Prenatal exposure to organophosphate pesticides and autism spectrum disorders in 11-year-old children in the French PELAGIE cohort

Mathilde Lizé, Christine Monfort, Florence Rouget, Gwendolina Limon, Gaël Durand, Hélène Tillaut, Cécile Chevrier

PRENATAL EXPOSURE TO ORGANOPHOSPHATE PESTICIDES AND AUTISM SPECTRUM DISORDERS IN 11-YEAR-OLD CHILDREN IN THE FRENCH PELAGIE COHORT

Authors :

Mathilde Lizé, Christine Monfort, Florence Rouget, Gwendolina Limon, Gaël Durand, Hélène Tillaut, Cécile Chevrier.

Affiliations :

Mathilde Lizé : Université Rennes 1, CHU Rennes, Irset (Institut de recherche en santé, environnement et travail) - UMR_S 1085, F-35000 Rennes, France. lize.math@gmail.com / mathilde.lize@chu-rennes.fr

Christine Monfort : Université Rennes 1, Inserm, EHESP, Irset (Institut de recherche en santé, environnement et travail) - UMR_S 1085, F-35000 Rennes, France. christine.monfort@univ-rennes1.fr

Florence Rouget : CHU Rennes, Université Rennes 1, Inserm, EHESP, Irset (Institut de recherche en santé, environnement et travail) - UMR_S 1085, F-35000 Rennes, France. florence.rouget@univ-rennes1.fr

Gwendolina Limon : LABOCEA (Laboratoire public Conseil, Expertise et Analyse in Brittany), F-29280 Plouzané, France. gwendolina.limon@labocea.fr

Gaël Durand : LABOCEA (Laboratoire public Conseil, Expertise et Analyse in Brittany), F-29280 Plouzané, France. gael.durand@labocea.fr

Hélène Tillaut : Université Rennes 1, Inserm, EHESP, Irset (Institut de recherche en santé, environnement et travail) - UMR_S 1085, F-35000 Rennes, France. helene.tillaut@univ-rennes1.fr

Cécile Chevrier : Université Rennes 1, Inserm, EHESP, Irset (Institut de recherche en santé, environnement et travail) - UMR_S 1085, F-35000 Rennes, France. cecile.chevrier@univ-rennes1.fr

Corresponding author:

Mathilde Lizé : lize.math@gmail.com / mathilde.lize@chu-rennes.fr, 00 33 658 73 75 57, 141 Avenue Aristide Briand 35000 Rennes, France / CHU Hôpital Sud Boulevard de Bulgarie 35200 Rennes, France.

ABSTRACT

BACKGROUND: Organophosphate (OP) pesticides act by inhibiting acetylcholinesterase activity at synaptic junctions and have already been linked with deleterious effects on neurodevelopment, including autism spectrum disorders (ASD).

OBJECTIVES: To investigate the association of prenatal exposure to OP pesticides with traits related to ASD in 11-year-old children.

METHODS: The "Childhood Autism Spectrum Test" (CAST) parent questionnaire was used to screen for autistic traits in 792 children from the French PELAGIE cohort. Prenatal maternal urine samples were collected <19 weeks of gestation in which metabolites of organophosphate insecticides were assessed for 185 of them. Negative binomial regression models were performed to explore the association between the CAST score and 8 groups of urine components, adjusted for potential ASD risk factors.

RESULTS: In these urine samples, dialkylphosphates (DAP) were detected most often (>80%), terbufos and its metabolites least often (<10%). No association with ASD was found for DAP, terbufos or its metabolites. Incidence rate ratios (IRRs) increased with maternal urinary diazinon concentrations, from 1.11 (95% CI: 0.87-1.42) to 1.17 (95% CI: 0.94-1.46). Higher CAST scores were statistically significantly associated with the maternal urine samples in which chlorpyrifos or two of its metabolites (chlorpyrifos-oxon and 3,5,6-trichloro-2-pyridinol) were detected. The IRR for exposure to chlorpyrifos or chlorpyrifos-oxon was 1.27 (95%CI: 1.05-1.52) among all children, and 1.39 (95%CI: 1.07-1.82) among boys.

CONCLUSION: These findings suggest an increase in autistic traits among 11-year-old children in association with prenatal maternal exposure to chlorpyrifos and possibly diazinon. These associations were previously suspected in the literature, in particular for chlorpyrifos. Further work establishing the causal mechanisms behind these risk association is needed.

KEY-WORDS:

Autism spectrum disorders, Autism, Organophosphate pesticides, Chlorpyrifos, Diazinon, Prenatal exposure, Children.

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The adults participating in this study provided their written informed consent. The Advisory Committee on Information Processing in Health Research (CCTIRS; 2015) and the French National Commission for Information Technology and Civil Liberties (CNIL; 2002, 2015) both approved this study.

Conflicts of interest:

The authors declare they have nothing to disclose.

1 Introduction

In the first decade of this century, organophosphate (OP) pesticides were the insecticides most widely used worldwide. They were — and still can be — found in various setting: agriculture, forestry, and even homes. In the general population, the main source of exposure is contaminated food, especially fruit and vegetables (Ye et al. 2015). Interiors are also contaminated through domestic use (Pesticides et santé – Nouvelles données (2021)). Exposure is ubiquitous in the general population and pregnant women are not exempt: in 2019, for example, Papadopoulou's team (Papadopoulou et al. 2019) looked at the dietary exposure to environmental pollutants in 6 longitudinal European cohorts and found higher urinary OP markers in those eating more fruit. In a study of the French PELAGIE cohort, Cartier et al. (2016) reported the presence of dialkylphosphate metabolites (metabolites of various OPs) in 91.3% of the urine samples of the pregnant women analyzed (n=231). Another French study in 2011 looked at the dietary exposure of pregnant women in the ELFE cohort to pesticides and highlighted daily exposure at risk of neurochemical effects, according to mechanistic data (de Gavelle et al. 2016). OP molecules, which are small and lipophilic, easily cross the placental barrier (Bradman et al. 2003).

