JAMA Pediatrics | Original Investigation

Association of Exposure to Diethylstilbestrol During Pregnancy With Multigenerational Neurodevelopmental Deficits

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IMPORTANCE Animal evidence suggests that endocrine disruptors affect germline cells and neurodevelopment. However, to date, the third-generation neurodevelopmental outcomes in humans have not been examined.

OBJECTIVE To explore the potential consequences of exposure to diethylstilbestrol or DES across generations—specifically, third-generation neurodevelopment.

DESIGN, SETTING, AND PARTICIPANTS This cohort study uses self-reported health information, such as exposure to diethylstilbestrol during pregnancy and attention-deficit/hyperactivity disorder (ADHD) diagnosis, from 47 540 participants enrolled in the ongoing Nurses' Health Study II. The 3 generations analyzed in this study were the participants (F1 generation), their mothers (F0 generation), and their live-born children (F2 generation).

MAIN OUTCOMES AND MEASURES Participant- and mother-reported exposure to diethylstilbestrol during pregnancy and physician-diagnosed child ADHD.

RESULTS The total number of women included in this study was 47 540. Of the 47 540 F0 mothers, 861 (1.8%) used diethylstilbestrol and 46 679 (98.2%) did not while pregnant with the F1 participants. Use of diethylstylbestrol by F0 mothers was associated with an increased risk of ADHD among the F2 generation: 7.7% vs 5.2%, adjusted odds ratio (OR), 1.36 (95% CI, 1.10-1.67) and an OR of 1.63 (95% CI, 1.18-2.25) if diethylstilbestrol was taken during the first trimester of pregnancy. No effect modification was observed by the F2 children's sex.

CONCLUSIONS AND RELEVANCE This study provides evidence that diethylstilbestrol exposure is associated with multigenerational neurodevelopmental deficits. The doses and potency level of environmental endocrine disruptors to which humans are exposed are lower than those of diethylstilbestrol, but the prevalence of such exposure and the possibility of cumulative action are potentially high and thus warrant consideration.

JAMA Pediatr. doi:10.1001/jamapediatrics.2018.0727
Published online May 21, 2018.

Editorial
Author Audio Interview
Supplemental content

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The prevalence of neurodevelopmental disorders, like attention-deficit/hyperactivity disorder (ADHD), has been increasing.[1,2] Children with ADHD have compromised quality of life[3] and substantial educational problems.[4] Most studies, but not all,[5] suggest the negative symptoms of childhood ADHD persist into adulthood[6-8] and considerably worsen the quality of life for those adults,[9,10] increasing their risk for obesity,[11,12] many other morbidity and mortality health indicators,[13-15] and even premature death.[16] Therefore, identifying the modifiable factors that contribute to these generational and multigenerational risks is of immense importance.

Endocrine-disrupting chemicals (EDCs) comprise potentially modifiable factors that have been linked to neurodevelopmental disorders,[17-21] including ADHD.[17] Interest in the potential multigenerational and transgenerational consequences of EDCs has increased given the hypothesized biological mechanism involving epigenetic reprogramming of the germline.[22-26] For example, exposure to di(2-ethylhexyl) phthalate, a known EDC, has been shown to alter third-generation behavior and stress responses, observed corticosterone levels, and pituitary gene expression and behavior in mice.[27] Wolstenholme et al[28] also reported changes in third- to fifth-generation social interactions following exposure to bisphenol A in mice.

Despite compelling evidence from toxicological studies, epidemiological evidence in humans of EDC-associated multigenerational neurodevelopmental outcomes is currently unavailable, likely because of the lack of large cohorts with relevant information across multiple generations.

In this study, we used data from the Nurses' Health Study II (NHSII) to examine the potential third-generation outcomes (ADHD) of diethylstilbestrol, a potent perinatal EDC. Between 1938 and 1971, diethylstilbestrol was given to pregnant women to prevent pregnancy complications. The exact number of women worldwide who used diethylstilbestrol is unknown, but between 5 and 10 million women in the United States were estimated to have used it.[29] Prescription of the drug started to phase out after a 1953 study found diethylstilbestrol had no actual treatment value,[30] and it was banned in 1971 after another study linked it to vaginal adenocarcinomas in female children of the women who used diethylstilbestrol.[31] Later, it was found to be associated with multiple other reproductive adverse outcomes in female offspring.[29] Only a few studies have considered EDCs and their outcomes for the third generation. For example, exposure to diethylstilbestrol during pregnancy was linked to hypospadias in the grandsons[32-34] and to delayed menstrual regularization in the granddaughters[35] of the exposed women. To our knowledge, no epidemiological study to date has assessed the multigenerational neurodevelopmental consequences of diethylstilbestrol or any other EDCs.

