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Authors

Schmidt, Rebecca J

Kogan, Vladimir

Shelton, Janie F

et al.

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Combined Exposures to Prenatal Pesticides and Folic Acid Intake in Relation to Autism Spectrum Disorder

Rebecca J. Schmidt, Ph.D.,[1,2] Vladimir Kogan, M.S.,[3] Janie F. Shelton, Ph.D.,[1] Lora Delwiche, M.S.,[1] Robin L. Hansen, M.D.,[2,4] Sally Ozonoff, Ph.D.,[2,5] Claudia C. Ma, M.S., M.P.H.,[6] Erin C. McCanlies, Ph.D.,[6] Deborah H. Bennett, Ph.D.,[1] Irva Hertz-Picciotto, Ph.D., M.P.H.,[1,2] Daniel Tancredi, Ph.D.,[4] Heather E. Volk, Ph.D., M.P.H.[3]

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

[2]Medical Investigation of Neurodevelopmental Disorders (MIND) Institute, University of California Davis, Sacramento, California, USA

[3]Departments of Preventive Medicine and Pediatrics, Zilkha Neurogenetic Institute, Keck School of Medicine, Children's Hospital Los Angeles, University of Southern California, Los Angeles, California, USA

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

[5]Department of Psychiatry and Behavioral Sciences, University of California Davis School of Medicine, Davis, California, USA

[6]National Institute for Occupational Safety and Health, CDC, Morgantown, West Virginia, USA.

CORRESPONDING AUTHOR:

Dr. Rebecca J. Schmidt

24123 Medical Sciences 1C

25One Shields Ave

26Davis, California 95616-8638

27(530) 752-3226 phone

28(530) 752-3239 fax

29rjschmidt@ucdavis.edu

30

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Vladimir Kogan	vkogan@usc.edu
Janie Shelton	jfshelton@ucdavis.edu
Lora Delwiche	llddelwiche@ucdavis.edu
Robin L. Hansen	rlhansen@ucdavis.edu
Sally Ozonoff	sozonoff@ucdavis.edu
Claudia C. Ma	iia4@cdc.gov

Erin C. McCanlies eim4@cdc.gov

Irva Hertz-Picciotto ihp@ucdavis.edu

Daniel Tancredi djtancredi@ucdavis.edu

Heather E. Volk hvolk1@jhu.edu

ABSTRACT

Background:

Maternal folic acid (FA) protects against developmental toxicity from certain environmental chemicals.

Objective:

To examine combined exposures to maternal FA and pesticides, in relation to autism spectrum disorder (ASD).

Methods:

Participants were California children born 2000-2007, enrolled in the CHARGE case-control study at age 2-5 years, clinically confirmed to have ASD (*n*=296) or typical development (*n*=220) and had information on maternal supplemental FA and pesticide exposures. Maternal supplemental FA and household pesticide product use were retrospectively collected in telephone interviews from 2003-2011. Mothers' addresses were linked to a statewide database of commercial applications to estimate agricultural pesticide exposure.

Results:

Above median FA intake (≥ 800µg) during the first pregnancy month and no known pesticide exposure was the reference group for all analyses. Compared with this group, ASD was increased in association with <800µg FA and any indoor pesticide exposure (adjusted OR=2.5; 95% CI: 1.3, 4.7) compared to low FA (OR=1.2; 95% CI: 0.7, 2.2) or indoor pesticides (OR=1.7; 95% CI:

1.1, 2.8) alone. ORs for the combination of low FA and regular pregnancy exposure (6+ months) to pet pesticides or outdoor sprays and foggers were 3.9 (1.4, 11.5) and 4.1 (1.7, 10.1), respectively. ORs for low maternal FA and agricultural pesticide exposure 3 months before or after conception were: 2.2 (0.7, 6.5) for chlorpyrifos, 2.3 (0.98, 5.3) for organophosphates, 2.1 (0.9, 4.8) for pyrethroids, and 1.5 (0.5, 4.8) for carbamates. Except for carbamates, these ORs were about two times greater than those for either exposure alone, or for the expected multiplicative or additive combined ORs.

Conclusions:

In this study population, associations between pesticide exposures and ASD were attenuated among those with high versus low FA intake during the first month of pregnancy. Confirmatory and mechanistic studies are needed.

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairments in social reciprocity and communication, and repetitive behaviors and/or restricted interests. ASD prevalence in the United States has increased over the past decade and is currently estimated to affect 1:68 children (Centers for Disease Control and Prevention 2016). Several epidemiologic studies have reported a reduced likelihood of ASD and autistic traits in children whose mothers took supplements containing folic acid (FA) near the time of conception and reduced risk for ASD and autistic traits (Braun et al. 2014a; Schmidt et al. 2011; Schmidt et al. 2012; Steenweg-de Graaff et al. 2014; Suren et al. 2013), yet not all studies have observed this association (Virk et al. 2015). Our previous work suggested that only genetically susceptible individuals (mothers and children with less efficient folate-dependent one-carbon metabolism genes) experienced reduced risk for ASD associated with maternal FA intake (Schmidt et al. 2011; Schmidt et al. 2012). Under the paradigm that autism etiology is multifactorial, we hypothesize that there are environmentally susceptible individuals that may experience an enhanced benefit from reduced ASD risk in association with maternal periconceptional FA intake; i.e., that nutrient status can modify risks associated with other environmental agents. Pesticides are neurotoxic by design (Rosas and Eskenazi 2008), and associations have been reported between ASD diagnoses or symptoms and organochlorine, organophosphate, and pyrethroid pesticide exposures during pregnancy (Braun et al. 2014b; Eskenazi et al. 2007; Keil et al. 2014; McCanlies et al. 2012; Roberts et al. 2007; Roberts and English 2013; Shelton et al. 2014). In animal studies, FA has been shown to protect against effects resulting from developmental exposure to a variety of environmental chemicals, including methomyl insecticide on reproductive outcomes in male rats (Shalaby et al. 2010) and effects of bisphenol A (BPA) on DNA methylation in mice (Dolinoy et al. 2007). To our knowledge, no previous study has

examined whether associations between pesticides and neurodevelopmental outcomes in children are modified by maternal FA intake. The goal of the present study was to be first to examine associations between ASD and combined exposures of maternal FA intake and pesticides, with the hypothesis that children with combined exposure to pesticides and low maternal periconceptional FA would have a greater risk of ASD than children with developmental exposure to pesticides and high maternal periconceptional FA or children with low FA and no pesticide exposure.

