Adjustment factors were chosen a priori based on a directed acyclic graph constructed using the  
249 published literature on risk factors for ASD (Supplemental Material, Figure S1). We adjusted for

250 the minimal sufficient set given by the Dagitty software (Textor et al. 2011). The set included  
251 socio-economic status, maternal body mass index (BMI) before pregnancy (continuous), season  
252 (categorical) and date of birth (continuous). Adjustment for date of birth was necessary because  
253 of the opposing time trends observed for ASD diagnosis and OP concentrations in our study  
254 population: 67% of the ASD children were indeed born after 2010 compared to 45% of the TD  
255 children (Table 1) while OP concentrations tended to decrease with time (Figure 1). As a  
256 measure of socio-economic status we used home ownership status (yes / no) rather than parental  
257 education since the number of parents of ASD children in the lowest education group “*high*  
258 *school, some college (no degree)*” was small (N = 6). In sensitivity analysis, we ran additional  
259 models adjusted for 1) the use of prenatal vitamins during the first month of pregnancy, 2)  
260 pyrethroid exposure estimated using the average concentrations of 3-phenoxybenzoic acid, a  
261 general metabolite of pyrethroid pesticides, also measured in the repeated urine samples  
262 collected during pregnancy, 3) gestational age (a potential mediator of the effects of OP on ASD  
263 and NTD), 4) maternal age, 5) paternal age. To investigate the impact of extreme values we also  
264 performed an analysis in which we removed those mothers in the top 2.5 percent of the OP  
265 pesticide urinary concentration. We explored potential sex specific effects by adding an  
266 interaction term between OP concentrations and sex in the regression models and by performing  
267 stratified analysis. Finally, we excluded 6 twins (3 pairs) and performed an analysis restricted to  
268 singleton births.

269 All analyses were performed using STATA/SE (College station, TX 77845, version 14) and SAS  
270 (Institute Inc. 2016. Sas 9.4. Cary, NC).

## 271 **Results**

### 272 *Study Population Demographics*

273 Among the 203 women included, 74 were recruited during their first trimester of pregnancy, 71  
274 during the second and 58 during their third trimester. The male to female ratio was 1.1, 2.5 and  
275 1.8 among TD, ASD and NTD, respectively. TD children tended to be born in the earlier years of  
276 the study than ASD and NTD children (Table 1), and included a higher proportion of white non-  
277 Hispanics (52%) compared to ASD and NTD (about 35%, Table 1). In the TD groups, 66% of  
278 the parents owned their home compared to 50% in the ASD group (p-value for Pearson's chi-  
279 squared test = 0.15). MARBLES mothers with missing outcomes or exposure tended to have a  
280 lower socio-economic status (a higher percentage of their parents did not own their house) and to  
281 have daughters, as compared with those included in our analyses (Supplemental Material, Table  
282 S3).

### 283 *OP concentrations in maternal urine*

284 TCPy, DMTP and DEP were detected in most of the urine samples with frequency of detection  
285 ranging from 89% for DEP to 95% for TCPy. The highest median was observed for TCPy (2.6  
286 µg/L) followed by DMTP (1.8 µg/L) and DEP (0.9 µg/L) (Table 2).

### 287 *Associations between the average pregnancy exposure and child diagnosis*

288 After adjustment for home owner status, maternal BMI before pregnancy, season and date of  
289 birth, the multinomial ORs for the associations between OP pesticide metabolites and the risk for  
290 the child developing ASD or NTD were below one and ranged between 0.80 (95%CI: 0.57; 1.12)  
291 for the association between TCPy and ASD and 0.99 (95%CI: 0.79; 1.25) for the association  
292 between DMTP and ASD. None of these associations was significant (all p-values  $\geq$  0.15, Table  
293 3). Additional adjustment for the use of prenatal vitamins during the first month of pregnancy,  
294 pregnancy pyrethroid exposure, gestational age at birth, maternal or paternal age did not change  
295 these null results (Supplemental Material, Table S4). Excluding the 6 twins of our study

296 population or the mothers in the top 2.5 percent of the OP pesticide concentrations led to similar  
297 results (Supplemental Material, Table S4).

