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Molecularly Tracing of Children Exposure Pathways to Environmental Organic Pollutants and the Autism Spectrum Disorder Risk

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Running Title: Childhood exposure to POPs and Autism Spectrum Disorder Risk.

Abstract:

Organic pollutants (OPs) including organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and polycyclic aromatic hydrocarbons (PAHs) have showed neuro-damaging effects, but studies concerning the autism spectrum disorder (ASD) risk are limited. A case-control study with ASD (n=125) and healthy control (n=125) children was conducted on the different land use settings across Punjab, Pakistan. Serum concentrations of 26 OCPs, 29 PCB congeners, 11 PBDEs and 32 PAHs were measured. Serum PCB77 (AOR = 2.00; 95% CI: 1.43, 2.18), PCB118 (AOR = 1.49; 95% CI: 1.00, 2.00), PCB128 (AOR = 1.65; 95% CI: 1.01, 1.91), PCB153 (AOR = 1.80; 95% CI: 1.55, 1.93) were significantly higher, but PCB187 (AOR = 0.37; 95% CI: 0.24, 0.49) was significantly lower in the ASD cases when compared to the controls. Serum BDE99 (AOR = 0.48; 95% CI: 0.26, 0.89) was significantly higher in the healthy controls than in the ASD cases. Among the analysed OCPs, p,p′-DDE (AOR = 1.50; 95% CI: 1.00, 1.85) was significantly elevated in the ASD cases with comparison in the controls. For PAHs, serum dibenzothiophene (AOR = 7.30; 95% CI: 1.49, 35.85) was significantly higher in the ASD, while perylene (AOR = 0.25; 95% CI: 0.06, 1.10) and fluorene (AOR = 0.21; 95% CI: 0.06, 0.72) were significantly higher in the controls. In addition, many of the serum pollutants were significantly associated with GSTT1, GSTM1 (null/present polymorphism) and presented the genotypic variation to respond xenobiotics in children. The children living in proximity to urban and industrial areas had a greater exposure to most of the studied pollutants when compared to the rural children, however children residing in rural areas showed higher exposure to OCPs. This comprehensive study documents an association between environmental exposure risk of several organic pollutants (OPs) from some contaminated environmental settings with ASD risk in children from Pakistan.

Keywords: Autism Spectrum disorder, Organic Pollutants, Estimated Daily Intake, Polychlorinated biphenyls, Organochlorine pesticide, Polybrominated diphenyl ethers, Polyaromatic hydrocarbons, Pakistan.

1. Introduction

Autism Spectrum Disorder (ASD) is a group of neurodevelopmental ailments categorized based on impaired social and verbal communication and restrictive and/or repetitive behavioral patterns. The causative factors of ASD are diverse and still an unresolved question (Marrus and Constantino, 2016), which may be caused by interplay between genes and environmental factors through the epigenetic modification and/or other toxic action on the neurodevelopmental process (Tordjman et al., 2014). Autism's etiology is so complex that a single factor could not be defined as its full causation, rather ASD is diverse and multifactorial disorder (Parellada et al., 2014; Tordjman et al., 2014). In the vast assortment of environmental pollution contributors to ASD, exposure to persistent organic pollutants (e.g., OCPs, PCBs, PAHs, PBDEs etc.) is claimed to be the potential one (Rossignol et al., 2014; Lyall et al., 2016; Ye et al., 2017; Brown et al., 2018;). The broad-spectrum use of such synthetic chemicals (including various pesticides, flame retardants, plasticizers, lubricants, refrigerants, fuels, solvents, and preservatives) has increased significantly over several decades and may have been directly linked to the rising numbers of neurodevelopment disorders including ASD (Lyall et al., 2016; Ye et al., 2017). Many of these organic pollutants are used as additives in a variety of consumer products and have capacity to be leached out in the environment. These organic pollutants have long half-lives and persist in the environment for very long periods, this leads to direct/indirect human exposure through various pathways such as dermal contact, ingestion of contaminated food and water, and inhalation of aerosols and dust (Dirtu and Covaci, 2010; West et al., 2016). The potential of these contaminants as a risk for human health has enhanced their importance and need for their eradication because many of them are causative agents for various health concerns including liver related conditions, neurodevelopmental and behavioral issues, hormonal ailments (Grandjean and Landrigan, 2014; Meeker and Stapleton, 2010).

Most of these compounds are lipophilic and deposit into fatty tissues of organisms, from there they may leach into the body and act as endocrine disrupting chemicals (EDCs) (Eqani et al., 2013; Ali et al., 2013a). Young children and pregnant women are particularly vulnerable to such environmental pollutants (Ali et al., 2013b; Lyall et al., 2017a). During early childhood, the human brain is in the critical phase of development and the blood-brain barrier is not fully established to protect its development, which may make it more vulnerable to toxic pollutants as compared to the adult brain (Bhutta and Anand, 2002; Lyall et al., 2016).

Glutathione S-transferase (GST) enzyme system is a robust detoxifying system to protect the body from oxidative stress caused by xenobiotics and endogenous toxins (Amen et al., 2020). Given that GST enzyme system plays a key role as an antioxidant for the detoxification of toxic compounds generated due to xenobiotics (heavy metals, OCPs, PCBs, PBDEs). The polymorphisms in GST genes may increase and/or decrease the individual susceptibility to oxidative stress and have role in the ASD associated with the toxic chemical exposures (Mandic-Maravic et al., 2019; Matelski and Van de Water, 2016). In humans, the GST gene superfamily has eight classes, among these, pi, mu and theta play very significant role in xenobiotics' detoxification (Josephy, 2010; Amen et al., 2020). Interestingly, existing data have shown that GSTM1 (Glutathione S-transferase Mu 1) and GSTT1 (Glutathione S-transferase Tau 1) null genotypes, alone and/or in combination with GSTP1 (Glutathione S-transferase Pi 1) polymorphism, may have associated with the risk of ASD by increasing and/or decreasing the enzyme capacity to detoxify the toxic compounds generated due to various environmental contaminants (Buyske et al., 2006; James et al., 2006; Mandic-Maravic et al., 2019).

The environmental pathways of human exposure to organic pollutants are multiple (air, water, dust, drinking water, food items) in developing countries including Pakistan (Zhang et al., 2008; Eqani et al., 2013; Ali et al., 2013a). These studies suggested that main sources of OCPs, PCBs, PBDEs and PAHs exposure includes the discharge of industrial wastewater, presence of obsolete pesticides dumping areas and foliar spray of OCPs on the agricultural land, combustion of electric materials, vehicle fuel, and various industrial processes. Human populations in these areas are reported to be exposed to several organic pollutants via complex routes, which include inhalation of contaminated air, dust ingestion/inhalation, and food intake (Eqani et al., 2013; Ali et al., 2013a and 2014, Sohail et al., 2018). However, few studies have documented the risk oriented exposure routes for legacy POPs and PAHs (Berghuis et al., 2015; Wang et al., 2015). Given many toxic chemicals like OCPs, PCBs, PBDEs and PAHs are neurotoxins and can affect the developing brains of children (Tang et al., 2003; Sharma et al., 2010; Pessah et al., 2019), and investigation of their major exposure scenario is critical for taking preventative action. The current study documented the exposure scenarios of the target organic pollutants on the different land use settings of Pakistan and developed their association with ASD. In addition to that, this work also highlighted the relation between null polymorphisms in GSTT1 and GSTM1 genes and levels of target pollutants in serum.

2. Methods

2.1. Sociodemographic Characteristics of Study Participants

A 15-point comprehensive Performa was designed to collect the information about sociodemographic characteristics of study participants. It was comprised of various features including information about residential settings, household monthly income, parent's occupation, number of siblings, consanguineous marriage of parents, presence of autistic features in other family members, parent's education, comorbidities and early infancy infections, vaccination history, smoking, alcohol or drug addiction of parents, maternal BMI, stress level during pregnancy and complications at the time of birth and gestation.

2.2. Land Use Settings for the Participants

The present investigation is a population-based case control study intended to identify the risk factors, environmental pathways and their linkage with ASD. The distal and fundamental driven force of children exposure to pollutants may come from the rapid urbanization, industrialization, and/or modern agricultural practices over the last several decades. Therefore, three cities with different land use settings i.e. urban residential (Islamabad), urban industrial (Lahore) and rural (Khanewal) were selected for sampling to identify the influence of residential land use and variable levels of pollutants exposure on ASD incidence.

2.3. Participants Selection

Children (aged: 4-16) were sampled from the study areas. These children belong to different socioeconomic groups. The socioeconomic groups are based on monthly income (Pakistani rupees-PKR) of parents and divided into 3 categories (High: Monthly Income $\geq$ 100,000 PKR, Moderate: $\geq$ 40,000 PKR, and Low < 40,000 PKR). The children were sampled randomly for the different socioeconomic groups. The sampled autistic children were already diagnosed for ASD [Using standard diagnostic tests including CARS (Childhood Autism Rating Scale) with CARS score of $\geq$ 30 and ADOS (Autism Diagnostic Observation Schedule) and met all the conditions for ASD diagnosis according to DSM-V (Diagnostic Schedule of Mental Disorders-V) criteria. The sampling strategy for cases and controls is shown in Figure 1. ASD positive children (n=125) were recruited from different hospitals and autism centers. The healthy children were sampled from different schools of same cities. These controls (n=125) were age, gender and location matched with the patients. Parents of the patients and healthy children were made aware

of the study outcome and their written informed consent was taken prior to the sample collection. Selection of patients was made very carefully by targeting only the specialized autism centers. Children (both autism and control) with no major infection or diseases were selected for the study. If any child had other nervous issues apart from ASD (like epilepsy, cerebral palsy, down syndrome etc.), he/she was not included in the present study.

2.4. Specimens Collection

Blood was drawn with the 5 mL BD syringes and stored in plain vacutainers. Soon after collection it was centrifuged at 4000 rpm for 10 minutes, separating the blood cells from serum, which was carefully extracted from the upper layer and kept at -80 ˚C till further investigation.