Methods

Study Design and Population

Interview data and biological specimens for this ongoing study were obtained from participants of the ongoing Childhood Autism Risks from Genetics and the Environment (CHARGE) population-based case-control study enrolled as described previously (Hertz-Picciotto et al. 2006). Eligible children include those between the ages of 2 and 5 years, born in California, living with at least one biologic parent who speaks English or Spanish, and residing in the catchment areas of a specified list of California Regional Centers that coordinate services for persons with developmental disabilities. Children with autism are identified through the California Regional Center System and general population controls are identified from state birth files and are frequency matched to the expected age, sex and catchment area distribution of the autism cases. Children with confirmed diagnoses were included in the present analyses if their mothers completed the original exposure questionnaire prior to November 2011, when revisions impacting diet and supplement data collection were implemented. Due to low enrollment of controls in the beginning of the study (from 1997 until 1999) and only three controls (no cases)

born in 2008 who completed the original questionnaire, only children born between 2000 and 2007 were used in analyses. The CHARGE Study protocol was approved by institutional review boards at the University of California, Davis, and the University of California, Los Angeles, and by the State of California Committee for the Protection of Human Subjects. Written informed consent was obtained before participation.

Diagnostic Classification

All children were assessed for cognitive function using the Mullen Scales of Early Learning (MSEL) (Mullen 1995) and for adaptive function using the Vineland Adaptive Behavior Scales (VABS) (Sparrow et al. 1984). The children of families recruited from the general population were screened for evidence of ASD using the Social Communication Questionnaire (SCQ) and if they scored above 15, they were evaluated for ASD, and if diagnosed they were included as cases. Children sampled from the general population were defined as typically developing (TD) controls if they received a score ≤ 15 on the SCQ and scored in the normal range on the MSEL and VABS, thereby showing no evidence of other types of cognitive or adaptive delays.

Diagnoses of ASD were confirmed by study personnel using the Autism Diagnostic Interview–Revised (ADI–R) (Lord et al. 1994; Lord et al. 1997), and the Autism Diagnostic Observation Schedule–Generic (ADOS–G) (Lord et al. 2000, 2003). ASD was defined by the ASD2 criteria of Risi et al. (2006) as meeting criteria a) on Social and Communication domains of the ADI-R prior to 36 months, b) on Social and within 2 points of Communication domain criteria on the ADI-R, c) on Communication and within 2 points on the Social domain criteria on the ADI-R or d) within 1 point on both Social and Communication domains on the ADI-R prior

to 36 months, and above the Social + Communication cutoff for ASD on the ADOS (Risi et al. 2006).

Exposure Measurement

Exposures in the CHARGE study were obtained through telephone interviews for the period 3 months prior to conception until the time of the interview (when the child was aged 2-5 years old). This study focuses on exposures during the index period, defined as the three months prior to conception, and during pregnancy. The date of conception was calculated by subtracting gestational age (reported by mothers) from the child's date of birth.

Maternal FA Intake

Maternal intake of FA and other nutrients were determined using data collected through telephone interviews on intake of multivitamins, prenatal vitamins, nutrient-specific vitamins, cereals, and other fortified foods or supplements (i.e., breakfast shakes and protein bars), for each month of the index period as described previously (Schmidt et al. 2012; Schmidt et al. 2014). Data included whether or not each item was consumed, and if so, the brand, dose, frequency and months consumed. From this information, we calculated a value of each nutrient for each product, and summed these into a total average value for each month for each woman. Nutrient amounts assigned to products were as reported by the manufacturer, or if this is not available, a standard amount was assigned based on the amount most commonly found in similar products. Total supplemental intake was quantified for the following nutrients: FA, vitamin B12, vitamin B6, vitamin D (ergocalciferol or cholecalciferol), calcium, iron, vitamin A (beta-carotene, retinol), vitamin E, and vitamin C. Total intake of choline, betaine, and zinc was quantified from sources with the information available. Total average FA intake (from all supplements and fortified sources) in the first month of pregnancy was the primary variable used

for all analyses below, given this month was most strongly associated with reduced ASD previously (Schmidt et al. 2012). Vitamins B6 and B12 in the first month were also explored for interaction with pesticide exposures, and confounding effects; the other nutrients were examined as potential confounders. Supplemental nutrient intake was quantified for all participants with interviews through November 2011, when the CHARGE questionnaire was modified.

Household Pesticide Exposure

The CHARGE parental telephone interview asked regarding the 3 months before pregnancy with the index child until the time of interview, “Did you or anyone in your household use…?” Items included: flea or tick soaps or shampoos on pets; sprays, dusts, powders or skin applications for fleas or ticks on pets; professional pest control or extermination; ant, fly or cockroach control products; and indoor foggers. Further questions addressed product type (spray, bait, etc.), brand name, whether the application was indoors, outdoors, or on a pet, and use of professional pest control services. We also obtained timing of pesticide use and combined product types to assign exposure by time period; however numbers of exposed were too small to examine combined exposure associations by specific timing in this study and exposure during the whole pregnancy period was used. Use of pesticide-containing poisoned bait containers were not included as they have a small surface area of pesticide, which would result in low volatilization, and thus limited exposure. Similarly, our primary analyses of indoor pesticides excluded use of flea and tick pet collars because of their limited release of pesticides into the environment; however, additional analyses were conducted including them in the ‘any indoor pesticides’ variable.

Commercial Agricultural Pesticide Exposure

The CHARGE study catchment area includes the northern part of the California Central Valley, a dense agricultural region with heavy pesticide usage, as well as urban and suburban areas surrounding Sacramento and parts of the San Francisco Bay Area. Commercial pesticide applicators in California are required to report to the Department of Pesticide Regulation the type, amount of active ingredient (in pounds), location, and application type (i.e. aerial, ground, ground injection) of every agricultural pesticide used. Pesticide use reports (PUR) are publically available for download by year {www.cdpr.ca.gov/docs/pur/purmain.htm}. The PUR data are available down to 1-square mile units known as the meridian township range section (MTRS), a parceling by the U.S. Geological Survey for the whole country. Thereby, each application is linked to each and every MTRS where it is applied.

Compounds recorded in the PUR database are identifiable by unique product codes, which we cross-linked with registration records from the Environmental Protection Agency (http://www.pesticideinfo.org/Search_Chemicals.jsp) to sort into chemical classes (e.g. organophosphate, pyrethroid, etc.).

Utilizing the address history data recorded for CHARGE study participants, we geocoded each address from 3 months prior to conception, by day, through delivery. Overall, 99% of addresses were successfully geocoded to obtain a longitude and latitude with a match of at least 80 percent in ArcMap (ArcGIS v10.0; ESRI) using the U.S. Rooftop search algorithm. Unmatched addresses were manually matched to the most likely address. For each day of pregnancy, the home was assigned to the MTRS in which it is located. For example, if a mother moved on day 46 of her pregnancy, the MTRS code would change from the previous home to her new home on day 47. This allowed correct addresses to be captured for women at each time period for the 1 in 5 participants that moved during their pregnancy.

Using a spatial model developed in ArcGIS, for each day, a circular buffer was drawn around each home with a radius of 1250, 1500, and 1750 meters. If the buffer intersected the centroid (center most point) of an MTRS where pesticides had been applied, the type and amount were linked to the home as a proximal exposure. This model generated an exposure profile by day of pregnancy. All records with no exposure identified were assigned to zero pounds applied. The daily exposure profile was then aggregated into time periods of interest for analysis, such as months and trimesters; for this study we used the 6-month period beginning 3 months prior to conception through the end of the third month of pregnancy (end of first trimester) to be consistent with the timing in the 1st month of pregnancy when FA intake is most associated with reduced likelihood of ASD, and would be most likely to modify the association between pesticides and ASD. In explorative analysis, we also examined exposure during all of pregnancy. Because two-thirds of participants experienced no pesticide applied within this proximity to their homes, analyses were conducted using binary variables for those “exposed” and “unexposed.”