298 Correlation between the second and third trimester-average concentrations ranged between 0.16  
299 for TCPy and 0.41 for  $\Sigma$ DMP (Supplemental Material, Table S5). When we looked at specific  
300 time windows during pregnancy (i.e., conducting separate analyses in samples collected during  
301 the 2<sup>nd</sup> trimester or the 3<sup>rd</sup> trimester) none of the OP metabolites was associated with the risk of  
302 having a child with ASD or NTD (all p-value  $\geq$  0.12, Table 3). After stratification for sex, among  
303 boys, OP concentrations tended to be associated with a decreased risk of ASD and all of the  
304 observed ORs were below 1 (Table 4). Among girls, DMTP tended to be associated with an  
305 increased risk of having a child with ASD (OR for a doubling in the DMTP concentration: 1.64  
306 (95%CI, 0.95; 2.82, p-values for interaction = 0.09, Table 4). No clear association was observed  
307 between  $\Sigma$ DMP and ASD risk among girls (OR = 1.38, 95%CI: 0.85; 2.25). These results have  
308 to be interpreted cautiously given the small number of females with ASD in our study population  
309 (n = 12). After stratification for sex none of the OP metabolite concentrations was strongly  
310 associated with NTD risk (Table 4).

## 311 **Discussion**

312 None of the OP metabolites assessed in the current study was significantly associated with  
313 increased risk of ASD or NTD when boys and girls were studied together. This null finding  
314 might be explained by the small sample size and the lower OP concentrations compared to  
315 previous studies looking at the associations with ASD, implying that a larger sample size would  
316 be needed to detect similar associations. Windows of exposure might also be an issue since we  
317 used urinary OP concentrations measured in urine samples collected at random time points

318 during the 2<sup>nd</sup> and 3<sup>rd</sup> trimesters while, for example, studies that relied on the annual Pesticide  
319 Use Report were able to re-construct exposure across the entire pregnancy (Shelton et al. 2014).  
320 After stratification for sex, our results suggested an association between DMTP urinary  
321 concentration and risk of ASD among girls but not among boys. No association was observed  
322 between  $\Sigma$ DMP, that included DMTP along with DMP and DMDTP, and ASD among girls.  
323 These results should be interpreted cautiously, and requires replication in another study with  
324 larger sample size, since only 12 girls with an ASD diagnosis were included in our analysis.

### 325 *Urinary concentrations of OP*

326 The decrease in OP concentrations with time (Figure 1) observed in our study population was  
327 likely a result of the U.S. EPA's action to restrict the manufacture and sale of OP products for  
328 the home use market. Compared to previous studies assessing OP metabolites during pregnancy  
329 and ASD, urinary concentrations tended to be lower in our study population. The median  $\Sigma$ DAP  
330 concentration was 40.8 nmol/L in our study population compared to 59.9 nmol/L in the HOME  
331 study (Millenson et al. 2017), 82 nmol/L in the Mount Sinai study (Engel et al. 2007) and  
332 between 107 to 141 nmol/L, depending on the trimester, in the CHAMACOS study (Raanan et  
333 al. 2015). Similarly, the median concentration for TCPy in our study population was 2.61 ng/ml  
334 compared to 3.5 to 4.6 ng/ml in CHAMACOS depending of the trimester (Eskenazi et al. 2007)  
335 and up to 7.6 ng/ml in the Mount Sinai cohort (Berkowitz et al. 2004). Differences in OP  
336 concentrations can be explained by the recruitments that occurred earlier in previous studies  
337 (1999-2000 in CHAMACOS, 1998-2002 in the Mount Sinai cohort and 2003-2006 in the HOME  
338 study) compared to ours (2006-2014). The U.S. Environmental Protection Agency indeed banned  
339 OP pesticides for most residential uses in the early 2000s (Clune et al. 2012) and a decrease in  
340 OP concentrations have been reported between 1988-1994 and 2003-2004 in NHANES (Clune et



341 al. 2012). The removal of OP pesticides from residential usage may have led to more intermittent  
342 exposures in our study population, which would imply greater misclassification relative to the  
343 true average concentrations within the time periods studied. In addition, sources of OP exposure  
344 may be substantially different compared to previous studies, leading to differences in the  
345 distribution of specific compounds.