OPs act mainly by inhibiting acetylcholinesterase activity in synaptic junctions, leading to acetylcholine accumulation and neuronal hyperexcitability. Their pathophysiological action also includes oxidative stress, altered axonal transport, and a decreased number of glial and neuronal cells (Terry 2012). Animal studies have shown the neurologic consequences of chronic exposure, in particular, in mice repeatedly exposed to low doses of chlorpyrifos: a 50% decrease in synaptic transmissions (electrophysiology) was correlated with a decreased number of synaptic junctions in the hippocampus (histology) (Speed et al. 2012). Worldwide, exposure to OP pesticides has become an important public health issue (Abreu-Villaça and Levin 2017), and human studies have confirmed neurodevelopmental risks. Based on several epidemiologic studies (Bouchard et al. 2011; Engel et al. 2007; Marks et al. 2010), the French INSERM (National Institute of Health and Medical Research) Expert reports on Pesticides and Health of 2013 and 2021 (Pesticides : Effets sur la santé; Pesticides et santé – Nouvelles données (2021)) concluded that exposure to OP pesticides during pregnancy is related to the occurrence of neurodevelopmental disorders in children. Results in 18 separate cohorts justify at least a strong presumption that OP exposure during pregnancy (without distinguishing between specific substances) is linked to the impairment of motor, cognitive, behavioral, and sensory capacities in the child.

In its fifth edition, the Diagnostic and Statistical Manual (DSM 5) (Messent 2013) clearly identifies the category of autism spectrum disorder (ASD) as a neurodevelopmental disorder The diagnosis is made clinically: "the essential characteristics of autism spectrum disorder are persistent deficits in reciprocal social communication and social interaction, a restricted and repetitive pattern of behaviors, interests and activities" (Grzadzinski et al. 2013). The revised definition has two domains; the one associated with language development in DSM 4 has been deleted. This broad definition includes

severe forms with intellectual disability as well as milder forms without intellectual disability, which may remain unrecognized during childhood. This delineation is consistent with the findings of various research teams, for whom the patterns of the autism spectrum may be seen as a continuum, with a continuous distribution of behavioral patterns in the general population tending towards, at one extreme, autism (Constantino 2011; Constantino et al. 2004; Robinson et al. 2011; Ronald et al. 2006). Worldwide ASD prevalence is reported to be increasing, with estimates across epidemiological studies currently between 1 and 2%. (Elsabbagh et al. 2012; Maenner et al. 2020). This increase may result from new risk factors or from the change in the definition between DSM 4 and 5, or both. Genetic factors are known to play a role in the development of ASD, particularly through the contribution of twin studies (Hallmayer et al. 2011), but they do not explain all of the variability. In the framework of the concept of the developmental origins of health and disease (DOHaD), environmental components are also suspected of involvement. This concept suggests that subtle changes in specific health parameters occurring early in life and even prenatally, in response to an event or toxic agent, can induce dysfunction or disease in later life (Barker 2007; Heindel et al. 2017; Suzuki 2018). Pregnancy is known to be a window of particular risk for the future child.

According to the INSERM expert report of 2021, the presumption of a link between OPs and ASDs is moderate, with two cohort results in favor: the CHAMACOS cohort (Center for the Health Assessment of Mothers and Children of Salinas) (Sagiv et al. 2018) and the CEHS (Children's Environmental Health Study) (Furlong et al. 2014). In the CHAMACOS cohort, which included 601 pregnant women between 1999 and 2000 in California, higher prenatal urine OP metabolite levels were associated with more ASD-related traits in 14-year-old adolescents using the "Social Responsiveness Scale" (SRS 2). When exposure to OP pesticides was assessed by residential proximity to agricultural OP use, however, this link did not appear. The case-control study by von Ehrenstein et al. (2019), conducted in California, is one of the largest epidemiologic studies on the subject (2,961 ASD cases and 35,370 controls, with children born between 1998 and 2010). ASD cases were selected from an administrative database, in which the diagnosis of autism was reported (according to DSM-IV). OP exposure was assessed by residential proximity to agricultural use of pesticides (home address of pregnant woman and the California State Mandated Pesticide Use Reporting program for agricultural use of pesticides) and focused on 11 pesticides. It reported a higher risk of ASD for 6 pesticides including 3 OPs: chlorpyrifos, diazinon, and malathion. In the Childhood Autism Risks from Genetics and Environment (CHARGE) case-control study, Shelton et al. (2014) also reported that the risk of ASD was associated with residential proximity during pregnancy to agricultural use of OPs (970 women included since 2003 in California). Cases were identified from administrative databases of the area completed with additional instruments (Autism Diagnostic Interview-Revised, Autism Diagnostic Observation Schedule). Millenson et al. (2017) explored this association in the HOME (Health Outcome and Measures of the Environment) cohort, which quantified urinary OP metabolites during pregnancy and used the quantitative SRS score to test 8-year-old children (224 pregnant women included in Ohio

between 2003 and 2006). They found prenatal urine concentrations were not significantly associated with this score.

Consequently, the variability of the results prevents us from generalizing the association, especially as all of these studies are from the United States (USA) (including three in California). Additional scientific knowledge appears necessary to judge the potential link between OPs and ASD. Our aim is to evaluate the impact of prenatal exposure to OPs on the development of ASD in children aged 11 years in a sample of the PELAGIE (Perturbateurs endocriniens: Etude Longitudinale sur les Anomalies de la Grossesse, l'Infertilité et l'Enfance, endocrine disrupters, longitudinal study of anomalies of fertility, pregnancy, and childhood) French mother-child cohort. We explore this link with maternal urine samples collected during pregnancy and parental screening questionnaire for ASD.

2 Methods

Study population

The PELAGIE cohort is a French longitudinal mother-child cohort that included 3,421 pregnant women before 19 weeks of gestation between 2002 and 2006. Among these pregnancies, 3,322 resulted in singleton live births (Chevrier et al. 2011). These women were recruited from 3 districts of Brittany during their first prenatal visit (generally between 8 and 12 weeks of gestation), by gynecologist-obstetricians or ultrasonographers. The initial aim of this cohort was to evaluate prenatal environmental exposures and their long-term consequences for the child. At inclusion, women completed a questionnaire about their socioeconomic characteristics, occupation, diet, and lifestyle. At the same time, they mailed to the researchers a sample of their first morning urine. At delivery, pediatricians and midwives at the maternity units collected information about the pregnancy, the delivery, and the health of the newborn. Several follow-up questionnaires were sent at different ages to collect further information on these families' lifestyles and their children's health and development.

The adults participating in this study provided their written informed consent. The Advisory Committee on Information Processing in Health Research (CCTIRS; 2015) and the French National Commission for Information Technology and Civil Liberties (CNIL; 2002, 2015) both approved this study. For the present follow-up of the cohort, we provided a written information specifically adapted to the 11-year-old children, explaining the objective of the cohort and the data protection rule, and we encouraged parents to discuss with them about their choice to participate or not.

