Maternal Serum Persistent Organic Pollutants in the Finnish Prenatal Study of Autism: A Pilot Study

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Abstract

Recent research emphasizes the contribution of environmental as well as genetic factors to the etiology of autism but studies testing associations between chemical exposures and autism have been limited. Prenatal exposure to persistent organic pollutants (POPs) has previously been associated with decrements in cognitive and developmental performance. We conducted a pilot study in the Finnish Prenatal Study of Autism (FiPS-A). Seventy-five cases with autism and 75 controls matched on sex, birth year, urbanization and maternal age were sampled from first-born children in the Finnish Maternity Cohort, which includes over 1 million births. The study sample included births occurring from 1991 to 2000. Subjects were followed up for autism through 2007. DDT, DDE, PCB-118, PCB-138, PCB-153, PCB-156, PCB-170, PCB-180, hexachlorobenzene, and BDE-47 were measured in archived maternal serum samples taken during pregnancy using gas chromatography-high resolution mass spectrometry. Correlations between pollutant measures were assessed and mechanistically-related weighting schemes for summarizing PCB levels were compared. Case and control differences were assessed using graphical and statistical methods. All analytes, with the exception of DDT and BDE-47, were detected above the limit of quantification in all samples. The correlation between levels of individual PCB congeners and weighted summary measures was high (0.71-1.00). Paired t-tests revealed no significant differences between cases and controls for log-transformed mean values of any analyte; however, in an adjusted model the odds ratio for autism was 1.91 (p=0.29) and 1.79 (p= 0.36) respectively, for subjects with total PCBs and DDE above the 90^{th} percentile of control values. Levels of prenatal PCB exposure in FIPS-A were similar to levels previously correlated with poorer neurodevelopmental measures in other populations. Further study in a

larger sample will be required to fully determine whether exposure to high POP levels are associated with autism diagnosis in the population.

Keywords: autism, persistent organic pollutants, PCBs, DDE, prenatal

1. Introduction

Autism is a developmental disorder involving impairments in language and social interaction; repetitive behaviors and restricted interests; and with an onset before age three. The developmentally dependent nature of the deficits involved in autism means that diagnosis is not typically made until after the first year of life. However, evidence suggests that neurodevelopmental events underlying the behavioral characteristics of autism have roots in the prenatal period (Arndt et al., 2005; Patterson, 2009), while recent heritability estimates suggest that environmental factors, in concert with genetics, play a substantial role in risk for the disorder (Hallmayer et al., 2011). Studies are limited in number that have addressed potential associations between autism spectrum disorders (ASDs) and prenatal exposure to chemicals in the environment, primarily focusing on exposure via air (Windham et al., 2006; Kalkbrenner et al., 2010; Roberts et al., 2007; Larsson et al., 2009; Volk et al., 2011).

Persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ether (PBDE), the pesticide dichlorodiphenyltrichloroethane (DDT), and its metabolite (dichlorodiphenyldichloroethylene) DDE, are lipophilic and bioaccumulate in the food chain. Associations between prenatal PCB or DDT/DDE exposure and lower scores on neurodevelopmental measures in humans have been reported in some (i.e. (Eskenazi et al., 2006; Jacobson and Jacobson, 1996; Jacobson and Jacobson, 2003; Jacobson et al., 1985; Park et al., 2010; Patandin et al., 1999; Stewart et al., 2000; Stewart et al., 2008; Torres-Sanchez et al., 2007)), though not all (i.e. (Bahena-Medina et al., 2011; Fenster et al., 2007; Gray et al., 2005; Winneke et al., 1998)) studies. While associations reported have primarily focused on general developmental or cognitive measures, studies examining biomarkers of exposure to PCBs, DDT or PBDE,

specifically, and autism have not to our knowledge been reported. Roberts *et al*. reported an increased risk of ASD among children whose mothers lived near sites where organochlorine pesticides as a group had been applied to fields in California during gestation (Roberts et al., 2007). Dicofol and endosulfan were the two pesticides primarily accounting for applications in this category. Dicofol in particular is chemically similar to DDT but is cleared from the body more quickly with lower bioaccumulation (Roberts et al., 2007). It may be hypothesized, therefore, that DDT could have a similar, and perhaps stronger, association with autism.