Key Points

Question Is exposure to diethylstilbestrol during pregnancy associated with adverse multigenerational neurodevelopmental outcomes?

Findings A cohort study of 47 450 women in the Nurses' Health Study II found significantly elevated odds for attention-deficit/hyperactivity disorder in the grandchildren (third generation) of users of diethylstilbestrol, a potent endocrine disruptor.

Meaning Exposure to endocrine disruptors during pregnancy may be associated with multigenerational neurodevelopmental deficits.

Methods

Data Collection

Study Population

In 1989, a total of 116 686 female registered nurses between the ages of 25 and 42 years enrolled in the NHSII prospective cohort by completing a mailed questionnaire. At baseline, participants resided in 14 US states (California, Connecticut, Indiana, Iowa, Kentucky, Massachusetts, Michigan, Missippi, New York, North Carolina, Ohio, Pennsylvania, South Carolina, and Texas), but they have since moved to all 50 states. The NHSII has a retention rate of more than 90%. Follow-up questionnaires are mailed to participants every 2 years to gather updated information on lifestyle and risk factors, medication use, and major illness occurrence. Our study was approved by the institutional review board of Brigham and Women's Hospital, Boston, Massachusetts, and the human subjects committee of Harvard T. H. Chan School of Public Health, Boston. Participants provided implied informed consent by completing and returning the questionnaire.

All NHSII participants (F1 generation) were born between 1946 and 1964, when diethylstilbestrol was still being prescribed. In 1993, the mailed questionnaire included questions about the use of diethylstilbestrol by the participant's mother (F0 generation) during her pregnancy with the F1 participant. We excluded from our analyses F1 participants who did not return the 1993, 2005, or 2013 questionnaires (the relevant surveys that collected outcome and exposure data) and who did not report any live-born children. Because children (F2 generation) with ADHD were identified only by their year of birth, we also excluded F1 participants with multiple pregnancies (eg, twins, triplets) and same-year births (from different pregnancies), as matching the response to the appropriate child born in the same year would not be possible. The final data set for the main analysis included 47 540 F0 mothers as well as 106 198 live-born F2 children.

Exposure Assessment

In 1993, a supplementary questionnaire was mailed to 2742 F1 participants who reported that their F0 mothers had been exposed to diethylstilbestrol while pregnant with the F1 participants. Among the 2317 participants (84.5%) who responded, 2032 (87.7%) reported being certain or somewhat certain of their mothers' exposure, 123 (5.3%) were not certain, and 162 (7.0%) reported no such exposure. Thus, we considered only those who were certain or somewhat certain of their mothers' exposure, excluding those who were not certain or had originally said yes but later reported no exposure. The 1993 questionnaire also included questions on trimester-specific diethylstilbestrol use—that is, any exposure during the first, second, or third trimester of pregnancy.

In 2001, a total of 29 070 F0 mothers received a questionnaire pertaining to their pregnancy with the F1 participants; it included a question about diethylstilbestrol use. We observed very good agreement between the mothers' responses to the 2001 survey and prior reports by the F1 in 1993 ($\kappa = 0.74$)[36,37] that did not vary by ADHD status.

Outcome Assessment

The outcome of interest was an F1 participant's report of whether any of her children ever received a doctor's diagnosis of ADHD. This question was first asked in the 2005 questionnaire, although at that time the date of birth of the child or children with ADHD was not asked. This question was repeated in the 2013 questionnaire, further requesting information on the birth years of the children with an ADHD physician diagnosis.