Occupational Pesticide Exposure

Parental occupational history information was collected during the CHARGE telephone interview. Occupational information included the place of employment, month and year of employment, which month(s) of pregnancy (or the postnatal period) the job was held, and the total hours worked at each job. This data was sent to the National Institute for Occupational Safety and Health (NIOSH) for analyses. Each job reported was assigned a North American Industry Classification System (NAICS) (U.S. Census Bureau 2007) and 2000 Standard Occupational Classification (SOC) (U.S. Census Bureau 2000) code. Occupational exposures were estimated qualitatively by two experienced industrial hygienists based on the NAICS and SOC codes as well as parents’ job history information, duties, tasks, and responsibilities. The

industrial hygienists independently assigned a qualitatively defined ordinal exposure level estimate to a selected list of chemical and physical agents including pesticides (insecticides, fungicides, and rodenticides) for each job (McCanlies et al. 2012). They were blinded to the children's case status (ASD or TD). After the industrial hygienist independently estimated exposure levels, they compared their estimates, any differences were discussed and a consensus on the estimated exposure levels determined. Based on the information provided in the database for each job, a code of 0 (none), 1 (exposure above background levels; no more than a few days per year), 2 (most likely exposed; exposure was unlikely to be daily), or 3 (definitely exposed; frequent or routine exposure) was entered to estimate both the frequency and intensity for each of the agents of interest. We only used the pesticide data for the current study. Few mothers had occupational exposure to pesticides during pregnancy or the 3 months before pregnancy. Therefore, we dichotomized occupational pesticide exposure during this period as regular vs. none or some, and only included occupational exposure with household and agricultural pesticide exposures when classifying women as having 'any pesticide' exposure, rather than analyzing it as a separate exposure.

Statistical Analysis

FA intake and prenatal pesticide exposures were dichotomized and evaluated separately and as combined four-level exposure variables (FA <800 µg and pesticide exposure, 800+ FA and pesticide exposure, and FA <800 µg and no pesticide exposure compared with FA 800+ and no pesticides as a common reference group) in logistic regression models with ASD vs. TD as the outcome. Several time intervals were considered for pesticide exposures using the information on the period from 3 months prior to conception through the end of pregnancy, with the primary

time of interest being exposure in the months near conception. Separate models were fitted for each time interval and pesticide class.

Total FA summed from all available sources (vitamins, supplements, cereals, etc.) in the first month of pregnancy (the time period during which FA was most strongly associated with ASD in this population (Schmidt et al. 2012)) was dichotomized as above or below 800 µg (the amount in most prenatal vitamins and the median for controls). We also examined combined associations when dichotomizing at 600 µg FA, the dietary reference intake for pregnancy (Institute of Medicine. Food and Nutrition Board 2000).

Household pesticides were classified as separate binary indicators (no exposure versus any) and when numbers allowed (with all cell sizes ≥ 5), we examined exposure by frequency defined as regular use (occurring in 6 or more months of pregnancy), some use (in less than 6 months of pregnancy) or no exposure (reference group). Regular use was examined separately given it would deliver a greater exposure than sporadic use, and would be more likely to include a susceptible time period if the fetus was not susceptible during the entire pregnancy. Additionally, in previous analyses of the association between household pesticides and ASD in CHARGE participants, associations were found primarily for regular users. Thus for this study regular exposure was considered ‘exposed’. Pesticide types included use of any flea products on indoor pets during pregnancy, and use of any professional or self-applied sprays or foggers indoors or outdoors during pregnancy. Pet flea and tick products were examined separately from indoor sprays and foggers to assess independent associations in combination with FA intake, but because effect estimates of these different types of pesticides were in the same direction, they were also examined in combination (any vs. no exposure to either type) for increased power.

Carbamate, organochlorine, organophosphate, and pyrethroid agricultural pesticides were measured at buffer distances of 1250, 1500, and 1750 meters around the residence. Commercial agricultural pesticide exposures were categorized into two levels representing any vs. no pesticide application in the specified area for the chosen prenatal time interval. We chose to use the 1250 m buffer distance for our primary analyses to reflect the most proximal exposure, and conducted sensitivity analyses using the 1500 and 1750 m buffers.

Potential confounders were identified by considering elements that may influence one's exposure to pesticides or FA supplements and risk for autism, especially attributes pertaining to socioeconomics such as home ownership and mother's education as these were confounders for associations between FA intake and ASD and between pesticides and ASD when their main effects were examined independently within the same parent study (Schmidt et al. 2012; Shelton et al. 2014). Other variables considered as potential confounders included maternal and paternal age, maximum education of parents, home ownership, type of insurance at delivery, maternal birthplace, education, smoking in 3 months before or during pregnancy, intention of getting pregnant when she did, intake of vitamins B6 and B12 from supplements in the first month of pregnancy, and child's sex, race/ethnicity, and year of birth. Changes of ten percent or greater in the beta estimates for the effects of interest (the doubly exposed category) were used as the criterion for confounder inclusion, both when each potential confounder was evaluated by itself, and when each was removed from a full model.

For each FA-pesticide exposure combination, we used the Akaike Information Criterion (AIC), a complexity-adjusted goodness-of-fit measure (Burnham et al. 2002), to compare the model with the two binary exposure variables (for pesticides and FA intake) as main effects versus the model with the four-level combined exposure classification, which is equivalent to

adding an interaction term to the main effects model. Expected joint effects under an additive model were calculated by adding the ORs of the groups with only one exposure and subtracting 1. Expected joint effects under a multiplicative model were calculated by multiplying the ORs of the groups with only one exposure. In addition, the relative excess risk due to interaction (RERI) and 95% CIs were calculated using the "ic" package in Stata Version 12.0 (Andersson et al. 2005; Hosmer and Lemeshow 1992). All other statistical analyses were performed using SAS software version 9.3 (SAS Institute Inc., Cary, North Carolina). Associations with vitamin B6-pesticide, vitamin B12-pesticide, and FA/vitamin B6-pesticide exposure combinations were evaluated in the same manner. Complete case analyses were conducted for all associations.