To highlight the exposure pathways of different pollutants among the studied children population, the paired water, dust, and food (rice, wheat and fish) were sampled from their ambient. Drinking water samples (n=15) of the studied areas (n=5 from each city), were obtained from the sources of local consumption, which include the government water supply, tap water, dig well and hand pumps. Composite samples of the locally cultivated/consumed rice (n=15) and wheat grains (n=15) were collected in zip-lock envelopes from the study areas (n=5 from each city). Indoor dust samples (n=15) were also collected from the selected houses in study areas (n=5 from each city) by following the reported methodology (Ali et al., 2013a). Briefly, the floors of residential living rooms were swept spanning 4 m^2 of surface, dust was then wrapped in aluminum foils and put in zip-lock envelopes in dark to avoid photodegradation. Pre-cleaned (acetone treated) 500 μm mesh strainers were used to sieve dust samples to maintain sample homogeneity and were then stored in polypropylene zipper bags in dark and moisture free place. To avoid any cross-contamination, the strainers were washed with acetone and hexane between samples. Fish is very important source which substantially contribute to the dietary exposure of organic pollutants. Contribution by this factor was assessed on the previously published data (Eqani et al., 2013). All samples were stored in the lab at -20 ˚C till the further analysis.

2.5. Analytical Measurements

The detailed methodology for the extraction and clean-up of serum and other environmental samples (water, dust, and food) are given as supplementary annexure I. OCPs, PCBs, and PBDEs were analyzed using an Agilent GC 6890N with a DB-5 MS fused silica capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness, J&W Scientific) equipped with a

Quattro micro GC tandem MS (Waters) in accordance with already established methods (Khairy et al., 2016). Briefly, for PCBs and OCPs the method was as follows; 1 μL of prepared extract was auto injected in the injection port set at 250 °C in splitless mode. Column flow rate was set at 1 mL min^{-1} in multiple reaction monitoring mode, with the starting oven temperature at 100 °C (1 min), ramping at 11 °C min^{-1} to 180 °C, then 3 °Cmin^{-1} to 260 °C and ultimately to 300 °C at rate of 20 °C min^{-1} with final holding time of 6 min. For PBDEs 1 μL extract was injected in the injection port set at 260 °C in splitless mode. Rate of column flow was 2 mL min^{-1}, with the instrument running in multiple reaction monitoring mode and the temperature program was as follows: initial temperature 140 °C for 2 mins, 180 °C at rate of 10 °C min^{-1} and then 3 °Cmin^{-1} to 220 °C and finally 310 °C at the rate of 10 °C min^{-1} for 5 min. For analysis of PAHs Agilent 6890 GC coupled to an Agilent 5973 MSD in EI+ selected ion monitoring (SIM) mode was used. Analysis and quality control protocols for PAH were those established previously (Khairy and Lohmann, 2012). The GC-MS program for PAHs was as follows: 1 μL extract was injected in the injection port in splitless mode with initial column flow rate of 1.9 mL min^{-1}. Initial temperature of oven at 60 °C for 3 min, 110 °C (2 min) at the rate of 5 °C min^{-1}, reaching 200 °C at 8 °C min^{-1} and finally attaining the temperature of 315 °C at 5 °C min^{-1} with final holding time of 10 min.

The 26 selected OCPs included in the current study were: hexachlorobenzene (HCB), alpha-hexachlorocyclohexane (α-HCH), beta-hexachlorocyclohexane (β-HCH), gamma-hexachlorocyclohexane (γ-HCH), delta-hexachlorocyclohexane (δ-HCH), heptachlor, heptachlor epoxide, aldrin, dieldrin, trans-chlordane, cis-chlordane, oxychlordane, trans-nonachlor, o,p′-DDE, p,p′-DDE, o,p′-DDD, p,p′-DDD/o,p′-DDT, p,p′-DDT, endrin, endrin aldehyde, endrin ketone, endosulfan-I, endosulfan-II, endosulfan sulfate and methoxychlor. The 29 PCB congeners included dioxin-like PCB-8, PCB-11, PCB-28, PCB-66, PCB-77, PCB-81, PCB-105, PCB-114, PCB-118, PCB-123, PCB-126, PCB-156, PCB-157, PCB-167, PCB-169 and PCB-189, non-dioxin-like PCB18, PCB-44, PCB-52, PCB-101, PCB-128, PCB-138, PCB-153, PCB-170, PCB-180, PCB-187, PCB-195, PCB-206 and PCB-209. The following 11 PBDEs were targeted: PBDE-2, PBDE-8, PBDE-15, PBDE-30, PBDE-28, PBDE-47, PBDE-49, PBDE-99, PBDE-100, PBDE-153 and PBDE-154. Additionally, 32 PAHs were analyzed including naphthalene, 2-methylnaphthalene, biphenyl, 1-methylnaphthalene, acenaphthylene, acenaphthene, dibenzofuran, fluorene, methylfluorene, dibenzothiophene, phenanthrene, anthracene, methyl phenanthrene/anthracene, methyl phenanthrene/anthracene 2, methyl phenanthrene/anthracene 3,

fluoranthene, pyrene, retene, methylpyrene, chrysene, 7-methylbenz(a)anthracene, benzo(c)phenanthrene, benzo(a)anthracene, 6-methylchrysene, 7,12-dimethylbenz(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, perylene, indeno(1,2,3-c,d)pyrene and dibenz(a,h)anthracene.

All the studied compounds were identified on the basis of comparative mass spectrum and retention time analysis of selected ions with calibration standards. Procedural blanks were used to calculate the limits of detection (LODs) which were estimated to be thrice of standard deviation of levels of OPs. However, for the undetected OPs in procedural blanks, calculation of LODs was based on quantity of analyte in each sample corresponding to lowest calibration standard. The LODs of studied compounds varied from 0.01-0.5 ng/µL and the detectable rates as the percentage of samples over the LOD are given in Table S2. Values below LODs were replaced with 0. Serum concentrations of organic pollutants were normalized on the basis of lipid weight and expressed as ng g^{-1} lipid weight. Bligh and Dyer method for lipid determination was used for the estimation of total lipids in the serum (Bligh and Dyer, 1959).

2.5.1. Quality Control and Quality Assurance

Glassware used for organic pollutants analysis was washed with inert soap, air dried and then rinsed with hexane, then with DCM (Dichloromethane) and finally with acetone, again air dried and muffled at 450 ˚C overnight before use. A series of standard solutions comprising of native compounds (0.001-1.00 ng μL^{-1}), surrogate standards (1 ng μL^{-1}) and injection standards (1 ng μL^{-1}) was used to establish a 6-point calibration curve, for quantification of analyzed compounds. Spiked blanks, matrix spikes and procedural blanks were analyzed with each sample batch in an identical manner to samples. Recoveries of surrogate standards range from 67-86% for PCBs, 63-91% for OCPs, 77-84% for PBDEs and 62-95% for PAHs. Spiked blank recoveries were 93-106% and matrix spike recoveries were 94-112 %.

2.6. GSTM1 and GSTT1 Null genotype analysis

BD vacutainer (4 mL) heparin tubes were used for plasma collection. 1 mL plasma was used for DNA extraction (50 µL) using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). High Resolution Melting (HRM) quantitative PCR was used for GSTT1 and GSTM1 null/present analysis. Detailed protocol explained previously (Amen et al., 2020).

2.7. Dietary and Non-Dietary Daily Intakes of Organic Pollutants

Various sources of human uptake of organic pollutants include exposures through consumer products, air borne dust particles, contaminated food ingestion and from drinking water. The current study included indoor dust, food (wheat, rice grains) and drinking water analysis for the calculation of estimated daily intakes of semi-volatile organic pollutants. Fish data was collected from literature and used for the calculation of Estimated Daily Intakes (EDIs). The following formula was used for the evaluation of estimated daily intake;

$$\text{EDI (ng kg}^{-1}\text{ day}^{-1}) = \frac{C_{op} * DC}{BW}$$

Where C_{op} is the concentration of organic pollutant in the analyzed source (ng g^{-1}/ng L^{-1}), DC is the daily intake rates (g person^{-1} day^{-1} and L person^{-1} day^{-1}) of wheat, rice, fish water and dust (Supplementary Annexure II). These daily consumption rates were calculated on the basis of comprehensive discussions from families of studied children by asking them about the portion and priorities of food intake in routine. Body Weight (BW, Kg person^{-1}) signifies the mean weight of the participants from the current study (25 Kg).

2.8. Statistical Analysis

All the statistical analysis was done by using IBM SPSS statistics (Version 19) software. The Kruskal-Wallis H test was used to compare the sociodemographic data between the ASD group and the control group. Unconditional binary logistic regression was used to analyze the risk factors associated with ASD. *p*-values of <0.05 were considered significant. Regression analysis was used to obtain the crude (ORs) and adjusted odds ratios (AORs) in this case-control study, in which the calculated ratio estimates the chances of pollutant concentrations and GST polymorphisms occurring in ASD population in relation to its rate of occurrence in the healthy controls. In the logistic regression models, organic pollutants concentrations were $\log_{10}$ transformed to reduce the influence of outliers and to normalize the data. The potential confounders including Body Mass Index (BMI), GSTT1 and GSTM1 null homozygous genotypes and the concentrations of studied organic pollutants were applied as adjustment factors in the final models. Factors like age group, maternal education level, parental age, number of siblings were preliminary considered in regression models, but finally excluded. It is because these variables were neither associated with any exposure nor associated with ASD outcome; they also did not change the estimate by >10%

when included or excluded in the calculation. Discrete regression models were applied to access the significant correlations/associations between GSTT1 and GSTM1 deletion/presence and pollutant's concentrations. In these models GSTT1/GSTM1 null/present genotypes were the dependent variables and concentrations of organic pollutants were independent variables. Children from different land-use settings were evaluated separately to check the effect of varied toxic exposures based on inhabited land's proximity to industrial, urban, and agricultural areas by calculating the estimated daily intakes of toxins. Correlations between concentrations of OPs in serum and other environmental matrices (dust, food and drinking water) was assessed by Pearson correlation.

3. Results

The present study involved 125 ASD cases and age and gender matched 125 controls. Sociodemographic features of participants are shown in Table 1. The average age of studied population was 9.2±2.9 years (mean ± S.D). Under-weight BMI (≤18.4) was more prevalent in ASD cases than in controls, and the male to female ratio was approximately 3:1. Based on land-use types, more ASD children resided in the industrial areas (70%) than in the rural (16%) and urban (14%) areas; in contrast, more controls lived in rural (44%) and urban (34%) than in industrial areas (22%) (Table 1). The GSTM1/GSTT1 null and/or positive genotype was not varied significantly among ASD cases vs controls (Table 1). The socioeconomic status (SES) of studied population showed that nearly 40% of ASD children belonged to rich families. Among the studied demographic characteristics, significant association between ASD and SES, BMI and land use settings were observed (Table 1).