#### 346 *Associations with ASD and NTD*

347 Among previous studies that investigated the associations between prenatal exposure to OP  
348 pesticides and ASD, one study did not report association (Millenson et al. 2017) while five  
349 studies reported increased risk of ASD or ASD like symptoms in association with OP exposure  
350 (Eskenazi et al. 2007; Furlong et al. 2014; Rauh et al. 2006; Roberts et al. 2007; Shelton et al.  
351 2014). The association observed in the Roberts et al study was no longer significant after  
352 correction for multiple testing (Roberts et al. 2007). Differences in study results might come  
353 from discrepancies in study design, outcome and exposure assessment. Our study population is  
354 indeed the first to enroll children with a high risk for ASD. Some of that elevated risk is due to  
355 genetic factors, which were not directly measured. Thus, environmental factors associated with  
356 ASD in these multiplex families (> 1 child with autism) might differ from those affecting ASD  
357 children in simplex families, due to greater genetic vulnerabilities, on average. Three studies,  
358 including ours, relied on clinical diagnosis of ASD (Roberts et al. 2007; Shelton et al. 2014),  
359 while the others assessed scale scores on the CBCL and SRS, two instruments completed by the  
360 parents that obtain data on symptoms common in ASD or in other types of pervasive  
361 developmental delay. Regarding exposure assessment, two studies examined agricultural  
362 pesticide uses near the homes (Roberts et al. 2007; Shelton et al. 2014). Although this approach  
363 provides valid estimates of agricultural exposures, it does not capture other sources of OP

364 pesticides such as dietary routes of exposure. The other studies measured OP metabolites in urine  
365 collected during pregnancy (Eskenazi et al. 2007; Furlong et al. 2014; Millenson et al. 2017;  
366 Rauh et al. 2006) or the parent compounds in umbilical cord blood collected at delivery (Rauh et  
367 al. 2006). However, they relied on a small number of biological samples (usually no more than  
368 2), which, considering the high variability reported for OP metabolite concentration in urine  
369 (intraclass correlation coefficients ranging from 0.14 to 0.31 for  $\Sigma$ DAP (Spaan et al. 2015) and  
370 of 0.41 for TCPy (Fortenberry et al. 2014)), is likely to lead to exposure measurement error. If  
371 this error is of classical type (i.e, the average of many replicate measurements is expected to  
372 approximate the true individual level) attenuation of the effect estimates would be expected  
373 (Perrier et al. 2016). Despite the fact that we collected more urine samples (on average 5 samples  
374 per mother) than previous studies, we did not demonstrate an association between prenatal  
375 urinary concentration of OP pesticide metabolite concentrations and ASD or NTD risk in our  
376 overall sample; however, our results were suggestive of an association between DMTP and ASD  
377 among girls. Among studies on organophosphate pesticides and child neurodevelopment, a few  
378 performed sex-stratified analysis (reviewed by (Gonzalez-Alzaga et al. 2014)). Among them,  
379 Bouchard et al. reported a negative association with child IQ that was not modified by child sex  
380 (p-value for interaction  $> 0.3$  (Bouchard et al. 2011)) while the others reported deleterious effects  
381 among boys but not among girls in ADHD (Marks et al. 2010), working memory (Horton et al.  
382 2012) and ASD like symptoms (Furlong et al. 2014).

### 383 *Limitations*

384 Urinary OP metabolite concentrations reflect direct exposure to OP parent compounds that were  
385 metabolized endogenously by the enrolled women, but also direct exposure to OP metabolites  
386 that are naturally present in the environment. These OP metabolites result from the photolysis of

387 the parent compounds in food or indoor dust (Clune et al. 2012), are non toxic and are mostly  
388 excreted unchanged in urine. The part of the urine concentrations from each source (parent  
389 compounds metabolized by the women versus OP metabolites naturally present in the  
390 environment) is unlikely to vary across diagnostic group and so this should not introduce  
391 differential measurement error with regard to the child's outcome in our study. Except for TCPy,  
392 a specific metabolite of chlorpyrifos, OP metabolites are not specific and reflect exposure to  
393 multiple OP parent compounds. While we adjusted for several confounders we cannot rule out  
394 residual bias due to unmeasured confounders or confounders measured with error. PON<sub>1</sub> enzyme  
395 has been associated with neurodevelopmental outcomes (Eskenazi et al. 2010) and is also  
396 involved in the detoxification pathway of several chemicals including some OP pesticides.  
397 Individuals with certain PON<sub>1</sub> polymorphisms might be more susceptible to the effects of these  
398 chemicals (Engel et al. 2007). We do not have information regarding PON<sub>1</sub> genetic  
399 polymorphism and enzyme activity, however, even with such information our small sample size  
400 may have limited our ability to examine the interaction between the polymorphism and OP  
401 pesticides. An additional limitation in our study design is the variable number of urine samples  
402 used to estimate average exposures, which we addressed using an ad hoc analytical weighting  
403 strategy. In simulation studies (Perrier et al. 2016), we have found that the analytical weighting  
404 strategy yields more accurate effect size estimates than a naive strategy that treats all exposure  
405 measurements as equally precise, but further methodological research is warranted to fully  
406 justify this approach