Results

Case and Control Characteristics and Exposure Frequencies

Of the 806 (466 ASD and 340 TD) participants born 2000 – 2007 and whose mothers were interviewed by November 2011, data on FA intake in the first month was available for 394 (85%) ASD and 282 (83%) TD; indoor pesticide exposure was available for 409 (88%) ASD and 303 (89%) TD; outdoor household pesticide exposure was available for 402 (91%) ASD and 303 (89%) TD; agricultural pesticide exposure was available for 428 (92%) ASD and 310 (91%) TD; and occupational pesticide exposure was available for 343 (74%) ASD and 255 (75%) TD (**Table 1**). Participants who had information available on both folic acid intake in the first month of pregnancy and at least one of the pesticides studied included 296 (64%) ASD and 220 (65%) TD (**Table 1**). Regardless of availability of folic acid and pesticide exposure information, case children were more likely to be born in the first years of the study compared to controls, and mothers of children with ASD were less likely to own their home than mothers of TD children

(**Table 1**). Parents of children with ASD were less likely to report taking 800 µg or more FA in the first month of pregnancy, and more likely to report any exposure to indoor household pesticides during pregnancy and any pesticide exposure (**Table 1**). For ASD and TD with interviews prior to Nov 2011, mothers of children with ASD were more likely to have vitamin B6 intake above the median in the first pregnancy month than mothers of TD, but this difference did not reach significance in the sample with folic acid and pesticide data. For those with folic acid and pesticide data, household outdoor pesticide exposure was significantly more common among mothers of children with ASD compared to mothers of TD children.

Household Pesticide Exposure by Maternal FA Intake

Home ownership, child's year of birth, and maternal vitamin B6 and vitamin D (natural log) intake in the first pregnancy month met confounder criteria and were thus included as adjustment variables in all models. Overall, adjusted ORs for ASD tended to be highest when mothers were exposed to pesticides and reported taking less than 800 µg FA in the first month of pregnancy in comparison with all other groups (**Figure 1**). Compared to women with above-median FA intake (800+ µg) during the first month of pregnancy and no indoor pesticide exposure, women with below-median FA intake and regular exposure to indoor sprays and foggers were more likely to have a child with ASD (OR=2.6, 95% CI: 1.3, 5.2) than those with either low FA (OR=1.3, 95% CI: 0.8, 2.3) or regular exposure to indoor sprays and foggers alone (OR=1.9, 95% CI: 1.1, 3.3) (**Table 2**). Similarly, women with below-median FA and regular exposure to pet flea and tick products were associated with higher risk of having a child with ASD (OR=3.9, 95% CI: 1.4, 11.5) than those with either low FA (OR=1.4, 95% CI: 0.8, 2.3) or regular exposure to pet flea and tick products alone (OR=1.6, 95% CI: 0.9, 3.1). Women with the combination of below-median FA intake and exposure to any indoor pesticides were associated

with elevated risk of having a child with ASD (OR=2.5, 95% CI: 1.3-4.7) compared to those with no exposure and high FA intake, which was greater than those exposed who had above-median intake (OR=1.7, 95% CI: 1.1-2.8). Finally, regular exposure to outdoor sprays and foggers in combination with lower FA was associated with elevated estimated risk (OR=4.1, 95% CI: 1.7, 10.1) that was over twice that of those with above-median FA intake and regular pesticide exposure, again compared with the lowest risk group (OR=1.8, 95% CI: 0.8-4.0). All ORs for the doubly exposed were greater than expected by additive or multiplicative models, with ORs from slightly greater, to over twice as great (**Table 2**). Inclusion of additional covariates produced similar results with generally increased ORs in all categories, and ORs for the doubly-exposed category that were greater than expected for most pesticide types (**See Tables S1-S2**). Effect estimates were similar but slightly attenuated in additional analyses including flea and tick collars (**See Table S3**). Results followed similar patterns when dichotomizing FA at 600 µg (**See Table S4 and Table S5**).

Agricultural Pesticide Exposure by Maternal FA Intake

The joint OR for low maternal FA intake and exposure to any agricultural pesticides 3 months before or after conception was: 2.0 (0.9, 4.2) which was greater than the OR for low FA intake and no pesticide exposure: 1.2 (0.7, 2.1) or the OR for high FA and pesticide exposure: 1.0 (0.6, 1.8). ORs for the combination of low maternal FA intake and exposure to individual agricultural pesticides 3 months before or after conception were: 2.2 (0.7, 6.5) for chlorpyrifos, 2.3 (0.98, 5.3) for organophosphates, 1.7 (0.8, 3.7) for pyrethroids, and 1.3 (0.4, 4.0) for carbamates (**Table 3, Figure 1**). Except for carbamates, these non-significant ORs were greater than those for agricultural pesticide exposure with higher FA intake or low FA with no pesticide exposure and were greater than expected by additive or multiplicative models. Results were

similar when examining agricultural pesticide exposure for pregnancy rather than in the peri-conceptional months (**See Table S6**). Results using the 1500 m buffer showed a similar pattern for greater, but slightly attenuated ORs in the combined low FA plus pesticide category; this pattern was only observed for chlorpyrifos when using the 1750 m buffer (**See Tables S7, S8**).

Only for agriculturally applied organophosphate pesticides was the AIC for the model with an interaction term between maternal first month FA intake and pesticide exposure less than the AIC for the model without an interaction term, indicating a better fitting model; for all other pesticide exposures, the model without an interaction term was the better fitting model (**See Table S9**). Maternal intake of vitamins B12 and B6 was highly correlated with maternal FA intake from supplements, and results for combinations of high (above median) and low vitamin B12/B6 in combination with pesticide exposures were relatively similar to those with FA, with greater ORs for doubly exposed than expected, but less consistency across types of pesticides (**See Tables S10-S13**). Because FA and vitamin B6 intake were correlated and each met criteria as a confounder for the other with similar patterns of when combined with pesticide exposure, we also examined joint associations of low (below median) maternal FA and vitamin B6 compared to either high maternal FA or vitamin B6 intake in combination with each pesticide; results were similar with regard to the observed combined exposure category having higher ORs than expected, with consistently higher ORs in all categories (**See Tables S14-15**).

<u>Occupational Pesticide Exposure</u>

Five (1.5%) of 343 mothers of children with ASD and 5 (2.0) of 255 mothers of children with TD had occupational pesticide exposure. Mothers of children with ASD were more likely than mothers of TD children to be classified with frequent/regular occupational exposure to

pesticides, with (n=4, 80% of exposed, compared to 1 (20% of exposed) regularly exposed. Because numbers exposed were so low, we did not examine occupational pesticide exposure separately in combination with FA, but the OR for joint exposure (to low FA and exposure to pesticides) in analyses including regular occupational exposure in combination with household or agricultural pesticide exposure (as any pesticide exposure) of 1.7 (0.8, 3.5) was attenuated in comparison with the OR of 2.1 (1.1, 4.1) for any pesticide exposure without regular occupational exposure, and only slightly greater than expected by multiplicative (1.6) or additive models (1.5) (**See Table S16.**).