The Childhood Autism Spectrum Test (CAST)

The CAST was developed in 2002 by the Autism Research Center at the University of Cambridge, with the aim of screening the general population for subtle and milder forms of ASD among children aged 4 to 11 years. The items were created from the International Classification of Diseases (ICD) 10, DSM 4, and the Autism Spectrum Screening Questionnaire (ASSQ) (Scott et al. 2002). This is a quick parental questionnaire with 37 items, assessing the presence of autistic traits in the child. The

maximum score is 31 (6 control items are not included in the scores). Each item response is binary: 0 (not in favor of autistic traits) or 1 (in favor). The total number of points out of 31 provides a counting measure: the higher the score the more likely that the child has autistic traits. It was constructed as a quantitative scale, assuming that autistic traits have a continuous distribution in the population (Williams et al. 2005). However, a cut-off point was investigated to delimit a category of behavior different from normal behavior. For the detection of ASD with a cut-off set at 15 by the authors (Williams et al. 2005), the sensitivity was 100%, the specificity 97%, and the positive predictive value 50%. In a Spanish validation study (Morales-Hidalgo et al. 2017), the sensitivity was 83.9%, the specificity 92.5%, and the positive predictive value 63%. The reproducibility of this test was evaluated by the weighted kappa coefficient at 0.82 (Williams et al. 2006).

In the PELAGIE cohort, when the child reached 11 years of age (from September 2015 through January 2018), the research team sent the CAST parental autism spectrum screening questionnaire to all families still eligible for follow-up, that is, who had completed at least one questionnaire since birth and had a valid postal address —1,541 families. Two questions were added to the CAST, to inquire if teachers or medical staff had reported concerns about the child and/or if the child had received any specific neurodevelopmental disorder diagnosis (questionnaire in supplemental material). We received 792 responses, for a participation rate of 51.4%. Four questionnaires were excluded because more than half of the items (≥ 16) were unanswered, and six mother-child pairs were excluded from the analysis: one because the child had trisomy 21 and five because the mothers had epilepsy. Figure 1 presents our study flow diagram.

Exposure measurement of organophosphate pesticides

At inclusion (<19 weeks of gestation), each woman provided a urine sample (first morning urine, more concentrated) in a 10 mL tube. The samples were sent in an opaque box at room temperature and then frozen at -20°C until analysis. The analyses were performed by liquid chromatography-electrospray ionization tandem mass spectrometry (LC/MS-MS) after solid phase extraction (SPE), by the LABOCEA (Laboratoire public Conseil, Expertise et Analyse in Brittany) laboratory (Details are shown in Supplemental material). Several compounds from the organophosphate insecticide family were investigated: 6 dialkylphosphates (DAP), metabolites of many OPs but not specific to any one substance, including dimethylphosphate (DMP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMDTP), diethylphosphate (DEP), diethylthiophosphate (DETP), and diethyldithiophosphate (DEDTP); diazinon, an OP pesticide; chlorpyrifos (CPF), an OP pesticide, and 2 of its specific metabolites: chlorpyrifos-oxon (the first human metabolite of CPF) and 3,5,6-trichloro-2-pyridinol (TCPY, an environmental and human metabolite of CPF) (PubChem Chlorpyrifos); terbufos, another OP pesticide, and its 5 specific environmental and human metabolites (terbufos-sulfone, terbufos-oxon, terbufos-oxon-sulfone, terbufos-oxon-sulfoxide, and terbufos-sulfoxide) (Terbufos| Food and Agriculture Organization of the United Nations). In parallel, to account for urine dilution, the urine creatinine was measured. These assays were initially performed in 2007-2008 for a

subcohort of 601 newborns randomly selected among the singleton livebirths in the PELAGIE cohort and subsequently for 69 participants in neuropsychological follow-ups at 6 or 11 years of age, in 2013 and 2017, selected from a second PELAGIE subcohort (Binter et al. 2020; Cartier et al. 2016). For the last round, in 2017, only the 6 DAP metabolites were measured. In total, the sample of the present study is composed of 185 mother-child pairs (n=145 in 2007-2008; n=24 in 2013; n=16 in 2017).

For the first round, only the limits of quantification (LOQ) were available, while the limits of detection (LOD) were available for the other rounds. For clarity's sake, we will use the term LOD in the following, including the LOQs for the first round. The laboratory detection limits and molar masses for each molecule are provided in Table 1. The molar masses (g/mol) used were taken from the Toxnet database. The urinary mass concentrations were all converted to molar concentrations to sum the dialkylphosphate metabolites: the sum of dimethylphosphate metabolites (DM: DMP + DMTP + DMDTP), the sum of diethylphosphate metabolites (DE: DEP + DETP + DEDTP) and the overall sum of dialkylphosphate metabolites (DAP: DE + DM). In the rest of our work, the dialkylphosphate metabolites are always used as a sum, to be comparable with the literature (Bouchard et al. 2011; Furlong et al. 2014; Millenson et al. 2017; van den Dries et al. 2019).

Covariates

At inclusion, we collected the mother's age at the beginning of pregnancy, her education level (primary/secondary school, baccalaureate, postsecondary), relationship status (couple married or living with partner, versus women not living with a partner), the socio-professional category of both parents, the place of residence during the pregnancy (rural, semiurban, urban), prepregnancy body mass index (<18.5, 18.5-25, >25 kg/m²), parity (no children before this pregnancy or at least one child), tobacco and alcohol consumption at the beginning of pregnancy (yes/no), fruit and vegetable consumption (<1 serving/day, 1 to 2, 2 to 3, ≥3), and folic acid supplementation (yes/no). At the maternity unit, we collected the delivery and health status of the newborn: maternal diabetes (previous or gestational diabetes), hypertension or preeclampsia, the child's sex, term or preterm birth (≥ 37 weeks of gestation, < 37), birth weight and fetal growth restriction (weight <10th percentile, Audipog curves (Mamelle et al. 1996)), need for neonatal hospitalization, Apgar score at 1 and 5 minutes of life (≤ 7, >7), and presence of a congenital malformation. The follow-up questionnaires at age 2 and 6 years collected the fathers' ages and the duration of breastfeeding (none, < 3 months, 4-6 months, ≥ 7 months). These covariates were selected a priori from the literature, based on known risk factors for ASD and for pesticide exposure.

Statistical Analysis

The CAST

The score is a strictly positive discrete count variable. We used Cronbach's alpha to check the questionnaire's internal consistency. Scores of questionnaires with unanswered items (maximum 15

unanswered items per questionnaire) were weighted so that the final score reflected the proportion of questions answered, as reported in a previous study (Robinson et al. 2011).