The Finnish Prenatal Study of Autism (FiPS-A) is based on a national birth cohort including the approximately 1.2 million births in Finland occurring from 1987-2005. A unique feature is availability of archived maternal serum samples taken during early gestation. A study of 112 placentae from male infants born between 1997-2001 in Finland found detectable levels of p,p'-DDE in 100% of samples, as well as a high prevalence of additional organochlorinated pesticides (Shen et al., 2005), suggesting that prenatal exposure to these substances is relevant for the Finnish population. We conducted a pilot study with the following aims: 1) to establish the feasibility of measuring POPs in prenatal maternal serum samples from the FiPS-A; 2) to evaluate associations of POPs with covariates in the general population; 3) to compare alternative weighting schemes for PCB congeners; and 4) to assess preliminary evidence for a relationship between exposure to prenatal POPs and autism.

2. Methods

2.1. Population

Complete details of the FiPS-A study design have been published elsewhere (Lampi et al., 2011). Briefly, the FiPS-A is based on the Finnish Maternity Cohort, a large national birth cohort including the approximately 1.2 million births in Finland from 1987-2005. We identified 1,132 childhood autism (ICD-code F84.0) cases diagnosed through 2007 by record linkage of the Finnish Medical Birth Registry (FMBR) with the Finnish Hospital and Outpatient Discharge Registry. Finnish registry diagnoses of childhood autism have been validated by the ADI-R, with 77/80 cases assessed (96%) meeting ICD-10 criteria (Lampi et al., 2010). Controls from the FMBR without ASD or severe/profound mental retardation were matched 4:1 to cases on date of birth (+/- 30 days), sex, place of birth, and residence in Finland. One control per case was selected for serologic analysis. Data on additional pre-, peri-, and neonatal variables are available from the FMBR.

For the present study, 75 subjects with autism and 75 controls matched on sex, birth year, urbanization and maternal age were selected from the total FiPS-A population. The purpose of matching was to select a comparison group similar on potential confounders given the limited sample size. The study sample was selected from first-born children born from 1991 to 2000 with sera available for analysis. For 66/75 case-control pairs a match according to these criteria was found from among the 4 matched controls identified for the parent study; for 9 pairs, a control meeting matching and inclusion criteria for the current study was identified from among other FiPS-A control subjects. Among 146 subjects where gestational age at blood draw was known, 79% of maternal samples were collected during the first trimester. Mean gestational age at blood draw did not significantly differ (p=0.70) between cases (11.1 weeks) and controls (10.8 weeks).

2.2. Laboratory analysis

DDT, DDE, 2,3',4,4',5-pentachlorobiphenyl (PCB-118), 2,2',3',4,4',5-hexachlorobiphenyl (PCB-138), 2,4,5,2',4',5'-hexachlorobiphenyl (PCB-153), 2,3,3',4,4',5-hexachlorobiphenyl (PCB-156), 2,2',3,3',4,4',5-heptachlorobiphenyl (PCB-170), 2,2',3,4,4',5,5'-heptachlorobiphenyl (PCB-180), hexachlorobenzene (HCB), and 2,2',4'4'-tetrabromodiphenyl ether (BDE-47) were measured in 200 µl aliquots from archived maternal serum using an extension (Koponen et al, manuscript under preparation) of previously published methods (Rantakokko et al., 2009). POPs selected for measurement were based on previous association with neurodevelopmental outcomes, environmental impact, and technical capabilities of the assay. The 6 PCB congeners measured account for approximately 80% of total PCBs on a mass basis. Briefly, after thawing the samples, ethanol and ^{13}C-labelled internal standards of each compound were added to serum to precipitate the proteins and equilibrate internal standards. Dichloromethane-hexane and activated silica were added and samples mixed. In this step extraction of POPs to organic solvent and binding of the sample water, ethanol and precipitate to activated silica were performed simultaneously. The upper dichloromethane-hexane layer was poured into a Solid Phase Extraction cartridge (SPE cartridge) containing silver nitrate silica and a mixture of sodium sulphate and silica. The lower semisolid layer in the test tube was extracted again with dichloromethane-hexane, and solvent was poured into the same SPE-cartridge. Elution of the SPE-cartridge was continued using additional dichloromethane-hexane. The eluate was concentrated to 20-30 µl and analyzed with gas chromatography (Agilent 6890) high resolution mass spectrometry (Waters Autospec Ultima).