Discussion

In this California study population, we found that associations between household and agricultural pesticide exposures and ASD in the child were reduced among women with higher (800+ mg/day) FA intake near the time of conception compared to associations among women with lower intake. This study provides the first evidence to our knowledge for attenuation of the association between gestational pesticide exposures and ASD by maternal FA intake. These findings are congruent with both human and animal studies demonstrating maternal FA's ability to alter effects of environmental toxicants on the developing offspring. In a prospective cohort study of 291 women in China, maternal pre-conception serum folate and B-vitamin sufficiency was shown to protect against adverse reproductive effects of 1,1,1-trichloro-2,2,bis(p-chlorophenyl)ethane (DDT) exposure (Ouyang et al. 2014). Human studies suggest that FA might reduce the potency of other contaminants, including arsenic, a potent neurotoxicant contained in a few pesticides unlikely to be captured in this study. In a double-blind placebo-controlled randomized trial of 200 adults, FA supplementation in highly arsenic-exposed

individuals appeared to enhance arsenic methylation, which may reduce its toxicity (Gamble et al. 2006). Another double-blind placebo-controlled randomized trial of over 600 adults in Bangladesh suggested higher doses of FA (800 µg/day) were needed to reduce blood arsenic concentration in populations containing folate replete individuals (Peters et al. 2015). Notably, a recent study of 57 cases and 55 controls in Bangladesh showed that 1st trimester inorganic arsenic exposure also significantly reduced protective effects of FA supplementation against neural tube defects (Mazumdar et al. 2015) suggesting that higher doses of FA might be needed to provide neuroprotection in those exposed to environmental contaminants.

Although non-causal explanations for the reduction ASD risk in association with pesticide exposures by FA cannot be ruled out, one can speculate that potential mechanisms could involve folate's antioxidant properties (Joshi et al. 2001), its role in DNA repair (Duthie 1999; Duthie et al. 2004), or its influence on DNA methylation (James et al. 2004; James et al. 2009) as shown in **Figure 2**. Folate's role as a major methyl donor could be relevant given that all other proposed pathways could lead to depletion of methyl groups necessary for DNA methylation (**Figure 2**), which could be critical near conception when the methylome is de-methylated and then re-established (Reik et al. 2001). Vitamin B6 also contributes to this one-carbon methylation pathway. Methylation pathways were proposed to explain reduced male reproductive effects of exposure to the insecticide methomyl in rats receiving FA (Shalaby et al. 2010) and maternal folate supplementation was shown to prevent effects of developmental exposure to BPA on DNA methylation in mice (Dolinoy et al. 2007). Evidence in human studies has suggested folic acid might alter susceptibility to arsenic toxicity through methylation pathways (Howe et al. 2014; Lambrou et al. 2012). In addition, a recent crossover study of 10 adults reported that changes in DNA methylation following 2 hours of controlled exposure to

PM2.5 were not observed in the same 10 loci when PM2.5 exposure followed four weeks of B vitamin supplementation, including high doses of FA (2.5 mg/d) and vitamin B6 (50 mg/d) (Zhong et al. 2017).

Methylation pathways are also congruent with studies providing evidence for altered DNA methylation linked to exposure to several types of pesticides (reviewed by Collotta et al. (2013)). This evidence includes associations between low-dose exposure to organochlorine pesticides and global DNA hypomethylation estimated by the percent 5-methyl-cytosine (%5-mC) in Alu and LINE-1 assays in 86 healthy Koreans (Kim et al. 2010), and a significant inverse linear relationship between plasma concentrations of DDT, DDE, -and other persistent organic pollutants (POPs) and blood global DNA methylation estimated in Alu repeated elements in 71 Greenlandic Inuit with high POP levels (Rusiecki et al. 2008). Maternal self-reported pesticide exposure was linked to placental DNA methylation changes using whole genome bisulfite sequencing in a cohort of 47 mothers of children with ASD (Schmidt et al. 2016). In rats, DDT exposure altered the methylation pattern in DNA extracted from the hypothalamus of young male rats, with significant hypomethylation of CpG islands in 6 genes compared with controls (Shutoh et al. 2009). Evidence for DNA methylation effects have also been observed for non-persistent pesticides, like organophosphates (Zhang et al. 2012a; Zhang et al. 2012b). Oxidative stress is another potential mechanism that could be induced by a variety of classes of pesticides and could be attenuated with folic acid through several pathways as shown in **Figure 2**. The reasons for a lack of FA attenuation of the association for carbamates are unclear, but could result from alternate mechanisms for this particular pesticide class.

<u>Study Limitations and Strengths</u>

A major limitation of this study was the reliance on self-reported FA and household pesticide exposure and the potential for recall bias to explain the observed associations, at least in part. For the higher OR in the group with combined exposure to be explained by recall bias, case mothers would have had to both under-report FA intake and over-report pesticide exposures. However, FA intake that was self-reported during pregnancy also was associated a reduced risk of ASD (n = 270 cases) in a prospective cohort of >85,000 Norwegian women (Suren et al. 2013). In addition, self-reported household pesticide use has been shown to be reliable in a case-control study of cutaneous melanoma in men and women of all ages living in Rome (163 cases and 113 controls) given the same pesticide questionnaire about a year apart (Fortes et al. 2009) and valid in 185 older male orchardists in Washington state recalling information 20-25 years later (Engel et al. 2001). Finally, patterns of associations with agricultural pesticide exposures, which were not self-reported, were similar to those for self-reported exposures in combination with FA.

For the household pesticide analyses, we combined all pesticide classes together; by not examining interaction effects by each pesticide type (e.g. pyrethroids) it is possible that individual effects of some pesticide types were diluted. Additionally, too few women were exposed to certain classes of agricultural pesticides, including organochlorines that have previously been linked to ASD, to produce stable estimates. Thus, interactions between FA and some specific pesticides could not be evaluated. However, the classes of pesticides examined – chlorpyrifos, organophosphates, pyrethroids, and carbamates, include several that are among the most widely prevalent exposures in the U.S.

Missing data was a limitation of our analyses. Though 88-92% had data available for each pesticide exposure other than occupational pesticide exposure, and 84% had data on folic

acid intake, when examining folic acid and pesticide exposure in combination, a high percentage (24-32%) of participants were missing data on one exposure or the other. Missing data was particularly an issue for occupational pesticide exposure where 36% cases and 38% controls were missing data. Though missingness appeared non-differential across case status, there was potential for bias due to missing data if the missingness was informative.

In addition, very few mothers in our study population reported occupations that were likely to result in regular pesticide exposure in the 3 months before and during pregnancy. Consequently, we were unable to thoroughly evaluate interactions between maternal FA intake and occupational pesticide exposure independently. Further, the strongest associations between household pesticides and ASD, and where we observed the greatest attenuation of ORs by FA, were for mothers with regular exposure during pregnancy, but we were unable to examine associations by frequency for all pesticide exposures and estimates for pesticides classified as 3-level exposures were imprecise due to small numbers of observations.

This study collected information on and evaluated numerous factors as potential confounders of the joint association of FA and pesticide exposures in relation to ASD, including most ASD risk factors identified in previous studies. ORs for the doubly-exposed category remained greater than expected for most pesticide types in full models adjusting for additional factors that did not meet criteria as confounders. However, confounding by other unmeasured factors is possible.

Strengths of this study include its extensive collection of environmental data to allow the examination of exposure combinations. Few other autism studies have collected information on nutrient intake and pesticide exposures, including timing and dose, on a large enough number of

participants to allow examination of their combined effects. In addition, this study included clinically-confirmed diagnostic classification using gold-standard standardized assessments.