Exposure data

Both the heterogeneous conditions of urine sampling and the analyses in 3 separate chronological series may have influenced the metabolite concentrations. Urinary concentrations of DAP, DM, DE, and diazinon were standardized by a previously published method based on regression residuals (Binter et al. 2020; Mortamais et al. 2012). We used an adjusted censored regression model to take multiple censored data into account to estimate the influence of sampling conditions: day of urine collection, duration of storage before freezing, duration of freezing before analysis, and urinary creatinine (Austin et al. 2000; Lubin et al. 2004). The year and the season of urine sampling were also included in the models, to avoid needing to correct for them, given that they influence the real exposure. For the DAP and DM sums, more than 70% of the values were above the LOD. Values below the LOD were considered left-censored data and were imputed by using a log-normal distribution (Jin et al. 2011). The parameter estimates (means and standard deviations) of the log-normal distribution were calculated from the concentration's distribution in the 2007-2008 measurement series, as it included the largest number of uncensored values. This log-normal distribution was then used to make imputations in each series. The DAP and DM sums were then used in tertiles and as continuous values. For metabolites detected or quantified in 30-70% of the urine samples, i.e., DE and diazinon, the values were used in 3 classes after standardization: < LOD, between LOD and median of quantified values, ≥ median of quantified values.

For the other metabolites, detected in less than 30% of the samples, composite exposure variables were created. The first variable corresponded to the detection in urine of CPF or CPF-oxon, the second to the detection of CPF or CPF-oxon or TCPY, and the last to the detection of terbufos or one of its 5 specific metabolites. These 3 variables were always used as binary forms in the analyses: at least 1 molecule detected or none. The molecules were not summed as for the DAPs because each composite variable could include both the parent molecule and its metabolites, which did not appear to be equivalent. We also explored the TCPY component alone, as a binary variable (detected/not detected), as it is the most common marker of CPF exposure used in the literature (Barr et al. 2005; Fortenberry et al. 2014).

Finally, 8 exposure groups were studied separately: DAP, DM, DE, Diazinon, CPF or CPF-Oxon, CPF or CPF-Oxon or TCPY, TCPY alone and Terbufos or at least 1 of these 5 metabolites.

Covariates and imputations of missing data

The selection of adjustment covariates was further refined by adding univariate associations for which $p<0.20$. To be included in the multivariable model, variable had to have every category representing at least 5% of individuals. Because there were few missing data for the selected covariates, with the maximum 4.9% for paternal age, we were able to use single imputation with mode or median.

Relations between CAST scores and organophosphate metabolite concentrations

After identification of the data's overdispersion negative binomial regression models were performed to explore the relation between prenatal maternal urinary concentrations of OP pesticides and/or their metabolites and the CAST score (Gardner et al. 1995; Sroka and Nagaraja 2018). For each group (=1 exposure variable), a first univariate model was performed, adjusting for urinary creatinine (crude model). Then a multivariable model was additionally adjusted for confounders and factors associated only with the CAST score. The continuous exposure variables were log-transformed and used as such in the models. For each exposure, we report the associated incidence rate ratio (IRR) with its 95% confidence interval (95% CI). The validity of the models was checked graphically by examining the residuals and the leverage points.

Complementary and sensitivity analyses

We conducted an additional analysis stratified by gender. The effect of the weighted CAST score was controlled by an analysis limited to the questionnaires without missing data. A second sensitivity analysis excluded the children in our dataset at higher risk of neurodevelopmental disorders, specifically, children born preterm, those with fetal growth restriction, and those with a 5-minute Apgar score ≤ 7.

The analyses were performed with R software (version 3.6.3). The NADA and survival packages were used for the variables with left-censored data and their analyses, and the MASS package was used for the negative binomial regression.

3 Results

Description of the population

Social and demographic characteristics of the 787 CAST respondents did not differ between families with and without a urine sample. Details are shown in Supplemental Material (Table S1). The mean score out of 31 points for the 787 responses was 5.7 (SD: 3.8) after weighting for questionnaires with missing items, similar to the mean for the questionnaires without any missing data (5.9, SD: 3.9). The minimum was 0 and the maximum 27 (2 individuals). Twenty-three individuals (2.9%) had a score above 15. The Supplemental Material (Table S2 and Figure S1) presents more details about the distribution of the CAST score.

The characteristics of the population with 185 mother-child pairs are described in Table 2. Mean maternal age at the onset of pregnancy was 30 years, 33 years for the fathers. They lived mostly in the countryside (65%), and 42% of the women were nulliparous before this pregnancy. Prepregnancy body mass index was normal for 70%, and 21% reported they had smoked at the beginning of the pregnancy. The average term of birth was 39 weeks of gestation, 4% of the newborns were preterm, and the average birth weight was 3400 grams. The notable neonatal events in our population included: 2 children who had a 5-minute Apgar score < 7 (one preterm at 31 weeks of gestation, the other at full term); one child had generalized tonic-clonic seizures, secondarily classified as familial idiopathic generalized seizures; and 7 children had congenital malformations, without apparent impact on their neurodevelopment:

auricular malformation (1), cryptorchidia (2), hypospadias (1), congenital hip luxation (2), and syndactyly (1).

Description of exposures

Table 3 presents the percentages of detection and concentration distributions for each metabolite. DAP and DM were quantified most often among the 185 samples (in 88.6% and 82.2% respectively), followed by DE in 49%, and diazinon (of the 169 determinations available, it was detected in 34.3%). Each of CPF, terbufos, and their metabolites (also only 169 determinations available) was detected in less than 20% of the samples (most below 10%). After standardization, the distributions were similar between the 3 series of measurements for DAPs and DEs. For DMs, however, the distribution of the 2013 series remained statistically different from that of 2007-2008 (p-value=0.001). Details about the distribution, the coefficients of censored regression models, and the effects of imputation for DAPs and DMs are presented in the Supplemental Material (Table S3, Figures S2-S5).

Relations between CAST scores and OP metabolite concentrations

The results of the adjusted analyses of the relations between the CAST score and the 8 exposure variables are presented in Table 4. These analyses were adjusted for the following variables: creatininuria, maternal education level, maternal BMI, maternal hypertension or preeclampsia, fruit and vegetable consumption in early pregnancy, duration of breastfeeding, and the child's sex.

No statistically significant associations were found between CAST scores and prenatal maternal urine concentrations of DAP, DM, DE, TCPY alone, or terbufos or its metabolites.

For diazinon, the crude model showed a positive association close to statistical significance with a p-value of 0.054 for the highest concentrations (> median). The IRR was 1.25 (95% CI: 0.98-1.55) for assays above the median and thus yielded a total CAST score multiplied by 1.25 for these individuals, compared to individuals with values below the LOD. After adjustment, the p-value increased to 0.16 for concentrations above the median. Despite the absence of statistical significance, CAST scores tended to rise along with maternal urinary diazinon concentrations: the IRR increased from 1.11 (95% CI: 0.87-1.42) to 1.17 (95% CI: 0.94-1.46) from concentrations below to those above the median.