3.1. Association of the Studied Organic Pollutants with ASD Risk

Overall 26 OCPs [out of which 2 congeners of para-para dichlorodiphenyldichloroethane and ortho-para dichlorodiphenyltrichloroethane (p,p′-DDD/o,p′-DDT) reported as the combined concentration because of their co-elution on gas chromatography], 29 PCB congeners, 11 PBDEs and 32 PAHs (co-eluting PAHs include methyl phenanthrene/methyl anthracene, methyl phenanthrene 2/ methyl anthracene 2, methyl phenanthrene 3/ methyl anthracene 3) were measured in the blood samples. When comparing the exposure biomarker concentrations among the study

297 groups, there were significant associations of various OCPs, PBDEs, PCBs, and PAHs with ASD
298 risk (Table 2 and Table S1).
299 Among the analyzed PCB congeners, PCB77 (mean: 2.65 ng g^{-1} lw (lipid weight) in cases vs
300 1.61 in controls), PCB118 (mean: 2.30 ng g^{-1} lw in cases vs 1.29 in controls), PCB128 (mean: 1.58
301 ng g^{-1} lw in cases vs 1.02 in controls), PCB153 (mean: 2.19 ng g^{-1} lw in cases vs 1.64 in controls)
302 were significantly higher in the ASD cases than in the controls, whereas PCB187 (mean: 0.42 ng
303 g^{-1} lw in cases vs 1.13 in controls) was significantly elevated in the controls when compared to the
304 ASD cases (Figure 2). Other analyzed PCB congeners did not vary significantly ($p>0.05$) between
305 the ASD and controls. Initially, the unadjusted odd ratios showed significant associations of
306 PCB118 (OR = 0.88; 95% CI: 0.79, 0.99), PCB128 (OR = 0.98; 95% CI: 0.92, 1.05) and PCB187
307 (OR = 0.76; 95% CI: 0.62, 0.93) with ASD. After adjustment of the confounding factors (BMI,
308 GSTT1, and GSTM1), PCB77 (AOR = 1.99; 95% CI: 1.43, 2.18), PCB118 (AOR = 1.49; 95% CI:
309 0.99, 2.00), PCB128 (AOR = 1.74; 95% CI: 1.55, 1.93), PCB153 (AOR = 1.80; 95% CI: 1.02,
310 1.92) and PCB187 (AOR = 0.37; 95% CI: 0.24, 0.49) were significantly associated with ASD risk
311 (Table 2). For the analyzed OCPs, p,p′-DDE (mean: 10.09 ng g^{-1} lw in cases vs 2.43 in controls)
312 was significantly higher in the ASD cases than in the controls. The regression model showed that
313 p,p′-DDE (AOR = 1.50; 95% CI: 1.00, 1.85) significantly associated with the ASD risk (Table 2),
314 all other OCPs showed no significant change both in crude and/or adjusted models (Table 2, Table
315 S1). Among the PBDEs, BDE99 (mean: 0.14 ng g^{-1} lw in cases vs 0.39 in controls) was
316 significantly higher in the controls than in the ASD cases (Table 2; Figure 2). BDE99 (mean: 0.14
317 ng g^{-1} lw in cases vs 0.39 in controls) inversely correlated with ASD risk, but not for other PBDEs
318 in both crude and adjusted models.
319 Significantly positive associations with ASD were found for dibenzothiophene (mean: 0.29 ng
320 g^{-1} lw in cases vs 0.12 in controls), while perylene (mean: 0.10 ng g^{-1} lw in cases vs 0.21 in
321 controls) and fluorene (mean: 0.17 ng g^{-1} lw in cases vs 0.32 in controls) were significantly higher
322 in the controls when compared to the ASD cases. Among the 32 PAHs, dibenzothiophene (AOR
323 = 7.30; 95% CI: 1.49, 35.85), perylene (AOR = 0.25; 95% CI: 0.06, 1.10) and fluorene (AOR =
324 0.21; 95% CI: 0.06, 0.72) significantly associated with ASD risk. Other PAHs were less likely
325 correlated with ASD risk (Table S1).
326 The concentrations and descriptive statistics of analyzed organic pollutants along with LODs
327 are shown in Table S2. In most of the cases, serum concentrations (except highest quartile values)

of the analyzed pollutants were less than the threshold values from the National Health and Nutrition Examination Survey (NHANES) (Crinnion, 2010; Jain, 2015), whereas the mean concentration of p,p′-DDE, PCB 66, 114, 105, 128, 157 and phenanthrene, methyl-phenanthrene/methyl-anthracene and methyl-phenanthrene 1/methyl-anthracene-1 were above the NHANES concentrations (Table S2).

3.2. Accumulation of the Studied Organic Pollutants by GSTT1 and GSTM1 Polymorphism

When analyzing GSTT1 genotype's correlation with organic pollutants in all participants, PCB66 (AOR = 0.42; 95% CI: 0.31, 0.75) and PCB156 (AOR = 0.42; 95% CI: 0.25, 0.96) were significantly higher but PCB81 (AOR = 1.74; 95% CI: 1.00, 1.87) and PCB118 (AOR = 1.98; 95% CI: 0.99, 2.13) were significantly lower in the GSTT1 null genotype than in the GSTT1 present genotype (Table 3; Table S3). Among the analyzed OCPs, δ-HCH (AOR = 0.25; 95% CI: 0.19, 0.43) and endrin (AOR = 0.47; 95% CI: 0.33, 0.79) were significantly elevated in the GSTT1 null genotype (Table 3; Table S3). For the analyzed PAHs, acenaphthylene (AOR = 0.31; 95% CI: 0.11, 1.02), 7-methylbenz(a)anthracene (AOR = 0.47; 95% CI: 0.09, 0.52) and 1-methylnaphthalene (AOR = 0.32; 95% CI: 0.02, 0.75) were significantly higher but benzo(a)pyrene (AOR = 2.19; 95% CI: 1.30, 2.65), 7,12-dimethylbenz[a]anthracene (AOR = 4.04; 95% CI: 0.99, 16.59) and benzo(b)fluoranthene (AOR = 5.76; 95% CI: 1.08, 7.91) were significantly lower in the GSTT1 null genotype in all participants (Table 3; Table S3).

Although no significant link ($p>0.05$) was observed between the GSTT1 genotypes and ASD risk (Table 2), the genotype related accumulation was observed for some pollutants either in the ASD cases or in the controls. For the ASD cases, PCB44 (AOR = 0.37; 95% CI: 0.23, 1.00) and PCB128 (AOR = 0.39; 95% CI: 0.18, 0.85) were higher but PCB167 (AOR = 4.55; 95% CI: 1.49, 7.90) was lower in GSTT1 null for the ASD cases. Among the investigated PAHs, 2-methyl naphthalene (AOR = 0.46; 95% CI: 0.18, 0.64), pyrene (AOR = 0.17; 95% CI: 0.08, 0.85) and 1-methylnaphthalene (AOR = 0.41; 95% CI: 0.12, 0.61) were significantly higher whereas 7,12-dimethylbenz[a]anthracene (AOR = 3.78; 95% CI: 1.13, 9.17) and benzo(a)pyrene (AOR = 1.90; 95% CI: 0.81, 2.65) were significantly lower in GSTT1 null genotype individuals in the ASD cases (Table 3). For the controls, only p,p′-DDT (AOR = 2.39; 95% CI: 1.04, 5.50) was significantly

lower in GSTT1 null genotype than in the controls (Table 3; Table S3). None of analyzed PBDEs were significantly associated with GSTT1 null/present genotype (Table 3; Table S3).

When the correlation of GSTM1 genotypes with organic pollutants were analyzed in all participants, PCB congeners of PCB77 (AOR = 0.33; 95% CI: 0.18, 0.98) was significantly higher in GSTM1 null genotype, while PCB52 (AOR = 1.58; 95% CI: 1.01, 2.32) and PCB101 (AOR = 1.68; 95% CI: 1.28, 2.59) were high in GSTM1 present genotype. In the studied OCPs, heptachlor (AOR = 0.49; 95% CI: 0.20, 1.00), dieldrin (AOR = 0.41; 95% CI: 0.17, 0.99) and p,p′-DDE (AOR = 0.36; 95% CI: 0.19, 1.00) positively associated with GSTM1 null genotype, whereas o, p′-DDD (AOR = 1.88; 95% CI: 1.55, 2.06) and endrin (AOR = 1.72; 95% CI: 1.41, 2.13) negatively associated to this genotype (Table 3). Among PBDEs, only BDE8 (AOR = 0.47; 95% CI: 0.30, 0.95) was positively associated with GSTM1 null genotype in all participants but not alone in the ASD cases or the controls (Table 3; Table S3). For the PAH analysis, dibenz(a,h)anthracene (AOR =4.59; 95% CI: 1.04, 8.22) was positively associated with GSTM1 positive genotype.

Similar to GSTT1, GSTM1 genotype specific pollutant accumulation was unbalanced between the ASD cases and controls. PCB77 (AOR = 0.29; 95% CI: 0.14, 0.96) was significantly higher in GSTM1 null genotype in ASD cases but not in the controls. Among the ASD positive individuals, PCB123 (AOR = 0.38; 95% CI: 0.07, 0.99) was high in the GSTM1 null genotype, but PCB66 (AOR = 1.72; 95% CI: 1.05, 1.95) was high in GSTM1 positive type. For the analyzed OCPs, heptachlor (AOR = 0.28; 95% CI: 0.13, 0.93), p,p′-DDE (AOR = 0.43; 95% CI: 0.18, 1.98), β-endosulfan (AOR = 0.40; 95% CI: 0.20, 0.83) and p, p′ -DDD/o, p′-DDT (AOR = 0.27; 95% CI: 0.14, 0.71) were significantly higher, but o,p′-DDD (AOR = 1.78; 95% CI: 1.02, 2.10), cis-chlordane (AOR = 1.76; 95% CI: 1.15, 2.41) and endrin (AOR = 1.98; 95% CI: 1.03, 2.16) were significantly lower in the GSTM1 null genotype individuals in the ASD cases. To PAHs, fluorene (AOR = 2.98; 95% CI: 1.36, 7.65) positively associated with GSTM1 [null/present] genotype but pyrene (AOR = 0.23; 95% CI: 0.05, 1.01) negatively associated in the ASD individuals (Table 3; Table S3).

