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Prenatal Dioxin Exposure and Neuropsychological Functioning in the Seveso Second Generation Health Study

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Abstract

Background: Prenatal 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure has been shown to alter sexual differentiation of the brain in animal models, impacting pubertal development, behavior, cortical dominance, and cognition. The effects of early life exposure to dioxin-like compounds on human neurodevelopment, however, are less clear and warrant further investigation.

Methods: The Seveso Women's Health Study (SWHS), initiated in 1996, is a well-characterized cohort of 981 Italian women who lived in proximity to an industrial accident in July 1976 that resulted in one of the highest residential TCDD exposures on record. In 2014–2016, we enrolled offspring born after the accident into the Seveso Second Generation Health Study. Children aged 7–17 years old (n=161) completed a neuropsychological assessment spanning executive function and reverse learning (Wisconsin Card Sort), non-verbal intelligence (Raven's Progressive Matrices), attention and hyperactivity (Connor's Continuous Performance (CPT)), and memory (Rey's Auditory Verbal Learning). We used multivariate regression with robust standard error estimates accounting for clustering of siblings to model the associations between these outcomes and prenatal exposure defined as TCDD measured in maternal serum collected soon after the explosion and estimated to pregnancy.

Results: The children (82 male, 79 female) averaged 13.1 (±2.9) years of age. Adjusting for covariates, a 10-fold increase in maternal serum TCDD was not adversely associated with reverse learning/set-shifting, memory, attention/impulsivity, or non-verbal intelligence. In sex-stratified models, prenatal TCDD was associated with more non-perseverative errors in boys but not in girls (p_{int}=0.04). TCDD was also associated with attention deficits on the CPT but only among children with the shortest breastfeeding histories.

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Declarations of interest: none

Conclusions: While overall, there were no significant associations, the observed differential neurotoxic sensitivities to TCDD by sex and lactation history may warrant confirmation in future studies.

Keywords

dioxins; prenatal exposure; Seveso; neurobehavioral effects; children's environmental health; TCDD

INTRODUCTION

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a widespread environmental pollutant commonly produced as an unwanted by-product of industrial combustion processes (Wikoff et al. 2012). TCDD is an endocrine disruptor that is highly lipophilic, chemically stable, and crosses the placenta (Chao et al. 2007; Nau et al. 1986). Perinatal exposure has been linked to altered immune function, glucose regulation, and steroidogenesis, as well as dental developmental anomalies in humans and animals (White and Birnbaum 2009). The diversity of health effects associated with TCDD is attributed to its high binding affinity for the aryl hydrocarbon receptor (AhR), a key transcription factor integral to many biological processes throughout human development, including cell growth, apoptosis, and detoxification (Mimura and Fujii-Kuriyama 2003).

TCDD's neurotoxicity is well documented in animals (Seegal and Schantz 1994; Seo et al. 1999) (Widholm et al. 2003). For example, studies in zebrafish have demonstrated TCDD's capacity to disrupt gene expression in highly conserved pathways of neuron development and brain growth (Carney et al. 2006; Hill et al. 2003; Nayyar et al. 2002). Other experimental evidence links perinatal TCDD exposure to altered neurogenesis (Fernandez et al. 2010; Mitsuhashi et al. 2010; Tanida et al. 2014), as well as to behavioral and cognitive effects such as hyperactivity and impaired learning, memory, executive function, and motor development in rodents and monkeys (Endo et al. 2012; Haijima et al. 2010; Hojo et al. 2002; Hojo et al. 2008; Nishijo et al. 2007; Schantz and Bowman 1989). Further, TCDD's action through the neuroendocrine pathways of AhR have been shown to alter sexual differentiation of the brain in animal models, impacting pubertal development, behavior, cortical dominance, and sex-specific changes in learning and memory (Mably et al. 1992; Nguyen et al. 2013; Petersen et al. 2006).

Epidemiological studies have associated perinatal background exposures to mixtures of TCDD and related members of the dioxins family (e.g. polychlorinated dibenzodioxins (PCDDs) and dibenzofurans (PCDFs)) with subtle neurodevelopmental decrements in cognition, attention and language skills in offspring (Caspersen et al. 2016a; Caspersen et al. 2016b; Halldorsson et al. 2009; Lee et al. 2007; Neugebauer et al. 2015). Additionally, background exposures to specifically dioxin-like congeners of polychlorinated biphenyls (PCBs) have been associated with delayed psychomotor development (Park et al. 2010), hyperactivity, and poorer attention in children and adolescents (Newman et al. 2009a; Patandin et al. 1999; Sagiv et al. 2012), albeit inconsistently (Sioen et al. 2013). Higher exposures to dioxins, such as from the 1979 mass contamination of rice bran oil with PCBs

and PCDFs in Yu-Cheng, Taiwan, have been linked to behavioral problems and lower cognition in perinatally exposed children compared to community-matched unexposed controls (Chen et al. 1992; Guo et al. 1995; Lai et al. 2001). Autistic traits and poorer cognitive and motor development have also been reported in Vietnamese infants born in areas with residual dioxin contamination decades after the wartime use of Agent Orange (Nishijo et al. 2014; Pham et al. 2015; Tai et al. 2016; Tran et al. 2016). These prior studies have assessed prenatal exposure to dioxins using proxies such as breastmilk levels (Nishijo et al. 2014; Pham et al. 2015; Tai et al. 2016; Tran et al. 2016), exposure registries (Chen et al. 1992; Lai et al. 2001), and estimates from dietary intake (Caspersen et al. 2016a; Caspersen et al. 2016b); none have collected contemporaneous biomarkers near the time of the mother's highest exposure.