The two binary variables including CPF (CPF or CPF-Oxon, and CPF or CPF-Oxon or TCPY), were associated with the CAST score. The children of exposed women were more likely to have a higher score. CPF or CPF-oxon exposure was associated with a higher CAST score (multiplied by 1.27, 95% CI: 1.05–1.52), reflecting the increasing presence of ASD traits (p=0.01). The association for the variable CPF or CPF-oxon or TCPY also favored an increased score but with a p-value at 0.058.

Complementary and sensitivity analyses

The results of the gender-stratified models are shown in the Supplemental Material (Table S4). For DAP, DM, DE, and terbufos or metabolites, all associations remained statistically non-significant, as did that with diazinon. For the exposure variables including CPF, the association with increasing IRRs was observed only among boys.

The results of the two sensitivity analyses are also reported in the Supplemental Material (Table S5). The associations pointed in the same direction. Once newborns at higher risk of neurodevelopmental disorders were excluded (subjects remaining in the sensitivity analysis =151), the crude model for diazinon became statistically significant for concentrations above the median (p=0.02), supporting an association in which prenatal maternal urinary diazinon concentrations increased with the CAST score (IRR: 1.34, 95% CI: 1.05-1.72). The strength of the association decreased after adjustment, with a p-value of 0.08 but IRRs remained above 1, in favor of a higher CAST score at higher exposure. When only complete questionnaires were considered (n=104), the association was statistically significant after adjustment, with an IRR of 1.40 (95% CI: 1.08-1.82) for levels above the median. For the composite variables including CPF, the positive relation with prenatal urine concentrations and CAST scores rising together remained statistically significant in these analyses, with both the IRRs and the associations of the same order of magnitude or even stronger.

4 Discussion

In our study, we found that detection of CPF and/or its metabolites in prenatal maternal urine samples was associated with higher CAST scores at age 11, compared with no detection of CPF or its specific metabolites. This relation was especially important among boys. The highest concentrations of diazinon in prenatal maternal urine samples were also associated with higher CAST scores, compared to no detection of diazinon. These two OP insecticides might therefore be involved in raising the risk of autistic traits in children. These associations were not found for dialkylphosphates or for terbufos and its specific metabolites.

This study used the CAST parent questionnaire as a screening measure for ASD among 11-year-old children. Other parental questionnaires are more often found in epidemiological studies, such as the ASSQ (Autism Spectrum Screening Questionnaire) and the SRS-2 (Social Responsiveness Scale). The SRS-2 contains 65 items, which is particularly long for a questionnaire destined to be used in a cohort and that is accompanied by a high probability of nonresponse or partial response. The ASSQ is a 27-item questionnaire, initially validated in a clinical sample and not in the general population (Ehlers et al. 1999). The items are very similar to those in the CAST, and there is also a threshold score requiring specialist advice (Autism Spectrum Screening Questionnaire (ASSQ) 2016). The team that created the CAST had in fact based it on the ASSQ (Scott et al. 2002). In our sample, we measured the internal consistency of the CAST with Cronbach's alpha coefficient, as previous studies have (Boucher et al. 2017; Morales-Hidalgo et al. 2017). The coefficient was 0.76, satisfactory according to standard interpretations (limit often set at 0.70), although we might have expected a higher value in view of the large number of items. Twenty-three individuals (2.9%) had a score above 15, which is in line with a worldwide prevalence of autism, around 2% (Elsabbagh et al. 2012; Maenner et al. 2020). The median score in our study was 4.3 for girls and 6 for boys, which is consistent with the results of the study by Williams et al. (2008). Similarly, they found that of the scores above 15, 80% were boys and only 20%

girls, ratios quite close to ours, where 20 boys (87%) had a score above 15, and only 3 girls (13%). The INMA Spanish mother-child cohort (Boucher et al. 2017) used this questionnaire for a large ASD screening involving 1,346 children and reported a mean score of 6.1 (SD: 3.2), similar to our results (mean: 5.7, SD: 3.8).

Although our findings are consistent with the existing literature using CAST, relatively few studies have done so. The validation studies by the Cambridge team are questionable as they did not look at the structure of the questionnaire. Psychometric validation studies of translated versions proposed a structure of a Mandarin version with 2 latent traits and only 27 items (Sun et al. 2014), while a Spanish study found the same structure and retained 28 items (Morales-Hidalgo et al. 2017). The two dimensions were related to social interactions and repeated/restricted interests and behavior, consistent with the two characteristic symptom groups in the DSM-5 and with factor analytic studies that conceptualize ASD symptoms and also find two dimensions for this entity (Frazier et al. 2012; Shuster et al. 2014). Although the questionnaire might cover two dimensions, the distribution of items is not clearly identified in the literature, so for comparability's sake, we have kept one dimension only. Because no French publication has validated the questionnaire, its cross-cultural validity has not been determined.

Our sensitivity analyses showed that the association between exposure and score was stronger when using only the full questionnaires. Nonetheless, we found no significant difference in the distribution of weighted scores and full questionnaire scores. An examination of the missing data did not identify any remarkable items. Despite the weighting, it appears that the incomplete questionnaires tend to have an inaccurately lower score and to be less representative of reality. It appears reasonable to suppose that if parents are undecided about an answer or have difficulty with an item it is because the child's behavior related to the item is at least somewhat unusual; otherwise there would be no reason not to answer it. As a result, the weighted scores (to deal with missing data) of children with ASD difficulties are likely to be underestimated, adding variability to our event of interest and increasing the standard deviation of the associations with exposures.

In determining our strategy for selecting adjustment covariates, we chose to consider all potential ASD risk factors available in our data and associated with our outcome. Our α risk was set at a threshold of 20%. This strategy led us to consider an adjustment set common to all our models. This facilitated comparisons. Some known risk factors for ASD, such as gestational diabetes and preterm birth, could not be included because sample sizes were too low. Particular neonatal events were explored, such as neonatal seizures or a low Apgar score, which might have promoted potential neonatal asphyxia, but again the sample sizes were very small, and they could not be used in the models. The potential impact of these factors was explored in our sensitivity analyses, which provided no major changes in the findings.