Public Health Implications

Use of indoor and outdoor pesticides around the household was commonly reported in our study. Based on previous studies linking maternal pesticide exposure to ASD or other adverse neurodevelopmental outcomes (Braun et al. 2014b; Eskenazi et al. 2007; Keil et al. 2014; McCanlies et al. 2012; Roberts et al. 2007; Roberts and English 2013; Shelton et al. 2014) and our results demonstrating that many maternal pesticide exposures were significantly associated with ASD even among women with high FA intakes, we would recommend that mothers avoid household pesticide use during pregnancy. However, it is more difficult to avoid agricultural pesticide exposures. In our California-based case-control study, children of women who were exposed to pesticides during pregnancy were less likely to be diagnosed with ASD if their mothers had high vs. low FA intake. Overall, our findings support the beneficial effects of FA supplementation during pregnancy.

Conclusion

These findings suggest that supplemental FA taken during the first month of pregnancy could potentially reduce, but not eliminate, the increased risk of ASD associated with maternal pesticide exposure before and during pregnancy. Larger studies, exposure measurements or markers that are prospectively collected, and research on potential mechanisms would be helpful in moving the field forward.

AUTHORS' CONTRIBUTIONS

RJS conceived of and designed the study, secured funding, provided study oversight, and drafted the manuscript; VK performed statistical analyses; RJS and HEV supervised VK in performing the statistical analyses; JFS conducted the retrospective agricultural pesticide exposure estimation for CHARGE participants utilizing the California State Pesticide Use Reporting data; DT provided statistical input and expertise for the statistical analysis plan; RLH and SO provided clinical oversight for the study and contributed clinical diagnoses; CCM and EM provided occupational pesticide exposure classification and data. IHP and LD provided the indoor pesticide and other data for the CHARGE study. DHB provided input on classification of pesticide exposures. All authors reviewed and approved the final manuscript.

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TABLE 1. Characteristics of Children with Autism Spectrum Disorder (ASD) and Typical Development (TD) and their Mothers in the CHARGE Case-control Study

	Cases and Controls with Interviews 2011 or Before			Cases and Controls with Information on Folic Acid and Pesticide Exposures		
Characteristic	ASD (N =466) *n* (%)	TD (N = 340) *n* (%)	*P* [a]	ASD (N = 296 [a]) *n* (%)	TD (N = 220 [a]) *n* (%)	*P* [a]
Child Sex			0.18			0.3663
Male	400 (85.8)	280 (82.4)		256 (86.49)	184 (83.64)	
Female	66 (14.2)	60 (17.6)		40 (13.51)	36 (16.36)	
Child's Race/Ethnicity			0.10			0.2059
Non-Hispanic White	241 (51.7)	175 (51.5)		146 (49.32)	110 (50.00)	
Hispanic	142 (30.5)	101 (29.7)		98 (33.11)	70 (31.82)	
Non-Hispanic Black	9 (1.9)	8 (2.4)		8 (2.70)	4 (1.82)	
Asian	28 (6.0)	9 (2.6)		16 (5.41)	5 (2.27)	
Mixed and Other	46 (9.9)	47 (13.8)		28 (9.46)	31 (14.09)	

Child's Birth Year			<0.0001		<0.0001
2000-2001	191 (41.0)	59 (17.4)		119 (40.20)	37 (16.82)
2002-2003	121 (26.0)	133 (39.1)		86 (29.05)	92 (41.82)
2004-2005	115 (24.7)	92 (27.1)		71 (23.99)	57 (25.91)
2006-2007	39 (8.4)	56 (16.5)		20 (6.76)	34 (15.45)
Maternal Age at Child's Birth (Years)			0.19		0.2399
<20	8 (1.7)	12 (3.5)		6 (2.03)	11 (5.00)
20-25	83 (17.8)	55 (16.8)		47 (15.88)	36 (16.36)
26-29	106 (22.8)	66 (19.4)		73 (24.66)	43 (19.55)
30-34	148	128		97	84

	(31.8)	(37.7)		(32.77)	(38.18)
35-39	103 (22.1)	63 (18.5)		63 (21.28)	39 (17.73)
40 or Older	18 (3.9)	16 (4.7)		10 (3.38)	7 (3.18)
Maternal Birthplace			0.14		0.4657
United States	353 (75.8)	277 (81.5)		224 (75.68)	176 (80.00)
Mexico	38 (8.2)	23 (6.8)		25 (8.45)	17 (7.73)
Other	75 (16.1)	40 (11.8)		47 (15.88)	27 (12.27)
Maternal Education			0.111		0.0671
High School Graduate or Less	65 (13.9)	53 (15.6)		40 (13.51)	38 (17.27)
Some College, Vocational, Associate Degree	180 (38.6)	107 (31.5)		116 (39.19)	65 (29.55)

Bachelor or Higher Degree	221 (47.4)	180 (52.9)		140 (47.30)	117 (53.18)	
Home Ownership			0.001			0.0184
No	152 (33.4)	76 (22.8)		96 (33.10)	51 (23.50)	
Yes	303 (66.6)	258 (77.2)		194 (66.90)	166 (76.50)	
Missing Information	11	6		6	3	
Insurance Delivery Type			0.08			0.5908
Private	380 (81.6)	292 (86.1)		245 (82.77)	186 (84.55)	
Government Program	86 (18.5)	47 (13.9)		51 (17.23)	34 (15.45)	
Intention to become pregnant			0.51			0.6725
Intended to become pregnant	292	228 (67.9)		187	150 (68.81)	

when they did	(64.3)		(64.71)		
Indifferent about becoming pregnant at that time	60 (13.2)	47 (14.0)	41 (14.19)	31 (14.22)	
Intended to become pregnant later	66 (14.5)	41 (12.2)	39 (13.49)	25 (11.47)	
Did not intend to become pregnant at all	36 (7.9)	20 (6.0)	22 (7.61)	12 (5.50)	
Missing Information	12	4	7	2	
Maternal Cigarette Smoking [b]			0.06		0.1632
No	395 (86.1)	305 (90.5)	255 (86.44)	199 (90.45)	
Yes	64 (13.9)	32 (9.5)	40 (13.56)	21 (9.55)	
Missing Information	7	3	1	0	
Folic Acid Pregnancy Month 1[c]			0.01		0.0492
< 800 µg	210 (53.3)	121 (42.9)	151 (51.01)	93 (42.27)	
800+ µg	184 (46.7)	161 (57.1)	145 (48.99)	127 (57.73)	
Missing Information	72	58			
Folic Acid Pregnancy Month 1[c]			0.0146		0.0827
< 600 µg	191 (48.48)	110 (39.01)	137 (46.28)	85 (38.64)	