In each batch of samples, 2 blanks were included, to control for possible laboratory contamination. Two control samples, Standard Reference Material 1589a (PCBs, Pesticides, PBDEs, Dioxins/Furans in human serum), from the National Institute of Standards and

Technology were included in each batch. Average recoveries of measured POPs in SRM 1589a were 92.5-104.8% of the certified values. The relative standard deviation of results measured from SRM 1589a ranged from 2.5 to 5.0% for all compounds except for p,p'-DDT for which it was 14.4% due to levels in SRM 1589a close to the limit of quantitation of the method.

2.3. Weighting schemes

PCBs have different modes of action with potential relevance for autism both within and across congeners. Because it is not precisely known which type of toxicity would be potentially most relevant for autism, we compared four different summary measures for PCBs: a sum of the total concentrations of the 6 measured congeners ("total PCBs") and three previously published weighting schemes: toxic equivalents (TEQ) for dioxin-like PCBs (Van den Berg et al., 2006), neurotoxic equivalent (NEQ) based on *in vitro* measures such as Ca^{+2} homeostasis (Simon et al., 2007) and a thyroid-hormone based TEQ (Yang et al., 2010).

2.4. Statistical analysis

Characteristics (birth year, maternal and paternal ages, sex, urbanicity, previous maternal pregnancies, and maternal socioeconomic status) of cases and controls were tabulated and compared using chi-square tests. To test for association of exposure with covariates in the general population which could potentially lead to confounding, means and standard deviations of the natural log of pollutant levels were calculated by category of covariates in controls, and differences across categories tested using ANOVA (f-test) and tests of linear trend.

Quantile-quantile (Q-Q) plots were used to graphically compare case and control distributions of pollutant levels. POP measures were log-transformed prior to statistical analyses. Means for each POP measure were compared between cases and matched controls using paired t-

tests. Correlations between individual pollutant measures and summary measures were calculated.

Because univariate and graphical comparisons suggested no differences in case and control distributions near the middle of the exposure range, but possible differences at the high end of the range, conditional logistic regression models used indicator variables for at or above (versus below) the 90^{th} percentile of control values for pollutants. Conditional logistic regression models were used to examine the associations between childhood autism and each POP measure. Unadjusted odds ratios (OR) and 95% confidence intervals (CI) were calculated for each POP measure. Due to high correlation (described below) between PCB congeners and summary measures, total PCBs were used as a single measure of PCB exposure in a multivariate model that also included DDE and HCB. DDT and BDE-47 were not included due to high numbers of observations with missing data. In all analyses, a two-sided p-value of <0.05 was considered statistically significant. Statistical analyses were performed with SAS statistical software (SAS Version 9.2, Cary, NC, USA).

3. Results

Birth year, parental ages, sex and urbanicity were similar for control and autism groups (Table 1). Autism mothers were more likely to have experienced a prior pregnancy and to have an upper white collar or "other" socioeconomic status, but these differences were not statistically significant ($p>0.05$).