In the control participants, only PCB118 (AOR = 0.47; 95% CI: 0.36, 1.00) positively associated with GSTM1 null genotype (Table 3; Table S3). For OCPs, the higher concentrations of dieldrin (AOR = 0.43; 95% CI: 0.35, 0.98) and β-HCH (AOR = 0.35; 95% CI: 0.28, 1.01) but the lower concentration of α-HCH (AOR = 2.47; 95% CI: 1.01, 6.05) in GSTM1 null genotype

individuals in the controls were observed (Table 3). Among the PAHs, perylene (AOR = 0.25; 95% CI: 0.09, 0.73), methyl-fluorene (AOR = 0.22; 95% CI: 0.03, 1.04), benzo(a)pyrene (AOR = 0.45; 95% CI: 0.34, 1.17) and biphenyl (AOR = 0.21; 95% CI: 0.14, 1.16) positively associated with GSTM1 null genotype, whereas acenaphthylene (AOR = 1.77; 95% CI: 0.76,6.73), fluoranthene (AOR = 4.86; 95% CI: 1.04, 6.28), benzo(e)pyrene (AOR = 1.91; 95% CI: 1.48, 4.20), acenaphthene (AOR = 1.90; 95% CI: 1.07, 5.34) and dibenz(a,h)anthracene (AOR = 1.93; 95% CI: 1.35, 2.46) were positively associated with GSTM1 positive genotype in the controls.

3.3. The Studied Organic Pollutant's Environmental Exposure Pathways

Evaluation of EDI linked the children's exposure to pollutants in the environmental samples in the studied land-use types. Contaminated food was the major source of the analyzed pollutants in all study areas, however, drinking water in Khanewal also contributed to OCP exposure among the residents. Food was the main exposure source for PCBs particularly in Khanewal compared to Lahore and Islamabad. Drinking water from Lahore and Khanewal was one of the important sources of DDTs. Drinking water also contributed to PBDE exposure for the residents in Lahore and Islamabad. Apart from the contaminated food, PAHs were mainly contributed by the contaminated dust in all land-use settings (Figure 3).

Contamination of the environmental samples of the analyzed pollutants were land-use type specific. The collective EDIs of organic pollutants from Islamabad (urban) in descending order were: ΣOCPs, ΣPAHs, ΣDDTs, ΣPCBs, ΣPBDEs and ΣHCHs, from Lahore (industrial) were: ΣOCPs, ΣDDTs, ΣPAHs, ΣPCBs, ΣHCHs and ΣPBDEs and from Khanewal (rural) were: ΣOCPs, ΣDDTs, ΣPAHs, ΣHCHs, ΣPCBs and ΣPBDEs. Collective EDIs for PAHs in Islamabad (68 ng kg^{-1} bw) was the highest, and then was Khanewal (64 ng kg^{-1} bw) and Lahore (52 ng kg^{-1} bw). Collective EDIs for PCBs in Lahore (29 ng kg^{-1} bw) was higher than in Islamabad (20 ng kg^{-1} bw) and Khanewal (15 ng kg^{-1} bw). Cumulative EDI for PBDEs was highest in Lahore (14 ng kg^{-1} bw) when compared to Islamabad (12 ng kg^{-1} bw) and Khanewal (7 ng kg^{-1} bw). OCPs cumulative EDI was highest in Khanewal (128 ng kg^{-1} bw) than Lahore (107 ng kg^{-1} bw) and Islamabad (80 ng kg^{-1} bw), in which HCHs ordered in Khanewal (17 ng kg^{-1} bw), Lahore (16 ng kg^{-1} bw) and Islamabad (5 ng kg^{-1} bw), and DDTs ordered in Khanewal (80 ng kg^{-1} bw), Lahore (67 ng kg^{-1} bw) and Islamabad (21 ng kg^{-1} bw) (Table 4). Roughly, these data may support that PAHs are mostly an

indicator for urban settings, PCBs and PBDEs for industrial settings and OCPs for rural settings, respectively.

The correlations of dust, food, and water to serum concentrations for analyzed organic pollutants are shown in Table 5. There were significant positive correlations between serum and environmental samples for some studied organic pollutants (OCPs, PCBs, PAHs, PBDEs) in all three regions. Although the EDI data estimated that food is the major exposure pathway for all OPs, correlation analysis of the water, food, and dust samples with the serum samples for each land use setting showed some different results. Among three land use settings, 96 significant Pearson Correlations ($p \leq 0.05$) (Table 5, Table S4) for internal-external exposure were observed, which may imply some traceability of these chemical's environmental exposure pathways. 53 (55%) correlations were observed in serum-water samples, following by 25 (26%) in serum-food and 18 (19%) in serum-dust, respectively. Beyond the EDI models (which consider uncooked food digestion but not the gaseous fraction of studied pollutants), additional contaminated water used for cooking and gaseous OPs inhalation should be additionally weighted in exposure scenarios for the estimation. From the viewpoint of health risk, there are 10 chemicals (5 PCBs, 3 PAHs along with p,p'-DDE and PBDE99) which have potential associations with ASD (Table 2). Among these risk drivers, PCB77, PCB118, p,p'-DDE and fluorene by dust, and PCB118, PCB187 and perylene by water, PCB153 by food have been observed at least in one land use setting. The observation supported that most (73%) of the potential hazard's environmental exposure have been tracked and there are comprehensive correlations from distal environmental, individual exposure and ASD risk.

4. Discussion

The current research has identified associative evidence of several classes of organic compounds with ASD risk in Pakistan. In addition, the environmental exposure factor analysis showed that the participants were commonly exposed to OCPs, PCBs, PAHs and PBDEs via drinking water, food, and dust.

4.1. Children Exposure to OPs Associated with ASD

Concentrations of serum PCBs in the current study were in accordance with the previous studies from Pakistan (Ali et al., 2013b; Ali et al., 2014). The overall PCB homolog distribution among the analyzed congeners showed the following trend tetra-CBs > penta-CBs > hexa-CBs >

di-CBs > tri-CBs > hepta-CBs > octa-CBs> nona-CBs > deca-CBs. The dominance of tetra and penta CBs was similar to previous results (Naqvi et al., 2018; Sohail et al., 2018) and is due to the fact that in Pakistan technical mixture of penta, tetra and tri CBs was predominantly used for industrial and commercial applications (Syed et al., 2014; Baqar et al., 2017). Concentrations of OCPs from the current study were similar (Ali et al., 2014) and/or lower than previously reported from same study areas (Bhalli et al., 2009; Ali et al., 2013b; Yasmeen et al., 2017). The predominance of ΣDDTs compared to other analyzed OCPs, was in accordance to the previous studies from Pakistan (Ali et al., 2014; Yasmeen et al., 2017). According to our results p,p′-DDE was significantly and positively associated with ASD and linked with excessive use of DDTs for the malarial control and crop protection (Eqani et al., 2013). Serum concentrations of PBDEs from the current research were in accordance with prior analysis from same areas (Ali et al., 2013b; Ali et al., 2014). Serum levels of PAHs from the current study showed lower levels of serum naphthalene and pyrene compared to another study reporting from the auto-mechanics, spray painters and petrol filling workers from Rawalpindi (Kamal et al., 2011; Rashid et al., 2017). The data concerning detailed profiling of serum PAHs from Pakistani population is still lacking but when compared to global scenario the present concentrations were similar to previously reported in China (Zhang et al., 2017) Saudi Arabia (Al-Daghri et al., 2013) and Canada (Neal et al., 2008) and lower than those reported from another study from China (Wang et al., 2015) and Hong Kong (Tsang et al., 2011).

Previous studies showed that childhood exposure to toxic chemicals might increase the risk of neurodevelopmental disorders including ASD (Cheslack-Postava et al., 2013; Lyall et al., 2017a, 2017b; Rosenquist et al., 2017). The present work showed that PCB 77, 118, 128 and 153 were significantly higher but PCB187 was lower among ASD cases compared to controls. The accumulation of most stable and high molecular weight PCBs may be linked to contaminated food ingestion. These congeners also have long half-lives, meaning that the correlations are more likely to reflect long-term exposure, which could explain the strong correlations for those PCBs and p,p'-DDE as compared to most PAHs. PAHs (and HCHs) are less persistent, so they will reflect recent exposure, which could explain the lack of correlation with ASD. The hypothesized mechanisms for PCB neurotoxicity include altered dopamine and thyroid hormone signaling, disruption of intracellular Ca^{2+} dynamics and oxidative stress induction (Liu et al., 2012; Pessah et al., 2019), the molecular specific effects showed that PCBs' toxicology is complex. Previous studies also

have associated prenatal p,p′-DDE exposure with poor learning outcomes (Rosenquist et al., 2017), and an increased risk of autism in association with maternal exposure to dicofol-contained the DDTs impurities (Roberts et al., 2007). Neurotoxicity induced by DDTs may be mainly accredited to higher production of reactive oxygen species (ROS), activation of various caspases and decrease in mitochondrial membrane potential (Sharma et al., 2010). According to our results, BDE-99 was significantly higher in control samples compared to ASD positive cases. Similar results were reported by Lyall et al. (2017b) showing higher PBDE serum levels of various congeners among general population compared to ASD positive cases. Among the analyzed 32 PAHs, dibenzothiophene was significantly positively associated with ASD, whereas perylene and fluorene showed negative association. PAHs exposure is known to disrupt gene expression, alter biochemical functions and induce oxidative stress leading to neuronal cells damage, necrosis, and cell death. Some PAHs can cross the blood-brain barrier and enter the brain, can cause inhibition of various essential enzymes involved in neuro-transmission and metabolic functions, leading to impairments in functioning of nervous system (Tang et al., 2003).