## 407 **Conclusion**

408 The MARBLES Study is a *high risk* longitudinal cohort which gave us the ability to  
409 prospectively assess prenatal OP exposure with the use of multiple urine samples collected

410 throughout pregnancy and clinically confirmed classifications of ASD and NTD at 3 years. We  
411 did not observe association between either prenatal exposure to OP pesticides and ASD or NTD  
412 when boys and girls were studied together. After stratification for sex, DMTP tended to be  
413 associated with an increased risk of having a child with ASD among girls. Given the relatively  
414 small sample size, both the association we observed among girls, as well as the lack of  
415 association in boys, needs to be replicated in studies with larger sample size.

416

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418 **Table 1:** Characteristics of the study population, n = 203 mother-child pairs of the MARBLES  
 419 cohort with OP assessments during pregnancy and diagnosis at 3 years

	TD		ASD		NTD		ASD versus TD <sup>a</sup>	NTD versus TD <sup>a</sup>
	N	%	N	%	N	%		
<b>Child sex</b>							0.03	0.20
Female	48	47	13	28	20	36		
Male	54	53	33	72	35	64		
<b>Year of birth</b>							0.01	0.19
2007-2008	33	32	4	9	11	20		
2009	24	24	11	24	13	24		
2010-2011	24	24	14	30	12	22		
2012-2014	21	21	17	37	19	35		
<b>Birth status</b>							0.44	0.10
Singleton	97	95	45	98	55	100		
Has a twin	5	5	1	2	0	0		
<b>Child race/ethnicity</b>							0.10	0.14
White non Hispanic	53	52	17	37	19	35		
Hispanic	27	26	17	37	17	31		
Other <sup>b</sup>	22	22	12	27	19	35		
<b>Birth season</b>							0.30	0.26
Warm months (May - Oct)	56	55	21	46	25	45		
Cold months (Nov - Apr)	46	45	25	54	30	55		
<b>Maternal age (years)</b>							0.39	0.52
< 30	26	25	8	17	10	18		
30 to 35	32	31	13	28	21	38		
> 35	44	43	25	54	24	44		
<b>Home owner</b>							0.15	0.34
Yes	67	66	23	50	31	56		
No	34	33	20	43	22	40		
Missing values	1	1	3	7	2	4		
<b>Parental education</b>							0.66	0.96
High school, some college (no degree)	17	17	6	13	9	16		
Bachelor degree	59	58	29	63	33	60		
Graduate or professional degree	26	25	9	20	13	24		
Missing values	0	0	2	4	0	0		
<b>Prepregnancy BMI (kg/m2)</b>							0.32	0.48
< 25	59	58	20	43	27	49		
25 to 30	23	23	13	28	13	24		
> 30	20	20	12	26	15	27		
Missing values	0	0	1	2	0	0		

420 <sup>a</sup> p-values for Pearson's chi-squared test

421 <sup>b</sup> include Black, Multiracial and Asian

422

423

**Table 2:** Maternal urinary concentrations of OP metabolites among 203 mother-child pairs of the MARBLES cohort (mean of the concentrations measured in repeated urine samples collected during pregnancy)