For all 6 PCB congeners, DDE, and HCB, levels above the limit of quantitation (LOQ) were observed in all samples. Levels above the LOQ were observed in only 7/150 samples for DDT and 35/150 for BDE-47. Associations between POP measures and covariates were

determined among controls (Table 2) in order to assess these associations in the general population. The only covariate showing a consistent significant association with POP measures was birth year; later birth year was associated with lower levels of these POPs. DDE levels were significantly higher (p=0.02) among control mothers who experienced a prior pregnancy, and showed a marginally significant (p=0.06) linear association with maternal age.

Mean levels of none of the pollutants differed significantly between cases and controls. However, 90th percentile levels were greater in cases for DDE, HCB and all PCBs (not shown; differences in the proportion >90th percentile for cases versus controls that were not statistically significant). DDE was minimally correlated with other pollutants ($|r|<0.1$). For HCB and total PCBs, r=0.76. The correlation between total PCBs and individual congeners ranged from 0.85 (PCB-118) to 1.00 (PCB-153); for total PCBs and composite TEQ measures, correlation ranged from 0.91 (TEQ-dioxin) to 1.00 (NEQ).

In unadjusted models, most ORs were above 1.0; however, given the small numbers of exposed subjects, all CIs were wide and included 1.0. The highest observed ORs (95% CI; p-value) were for PCB-138: 2.25 (0.69, 7.31; 0.18), PCB NEQ: 2.20 (0.76, 6.33; 0.14), total PCBs: 2.00 (0.68, 5.85; 0.21), and DDE: 2.00 (0.60, 6.64; p=0.26). ORs (95% CI; p-value) for total PCBs $\geq$90th percentile were 1.91 (0.57, 6.39; p=0.29) for total PCBs, 0.89 (0.28, 2.76; p=0.83) for HCB, and 1.79 (0.51, 6.21; p=0.36) for DDE (Table 3).

4. Discussion

In this pilot study, we measured levels of 10 POPs in archived maternal serum samples corresponding to 75 cases with autism and 75 matched controls born in Finland between 1991 and 2000. We first demonstrated that 6 PCB congeners, DDE and HCB are quantifiable in these archived serum samples, with DDT and BDE-47 quantified in a limited number of samples.

We explored associations of POP levels with potential confounding variables among controls. Factors previously found to be associated with higher levels of adipose PCBs in a Finnish general population included older age, lactation history, geographic area, and frequency of fish consumption (Kiviranta et al., 2005). In the present study, total PCBs, HCB and DDE levels all decreased significantly with later year of birth, which is indicative of the time of serum sampling. This is consistent with the interpretation that cross-sectional associations between higher levels of POPs including PCBs and older age was driven by decreased environmental exposure over time (Kiviranta et al., 2005). The concurrent temporal trends of increasing autism diagnoses and decreasing POP exposures, in our view, should not necessarily be taken as evidence against a potential association. Increased rates of autism diagnoses may have many causes (Fombonne, 2002), including increased awareness, changes in diagnostic criteria, and increases in other environmental exposures.

The maternal POP levels observed here were similar to those of certain other populations where associations with other neurodevelopmental disorders have been observed. Using correction factors previously developed to facilitate comparison across 10 studies of PCBs and neurodevelopment from North America and Europe (Longnecker et al., 2003), we estimated a median of 57 ng/g lipid for PCB-153 in controls from the present study. This was on the lower end of the distribution, but well within the range of 30-450 ng/g lipid PCB-153 reported among those 10 studies, which had sample collections ranging from 1959-1998. Our median level was closest to those observed in studies of populations from the U.S. in North Carolina (Rogan et al., 1986), New York (Darvill et al., 2000), and Massachusetts (Korrick et al., 2000). In each of these three studies, significant associations have been observed between prenatal PCB exposure levels and decrements in neurodevelopmental measures, including, for example, hypotonicity and

hyporeflexia (Rogan et al., 1986), attention-related measures (Sagiv et al., 2008) on the Neonatal Behavioral Assessment Scale in newborn infants; and intelligence scores on the Fagan Test of Infant Intelligence at 6 and 12 months (Darvill et al., 2000).