Maternal reports of ADHD have been found to be reliable.[38] Moreover, in a validation study involving 92 F1 participants who reported in the 2005 questionnaire of having a child diagnosed with ADHD, we found that the F2 children scored high on the ADHD Rating Scale-IV.[39] All girls scored above 90%, 81.1% of boys scored above 80%, and 63.8% of boys scored above 90%.[40]

For our main analysis, we included information on F1 participants who provided concordant responses (92.6% concordance) in the 2005 and 2013 questionnaires to the question of whether any of their children had been diagnosed with ADHD by a physician, using the child-specific responses from the 2013 questionnaire to minimize any potential outcome misclassification. We also conducted analyses of all participants who responded to the 2013 questionnaire to include their children, who may have received a diagnosis after 2005.

Covariates

Potential confounders of the association between F0 mothers' diethylstilbestrol exposure and F2 children's ADHD could only be variables that preceded diethylstilbestrol exposure[41] (ie, F0 mother-level variables). Therefore, we did not consider participant or child variables as covariates in the health models except for self-identified participant race/ethnicity, which was assumed to reflect the mother's race/ethnicity. In the 1999 questionnaire, participants were asked whether their mothers smoked during pregnancy. In the 2005 questionnaire, participants were asked about their family socioeconomic status at their birth as well as their mother's lifestyle, educational level, and occupation. We adjusted for the participant's race (white, African American, Asian, or other), ethnicity (Hispanic—yes or no), and year of birth (and squared year of birth to account for nonlinear confounding); mother's smoking during pregnancy (yes or no); mother's home ownership at the time of pregnancy (yes or no); mother's education (<9 years, 1-3 years of high school, 4 years of high school, 1-3 years of college, or ≥4 years of college); father's educational level (same categories); mother's occupation (professional, executive/manager, sales/clerical work, mechanic/skilled, machine operator/driver, service worker, laborer/unskilled work, farming, military, or housekeeping); and father's occupation (same categories).

Because of previous reports of an association between F0 mother's diethylstilbestrol exposure and F1 participant's depression[37] as well as between depression and ADHD,[42] in a sensitivity analysis we adjusted for the mother's depression history, which was reported by the participants in the 2005 survey. However, this sensitivity analysis was only a secondary analysis, as information was not available on the timing of the diagnosis of the mother's depression relative to her index pregnancy. In a secondary analysis, we adjusted for the mother's age when the participant was born because age at delivery was unknown for 1928 F0 mothers (4.1%) in our study. We included a missing indicator for other covariates with missing responses, which arose mostly when the participant did not know the response for her mother.

Statistical Analysis

Data Analysis

In utero exposure to diethylstilbestrol is known to affect women's reproductive ability, which could affect the number of grandchildren within the F0 generation and the likelihood that any F2 child has ADHD—that is, the distribution of ADHD given diethylstilbestrol potentially may depend on the number of children within the F1 generation—for which our data provide evidence (results not shown). This structure is defined as *informative clustering*.[43-45] Standard generalized estimating equation approaches are not appropriate for this analysis and would result in invalid estimates and inferences.[43-45] We used cluster-weighted generalized estimating equations with a logit link to account for multiple children within the F1 generation, adjusting for potential confounders with the inverse of the cluster size (ie, number of children per participant) as the weights to account for informative clustering.[45] We also assessed potential effect modification by F2 children's sex by including in the model an interaction term between F0 mothers' diethylstilbestrol use and F2 children's sex.

We considered any diethylstilbestrol exposure during pregnancy and trimester-specific exposures. These exposures were not mutually exclusive and were modeled as 3 binary variables along with another variable for those who did not know about trimester-specific timing; all were included simultaneously in 1 model. Results were similar when we repeated this analysis using separate models for each trimester and excluding the 302 F0 mothers (with 625 F2 children) who did not know the trimester of exposure.

Our results are reported as odds ratios (ORs) and 95% CIs. Statistical significance was assessed at $\alpha = .05$; all *P* values presented are for 2-tailed tests. All analyses were conducted using SAS statistical software, version 9.4 (SAS Institute Inc).