Despite a large body of research examining PCBs and dioxins on child neurodevelopment, the particular contribution of TCDD, the most toxic congener of this family of planar halogenated hydrocarbons, is not well studied. Furthermore, questions remain about the sex-specific effects of TCDD on human neurodevelopment since some studies have reported no sex differences or conflicting results as to which sex appears more sensitive (Caspersen et al. 2016a; Caspersen et al. 2016b; Guo et al. 2004; Petersen et al. 2006; Sagiv et al. 2012). In the present study, we examine the neuropsychological functioning of children whose mothers were exposed to TCDD as a result of an explosion in Seveso Italy on July 10, 1976 and who participated in the Seveso Women's Health Study (Eskenazi et al. 2000a; Warner et al. 2012). We measured TCDD in blood collected near the time of exposure as well as estimated serum levels to the time of pregnancy. We also explore whether these potential neurotoxic effects are modified by child sex and breastfeeding histories since lactation could be an important source of postnatal exposure.

METHODS

Study Population

Recruitment of the Seveso Women's Health Study (SWHS) has been described previously (Eskenazi et al. 2000b). Briefly, this historical cohort study recruited 981 women who were newborn to 40 years of age on July 10, 1976, resided at that time in the highest contaminated areas (Zones A and B) in Seveso, Italy, and had an adequate amount of stored serum collected soon after the explosion for analysis of TCDD. In 2014–2016, we enrolled 611 children of SWHS participants who were born after the explosion in the Seveso Second Generation Health Study (66.4% of 920 alive and eligible) (Eskenazi et al. 2018). Participants in the offspring study were 2–38 years old at enrollment, and completed a personal interview, a fasting blood draw, and anthropometry during their study visit. Participants who were 7–17 years old at the time of their enrollment were invited to participate in an assessment of neuropsychological functioning. This sample included 161 children (65% of 249 eligible) born to 120 mothers.

Procedures

The study was approved by the Institutional Review Boards of the participating institutions. We obtained, prior to participation, written informed consent from all mothers, oral assent

from children aged 7–12 years, and written assent from children aged 13–17 years. Information on self-reported demographic, lifestyle factors and medical history were obtained from a questionnaire administered to the mother in private by a trained nurse-interviewer. Meanwhile, a second trained interviewer conducted a brief medical exam with the child, which included anthropometric and blood pressure measurements, and administered the neuropsychological assessment in a quiet, private room. All interviewers were blinded to participants' maternal serum TCDD levels and exposure zones of residence.

Neuropsychological Assessment

The neuropsychological assessment targeted domains hypothesized to be sensitive to dioxin exposure based on the findings from previous toxicological and human studies (Endo et al. 2012; Guo et al. 1995; Haijima et al. 2010; Hojo et al. 2002; Hojo et al. 2008; Newman et al. 2009a; Nishijo et al. 2007; Patandin et al. 1999; Sagiv et al. 2012; Schantz and Bowman 1989; Sioen et al. 2013; Tanida et al. 2014). The main areas of interest included executive functioning and reversal learning, non-verbal intelligence, attention and hyperactivity, and memory. Every child was administered the neuropsychological assessment in the same order by one of two interviewers under identical conditions. The entire assessment included both computer and paper-based administration with a total duration of 50 minutes. The battery is described below with tests in order of administration.

Rey's Auditory Verbal Learning Test (RAVLT)—In this task, children listened to an audio recording of the test administrator reading a list of 15 semantically unrelated items. Immediately afterward, they were asked to recall as many of the words as they could. This process was repeated twice more and the child's immediate recall score was calculated as the sum of the number of words recalled across each of the three trials (i.e. scores could span 0–45). In the delayed recall task, conducted thirty minutes later, the children were asked to freely recall as many of the words as they could without hearing the list a fourth time (i.e. scores could span 0–15).

Ravens Progressive Matrices Test (RPM)—In the RPM, children worked through a series of 60 visual problems requiring recognition of spatial, numerical, and stylistic patterns (Muriel Lezak 2012). The multiple choice test, split into 5 sets of 12 puzzles of progressing difficulty, was administered with pen and paper. The RPM measures non-verbal intelligence, abstract reasoning, and problem-solving ability. Given the time constraints of the study visit, we implemented a 20-minute timed version of the exam. Those matrices the child left blank, perhaps due to the time limit, were counted as incorrect. Prior research indicates that this adaptation is a reasonable predictor of performance on the untimed version (Hamel and Schmittmann 2006).

Connor's Continuous Performance Test II (CPT)—The children completed the CPT, a computerized task that assesses impulse control, vigilance, and attention deficit hyperactivity disorder (ADHD)-like behaviors (Conners 2000). We examined continuous *t*-scores (standardized to a non-clinical population; mean=50 with a SD=10) for commission errors (false positives), omission errors (non-response, false negatives), and overall and between-set hit rate standard errors. Variability in hit rate, a measure of sustained attention

on the test, reflects inconsistent performance, a symptom of ADHD (Epstein et al. 2003). We also examined the ADHD Confidence Index score, a continuous measure of the probability that the child would be diagnosed as having clinical ADHD.