PCB-187, BDE-99, perylene and fluorene showed negative association with ASD incidence. The reasons for these inverse associations are not apparent. Although the data is adjusted for prospective covariates, it is possible that these inverse associations may be linked to unmeasured confounding factors by some shared influences on the level of these pollutants and ASD, instead of a true protective association. It is also likely that such outcomes are due to chance. Out of a large array of compounds analyzed only a few showed significant associations, most of the analyzed organic pollutants showed no associations with ASD outcome, which suggest that exposure to these contaminants may be unrelated to ASD risk specifically. In the spectrum of autism there is complex heterogeneity, which manifest as a wide continuum of phenotypic features. We did not have the ability to assess the influence of heterogeneity within ASD, and some associations which were found could plausibly vary according to phenotypically distinct ASD subgroups. Another reason for inverse association could be that the metabolic rates vary from individual to individual. Some individuals have very high metabolic rates. Aging, use of various drugs and disease could affect the metabolic rate. According to Cheng and coworkers (Cheng et al., 2017) about 30% of children with ASD may experience metabolic abnormalities. Given the difference in metabolic rates of ASD patients and controls, the excretion rate of the observed

pollutants remains different, leading to their significantly varying levels of toxic chemicals into the different human samples among ASD and control individuals.

Although usually thought as persistent, glutathione-S-transferase (GST) dose play a significant role in the detoxification of the investigated OPs. Serum concentrations of about 41 measured chemicals associated with GSTM1 and/or GSTT1. GSTM1 and GSTT1 show polymorphisms and depict a range of vulnerability to xenobiotics accumulations among the populations, which may cause impaired enzyme functioning, leading to affect the detoxification potential of the body, and ultimately inducing oxidative stress. Although a direct correlation with ASD was not observed, 7 of 10 ASD-related OPs were associated to GSTM1 and/or GSTT1. These data may further supported the associations between some OPs and the ASD risk, in which the GST detoxification and related oxidative stress can further affect the development of neuron energy production process, inflammatory responses, production of ATPs and neuronal signaling causing ASD (Buyske et al., 2006; Chauhan and Chauhan, 2006).

4.2. Exposure Factors and Environmental Traceability of ASD Risk

Generally, agronomic intensification, enhanced industrial development and rapid urbanization have characterized the investigated pollutants' environmental variation, children exposure and ASD risk. The overall cumulative EDIs showed higher EDIs for OCPs and PAHs compared to PCBs and least for PBDEs. This can be explained by continued illegal use of many OCPs (e.g. DDTs) and past excessive use of these banned chemicals (Eqani et al., 2013). The increased exposure of PAHs is due to burning of biomass, wood, coal and petroleum products for heating and fuel purposes (Kamal et al., 2011). PCBs low exposure is due to less use of PCBs after the ban of Stockholm convention, the key exposure is due to improper handling of old e-waste, various industrial and consumer products (Ali et al., 2013b). Although they have been banned by the Stockholm convention, the persistence and bioaccumulation of PCBs explains their extensive occurrence into serum samples in the present study. Limited occurrence of PBDEs were due to the lesser use of sophisticated consumer products containing flame retardants, such exposures are usually high in developed countries (Ali et al., 2013b).

The spatial distribution patterns in the study areas showed that ΣPCB was higher in Lahore than in Islamabad, and the lowest is in Khanewal. This points to the fact that Lahore is densely urbanized and industrialized region with excessive rate of industrialization and chemical

contamination. Islamabad is mostly urbanized and increased urban activities, the improper e-waste handling are the main contributory sources of PCBs in Islamabad. PCBs in Khanewal are mainly accredited to the semi-volatile nature of PCBs, which can travel long distances and reach the rural areas (Naqvi et al., 2018). Although EDIs of ΣPCB showed food as the main exposure source, the exposure factor analysis showed that contaminated water exposure for PCBs was more common than OCPs and PAHs in all three land use settings. Although the ΣPCB concentration was lower, the serum-food correlations of PCBs were much greater in Khanewal than in Lahore and Islamabad. The fact supported that ingestion of PCBs via food in Khanewal was more tensely than in Lahore and Islamabad, which may imply the contaminated water irrigation in farming. The most apparent serum-dust correlations of PCBs in Islamabad may be due to the chemicals' grasshopper transportation and mountain front precipitation, in this case the inhalation of the fine fraction of dust may have play the key role of children exposure by combination of dermal contact (Sohail et al., 2018).

The spatial distribution of analyzed OCPs showed higher levels of ΣOCPs in Khanewal than in Islamabad and Lahore. The higher concentrations of OCPs in Khanewal can be justified by the fact that Khanewal is well known for its agricultural activities and cotton growing area. Massive application of pesticides in the region leads to increased exposure of OCPs to the inhabitants, including illegal use of the banned pesticides. OCPs in Lahore and Islamabad were mainly contributed by the outdated pesticides dumped near demolished factories. In Lahore, the serum-water exposure correlation of OCPs was more apparent than in the other two areas, which may be supported by the inappropriate handling and storage of banned pesticides in the demolished units resulted in leakage and increased contamination of surrounding areas (Eqani et al., 2013; Sohail et al., 2018). The main source for OCP uptake was food, however dust exposure from Lahore and contaminated water from Khanewal also contributed to OCP exposure among the residents.

The spatial distribution showed ΣPBDE levels were approximately similar in Lahore and Islamabad but were lower in Khanewal. This can be explained by increased industrialization and urbanization in these areas compared to Khanewal. Low levels of PBDEs compared to other analyzed OPs show low exposure to PBDEs in the analyzed population. Contaminated food was the major source of exposure, but dust and water also contributed to PBDEs exposure among the inhabitants of study areas.

The EDI for ΣPAHs was basically similar in Islamabad and Khanewal and lower in Lahore. Due to increased urbanization in Islamabad diesel and gasoline combustion from vehicular discharge is the main contributory source to PAHs exposure for inhabitants of Islamabad, which may have supported the observation of more common serum-food exposure correlations of PAHs in Islamabad than in Khanewal and Lahore. Similar to Islamabad traffic exhaust due to high traffic influx in the Lahore was the major contributory source to atmospheric PAHs levels in addition to emissions from industries and brick kilns (Kamal et al., 2011). In contrast, the major contribution to the PAHs exposure in Khanewal is due to anthropogenic activities involving burning of biomass, wood and coal for cooking and heating purposes.

4.3. Strengths and Limitations:

The current study has several strengths including the systematic and quantitative measurements of individual chemicals in serum and the comprehensive environmental pathway samples; the land-use types based ASD-health cross-section study design, and the susceptibility assessment of GSTT1/GSTM1 genotypes for each participant. Therefore, this work has added to the few studies to address the probable environment-gene interactions linking GSTs polymorphism with OPs and ASD. The present study has several limitations. First, our study lacks the multiple clinical diagnosis data about various stages and classification of ASD, which may be associated to varying levels of toxin exposures. A second limitation is the use of only one-time monitoring to evaluate the juvenile exposures, therefore the results should be interpreted carefully given the possibility of chance findings. However, for long-lived POPs (Persistent Organic Pollutants) in human serum (PCBs and OCPs), the results are more likely to reflect past exposure. Another limitation is that the present investigation is a case-control association study and can show some associations only but not causations.

5. Conclusion

Our results demonstrated the significant associations of ASD with selected studied PCBs, OCPs and PAHs in children from Pakistan. For the first time, the exposure-hazard correlations were traced to the children's inhabited land settings, which is characterized on the basis of the indigenous environmental polluted samples including water, indoor dust, and food. Importantly, the exposure pathway analysis showed that water was more critical in the semiarid areas where

water needs to be efficiently used. It is interesting to note that the ASD related OPs are mostly exposure factor traceable for some scenarios, which is useful for the primary prevention to against OPs-related ASD risk. The present study adds relevant information that would be helpful to associate the distal risk aspects of urban expansion, industrial and agronomic activities with the susceptibility to health outcome by conducting the exposure pathway analysis of toxicants.

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799

800 **Table 1.** Socio-demographic and individual characteristics of the study population.

Factors	**Study Population** (*n*=250)		***p*-value**
Gender	**Autism**	**Control**	0.55
Male	89 (72%)	98 (78%)	
Female	36 (28%)	27 (22%)	
BMI			
≤18.4 (Under-weight)	91 (72%)	63 (50%)	**0.01**
18.5-24.9 (Normal)	30 (24%)	59 (48%)	
>25 (Over-weight)	4 (4%)	3(2%)	
Monthly Income/SES			**0.01**
High	47 (38%)	11 (8%)	
Moderate	40 (32%)	75 (60%)	
Low	38 (30%)	39 (32%)	
Land Use Settings			**0.00**
Islamabad (Urban)	17 (14%)	43 (34%)	
Lahore (Industrial)	88 (70%)	27 (22%)	
Khanewal (Rural)	20 (16%)	55 (44%)	
GSTT1 genotype			0.63
Positive	110 (88%)	97 (78%)	
Null	15 (12%)	28 (22%)	
GSTM1 genotype			0.67
Positive	56 (45%)	52 (42%)	
Null	69 (55%)	73 (58%)	

801 *p*-values are based on Kruskal-Wallis H test.

802

Table 2. Associations of sociodemographic factors and only significantly associated organic pollutants with ASD risk (relative to general population controls).

Variables	OR[a] (95% CI)	Un-adjusted p-value [c]	AOR[b] (95% CI)	Adjusted p-value [c]
GSTT1	1.81 (0.82, 4.03)	0.14	1.35 (0.55, 3.31)	0.51
GSTM1	1.23 (0.66, 2.29)	0.51	0.87 (0.42, 1.79)	0.71
BMI	0.95 (0.88, 1.04)	0.28	**0.75 (0.56, 0.99)**	**0.03**
Age	1.21 (0.66, 2.23)	0.54	0.96 (0.85, 1.08)	0.49
Gender	0.99 (0.90, 1.10)	0.97	1.11 (0.52, 2.37)	0.79
PCB77	0.95 (0.88,1.03)	0.20	**2.00 (1.43,2.18)**	**0.00**
PCB118	0.88 (0.79,0.99)	0.05	**1.49 (1.00,2.00)**	**0.05**
PCB153	0.93 (0.85,1.02)	0.13	**1.80 (1.55,1.93)**	**0.03**
PCB128	0.98 (0.92,1.05)	0.02	**1.65 (1.01, 1.91)**	**0.01**
PCB187	0.76 (0.62,0.93)	0.03	**0.37 (0.24,0.49)**	**0.00**
p,p'-DDE	1.03 (0.10,1.06)	0.11	**1.50 (1.00,1.85)**	**0.03**
PBDE99	0.52 (0.30,0.88)	0.02	**0.48 (0.26,0.89)**	**0.02**
Fluorene	0.30 (0.12,0.73)	0.01	**0.21 (0.06,0.72)**	**0.01**
Dibenzothiophene	1.26 (0.69,2.30)	0.05	**7.30 (1.49, 35.85)**	**0.01**
Perylene	0.77 (0.35,1.70)	0.52	**0.25 (0.06,1.10)**	**0.04**

a. OR: Unadjusted crude odd ratios

b. AOR: Adjusted odd ratios, adjusted for GSTT1 and GSTM1 presence/absence and BMI categories

c. *p*-values indicate significance levels of variables between ASD cases relative to controls

Table 3. Associations of significantly varying organic pollutants with GSTT1 and GSTM1 null genotype.