Sensitivity Analyses

As stated, we repeated the analyses using the ADHD-related responses to only the 2013 questionnaire. In addition, we conducted sensitivity analyses to assess the outcome of potential unmeasured confounding by calculating the E value—that is, the minimal strength at which a confounder would need to be associated with both the outcome and the exposure of interest to fully explain the observed association.[46]

Results

In the main analysis, we included information on 47 540 F0 mothers, 861 (1.8%) of whom were exposed to diethylstilbestrol while pregnant with the F1 participants and 46 679 (98.2%) who were not. **Table 1** presents descriptive characteristics of the mothers and participants by diethylstilbestrol use. The F1 participants had a median of 2 children (range, 1-12) if their F0 mothers did not use diethylstilbestrol while pregnant and a median of 2 children (range, 1-5) if their mothers used diethylstilbestrol. **Table 2** presents descriptive characteristics of the F1 participants and their F0 mothers by the ADHD status of the F2 children. The total number of children was 106 198, and 5587 (5.3%) of them were diagnosed with ADHD. The median F2 year of birth was 1983 (interquartile range, 1978-1988). The grandmothers of 137 F2 children with ADHD (2.5%) were exposed to diethylstilbestrol while pregnant with the F1 participants.

We observed harmful associations between the F0 mothers' diethylstilbestrol use while pregnant and the F2 child's ADHD diagnosis (**Table 3**). Use of diethylstilbestrol by F0 mothers was associated with increased odds of ADHD among the F2 generation (adjusted OR, 1.36; 95% CI, 1.10-1.67). Further

Table 1. Characteristics of 47 540 F1 Participants and Their F0 Mothers, by F0 Diethylstilbestrol Use During Pregnancy

Characteristic	Diethylstilbestrol Use During F0 Pregnancy, No. (%)	
	Yes (n = 861)	No (n = 46 679)
F1 race		
White	822 (95.5)	44 338 (95.0)
African American	6 (0.7)	509 (1.1)
Asian	10 (1.2)	633 (1.4)
Other	23 (2.7)	1199 (2.6)
F1 ethnicity		
Hispanic	6 (0.7)	582 (1.2)
F0 educational level, y		
<9	28 (3.3)	3228 (6.9)
1-3 High school	90 (10.5)	5332 (11.4)
4 High school	394 (45.8)	22 921 (49.1)
1-3 College	244 (28.3)	10 263 (22.0)
≥4 College	95 (11.0)	4199 (9.0)
F1 Did not know	10 (1.2)	736 (1.6)
Missing	5 (0.6)	244 (0.5)
F1 father's educational level, y		
<9	62 (7.2)	5001 (10.7)
1-3 High school	90 (10.5)	6008 (12.9)
4 High school	283 (32.9)	17 511 (37.5)
1-3 College	185 (21.5)	7568 (16.2)
≥4 College	227 (26.4)	9198 (19.7)
F1 did not know	14 (1.6)	1393 (3.0)
Missing	3 (0.3)	261 (0.6)
F0 occupation		
Professional	123 (14.3)	5094 (10.9)
Executive, manager	12 (1.4)	284 (0.6)
Sales/clerical work	73 (8.5)	4502 (9.6)
Mechanic/skilled	4 (0.5)	355 (0.8)
Machine operator/inspector/driver	15 (1.7)	594 (1.3)
Service worker	13 (1.5)	893 (1.9)
Laborer/unskilled worker	18 (2.1)	1542 (3.3)
Farming	7 (0.8)	728 (1.6)
Military	0 (0)	12 (0)
No work outside the home	594 (69.0)	32 060 (68.7)
F1 did not know	2 (0.2)	615 (1.3)
F1 father's occupation[a]		
Professional	184 (21.4)	8186 (17.5)
Executive, manager	138 (16.0)	5011 (10.7)
Sales/clerical work	87 (10.1)	4706 (10.1)
Mechanic/skilled	188 (21.8)	10 891 (23.3)
Machine operator/inspector/driver	65 (7.5)	4273 (9.2)
Service worker	35 (4.1)	1829 (3.9)
Laborer/unskilled worker	74 (8.6)	5573 (11.9)
Farming	52 (6.0)	3983 (8.5)
Military	50 (5.8)	2310 (4.9)
No work outside the home	1 (0.1)	186 (0.4)
F1 did not know	5 (0.6)	831 (1.8)
F0 smoking during pregnancy		
Yes	282 (32.9)	10 857 (23.3)
No	478 (55.5)	29 440 (63.1)
F1 did not know	76 (8.8)	4205 (9.0)
Missing	25 (2.9)	2177 (4.7)
F0 Lifetime history of depression	142 (16.5)	4985 (10.7)
F1 Total No. of children, mean (SD)	2.07 (0.8)	2.24 (0.9)

Abbreviations: F0, mothers of participants in the Nurses' Health Study II; F1, participants in the Nurses' Health Study II.