Cases with autism in this population have numerically higher percentages of observations above the 90th percentile of control distributions for some maternal POPs, suggesting there may be a threshold for some pollutants above which higher levels are associated with increased risk of autism. Although these associations were not statistically significant in this small study, total PCBs at or above the 90th percentile were associated with an odds ratio for autism of 1.91 (95% CI, (0.57, 6.39)).

The strengths of this study include biomarker-based exposure assessments using archived maternal serum samples during pregnancy. The subsample of subjects in this study were drawn from the FiPS-A, based on a large, population-based national birth cohort including all childhood autism cases diagnosed in Finland assessed through psychiatric registries. This population-based design reduces the chance of selection bias in the study. The register diagnosis of childhood autism has been validated by directly administered ADI-R interviews. Finally, data on potential confounders available through population registers were used for matching and analysis of relationships between exposures and outcome.

We note potential limitations. Since the sample size was limited, a larger study will be required to determine whether maternal POP exposure in the upper decile is significantly associated with autism in this population. It is also possible that controls were matched to cases more closely than necessary to address confounding, thereby decreasing observed differences in exposure levels between groups, or that an unmeasured confounder biased the observed

association. However, it remains that correlation does not equal causation, and the absence of observed correlation does not imply the absence of causation.

In conclusion, we showed that POPs are present at detectable levels in prenatal serum samples from FiPS-A study subjects. This pilot study suggests that qualitatively higher levels of total PCBs may be associated with risk of childhood autism; however, a larger study will be required to substantiate this finding given the lack of statistical significance. A larger study would also allow subgroup analyses, for example, by child sex. Future studies should also address issues of multiple comparisons among correlated exposures, using methods such as semi-Bayes hierarchical models (Greenland and Poole, 1994). The identification of environmental risk factors for autism may have substantial public health importance given their potential modifiability. Despite declining levels over time, 6 PCB congeners, DDE and HCB were still detected in 100% of the Finnish samples tested; additionally, a nationally-representative study from the United States found detectable levels of PCBs, DDE, HCB and BDE-47 in 96-100% of samples from pregnant and non-pregnant women of childbearing age in 2003-2004 (Woodruff et al., 2011). While tens of thousands of industrial chemicals remain in use, information on their potential neurodevelopmental effects is absent for the vast majority (Grandjean and Landrigan, 2006). In our view, this study may represent an important initial step towards investigating environmental risk factors for autism.

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Conflict of interest statement:

The authors declare that there are no conflicts of interest.

Table 1. Demographic characteristics for 75 subjects with childhood autism and 75 matched controls.

Characteristic	Autism (N=75)	Controls (N=75)
	Mean (sd)	*Mean (sd)*
Birth year	1995 (2.6)	1995 (2.7)
Maternal age	31.3 (4.1)	30.7 (3.7)
Paternal age	32.4 (5.6)	32.4 (5.7)
	%	%
Male	84%	84%
Urban (>50,000 inhabitants)[a]	80%	79%
Rural (<=50,000 inhabitants)[a]	20%	21%
Previous pregnancies >=1	29%	21%
Socioeconomic status[b]		
Upper white collar	33%	28%
Lower white collar	43%	51%
Blue collar	13%	13%
Other	10%	7%

[a]data missing for n=1 observation (cases), percentages calculated out of non-missing values.

[b]data missing for 8 cases and 8 controls, percentages calculated out of non-missing values.

Table 2. Associations of subject characteristics with persistent organic pollutant levels among 75 controls.