Organic pollutants	GSTT1						GSTM1					
	Total		ASD		Control		Total		ASD		Control	
	AOR[a] (95% CI)[b]	p-Value[c]	AOR[a] (95% CI)[b]	p-Value[c]	AOR[a] (95% CI)[b]	p-Value[c]	AOR[a] (95% CI)[b]	p-Value[c]	AOR[a] (95% CI)[b]	p-Value[c]	AOR[a] (95% CI)[b]	p-Value[c]
PCB52	0.99(0.88,1.13)	0.92	1.43(0.92,2.24)	0.11	0.43(0.41,1.04)	0.94	**1.58(1.01,2.32)**	**0.036**	1.06(0.90,1.25)	0.483	1.20(0.96,1.49)	0.110
PCB44	0.95(0.84,1.08)	0.45	**0.37(0.23,1.00)**	**0.05**	0.59(0.36,1.36)	0.95	0.98(0.89,1.09)	0.748	1.01(0.86,1.20)	0.887	1.04(0.84,1.29)	0.698
PCB66	**0.42(0.31,0.75)**	**0.04**	0.86(0.66,1.13)	0.28	0.56(0.13,1.32)	0.97	1.05(0.94,1.18)	0.373	**1.72(1.05,1.95)**	**0.016**	0.91(0.68,1.22)	0.517
PCB81	**1.74(1.00,1.87)**	**0.03**	1.27(0.82,1.95)	0.28	0.61(0.42,1.42)	0.94	0.98(0.86,1.11)	0.705	0.91(0.75,1.10)	0.318	1.09(0.82,1.43)	0.560
PCB77	0.94(0.84,1.05)	0.24	0.98(0.77,1.24)	0.86	0.38(0.29,1.23)	0.95	**0.33(0.18,0.98)**	**0.018**	**0.29(0.14,0.96)**	**0.015**	0.90(0.66,1.23)	0.498
PCB101	1.00(0.89,1.12)	0.99	1.08(0.81,1.46)	0.60	0.65(0.35,1.03)	0.95	**1.68(1.28,2.59)**	**0.012**	1.11(0.95,1.30)	0.172	1.20(0.95,1.51)	0.135
PCB123	0.95(0.82,1.10)	0.51	0.83(0.50,1.38)	0.46	1.22(0.41,2.41)	0.94	0.99(0.88,1.11)	0.828	**0.38(0.07,0.99)**	**0.039**	1.27(0.93,1.74)	0.130
PCB118	**1.98(0.99,2.13)**	**0.04**	1.03(0.66,1.61)	0.90	0.29(0.14,0.76)	0.95	0.94(0.83,1.05)	0.284	1.15(0.96,1.39)	0.135	**0.47(0.36,1.00)**	**0.049**
PCB128	0.92(0.74,1.13)	0.41	**0.39(0.18,0.85)**	**0.01**	0.00(0.00,0.01)	0.99	1.09(0.90,1.31)	0.389	1.13(0.88,1.45)	0.347	1.40(0.67,2.96)	0.373

PCB167	1.17(0.87,1.57)	0.29	**4.55(1.49,7.90)**	**0.00**	0.24(0.04,0.72)	0.95	1.07(0.87,1.32)	0.497	1.28(0.94,1.75)	0.121	1.12(0.64,1.94)	0.699
PCB156	**0.42(0.25,0.96)**	**0.03**	0.84(0.37,2.50)	0.21	0.76(0.41,1.04)	0.94	1.32(0.78,2.23)	0.293	1.27(0.82,5.25)	0.124	1.07(0.44,2.61)	0.882
α-hexachlorocyclohexane	0.91(0.78,1.06)	0.22	0.91(0.72,1.15)	0.42	0.74(0.20,3.69)	0.44	1.06(0.91,1.23)	0.465	1.20(0.93,1.56)	0.165	**2.47(1.01,6.05)**	**0.047**
β-hexachlorocyclohexane	1.00(0.90,1.10)	0.95	1.02(0.88,1.18)	0.83	0.18(0.02,1.59)	0.12	1.02(0.95,1.10)	0.623	1.09(0.97,1.23)	0.141	**0.35(0.28,1.01)**	**0.052**
δ-hexachlorocyclohexane	**0.25(0.19,0.43)**	**0.05**	0.88(0.71,1.10)	0.26	0.82(0.62,1.07)	0.14	0.95(0.85,1.06)	0.334	0.93(0.72,1.19)	0.564	0.96(0.82,1.12)	0.602
Heptachlor	1.10(0.92,1.31)	0.30	1.07(0.86,1.33)	0.53	1.12(0.31,4.04)	0.86	**0.49(0.20,1.00)**	**0.048**	**0.28(0.13,0.93)**	**0.009**	0.72(0.44,1.18)	0.195
p,p'-DDE	1.01(0.98,1.04)	0.60	1.03(0.98,1.07)	0.24	0.87(0.55,1.37)	0.54	**0.36(0.19,1.00)**	**0.035**	**0.43(0.18,1.98)**	**0.003**	1.02(0.88,1.17)	0.802
Cis-chlordane	0.99(0.84,1.17)	0.91	1.33(0.00,1.25)	0.99	0.80(0.49,1.33)	0.40	1.13(0.98,1.30)	0.101	**1.76(1.15,2.41)**	**0.007**	1.23(0.94,1.60)	0.129
Dieldrin	1.07(0.93,1.24)	0.35	1.11(0.87,1.42)	0.39	1.14(0.42,3.10)	0.79	**0.41(0.17,0.99)**	**0.031**	0.91(0.73,1.14)	0.404	**0.43(0.35,0.98)**	**0.040**
o,p'-DDD	1.03(0.97,1.09)	0.33	1.04(0.95,1.15)	0.38	0.58(0.32,1.05)	0.07	**1.88(1.55,2.06)**	**0.044**	**1.78(1.02,2.10)**	**0.007**	1.12(0.94,1.34)	0.199
p,p'-DDD/*o,p'*-DDT	0.93(0.81,1.07)	0.31	0.98(0.79,1.24)	0.89	1.03(0.45,2.36)	0.94	0.93(0.81,1.06)	0.266	**0.27(0.14,0.71)**	**0.031**	1.10(0.80,1.52)	0.544

p,p'-DDT	1.01(0.98,1.03)	0.62	0.99(0.96,1.02)	0.40	**2.39(1.04,5.50)**	**0.04**	1.01(0.99,1.03)	0.339	1.02(0.99,1.05)	0.131	1.03(0.95,1.13)	0.473
Endrin	**0.47(0.33,0.79)**	**0.02**	0.84(0.67,1.04)	0.10	0.91(0.40,2.07)	0.83	**1.72(1.41,2.13)**	**0.043**	**1.98(1.03,2.16)**	**0.030**	1.04(0.78,1.37)	0.808
β-endosulfan	1.18(0.87,1.59)	0.28	1.08(0.66,1.77)	0.75	1.13(0.70,1.48)	0.18	0.81(0.64,1.03)	0.088	**0.40(0.20,0.83)**	**0.014**	0.79(0.55,1.14)	0.207
PBDE8	0.31(0.07,1.31)	0.11	0.35(0.03,4.63)	0.42	0.28(0.04,2.12)	0.21	**0.47(0.30,0.95)**	**0.044**	0.40(0.03,5.81)	0.501	0.22(0.02,1.23)	0.209
Acenaphthylene	**0.31(0.11,1.02)**	**0.05**	0.00(0.00,1.59)	0.06	0.00(0.00,1.02)	1.00	1.35(0.29,2.06)	0.42	1.04(0.03,2.22)	0.84	**1.77(0.76,6.73)**	**0.03**
Biphenyl	1.67(0.22,12.50)	0.62	1.04(0.02,2.56)	0.98	1.18(0.02,2.50)	1.00	0.88(0.28,2.76)	0.83	1.12(0.35,6.44)	0.58	**0.21(0.14,1.16)**	**0.04**
Benzoapyrene	**2.19(1.30,2.65)**	**0.02**	**1.90(0.81,2.65)**	**0.04**	0.54(0.14,1.65)	0.99	1.36(0.43,5.53)	0.51	0.44(0.08,2.44)	0.35	**0.45(0.34,1.17)**	**0.05**
Benzoapyrene	**2.19(1.30,2.65)**	**0.02**	**1.90(0.81,2.65)**	**0.04**	0.54(0.14,1.65)	0.99	1.36(0.43,5.53)	0.51	0.44(0.08,2.44)	0.35	**0.45(0.34,1.17)**	**0.05**
7 Methylbenzaanth	**0.47(0.09,0.52)**	**0.01**	0.01(0.00,2.62)	0.10	0.95(0.00,1.52)	0.99	1.16(0.35,6.84)	0.56	0.79(0.48,2.70)	0.20	0.01(0.72,1.59)	0.59
1 methylnaphthlene	**0.32(0.02,0.75)**	**0.00**	**0.41(0.12,0.61)**	**0.02**	0.83(0.12,1.75)	1.00	1.07(0.62,6.91)	0.24	0.80(0.50,1.65)	0.24	1.24(0.07,2.36)	0.12
Fluorene	0.58(0.34,1.39)	0.56	1.02(0.28,2.18)	0.75	1.09(0.28,1.39)	1.00	1.39(0.83,6.87)	0.11	**2.98(1.36,7.65)**	**0.03**	1.41(0.48,1.84)	0.27
7,12-Dimethylb	**4.04(0.99,16.59)**	**0.05**	**3.78(1.13,9.17)**	**0.04**	1.08(0.13,1.59)	1.00	0.55(0.21,1.43)	0.22	0.34(0.09,1.32)	0.12	0.03(0.02,1.28)	0.15