Wisconsin Card Sorting Test (WCST)—Lastly, we administered a computerized version of the WCST. This test evaluates set-shifting— a complex skill pertinent to executive function involving strategic planning, cognitive shifting/learning, and impulse control (Muriel Lezak 2012). Subjects are tasked with sorting cards, one at a time, into one of four piles based on the shape, color, or number of symbols on the card. However, the sorting rule is concealed and must be discovered by the subject through trial and error. As the test progresses, the current sorting rule changes without notice, requiring subjects to recognize and adapt their sorting accordingly. We examined raw scores for the number of trials taken to complete the first set and t-scores for total errors, non-perseverative errors, and perseverative errors (Kongs 2000).

TCDD Analysis

In utero TCDD exposure was defined in two ways: 1) the initial (1976) maternal serum TCDD level and 2) maternal serum TCDD extrapolated to the time of pregnancy. The former examines the hypothesis that the primary dose in 1976 resulted in a persistent and, if involving the epigenetics of her oocytes, possibly heritable change to the woman's reproductive system impacting the health of her offspring. The latter examines an alternative hypothesis that the toxicologically-relevant dose is the maternal body burden at the time of the pregnancy, which is influenced by initial dose, age, and other covariates (Eskenazi B Submitted; Warner et al. 2014).

Archived maternal serum samples collected in 1976 were stored at −20°C until shipped to the Centers for Disease Control and Prevention (CDC) for analysis (Eskenazi et al. 2000b; Eskenazi et al. 2004b). TCDD was measured by high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) methods (Patterson et al. 1987) and adjusted for blood lipid concentrations prior to statistical analysis (Akins et al. 1989). Serum TCDD levels were reported in picograms per gram lipid or parts per trillion (ppt). Samples below the limit of detection (average LOD = 18.8 ppt) were assigned a value equal to one-half of the LOD (Hornung and Reed 1990).

Maternal serum TCDD at pregnancy was estimated from a first order kinetic model based on TCDD concentrations in maternal serum collected in 1976, as well as from additional blood samples collected from a subsample of the mothers at the 1996 or 2008 follow up visits (Eskenazi et al. 2018; Warner et al. 2014). Serum TCDD in 1996 and 2008 were also measured with HRGC/HRMS methods with an average LOD of 1ppt (Warner et al. 2014). The pregnancy TCDD estimate was extrapolated from the measurement collected closest to the pregnancy; as a result, estimates were extrapolated from 1976 blood samples for 15 children, from 1996 samples for 142 children, and from 2008 samples for 4 children. The median time between the closest TCDD measure and pregnancy was 5.5 years (IQR: 3.0–8.5 years), and 80% of the children had a maternal TCDD measurement within one TCDD half-life (9.0 years) of their births.

Statistical Analysis

Because the maternal serum TCDD distribution was approximately log-normal, the TCDD levels in 1976 and those extrapolated to the time of pregnancy were $\log_{10}$-transformed. The transformed serum TCDD levels were analyzed as a continuous exposure variable. For each endpoint, we assessed the shape of the dose-response function with locally weighted scatterplot smoothing (LOWESS) and restricted cubic splines in Stata 13 (StataCorp, College Station, TX). We constructed multivariable linear regression models for each neuropsychological outcome and we fit the regression models with variances determined by the Huber-White sandwich estimator to account for clustering of siblings in the sample (Huber 1967).

Covariates were identified *a priori* as confounders in the relationship between *in utero* TCDD exposure and neurocognitive performance with directed acyclic graphs (DAGs) informed by the literature (Supplementary Figures 1 and 2). Variables considered included the child's exact age at exam (in months), educational attainment of the primary wage earner in the child's home (<highschool, highschool, and > highschool) as a proxy for socioeconomic status, birth order, the short form of the Home Observation for Measurement of the Environment [HOME (continuous measure combining maternal report and interviewer observations, standardized within our sample using z-scores)] (Mott 2004), months breastfed, maternal smoking during pregnancy (yes/no), the mother's performance on an abbreviated 12-item Raven's Continuous Matrices test (continuous) (Arthur and Day 1994; Bilker et al. 2012), and maternal age at pregnancy. We examined whether there was any significant variability associated with either of the assessment's administrators or testing laptops. We also examined models adjusted for additional predictors of the outcome such as the child's video and computer game usage (maternal report of average hours per a week) and collected information on color-blindness as well as illness and medications used in the 24 hours preceding the assessment.

We evaluated child sex as a pre-specified effect modifier in both stratified regression models and in interaction models with the cross-product term of $\log_{10}$TCDD*sex. We also considered modification by breastfeeding (0–1 month and ≥1 month), as lactation is possibly neuroprotective but also a significant source of postnatal exposure (Abraham 2017; Mortensen et al. 2002). In sensitivity analyses, we also considered breastfeeding duration stratified at 0–3 month and ≥3 months, the median duration. Main effects of TCDD were assessed for statistical significance at the 5% level and interactions were considered significant if the Wald test p-value for the cross-product term was <0.2.