		Total PCBs[a] ln(pg/ml)		HCB[a] ln(pg/ml)		DDE[a] ln(pg/ml)	
	N	Mean (SD)	p-value	Mean (SD)	p-value	Mean (SD)	p-value
Maternal age							
25-29	39	6.93 (0.48)	0.41	5.05 (0.40)	0.90	6.76 (0.71)	0.16[b]
30-34	22	7.06 (0.40)		5.02 (0.37)		6.95 (0.75)	
35+	14	7.10 (0.56)		5.01 (0.41)		7.25 (1.16)	
Paternal age							
25-29	25	6.97 (0.53)	0.54	5.06 (0.42)	0.84	6.77 (0.91)	0.44
30-34	29	7.00 (0.44)		5.04 (0.37)		6.84 (0.61)	
35-39	14	6.94 (0.50)		4.95 (0.41)		7.18 (1.20)	
40+	7	7.25 (0.35)		5.06 (0.38)		7.09 (0.30)	
Birth year							
1991-1993	23	7.27 (0.45)	<0.001[c]	5.32 (0.32)	<0.001[b]	7.19 (0.78)	0.01[b]
1994-1996	25	7.01 (0.45)		5.03 (0.36)		7.04 (0.85)	
1997-2000	27	6.77 (0.40)		4.80 (0.31)		6.54 (0.74)	
Infant sex							
Male	63	7.01 (0.49)	0.66	5.02 (0.39)	0.53	6.96 (0.85)	0.21
Female	12	6.95 (0.42)		5.10 (0.43)		6.63 (0.68)	
Geographic							
Urban	59	7.01 (0.47)	0.88	5.01 (0.42)	0.75	6.94 (0.86)	0.48
Rural	16	6.99 (0.49)		5.04 (0.38)		6.77 (0.74)	
Previous pregnancies							
None	58	6.99 (0.44)	0.89	5.02 (0.35)	0.75	6.77 (0.65)	0.02
One or more	15	6.97 (0.57)		4.99 (0.49)		7.31 (1.26)	
Socioeconomic status							
Upper white collar	19	7.14 (0.52)	0.35	5.11 (0.37)	0.75	6.99 (0.69)	0.57
Lower white collar	34	7.01 (0.41)		5.00 (0.34)		6.87 (0.74)	
Blue collar	9	6.93 (0.43)		5.03 (0.39)		6.71 (0.77)	
Other	5	6.75 (0.44)		4.96 (0.33)		6.55 (0.48)	

[a]PCBs, polychlorinated biphenyls; HCB, hexachlorobenzene; DDE, dichlorodiphenyldichloroethylene. Total PCBs calculated as the sum of 6 congeners (PCB-118, PCB-138, PCB-153, PCB-156, PCB-170, and PCB-180).

[b]Test for linear trend, p=0.06.

[c]Test for linear trend, p<0.01.

Table 3: Comparison between childhood autism cases and controls with persistent organic pollutant levels at or above the 90th percentiles of control distributions.

Pollutant	n≥90th percentile (vs. n=8 controls)	OR[a] (95% CI)	OR[b] (95% CI)
HCB	10	1.29 (0.48, 3.45)	0.89 (0.28, 2.76)
DDE	12	2.00 (0.60, 6.64)	1.79 (0.52, 6.21)
PCB-118	8	1.00 (0.32, 3.10)	
PCB-138	13	2.25 (0.69, 7.31)	
PCB-153	12	1.80 (0.60, 5.37)	
PCB-156	13	1.83 (0.68, 4.96)	
PCB-170	12	1.67 (0.61, 4.59)	
PCB-180	13	1.83 (0.68, 4.96)	
Total PCBs	13	2.00 (0.68, 5.85)	1.91 (0.57, 6.39)
TEQ_{dioxin}	11	1.75 (0.51, 5.98)	
$TEQ_{thyroid}$	12	1.80 (0.60, 5.37)	
NEQ	13	2.20 (0.76, 6.33)	

[a]Odds ratios from unadjusted conditional logistic regression model comparing childhood autism cases and matched controls.

[b]Odds ratios from conditional logistic regression model comparing childhood autism cases and matched controls; mutually adjusted for other pollutants in column.