enzaanthracene												
Floranthene	1.32(0.42,2.94)	0.40	1.01(0.18,1.67)	0.34	1.08(0.13,1.290	1.00	1.14(0.48,2.74)	0.75	0.63(0.16,2.38)	0.49	**4.86(1.04,6.28)**	**0.05**
Benzobjkfloranthene	**5.76(1.08,7.91)**	**0.04**	1.34(0.11,3.02)	0.48	0.58(0.11,1.91)	1.00	1.08(0.44,2.65)	0.87	0.96(0.25,3.73)	0.96	0.96(0.10,1.45)	0.06
Pyrene	**0.20(0.02,0.59)**	**0.01**	**0.17(0.08,0.85)**	**0.04**	0.63(0.08,1.590	1.00	0.50(0.17,1.44)	0.20	**0.23(0.05,1.01)**	**0.05**	0.06(0.01,1.09)	0.25
TwoMethylnaphthalene	0.54(0.11,2.73)	0.46	**0.46(0.18,0.64)**	**0.04**	0.36(0.18,1.27)	1.00	0.74(0.27,2.06)	0.57	1.28(0.28,5.89)	0.75	0.21(0.17,2.63)	0.18
MethylFlorene	1.32(0.36,4.84)	0.68	0.64(0.02,1.96)	0.79	0.21(0.02,1.44)	1.00	0.77(0.33,1.78)	0.54	1.14(0.57,2.70)	0.33	**0.22(0.03,1.04)**	**0.06**
BenzoePyrene	0.38(0.01,6.68)	0.62	0.00(0.00,1.32)	0.33	1.06(0.24,1.68)	1.00	0.38(0.02,5.97)	0.49	0.11(0.00,1.75)	0.24	**1.91(1.48,4.20)**	**0.03**
Acenaphthene	1.04(0.22,4.89)	0.96	0.34(0.03,4.08)	0.39	1.01(0.03,4.89)	1.00	1.42(0.65,6.07)	0.23	1.15(0.36,1.37)	0.57	**1.90(1.07,5.34)**	**0.06**
Perylene	1.25(0.79,3.07)	0.08	1.40(0.08,1.61)	0.99	0.13(0.08,0.37)	1.00	0.51(0.14,1.89)	0.31	0.64(0.05,9.00)	0.74	**0.25(0.09,0.73)**	**0.02**
Dibenz(a,h)anthracene	1.16(0.12,11.25)	0.90	1.10(0.23,1.69)	0.56	1.27(0.23,3.25)	1.00	**4.59(1.04,8.22)**	**0.04**	0.39(0.05,3.05)	0.37	**1.93(1.35,2.46)**	**0.02**

a. AOR: Adjusted odd ratios, adjusted for concentrations of organic pollutants
b. 95% CI: 95% Confidence Interval for odd ratios
significant values are shown in bold
c. *p*-values indicate significance levels of analyzed organic pollutants between GSTM1/GSTT1+ relative to GSTM1/GSTT1 null genotypes

Table 4. Daily intake of analyzed organic pollutants from various sources (food, water and dust) compared among different land-use settings. Units are in (ng kg^{-1} day^{-1})

Organic Pollutant	EDI[a] food	EDI[a] water	EDI[a] dust
Σ_{29}PCBs			
Islamabad (Urban)	20.32	0.40	0.23
Lahore (Industrial)	28.76	0.92	0.24
Khanewal (Rural)	15.12	0.07	0.23
Σ_{26}OCPs			
Islamabad (Urban)	79.16	1.59	0.16
Lahore (Industrial)	104.88	2.26	0.18
Khanewal (Rural)	123.80	4.62	0.20
Σ_{4}HCHs			
Islamabad (Urban)	5.16	0.32	0.02
Lahore (Industrial)	15.60	0.41	0.02
Khanewal (Rural)	16.72	0.90	0.03
Σ_{5}DDTs			
Islamabad (Urban)	21.28	0.15	0.06
Lahore (Industrial)	67.08	0.45	0.07
Khanewal (Rural)	79.88	0.78	0.07
Σ_{11}PBDEs			
Islamabad (Urban)	10.84	1.26	0.11
Lahore (Industrial)	12.04	2.11	0.06
Khanewal (Rural)	6.52	0.57	0.04
Σ_{32}PAHs			
Islamabad (Urban)	67.00	0.37	0.94
Lahore (Industrial)	50.72	0.26	1.17
Khanewal (Rural)	63.24	0.25	1.04

a. EDI: Estimated daily intakes

Table 5. Correlations of serum to water, dust and food concentrations of analyzed PCB congeners in different study areas

Organic Pollutants	Islamabad (Urban)		Lahore (Industrial)		Khanewal (Rural)	
	Pearson Correlation coefficient	**P-value**	**Pearson Correlation coefficient**	**P-value**	**Pearson Correlation coefficient**	**P-value**
$PCB8_{water}$	.09	.91	**.95**	**.00**	.99	.11
$PCB11_{dust}$	.81	.09	**.70**	**.02**	.05	.95
$PCB11_{water}$	.58	.31	**.97**	**.00**	.24	.76
$PCB18_{dust}$	.35	.50	**.68**	**.01**	.36	.64
$PCB18_{water}$	.54	.27	**.67**	**.01**	.25	.75
$PCB81_{dust}$	**.90**	**.00**	.36	.48	.59	.12
$PCB77_{dust}$	**.63**	**.03**	.33	.46	**.68**	**.04**
$PCB101_{dust}$	**.67**	**.01**	.85	.15	**.65**	**.04**
$PCB123_{dust}$	**.66**	**.01**	.83	.08	.24	.64
$PCB123_{food}$	-.07	.81	**.92**	**.03**	.68	.14
$PCB118_{dust}$	**.58**	**.02**	.31	.45	.19	.68
$PCB118_{water}$	**.56**	**.03**	**.83**	**.01**	.52	.23
$PCB114_{dust}$	**.61**	**.01**	.41	.28	.22	.59
$PCB114_{water}$	**.60**	**.01**	**.83**	**.01**	.54	.16
$PCB105_{dust}$	**.61**	**.01**	.44	.20	.22	.57
$PCB105_{food}$	.10	.70	.34	.33	**.71**	**.03**
$PCB105_{water}$	**.62**	**.01**	**.86**	**.00**	.54	.13
$PCB153_{food}$	-.19	.63	.04	.94	**.64**	**.03**
$PCB138_{food}$	-.47	.35	**.97**	**.03**	.56	.07
$PCB167_{food}$	-.18	.67	.69	.51	**.58**	**.04**
$PCB156_{food}$	-.12	.77	.73	.27	**.58**	**.03**
$PCB157_{food}$	-.07	.85	.74	.15	**.59**	**.02**
$PCB169_{food}$	-.02	.96	.66	.15	**.77**	**.00**
$PCB169_{water}$	.52	.10	.19	.71	**.77**	**.00**
$PCB187_{water}$	**.69**	**.00**	.34	.46	**.78**	**.00**
$PCB180_{water}$	**.64**	**.02**	.59	.12	**.73**	**.00**
$PCB170_{dust}$	**.58**	**.04**	.52	.15	.27	.31
$PCB170_{water}$	**.66**	**.01**	.63	.07	**.80**	**.00**
$PCB189_{dust}$	**.57**	**.03**	.52	.12	.17	.51
$PCB189_{water}$	**.66**	**.01**	.63	.05	**.77**	**.00**

$PCB195_{water}$	**.68**	**.01**	.37	.33	**.56**	**.01**
$PCB206_{food}$	-.14	.66	.59	.10	**.98**	**.02**
$PCB209_{water}$	.32	.34	.39	.27	**.56**	**.01**
HCB_{water}	**.99**	**.01**	.06	.92	**.94**	**.02**
α-HCH_{food}	.12	.92	.24	.64	**.89**	**.04**
γ-HCH_{water}	**.99**	**.01**	.17	.69	.04	.96
$Heptachlor_{food}$	**.97**	**.03**	.09	.84	.72	.17
$Heptachlor_{water}$	.74	.26	.43	.29	**.96**	**.01**
$Aldrin_{food}$	**.94**	**.02**	.78	.22	.70	.19
$Aldrin_{water}$	.53	.36	.57	.43	**.96**	**.01**
$Oxychlordane_{food}$	**.96**	**.00**	.58	.60	.30	.62
o,p'-DDE_{water}	.42	.40	**.98**	**.02**	.73	.16
p,p'-DDE_{dust}	**.84**	**.03**	.75	.15	.23	.71
$Cischlordane_{food}$	.49	.27	-.09	.84	**.89**	**.04**
$Cischlordane_{water}$	**.75**	**.05**	.73	.06	.31	.61
$EndosulfanI_{food}$	**.89**	**.02**	.80	.10	.17	.78
$EndosulfanI_{water}$	-.63	.18	**.97**	**.01**	.78	.12
$Transnonachlor_{food}$	.66	.11	**.83**	**.04**	.06	.93
$Transnonachlor_{water}$	-.33	.47	**.95**	**.00**	.80	.10
$Dieldrin_{water}$	-.45	.27	**.93**	**.01**	**.90**	**.04**
p,p'-DDD/o,p'-DDT_{food}	.34	.33	**.76**	**.05**	.38	.62
p,p'-DDD/o,p'-DDT_{water}	.40	.26	**.83**	**.02**	.53	.47
p,p'-DDT_{water}	**.73**	**.01**	**.97**	**.00**	.79	.11
$Endrin_{water}$	.40	.23	**.97**	**.00**	.72	.17
$EndosulfanII_{water}$	.41	.19	**.97**	**.00**	.76	.14
Endrin $Aldehyde_{dust}$	-.35	.24	.46	.16	**.96**	**.01**
Endrin $Aldehyde_{water}$	.45	.12	**.97**	**.00**	.85	.07
Endosulfane $Sulphate_{dust}$	.36	.42	-.39	.21	**.93**	**.02**
Endosulfane $Sulphate_{water}$	**.82**	**.02**	**.97**	**.00**	.83	.08
Endrin $Ketone_{water}$	**.74**	**.04**	**.97**	**.00**	**.96**	**.01**
$Methoxychlor_{water}$	**.81**	**.02**	**.97**	**.00**	.66	.22
$PBDE28_{food}$	.34	.51	.66	.34	-.91	.00
$PBDE28_{water}$	-.09	.87	.00	.91	-.97	.03
$PBDE100_{water}$	-.04	.93	-.17	.78	-.91	.01