RESULTS

Descriptive characteristics of the mother-child pairs included in this analysis are presented in Table 1. All children were Caucasian and the mean age at assessment was 13.1 (±2.9) years. Approximately half of the children were female (49%) and the majority (67.3%) were breastfed past the first month of infancy. As reported by the mother, 13 children had been diagnosed with a learning disability and 10 children with ADHD but none was on medication. The 120 SWHS mothers of the children in this sample were all relatively young

at the time of the Seveso accident (mean=7.6 years old in July 1976 with an age range of newborn to 17 years old). Only 7% of the women reported smoking during pregnancy.

Distributions of maternal 1976 TCDD and levels estimated to the time of pregnancy are included in Supplementary Table 1. The median (IQR) of maternal 1976 and estimated pregnancy TCDD levels were 74.6 (40.4, 214.0) ppt and 4.5 (2.7, 9.2) ppt, respectively. Maternal TCDD levels in 1976 and estimated to pregnancy were strongly correlated (r=0.69). Distributions of the neuropsychological outcomes are available in Supplementary Table 2. On the timed RPM, 55 children were unable to complete all 60 matrices within the time allotted. These children did not significantly differ in age or TCDD exposure from those who completed the test.

The lowess plots and models using restricted cubic splines, did not show evidence of non-linearity in the association of TCDD and neuropsychological performance so we proceeded with linear multivariate regression, with both outcome and exposure included as continuous variables. Tables 2 and 3 report adjusted beta coefficients and 95% CI for models regressing differences in test performance on maternal TCDD levels in 1976 serum and estimated pregnancy levels, respectively. A 10-fold increase in maternal 1976 serum TCDD was not significantly associated with performance on the WCST, CPT, RAVLT, or RPM (Table 2). We observed similarly null associations with all neuropsychological measures when we used maternal serum TCDD estimated to pregnancy (Table 3).

Sex differences

Significant heterogeneity by sex was observed between maternal 1976 TCDD and several measures of the WCST, namely total errors, trials to complete the first sorting set, and nonperseverative errors (Table 2). The coefficients on maternal 1976 TCDD indicated a dose-related pattern of poorer performance in boys but better performance in girls on these WCST measures (Table 2); however, only the observed beneficial association between 1976 TCDD and trials to complete first set in girls was significant (adj-β=−7.70, 95% CI −14.08, −1.33). With maternal TCDD estimated to pregnancy, the sex differences on the WCST were similar though none of the interactions were significant (Table 3).

Interaction by duration of breastfeeding

In models examining interaction and stratification by duration of breastfeeding (0–1 month versus ≥1 month), we observed no evidence that breastfeeding duration modifies the relationship between maternal 1976 TCDD and any of the neurodevelopmental measures (Table 4). However, there was evidence of interaction between breastfeeding duration and maternal TCDD estimated to the time of pregnancy for several scales of the CPT, including commission and omission errors, hit rate standard error overall, and the ADHD confidence index (Table 4). In stratified models, estimated pregnancy TCDD was significantly adversely associated with hit rate standard error overall (adj-β=−6.40, 95% CI 0.44, 12.37) and the ADHD confidence index (adj-β=10.91, 95% CI 0.22, 21.59) only among children with the shortest breastfeeding histories (Table 4). Coefficients in the group with longer breastfeeding, were null, indicating no adverse association with higher TCDD. Interactions of TCDD and breastfeeding with respect to the hit rate SE overall and ADHD confidence

index persisted with some attenuation when the breastfeeding cutpoint was set to the median duration of ≥3 months.

DISCUSSION

This study examined the relationship of *in utero* exposure to TCDD and child neurodevelopment in a unique cohort of Italian children born to women exposed as a result of a 1976 explosion in Seveso, Italy. Although we previously found no association between postnatal TCDD exposure and working memory in the SWHS women (Ames et al. 2017), we hypothesized that those exposed *in utero*, a critical period of brain development, may be more sensitive to TCDD's neurotoxicity. Using serum TCDD levels measured in maternal samples collected soon after the explosion and near the time of the pregnancy, we observed no adverse relationships of maternal TCDD exposure with performance of their 7 to 17 year old children on several tasks of working verbal memory, attention, impulsivity, non-verbal intelligence, and reverse learning. While sensitivities among certain subgroups (e.g. children with shorter lactation histories and boys) merit closer attention, to date, there is limited evidence that TCDD is associated with adverse neuropsychological functioning in Seveso women and their children.