600+ µg	203 (51.52)	172 (60.99)		159 (53.72)	135 (61.36)	
Missing Information	72	58				
Vitamin B12 Pregnancy Month 1[c]			0.1622			0.1307
< 8 µg	213 (50.71)	141 (45.48)		148 (50.17)	95 (43.18)	
8 + µg	207 (49.29)	169 (54.52)		147 (49.83)	125 (56.82)	
Missing Information	46	30		1	0	
Vitamin B6 Pregnancy Month 1[c]			0.0278			0.1155
< 2.83 mg	245 (58.19)	155 (50.00)		170 (57.43)	111 (50.45)	
2.83 + mg	176 (41.93)	155 (50.00)		126 (42.57)	109 (49.55)	
Missing Information	45	30				
Occupational Pesticide			0.8493			0.7407
None	345 (98.29)	256 (98.08)		227 (99.13)	163 (98.79)	
Any	6 (1.71)	5 (1.92)		2 (0.87)	2 (1.21)	
Missing Information	115	79		67	55	
Household Indoor Pesticide Exposure [d]			0.005			0.0109

No	220 (53.8)	195 (64.4)		165 (55.74)	147 (66.82)	
Yes	189 (46.2)	108 (35.6)		131 (44.26)	73 (33.18)	
Missing Information	57	37				
Household Outdoor Pesticide Exposure [e]			0.07			0.0189
No	248 (61.7)	207 (68.3)		179 (60.47)	155 (70.45)	
Yes	154 (38.3)	96 (31.7)		117 (39.53)	65 (29.55)	
Missing Information	64	37				
Agricultural Pesticide Exposure [f]			0.75			0.3051
No	351 (82.0)	257 (82.9)		240 (81.08)	186 (84.55)	
Yes	77 (18.0)	53 (17.1)		56 (18.92)	34 (15.45)	
Missing Information	38	30				
Any Pesticide Exposure [g]			0.04			0.0579
No	124 (35.4)	116 (43.8)		110 (37.16)	100 (45.45)	
Yes	226 (64.6)	149 (56.2)		186 (62.84)	120 (54.55)	
Missing Information	116	75				

Abbreviations: ASD, Autism Spectrum Disorder; CHARGE, Childhood Autism Risks from Genetics and Environment; TD, Typical Development.

[a] Limited to those with information on both maternal folic acid intake and at least one type of pesticide exposure.

[a] *P* values derived from chi-squared tests comparing category proportions between the ASD group and the TD.

[b] Mother reported smoking any tobacco product before or during pregnancy.

[c] Average folic acid consumed per day summed from prenatal vitamins, multivitamins, folic acid supplements, other supplements, and breakfast cereals.

[d] Maternally-reported exposure to professionally- or self-applied pesticide sprays or foggers, or pet pesticides (flea/tick shampoos, pouches, not collars), inside the home during pregnancy.

[e] Maternally-reported exposure to professionally- or self-applied pesticide sprays or foggers outside the home during pregnancy

[f] Exposure to carbamate, organochlorine, organophosphate, and pyrethroid pesticides applied to agricultural fields within a 1250 m buffer around the mother's home during the period from 3 months before through the 3rd month after conception based on linkage of her address(s) to the California Pesticide Use Report.

[g] Maternal exposure to any indoor or outdoor household pesticides, or agricultural pesticides, as defined above.

TABLE 2. Combinations of Household Pesticide Exposure and Maternal Folic Acid Intake the First Month of Pregnancy in Relation to Risk for Autism Spectrum Disorders (ASD)

Pesticide Exposure During Pregnancy		Maternal Folic Acid Intake [a]	ASD No. (%)	Typically Developing No. (%)	Expected Joint OR: Multiplicative Model [b]	Expected Joint OR: Additive Model [c]	OR [d] (95% CI)	RERI (95% CI)
Indoor Sprays or Foggers	None	800 + µg	107 (32.4)	116 (46.8)			1.0	
		< 800 µg	120 (36.4)	84 (33.9)			1.3 (0.8, 2.3)	
	Any	800 + µg	49 (14.9)	27 (10.9)			1.9 (1.1, 3.3)	
		< 800 µg	54 (16.4)	21 (8.5)	2.6	2.2	2.6 (1.3, 5.2)	0.4 (-1.4, 2.1)
Pet Flea and Tick Products	None	800 + µg	127 (36.3)	118 (46.1)			1.0	
		< 800 µg	149 (42.6)	95 (37.1)			1.4 (0.8, 2.3)	
	Some [e]	800 + µg	8 (2.3)	12 (4.7)			0.8 (0.3, 2.1)	
		< 800 µg	8 (2.3)	8 (3.1)	1.1	1.2	1.0 (0.3, 2.9)	-0.2 (-1.5, 1.1)
	Regular [f]	800 + µg	33 (9.4)	18 (7.0)			1.6 (0.9, 3.1)	
		< 800 µg	25 (7.1)	5 (2.0)	2.3	2.0	3.9 (1.4, 11.5)	2.0 (-2.2, 6.2)
	Any	800 + µg	41 (11.7)	30 (11.7)			1.3 (0.8, 2.3)	
		< 800 µg	33 (9.4)	13 (5.1)	1.8	1.7	2.1 (0.99, 4.7)	0.6 (-1.1, 2.2)
Any Indoor [g] Pesticides	None	800 + µg	81 (24.4)	90 (36.9)			1.0	
		< 800 µg	100 (30.1)	75 (30.7)			1.2 (0.7, 2.2)	
	Any	800 + µg	77 (23.2)	50 (20.5)			1.7 (1.1, 2.8)	
		< 800 µg	74 (22.3)	29 (11.9)	2.0	1.9	2.5 (1.3, 4.7)	0.6 (-0.8, 1.9)
Outdoor Sprays or Foggers	None	800 + µg	96 (30.9)	95 (39.9)			1.0	
		< 800 µg	100 (32.2)	73 (30.7)			1.1 (0.6, 2.0)	
	Some [e]	800 + µg	34 (10.9)	31 (13)			1.5 (0.8, 2.7)	
		< 800 µg	18 (5.8)	19 (8.0)	1.7	1.6	0.9 (0.4, 2.1)	-0.7 (-1.8, 0.5)
	Regular [f]	800 + µg	23 (7.4)	12 (5.0)			1.8 (0.8, 4.0)	

		< 800 µg	40 (12.9)	8 (3.4)	2.0	1.9	4.1 (1.7, 10.1)	2.0 (-1.4, 5.3)
	Any	800 + µg	57 (18.3)	43 (18.1)			1.6 (1.0, 2.7)	
		< 800 µg	58 (18.7)	27 (11.3)	1.8	1.7	2.0 (1.0, 3.8)	0.2 (-1.0, 1.5)
Any Household Indoor or Outdoor Pesticides	None	800 + µg	62 (20.0)	67 (28.6)			1.0	
		< 800 µg	77 (24.8)	58 (24.8)			1.2 (0.6, 2.3)	
	Any	800 + µg	87 (28.1)	67 (28.6)			1.6 (1.0, 2.7)	
		< 800 µg	84 (27.1)	42 (18.0)	2.0	1.8	2.1 (1.1, 3.9)	0.2 (-0.9, 1.3)
Any Household or Agricultural Pesticides [h]	None	800 + µg	47 (16.5)	53 (24.8)			1.0	
		< 800 µg	60 (21.1)	45 (21.0)			1.2 (0.6, 2.5)	
	Any	800 + µg	94 (33.0)	70 (32.7)			1.7 (1.0, 2.9)	
		< 800 µg	84 (29.5)	46 (21.5)	2.0	1.9	2.1 (1.1, 4.1)	0.2 (-1.0, 1.4)

Abbreviations: CI, confidence interval; OR, odds ratio; RERI, relative excess risk due to interaction.