7.Methylbenz-a-anthracene$_{water}$	**.95**	**.01**	.43	.47	.26	.42
Benzo-c-phenanthrene$_{water}$	**.95**	**.00**	-.43	.46	.17	.59
1-methylnaphthlene$_{water}$	**.87**	**.01**	.50	.39	.18	.65
Fluorene$_{dust}$	**.92**	**.01**	.76	.14	.21	.79
7-12 Dimethylbenz-a-anthracene$_{water}$	**.72**	**.04**	.06	.79	.10	.84
Benzo-a-anthracene$_{food}$	.32	.39	**.99**	**.00**	.67	.10
Chrysene$_{food}$	**.73**	**.03**	.67	.21	.63	.13
pyrene$_{food}$	**.66**	**.04**	.67	.21	.36	.43
methylPhen/Anthra$_{food}$	**.66**	**.03**	.54	.35	.74	.09
2-Methylnaphthalene$_{food}$	**.65**	**.02**	.28	.64	**.80**	**.02**
Naphthalene$_{food}$	**.56**	**.04**	.85	.07	.61	.11
MethylFlorene$_{food}$	**.57**	**.03**	.34	.57	.36	.43
Acenaphthene$_{dust}$	-.09	.71	-.17	.75	**.94**	**.02**
perylene$_{water}$	.11	.66	.22	.72	**.95**	**.01**
Indeno1,2,3 c,d-pyrene$_{water}$	.11	.66	.09	.89	**.95**	**.01**
Dibenz-a,h-anthracene$_{water}$	.07	.78	.21	.74	**.94**	**.02**

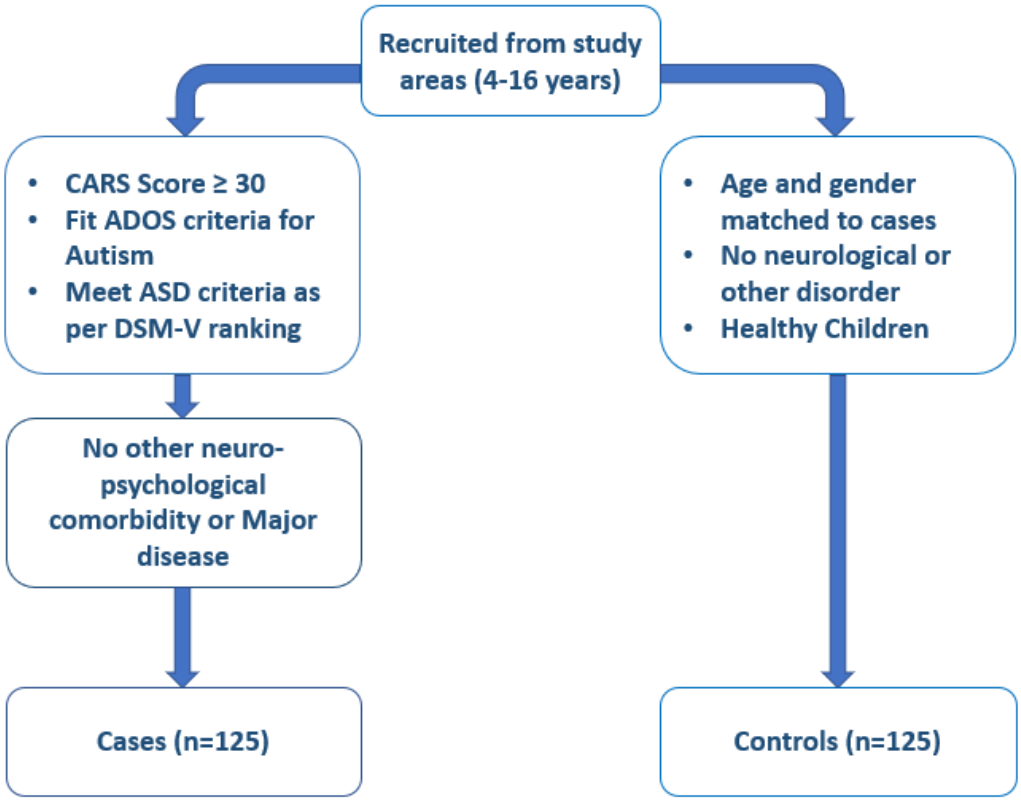

Figure 1. Flowchart showing sampling criteria for study participants

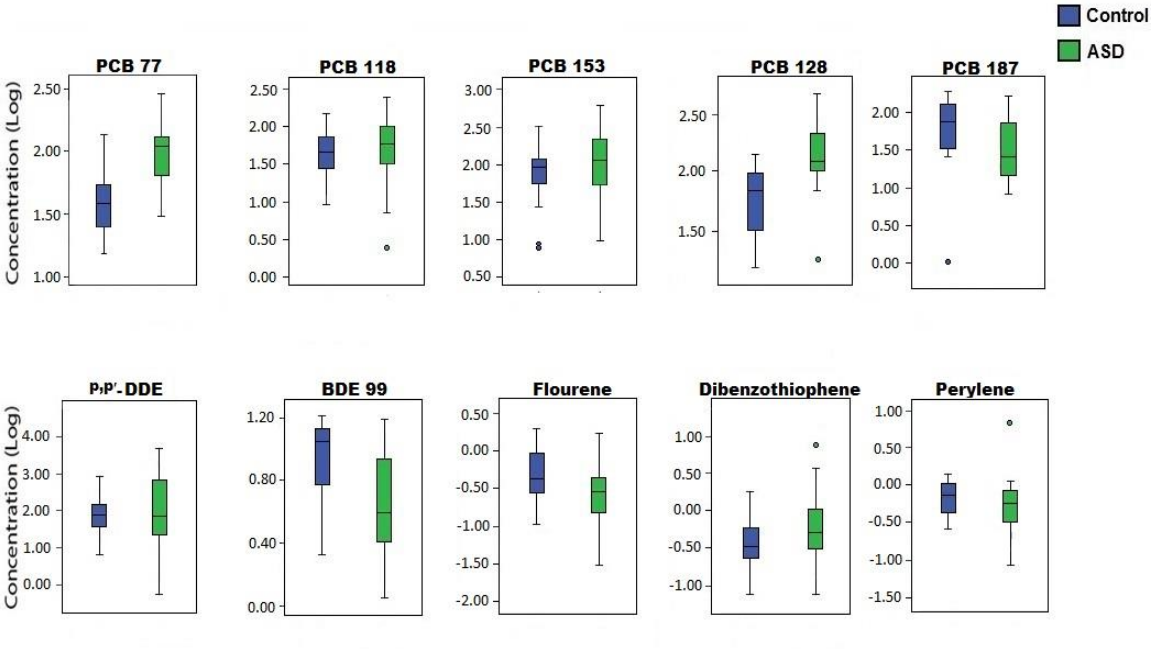

Figure 2. Log transformed concentrations (mean ± SD) of significantly varying organic pollutants in serum samples of autistic vs control children. Whiskers represent the SD. Dots represent outliers.

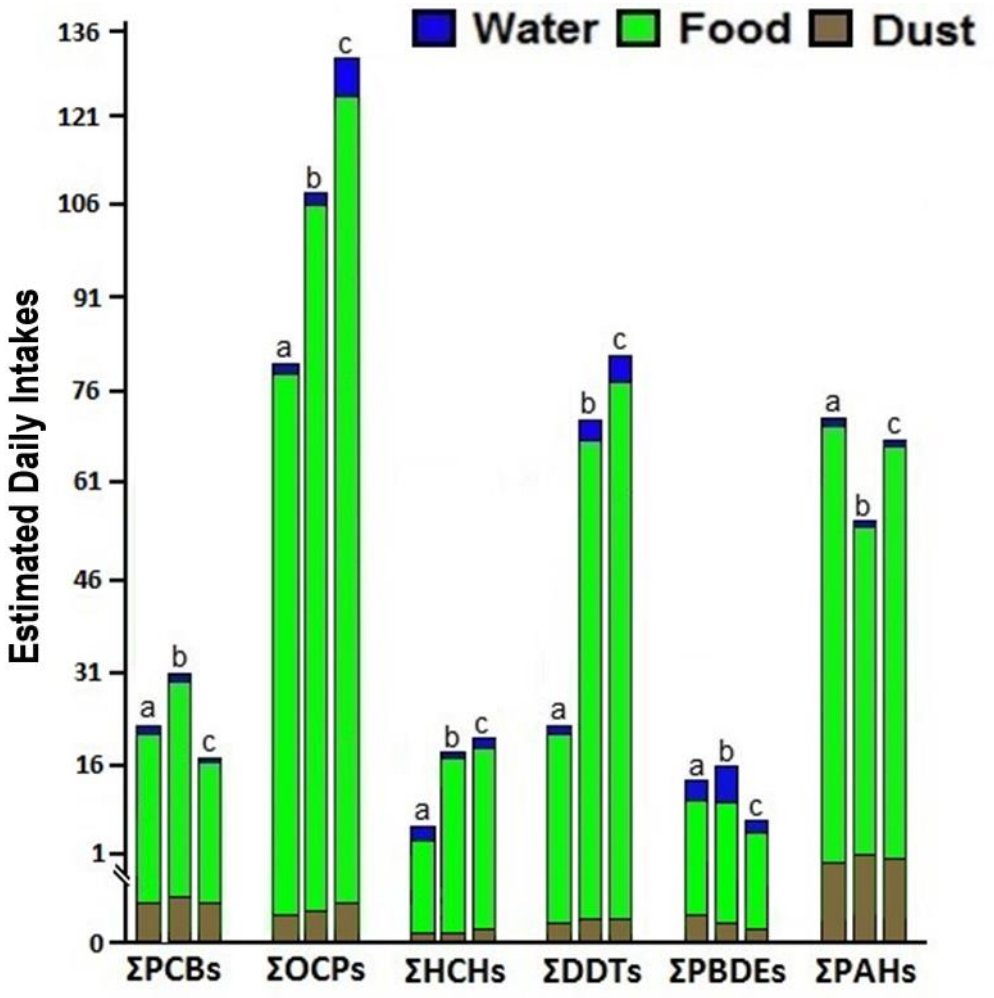

Figure 3. Estimated daily Intakes (ng kg^{-1} day^{-1}) of analyzed pollutants. Columns abbreviated a, b, c indicate cumulative EDIs (dust, food and drinking water) for children from Islamabad, Lahore and Khanewal respectively.