[a] F1 father's occupation categories are not mutually exclusive.

adjusting for F0 mothers' age at delivery (excluding 1928 F0 mothers and 4368 F2 children with unknown age) yielded the same results (OR, 1.35; 95% CI, 1.09-1.66; P = .005). We observed no difference in the effect estimate when we included information on F0 mothers' depression history in the main model (OR, 1.33; 95% CI, 1.08-1.63; P = .007).

Information on sex was available for only 100 786 F2 children, 48 831 (48.5%) of whom were female. Of the 5338 F2 children with ADHD who had available sex information, 3977 (74.5%) were male. We found no effect modification by children's sex (P = .62 for the interaction).

In the trimester-specific analysis, we observed that the association was limited to diethylstilbestrol use during the first trimester (OR, 1.63; 95% CI, 1.18-2.25). The ORs for the other trimesters were not substantially different from 1 (Table 3).

In sensitivity analyses, among the 51 561 F0 mothers and 115 806 F2 children (8699 [7.5%] of whom had ADHD) identified when only the ADHD reported on the 2013 questionnaire was considered, we found an OR of 1.28 (95% CI, 1.08-1.52; P = .005) for any diethylstilbestrol use during pregnancy. Our sensitivity analyses of potential unmeasured confounding are presented in the eMethods and eFigure in the Supplement. Briefly, we do not believe that the estimated associations can be fully attributed to unmeasured confounding.

Discussion

We conducted a large-scale cohort analysis to assess the association between use of diethylstilbestrol during pregnancy and third-generation ADHD. The observed associations were robust to covariate adjustment and sensitivity analyses. Despite animal evidence of adverse multigenerational consequences—including neurodevelopmental disorders—of EDC exposure,[27,28,47] to date only a few studies have explored the potential multigenerational implications of EDC exposure in humans. These studies have only considered diethylstilbestrol exposure, and none has studied neurodevelopmental outcomes. Some studies have reported increased risk of hypospadias in grandsons of women exposed to diethylstilbestrol

Table 2. Characteristics of the 47 540 F1 Participants and Their F0 Mothers, by 106 198 F2 Children's ADHD Status

Characteristic	F2 ADHD Status, No. (%)	
	Yes (n = 5587)	No (n = 100 611)
F1 race		
White	5376 (96.2)	95 777 (95.2)
African American	25 (0.4)	1022 (1.0)
Asian	26 (0.5)	1303 (1.3)
Other	160 (2.9)	2509 (2.5)
F1 ethnicity		
Hispanic	48 (0.9)	1221 (1.2)
F0 educational level, y		
<9	304 (5.4)	6899 (6.9)
1-3 High school	655 (11.7)	11 133 (11.1)
4 High school	2601 (46.6)	49 718 (49.4)
1-3 College	1365 (24.4)	22 236 (22.1)
≥4 College	586 (10.5)	9103 (9.0)
F1 did not know	76 (1.4)	1522 (1.5)
Missing	31 (0.6)	508 (0.5)
F1 father's educational level, y		
<9	498 (8.9)	10 645 (10.6)
1-3 High school	649 (11.6)	12 788 (12.7)
4 High school	1969 (35.2)	37 810 (37.6)
1-3 College	1034 (18.5)	16 415 (16.3)
≥4 College	1281 (22.9)	20 084 (20.0)
F1 did not know	156 (2.8)	2869 (2.9)
Missing	26 (0.5)	565 (0.6)
F0 occupation		
Professional	655 (11.7)	10 995 (10.9)
Executive, manager	33 (0.6)	603 (0.6)
Sales/clerical work	577 (10.3)	9346 (9.3)
Mechanic/skilled	34 (0.6)	748 (0.7)
Machine operator/inspector/driver	59 (1.1)	1218 (1.2)
Service worker	100 (1.8)	1826 (1.8)
Laborer/unskilled worker	141 (2.5)	3208 (3.2)
Farming	77 (1.4)	1608 (1.6)
Military	2 (0)	31 (0)
No work outside the home	3832 (68.6)	69 862 (69.4)
F1 did not know	82 (1.5)	1263 (1.3)
F1 father's occupation[a]		
Professional	1127 (20.2)	17 652 (17.5)
Executive, manager	672 (21.0)	10 941 (10.9)
Sales/clerical work	622 (11.1)	10 115 (10.1)
Mechanic/skilled	1288 (23.1)	23 257 (23.1)
Machine operator/inspector/driver	452 (8.1)	9116 (9.1)
Service worker	193 (3.5)	3996 (4.0)
Laborer/unskilled worker	533 (9.5)	12 012 (11.9)
Farming	353 (6.3)	9045 (9.0)
Military	336 (6.0)	4836 (4.8)
No work outside the home	23 (0.4)	410 (0.4)
F1 did not know	107 (1.9)	1700 (1.7)
F0 smoking during pregnancy		
Yes	1527 (27.3)	23 104 (23.0)
No	3254 (58.2)	63 939 (63.6)
F1 did not know	528 (9.5)	8864 (8.8)
Missing	278 (5.0)	4704 (4.7)
F0 lifetime history of depression	839 (16.5)	10 316 (10.7)
F1 total No. of children, mean (SD)	2.51 (0.9)	2.60 (1.0)