	Whole study population						TD			ASD			NTD			ASD versus TD <sup>a</sup>	NTD versus TD <sup>a</sup>			
	LOD	%> LOD	N	Percentiles			N	Percentiles			N	Percentiles			N			Percentiles		
				5	50	95		5	50	95		5	50	95				5	50	95
TCPy (ng/mL)	0.1	95	203	<LOD	2.61	15.74	102	<LOD	2.65	18.3	46	0.34	2.36	9.03	55	0.41	2.69	18.12	0.11	0.77
DMP (ng/mL)	0.35	51	203	<LOD	0.44	10.07	102	<LOD	<LOD	12.0	46	<LOD	0.50	5.23	55	<LOD	0.46	9.37	0.62	0.55
DMTP (ng/mL)	0.25	94	203	<LOD	1.80	18.4	102	<LOD	1.90	23.1	46	0.35	2.05	18.4	55	<LOD	1.60	15.94	0.65	0.13
DMDTP (ng/mL)	0.25	34	203	<LOD	<LOD	5.88	102	<LOD	<LOD	9.83	46	<LOD	<LOD	3.64	55	<LOD	<LOD	4.27	0.03	0.08
ΣDMP (μmol/mL)			203	6.37	26.0	225	102	6.73	23.51	296	46	6.57	27.91	164	55	5.89	26.0	193	0.78	0.10
DEP (ng/mL)	0.25	89	203	<LOD	0.91	13.4	102	<LOD	1.13	13.4	46	<LOD	0.79	10.2	55	<LOD	0.83	17.0	0.06	0.06
DETP (ng/mL)	0.25	34	203	<LOD	<LOD	1.70	102	<LOD	<LOD	1.93	46	<LOD	<LOD	1.55	55	<LOD	<LOD	1.87	0.01	0.09
ΣDEP (μmol/mL)			203	2.23	8.39	115	102	2.23	10.12	117	46	2.23	6.58	67.8	55	2.23	7.56	122	0.01	0.01
ΣDAP(μmol/mL)			203	11.71	40.8	304	102	12.09	43.24	345	46	12.09	40.03	210	55	10.33	35.1	312	0.30	0.04

<sup>a</sup> p-value for Wilcoxon rank test

Abbreviations: DEP: Diethylphosphate, DETP: Diethylthiophosphate, DMDTP: Dimethylthiophosphate, DMP: Dimethylphosphate, DMTP: Dimethylthiophosphate, TCPy: 3,5,6-trichloro-2-pyridinol, ΣDAP: molar sum of DEP, DETP, DMP, DMTP and DMDTP, ΣDEP: molar sum of DEP and DETP, ΣDMP: molar sum of DMP, DMTP, and DMDTP

**Table 3:** Adjusted<sup>a</sup> Multinomial Odds Ratios for Autism Spectrum Disorder and Non Typically Developing children in relation to OP pesticide metabolite urinary concentrations<sup>b</sup>, MARBLES study

	ASD versus TD		NTD versus TD	
	OR	95%CI	OR	95%CI
<i>A) Whole pregnancy exposure (N = 101 TD, 42 ASD, 53 NTD<sup>c</sup>)</i>				
TCPy	0.80	[0.57; 1.12]	0.99	[0.74; 1.32]
DMTP	0.99	[0.79; 1.25]	0.86	[0.69; 1.06]
DEP	0.94	[0.74; 1.20]	0.92	[0.74; 1.15]
ΣDMP	0.92	[0.73; 1.15]	0.86	[0.69; 1.06]
ΣDEP	0.91	[0.72; 1.16]	0.91	[0.73; 1.13]
ΣDAP	0.89	[0.68; 1.17]	0.85	[0.66; 1.08]
<i>B) Analysis restricted to samples collected in the 2<sup>nd</sup> trimester of pregnancy (n = 66 TD, 28 ASD, 40 NTD<sup>c</sup>)</i>				
TCPy	0.77	[0.53; 1.13]	0.90	[0.65; 1.25]
DMTP	1.11	[0.83; 1.48]	0.96	[0.74; 1.25]
DEP	1.03	[0.76; 1.38]	0.89	[0.68; 1.18]
ΣDMP	1.01	[0.76; 1.35]	0.94	[0.72; 1.22]
ΣDEP	0.94	[0.70; 1.28]	0.85	[0.64; 1.12]
ΣDAP	1.00	[0.72; 1.40]	0.89	[0.66; 1.21]
<i>C) Analysis restricted to samples collected in the 3<sup>rd</sup> trimester of pregnancy (n = 98 TD, 39 ASD, 48 NTD<sup>c</sup>)</i>				
TCPy	0.95	[0.71; 1.27]	1.06	[0.82; 1.36]
DMTP	1.01	[0.82; 1.24]	0.86	[0.71; 1.04]
DEP	0.92	[0.74; 1.16]	0.97	[0.80; 1.18]
ΣDMP	0.97	[0.78; 1.19]	0.86	[0.70; 1.04]
ΣDEP	0.91	[0.73; 1.13]	0.95	[0.79; 1.16]
ΣDAP	0.93	[0.73; 1.19]	0.88	[0.71; 1.10]