[a] Average daily intake during first month of pregnancy.

[b] Expected combined OR for multiplicative model calculated as the product of the ORs for no pesticide exposure and folic acid < 800 µg, pesticide exposure and folic acid 800+ µg.

[c] Expected combined OR for additive model calculated as 1 + (the OR for no pesticide exposure and folic acid < 800 µg – 1) + (the OR for pesticide exposure and folic acid 800+ µg – 1).

[d] ORs adjusted for home ownership, child's birth year, and maternal vitamin B6 and vitamin D (natural log) intake during the first month of pregnancy.

[e] Exposure to pesticides reported for <6 months of pregnancy.

[f] Exposure to pesticides reported for 6+ months of pregnancy.

[g] Maternally-reported exposure to professionally- or self-applied pesticide sprays or foggers, or pet pesticides (flea/tick shampoos, pouches, not collars), inside the home during pregnancy.

[h] Any household indoor or outdoor pesticide exposure during pregnancy; or agricultural pesticide exposure months 3 months before through 3rd month of pregnancy.

TABLE 3. Combinations of Agricultural Pesticide Exposure and Maternal Folic Acid Intake the First Month of Pregnancy in Relation to Risk for Autism Spectrum Disorders (ASD)

Periconceptional Agricultural/Commercial Pesticide Exposure [a]		Maternal Folic Acid Intake [b]	ASD No. (%)	Typically Developing No. (%)	Expected Joint OR: Multiplicative Model [c]	Expected Joint OR: Additive Model [d]	Observed OR [e] (95% CI)	RERI (95% CI)
Chlorpyrifos	None	800 + µg	159 (45.6)	132 (52.8)			1.0	
		< 800 µg	165 (47.3)	101 (40.4)			1.3 (0.8, 2.2)	
	Any	800 + µg	12 (3.4)	11 (4.4)			1.1 (0.4, 2.6)	
		< 800 µg	13 (3.7)	6 (2.4)	1.4	1.4	2.2 (0.7, 6.5)	0.8 (-1.6, 3.2)
Organophosphates	None	800 + µg	145 (41.6)	120 (48)			1.0	
		< 800 µg	150 (43)	96 (38.4)			1.2 (0.7, 2.0)	
	Any	800 + µg	26 (7.5)	23 (9.2)			0.8 (0.5, 1.6)	
		< 800 µg	28 (8.0)	11 (4.4)	1.0	1.0	2.3 (0.98, 5.3)	1.2 (-0.6, 3.0)
Pyrethroids	None	800 + µg	149 (42.7)	124 (49.6)			1.0	
		< 800 µg	153 (43.8)	96 (38.4)			1.2 (0.7, 2.1)	
	Any	800 + µg	22 (6.3)	19 (7.6)			0.9 (0.5, 1.8)	
		< 800 µg	25 (7.2)	11 (4.4)	1.1	1.2	2.1 (0.9, 4.8)	0.9 (-0.7, 2.6)
Carbamates	None	800 + µg	160 (45.9)	138 (55.2)			1.0	
		< 800 µg	169 (48.4)	102 (40.8)			1.4 (0.8, 2.3)	
	Any	800 + µg	11 (3.2)	5 (2.0)			1.5 (0.5, 4.5)	
		< 800 µg	9 (2.6)	5 (2.0)	2.0	1.8	1.5 (0.5, 4.8)	-0.3 (-2.7, 2.0)
Any Agricultural Pesticides	None	800 + µg	137 (39.3)	117 (46.8)			1.0	
		< 800 µg	145 (41.6)	91 (36.4)			1.2 (0.7, 2.1)	

	Any	800 + µg	34 (9.7)	26 (10.4)			1.0 (0.6, 1.8)	
		< 800 µg	33 (9.5)	16 (6.4)	1.2	1.2	2.0 (0.9, 4.2)	0.7 (-0.6, 2.1)

Abbreviations: CI, confidence interval; OR, odds ratio; RERI, relative excess risk due to interaction.

[a] Any during the period 3 months before or after conception, using a 1250 m buffer.

[b] Average daily intake during first month of pregnancy.

[c] Expected combined OR for multiplicative model calculated as the product of the ORs for no pesticide exposure and folic acid < 800 µg, pesticide exposure and folic acid 800+ µg.

[d] Expected combined OR for additive model calculated as 1 + (the OR for no pesticide exposure and folic acid < 800 µg – 1) + (the OR for pesticide exposure and folic acid 800+ µg – 1).

[e] ORs adjusted for home ownership, child's birth year, and maternal vitamin B6 and vitamin D (natural log) intake during the first month of pregnancy.

Figure Captions

FIGURE 1. ASD Odds Ratios for Pesticide and Folic Acid Exposure Combinations.
Odds ratios (aOR) and 95% confidence intervals (bars) for the association between ASD and combinations of exposures to pesticides and average maternal folic acid intake (<800, 800+ µg/day) during the first month of pregnancy were adjusted for home ownership, child's year of birth, maternal intake of vitamins B6 and D (natural log) in the first month of pregnancy. In all comparisons, the reference group was those with above-median FA intake (800+ µg) during the first pregnancy month *and* no pesticide exposure.

FIGURE 2. Pathways Connecting Folic Acid to Potential Mechanisms of Environmental Contaminants.
Abbreviations: CNV, Copy number variation; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate.
Folic acid inputs into the folate cycle through conversion to THF which augments folate's essential role as a donor and acceptor of one-carbon units, important for the biosynthesis of nucleic acids, proteins, and methyl groups (Crider et al. 2012). During development, biosynthesis of nucleic acids is necessary for DNA synthesis, repair, and cell division, and methyl groups are important for regulation of gene expression (Crider et al. 2012). Environmental contaminants like pesticides can trigger immune responses and inflammation (Voccia et al. 1999) that induce cellular proliferation and DNA synthesis; similarly, pesticides can induce DNA damage (Corsini et al. 2008; Undeger and Basaran 2005) that requires repair; both of these folate-dependent processes necessitate biosynthesis of nucleic acids which could deplete folate at a time during early pregnancy when demand is high, but could potentially be countered with high folate quantities. Environmental contaminants can also induce oxidative stress (Abdollahi et al. 2004); in response, homocysteine is permanently removed from the methionine cycle through degradation into cysteine in the transsulfuration cycle, where it is converted to cysteine and then

glutathione, a universal antioxidant (Schmidt and LaSalle 2011). This diversion of the methionine cycle towards glutathione antioxidant reactions and away from DNA synthesis, repair, and methylation, may be countered by high folate supply, driving conversion of homocysteine to methionine, and the biosynthesis of methionine to SAM which serves as a methyl-donor for methylation reactions that are especially critical during key periods of growth and re-methylation at the start of development.