Abbreviations: ADHD, attention-deficit/hyperactivity disorder; F0, mothers of participants in the Nurses' Health Study II; F1, participants in the Nurses' Health Study II; F2, live-born children of the F1 participants.

[a] F1 father's occupation categories are not mutually exclusive.

during pregnancy.[32-34] Titus-Ernstoff et al[35] found delayed menstrual regularization, higher odds of irregular menstrual periods, and fewer live births among women whose grandmothers used diethylstilbestrol during pregnancy. Birth defects have also been found in grandchildren of women who used diethylstilbestrol when pregnant.[48]

Oocytes that will develop into the F2 child grow while the F1 mother is still in utero. Thus, third-generation implications of diethylstilbestrol (and other EDC) exposures could relate to direct exposure of F1's oocytes developing during the F0 pregnancy. This does not imply transgenerational inheritance of epigenetic changes (ie, effects that do not derive from direct exposures, which would require F3 assessment), but animal studies do suggest that epigenetics play a role. Evidence from animal studies suggests that the biological pathway for multigenerational and transgenerational consequences of EDCs involves molecular alterations to the germline, mediated through epigenetic mechanisms, to promote outcomes to subsequent generations[22-26]; this process is known as *epigenetic transgenerational inheritance*.[22,49] As described earlier, F0 exposure to EDCs—specifically to di(2-ethylhexyl) phthalate and bisphenol A—has been linked to behavioral changes and altered social interactions among mice in later generations.[27,28] Direct epidemiological evidence of multigenerational and transgenerational neurodevelopmental outcomes of EDCs is lacking, but 2 studies have reported increased risk for ADHD and autism spectrum disorders among boys with hypospadias,[50,51] which in F2 has also been linked to F0 diethylstilbestrol exposure during pregnancy.[34] Note that diethylstilbestrol is similar structurally and functionally to bisphenol A, albeit more potent.[52] However, Newbold et al[47] have observed multigenerational consequences of diethylstilbestrol even at very low diethylstilbestrol estrogenic doses comparable to environmental EDC exposures.

Our trimester-specific analyses indicated that first-trimester F0-generation diethylstilbestrol use is associated with significantly increased risk for F2-generation ADHD. The associations with diethylstilbestrol use during the second and third trimesters were slightly weaker and not significant. The

Table 3. Odds Ratios and 95% CIs for F2 Children's ADHD, by F0 Diethylstilbestrol Use During Pregnancy

Exposure	Children, No.	ADHD Cases, No. (%)	OR (95% CI)	*P* Value
Any diethylstilbestrol				
Unexposed	104 414	5450 (5.2)	1 [Reference]	
Exposed (crude)[a]	1784	137 (7.7)	1.43 (1.16-1.76)	.001
Exposed (adjusted)[b]			1.36 (1.10-1.67)	.004
By trimester[b]				
Unexposed	104 414	5450 (5.2)	1 [Reference]	
First	950	82 (8.6)	1.63 (1.18-2.25)	.003
Second	519	33 (6.4)	0.68 (0.35-1.34)	.27
Third	338	27 (8.0)	1.41 (0.72-2.82)	.32
F1 did not know	625	42 (6.7)	1.15 (0.80-1.65)	.46

Abbreviations: ADHD, attention-deficit/hyperactivity disorder; F0, mothers of participants in the Nurses' Health Study II; F1, participants in the Nurses' Health Study II; F2, live-born children of the F1 participants; OR, odds ratio.