<sup>a</sup> Adjustment factors were: home ownership, prepregnancy BMI, season and date of birth

<sup>b</sup> OR represents the change in the odds of the outcomes for a doubling in the OP concentrations standardized for specific gravity

<sup>c</sup> Analysis restricted to the mother-child pairs with OP concentrations, diagnosis at 3 years and non-missing covariates.

**Abbreviations:** DEP: Diethylphosphate, DMTP: Dimethylthiophosphate, TCPy: 3,5,6-trichloro-2-pyridinol, ΣDAP: molar sum of DEP, DETP, DMP, DMTP and DMDTP, ΣDEP: molar sum of DEP and DETP, ΣDMP: molar sum of DMP, DMTP, and DMDTP.

DMDTP, DETP, DETP and DMP were not studied separately because they were detected in less than 60% of the urine samples analyzed.

**Table 4:** Adjusted<sup>a</sup> Multinomial Odds Ratios for Autism Spectrum Disorder and Non Typically Developing children in relation to OP urinary concentrations<sup>b</sup> among boys and girls studied separately

	Girls (N = 48 TD, 12 ASD, 18 NTD) <sup>c</sup>		Boys (N = 53 TD, 30 ASD, 35 NTD) <sup>c</sup>		P-value for interaction
	OR	95%CI	OR	95%CI	
<i>A) ASD versus TD</i>					
TCPy	1.09	[0.54; 2.22]	0.69	[0.45; 1.06]	0.56
DMTP	1.64	[0.95; 2.82]	0.84	[0.63; 1.11]	0.09
DEP	1.15	[0.66; 1.99]	0.83	[0.61; 1.13]	0.65
∑DMP	1.38	[0.85; 2.25]	0.78	[0.58; 1.05]	0.24
∑DEP	1.07	[0.62; 1.84]	0.80	[0.59; 1.09]	0.65
∑DAP	1.29	[0.73; 2.27]	0.75	[0.54; 1.06]	0.44
<i>B) NTD versus TD</i>					
TCPy	1.02	[0.63; 1.67]	0.91	[0.62; 1.34]	0.63
DMTP	0.89	[0.60; 1.31]	0.81	[0.61; 1.07]	0.78
DEP	0.96	[0.66; 1.42]	0.83	[0.62; 1.12]	0.51
∑DMP	0.90	[0.62; 1.30]	0.80	[0.61; 1.06]	0.73
∑DEP	0.92	[0.63; 1.35]	0.83	[0.62; 1.11]	0.63
∑DAP	0.90	[0.59; 1.38]	0.78	[0.56; 1.07]	0.64

<sup>a</sup> Adjustment factors were: home ownership, prepregnancy BMI, season and date of birth

<sup>b</sup> OR represents the change in the odds of the outcomes for a doubling in the OP concentrations standardized for specific gravity