The urine samples were analyzed from 1 to 10 years after freezing, and it is difficult to judge the stability over time of urinary metabolites frozen at -20°C (Rotter et al. 2017). The coefficients of the

censored regression models and the distributions of the three chronological series of chemical measurements suggest that DAP and DM levels tend to decrease over time, for an underestimation of real exposure, while the DE concentrations increased, perhaps favoring overestimation. In addition, during the first series of analyses, the relatively high LOQs might have resulted in a classification bias, which could have led to underestimating exposure. Our standardization nonetheless took the variations of sampling conditions into account, even though the distribution of DMs in the 2013 series differed from the first series ($p<0.0001$).

In humans, OP pesticides are readily absorbed from diverse routes; they are then rapidly distributed into the body, possibly achieving the fetus by crossing the placental barrier (Whyatt and Barr 2001). 80-90% of metabolites are eliminated within the first 48 hours and the remaining 10-20% within a few days. Urine samples collected only once during pregnancy represent a small window of exposure and may not be representative of the entire pregnancy. Exposures to OPs may fluctuate, with for example higher exposure in spring due to greater agricultural use. Repeated determinations during the pregnancy might have enabled a better estimation of real exposure (Bouchard et al. 2011; Millenson et al. 2017). The women were included during the first trimester of pregnancy with a urine sample before 19 weeks of gestation. By this time, many key steps in brain development have already taken place (e.g., neural tube closure by day 30); neuroblast proliferation and differentiation continue, as do axonal growth and migration of interneurons to join the pyramidal neurons (around 18 weeks). Thus, although only one sampling is taken, it occurs during an important window for fetal brain development (Stiles and Jernigan 2010).

Urinary metabolites from the biotransformation of OPs are considered to be the most sensitive markers for measuring exposure to this family (Fréry et al. 2017). Dialkylphosphates are the most commonly used because they are metabolites of more than 20 OP pesticides. We did not find a statistically significant relation between their concentration and higher CAST scores. The CHAMACOS study in California (Sagiv et al. 2018) found mean prenatal urinary concentrations of 124.6 nmol/L for DAP, 92.6 nmol/L for DM, and 20.3 nmol/L for DE, which are higher than the mean levels in our analysis (respectively 83.4 nmol/L, 47.4 nmol/L, and 36 nmol/L). These higher mean concentrations produced a statistically significant association in which urinary OP metabolite concentrations increased with the SRS score, indicating an association with more autistic traits. In a New York City study of 404 mother-child pairs (Furlong et al. 2014), mean maternal urinary concentrations were similar to those in our study: 76.9 nmol/L for DAP, 41.7 nmol/L for DM, and 17.4 for DE. A study of 224 mothers in Ohio (Millenson et al. 2017) found a lower median prenatal DAP concentration — 60 nmol/L. Consistent with our study, neither of these two studies found a statistically significant association between DAP and ASD in their samples as a whole. This could suggest that lower concentrations of DAPs or their corresponding OP mixtures are not associated with ASD risk, or alternatively that their sample sizes were too small to observe an association.

CPF and diazinon have already been incriminated in studies focusing on neurodevelopmental disorders. For example, the authors of a study of 254 children born between 1998 and 2002 in New York City (Rauh et al. 2006) reported a deleterious effect of CPF, with a statistically significant decrease in developmental scores of the Bayley Scales of Infant Development II (-6.5 ± 2.2, p=0.003) at 3 years of age, for children with the highest levels of CPF in cord blood (>6.17pg/g plasma). More specifically, the CHARGE study (Shelton et al. 2014) looked at the association between ASD and residential proximity during pregnancy to agricultural areas in California where OP pesticides were used. This study reports that CPF was the most widely used OP insecticide in California, with diazinon in third place. When the home during pregnancy was within 1.75 km of an area where CPF was applied, the child's risk of developing ASD increased (OR: 1.8, 95% CI: 1.1-3.0). A California case-control study with 10 controls for each confirmed case (n=38,331 in total) also found a positive association between prenatal exposure (geographical data of residential proximity to agricultural pesticide use) and risk of autism (von Ehrenstein et al. 2019). The pesticides studied and significantly associated with ASD were CPF (OR: 1.1, 95% CI: 1.05-1.2) and diazinon (1.1, 1.01-1.2).

In the studies incriminating CPF, the biomarkers of exposure used were CPF itself in blood samples or TCPY in urine samples (Chen et al. 2017; Dalsager et al. 2019; Fortenberry et al. 2014; Guo et al. 2019). CPF-oxon has not previously been used to measure exposure to CPF, while it has been reported that CPF and CPF-oxon can be recoverable from urine, but mainly in case of very high exposure (Chlorpyrifos | Toxicological Profile | ATSDR). In vitro and animal studies have often studied this specific CPF metabolite, thus demonstrating that it is an active metabolite with anti-acetylcholinesterase activity and its own neurotoxicity (Cole et al. 2012; Jameson et al. 2007; Jiang et al. 2010). In addition, CPF-oxon is a mammalian-specific, nonenvironmental metabolite. Thus, its detection in human sample is evidence of direct contact with CPF, unlike TCPY, which can be metabolized in the environment. It should be noted that in our study, the strength of our evidence is limited due to the binary nature of our variable.

Detection of TCPY was relatively sparse in our study (9.5%), and the median of the detected values was 1.48 nmol/L, lower than in most studies. The detection percentage for TCPY in a Danish work that included pregnant women between 2010 and 2012 was 90.5% with a median concentration of 8.11 nmol/L (Dalsager et al. 2019), and in a Mexican study (including pregnant women in 1994–2005) 90%, with a median concentration of 8.87 nmol/L (Fortenberry et al. 2014). The low detection of TCPY in the PELAGIE cohort may explain why we were unable to find an association using this component alone, as in previous studies.

The lack of significant results for terbufos and its metabolites is consistent with the literature. None of the epidemiologic studies cited above associated this pesticide with neurodevelopmental toxicity. It has rather been thought to be involved in risks of carcinogenesis (Bonner et al. 2010) or infertility (Greenlee et al. 2004).

In 2008, shortly after inclusion ended in the PELAGIE cohort, OP insecticides accounted for 13% of global insecticide sales in France. The sale of insecticides did not decrease between 2009 and 2016, either in France or worldwide; 3 tons were sold in France in 2016 (Aillery et al.). European sales restrictions (e.g., for diazinon in 2007) have led to changing uses within the OP family, and the components measured in the PELAGIE cohort are no longer necessarily the majority substances on the market. In 2012, 207,296 kg of CPF were sold in France, compared with only 1,603 in 2019. In fact, the year before its sale became illegal in the EU. This ban is nonetheless not exhaustive outside the European Union, and CPF has been replaced by another OP insecticide: Phosmet. In 2012 in France, 3,553 kg of Phosmet were sold, compared with 243,796 kg in 2019. In 2021, the European Food Safety Authority (EFSA) published a report on Phosmet, reporting the same anti-acetylcholinesterase activity as other OP pesticides in studies of rats (European Food Safety Authority (EFSA) et al. 2021) and highlighting the lack of sufficient data on developmental and neurotoxic risks. Research on the developmental effects of exposure to OP pesticides is therefore still relevant, and the use of new pesticides and metabolites on the market will need to be monitored.