[a] Crude model: cluster-weighted generalized estimating equations without including any covariates. A crude model ignoring any F2-generation clustering within the F1 generation yielded an OR of 1.51 (95% CI, 1.27-1.80).

[b] Adjusted model: cluster-weighted generalized estimating equations for F1 race, F1 ethnicity, F1 year of birth and squared year of birth, F0 smoking during pregnancy with F1, whether F0 owned her home at the time of pregnancy, F0 education, F1 father's education, F0 occupation, and F1 father's occupation.

attenuated associations with the other trimesters could result from the smaller number of exposed cases (33 for the second trimester and 27 for the third trimester vs 82 for the first trimester). Although additional studies are warranted, these findings could suggest that the first trimester is a critical exposure window. The early phase of gestation is thought to be especially sensitive to maternal influences, resulting in embryonic and germ cell reprogramming, because during this period a wave of genome demethylation followed by de novo remethylation occurs together with the establishment of imprints and determination of sex.[53,54]

Limitations

We were in the unique position to access information across 3 generations in a well-defined nationwide cohort comprising a large number of F2-generation ADHD cases and extensive follow-up. Our findings, nevertheless, should be interpreted in light of our limitations.

Outcome misclassification is possible. The ADHD assessment was based on maternal reports of a doctor's diagnosis. Maternal reports have been found to be highly reliable,[38] and we found good agreement in the internal validation study. In addition, in a sensitivity analysis including all 2013 questionnaire responses, we observed only a slight attenuation, suggesting that any bias from misclassification was likely minimal.

Some exposure misclassification is likely, as exposure assessment depended on the F1 participants' report on whether their F0 mothers used diethylstilbestrol. To minimize potential exposure misclassification, we excluded from our analyses any women whose exposure was uncertain. Furthermore, the small validation study in our data comparing F1 responses with those directly obtained from F0 mothers showed very good agreement. Any exposure or outcome misclassification is likely nondifferential, as information on exposure was collected 12 to 20 years prior to the question about the outcome and in 1993 (when the question on diethylstilbestrol use was asked) there was no toxicological or epidemiological evidence of EDC multigenerational outcomes. Indeed, in our validation study, we found that agreement between F0-generation and F1-generation diethylstilbestrol reporting did not vary by ADHD status. Any potential resulting bias, thus, would likely be toward the null.

Some residual confounding is possible given that we used F1-provided information on F0 factors. However, we included information about several socioeconomic status factors, including smoking during pregnancy, and conducted sensitivity analyses; therefore, our findings are not likely to be completely attributable to residual confounding. Finally, although confounding by indication is possible, the observed different consequences across trimester-specific diethylstilbestrol use suggest that this is unlikely.[55,56]

Conclusions

We found evidence in humans of increased ADHD risk in the third generation following diethylstilbestrol exposure during F0 pregnancy. The levels of a high number of EDCs to which humans are daily exposed are lower than that of the prescribed diethylstilbestrol; thus, extending this work to lower levels of exposure is warranted. Because EDCs are ubiquitous, the high prevalence of exposure and the possibility of cumulative consequences must be considered.

ARTICLE INFORMATION

Accepted for Publication: March 7, 2018.

Published Online: May 21, 2018. doi:10.1001/jamapediatrics.2018.0727

Author Contributions: Dr Kioumourtzoglou had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Obtained funding: Weisskopf.
Administrative, technical, or material support: Kioumourtzoglou, O'Reilly.
Study supervision: Ascherio, Weisskopf.

Conflict of Interest Disclosures: None reported.

Funding/Support: This study was funded in part by training grant T32 ES 007069 from the National Institutes of Health (NIH), by grants P30 ES000002 and P30 ES009089 from the National Institute of Environmental Health Sciences, and by the Escher Fund for Autism. The Nurses' Health Study II is also supported by infrastructure grant UM1 CA176726 from the NIH.

Role of the Funder/Sponsor: The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

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