Although studies have found delays in cognitive and psychomotor skills observable at 6–12 months of age related to low levels of prenatal dioxin exposure (Halldorsson et al. 2009; Koopman-Esseboom et al. 1996; Nakajima et al. 2017; Park et al. 2010), the associations in two of these earlier studies attenuated or disappeared by later infancy (Koopman-Esseboom et al. 1996; Nakajima et al. 2017). In somewhat older children 3 to 8 years of age, prenatal exposures to background levels of dioxin-like chemicals have been associated with no (Caspersen et al. 2016a; Forns et al. 2012; Lanting et al. 1998) to subtle but statistically significant deficits in language development, attention, and processing speed as well as atypical gender-related play behavior (Caspersen et al. 2016b; Sagiv et al. 2012; Vreugdenhil et al. 2002). However, some of the most persistent cognitive impairments were reported in Yucheng, where 6–9 year old children were assessed following prenatal exposures to PCBs and furans in contaminated cooking oil (Chen et al. 1992; Guo et al. 1995); while maternal exposures were not biologically measured in these Taiwanese studies, previous work reports mean blood PCDFs levels in Yucheng patients of 0.76 ppb, approximately 10^5 times higher than background in Taiwan (Kashimoto et al. 1985). Taken together with our study, the literature suggests that neurodevelopmental decrements associated with *in utero* exposure may diminish as children grow older but the heterogeneity across these studies in populations, exposure profiles, and neuroassessment measures limits drawing firm conclusions.

Our study also found that child's sex may modify the relationship between TCDD and performance on the WCST with boys doing worse and girls doing better, although none of the adverse associations in boys were significant. Sex differences are plausible given that TCDD's activation of AhR could result in endocrine disruption of the hypothalamic-pituitary-gonadal and thyroid axes during fetal brain development (Cao et al. 2008; Weiss 2012; Wilhelm et al. 2008). Furthermore, reports of sex-patterned alterations to neurogenesis (Hojo et al. 2006; Ikeda et al. 2005; Zareba et al. 2002) and neurobehavior in rodent models

(Hojo et al. 2002; Nishijo et al. 2007) suggest that perinatal dioxin exposure may play alternately anti-estrogenic and anti-androgenic roles in a sex-specific manner. Additional epidemiological evidence support the hypothesis that prenatal dioxins may have sexually dimorphic effects on human neurodevelopment (Caspersen et al. 2016b; Nowack et al. 2015; Winneke et al. 2014); while two of these studies corroborate greater attention and cognitive difficulties specifically among prenatally exposed boys (Guo et al. 1995; Sagiv et al. 2012), the literature also documents neurobehavioral effects specific to girls or opposing effects across sexes (Caspersen et al. 2016b; Nowack et al. 2015; Winneke et al. 2014).

In general, female children of mothers with higher 1976 TCDD levels performed better on the WCST, though only the relationship with the WCST's time to complete the first set was significant. Several studies have also noted beneficial associations between prenatal dioxins and neurodevelopment, including reports of less autistic-like behavior in perinatally-exposed humans and animals (Negishi et al. 2006; Nowack et al. 2015), improved spatial learning in animals (Schantz and Bowman 1989; Schantz et al. 1996), and faster reaction times on the CPT among girls (Sagiv et al. 2012). However, a possible biological explanation for these unexpected favorable relationships remain unknown and these findings could be due to random error or residual confounding.

We also found that TCDD estimated at pregnancy was adversely associated with certain measures of attention (overall hit rate standard error and the ADHD confidence index on the CPT) only among children of women who breastfed for shorter time but not among children of those who breastfed longer. This finding appears consistent with previous work linking prenatal dioxins to poorer divided-attention (Neugebauer et al. 2015) and ADHD-like inattention (Sagiv et al. 2012). Although these studies did not examine effect modification by breastfeeding, their study participants reported, on average, shorter histories of breastfeeding than in our sample. Thus, if breastfeeding protects against the neurotoxic effects of TCDD, the relative difference in breastfeeding histories could explain why their studies observed associations at the marginal level while the present study did not.

Numerous studies have demonstrated the beneficial effects of breastfeeding on neurodevelopment (Abraham 2017; Mortensen et al. 2002). Our concern was whether postnatal exposure via lactation to a lipophilic chemical could outweigh the benefits. For example, dioxin levels in breast milk, without accounting for duration of breastfeeding, were found to be associated with deficits in psychomotor and cognitive skills and increased autistic traits in young Vietnamese children (Nishijo et al. 2014; Tai et al. 2016; Tran et al. 2016). However, our present findings, assuming no unmeasured confounding, suggest that the benefit of breastfeeding may negate the potential neurotoxic influence of perinatal dioxin exposure. This agrees with prior research on other endocrine-disrupting compounds (EDCs) indicating that prenatal exposures have a more pronounced deleterious influence on neurodevelopment in childhood than lactational exposures incorporating breastfeeding duration (Jacobson and Jacobson 1996; Neugebauer et al. 2015; Patandin et al. 1999; Vreugdenhil et al. 2002).

Our study has some limitations. Though our findings, particularly with respect to sex and lactation differences, are suggestive, the small sample size, made effectively smaller in

stratified analyses and when taking sibling covariance into account, limits conclusive inference. Therefore, we cannot rule out the possibility of sampling error in explaining the significant findings. The number of outcomes examined also raises the possibility of observing type I error from multiple comparisons. We did not correct for multiple comparisons given the exploratory nature of the stratified analyses in this relatively small sample size (Althouse 2016). Likewise, analyses may have been underpowered given the wide age range. For example, while the adverse direction of the coefficients between maternal estimated pregnancy TCDD levels and correct responses on the RPM were consistent with studies of this relationship in Yucheng (ages 6–9, n=118) and Mohawk children (ages 10–16, n=271), our study was relatively smaller within these age groups and exhibited wider confidence intervals spanning the null (Guo et al. 1995; Newman et al. 2009b). Despite these power limitations, we did observe patterns across related psychometric domains and redundancies of the assessment that were qualitatively consistent with each other.