To conclude, we found a statistically significant association between prenatal maternal exposure to CPF and an increase in autistic traits (measured with the CAST score) for 11-year-old children, especially among boys. The detection of high concentrations of diazinon was also associated with a higher CAST score. These two OP insecticides might therefore be involved in a growing risk of autistic traits in children. These associations have already been suggested in the literature, with CPF implicated as a risk factor for autistic traits or neurodevelopmental disorders. Further work appears to be needed to explore the biological plausibility of the underlying pathophysiological mechanisms.

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6 Tables

Table 1: List of organophosphate insecticides and their metabolites screened in maternal urine samples, with their limit of quantification (LOQ) or detection (LOD) and molar mass.

MOLECULES	LOQ (measurement series in 2007-2008) µg/L	LOD (measurement series in 2013-2017) µg/L	Molar mass g/mol
DMP	0.2	0.06	126.048
DMTP	1	0.32	141.101
DMDTP	0.45	0.13	158.17
DEP	1.25	0.2	154.101
DETP	1.7	0.1	170.168
DEDTP	0.02	0.005	186.235
Diazinon°	0.001	0.001	304.35
Chlorpyrifos°	0.035	0.01	350.6
Chlorpyrifos-oxon	0.002	0.001	334.5
TCPY	0.15	0.042	198.43
Terbufos°	0.002	0.001	288.4
Terbufos-sulfone	0.01	0.003	320.4
Terbufos-oxon	0.009	0.003	272.4
Terbufos-oxon-sulfone	0.045	0.015	304.4
Terbufos-oxon-sulfoxide	0.03	0.01	288.4
Terbufos-sulfoxide	0.008	0.003	304.4

° parent molecules, LOQ: limits of quantification, LOD: limits of detection
DMP: dimethylphosphate, DMTP: dimethylthiophosphate, DMDTP: dimethyldithiophosphate, DEP: diethylphosphate, DETP: diethylthiophosphate, DEDTP: diethyldithiophosphate, TCPY: 3,5,6-trichloro-2-pyridinol

Table 2: Characteristics of the study population (n=185)

Characteristics	*n (%)/mean (SD)*	*Missing data n (%)*
Place of residence		0 (0)
Rural	122 (65.9)	
Semiurban	22 (11.9)	
Urban	41 (22.2)	
Parity before this pregnancy		0 (0)
No children	78 (42.2)	
At least 1 child	107 (57.8)	
Maternal age (in years)	30,5 (3.8)	0 (0)
Paternal age (in years)	33,5 (4.6)	9 (4.9)
Maternal education level		1 (0.5)
Primary / Secondary school	22 (12)	
Baccalaureate	28 (15.2)	
Postsecondary	134 (72.8)	
Maternal BMI (kg/m²)		1 (0.5)
<18.5	19 (10.3)	
18.5 – 25	130 (70.7)	
≥25	35 (19)	
Alcohol use in early pregnancy	20 (10.9)	2 (1.1)
Tobacco use in early pregnancy	39 (21.4)	3 (1.6)
Fruit and vegetable consumption in early pregnancy		1 (0.5)
< 2 servings per day	63 (34.2)	
≥2 servings per day	121 (64.8)	
Maternal diabetes during pregnancy	5 (2.8)	6 (3.2)
Maternal hypertension during pregnancy or preeclampsia	10 (5.5)	4 (2.2)
Folic acid supplementation	36 (19.5)	0 (0)
Term (Weeks of gestation)	39,3 (1.7)	0 (0)
<37	8 (4.3)	
Gender of the child		0 (0)
Male	95 (51.4)	
Female	90 (48.6)	
Birth weight (in grams)	3387 (512)	0 (0)
<10th percentile (FGR)	11 (5.9)	
Apgar ≤ 7 at 1 minute	8 (4.3)	2 (1.1)
Apgar ≤ 7 at 5 minutes	2 (1.1)	2 (1.1)
Neonatal hospitalization	13 (7)	0 (0)
Congenital malformation	7 (3.8)	0 (0)
Duration of breastfeeding (months)	4.3 (6.3)	8 (4.3)
CAST score (weighted)	5.3 (3.6)	0 (0)

n: sample size, SD: standard deviation, BMI: body mass index, FGR: fetal growth restriction.
Congenital malformations : auricular malformation (1), cryptorchidia (2), hypospadias (1), congenital hip luxation (2), syndactyly (1)

Table 3: Description of the urinary quantifications of organophosphate metabolites (n=185 for DAP, DM, DE; n=169 for chlorpyrifos, diazinon, terbufos, and their metabolites)							
Exposure variables	**Detection % (n)**	**PERCENTILES (nmol/L)**					
		Min	**25**	**50**	**75**	**95**	**Max**
DAP	88.6 (164)	0.17	8.03	33.31	81.85	260.79	3760.61
DM	82.2 (152)	0.15	4.99	24.09	73.81	258.45	1950.18
DE	49.0 (91)	0.01	<LOD	<LOD	3.87	42.12	123.77
Diazinon°	34.3 (58)	0.001	<LOD	<LOD	0.003	0.03	0.59
CPF°	12.4 (21)	0.04	<LOD	<LOD	<LOD	0.38	2.52
CPF-oxon	17.7 (30)	0.006	<LOD	<LOD	<LOD	0.06	1.89
TCPY	9.5 (16)	0.50	<LOD	<LOD	<LOD	1.19	27.18
CPF or CPF-oxon *	26.6 (45)						
CPF or CPF-oxon or TCPY *	32.5 (55)						
Terbufos°	6.5 (11)	0.02	<LOD	<LOD	<LOD	0.02	2.38
Terbufos-sulfone	6.5 (11)	0.03	<LOD	<LOD	<LOD	0.05	0.54
Terbufos-oxon	7.7 (13)	0.01	<LOD	<LOD	<LOD	0.08	0.34
Terbufos-oxon-sulfone	9.5 (16)	0.14	<LOD	<LOD	<LOD	0.53	2.11
Terbufos-oxon-sulfoxide	3.5 (6)	0.14	<LOD	<LOD	<LOD	<LOD	5.27
Terbufos-sulfoxide	8.9 (15)	0.03	<LOD	<LOD	<LOD	0.09	6.01
Terbufos or 1 metabolite *	26.6 (45)						

*° parent molecules, * composite variables (at least one component detected, binary variable)*
DAP: dialkylphosphate, DMP: dimethylphosphate, DEP: diethylphosphate,
TCPY: 3,5,6-trichloro-2-pyridinol, CPF: chlorpyrifos
Concentrations for DAP, DM, DE and diazinon have been standardized, DAP and DM are also imputed.