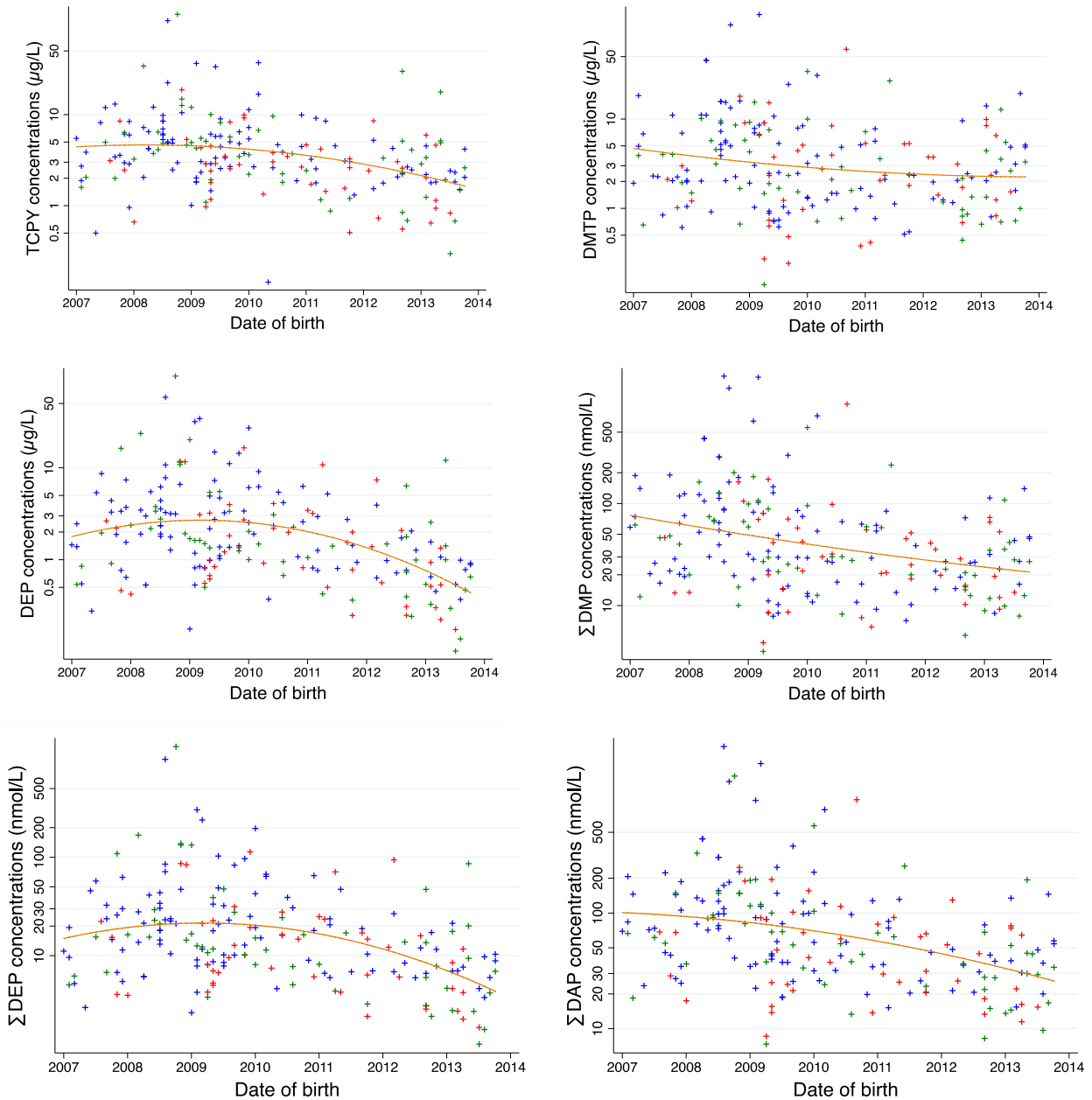
<sup>c</sup> Analysis restricted to the mother-child pairs with OP concentrations, diagnosis at 3 years and non-missing covariates.

Abbreviations: DEP: Diethylphosphate, DMTP: Dimethylthiophosphate, TCPy: 3,5,6-trichloro-2-pyridinol, ∑DAP: molar sum of DEP, DETP, DMP, DMTP and DMDTP, ∑DEP: molar sum of DEP and DETP, ∑DMP: molar sum of DMP, DMTP, and DMDTP.

DMDTP, DETP, DETP and DMP were not studied separately because they were detected in less than 60% of the urine samples analyzed.



**Figure 1:** Average OP metabolite concentrations according to the child date of birth (n = 203 mother child pairs from the MARBLES study)



Legend: Blue crosses represent TD children, red crosses ASD children and green crosses NTD children.

Abbreviations: DEP: Diethylphosphate, DMTP: Dimethylthiophosphate, TCPy: 3,5,6-trichloro-2-pyridinol, ΣDAP: molar sum of DEP, DETP, DMP, DMTP and DMDTP, ΣDEP: molar sum of DEP and DETP, ΣDMP: molar sum of DMP, DMTP, and DMDTP.

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