The reliance on estimated levels of dioxin during pregnancy is likely a source of exposure misclassification but we expect bias to be non-differential with respect to the outcome. Another limitation is that although our neuroassessment contained a diverse cross-section of psychometric domains with hypothesized sensitivity to TCDD based on the literature (Endo et al. 2012; Haijima et al. 2010; Hojo et al. 2002; Hojo et al. 2008; Newman et al. 2009a; Nishijo et al. 2007; Patandin et al. 1999; Sagiv et al. 2012; Schantz and Bowman 1989; Sioen et al. 2013; Tanida et al. 2014), we possibly still omitted the most sensitive endpoints of cognition and behavior. For example, we could not consider play behavior, psychomotor skills, and language development— outcomes reported in previous epidemiological studies of prenatal dioxin exposure— due to the older ages of our sample (Caspersen et al. 2016b; Nakajima et al. 2017; Nowack et al. 2015; Winneke et al. 2014).

We found little evidence of selection bias despite the relatively low participation among eligible 7–17 year old children in the 2014 SWHS follow up (65%). Among non-participant children (n=88 children, 68 mothers), maternal 1976 levels were higher than those among participants (median (IQR) =157.5(50.8, 393.5) ppt vs. 74.6 (40.4, 214.0) ppt) but estimated exposures in pregnancy were not significantly different (median (IQR)=4.9 (3.0, 9.1) ppt vs. 4.5 (2.7, 9.2) ppt). Non-participant children also tended to be younger and to have mothers who were younger at the time of the accident, explaining the higher 1976 levels (Eskenazi et al. 2004a). Furthermore, participation was unlikely to be related to the outcome as the prevalence of maternally reported ADHD and learning disability in the analytic sample (7% and 10%, respectively) was only slightly higher than rough estimates of ADHD prevalence previously reported in Italian children (5%) (Donfrancesco et al. 2015). Nevertheless, if the mothers' 1976 levels are more relevant, exclusion of these children with higher maternal 1976 levels could have led us to underestimate the relationship between the mother's highest level of exposure and neurodevelopment in the Seveso second generation.

Due to study constraints, we were unable to administer the neuropsychological assessment to SWHS children aged 18+, many of whom were born closer to the time of their mother's initial exposure. Thus, it is possible that the findings in our sample of 7–17 year old children, who were born several TCDD half-lives after the accident, are not generalizable to

these older children who may have experienced the highest *in utero* exposures. Given that some outcomes were related to pregnancy TCDD levels in the present study, a neuropsychological examination of these older children may be warranted.

This study also has several strengths. The unique exposure scenario of the Seveso population, which resulted in a wide distribution of TCDD exposures independent of other dioxin-like compounds (Warner et al. 2005), allows isolation of the particular neurotoxic properties of TCDD. Further, given the substantial decline in environmental dioxins since 1976, the possibility of high postnatal exposures beyond lactation in this study population is low. These conditions allowed us to consider maternal highest lifetime dose of TCDD and levels estimated at the time of pregnancy as two potentially distinct biological pathways impacting the fetus. While we observed suggestive differences in the associations between 1976 and estimated pregnancy exposure models of TCDD with the children's neuropsychological outcomes, the pattern was not consistent and is difficult to interpret without more research.

CONCLUSIONS

The Seveso second generation cohort is unique in its wide range of biologically measured TCDD exposures and affords one of the only epidemiological opportunities to evaluate the specific effects of TCDD exposure *in utero* on neuropsychological function. While we found limited evidence of an adverse association between prenatal dioxin exposure and neuropsychological functioning in 7–17 year old children, the neurotoxicological profile of TCDD warrants further research. In particular, our findings of susceptibility differences by sex and lactation histories, particularly with respect to psychometric measures of learning and attention, should be explored in future work. Additional research that includes the adult children in the second generation, many of whom received the highest prenatal exposures, may also better reveal the long-term neuropsychological effects of prenatal TCDD exposure across the life-course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1:

Characteristics of mothers and children in the study sample, Seveso Second Generation Health Study, Seveso, Italy, 2014–2016

Characteristic	N	%
Maternal characteristics	120	(100)
Age at explosion		
0–10	80	(66.7)
11–20	40	(33.3)
Maternal age at delivery		
24–29	29	(18.01)
30–34	76	(47.2)
35–43	56	(34.78)
Total parity		
0	35	(21.7)
≤1	126	(78.3)
Maternal education		
<Highschool	28	(23.3)
Highschool	34	(28.3)
>Highschool	58	(48.3)
Smoking during pregnancy		
Smoker	11	(6.8)
Non-smoker	150	(93.2)
Household characteristics		
Primary wage earner education		
<Highschool	37	(30.8)
Highschool	43	(35.8)
>Highschool	40	(33.4)
Children characteristics	161	(100)
Child sex		
Female	79	(49.1)
Male	82	(50.9)
Child age at assessment		
7–10	29	(18.0)
11–13	49	(30.4)
14–17	83	(51.6)
Low birthweight (<2500g)	17	(10.6)
Preterm (<37wks)	14	(8.7)
Breastfeeding duration (months)		
0–1 month	52	(32.7)
> 1 month	109	(67.3)
Sibling groups in neuro sample		
One child groups	83	(51.6)