Table 4: Associations between the CAST score in 11-year-old children and prenatal maternal urinary concentrations of organophosphate metabolites.		
Exposure variables	**Minimal adjustment IRR (95% CI)**	**Complete adjustment IRR (95% CI)**
DAP n =185		
1st tertile	*Ref*	*Ref*
2nd tertile	0.95 (0.77, 1.18)	0.93 (0.75, 1.14)
3rd tertile	0.93 (0.75, 1.15)	0.91 (0.74, 1.13)
Continuous µmol/L	1.01 (0.75, 1.36)	1.06 (0.77, 1.45)
DM n=185		
1st tertile	*Ref*	*Ref*
2nd tertile	0.90 (0.73, 1.11)	0.93 (0.76, 1.15)
3rd tertile	0.91 (0.74, 1.12)	0.92 (0.74, 1.14)
Continuous µmol/L	0.75 (0.49, 1.12)	0.68 (0.43, 1.05)
DE n=185		
<LOD	*Ref*	*Ref*
≥LOD - < median	0.86 (0.69, 1.06)	0.88 (0.71, 1.09)
≥ median	0.93 (0.75, 1.14)	0.94 (0.76, 1.15)
Diazinon° n=169		
<LOD	*Ref*	*Ref*
≥LOD - < median	1.03 (0.80, 1.31)	1.11 (0.87, 1.42)
≥ median	1.25 (0.99, 1.57)	1.17 (0.94, 1.46)
Terbufos/metabolites n=160		
Not detected	*Ref*	*Ref*
Detected	1.03 (0.84, 1.25)	1.02 (0.84, 1.23)
CPF/Oxon n=169		
Not detected	*Ref*	*Ref*
Detected	1.29 (1.07, 1.57)	1.27 (1.05, 1.52)
CPF/Oxon/TCPY n=169		
Not detected	*Ref*	*Ref*
Detected	1.18 (0.98, 1.43)	1.19 (0.99, 1.42)
TCPY n=169		
Not detected	*Ref*	*Ref*
Detected	1.00 (0.74, 1.35)	1.04 (0.77, 1.39)

° Parent molecules °°5 specific metabolites of terbufos: terbufos-sulfone, terbufos-oxon, terbufos-oxon-sulfone, terbufos-oxon-sulfoxide and terbufos-sulfoxide
DAP: dialkylphosphates, DM: dimethylphosphates, DE: diethylphosphates, CPF: chlorpyrifos, oxon: chlropyrifos-oxon, TCPY: 3,5,6-trichloro-2-pyridinol, LOD: limit of detection, IRR: incidence rate ratio, 95% CI: 95% confidence interval
Minimal adjustment variable: urinary creatinine
Complete adjustment variables: urinary creatinine, maternal education level, maternal BMI, maternal hypertension or preeclampsia, fruit and vegetable consumption at the beginning of pregnancy, duration of breastfeeding, sex of the child.
Analysis used a negative binomial regression

7 Figure

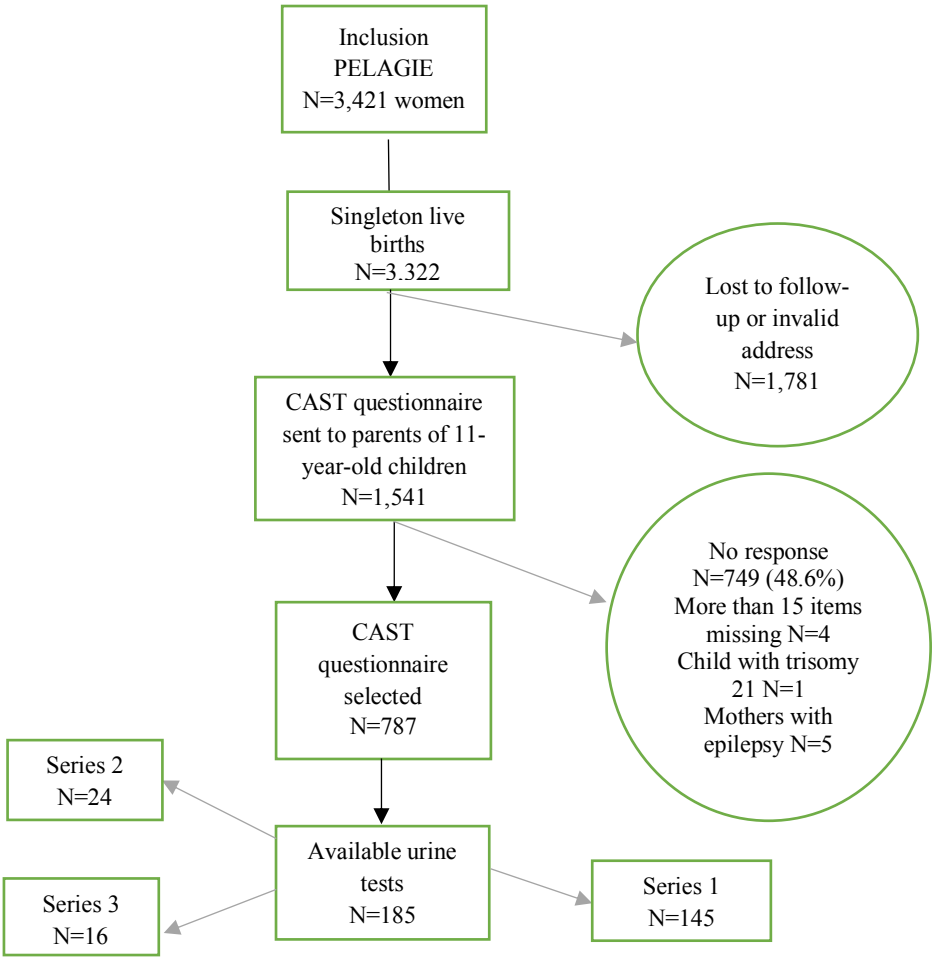

Figure 1: Flowchart from inclusion in PELAGIE cohort (n=3,421) to our study population (n=185).