Characteristic	N	%
Two sibling groups	33	(41.0)
Three sibling groups	4	(7.4)
Diagnosed with ADHD (maternal report)	11	(6.9)
Diagnosed with learning disability (maternal report)	15	(9.4)

Table 2:

Neuropsychological outcomes associated with a 10-fold increase in maternal serum TCDD levels in 1976 (Log_{10}TCDD) for all children, stratified by child sex, Seveso Second Generation Health Study, Seveso Italy 2014–2016

Outcome	Direction of poorer performance[c]	All children n=161 adj-β[a] 95% CI	Girls n=79 adj-β[a] (95% CI)	Boys n=82 adj-β[a] (95% CI)	p_{int}
Wisconsin Card Sorting Test (WCST)					
Total errors T-score	(−)	1.32 (−1.81, 4.45)	4.78 (−0.46, 10.01)	−1.84 (−5.85, 2.16)	0.01
Trials to complete first set	(+)	−1.71 (−6.33, 2.90)	−7.70 (−14.08, −1.33)*	2.70 (−3.90, 9.30)	0.01
Perseverative errors T-score	(−)	0.53 (−3.37, 4.43)	1.72 (−5.00, 8.45)	−0.29 (−5.78, 5.20)	0.29
Non-perseverative errors T-score	(−)	1.22 (−2.06, 4.49)	3.56 (−2.02, 9.14)	−1.46 (−5.49, 2.56)	0.04
Connor's Continuous Performance Test (CPT)[b]					
Commission errors T-score	(+)	−0.69 (−3.62, 2.24)	−1.99 (−6.11, 2.12)	0.56 (−3.87, 4.99)	0.22
Omission errors T-score	(+)	−1.08 (−4.37, 2.21)	−1.54 (−7.03, 3.94)	−0.05 (−3.50, 3.40)	0.34
Hit rate SE overall T-score	(+)	0.47 (−2.44, 3.39)	0.35 (−4.41, 5.12)	1.32 (−2.24, 4.88)	0.41
Hit rate SE between sets T-score	(+)	−0.15 (−3.09, 2.80)	−0.33 (−5.26, 4.59)	0.57 (−3.00, 4.14)	0.61
ADHD confidence index	(+)	−0.20 (−5.27, 4.87)	1.32 (−7.23, 9.87)	0.09 (−5.72, 5.91)	0.75
Rey's Auditory and Verbal Learning Test (RAVLT)					
Immediate recall	(−)	1.08 (−0.50, 2.66)	1.04 (−1.49, 3.58)	1.01 (−0.98, 3.00)	0.76
Delayed recall	(−)	0.32 (−0.34, 0.98)	0.28 (−0.70, 1.27)	0.30 (−0.64, 1.24)	0.93
Ravens Progressive Matrices (RPM)					
Total correct	(−)	0.10 (−2.03, 2.24)	0.28 (−3.45, 4.01)	−0.11 (−2.71, 2.49)	0.97

[a] Adjusted for child age at assessment, maternal age at pregnancy, highest level of education in household, maternal shortened Ravens score, total parity, and child sex. Child sex was removed from sex-stratified models.

[b] Excluding child with apparent non-effort or computer malfunction during assessment

[c] (+) higher scores indicate poorer performance on test, (−) lower scores indicate poorer performance on test

* p<0.05

Table 3:

Neuropsychological outcomes associated with a 10-fold increase in maternal serum TCDD levels estimated at the time of pregnancy (Log_{10}TCDD), stratified by child sex, Seveso Second Generation Health Study, Seveso Italy 2014–2016

Outcome	Direction of poorer performance[c]	All children n=161 adj-β[a] 95% CI	Girls n=79 adj-β[a] (95% CI)	Boys n=82 adj-β[a] (95% CI)	p_{int}
Wisconsin Card Sorting Test (WCST)					
Total errors T-score	(−)	−0.13 (−3.52, 3.26)	1.02 (−4.19, 6.22)	−2.51 (−6.95, 1.92)	0.56
Trials to complete first set	(+)	0.56 (−3.77, 4.89)	−1.91 (−7.60, 3.78)	3.00 (−3.71, 9.71)	0.40
Perseverative errors T-score	(−)	0.95 (−3.70, 5.59)	1.91 (−5.30, 9.12)	−1.67 (−8.25, 4.91)	0.60
Non-perseverative errors T-score	(−)	−0.48 (−3.75, 2.80)	0.04 (−4.24, 4.33)	−2.04 (−6.49, 2.42)	0.60
Connor's Continuous Performance Test (CPT)[b]					
Commission errors T-score	(+)	−1.52 (−4.80, 1.76)	−1.97 (−6.80, 2.86)	−0.66 (−5.58, 4.25)	0.82
Omission errors T-score	(+)	0.06 (−2.47, 2.58)	0.61 (−3.49, 4.72)	0.87 (−1.94, 3.68)	0.95
Hit rate SE overall T-score	(+)	2.47 (−0.49, 5.43)	3.26 (−1.72, 8.25)	2.87 (−0.41, 6.15)	0.84
Hit rate SE between sets T-score	(+)	1.07 (−2.27, 4.42)	0.84 (−4.31, 5.99)	2.47 (−2.03, 6.97)	0.63
ADHD confidence index	(+)	3.92 (−1.17, 9.01)	6.60 (−1.93, 15.12)	3.50 (−1.94, 8.94)	0.49
Rey's Auditory Verbal Learning Test (RAVLT)					
Immediate recall	(−)	1.02 (−0.65, 2.68)	0.10 (−2.56, 2.75)	1.66 (−0.55, 3.87)	0.58
Delayed recall	(−)	0.46 (−0.19, 1.12)	0.02 (−0.93, 0.96)	0.81 (−0.18, 1.79)	0.49
Ravens Progressive Matrices (RPM)					
Total correct	(−)	−1.95 (−4.02, 0.11)	−2.86 (−5.81, 0.10)	−2.02 (−5.23, 1.20)	0.50

[a] Adjusted for child age at assessment, maternal age at pregnancy, highest level of education in household, maternal shortened Ravens score, total parity, and child sex. Child sex was removed from sex-stratified models.

[b] Excluding child with apparent non-effort or computer malfunction during assessment

[c] (+) higher scores indicate poorer performance on test, (−) lower scores indicate poorer performance on test

* p<0.05

Table 4:

Neuropsychological outcomes associated with a 10-fold increase in maternal serum TCDD levels (Log_{10} TCDD), stratified by breastfeeding duration (0–1 month vs. >1 month), Seveso Second Generation Health Study, Seveso Italy 2014–2016

		Maternal serum TCDD levels in 1976			Maternal serum TCDD levels estimated to pregnancy		
Outcome	**Direction of poorer performance[c]**	**0–1 Month adj-β[a] (95% CI)**	**>1 Month adj-β[a] (95% CI)**	**P_{int}**	**0–1 Month adj-β[a] (95% CI)**	**>1 Month adj-β[a] (95% CI)**	**P_{intt}**
Wisconsin Card Sorting Test (WCST)							
Total errors T-score	(−)	0.41 (−6.75, 7.57)	2.06 (−1.69, 5.81)	0.94	−0.75 (−8.17, 6.66)	0.35 (−3.52, 4.23)	0.93
Trials to complete first set	(+)	2.73 (−8.47, 13.93)	−3.57 (−8.86, 1.72)	0.66	−1.41 (−9.77, 6.95)	1.05 (−3.72, 5.82)	0.91
Perseverative errors T-score	(−)	0.86 (−9.50, 11.23)	1.59 (−2.79, 5.96)	0.86	−0.79 (−10.97, 9.39)	3.12 (−2.73, 8.97)	0.56
Non-perseverative errors T-score	(−)	1.43 (−5.23, 8.09)	0.46 (−3.41, 4.34)	0.26	0.59 (−4.49, 5.66)	−1.91 (−6.20, 2.38)	0.37
Connor's Continuous Performance Test (CPT)[b]							
Commission errors T-score	(+)	0.94 (−4.86, 6.73)	−0.63 (−4.18, 2.91)	0.60	1.41 (−4.13, 6.94)	−3.28 (−7.40, 0.83)	0.12
Omission errors T-score	(+)	1.08 (−3.65, 5.81)	−2.02 (−6.53, 2.49)	0.59	2.42 (−2.71, 7.55)	−1.98 (−5.17, 1.22)	0.10
Hit rate SE overall T-score	(+)	2.93 (−2.31, 8.18)	−0.36 (−4.04, 3.33)	0.31	6.40 (0.44, 12.37)*	−0.22 (−3.99, 3.55)	0.05
Hit rate SE between sets T-score	(+)	1.31 (−3.43, 6.06)	−1.03 (−4.88, 2.81)	0.21	2.20 (−3.44, 7.84)	−0.11 (−4.43, 4.20)	0.53
ADHD confidence index	(+)	4.85 (−5.13, 14.83)	−2.61 (−8.99, 3.77)	0.20	10.91 (0.22, 21.59)*	−1.92 (−7.74, 3.89)	0.02
Rey's Auditory Verbal Learning Test (RAVLT)							
Immediate recall	(−)	−0.21 (−2.73, 2.31)	1.71 (−0.19, 3.61)	0.51	0.18 (−2.23, 2.59)	1.67 (−0.80, 4.14)	0.32
Delayed recall	(−)	0.05 (−1.02, 1.12)	0.46 (−0.36, 1.29)	0.69	0.50 (−0.28, 1.28)	0.44 (−0.59, 1.47)	0.94
Ravens Progressive Matrices (RPM)							
Total correct	(−)	0.52 (−3.11, 4.15)	0.29 (−2.44, 3.03)	0.73	−3.42 (−7.15, 0.31)	−0.61 (−3.56, 2.34)	0.30

[a] Adjusted for child age at assessment, maternal age at pregnancy, highest level of education in household, maternal shortened Ravens

[b] Excluding child with apparent non-effort or computer malfunction during assessment

[c] (+) higher scores indicate poorer performance on test, (−) lower scores indicate poorer performance on test

* $p<0.05$